Development and validation of a non-invasive glucocorticoid monitoring protocol in reptiles

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"Without patience I could never have succeeded."

— Jane Goodall

Abstract

Glucocorticoids (cortisol and corticosterone) are the main hormones secreted by animals via activation of the hypothalamo-pituitary-adrenal (HPA) axis in response to a stressor. Measuring glucocorticoids as indicators of a stress response can be a useful tool for assessing wildlife health and how an individual or population may be coping in its environment. Blood sampling is currently the most common method used for measuring glucocorticoids in animals; however use of 'non-invasive' methods for measuring glucocorticoids are becoming increasingly popular due to animal welfare concerns and practicality. Glucocorticoids can be measured from alternatives such as faeces, saliva, skin, hair and feathers. Non-invasive techniques in reptiles, however, have been limited mainly to faecal and shed skin samples, but these methods are not suitable for assessing acute responses that reflect glucocorticoid levels circulating in the blood. Saliva, often used for measuring cortisol in mammals, has not been tested as a method in reptiles for measuring corticosterone. Different saliva collection methods were tested in water dragons (*Intellagama lesueurii lesueurii*) and leopard tortoises (*Stigmochelys pardalis*) to determine which method would yield the required volumes for corticosterone assay in each species. Each species was grouped into saliva and blood sampling groups, and corresponding samples were collected from animals following a standard stress protocol. Corticosterone responses to the stress protocol in each sample type were compared. Finally, blood samples were taken following either an initial blood sample or saliva sample to determine if saliva sampling resulted in a smaller corticosterone response. Corticosterone was successfully measured in reptile saliva, and a full corticosterone response was measured in saliva for both water dragons and leopard tortoises. Sampling type did not affect corticosterone responses. The ability to measure corticosterone from saliva in reptiles provides a new management tool for assessing and further understanding reptilian stress responses.

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1. General Introduction

1.1 Introduction

Measuring circulating glucocorticoids as indicators of a stress response can be a useful tool for assessing wildlife health (Alaasam *et al.*, 2024; Sheriff *et al.*, 2011) especially in the light of climate change and how individuals, populations, or species are able to cope with environmental change (Cockrem, 2022). Furthermore, knowledge of animal coping styles (the capacity to appropriately respond to new situations), has implications for animal welfare and conservation management practice, including captive breeding programs or wildlife translocations (Williams-Kelly *et al.*, 2023). Non-invasive procedures for measuring glucocorticoids have been developed for mammals (Cook, 2012), amphibians (Narayan, 2013) and birds (Aharon-Rotman *et al.*, 2021). For reptiles, the range of available sample types is more limited; the most effective method to measure glucocorticoids are plasma assays, followed by faecal metabolites, and even less commonly used, keratinised tissues (Carbajal *et al.*, 2024). However, faecal metabolites and keratinised tissue concentrations are not always reflective of plasma concentrations at a given point in time (Romero and Beattie, 2022), thus plasma still remains the most widely used method.

1.2 Stress

1.2.1 Definition of stress

Stress in animals can be described as a state in which the neuroendocrine stress system is activated in response to stimuli which the animal perceives to be a threat (Cockrem, 2022), either to the animal's ability to cope in its environment (Greenberg, 2002), or maintain homeostasis (Smith and Vale, 2022). Stimuli that activate the neuroendocrine stress system can be referred to as stressors. It is generally accepted that there is an increase in glucocorticoid concentrations in the blood when an animal is responding to a stressor. A stressor can be present briefly (acute stressor) or persist for longer periods of time (chronic stressor). The source of a stressor can originate from a change or disruption to an animal's physiology (physical stressor), or through sensory input being perceived as adverse (emotional stressor) (Chrousos, 2009; Cockrem, 2007). An animal's physiological and behavioural reaction to a stressor is referred to as a "stress response".

1.2.2 Stress responses

Stressors triggering a stress response can come in many forms including predators (Thaker *et al.*, 2009), conspecifics (Creel, 2001), environmental changes (Romero and Wikelski, 2001), disease (Lind *et al.*, 2018), reproductive state (Anderson *et al.*, 2014), and human interaction (Langkilde and Shine, 2006; Romero and Wikelski, 2002). Animals may perceive the same stressor differently, however; what is perceived to be a stressor and elicits a stress response in one animal may not elicit a stress response in another (Cockrem, 2007). A stress response can involve different behavioural and physiological actions. In vertebrates, physiological actions include the activation of multiple neuroendocrine pathways. These pathways can vary slightly between vertebrate groups (Tort *et al.*, 2020), but mainly involve the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system. The HPA axis (or the hypothalamic-pituitary-interrenal [HPI] axis in fish and amphibians) results in increased secretion of glucocorticoids (cortisol or corticosterone) into the blood and acts within minutes to hours. The sympathetic nervous system results in increased secretion of catecholamines (adrenaline and noradrenaline) and acts within seconds to minutes – this system is often called the "fight or flight" response (Cockrem, 2013; Jessop *et al.*, 2013; Ulrich-Lai and Herman, 2009). It is important to note however, that behavioural changes and response of the sympathetic nervous system can occur in the presence of a stimulus that is not a perceived or real threat (i.e., not a stressor); therefore activation of the HPA axis is considered the main component of a stress response (Cockrem, 2007).

1.3 Hypothalamic-pituitary-adrenal axis and corticosterone in reptiles

1.3.1 Hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis (Fig. 1.1) consists of a cascade of neural and endocrine processes which primarily results in the secretion of increased glucocorticoids into the bloodstream, and can be considered to be part of the ‘neuroendocrine stress system’ (Cockrem, 2022). Neural inputs from external and internal stimuli act on the paraventricular nucleus (PVN) of the hypothalamus, which promotes secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). These hormones act on the anterior pituitary gland to promote secretion of adrenocorticotropic hormone (ACTH). ACTH is then released into the bloodstream, acting on the adrenal cortex to secrete glucocorticoids, which enter the bloodstream and are circulated throughout the body (Romero and Butler, 2007; Ulrich-Lai and Herman, 2009).

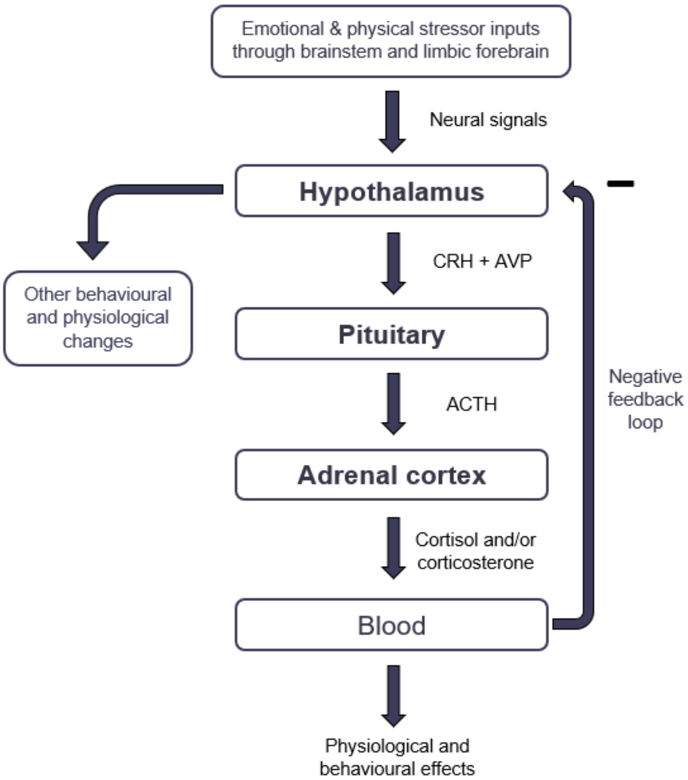

Fig. 1.1. Basic overview of HPA axis components and interactions. Derived from a combination of diagrams in Cockrem (2022) and Sheriff *et al.* (2011).

1.3.2 Glucocorticoid secretion and actions

The primary glucocorticoid released during activation of the HPA axis in reptiles is corticosterone. Corticosterone is also the primary glucocorticoid in birds, amphibians, and some rodents (e.g. mice and rats). Cortisol is the primary glucocorticoid in fish and most mammals (excluding some rodents). While possessing slightly different structures, corticosterone and cortisol perform the same functions and bind to the same proteins within vertebrates (Romero and Butler, 2007). Once secreted by the adrenal cortex, glucocorticoids enter the bloodstream and are circulated around the body. Most circulating glucocorticoids in plasma are bound to corticosteroid binding globulin (CBG), however CBG is too large to move through the capillaries into tissues. The 'bound' portion of glucocorticoids in the blood therefore remain in circulation. The remaining unbound portion available to diffuse into tissues is usually referred to as the 'free' portion (Sheriff *et al.*, 2011).

The main function of glucocorticoids is to increase glucose concentrations in the blood by mobilisation of energy stores (hence the 'gluco' in 'glucocorticoid'). They are also part of a number of normal physiological processes (e.g. circadian rhythms, reproduction, immune system) (Cockrem, 2022; Dayger *et al.*, 2018). In response to a stressor, increased glucocorticoids have five main general effects: increased blood glucose, inhibited growth, inhibited reproduction, immune system modulation and altered behaviour (Anderson *et al.*, 2014; Romero and Butler, 2007; Ulrich-Lai and Herman, 2009). These effects aim to help mediate recovery from the stressor and return the animal to homeostasis, replenish energy expenditure by the sympathetic nervous system response, and prepare the animal for any further stressors (Romero and Beattie, 2022). Glucocorticoids also act back on the hypothalamus and other areas of the brain in a negative feedback loop to decrease further glucocorticoid release and slowly bring an end to the stress response.

While effects of elevated glucocorticoids are often considered to increase fitness and survival of the animal in response to a stressor, constant activation of the HPA axis by a chronic stressor or when negative feedback signals are weak (resulting in elevated glucocorticoid concentrations for a longer duration) can be detrimental to the animal. Chronic exposure to increased glucocorticoids can have detrimental effects, which includes changes to the structure and function of parts of the brain (including those involved in stress responses), reproductive issues, immunosuppression, and overall decreased fitness. Often, the effects of chronically increased secretion of glucocorticoids can be worse than the effects of the original stressor (Romero and Beattie, 2022; Sheriff *et al.*, 2011; Ulrich-Lai and Herman, 2009).

1.3.3 Glucocorticoid measurement

As cortisol and corticosterone are the main glucocorticoids released from activation of the HPA axis, measured concentrations of these hormones are commonly used as biomarkers of a stress response (MacDougall-Shackleton *et al.*, 2019). Cortisol and corticosterone can be measured in serum, or in plasma obtained after blood is centrifuged. Increased concentrations can be measured as soon as 1 minute after onset of a stressor, with peak concentrations usually observed after 15 – 30 minutes. In reptiles, the increase in corticosterone can be slower; Cockrem's (2013) review noted initial corticosterone increases in reptiles occurred 10-15 minutes after a stressor (handling). After the initial increase and peak, concentrations slowly return to basal levels, usually within 60 – 90 minutes, although this can depend on the severity of the stressor (Sheriff *et al.*, 2011). The most common methods of measuring glucocorticoids in serum and plasma are radioimmunoassay (RIA) and enzyme immunoassay (EIA) (Sheriff *et al.*, 2011). Glucocorticoids and their metabolites can also be measured in non-blood tissues including saliva, faeces, urine, feathers, hair, claws, and skin (Baxter-Gilbert *et al.*, 2014; Berkvens *et al.*, 2013; Cockrem, 2022; Kechnebbou *et al.*, 2019). However, with the exception of saliva, most of these are not always reflective of blood concentrations (especially when considering daily rhythms) and correlations between blood and non-blood tissues must be proved (Romero and Beattie, 2022). Rather than high or low concentrations of glucocorticoids, an animal's pattern (magnitude and duration) of cortisol/corticosterone response and how this pattern of response changes with stressors is probably a more appropriate indication as to how an animal is coping (e.g. to changes in their environment, predation). Understanding these changes in patterns may also help researchers understand the effects that stressors have on factors such as survival and reproductive success (Romero and Beattie, 2022; Sheriff *et al.*, 2011).

1.4 Corticosterone in reptiles

1.4.1 Current methods of corticosterone measurement in reptiles

1.4.1.1 Blood

Blood is the most common sample type used for measurement of corticosterone in reptiles. Corticosterone is normally measured in plasma which is obtained after centrifugation of whole blood. If baseline concentrations of glucocorticoids prior to exposure to a stressor are being measured, two important factors to consider are capture and sampling time. Capture and blood sampling are stressful in themselves, therefore minimising blood sampling time (and therefore the time in which glucocorticoid concentrations can increase from baseline) is crucial so that data is not misinterpreted (Romero and Reed, 2005). The time needed to collect a baseline sample varies between species and research situations, although most samples are taken within 10 minutes of capture (Fig. 1.2). Due to the temperature-dependent effects and metabolism of reptiles, this time period may be slightly longer in reptiles compared to birds and mammals (Pough, (1980) in Tylan *et al.* (2020)). Romero and Reed (2005)'s review of five bird species and marine iguanas (*Amblyrhynchus cristatus*) concluded that in samples collected within 3 minutes of capture, corticosterone concentrations were not significantly different from baseline levels. A study of five species of viviparous scincid lizards measured corticosterone at 5 minutes, 30 minutes, 1 hour and 2 hours, and found that corticosterone concentrations were not significantly increased after 5 minutes but were after 30 minutes. Additional measurements between 5 and 30 minutes were not taken, so the point at which the increase in corticosterone began was not determined (Langkilde and Shine, 2006). Cash *et al.* (1997) showed there was no correlation between increased corticosterone concentrations and sampling time in samples collected within 10 minutes of capture in red-eared slider turtles (*Trachemys scripta elegans*).

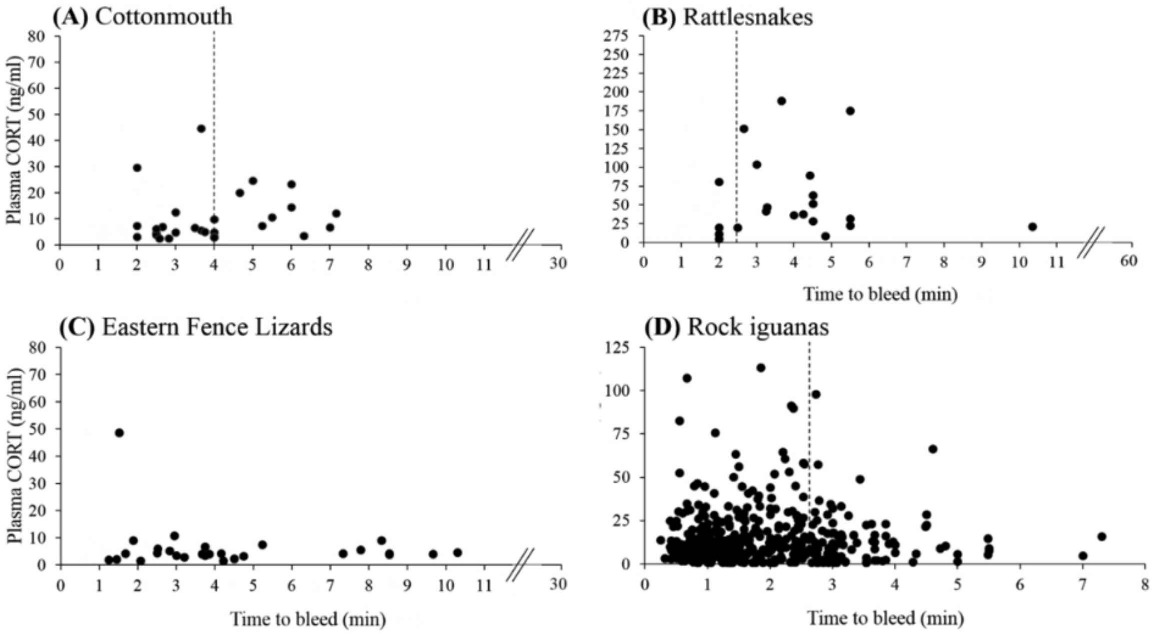

Fig. 1.2. Corticosterone concentrations in relation to time taken to collect a blood sample in four species of reptile. (A) cottonmouth (*Agkistrodon piscivorus*), (B) rattlesnake (*Crotalus oreganus*), (C) eastern fence lizard (*Sceloporus undulatus*), and (D) rock iguana (*Cyclura cychlura*). Vertical dotted lines represent the point at which corticosterone concentrations were significantly different from initial samples. Adapted from Tylan *et al.* (2020).

The choice of venipuncture site can be an important factor in minimising sample collection time and for maximising the quality of blood collected. Redrobe and MacDonald (1999) describe preferred venipuncture sites for different reptile groups. The ventral coccygeal vein (distal to the cloaca) is the preferred vessel for lizards and snakes as it is easy to collect blood from this vein. Crocodilians can also have blood collected from the ventral coccygeal vein, but this may be impractical for larger species, in which case the supravertebral vein is preferred as it is easier to access when the animal is restrained (Nardini *et al.*, 2013). Chelonians (tortoises and turtles) present a challenge for blood collection as accessible vessels (such as the coccygeal vein) also have lymph vessels nearby, and blood collected from these vessels can often be diluted with lymph fluid, potentially affecting corticosterone concentrations. For this reason, the coccygeal vein mentioned above for other reptile groups is not preferred for chelonians due to the proximity of lymph vessels. The preferred site for chelonian blood collection is the jugular vein. For this site, lymph dilution is low, blood collection is often faster than from the coccygeal vein, and has a lower risk of infection (Redrobe and MacDonald, 1999). The brachial vein can also be used but, like the coccygeal vein, lymph dilution can occur.

1.4.1.2 Faeces

Faeces is the most common sample for non-invasive corticosterone measurement in reptiles as faecal samples are relatively easy to collect with little or no disturbance of animals. Corticosterone has been measured in faeces in all extant reptile groups, except tuatara (*Sphenodon punctatus*) (Borgmans *et al.*, 2018; Kalliokoski *et al.*, 2012; Kechnebbou *et al.*, 2019; Rittenhouse *et al.*, 2005). Circulating corticosterone is metabolised by the liver and corticosterone metabolites are excreted into the intestine, after which they are referred to as faecal corticosterone metabolites (FCM), or as faecal corticosterone. The first study in which faecal FCMs were measured in reptiles was by Rittenhouse *et al.* (2005), who tested effects of radiotransmitter attachment on corticosterone concentrations in three-toed box turtles (*Terrapene carolina triunguis*). Faecal corticosterone concentrations were not significantly increased by the procedure of fitting a radiotransmitter. Kalliokoski *et al.* (2012) collected faecal samples from green iguanas (*Iguana iguana*) during acclimatisation and two experimental periods (separated by rest periods) and found FCM concentrations were significantly higher during the experimental periods. Kechnebbou *et al.* (2019) compared faecal corticosterone with plasma corticosterone in free-living spiny-tailed lizards (*Uromastyx acanthinura*) to determine whether corticosterone concentrations changed in relation to proximity to roads or urban areas. Individual lizards were captured at their burrows and had blood samples taken before being released. Burrows were revisited on the same day and the freshest faecal samples collected. It was assumed that faecal samples had been deposited on the same day as blood was collected. Both plasma and faecal corticosterone concentrations showed the same increase in corticosterone concentrations with increasing proximity to roads and urban areas. Faecal corticosterone was also highly correlated with plasma corticosterone ($r = 0.90$, $P < 0.0001$) (Fig. 1.3).

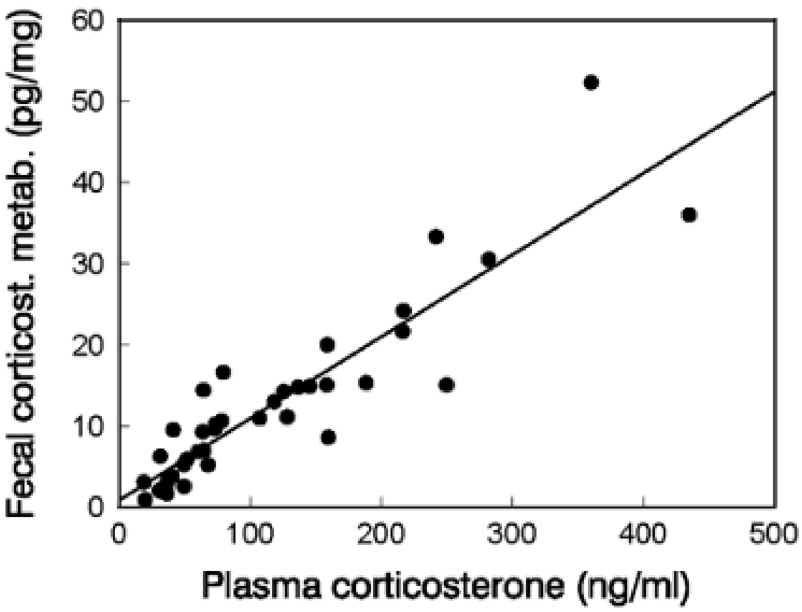

Fig. 1.3. Correlation between faecal and plasma faecal corticosterone metabolite (FCM) concentrations measured from the same individual (*Uromastyx acanthinura*). From Kechnebbou *et al.* (2019).

FCMs accumulate in faeces for as long as the faeces remains in the intestinal tract, so FCM concentrations are highly correlated with gut passage time (Palme, 2019). It is important to have knowledge of gut passage time for interpreting data and determining how suitable a species may be for measuring FCMs. Species with longer gut passage times and inconsistent periods may not be suitable as FCMs would accumulate over multiple stressful events and changes in concentrations could not be attributed to any one event (Miller *et al.*, 2013). For example, snake species have highly varied gut passage times, ranging from 2.6 days (western ratsnakes *Pantherophis obsoletus*) to 183 days (gaboon adders *Bitis gabonica*) under the same conditions (Lillywhite *et al.*, 2002). As faeces cannot always be collected immediately, exact time of excretion is unknown (unless the animals are constantly monitored) and could cause inconsistencies in data interpretation. Hormones also degrade over time so if faecal samples are not collected quickly enough after defaecation, measured concentrations could be inaccurate. Ganswindt *et al.* (2014) found that corticosterone concentrations in Nile crocodile (*Crocodylus niloticus*) faeces decreased by 8% over 24 hours outdoors. This could also be true of those species with longer gut passage times. While FCMs may be continuously secreted, the oldest secreted FCMs may begin to degrade within the gut, giving lower concentrations of FCMs for that passage period (Miller *et al.*, 2013).

1.4.1.3 Keratinous tissues

A small number of studies have quantified corticosterone concentrations in shed skin and claws of reptiles. Hormones can be incorporated into keratinous tissues as they grow (Hunt et al. (2016) as cited in Zena *et al.* (2022)) and retain the hormones over the course of weeks, months, and years. These tissues therefore could be potentially be used as samples for the measurement of stress over longer periods. Shed skin was first used in a study by Berkvens *et al.* (2013) who validated a relationship between shed skin corticosterone and faecal corticosterone in the African house snake (*Lamprophis fuliginosus*). The study found that shed skin corticosterone concentrations did appear to be associated with faecal concentrations during the growth period of the next shed skin (Fig. 1.4). Subsequent studies using Komodo dragons (*Varanus komodoensis*) and Argentine black and white tegus (*Salvator merianae*) also successfully measured corticosterone from shed skin, both showing seasonal variation with higher corticosterone concentrations in the spring (Carbajal *et al.*, 2018; Zena *et al.*, 2022). Zena *et al.* (2022) compared shed skin corticosterone with plasma corticosterone over several months and found plasma corticosterone negatively correlated with shed skin corticosterone within the same month of collection. They suggested this relationship could be related to the skin producing glucocorticoids separate to the HPA axis (this occurs in mammals). Lastly, claws were collected from wild midland painted turtles (*Chrysemys picta marginata*) and corticosterone was measured from these samples, although no comparisons with blood or other tissues were made (Baxter-Gilbert *et al.*, 2014).

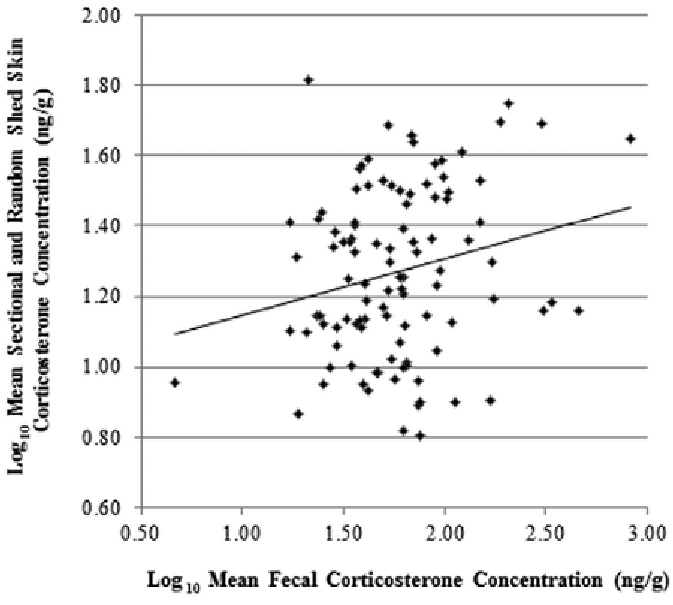

Fig. 1.4. Log_{10} corticosterone concentrations in faeces vs shed skin in African house snakes (*Lamprophis fuliginosus*) (β=0.17, 95% CI:0.05–0.29, p=0.007). From Berkvens *et al.* (2013).

1.4.2 Variation in the corticosterone responses of reptiles

1.4.2.1 Corticosterone, individual variation, and personality

Individual animals differ in their behavioural and physiological responses and use different strategies in order to cope with a particular stressor (Cockrem, 2013; Koolhaas *et al.*, 2010; Koolhaas *et al.*, 1999; Thaker *et al.*, 2009). In the past, most studies focussed on mean results; only more recently has the importance of individual variation been taken into consideration (Cockrem, 2013; Koolhaas *et al.*, 2010). Individual variation arises from a combination of different factors including genotype, phenotype, development, past experiences, and physiology. Across a population or species, the variation in responses and coping mechanisms can provide the basis for natural selection and evolution. An individual's coping mechanisms is often referred to as an animal's personality and can involve a suite of behaviours and physiological changes in response to stimuli from the environment. Animal personalities are often categorised as being either proactive or reactive. Proactive individuals tend to display active or bold behaviours such as biting, charging, or running away (fight or flight responses) and tend to have more rigid routines. Reactive individuals tend to display passive or shy behaviours such as hiding and freezing and tend to react to stressors quicker. It has also been suggested that reactive individuals usually have more behavioural flexibility and are better at adapting to new situations than proactive individuals (Brashears *et al.*, 2020; Cockrem, 2022; Koolhaas *et al.*, 1999; Rodríguez-Prieto *et al.*, 2011).

Generalisations have been made regarding personality and glucocorticoid responses, in that reactive individuals tend to have above average baseline glucocorticoid concentrations and responses to a stressor (e.g. a predator), and proactive individuals show the opposite (Brashears *et al.*, 2020; Koolhaas *et al.*, 1999). However, the relationship between personality and glucocorticoid responses to stressors can be complex and relationships can be strong, weak, or non-existent (Koolhaas *et al.*, 2010). In contrast to the generalised relationship between coping styles and glucocorticoid concentrations and responses, Brashears *et al.'s* (2020) study showed the opposite. Three python species (Ball pythons (*Python regius*), Children's pythons (*Antaresia children*) and Bismarck ring pythons (*Bothrochilus boa*)) were subjected to a 'striking test' (being tapped on the head or body) after a confinement period to elicit a behavioural response. Blood samples for the measurement of corticosterone responses were taken before and after confinement to determine any relationship between behaviour and corticosterone responses. An additional experiment involved confinement and strike tests alongside administration of ACTH to determine maximum corticosterone responses. All three species responded differently behaviourally. Ball pythons balled and hid their heads and never showed any striking behaviour. Children's pythons usually tried to flee, but some strikes did occur. Most Bismarck ring pythons remained coiled and displayed striking behaviour. When considering coping styles, ball pythons could be considered to have reactive coping (hiding), whilst Children's pythons and Bismarck ring pythons could be considered to have proactive coping (fleeing and striking).

In contrast to the generalisations that proactive individuals have the lowest glucocorticoid levels and responses, ball pythons (the most reactive of the three species) had the lowest corticosterone response to confinement (average increase of 8 ng/ml), compared to the proactive species, Children's pythons and Bismarck ring pythons (average increases of 16 ng/ml and 15 ng/ml respectively – Fig. 1.5). Furthermore, the relationship between striking behaviour and corticosterone concentrations between species was highly correlated in individuals.

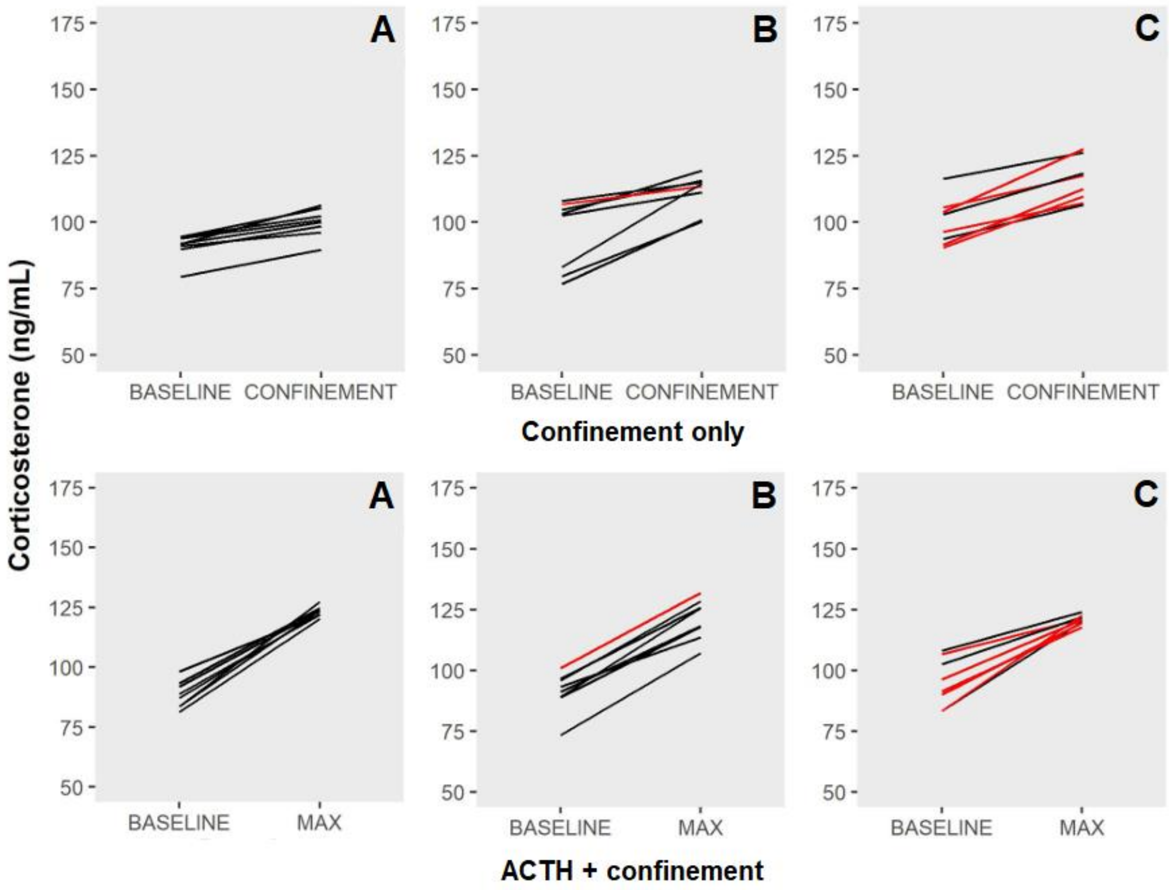

Fig. 1.5. Individual corticosterone responses to confinement (top row) and ACTH administration plus confinement (bottom row) for three species of python (A: *Python regius*, B: *Antaresia childreni*, C: *Bothrochilus boa*). Red lines indicate individuals who struck at least once during the experiments. Graph adapted from Brashears (2020).

1.4.2.2 Corticosterone and environmental conditions

Changes in the environment and differences between environments may affect baseline corticosterone concentrations and corticosterone responses to stressors. Environmental temperature is an important factor when considering the ectothermic nature of reptiles, especially in environments where conditions may make it difficult to thermoregulate effectively. The relationship between glucocorticoid concentrations and temperature is therefore highly varied amongst species (Jessop *et al.*, 2016), with some species demonstrating higher corticosterone concentrations with increases in temperature (Gangloff *et al.*, 2016; Sykes and Klukowski, 2009) – see Fig. 1.6, and others with increased concentrations in response to decreases in temperature (Dupoue *et al.*, 2013).

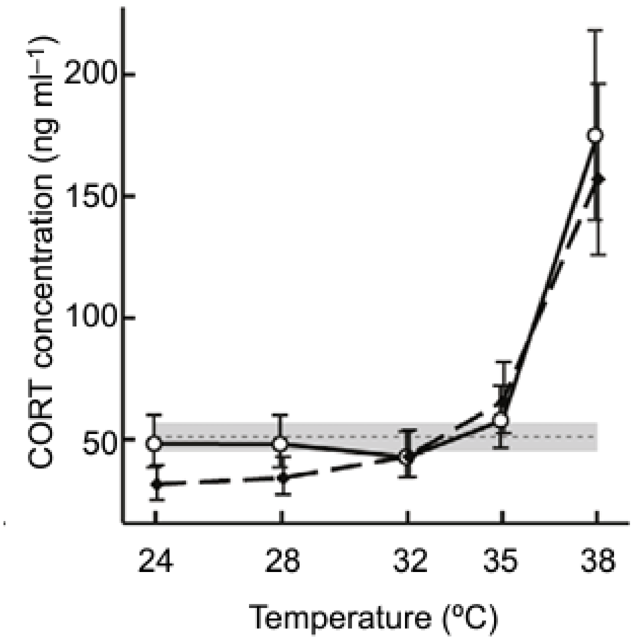

Fig. 1.6. Mean corticosterone responses to increasing temperatures in two populations of garter snakes (*Thamnophis elegans*) inhabiting different habitats. White circles = lakeshore habitat, black circles = meadow habitat. Grey shaded area and dotted line is mean baseline corticosterone taken from the same individuals in their normal habitat before the experiment. Adapted from Gangloff *et al.* (2016).

Changes in environmental temperature can also cause variation in corticosterone increased concentrations through secondary effects. Marine iguanas (*Amblyrhynchus cristatus*) in the Galapagos during a severe El Niño year in 1998 (where changes in water temperature caused a reduction in their food source) showed higher baseline corticosterone concentrations and responses to capture in 5 out of the 6 islands studied compared to 1999 after the El Niño ended and food availability increased (Fig. 1.7) (Romero and Wikelski, 2001).

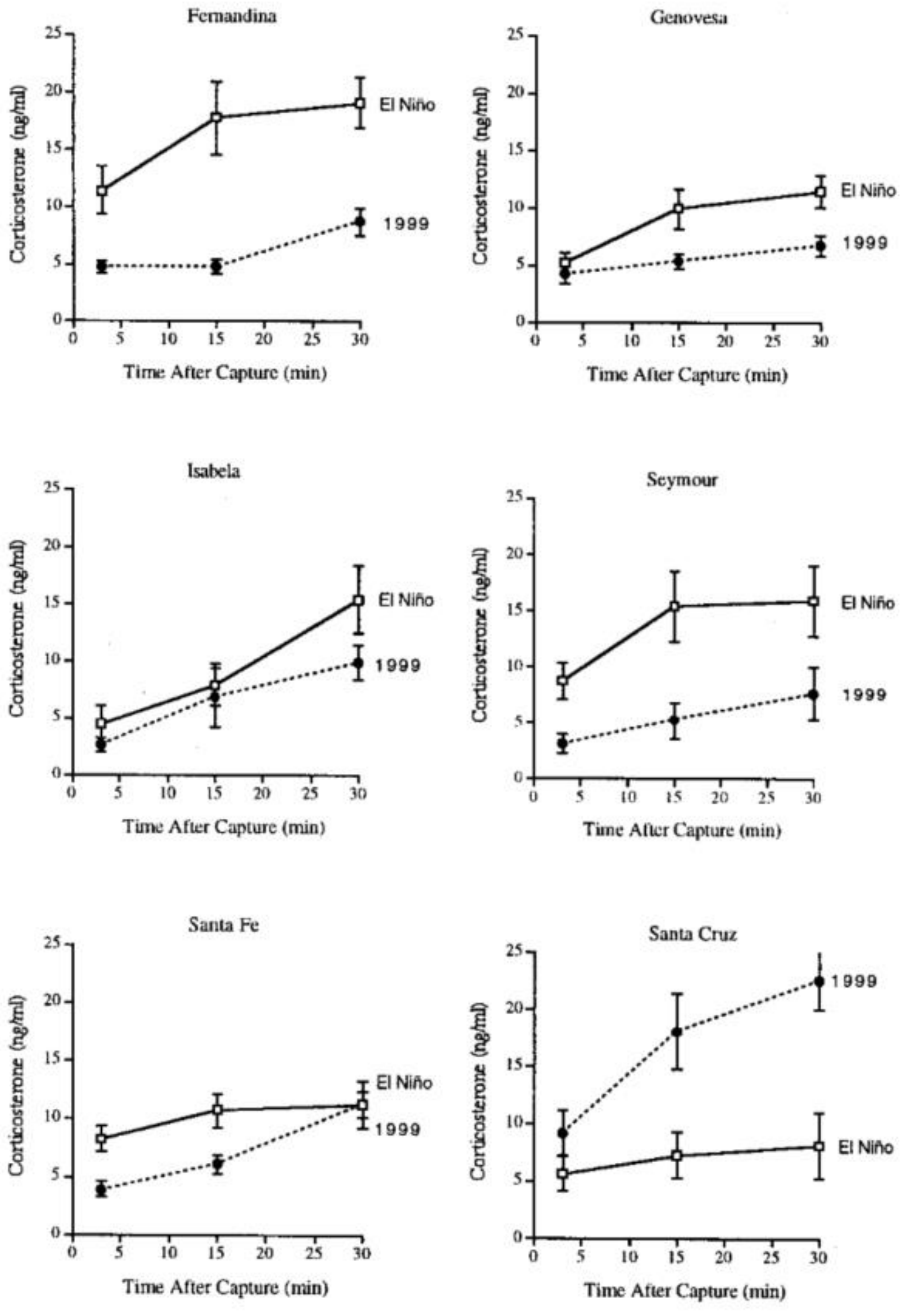

Fig. 1.7. Mean corticosterone responses to capture in marine iguanas (*Amblyrhynchus cristatus*) living on different islands in the Galapagos during the 1998 El Niño (limited food availability) and 1999 after the El Niño had ended (Romero and Wikelski, 2001).

Variation in inhabited environments and ecotypes within a species can also affect corticosterone responses. Two populations of garter snakes (*Thamnophis elegans*) occupying nearby but different habitats show differences in corticosterone responses to stressors. Garter snakes around Eagle Lake (California, USA) have two different ecotypes: lake shore type and meadow type. Lake shore snakes exhibit a fast-paced lifestyle, while the meadow snakes exhibit a slow-paced lifestyle (Palacios *et al.*, 2012). On average, snakes in the meadow habitat had a larger corticosterone response to capture and handling than snakes in the lakeshore habitats (Fig. 1.8).

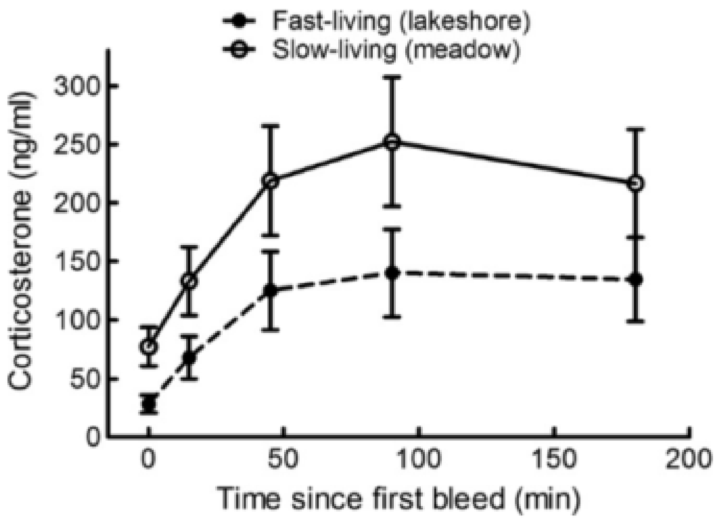

Fig. 1.8. Mean corticosterone responses to capture and restraint in two different ecotypes of the garter snake (*Thamnophis elegans*). Time 0 = baseline corticosterone measurements (Palacios *et al.*, 2012).

1.4.2.3 Corticosterone and annual cycles of reproduction

Romero's (2002) review of seasonal changes in plasma glucocorticoid concentrations in vertebrate species indicated that most reptile species show both daily and seasonal patterns of glucocorticoid concentrations (Romero notes their review is not indicative of all species however, only those that were included in the review). Some reptile species have seasonal variation unrelated to breeding status, or no consistent variation at all (Tyrrell and Cree, 1998). Daily fluctuations in corticosterone concentrations are present in most reptile species, with higher concentrations during the day and lower concentrations at night. Some reptiles retain the ability to secrete corticosterone during the night and as such daily fluctuations are not entirely dependent on temperature (Jessop *et al.*, 2000; Romero and Wikelski, 2006).

For the species in Romero's review that showed variations across seasons, peak glucocorticoid concentrations tended to occur during the breeding period (Fig. 1.9). In males, activation of the HPA axis generally seems to suppress certain behaviours (e.g. territorial) during the breeding season, and changes in corticosterone often coincide with changes in testosterone, though relationships vary between species (Moore and Mason, 2001).

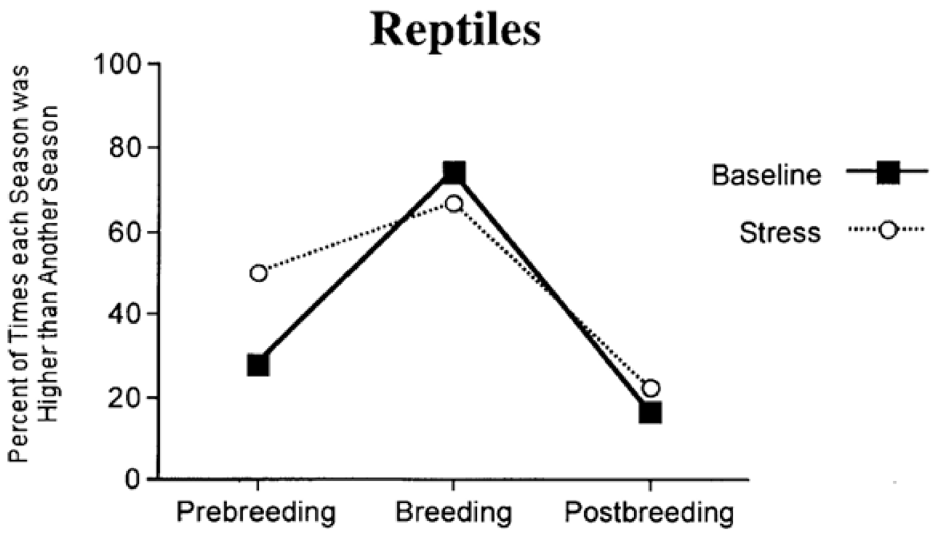

Fig. 1.9. Graphical summary adapted from Romero's (2002) review depicting the overall pattern of seasonal corticosterone concentrations from a wide variety of reptile studies. Each value for baseline (black squares) and stress (white circles) represents the total percentage of reviewed studies where one season had higher corticosterone concentrations than another.

Anderson *et al.* (2014) found that gravid female tuatara (*Sphenodon punctatus*) had significantly higher baseline corticosterone concentrations than non-gravid females and males during the nesting period, as had been found in previous studies. In an earlier study, Cree and Tyrrell (2001) found that corticosterone concentrations were highest during nest digging and oviposition, after which corticosterone concentrations drop significantly. This led Cree and Tyrell to suggest that corticosterone may have a potential role in oviposition timing in tuatara. Marine iguanas also follow the same pattern; corticosterone concentrations increase significantly during arrival and digging at the nesting site, before dropping after oviposition (Fig. 1.10) (Rubenstein and Wikelski, 2005).

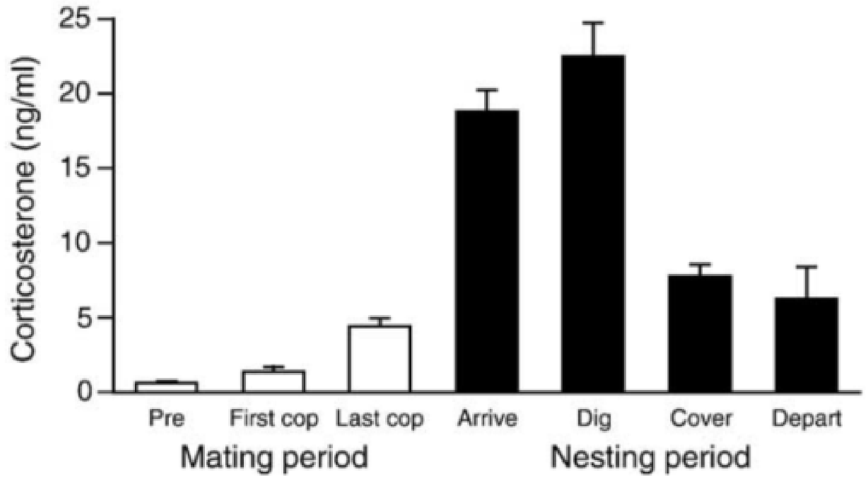

Fig. 1.10. Mean corticosterone concentrations during different stages of the breeding season in marine iguanas (*Amblyrhynchus cristatus*). Breeding season is separated into the mating period and the nesting period. First cop = first copulation, last cop = last copulation. Adapted from Rubenstein and Wikelski (2005).

Hormonal and behavioural responses during the breeding period to stressors can depend on multiple factors including reproductive state, environmental conditions, and physical threats. While gravid females during nesting have relatively high baseline corticosterone concentrations, their responses to stressors (e.g. capture and restraint) can be dampened during this period, even when presented with potentially harmful stressors. Corticosterone responses of gravid nesting female tuatara were only one fifth those of non-gravid females and males during the same part of the season in response to capture and restraint, as was reported in green turtles in response to capture and high temperatures (*Chelonia mydas*) (Anderson *et al.*, 2014; Jessop *et al.*, 2000). Causes of this dampened response are varied; Anderson *et al.* (2014) stated possible reasons as “adaptive interruption of certain stress response reactions (e.g. flight response) to nest successfully, adaptive maternal effects and/or programming, lack of awareness towards certain stressors, or an inability to mount a further corticosterone response”. It appears the dampened response may aid in increasing overall reproductive success in the presence of threats that would otherwise cause interruptions to breeding attempts (Jessop *et al.*, 2000; Moore and Jessop, 2003).

1.4.2.4 Corticosterone and physiological variation

Physiological sources of variation outside of reproduction may arise from factors including body condition, disease, and transgenerational effects. Baseline corticosterone and corticosterone responses can vary with changes in body condition, with higher glucocorticoid concentrations often being related to lower body condition indices, since actions of glucocorticoids include mobilisation of energy stores (Dayger *et al.*, 2013). Graph (A) in Figure 1.11 shows the relationship between body condition index and baseline glucocorticoid concentrations in free-ranging pigmy rattlesnakes (*Sistrurus miliarius*); corticosterone concentrations decreased as body condition increases. As with other sources of variation, this relationship is highly species and context dependent; some studies have shown a relationship between body condition and glucocorticoid concentrations only when an animal is below a specific body condition threshold, and others showed no relationship at all (Moore *et al.*, 2000; Romero and Wikelski, 2001; Spence *et al.*, 2020). Corticosterone concentrations have also been shown to increase with increasing severity of disease; Lind *et al.* (2018) found that pygmy snakes with severe clinical infections of snake fungal disease (*Ophidiomyces ophiodiicola*) had baseline corticosterone concentrations almost twice that of mildly symptomatic and asymptomatic snakes, and that infection scores were positively correlated with corticosterone concentrations (Fig. 1.11).

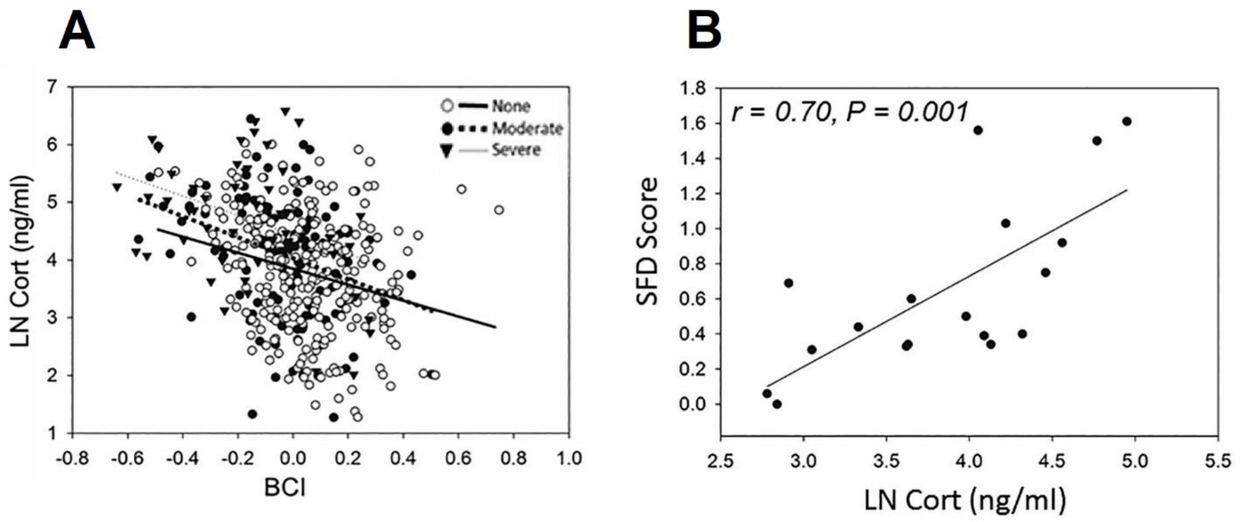

Fig. 1.11. (A) Relationship between corticosterone concentrations and body condition index (BCI) in different severities of disease, and (B) relationship between scored severity of snake fungal disease (SFD) and corticosterone in free-ranging pigmy rattlesnakes (*Sistrurus miliarius*). Adapted from Lind *et al.* (2018).

Variation in corticosterone can also be due to maternal effects passed down while females are gravid. When considering the two ecotypes of the garter snakes previously mentioned (lakeshore and meadow) it could be assumed that the juveniles of these ecotypes would follow the same general baseline corticosterone pattern (lakeshore = low baseline concentrations, meadow = high baseline concentrations). However, Robert *et al.* (2009) found the opposite; juveniles born from lakeshore ecotype mothers had higher baseline corticosterone than the meadow ecotype juveniles. This could be in part due to the faster-paced lifestyle and growth rates of the lakeshore type snakes that have higher energy requirements (and therefore higher corticosterone secretion) for faster growth during the juvenile period. Similarly, other studies have shown that juveniles of females treated with corticosterone during pregnancy show variations in survival and morphology traits compared to non-treated mothers, although no corticosterone measurements from juveniles were taken (Meylan *et al.*, 2010; Palacios *et al.*, 2022).

1.4.2.5 Corticosterone and social status

Lastly, differences in social hierarchy within a group can be a source of variation in baseline corticosterone and corticosterone responses. Multiple studies of captive animals have found that in general, subordinate males have higher baseline corticosterone concentrations and larger responses than dominant males during or after agonistic interactions, (Creel, 2001; Schuett and Grober, 2000; Yang and Wilczynski, 2003), or while being housed together (Barry *et al.*, 2010). Maintenance of hierarchies by dominant individuals may continually expose subordinate individuals to agonistic encounters and to dominance behaviours, which may contribute to these differences (Greenberg *et al.*, 1984). Furthermore, a study of captive green anole lizards (*Anolis carolinensis*) by Plavicki *et al.* (2004) demonstrated that these differences may also exist outside of social interactions; dominant males had a smaller response and recovered faster than subordinate males when subjected to forced movement (chased around an enclosure with a small stick for 10 minutes).

However, many of these studies were carried out in captivity, and therefore results (as in Fig. 1.12) may not reflect relationships between social status and glucocorticoid responses in the wild. The social context in a captive setting where individuals are paired up may be substantially different to that of a wild population with a stable hierarchy. In a captive setting, subordinate individuals may not be able to avoid dominant individuals (by either hiding or fleeing), which may exacerbate their response (Creel, 2001).

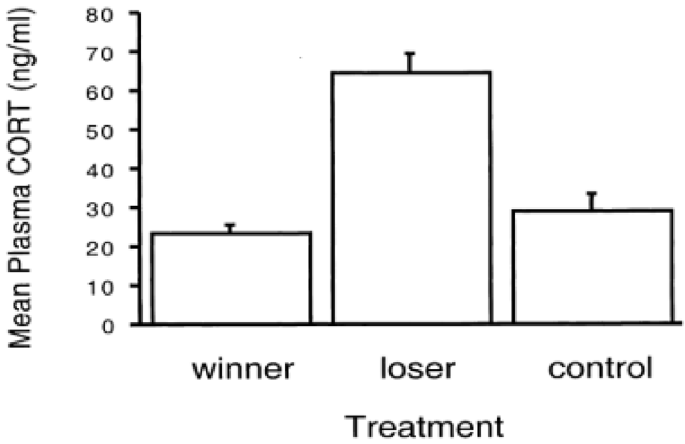

Fig. 1.12. Mean corticosterone concentrations of copperhead snakes (*Agkistrodon contortrix*) following an agonistic interaction with another snake. 'Winners' of these interactions had significantly lower corticosterone concentrations than their 'loser' counterparts, and slightly less (but not significantly) than control snakes. Adapted from Schuett and Grober (2000).

1.4.3 Variation in corticosterone responses across reptile taxa

Cockrem's (2013) review of individual variation included a comparison of maximum mean corticosterone concentrations in response to various methods of capture across the four main reptile groups: testudines (turtles and tortoises), crocodilia (crocodiles and alligators), squamata (lizards and snakes) and sphenodontia (tuatara). Testudines and sphenodontia have relatively low concentrations of corticosterone, crocodilians have moderate concentrations, and the squmates have moderate-high concentrations, with snakes having the highest concentrations.

Studies published since 2013 follow a similar pattern; maximum corticosterone concentrations in response to capture ranged from 2.5 – 9 ng/mL in 4 studies of testudines (average 4.6 ng/mL) (Boers *et al.*, 2020; Currylow *et al.*, 2017b; Hunt *et al.*, 2016a; Hunt *et al.*, 2016b), 3.4 – 70 ng/mL in 4 studies of crocodilians (average 35.4 ng/mL) (Finger Jr *et al.*, 2015; Finger *et al.*, 2018; Finger *et al.*, 2021; Kohno *et al.*, 2020), 5.6 – 25 ng/mL in 2 studies of tuatara (average 15.3 ng/mL) (Anderson *et al.*, 2014; Anderson *et al.*, 2015), and 12 – 260 ng/mL in 8 studies of squamates (average 75.8 ng/mL) (Claunch *et al.*, 2022; Dayger *et al.*, 2013; Hews and Baniki, 2013; Holding *et al.*, 2014; Jessop *et al.*, 2015; McCallie and Klukowski, 2022; Racic *et al.*, 2020; Sparkman *et al.*, 2014). As was found by Cockrem (2013), in these more recent studies testudines had the lowest corticosterone concentrations, followed by crocodilians and tuatara, with squamates having the highest. When splitting the squamates into lizards and snakes, snakes had the highest corticosterone concentrations.

Fig. 1.13 shows relationships between plasma corticosterone and body temperature in a testudine and a squamate; the tawny dragon lizard (*Ctenophorus decresii*) has much higher corticosterone concentrations (reaching above 30 ng/mL) compared to the green sea turtle (*Chelonia mydas*), which did not exceed 4 ng/mL in similar temperatures.

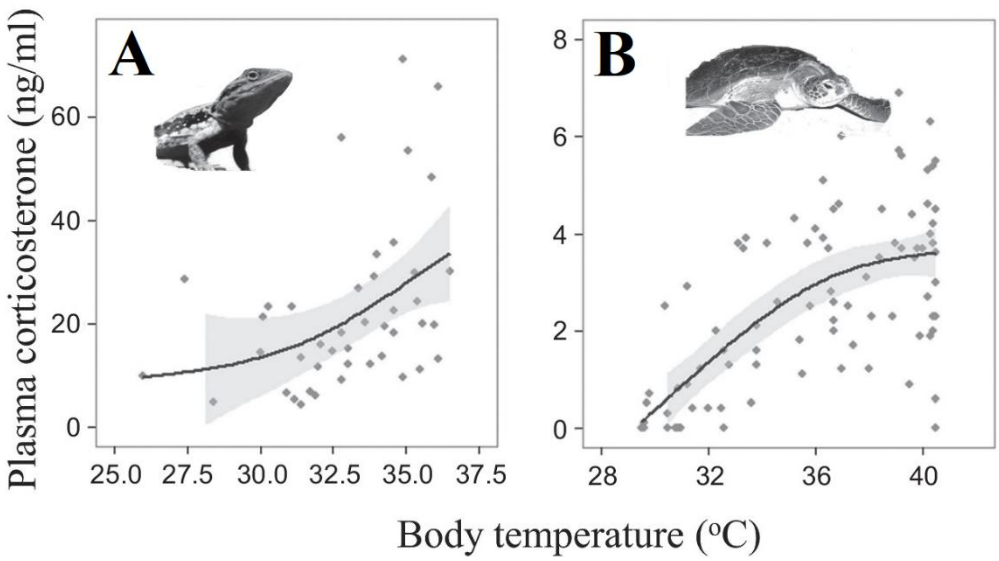

Fig. 1.13. Relationship between plasma corticosterone concentrations and body temperature in (A) a squamate (*Ctenophorus decresii*), and B) a testudine (*Chelonia mydas*). Shaded areas represent 95% confidence error bands of fitted models. Adapted from Jessop *et al.* (2016).

1.5 Saliva collection and glucocorticoid measurement

1.5.1 Saliva collection methods in reptiles and other taxa

A wide range of methods have been reported for the collection of saliva from different species of animals in different situations. Saliva sampling techniques in the literature are highly varied, with a large variety of collection devices used and sampling techniques customised to suit the needs of the experiment. Michalke *et al.* (2015) categorised saliva sampling into five categories: draining (passive drool), spitting, suction, rinsing, and absorption. Draining and spitting are used mainly in human studies as these collection methods require voluntary action by the subject. Absorption and suction are the most common methods used in animals where saliva cannot voluntarily be given. These two methods introduce some degree of stimulation as chewing or biting on the sample apparatus can increase salivary flow. Methods of saliva collection in reptiles and other vertebrate taxa are outlined below.

Chen *et al.* (2019) mentions there are "no standard methods to get saliva samples from reptiles", and that more research was needed. One of the first studies involving collection of reptile saliva was that of Van Denburgh (1898). Van Denburgh collected saliva from gila monsters (*Heloderma suspectum*) to test the effects of the venom component of saliva on pigeons, and to determine which gland(s) venom was secreted from. Saliva was collected by having the gila monsters repeatedly bite down on a piece of soft rubber wrapped in filter paper. The filter paper was then placed in water to allow the saliva to diffuse out, and the resulting fluid used for experiments. Further studies have been carried out with the saliva of helodermid lizards, as a peptide found in the venom of gila monsters (exendin-4) has become useful in the treatment of type 2 diabetes (Eng *et al.*, 1992). While most of the older studies do not mention exact methods of saliva collection, a more recent study by Sanggaard *et al.* (2015) encouraged gila monsters and Mexican beaded lizards (*Heloderma horridum*) to bite down on a soft rubber tube (similar to Van Denburgh), and collected saliva by pipetting directly from the mouth. Akin to the helodermid lizards, Komodo dragons (*Varanus komodoensis*) have bacterial variants within their saliva, and some studies have suggested they may also produce venom (Fry *et al.*, 2009). Montgomery *et al.* (2002) restrained captive Komodo dragons and used a cotton swab to collect saliva from the gumline and dorsal palette. The saliva was used to determine the presence of aerobic bacteria species. Some dragons that were actively salivating throughout restraint also had saliva removed by using a catheter-tipped syringe, although only small amounts were collected with this method. Subsequent studies used similar methods, either swabbing gums or pipetting directly from the mouth (Goldstein *et al.*, 2013).

Chen *et al.* (2019) investigated effects of pesticide use on Mongolian racerunners (*Eremias argus*) by measuring enzymic activity in a range of tissues including saliva. However, the lizards were euthanised and the saliva collection method was not mentioned. Similarly, saliva was collected from common wall lizards (*Podarcis muralis*) to test for biomarkers following pesticide exposure (Mingo *et al.*, 2017). Lizards were encouraged to bite a Copan 155C swab which was then rotated in the mouth. The yield of saliva was not reported in this study. Other species of reptiles have had saliva sampled for DNA collection. Buccal swabbing with either small cotton-tipped dry swabs or small wooden sticks have been used in common wall lizards, Chinese crocodile lizards (*Shinisaurus crocodilurus*), tuatara (*Sphenodon punctatus*), and green turtles (*Chelonia mydas*) (Huang *et al.*, 2014; Lanci *et al.*, 2012; Miller, 2006; Schulte *et al.*, 2011). Saliva for antibody studies was collected from Philippene crocodiles (*Crocodylus mindorensis*) by swiping cotton buds over the tongue multiple times (Groffen *et al.*, 2013). Saliva sampling methods for DNA analysis focus on small volumes of saliva containing buccal cells from the wall of the mouth and may not be suitable for saliva collection for hormone measurements.

Saliva collection from adult humans involves collection of saliva from a passive drool or from a swab placed in the mouth. Babies are not able to voluntarily provide saliva, so a variety of other methods have been used for saliva collection. Santiago *et al.* (1996) used a plastic tube attached to a syringe to aspirate saliva from the mouths of neonatal infants. In other primate species in which salivary cortisol has been measured, saliva has usually been collected from captive animals that have been trained to provide saliva, or from animals conditioned to a sampling apparatus. In these situations, the degree of stress associated with sample collection and hence effects of sampling on baseline cortisol levels are reduced in comparison with collection of samples from animals not familiar with the sampling procedure. Studies of a range of primate species (bonobos (*Pan paniscus*), chimpanzees (*Pan troglodytes*), orangutans (*Pongo pygmaeus*), western gorillas (*Gorilla gorilla*), Hamadryas baboons (*Papio hamadryas*), rhesus macaques (*Macaca mulatta*)) have used cotton ropes, cotton rolls and fixed pieces of gauze as the absorbent medium, with the sample collection material attached to a pole mount presented through enclosure bars (Fig. 1.14). Flavouring is often added onto the sample medium to encourage chewing or licking and to stimulate saliva production (Behringer *et al.*, 2012; Lutz *et al.*, 2000; Pearson *et al.*, 2008). Other studies used specialised salivary swabs in their studies (e.g. Salivette® (Sarstedt) and SalivaBio Oral Swab (Salimetrics®)), with swabbing movements made by the researchers. For example, Verspeek *et al.* (2021) used salivettes and swiped them through the mouth of bonobos for 20-30 seconds.

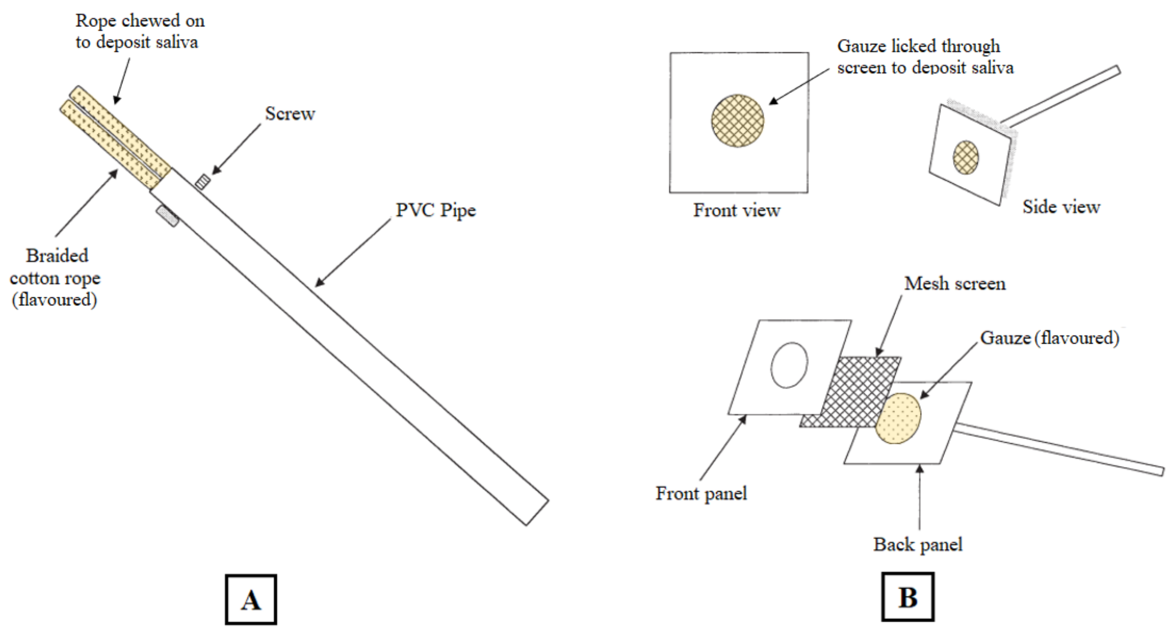

Fig. 1.14. Diagrams of the pole apparatus (A) and the screen apparatus (B) used by Lutz *et al.* (2000) to collect saliva from rhesus macaques through their cage. Both the cotton rope and gauze were flavoured with drink crystals (orange colouring) to encourage licking or chewing on the apparatus.

Swabs have been used for the collection of saliva from other large mammals, with swabs brushed through the buccal area or left in the mouth for the animal to chew on. Saliva has been collected for cortisol assay using a variety of absorbent methods in a range of species including Asian elephants (*Elephas maximus*), horses (*Equus ferus caballus*), reindeer (*Rainger tarandus*), dogs (*Canis familiaris*) (Fig. 1.15), pigs (*Sus domesticus*), and even bottlenose dolphins (*Tursiops truncates*) (Dreschel and Granger, 2009; Lensen *et al.*, 2015; Pedernera-Romano *et al.*, 2006; Plangsangmas *et al.*, 2020; Rehbinder and Hau, 2006; Rickert *et al.*, 2022; Scheidegger *et al.*, 2016). Captive dolphins have been trained to come to the surface of their pool and open their mouths until given direction to close them. Pedernera-Romano *et al.* (2006) took saliva samples with a cotton swab under the tongue as it was presumed this would be the area with the highest volume of pooled saliva. However, Rickert *et al.* (2022) argued that the base of the tongue would also contain pooled water which would dilute the sample. The latter therefore collected saliva with a salivette from the side of the tongue, as they postulated that this area would contain the least diluted sample (Fig. 1.16). Saliva sample collection methods used for smaller mammals are more relevant to small reptile species that methods used in large mammals. Saliva has been collected from small mammal species including mice (*Mus musculus*), guinea pigs (*Cavia porcellus*), tree shrews (*Tupaia belangeri*), hedgehogs (*Erinaceus europaeus*) and marmosets (*Callithrix jacchus*) (Ash *et al.*, 2018; Fenske, 1996; Nemeth *et al.*, 2016; Nohara *et al.*, 2016; Ohl *et al.*, 1999; Rasmussen *et al.*, 2021). Most studies used absorbent materials, with a variety of saliva collection methods. Ash *et al.* (2018) used a SalivaBio Oral Swab (Salimetrics®) coated in banana which was presented to marmosets to chew on, whereas Fenske (1996) and Nemeth *et al.* (2016) both inserted cotton buds (Q-tips) into the cheek pouch of guinea pigs, although the time for which the buds were held varied. Ohl *et al.* (1999) gave cotton rolls (twisted gauze sponges) to tree shrews and encouraged them to chew on them for at least 5 seconds. Suction methods have also been used; for example, Rasmussen *et al.* (2021) used single-use pipettes to aspirate saliva from the corner of the mouth and cheek in hedgehogs.

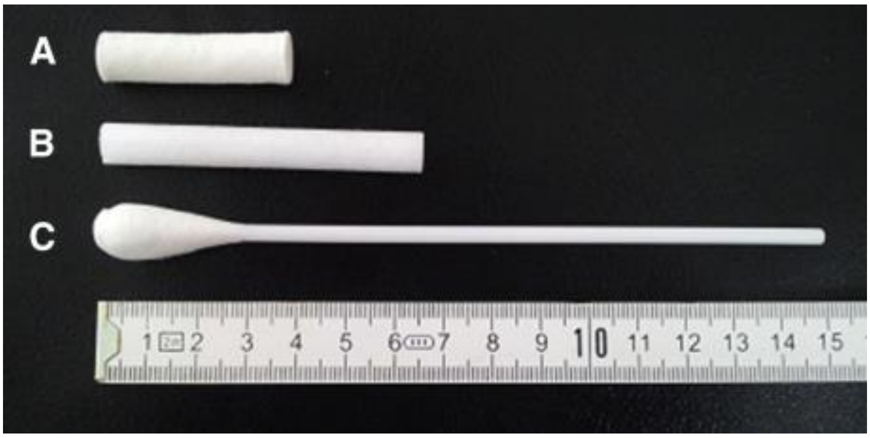

Fig. 1.15. Three types of saliva swabs tested by Lensen *et al.* (2015) on dogs: Cotton swab (A), synthetic swab (B) and cotton bud (C). Tape measure for size reference.

Fig. 1.16. Saliva sampling of a dolphin using a Salivette (Rickert *et al.*, 2022). The white arrow points to the swab being swiped through the side of the mouth.

Some bird species do not produce saliva, and there have not been any avian studies in which glucocorticoids were measured in saliva. Avian saliva has been used for the measurement of other biomarkers, however. Stocker *et al.* (2021) measured salivary mesotocin concentrations in common ravens (*Corvus corax*) by training birds to take a salivary swab, place it in their throat pouch, and return it to the handler. In a different study, researchers collected saliva from chickens (*Gallus domesticus*) by rinsing 5 ml of saline into the oral cavity and collecting the wash-out fluid into a tube. From this sample they were able to test the antiviral properties of chicken saliva against H5N1 avian influenza virus (Limsuwat *et al.*, 2014).

The collection of saliva from anurans has generally involved some form of absorbent material inserted into the mouth. Janin *et al.* (2012) measured salivary corticosterone in common toads (*Bufo bufo*). A small metal spatula was used to gently open the mouth, after which a cotton ball was inserted into the mouth and left for the toad to chew on for 15 seconds. Other studies using similar methodology to collect saliva for corticosterone measurements in yellow-bellied toads (*Bombina variegata*) and saliva for testosterone measurements in common toads. In both studies the cotton balls were left for a longer collection period of at least 30 seconds (Cayuela *et al.*, 2017; Touzot *et al.*, 2020). Hammond *et al.* (2018) conducted a study to validate salivary corticosterone in three species of frogs (*Rana catesbeiana, Rana clamitans, Rana pipiens*) using small pieces of salivary swab (Salimetrics®' SalivaBio Infant's Swab), held in the mouth by a pair of sterile forceps. These small pieces of swab yielded between 30 and 75µL of saliva. It was found for each species that salivary corticosterone was reliable in reflecting elevated plasma concentrations in response to stressors. A follow-up study aimed to find a relationship between glucocorticoids and infection with chytrid fungus using the same method and swabs (Hammond *et al.*, 2020). These anuran studies provide valuable examples of saliva collection outside of the reptile group, as the species used have small mouths and need to be handled during the duration of sampling.

It can be concluded from this review of the techniques used for reptiles and across other taxa, that absorbent methodology is the most effective method for saliva collection, most importantly for field collection where laboratory equipment may not be readily available. As there is no capacity for training the animals to be used in the current study, restraint will need to be applied. While restraint provides an external stressor that may affect baseline corticosterone levels, it provides collection options that need more involved manipulations, such as suction with a small syringe. The anatomy and saliva consistency of each study species will need to be considered, and there may be differences between species in the most appropriate saliva collection method.

1.5.2 Validation of salivary glucocorticoids and comparisons with plasma glucocorticoid concentrations

Using saliva for measuring glucocorticoids needs to be validated for each study for it to have any biological relevance. Validation requires a correlation between concentrations of glucocorticoids in saliva and plasma (Kivlighan *et al.*, 2004; Romero and Beattie, 2022). A common method for validating salivary glucocorticoids involves administering adrenocorticotropic hormone (ACTH), commonly known as the 'ACTH challenge' (Millspaugh *et al.*, 2002). As ACTH acts on the adrenal glands to release cortisol or corticosterone into the blood, administering ACTH in this way creates an artificial glucocorticoid response which can be measured. Administered ACTH is in much higher concentrations than what would naturally be secreted, so the glucocorticoid responses that follow are unnaturally high.

Peeters *et al.* (2011) conducted a study to confirm previous hypotheses that saliva is a reliable alternative for cortisol measurement in horses (*Equus ferus caballus*). Blood and saliva baseline samples were collected from horses before the administration of ACTH, and subsequent blood and saliva samples were taken at time intervals up to 500 minutes after ACTH administration. Fig. 1.17 shows the glucocorticoid response to the ACTH challenge. While cortisol concentrations in saliva are much lower compared to serum, the response pattern is similar. The regression analysis of the two sampling procedures showed an adjusted r^2 value of 0.80 ($P<0.0001$), and Peeters stated "80% of variability in salivary cortisol could be explained by total serum cortisol concentration". This strong correlation means that in the context of this study, salivary cortisol could reliably be used as an alternative to serum cortisol. A study by Cook *et al.* (1996) compared serum and salivary glucocorticoid concentrations in pigs following ACTH administration, and also following a physical stressor. In the ACTH challenge, salivary cortisol was closely correlated to serum cortisol ($P < 0.025$). Similarly, after the physical stressor (application of a snare), salivary cortisol was also closely correlated with serum cortisol ($P < 0.05$) and followed a similar response pattern (Fig. 1.18).

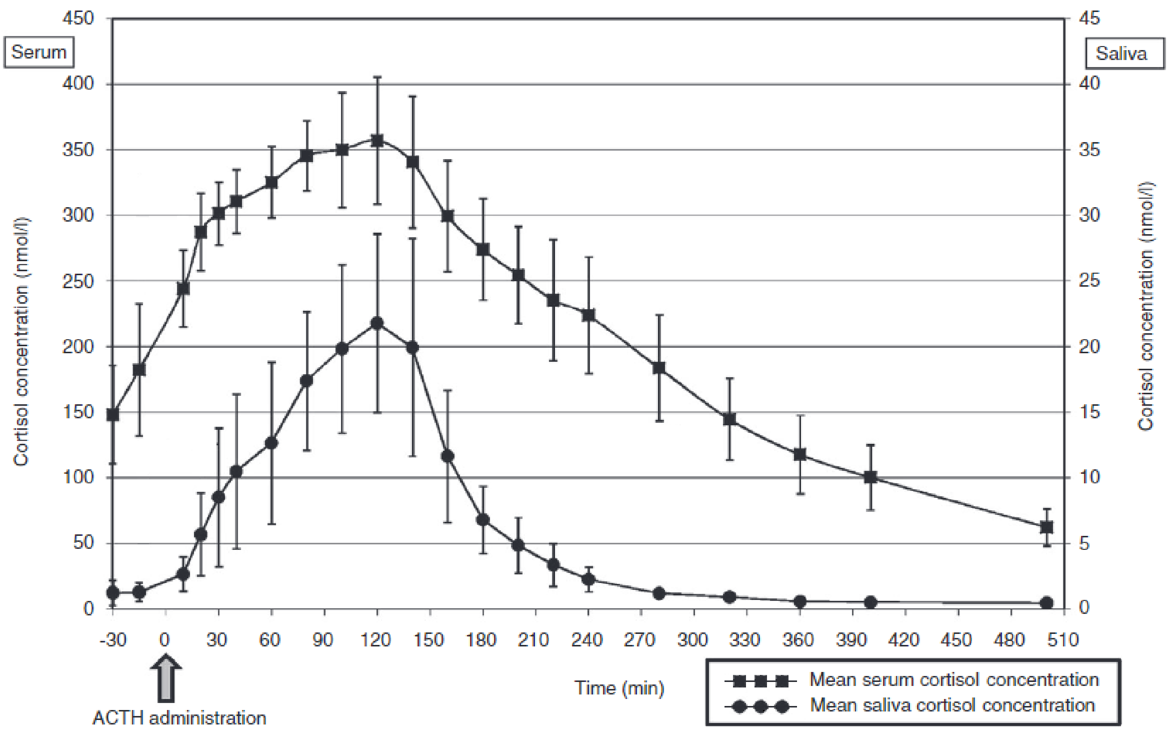

Fig. 1.17. Cortisol response during an ACTH challenge comparing mean serum cortisol (left Y axis) and saliva cortisol (right Y axis) concentrations from five horses. Adapted from Peeters *et al.* (2011).

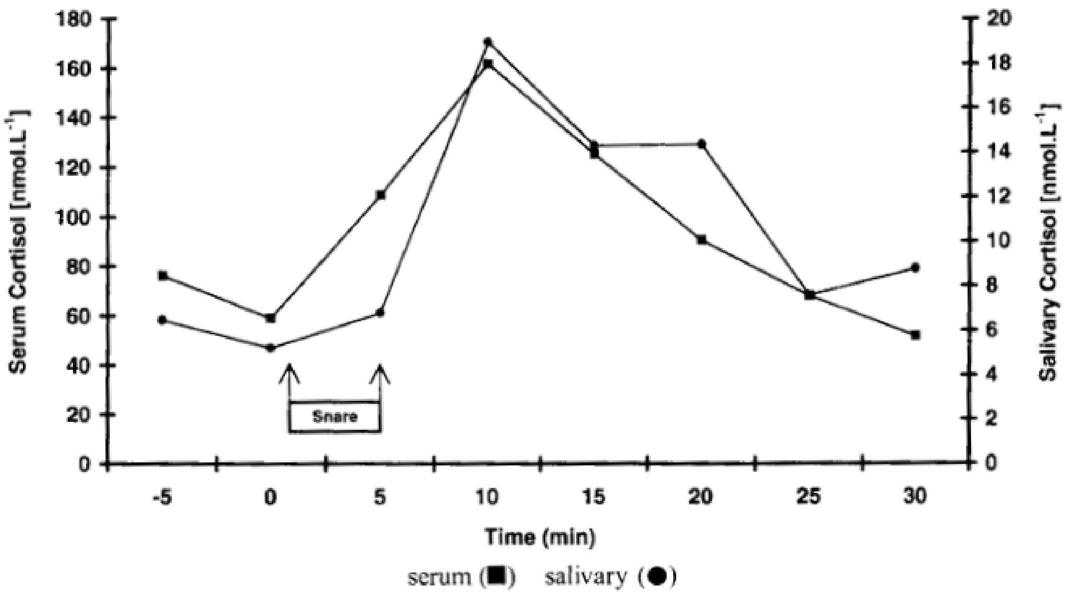

Fig. 1.18. Mean serum (left Y axis) and saliva (right Y axis) cortisol concentrations in response to application of a snare. Snare was applied at 0 minutes and removed at 5 minutes. Adapted from (Cook 1996).

Validations of salivary glucocorticoids have also been carried out without comparison to plasma glucocorticoids. Hammond *et al.* (2018) carried out ACTH challenges on three species of amphibians to validate the use of saliva for measuring corticosterone responses: American bullfrog (*Rana catesbeiana*), green frog (*Rana clamitans*) and northern leopard frog (*Rana pipiens*) (Fig. 1.19). The ACTH challenge produced a significant corticosterone response in all three species which peaked around 30 minutes after ACTH administration. Following the ACTH challenge, Hammond carried out a handling challenge to provide a more biologically relevant salivary corticosterone response to a stressor. Corticosterone concentrations peaked around 30 minutes after initial handling (as in the ACTH challenge), although concentrations were much lower compared to the ACTH challenge, which is expected.

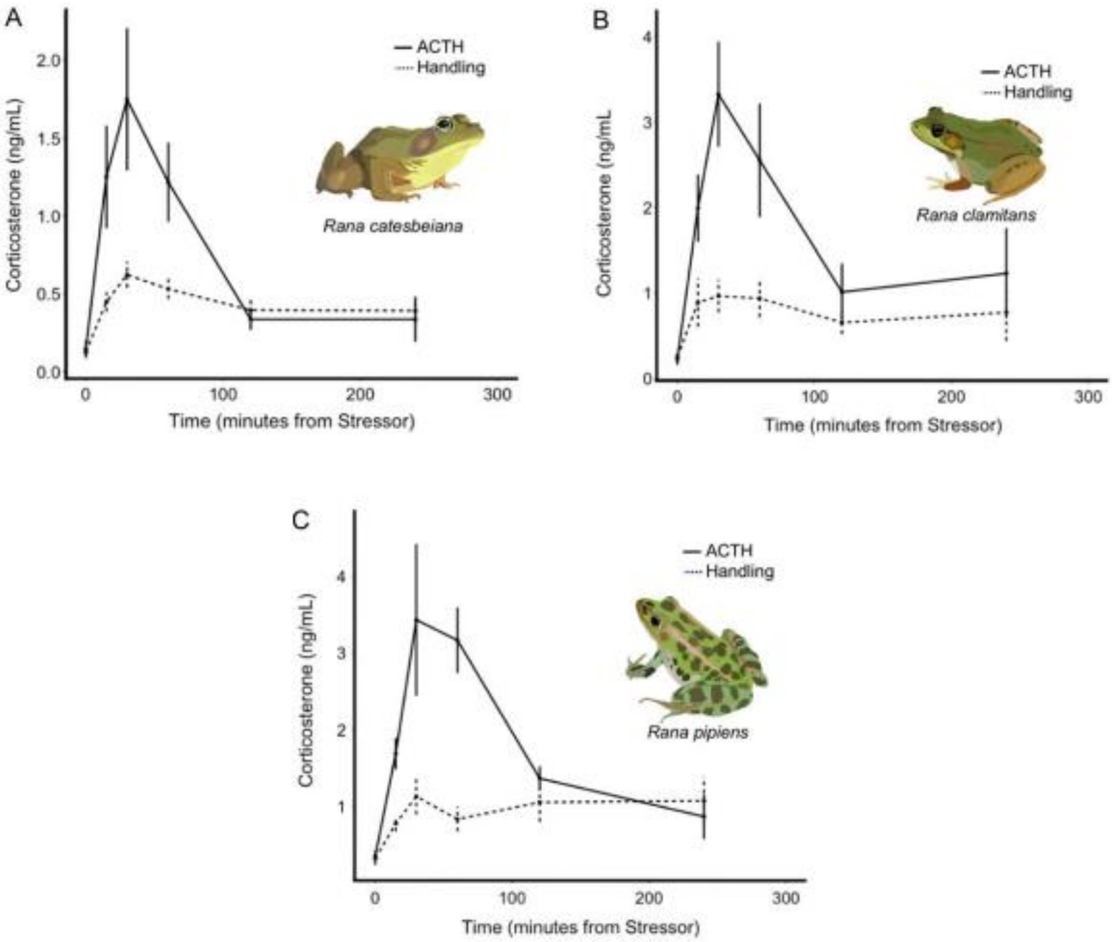

Fig. 1.19. Salivary corticosterone response to ACTH administration (solid line) and handling (dotted line) in American bullfrog (A), green frog (B) and northern leopard frog (C) (Hammond *et al.*, 2018).

In the examples shown above, concentrations of salivary glucocorticoids are much lower compared to the concentrations of plasma glucocorticoids, typical for most steroid hormones present in saliva (Kivlighan *et al.*, 2004). Glucocorticoids in plasma are present in two forms: bound and free. Within blood approximately 90% of circulating glucocorticoids are bound to corticosteroid-binding globulin (CBG). The remaining glucocorticoids not bound are "free" circulating and are considered to be the "biologically active" portion still available to be bound to receptors and move into other tissues. It is this free portion that passively diffuses into the acinar cells of the salivary glands and through to the saliva (Sheriff *et al.*, 2011; Vining *et al.*, 1986). The difference is evident in the ACTH challenge carried out by Cook *et al.* (1996) (mentioned above), where the mean salivary cortisol concentration was 8.97% of plasma concentration. The percentage of bound glucocorticoids can vary from species to species however, supporting the need for salivary glucocorticoids to be validated for each species (Sheriff *et al.*, 2011). Because only the free portion of glucocorticoids are free to move into saliva, salivary glucocorticoids are much more highly correlated to the free portion of glucocorticoids in plasma. A review by Lac (2001) determined correlations with whole plasma are usually high across studies ($r^2 = 0.6$ to $r^2 = 0.9$) but the correlations are even higher when comparing salivary glucocorticoids to only the free portion of glucocorticoids in plasma (up to $r^2 = 0.97$). This suggests that salivary glucocorticoids are a more suitable representation of biologically active glucocorticoids compared to whole plasma glucocorticoid concentrations.

Due to the time it takes for glucocorticoids to diffuse through the salivary gland tissues and into saliva, there is usually a time delay in the salivary glucocorticoid response when compared to the plasma glucocorticoid response. This delay can be anywhere from two to thirty minutes behind the plasma response and provides a greater window of time to collect saliva samples to measure baseline concentrations before they begin to increase (Peeters *et al.*, 2011). This is especially useful for wild animals where capture and restraint can elevate plasma glucocorticoid concentrations quickly and can give inaccurate measurements of baseline concentrations. A study by Hernandez *et al.* (2014) showed salivary cortisol concentrations peaked ten minutes after plasma cortisol after social separation in domestic cows (Fig. 1.20).

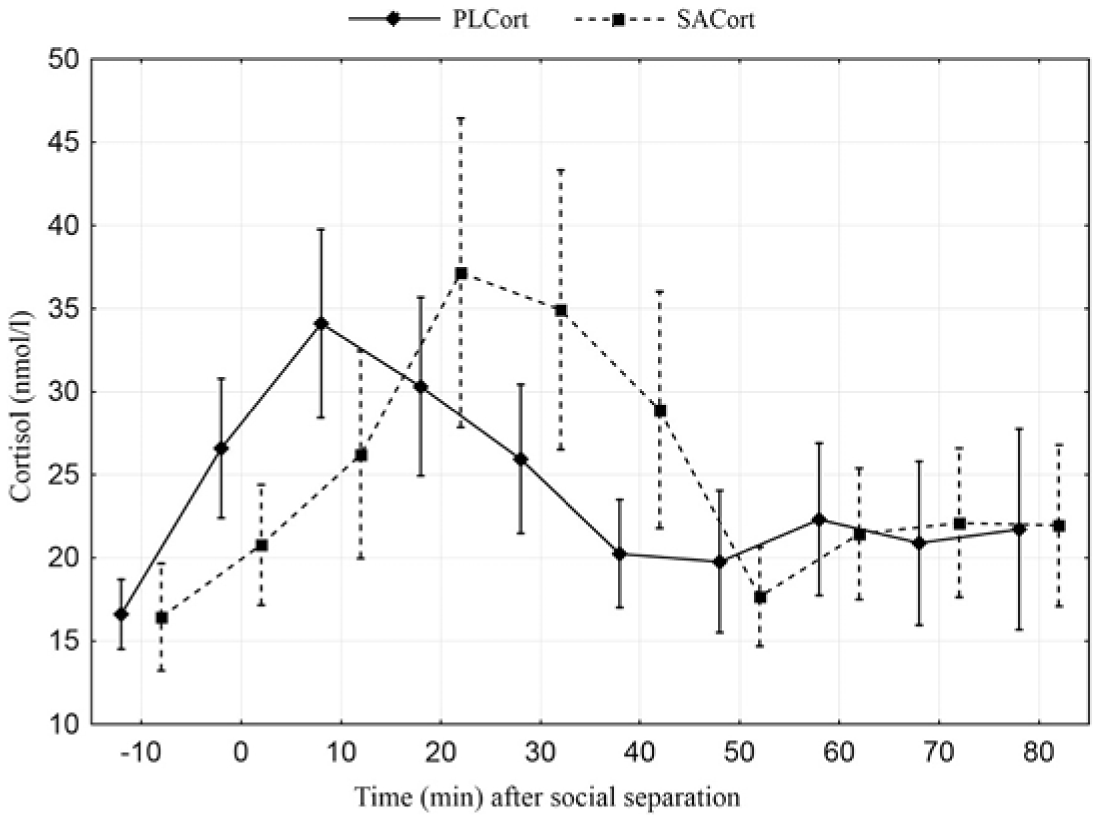

Fig. 1.20. Mean plasma (PLCort) and salivary (SACort) cortisol concentrations measured from cows over a 90-minute period. Salivary cortisol concentrations have been multiplied by a factor of 10 for clarity and comparison to plasma cortisol concentrations. Graph points have been slightly time-shifted to avoid overlapping error bars.

Although using saliva to measure glucocorticoids may be a preferred method for many species, there are some disadvantages. When considering smaller species with smaller volumes of saliva, collecting enough for assay can be difficult. Similarly, species with more viscous saliva may have only a small amount absorb into a swab or be suctioned, which may not be enough for assay. Lac's (2001) review suggests 50 µL of saliva as a minimum for assay, although newer specialised assay protocols can require as little as 25 µL as a standard test volume for successful assay (Granger *et al.*, 2007; Harmon *et al.*, 2007). However, even with sufficient volumes of saliva for assay, concentrations of glucocorticoids can be too low for reliable measurement. Rehbinder and Hau (2006) assayed cortisol and cortisol immunoreactive metabolites (C-CIM) taken from reindeer (*Rainger tarandus*). Samples were collected from serum, faeces, urine and saliva. All salivary C-CIM concentrations were below 10 nmol/L (exact concentrations could not be determined) and were deemed unreliable. In contrast, concentrations from all but one urine sample ranged between 48 and 178 nmol/L.

Conversely, other factors can cause glucocorticoid concentrations in saliva to be over-quantified. Where saliva flow or volume of a study participant is low, oral stimulants may be used to increase saliva production. This is often used in human babies and animal species where passive drool is not possible. Oral stimulants can also be used to entice animals to interact with a sampling apparatus (e.g. Lutz *et al.* (2000). Schwartz *et al.* (1998) found that in some cases, the use of some stimulants (e.g. flavoured powdered drink crystals) can affect cortisol concentrations in humans. The drink crystals contain citric acid which lower the pH of saliva, and this low saliva pH can be attributed to an increase in salivary cortisol concentrations. Dreschel and Granger (2009) found *in vitro,* increasing concentrations of citric acid added to dog (*Canis familiaris*) saliva increased cortisol concentrations (Fig. 1.21). This effect was not observed *in vivo* however, possibly due to buffering effects of fresh dog saliva. Other studies found no statistically significant effects of citric acid on glucocorticoid concentrations (Brorsson *et al.*, 2014; Kidd *et al.*, 2009). Kidd, however, found that the use of cotton and polyester swabs increased salivary cortisol concentrations when compared to plain saliva obtained via passive drool. Kidd stated, “the discrepancy was presumed to be due to cross-reactivity of swab constituents with the assay antibody”. This adds to the importance of validating saliva collection protocols for each method and species being used.

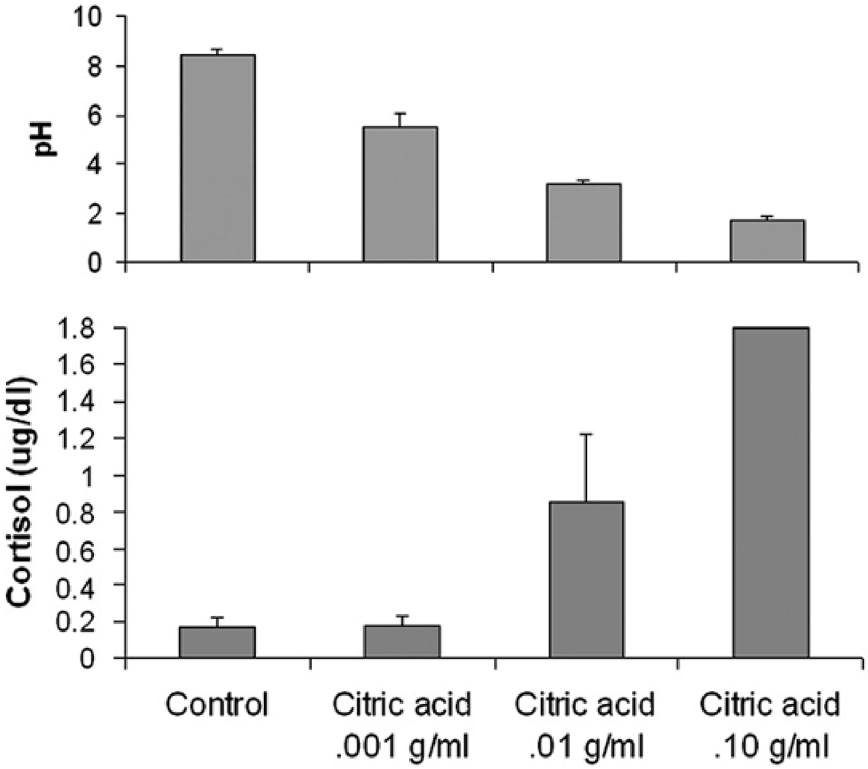

Fig. 1.21. Cortisol concentrations and pH measured from dog saliva. The addition of citric acid decreases saliva pH and increases salivary cortisol concentrations (Dreschel and Granger, 2009).

Another factor which may unintentionally increase measurements of salivary glucocorticoids is blood leakage into the oral cavity. Some aspects of saliva collection may cause microinjuries in the mouth, and due to the higher concentrations of glucocorticoids in blood, the contamination of saliva with leaked blood can elevate salivary glucocorticoid concentrations and affect validity of the study. Kivlighan *et al.* (2004) noted many studies excluded samples that were visibly contaminated with blood and subsequently conducted a study to test the effects of microinjury to the oral cavity on cortisol concentrations in humans. Study participants brushed their teeth prior to providing saliva samples for assay, which caused minor to moderate levels of microinjury. While haemoglobin was measured in the saliva samples (measure by transferrin assay), the level of microinjury was not enough to increase cortisol concentrations. Kivlighan concluded that at the lowest levels of blood leakage, effects on glucocorticoid levels is likely to be small. However, they suggested sampling not be carried out within 45 minutes of microinjury, and that samples be inspected immediately after collection and excluded if visibly contaminated.

1.6 Outline of thesis

The study outlined in the following chapters was planned to determine whether salivary corticosterone assays are a valid alternative to blood corticosterone assays in eastern water dragons (*Intellagama lesueurii lesueurii*) and African leopard tortoises (*Stigmochelys pardalis*). The study was divided into three main parts: Part 1 compared saliva collection methods to determine highest saliva yields for the two study species to be used in subsequent experiments, and additionally in two species of bearded dragon (*Pogona vitticeps* and *Pogona barbata*). Saliva samples were also used to determine whether corticosterone is measurable from saliva in the study species used, before the subsequent experiments were carried out. Part 2 compared the salivary and plasma corticosterone responses to capture and handling in both species by applying a stress protocol prior to taking samples of saliva or plasma at multiple time intervals. Part 3 compared the plasma corticosterone response to either an initial blood or saliva sample in both species. The results of this study and its implications will help to improve the current hormone sampling protocol for reptiles and will provide a foundation for future research into non-invasive stress monitoring techniques.

2. Methods

2.1 Study site

All animals used in this study were permanently housed at the Ti Point Reptile Park, Leigh, New Zealand (36º 30' S, 174º 79' E). Ti Point Reptile Park is New Zealand's only reptile-specific wildlife park and houses several reptile species from New Zealand, Australia, Asia, Africa and America. Enclosures were scattered amongst native New Zealand bush and each enclosure caters to individual species' needs. Study species were housed in six different enclosures around the park. Enclosures did not have official names so were named based on identifying features: tortoise, hexagon, top, platform, indoor, and pool. Four of the six enclosures housed more than one species over the course of the project.

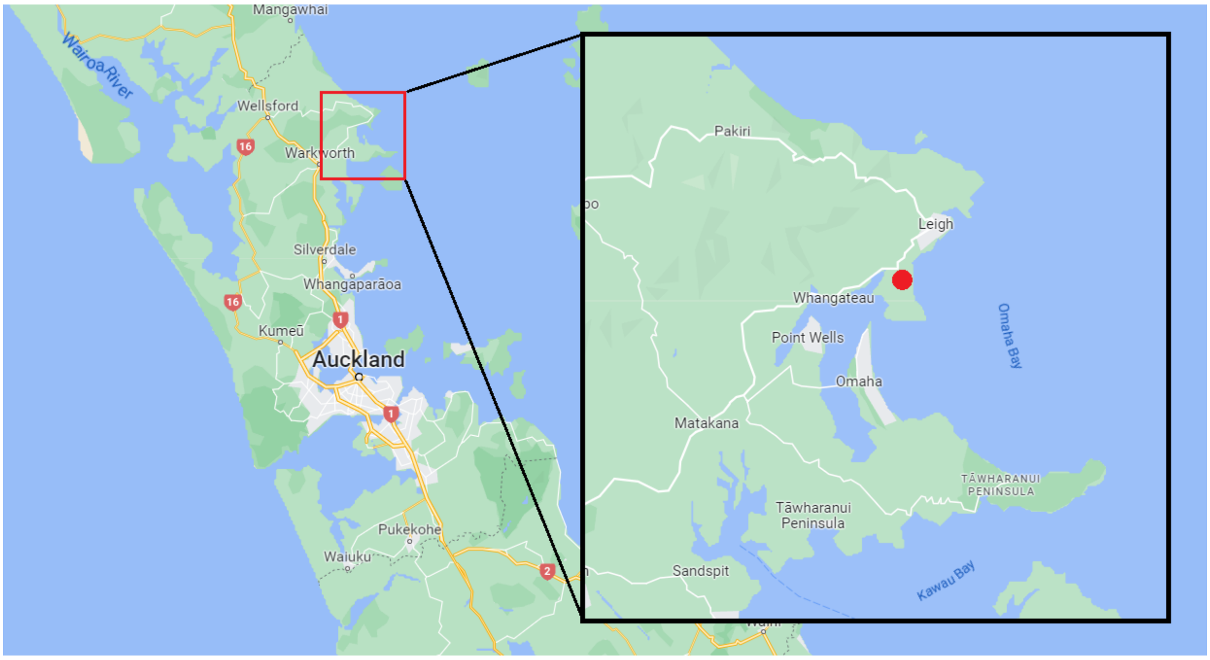

Fig. 2.1. Map showing the location of Ti Point Reptile Park (marked with ●) near Leigh, New Zealand (Google Maps, 2023).

2.2 Study Species

Four species of reptiles were used in this study: Eastern water dragon (*Intellagama lesueurii lesueurii*), leopard tortoise (*Stigmochelys pardalis*), central bearded dragon (*Pogona vitticeps*) and eastern bearded dragon (*Pogona barbata*). Water dragons and leopard tortoises were used in all three parts of the study, while the central and eastern bearded dragons were only used in Part 1 (see section 2.3). These species were used as the park housed appropriate numbers of these reptiles readily available for sampling.

2.2.4 Enclosures housing study animals

Study animals were housed across six different enclosures within the park; four of the enclosures housed more than one species. The enclosures were given short names based on their most prominent feature for the purpose of the study:

Tortoise

Housed only leopard tortoises. A grassy enclosure with varieties of succulents and cacti, large rock arrangements (providing hiding places), and a small temperature-controlled hut with heat lamps and ventilation. The hut featured sliding panels to allow tortoises to move in and out freely but could be closed to keep tortoises in at night and during bad weather. The rear side of the hut included a viewing window from the walkway behind to give visitors the opportunity to view tortoises if they were not outside.

Hexagon

Housed inland bearded dragons, blue-tongue skinks (*Tiliqua scincoides scincoides*) and red-eared terrapins (*Trachyemys scripta elegans*). The enclosure contained a combination of grass and dirt with a small rocky pool in the middle. Large logs, branches and plants were also scattered around the enclosure to provide basking and hiding spots. A small temperature-controlled hideaway with heat lamps housed the bearded dragons at night but was left open during the day to allow free movement. During the study, some of the inland bearded dragons and blue-tongue skinks were moved into the 'top end' enclosure by park staff.

Top end

Housed most of the water dragons kept at the park, plus coastal bearded dragons, and inland bearded dragons (after being moved from 'hexagon'). The enclosure also included other species such as blue-tongue skink, chaco tortoise (*Chelonoidis chilensis*), red-eared terrapin, and Reeve's turtle (*Mauremys reevesii*). This was the largest enclosure in the park and featured a grassy field with a large tree in the centre. The enclosure included multiple rocky outcrops, log arrangements and concrete slab arrangements to provide hiding and basking spots for the lizard species. The concrete tortoise house near the entrance of the enclosure had a small pool attached. An inaccessible water dragon burrow beneath the tortoise house meant the total number of water dragons within the enclosure was unknown (park staff estimated 25 individuals).

Platform

Housed water dragons, Cunningham's skinks (*Egernia cunninghami*), and a Murray River turtle (*Emydura macquarii*). This enclosure was on a slope in amongst the native bush and featured a platform extending into the middle of the enclosure from which park visitors could observe animals. The lowest part of the enclosure included a pool, large bushes, feeding platforms, and multiple hiding or basking spots constructed from rocks, concrete slabs and old brick pipes.

Indoor

Housed water dragons and a Reeve's turtle. This was the only indoor enclosure used in the experiment and had a large glass viewing window at the front for visitors. The enclosure consisted mostly of dirt substrate, but also contained a large pool, several plants and logs, and a large artificial boulder that took up most of the rear half of the enclosure. A skylight provided natural UV exposure to the reptiles, as well as and heat and UV lamps at the front of the enclosure.

Pool

Housed water dragons only. This enclosure was attached to the outside of the indoor enclosure building. It had a large, deep pool with aquatic plants on one side that extended to the enclosure wall. Around the pool were additional plants and logs for climbing and basking. The rest of the enclosure was grass, except for a covered rocky area with heat and UV lamps at the top end.

2.2.5 Identification and measurement

Prior to the commencement of saliva collection, study animals in each of the six enclosures were captured, measured and assigned an identifier consisting of a letter (water dragon [W], bearded dragon [B], leopard tortoise [T]) and a number (starting at 1). Animals were handled as per recommendations from park staff and Auckland Zoo veterinarian Dr Jakob-Hoff who administered blood collection training. Similar to Cree *et al.* (2000), a silver non-toxic permanent marker was used to write this identifier on each individual. Water dragons were labelled on the base of the tail on each side (Fig. 2.2A), while Eastern and Inland bearded dragons were labelled on the dorsal side of the tail between the hind legs. Tortoises were labelled on the rear of the carapace (Fig. 2.2B). This allowed for quick identification precapture, which was a non-harmful, temporary identification which would wear off over time or during shedding. These markings were reapplied when necessary over the course of the study to maintain identification. Other identifying characteristics (e.g. missing toes or claws, unique scale or shell markings) were noted and use to re-identify individuals if their label had shed or worn off.

Once labelled, individuals were weighed inside a black drawstring cotton bag, with 2 kg spring weights accurate to 10 g. Tortoises larger than 2000 g were weighed using an electronic scale accurate to 10 g. Lizards had snout-vent length (mm) and vent-tail length (mm) measurements taken using a clear 400 mm ruler pressed against the ventral body surface. Tortoises were measured using the straight midline carapace length (mm) using callipers. Animals were then left to recover for 2-3 weeks before commencing the next stage of the study.

Fig. 2.2. Location of identification markings on (A) water dragon and (B) leopard tortoise.

2.3 Development of a saliva collection protocol (part 1)

The aim of part 1 of the study was to test and compare yields of different saliva collection apparatus, as results of parts 2 and 3 relied on collecting enough saliva for successful hormone assay; Lac (2001) suggested a minimum of 50 μL. The method with the highest yield for each species would then be used as the primary saliva collection method for that species for the remainder of the study.

2.3.1 Saliva collection apparatus

After considering the methods used in the literature (see section 1.5.1) and what was available, three different saliva collection methods were selected to use across the three study species:

1. **SalivaBio infant swab** (Salimetrics®) – a thin, long polyester swab designed for collecting saliva from human infants. The swabs were very durable and resistant to tearing, which was crucial for the lizard species due to their ability to chew through some soft materials. From here on referred to as 'SI swab'.

2. **FLOQSwabs®** (Copan) – a long, thin, plastic handle with a small, flocked, nylon tip. Due to the flocking, the swab tips have a larger surface area than standard cotton swabs of similar sizes and are suitable for absorbing more viscous fluids. Often the thin plastic shaft of the swab was wrapped in a small amount of paraffin wax film to prevent bending or breakage. From here on referred to as a 'FLOQ swab'.

3. **3 mL syringe** (Sigma-Aldrich®) – standard 2-piece plastic syringe, with a long, small tip suitable for extracting saliva from small oral cavities.

Apparatus not used

Other methods of collecting saliva were also initially considered; these included plain cotton buds, sorbettes (Stratech), wet-strengthened filter paper (Whatman™), and plastic bloodgas capillary tubes (Webers GmbH). These sample collection items were not strong enough when being bitten by both dragon species and tortoises, which either presented a safety hazard for the animals or resulted in the apparatus not being able to absorb saliva. These methods were therefore not considered further.

2.3.2 Saliva collection protocol

Only two methods were used for each species (Table 2.1), which varied between lizards and tortoises due to anatomical differences. The 3 mL syringes did not suit tortoise anatomy; a longer apparatus was needed to reach the tortoise's mouth while retreated into its shell. The long, thin, plastic handle of the FLOQ swabs allowed for sampling even while retreated and could not be bitten through. The small, flocked swab head also suited the size of the smaller tortoise mouth. FLOQ swabs were not used with water dragons or bearded dragons as the upper part of the plastic handle could have been bitten off easily and as such presented a safety hazard.

Table 2.1. Method, sample size and sample collected duration for each species used for comparing saliva collection methods.

Species	Method	Sample size	Collection duration
Water dragon	SI swab	10	5 min
	3 mL syringe	10	5 min
Bearded dragon	SI swab	10	5 min
	3 mL syringe	10	5 min
Leopard tortoise	SI swab	10	5 min
	FLOQ swab	10	5 min

Where there were more than 10 individuals present for a group (e.g., water dragon, leopard tortoise), animals were selected at random to be sampled and were not identified until capture. All water dragons sampled for this part of the study were housed in the ‘Top’ enclosure. Bearded dragons were sampled first from the ‘House’ enclosure containing the inland bearded dragons (n = 8), and the remaining two samples were taken from randomly selected coastal bearded dragons in the adjacent ‘Top’ enclosure. Due to the uncertainty of which animals would be available for sampling, different animals were used for different sampling methods each species (some animals were sampled for both methods, however).

Each saliva collection method followed the same protocol. Once selected, individuals were approached carefully and either caught by hand or with a small hand net and taken to the sampling area. The total ‘catch time’ from first approach to the start of saliva sampling was recorded, as well as ambient temperature. Animals were restrained by one person (as recommended by Auckland Zoo veterinarians), while the other person began the saliva collection (see Figs. 2.3, 2.4 and 2.5). Saliva sampling began when the apparatus was first inserted into the mouth and was timed for a total of five minutes. At the end of the five minutes the sampling finished, and the animal was released back into the enclosure where it was initially captured. The saliva sampling duration of 5 minutes was chosen so that maximum sampling times would be consistent across sampling protocols in Part 3 (see section 2.6).

When sampling with both SI swabs and FLOQ swabs, most water dragons and bearded dragons opened their mouths voluntarily or did so after a light touch on the snout (opening the mouth as an intimidation/defence mechanism has previously been displayed in lizards (Miranda *et al.*, 2023)). When animals did not open their mouths (e.g., most leopard tortoises), a small spatula was used to gently pry the mouth open. Wooden spatulas were used for dragons, whilst a smaller metal spatula was required for tortoises. Once the swab was placed in the mouth, many individuals either chewed or tried to spit out the swab. The swab often needed to be re-inserted into the mouth over the five-minute sampling duration.

For the 3 mL syringe, a small wooden spatula was needed to keep the mouth open and was held in place towards the back of the mouth while saliva was being suctioned. Due to the small amount of saliva present in the mouth cavity, syringes were often filled mostly with air. Multiple syringes were needed for each individual to be able to sample for the full five minutes; per sample, an average of three syringes were needed for water dragons, and four syringes for bearded dragons.

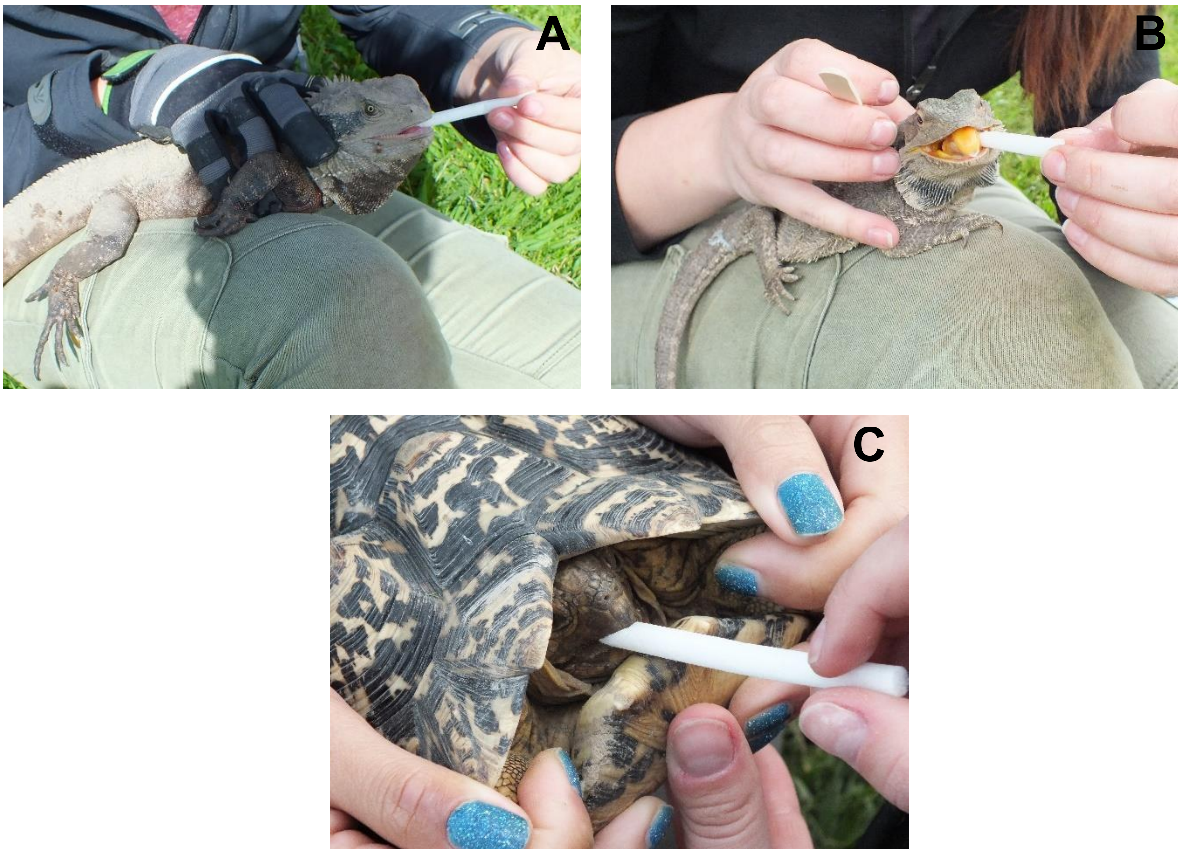

Fig. 2.3. Saliva collection from a water dragon (A), bearded dragon (B) and leopard tortoise (C) using the SalivaBio Infant swab

Fig. 2.4. Saliva collection from a water dragon (A) and bearded dragon (B) using a 3 mL syringe.

Fig. 2.5. Saliva collection from a leopard tortoise using a FLOQ swab reinforced with paraffin wax film.

2.3.3 Saliva measurement

Saliva samples were centrifuged onsite using a fixed-speed microcentrifuge at 6000 rpm for 10 minutes. Following centrifuging, swabs were removed from tubes and the samples were placed in a small, chilled container for transport back to the laboratory. Samples were refrigerated overnight at the laboratory and were measured within 24 hours of collection.

Saliva volume was measured using autopipettes as saliva samples were too viscous to be measured with a microlitre syringe. To provide a starting point for measurement with the autopipettes, saliva samples were weighed in the tubes on a fine precision scale. Saliva sample weights were calculated by subtracting the average weight of an Eppendorf tube from the total weight of the saliva in the tube. With no literature on the viscosity of reptile saliva, the assumption was made that reptile saliva shares a 1 mL = 1 g ratio with water, as did for human saliva in Sreebny and Vissink (2010). Saliva weights were converted to microlitres and provided a volume estimate for measurement with the autopipettes. Different volume autopipettes were selected for measurement based on the estimated volumes (2-20 μL and 20-200 μL). Using the estimated volume, the autopipette was adjusted in 1 μL increments until the correct volume was measured. As the autopipettes measured to a minimum of 2 μL, any saliva volumes less than this were recorded as 0 μL (a null collection).

2.4 Corticosterone measurement

Saliva samples were frozen and sent in a chilled container to the Institute of Veterinary and Biomedical Sciences (Massey University, Palmerston North, New Zealand). Corticosterone concentrations in saliva were measured by radioimmunoassay.

Saliva sample preparation

Each saliva sample was assayed neat or diluted in PBSG (0.1M phosphate-buffered saline with 0.1% gelatin, pH 7.0) by a factor of 2.

Radioimmunoassay of corticosterone

Corticosterone concentrations in saliva were measured by radioimmunoassay. Samples were assayed in duplicate. 10 μL of saliva was incubated with 20 μL of iodinated corticosterone and 20 μL of antiserum (^{125}I- corticosterone and antiserum ImmuChem™ Double Antibody Corticosterone ^{125} I RIA kit, MP Biomedicals, USA; 4 000 cpm) for 2 hours at room temperature (22-25°C). 50 μL of precipitant solution (MP Biomedicals, USA), was added and each sample vortexed thoroughly, incubated for 15 minutes at room temperature to separate bound and free corticosterone, then centrifuged for 15 minutes at 2 000 g at 4°C. 20 μL starch (25 g/L starch (Sigma) plus 0.05 g/L neutral red (BDH) in PBSG) was added to increase adhesion of the pellet to the tube. The tubes were centrifuged for 15 minutes at 2 000 g at 4°C and the supernatant aspirated off. The pellets were counted on a PerkinElmer Wallac Wizard 1470 gamma counter for 5 minutes each.

The cross-reactivity of the corticosterone antibody with other steroids was tested by MP Biomedicals. Cross-reactions are as follows: deoxycorticosterone (0.34%), testosterone (0.10%), cortisol (0.05%), aldosterone (0.03%), progesterone (0.02%), androstenedione (0.01%), 5α-dihydrotestosterone (0.01%) and cholesterol, 11-deoxycortisol, dehydroepiandrosterone, dehydroepiandrosterone-sulphate, 20α-dihydroprogesterone, oestrone, oestradiol-17α, oestradiol-17β, oestriol, pregnenolone, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone (<0.01%).

Parallelism and hormone additions

Serial dilutions of water dragon, bearded dragon and leopard tortoise saliva in PBSG were parallel to the corticosterone standard curve. The quantitative recovery of corticosterone was measured by adding different amounts of standard corticosterone to two aliquots of saliva in PBSG. The recoveries of added corticosterone were 97.6 ± 1.3% and 108.3 ± 4.3% for water dragon saliva, 107.2 ± 2.9% and 102.4 ± 4.5% for bearded dragon saliva and 106.0 ± 2.9%, and 101.2 ± 7.9% for leopard tortoise saliva.

Assay sensitivity

The sensitivity of the corticosterone assay was the minimum hormone level that could be consistently distinguished from zero. It was determined as the hormone concentration at the mean - 2 standard deviations from the zero hormone point on the standard curves. The assay sensitivity was 0.05 ng/mL for salivary corticosterone in all three species.

Intra- and inter-assay variation

Solutions of corticosterone in PBSG at concentrations that gave approximately 80, 50 and 20% binding on the standard curve were used as low, medium and high-quality controls in every assay. The mean concentrations of corticosterone in these solutions were 234.9± 19.8, 574.2 ± 47.2 and 1769.5 ± 127.7 pg/mL respectively. The intra-assay coefficient of variation for each solution was determined by conducting an assay with twenty duplicates of each solution. The intra-assay coefficients of variation for corticosterone were 8.4%, 6.0% and 7.2% for low, medium and high solutions respectively. Inter-assay coefficients of variation were calculated from duplicates of the solutions included at the beginning and end of each assay. The inter-assay coefficients of variation for ten assays were 7.9%, 8.4% and 11.5% for low, medium and high solutions respectively.

2.5 Saliva and plasma corticosterone responses to capture and restraint (part 2)

Part 2 of the study had two aims: 1) To assess whether the course and magnitude of salivary corticosterone corresponds to plasma corticosterone in two species of reptile (eastern water dragons and leopard tortoises) in response to a stressor (capture, handling and restraint); and 2) to determine the variation between individual animals for plasma and salivary corticosterone responses to a stressor. The stress protocol used in this study is based on the methodology described in Romero and Wikelski (2001) and Cash *et al.* (1997).

For both species, individuals were assigned to one of two sample groups:

Group 1: Baseline blood sample (0 min), initial stressor, blood samples at 30 min and 60 min
Additional stressors at 10, 20, 40 and 50 min.

Group 2: Baseline saliva sample (0 min), initial stressor, saliva samples at 30 min and 60 min
Additional stressors at 10, 20, 40 and 50 min.

For both Groups 1 and 2, capture duration was timed as first approach to beginning of sampling. While dragons tended to run away, tortoises often retreated into their shells, resulting in their mouths/limbs being inaccessible for sampling. Capture time for tortoises was therefore often spent trying to coax them out of their shells. Where a capture attempt exceeded 5 minutes, the individual was left for the remainder of the sampling period and a new animal was approached. This was done to minimise the possibility of corticosterone concentrations increasing before a baseline sample could be collected. Capture methods followed those outlined in Section 2.3.2. On capture, animals were taken to the sampling station within the enclosure and sampled according to their assigned group (Table 2.2). Baseline (0 min) was started at the beginning of sample collection and sample collection did not exceed five minutes.

Table 2.2. Sampling groups and corresponding sample sizes for each species.

Species	Group	Sample size
Water dragon	1 (blood)	10
	2 (saliva)	10
Leopard tortoise	1 (blood)	8
	2 (saliva)	10

Following blood or saliva collection, the animal was placed into a small black cotton drawstring bag and subjected to a stressor (flipped upside down five times inside the bag). A hole was left at the top of the bag to allow airflow. Up to three individuals were being sampled at any one time and were identified by different coloured drawstrings on the bags (Fig. 2.6). The animal was then placed on the ground (or in a container if necessary) and subjected to subsequent stressors (flipping inside the bag) at 10 and 20 minutes. These additional stressors were carried out to provide a consistent handling stressor to reduce the influence of the black bag the animals were placed in. A second sample was collected at 30 minutes, the animal placed back in the bag back on the ground. The animal was subjected to subsequent stressors (flipping inside the bag) at 40 and 50 minutes. A third and final sample of blood or saliva was collected at 60 minutes, and the animal released back to where they were captured from.

[N.B. if samples at any time were unable to be collected, the animal was placed back in the bag, had the stressors applied, and was left to the next allotted collection time where collection was attempted again. Therefore, some animals may have results for only two times e.g., 0 min + 30 min, 0 min + 60 min, or 30 min + 60min].

Fig. 2.6. Restraint of water dragons in breathable cotton bags between sampling times. Different drawstring colours enabled easy sampling identification.

2.5.1 Blood collection

Identification was checked to determine total blood volume that could be taken across the three samples. The total amount of blood (in millilitres) to be collected in small reptiles is suggested to be between 0.5% and 0.8% of the reptile's total body weight in grams (Sykes IV and Klaphake, 2008). For example, a 100 g water dragon may have 0.5 – 0.8 mL (500 – 800 µL) taken during a sampling period. Blood samples were limited to less than the suggested 0.5%; approximately 100-150 µL per sample was collected (depending on reptile weight) as these volumes are sufficient for radioimmunoassay. The smallest reptile used for blood collection in Part 2 was 190 g (suggested blood volume collection of 950 µL) but only 350 µL was collected in total from this animal over the course of the sampling period.

Water dragon

Water dragons were restrained and held vertically with the ventral surface facing the blood collector. Blood was collected using a 25 g x 5/8" needle and 1 mL syringe (Terumo®). The venipuncture site was cleaned with sterile 70% isopropyl alcohol prep pads prior to blood collection. Blood was taken from the ventral coccygeal vein approximately two thumb-widths from the base of the tail at a 90^{o} angle to the skin's surface. After withdrawal of the needle, gentle pressure was applied for 30 seconds over the puncture site with a sterile gauze swab.

Leopard tortoise

Leopard tortoises were restrained with forelimbs held out of the way to enable access to the head or side of the forelimb. Blood was collected using a 25 g x 1" needle and 1 mL syringe (Terumo®). Multiple venipuncture sites were available for tortoises: right jugular vein, dorsal coccygeal sinus and right brachial vein. The ideal site for each animal was determined after capture, however due to the tortoises often retreating into their shells, the dorsal coccygeal sinus was the most commonly used (Fig. 2.7). The venipuncture site was cleaned with sterile 70% isopropyl alcohol prep pads and blood was taken from the selected vein. After withdrawal of the needle, gentle pressure was applied for 30 seconds over the puncture site with a sterile gauze swab.

After collection, blood was syringed into a labelled, heparinised 1.5 mL Eppendorf tube and placed inside a cooler. After all samples were collected, blood was centrifuged onsite with a fixed-speed microcentrifuge at 6000 rpm for 10 minutes and then placed back into the cooler for transport.

Fig. 2.7. Blood collection from the dorsal coccygeal vein of a leopard tortoise

2.5.2 Saliva collection

Saliva collection used the most effective method for each species determined in Part 1(see section 3.1); SI swabs were used for water dragons (Fig. 2.3A) and FLOQ swabs for leopard tortoises (Fig. 2.5). While FLOQ swabs did not yield significantly more saliva than the SI swabs, a 'custom' FLOQ swab was used for Part 2 by combining two FLOQ swabs together at the top of the shaft using paraffin wax film. This created a 'double' swab head and enabled a greater yield of saliva from tortoises compared to using a single swab.

Animals were restrained and saliva collected using the same methods outlined in Section 2.4. Following sampling, swabs were placed into labelled 1.5 mL Eppendorf tubes. Both SI and FLOQ swabs needed to be cut down to fit inside the tubes; SI swabs were cut to approximately 3cm, making sure all the wetted length was included. The swabs were suspended at the top of the tube, so saliva was not reabsorbed into the swab after centrifugation. FLOQ swab heads were cut off leaving approximately 1 cm of the shaft and placed into the Eppendorf tubes with the shaft at the bottom so saliva could not be reabsorbed. Samples were then placed into a cooler until they could be centrifuged. Saliva swabs were centrifuged frequently throughout sampling to avoid the saliva drying out in the swab. Samples were centrifuged onsite with a fixed-speed microcentrifuge at 6000 rpm for 10 minutes. The swabs were then removed to prevent reabsorption and the tubes placed back into the cooler for transport to the laboratory.

2.5.3 Sample measurement

At the laboratory, blood was centrifuged again at 5000 rpm for 15 minutes. The plasma was syringed off the erythrocyte layer and measured with a 250 µL fixed-needle glass syringe (Hamilton®). Plasma was then placed into labelled 1 mL cryotubes and frozen at -80ºC until radioimmunoassay. Saliva samples were measured within 24 hours at the laboratory using the same methods as described in Section 2.4.2, placed into labelled cryotubes and placed in the freezer at -80ºC until radioimmunoassay.

2.6 Plasma corticosterone response to saliva collection compared with blood collection (part 3)

Part 3 of the study aimed to compare the plasma corticosterone response to a saliva collection procedure compared to a blood collection protocol. Full corticosterone responses to each method type for individual animals was not possible for this part, as blood samples at latter time periods may have interfered with the corticosterone response to the 'original' stressor (i.e., blood samples taken for corticosterone measurement after a saliva sampling protocol could potentially affect the overall corticosterone response). Therefore, only one blood sample was taken from each individual following sampling type.

For both species, animals were assigned to one of four sample groups:

Group 1: Blood collection procedure (0 min), blood collection for assay at 15 min

Group 2: Blood collection procedure (0 min), blood collection for assay at 30 min

Group 3: Saliva collection procedure (0 min), blood collection for assay at 15 min

Group 4: Saliva collection procedure (0 min), blood collection for assay at 30 min

By grouping individuals this way, a mean corticosterone 'response' over 30 mins could be obtained.

For each group:

Each step was timed (first approach to capture time, capture till sample collection completed, 15- or 30-min latency to final blood collection). Individual animals were captured at random by hand and restrained. If capture attempt exceeded five minutes the animal was left and a new individual approached. On capture, individuals were identified and sampled immediately according to their assigned group (Table 2.3). Blood and saliva sampling were carried out using the methodology outlined in Sections 2.5.1 and 2.5.2 respectively. Once the initial sample had been collected, the individual was placed into a black cloth drawstring bag, which was placed on the ground (or in container if necessary). Depending on the individual's assigned group, animal was left in the bag for either 15 min or 30 min. No other additional stressors (e.g. manipulation) were added. Once either 15 min or 30 min had elapsed, a blood sample was taken and the animal released back into the enclosure.

Table 2.3. Sampling groups and corresponding sample sizes for each species.

Species	Group	Sample size
Water dragon	1 (blood sample + 15 min)	6
	2 (blood sample + 30 min)	6
	3 (saliva sample + 15 min)	6
	4 (saliva sample + 30 min)	6
Leopard tortoise	1 (blood sample + 15 min)	5
	2 (blood sample + 30 min)	5
	3 (saliva sample + 15 min)	6
	4 (saliva sample + 30 min)	6

2.7 Statistical analysis

Statistical analysis and graphing were performed using GraphPad Prism 9.0 (GraphPad Software, Inc). Descriptive statistics were used to calculate means and standard error in plasma and saliva corticosterone concentrations. Total integrated plasma responses were calculated using Area under the curve (AUC). Relationships between environmental & individual variables and total integrated plasma response were determined using linear regression. A one-way repeated measure ANOVA with post-hoc Šidák's testing was carried out on both plasma and saliva responses in water dragons. Because corticosterone could not be measured from all leopard tortoise samples, no ANOVA was carried out on leopard tortoise plasma and saliva corticosterone responses. Two-tailed unpaired T-tests were used to compare means of corticosterone responses between sampling types at 15 and 30 min in part 3. Linear regression was used to test if blood collection time significantly affected baseline plasma corticosterone responses in both water dragons and leopard tortoises.

3. Results

3.1 Development of a saliva collection protocol (part 1)

The SalivaBio infant (SI) swabs yielded significantly more saliva in both water dragons and bearded dragons compared to extracting saliva with the 3 mL syringes. For leopard tortoises, both the SI swab and FLOQ swab yielded similar volumes of saliva (Figs 3.1 & 3.2; see Table 3.1 for statistics).

Table 3.1. Results of Mann-Whitney test comparing yields of each saliva collection method for each species

Species	Method	min (μL)	max (μL)	mean ±SE (μL)	*p*-value
Water dragon	SI swab	17	146	58.5 ± 11.9	0.0035*
	3 mL syringe	7.5	40	23.2 ± 3.6	
Bearded dragon	SI swab	29	107	62.3 ± 6.9	<0.0001*
	3 mL syringe	0	8	2.9 ± 0.9	
Leopard tortoise	SI swab	5	49	17.8 ± 4.2	0.6706
	FLOQ swab	2	56	18.5 ± 5.9	

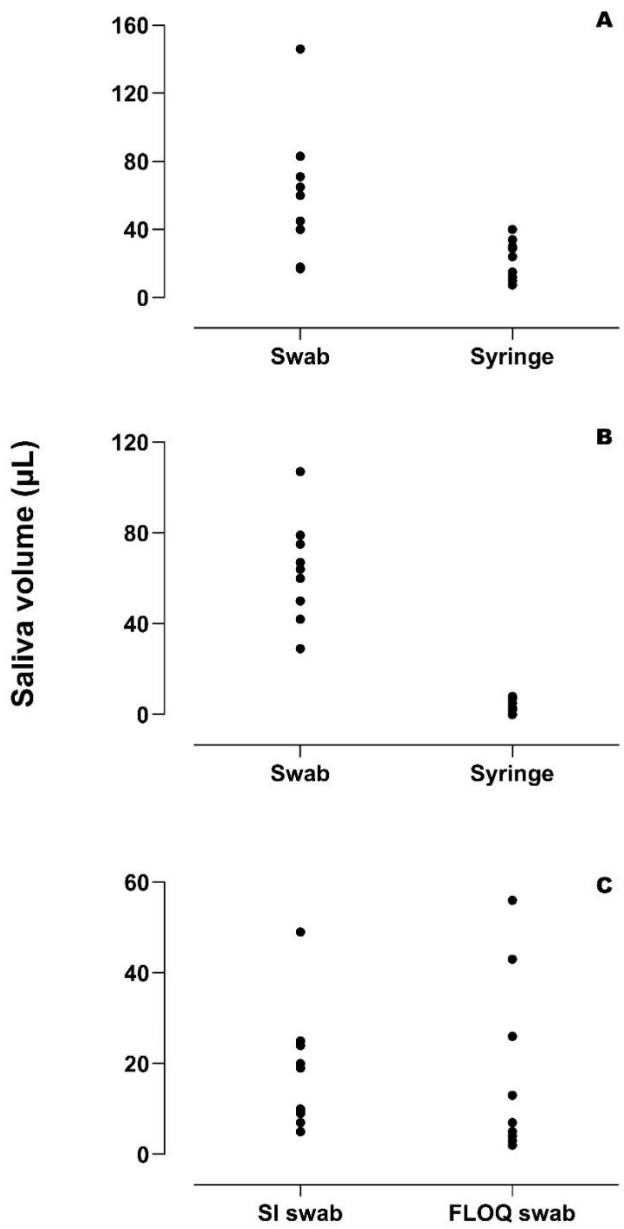

Fig. 3.1. Individual saliva volumes collected using two collection methods for water dragons (A), bearded dragons (B), and leopard tortoises (C) over five minutes.

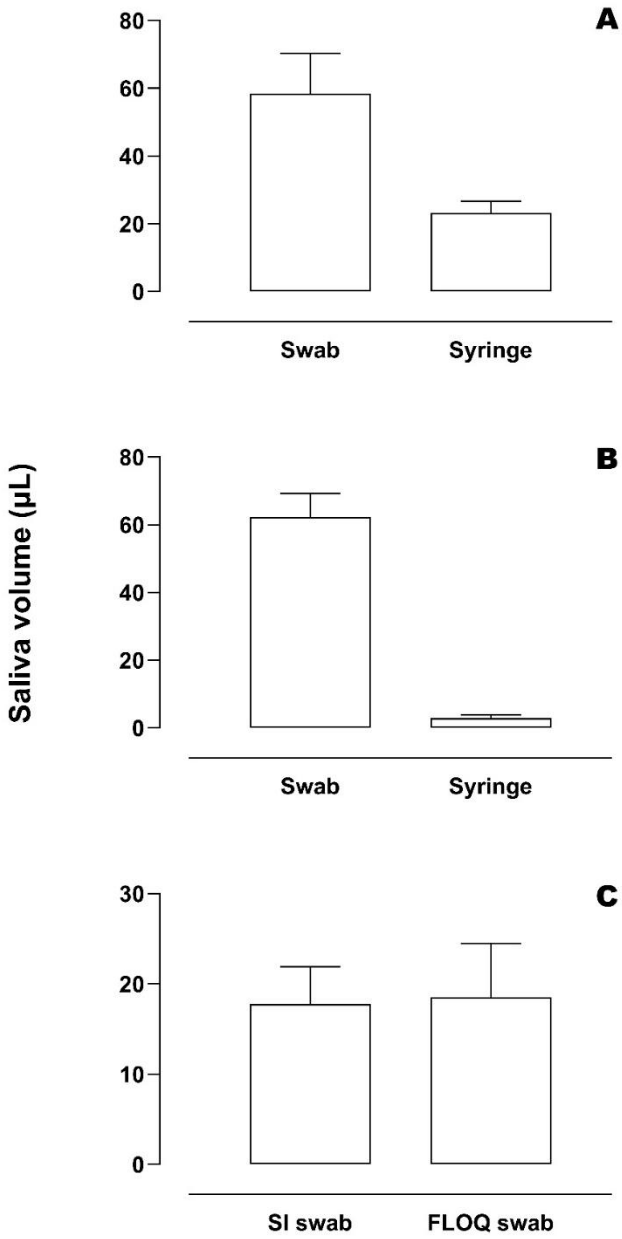

Fig. 3.2. Mean ± SE saliva volumes collected using two collection methods for water dragons (A), bearded dragons (B), and leopard tortoises (C) over five minutes.

No variables correlated with saliva volume collected in five minutes in water dragons (Table 3.2 & Fig. 3.3). However, both carapace length and weight (size variables) correlated with saliva volume collected in five minutes in leopard tortoises (Table 3.3 and Fig. 3.4).

Table 3.2. Results of linear regression of relationships between saliva volume and sampling variables using the SI swab in water dragons

Variable	r^2	*F*	Degrees of freedom	*p-value*
Temperature	0.3472	4.256	1, 8	0.073
Capture time	0.0018	0.0144	1, 8	0.9075
Snout-vent length	0.9075	2.448	1, 8	0.1563
Weight	0.1723	1.666	1, 8	0.2329

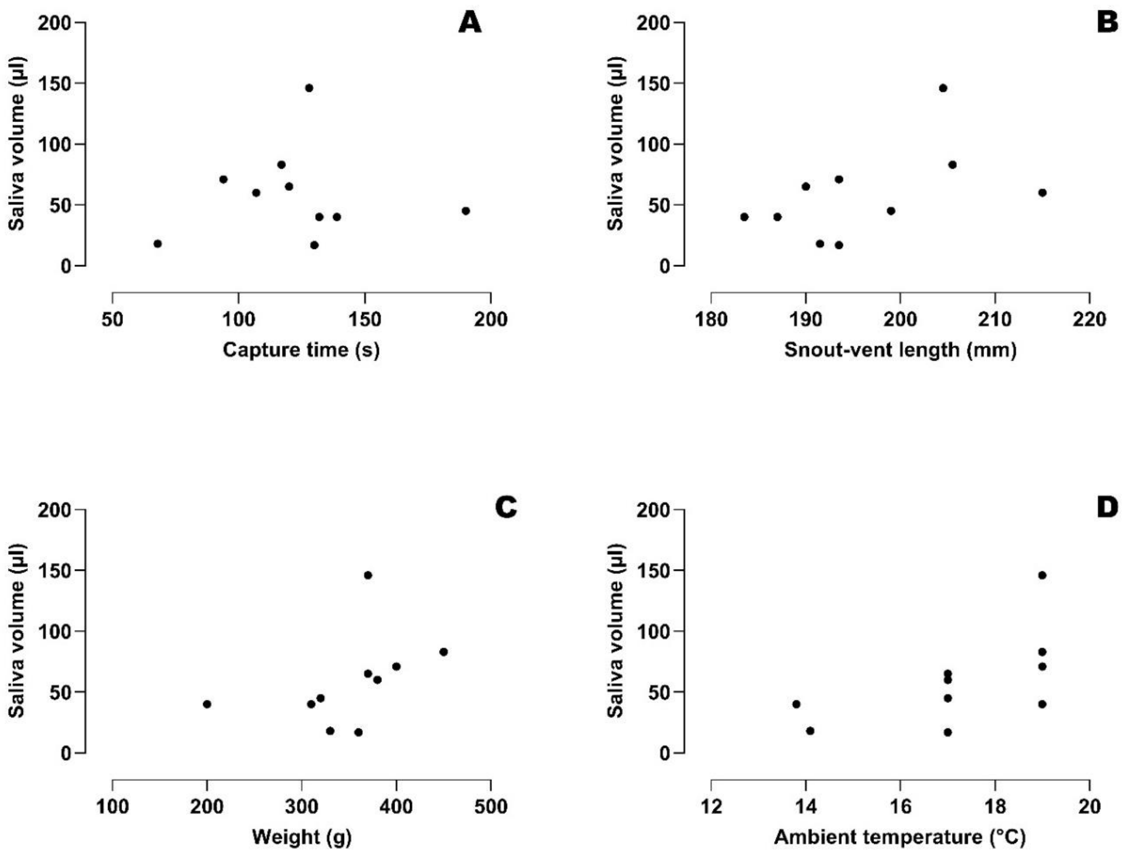

Fig. 3.3. Individual saliva volumes collected from water dragons using a SI swab over a five minute period in relation to capture time (A), snout-vent length (B), weight (C) and ambient temperature (D).

Table 3.3. Results of linear regression of relationships between saliva volume and sampling variables using the FLOQ swab in leopard tortoises

Variable	r^2	F	Degrees of freedom	*p-value*
Temperature	0.2772	3.068	1, 8	0.1180
Capture time	0.0705	0.6069	1, 8	0.4584
Carapace length	0.4947	7.831	1, 8	0.0233*
Weight	0.4194	5.780	1, 8	0.0429*

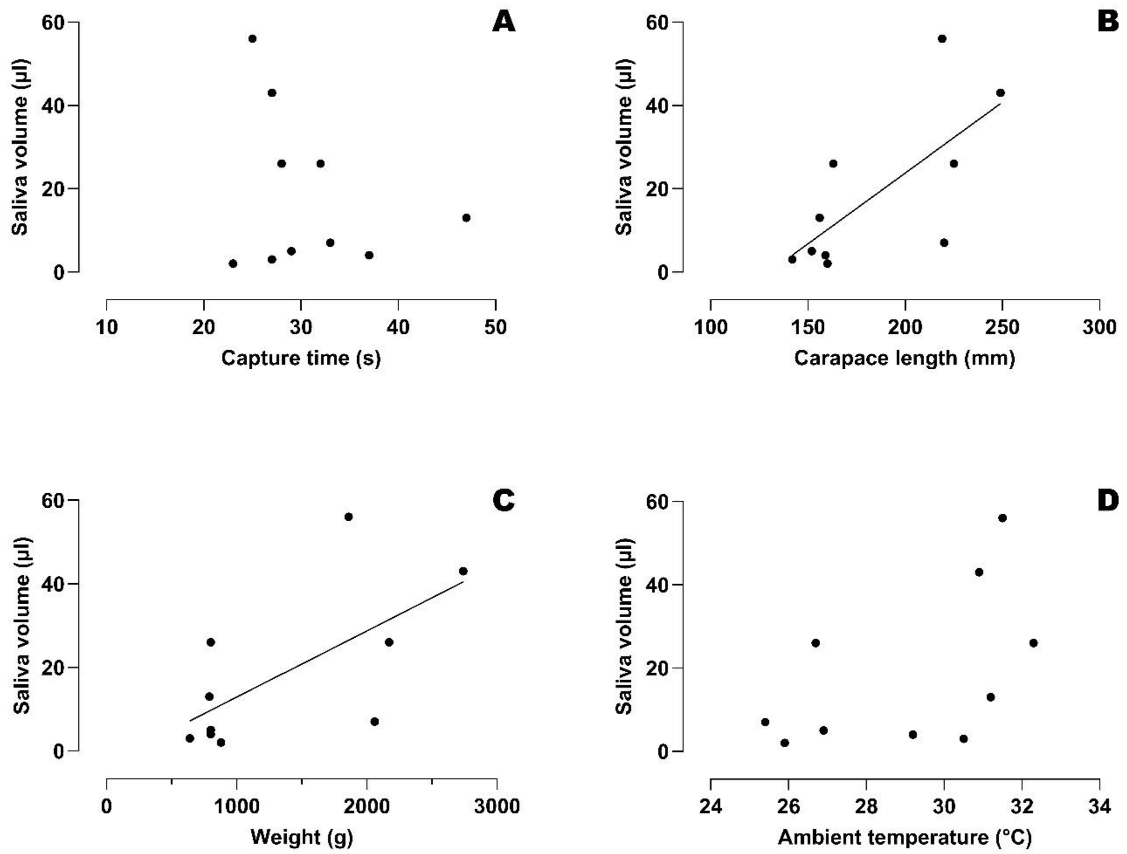

Fig. 3.4. Individual saliva volumes collected from leopard tortoises using a FLOQ swab over a five minute period in relation to capture time (A), carapace length (B), weight (C) and ambient temperature (D). Regression lines are shown where the relationship was significant.

Varying saliva collection times did not significantly affect collected saliva volumes in both water dragons and leopard tortoises (Table 3.4 & Fig. 3.5).

Table 3.4. Results of linear regression of relationships between saliva volume and sampling time using the SI swab in water dragons, and the FLOQ swab in leopard tortoises

Species	r^2	F	Degrees of freedom	*p-value*
Water dragon	0.0025	0.0203	1, 8	0.8903
Leopard tortoise	0.2964	3.370	1, 8	0.1037

Preliminary assay on the six samples collected from each species during saliva sampling confirmed corticosterone could be measured from reptile saliva in all three species. Water dragon samples varied from 0.16-0.61 ng/mL (n=5); bearded dragons 0.24-0.72 ng/mL (n=6), and leopard tortoises 0.06-0.14 ng/mL (n=2). Assay confirmed an approximate volume of 50 μL saliva was needed for successful assay.

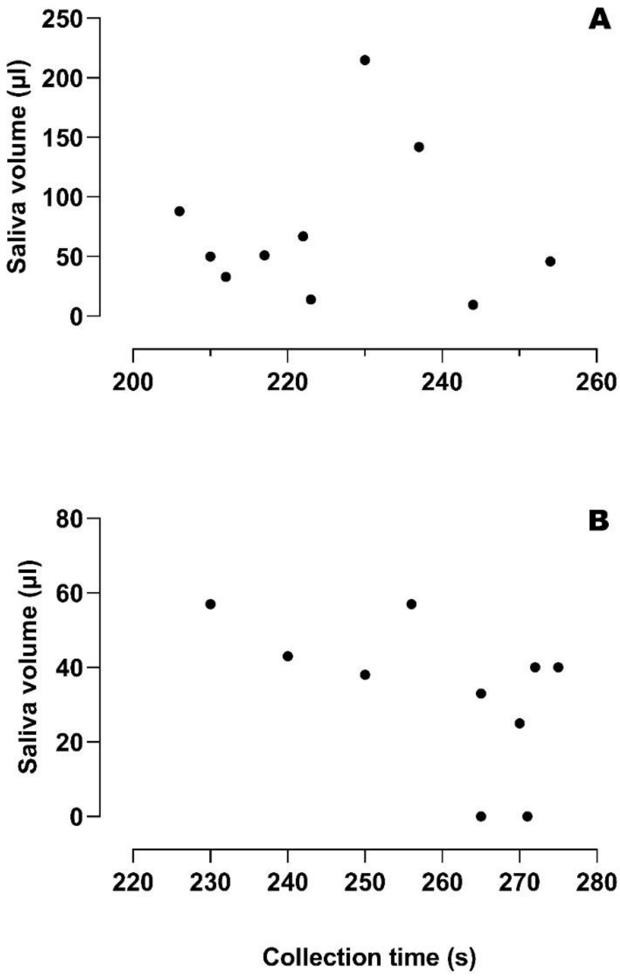

Fig. 3.5. Individual saliva volumes collected from water dragons using a SI swab (A) and from leopard tortoises using a FLOQ swab (B) across differing saliva collection times when the total interaction (including capture) was limited to five minutes.

3.2 Saliva and plasma corticosterone responses to capture and restraint (part 2)

A whole corticosterone response could be measured for each water dragon from both plasma and saliva. Individual variation in responses was observed (Fig. 3.6), with some dragons already showing a decrease in plasma corticosterone concentrations by the 60 min sampling time. Saliva corticosterone responses, however, increased through each sampling time for all dragons. Mean responses for both plasma and saliva showed an overall increase in corticosterone concentrations between 0 min and 60 min (Fig. 3.7). A one-way repeated measures ANOVA showed that there was a significant increase in plasma corticosterone concentrations over the sampling period ($F_{1.554,\ 13.98}$=15.40, p=0.0006). Šidák's post-hoc testing revealed the most significant increase in plasma corticosterone occurred between 0 and 30 min (adjusted p=0.0017). Saliva corticosterone also showed a significant increase over the 60 min sampling timeframe ($F_{1.208,\ 10.87}$ = 22.28, p=0.0004). Both 0-30 min and 30-60 min timeframes were significant (adjusted p=0.0024 and 0.0058 respectively), but 0-30 min showed the larger increase.

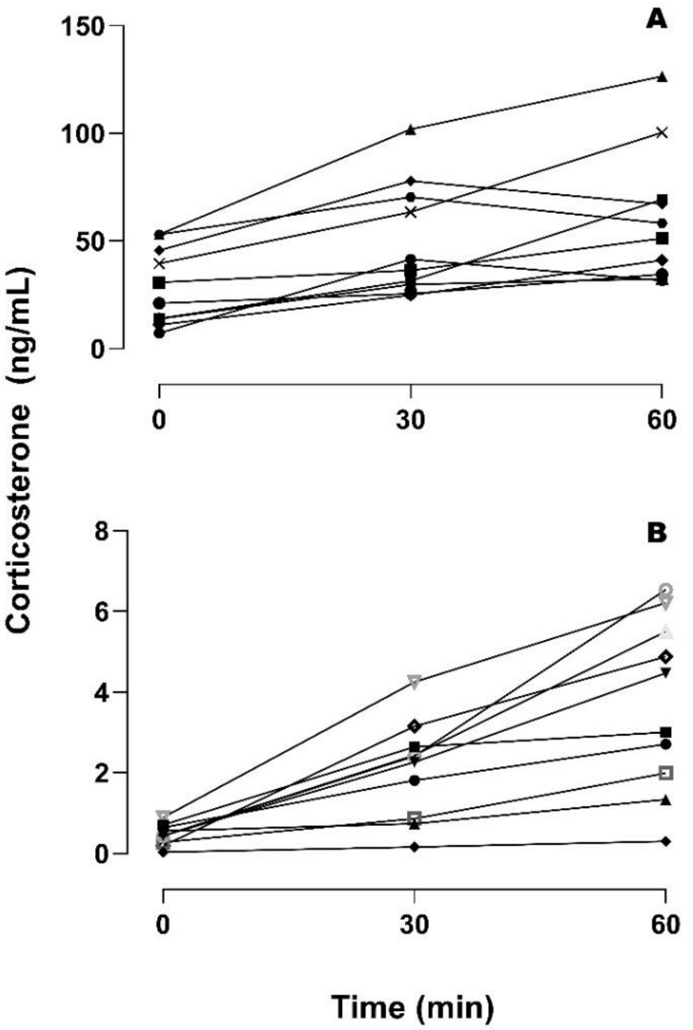

Fig. 3.6. Individual (A) plasma corticosterone and (B) saliva corticosterone responses of water dragons to capture and restraint

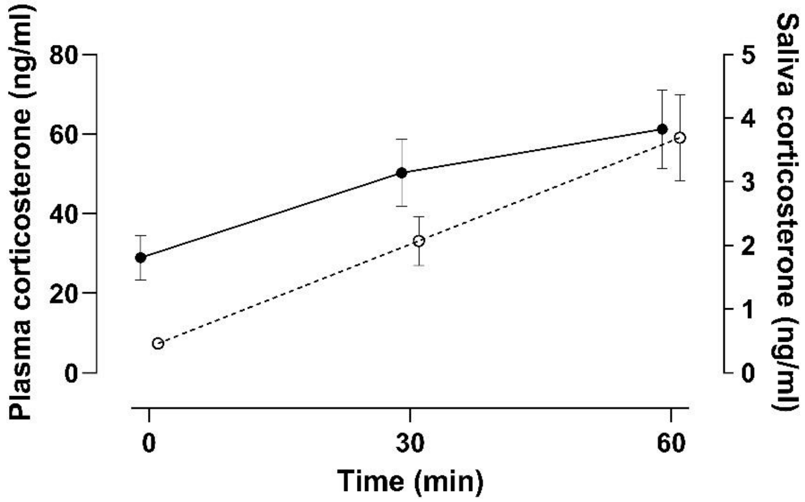

Fig. 3.7. Comparison of mean ± SE plasma corticosterone (—●—, n=10) and saliva corticosterone (--○--, n=10) responses of water dragons to capture and restraint

Saliva corticosterone concentrations were magnitudes lower than plasma corticosterone concentrations; between 1.6% and 6.0% that of plasma corticosterone concentrations (Table 3.5). Size, handling time, and ambient temperature did not affect the total integrated plasma response of water dragons (Fig. 3.8 & Table 3.6).

Table 3.5. Mean corticosterone concentrations at 0, 30 and 60 min in plasma and saliva sampled from water dragons.

	Mean corticosterone ±SE (ng/mL)		
Time (min)	Plasma	Saliva	Saliva % of plasma
0	28.91 ± 5.63	0.46 ± 0.08	1.6%
30	50.25 ± 8.37	2.07 ± 0.39	4.1%
60	61.21 ± 9.89	3.69 ± 0.68	6.0%

Table 3.6. Results of linear regression of relationships between total integrated plasma response and sampling variables in water dragons

Variable	r^2	F	Degrees of freedom	p-value
Temperature	0.2116	2.147	1, 8	0.1810
Handling time	0.0788	0.6840	1, 8	0.4322
Snout-vent length	0.2661	2.900	1, 8	0.1270
Weight	0.1734	1.678	1, 8	0.2313

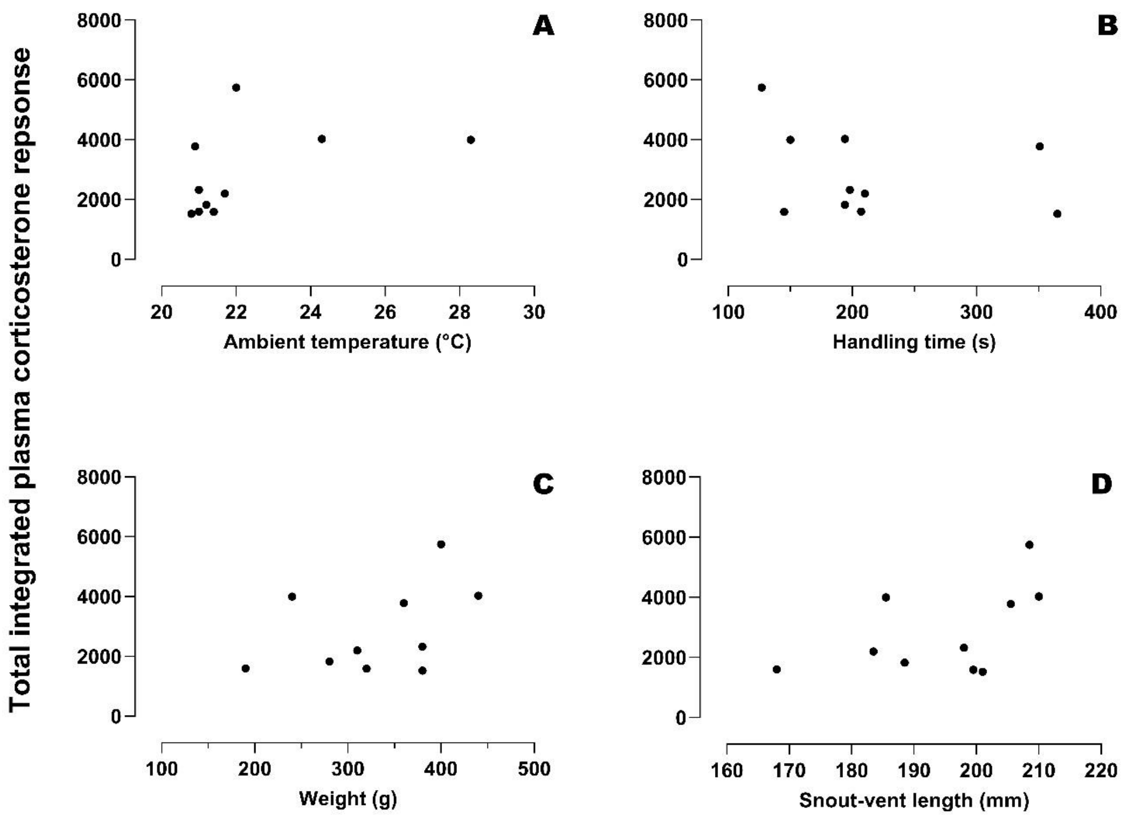

Fig. 3.8. Total integrated plasma corticosterone responses of water dragons in relation to (A) ambient temperature, (B) total handling time for blood collection, (C) weight, and (D) snout-vent-length.

Only two full plasma corticosterone responses were measured out of the 10 leopard tortoises (Fig. 3.9). However, individual variation in responses can still be seen. There is also an increase in mean corticosterone concentrations from 0 to 60 min. Only one full corticosterone response could be measured from saliva out of 10 leopard tortoises due to the low volume of saliva. Again, some individual variation can be seen. A one-way unmatched ANOVA showed that there was a significant difference in plasma corticosterone concentrations between 0 min and 60 min ($F_{2, 15}$=4.609, p=0.0275). Mean saliva corticosterone concentrations increased slightly between 0 and 30 min and then decreased slightly at 60 min (Fig. 3.10), but no changes were significant. Saliva corticosterone concentrations were also magnitudes lower than plasma corticosterone, even more so than observed in water dragons, between 0.4 – 1.0%, (Table 3.7).

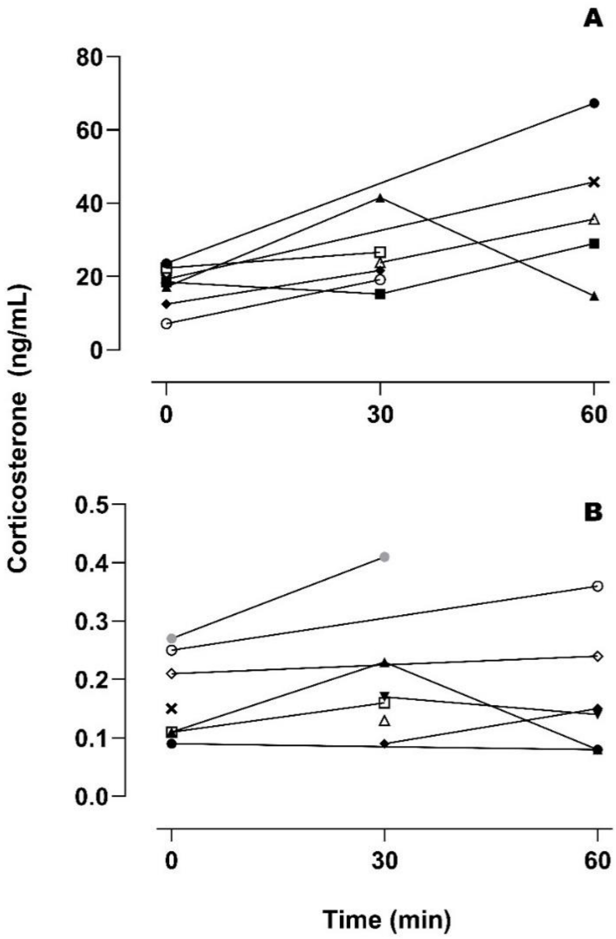

Fig. 3.9. Individual (A) plasma corticosterone and (B) saliva corticosterone responses of leopard tortoises to capture and restraint. Full responses could not be collected from most individuals due to saliva samples being too small for successful assay

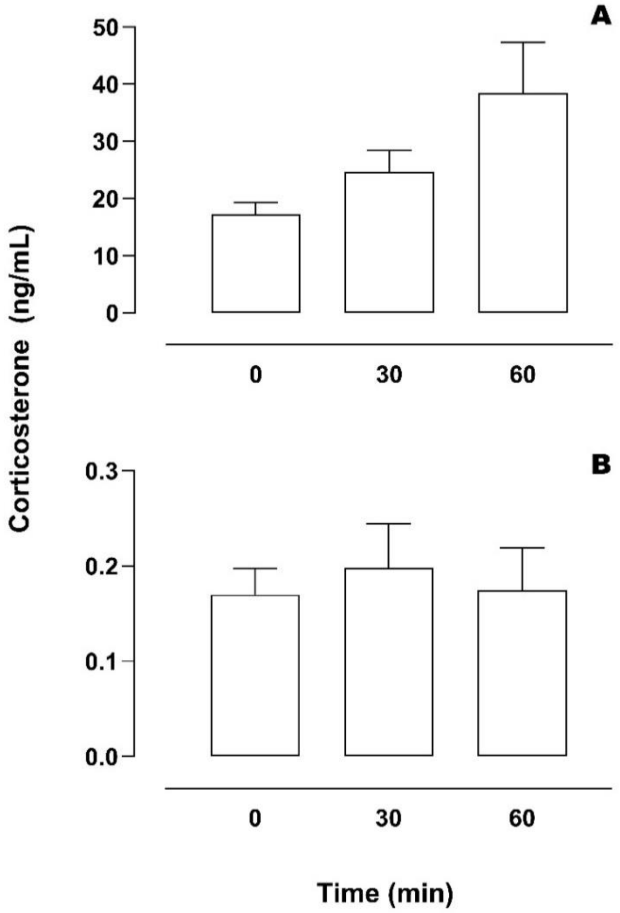

Fig. 3.10. Mean ± SE (A) plasma corticosterone (n=8) and (B) saliva corticosterone (n=10) responses of leopard tortoises to capture and restraint.

Table 3.7. Mean corticosterone concentrations at 0, 30 and 60 min in plasma and saliva taken from leopard tortoises.

	Mean corticosterone ±SE (ng/mL)		
Time (min)	Plasma	Saliva	Saliva conc. % of plasma conc.
0	17.23 ± 2.16	0.17 ± 0.02	1.0%
30	24.67 ± 3.73	0.20 ± 0.03	0.8%
60	38.49 ± 8.79	0.17 ± 0.04	0.4%

Mean saliva corticosterone concentrations were much higher in water dragons compared to leopard tortoises. (Fig. 3.11). Corticosterone was measured from each water dragon saliva sample, with samples ranging 18.5 – 210 μL, and mean volume decreased throughout the 60 min in water dragons, and corticosterone concentrations varied across individual water dragons. Saliva volumes also varied in leopard tortoises (Fig. 3.12); however, leopard tortoise mean saliva volume decreased by 30 min before increasing slightly by 60 min (Fig. 3.13).

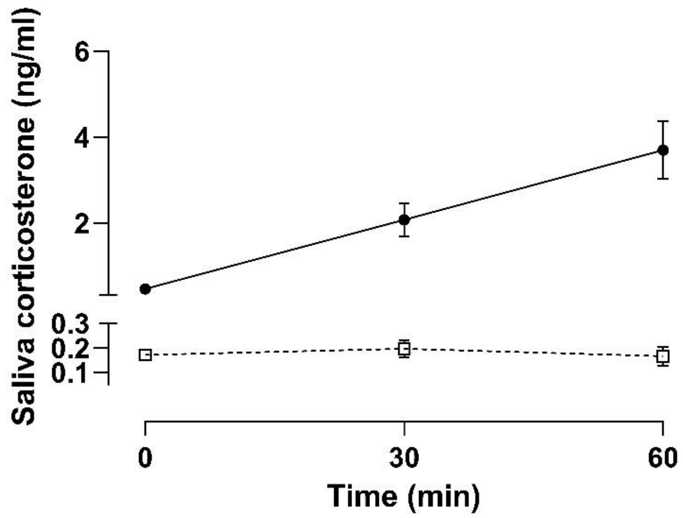

Fig. 3.11. Comparison of mean ± SE saliva corticosterone responses in water dragons (—●—, n=10) and leopard tortoises (--□--, n=10) to capture and restraint.

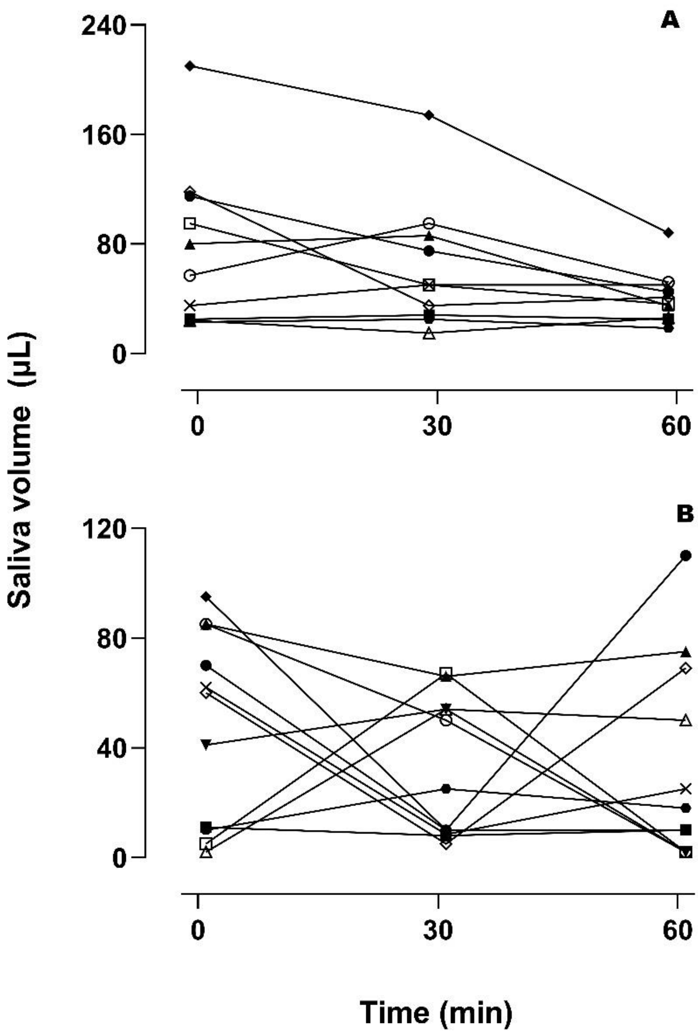

Fig. 3.12. Individual saliva volumes collected from (A) water dragons and (B) leopard tortoises.

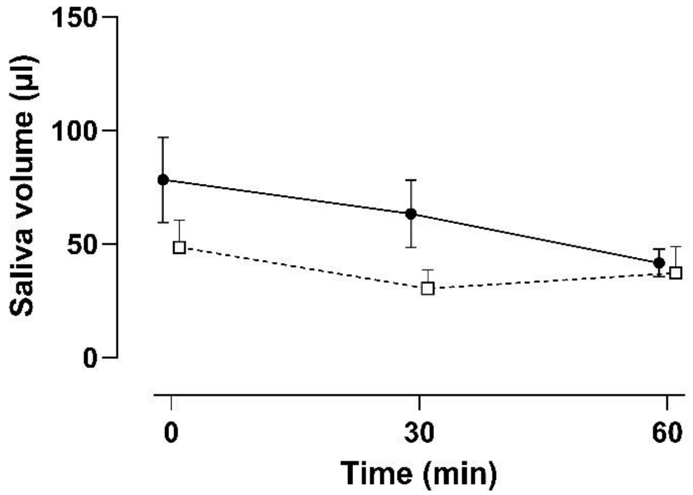

Fig. 3.13. Mean ± SE saliva volume collected from water dragons (—●—, n=10) and leopard tortoises (--□--, n=10).

3.3 Plasma corticosterone response to saliva collection compared with blood collection (part 3)

Both water dragons and leopard tortoises showed individual variation in their responses to both sampling types at both 15 and 30 min (Fig. 3.14). Mean plasma corticosterone concentrations in water dragons following saliva collection was higher compared to blood collection at 15 min, but not significantly different ($t[10]=1.912$, $p=0.0849$) Fig. 3.15[A]). Corticosterone concentrations at 30 min were similar ($t[10]=0.721$, $p=0.4874$). In contrast, leopard tortoise mean plasma corticosterone concentrations following saliva collection was lower compared to blood collection, but also not significant ($t[5]=2.149$, $p=0.0843$) (Fig. 3.15[B]). As with water dragons, corticosterone concentrations at 30 min were similar in leopard tortoises ($t[9]=0.621$, $p=0.549$).

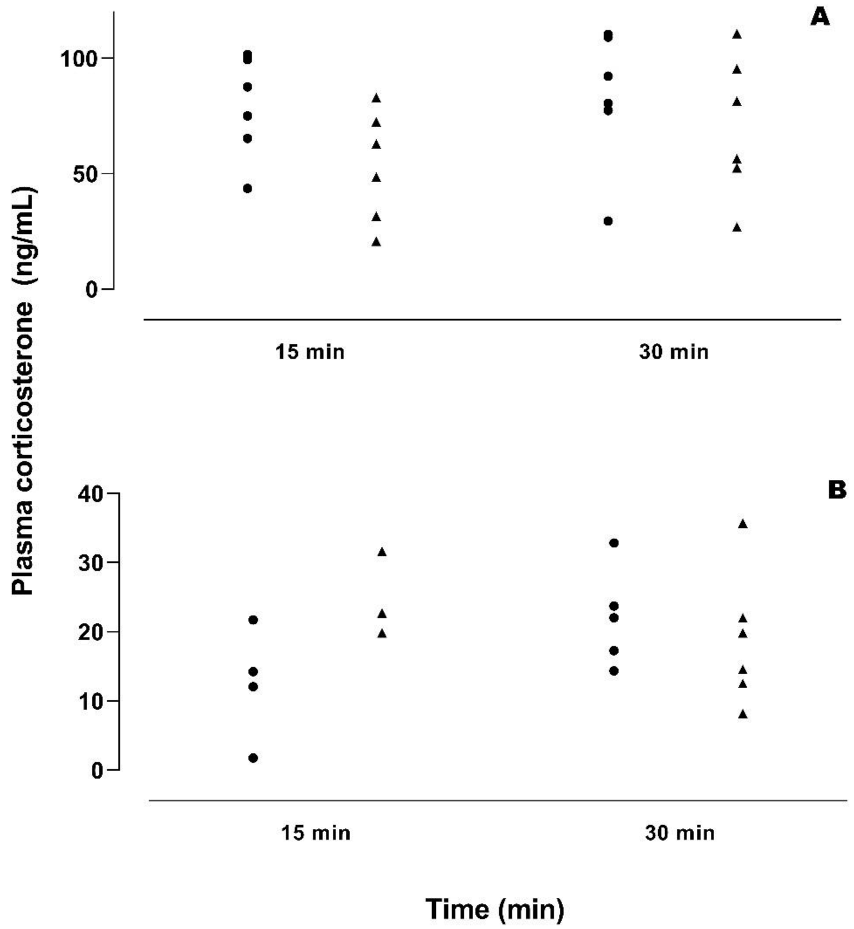

Fig. 3.14. Individual plasma corticosterone concentrations at 15 and 30 minutes following an initial saliva sample (●) or initial blood sample (▲) in water dragons (A) and leopard tortoises (B).

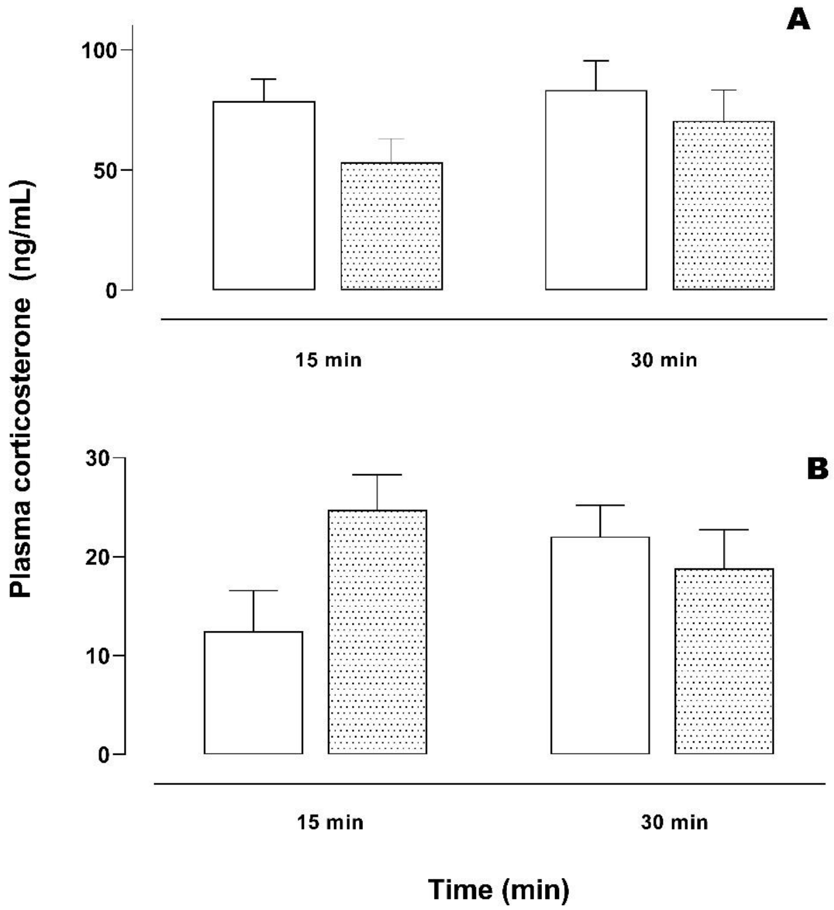

Fig. 3.15. Mean ± SE plasma corticosterone concentrations at 15 and 30 minutes following an initial saliva sample (white boxes) or initial blood sample (shaded boxes) in water dragons (A) and leopard tortoises (B).

Initial blood sampling time in sample groups 1 and 2 for both species showed no significant effect on baseline corticosterone concentrations (Fig. 3.16, see Table 3.8 for statistics).

Table 3.8. Results of linear regression between blood collection time and baseline plasma corticosterone concentrations.

Species	r^2	F	Degrees of freedom	p-value
Water dragon	0.1629	1.946	1, 10	0.1932
Leopard tortoise	0.2595	2.453	1, 7	0.1613

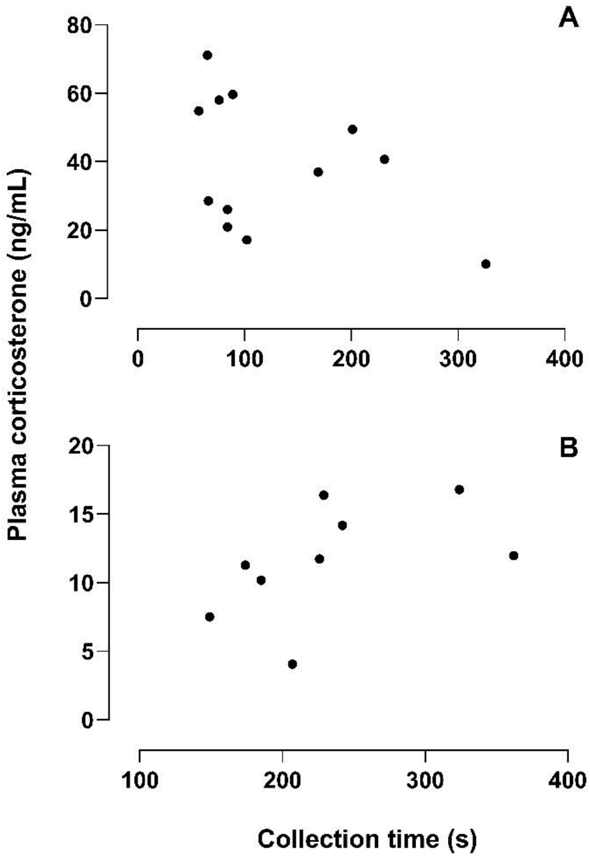

Fig. 3.16. Individual baseline plasma corticosterone concentrations compared to sample collection time

4. Discussion

4.1 Saliva collection and initial assays

Due to Part 2 of the study relying on successful assay of salivary corticosterone for comparison with plasma corticosterone concentrations, finding the best saliva collection method for each study species was essential. Lac's (2001) study suggested a minimum of 50 µL for successful assay, which is still a standard target volume used in more recent salivary glucocorticoid studies (Stocker *et al.*, 2023; Survilienė *et al.*, 2022). Many of the absorption methods mentioned in the literature for reptiles (e.g., filter paper, cotton swabs, wooden sticks – see Section 1.5.1) did not mention the volume of saliva collected, but given the methods used and purpose of collection (e.g., DNA sampling) likely would have had limitations in the scope of this study and would not have collected the volume of saliva required for corticosterone assay. Additionally, some of the methods may have posed safety risks to the reptiles due to fragility of the apparatus if the reptile was to chew or bite down. Additionally, any injuries to the oral cavity may have caused contamination of the saliva with blood and given inaccurate corticosterone measurements (Kivlighan *et al.*, 2004). Out of all the absorption methods evaluated prior to sampling, only the SI swab was deemed sturdy enough to be used with both water and bearded dragons. For both water dragons and bearded dragons, the SI swab was clearly the most effective method compared to syringing. The swab allowed the lizards to chew, stimulating the production of saliva over the course of the sampling period (as seen in Lutz *et al.* (2000)). Chewing was not often observed during sampling with the syringes as the mouth had to be constantly held open to syringe saliva from the mouth. Mean saliva volume using the SI swab in leopard tortoises was much lower than both water dragons and bearded dragons. Leopard tortoise saliva seemed to be much more viscous than water dragon or bearded dragon saliva and sometimes would 'adhere' to the swab. Viscous saliva has been observed in other terrestrial tortoise species, especially those that live in arid environments where keeping the oral cavity lubricated is important (Alsafy *et al.*, 2024; Rashwan *et al.*, 2024). Although the FLOQ swab was much smaller, mean saliva volumes were very similar when compared to the SI swab. The flocked head of the FLOQ swab provided a large surface area and appeared much easier for the viscous tortoise saliva to adhere to. However, mean saliva volume was still less than half of the recommended 50 µL, which led to the decision to join two FLOQ swabs together to increase saliva yield for the subsequent parts of the project.

Physical and environmental variables did not correlate with saliva volume in reptiles. However, carapace length and weight did correlate with saliva volume in leopard tortoises; the bigger tortoises yielded the most saliva. Other variables were not recorded however, including time from last feeding, which may have impacted saliva volume if feeding was recent before sampling. Feeding times varied for water dragons between enclosures, and it was unclear whether individuals had indeed eaten as not all dragons will have emerged from their burrows at feeding time. Tortoises, however, were gathered up for feeding time (which also varied), and notes were recorded if sampling took place after feeding. It did not appear that recent feeding affected saliva volumes, however.

Preliminary assay of saliva samples in Part 1 confirmed that 50 µL was the suggested needed saliva volume for measuring corticosterone in reptile saliva - some samples were too small for successful assay. It was also confirmed that corticosterone could be measured in reptile saliva. No published journal articles describing the measurement of corticosterone in reptile saliva were found in the literature. A recent review by Carbajal *et al.* (2024) also confirmed that salivary corticosterone had still not been explored in reptiles, while noting that their previous research measuring salivary sex hormones suggested that salivary corticosterone may also be a viable non-invasive measurement of glucocorticoids in reptiles.

4.2 Corticosterone responses

Corticosterone responses were successfully measured in both plasma and saliva for all water dragons. The mean saliva corticosterone response followed a similar pattern of increase to mean plasma corticosterone, increasing throughout the 60-minute sampling period. Saliva corticosterone was significantly elevated at 30 min, and by 60 min was eight times higher than at baseline. It is known that due to the time taken for corticosterone to diffuse into saliva, saliva corticosterone takes longer to start increasing compared to plasma corticosterone (Peeters *et al.*, 2011). Sampling at shorter intervals between 0 and 30 minutes would help pinpoint how long it takes saliva corticosterone to start increasing after capture and restraint in water dragons compared to plasma corticosterone. Continuing sampling past 60 minutes would be needed to determine how long it would take for saliva corticosterone concentrations to peak. As this was the first study to measure saliva corticosterone responses in a lizard, there are no comparative studies in the literature.

In comparison, plasma corticosterone concentrations only reached two times higher at 60 min than at baseline, but the increase was still significant, representing a clear response to capture, handling, and blood sampling. Plasma corticosterone concentrations here are consistent with plasma corticosterone responses of other reptile species within the literature (see Cockrem's 2013 review and Section 1.4.3). Temperature, handling time, weight and length did not affect the magnitude of the corticosterone response (total integrated plasma corticosterone) of water dragons. While total integrated corticosterone responses have not previously been reported, there are many which investigated the effects of temperature and body condition on corticosterone levels and responses in other reptiles (see Section 1.4.2).

Only two full plasma corticosterone responses and one full saliva corticosterone response could be measured for leopard tortoises. This issue was due to the behaviour and anatomy of the tortoises; tortoises often retreated into their shells after each sample collection and remained so after being removed from the bags for the next sample. Time had to be spent attempting to coax the tortoises out of their shells to access limbs for repeated blood and saliva sampling. The remainder of plasma responses only had two samples collected each (0 and 30 min, 0 and 60 min, or 30 and 60 min). Saliva sampling had the added issue of saliva volumes not being large enough for successful assay. Half of the 30 tortoise saliva samples measured less than 20 µL, and only one of these (18 µL) was successfully assayed. This resulted in only one full saliva corticosterone response, with the remainder of individuals either having one or two samples that could either be collected and/or measured. Being unable to obtain subsequent samples has been reported in the literature for other tortoise species (Currylow *et al.*, 2017a; Drake *et al.*, 2012). A larger sample size or using larger tortoises may have improved the ability to access limbs for restraint to obtain samples successfully. Despite the challenges, this is the first study to measure saliva corticosterone in a tortoise species.

Mean plasma corticosterone concentrations fit within ranges already reported in the literature for reptiles (Goessling and Mendonça, 2021; Gregory and Schmid, 2001), although were higher than those reported for some species (Lance *et al.*, 2001). While significant elevations in mean plasma corticosterone were observed by 60 min, no significant change in saliva corticosterone was observed. Gregory *et al.* (1996) found that plasma corticosterone concentrations also did not begin to rise significantly until 60 mins in loggerhead turtles (*Caretta caretta*). In addition, corticosterone did not peak until the 180 min sample. It may be that saliva corticosterone in leopard tortoises need a longer sampling time to allow enough passive diffusion of corticosterone into saliva for it to be detected. Further studies using saliva corticosterone in tortoises therefore should incorporate sampling over a longer period.

Both plasma and saliva corticosterone mean concentrations were higher in water dragons than in leopard tortoises. This is consistent with previous studies that found that testudines have lower corticosterone concentrations and responses compared to squamates (Fazio *et al.,* 2014 & see Section 1.4.3). Mean saliva corticosterone concentrations were also much lower than mean plasma concentrations in both water dragons and leopard tortoises. This is consistent with the literature as saliva corticosterone represents the "free" portion circulating in the blood available to diffuse into saliva (see Section 1.5.2). What is inconsistent, however, is the ratio of corticosterone in saliva compared to plasma seen in both species. Mean saliva corticosterone ranged between 1.6 - 6% compared to mean plasma corticosterone in water dragons, and 0.4 – 1% in leopard tortoises. These percentages are much lower than the ~10% in current literature (Kivlighan *et al.*, 2004; Sheriff *et al.*, 2011). Perhaps the lower concentrations of corticosterone in saliva are a result of the lower metabolic requirements of reptiles compared to mammals, where most of research on salivary glucocorticoids has been conducted (Neuman-Lee *et al.*, 2020). More research needs to be done into the physiology underlying the diffusion of corticosterone and other hormones into saliva from blood.

Individual variation in responses was observed in both saliva and plasma corticosterone responses in water dragons. Baseline plasma corticosterone concentrations varied from 7.2 – 52.9 ng/mL, and concentrations at 60 min after capture and handling ranged from 31.6 –

126.4 ng/mL. Some dragons had higher baseline corticosterone concentrations compared to other dragons after capture and handling. This variation was also seen in saliva corticosterone responses, ranging from 0.03 – 0.89 ng/mL at baseline and 0.3 – 6.5 ng/mL at 60 min following capture and handling. Variation in water dragon plasma corticosterone responses has been observed by Baird *et al.* (2014), who found variation in male dragons' responses following interactions with other male dragons. Winners of new territory saw increases of corticosterone by 7.8 times from baseline (41.8 -326.3 pg/mg), whereas the loser of the territory only had an increase of 1.7 times from baseline (69.8 -119.3pg/mg). Corticosterone concentrations were expressed in pg/mg of freeze-dried plasma so comparisons could not be made with results from this study (measured in ng/mL).

While samples were missing, individual variation could also be seen in leopard tortoise responses. Most tortoises who only had two samples measured saw increased corticosterone concentrations in both plasma and saliva, although some saliva concentrations stayed very similar. The few tortoises that had a full response measured showed interesting patterns. One tortoise's plasma corticosterone increased through to 30 min and then decreased at 60 min to less than baseline, which was also seen in the tortoise with the full saliva corticosterone response. Another tortoise's plasma corticosterone decreased below baseline at 30 min and then increased to just above baseline at 60 min. Currylow *et al.* (2017a) found individual variation in radiated tortoises (*Astrochelys radiata*), with plasma concentrations ranging from 0.055 - 12.16 ng/mL. Differences in corticosterone responses could also be seen between individuals who were classed as "bold/proactive" (larger, better condition, more active in their home range) and "reactive" (smaller, lower condition, moving less within their range) based on observed behaviours. Bolder tortoises showed smaller corticosterone responses to handling whereas the reactive tortoises had larger corticosterone responses. In the context of wildlife management, observing variation in personalities and coping styles within a population is crucial to understand how well suited that population may be to deal with environmental changes (Cockrem, 2022).

4.3 Comparison of corticosterone responses to saliva sampling and blood sampling

In both species of reptile, there were no significant differences in plasma corticosterone at 15 or 30 min between samples collected after initial blood sampling and samples collected after initial saliva sampling. This suggests that capture, restraint and sampling is the main stressor event, with corticosterone responses to this stressor not influenced by factors such as sampling time or the amount of manipulation needed to obtain a sample. For example, blood sampling from water dragons was much quicker than saliva sampling (average of 2 min for blood sampling versus 5 min for saliva sampling) and required less restraint. Mean corticosterone concentrations at 15 min were slightly lower following blood sampling, but this difference was not significant and was also not present in the 30 min sample group. Tortoises were similar in that blood sampling took slightly less time (average of 4 min for blood sampling versus 5 min for saliva sampling) but also required much more restraint and manipulation. If blood could not be taken from one body part (usually the dorsal coccygeal vein in the tail was sampled first) a different vein would be attempted (usually the brachial vein). This resulted in different body parts being manipulated to try and obtain a blood sample. Most tortoises would continually attempt to withdraw all body parts into their shell. Corticosterone concentrations were slightly higher at 15 min following blood sampling but not significantly, and again this slight difference was not present in the 30 min sample group. The use of a cloth bag to keep animals contained may have reduced the influence of initial sampling type by providing the same stressor to both groups between initial sampling and the 15- or 30-min sampling.

So, while saliva sampling is considered a "non-invasive" method of measuring glucocorticoids, the animal may not perceive saliva sampling to be less of a stressor compared to blood sampling. Sampling time and the degree of restraint needed varies from species to species, and this needs to be taken into consideration in relation to determining the most effective method, due to the extra time it may take to obtain a large enough saliva sample compared to the time required for blood sampling. However, saliva sampling may be less risky for reptiles from a welfare perspective, in that it carries less risk of introducing pathogens into the animal via the venipuncture site. The finding of no significant difference between sampling methods in tortoises is supported by Kahn *et al.* (2007) who found plasma corticosterone response to capture, restraint (trap) and blood sampling in gopher tortoises was not significantly different from responses in tortoises that were only captured and sampled before immediate release.

5. Conclusion and directions for further research

This is the first study where corticosterone has been measured in reptile saliva (and in two separate reptile orders; squamata and testudines). It is also the first time that a whole corticosterone response has been measured from saliva in a lizard species, and in a reptile species. Successful saliva sampling is crucial for subsequent assay of corticosterone, especially in smaller species where saliva volumes may be low. In some larger species this may not be an issue and, as for mammals, multiple options may be available. For smaller species, however, finding the most effective method specific to each species is key. This project was intended to lead on to and be adapted for further stress-related research with endemic New Zealand Duvaucel's geckos (*Hoplodactylus duvaucelii*). Being a small species, however, obtaining a large enough saliva sample may be challenging (as for the juvenile leopard tortoises).

While saliva sampling is considered a 'non-invasive' sampling method, some individuals may not perceive it to be less stressful due to extra manipulation and the time it may take to obtain a large enough sample when compared to blood sampling. However, it is still a worthwhile alternative when considering it poses less risks to individuals from a welfare perspective e.g. introducing pathogens into the venipuncture site. In addition, the latency for corticosterone to enter saliva from the blood provides a longer timeframe to collect baseline concentrations after first approach of a reptile. Additional research needs to be done to determine the length of this latency period in different species.

While the use of saliva for measuring corticosterone needs to be validated for each species, this study provides a basis for future saliva corticosterone measurement in other reptile species. Using saliva instead of blood for corticosterone measurement may enable easier access to monitoring wild populations of reptiles in the field, especially in remote areas where equipment and facilities for blood sampling may be limited. Given that 20% of reptile species are classified as threatened by the IUCN (Böhm *et al.*, 2013), developments in monitoring techniques such as this may be crucial for those in remote areas or are sensitive to environmental changes (e.g., due to climate change) and where approval for invasive sampling may be more difficult to obtain due to their conservation status or management frameworks.

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