Study site and animals

The study was conducted at the National Zoological Gardens of South Africa (NZG), Pretoria, South Africa (25.73913° N, 28.18918° E) on the edible bullfrog (*Pyxicephalus edulis*). According to the IUCN Red List of Threatened Species (2016), *P. edulis* is currently listed as least concerned. The animals were housed individually in a plastic container containing a dark plastic cup for hiding purposes. To keep individuals hydrated, the container was lined with permeable aquarium filter foam to which clean water was added when required; this ensured that *P. edulis* individuals remained hydrated throughout the study period while limiting the area of contact with water and the possible loss of dermal secretion. A water bowl was not provided within the containers as prolonged periods within the bowl may lead to the unintentional removal of dermal secretion. A dry area was also included into the container should individuals need respite from the moist, permeable surface. All individuals were allowed 15 days to acclimatise to their new environment prior to the beginning of the biological and physiological validation periods respectively. Individuals were fed by the NZG amphibian conservation staff according to a fixed schedule; we ensured feeding and sample collection events did not occur on the same day. All individuals were housed in the Endocrine Laboratory, NZG, at a constant temperature of 24.34±0.93˚C (median±standard deviation (SD)). The entire study was performed with the approval of the NZG Animal Use and Care Committee (Reference NZG/RES/P16/19).

Table 1. The number of individuals per treatment group, as well as the blotting interval for each group, during the mucus secretion rate experiment

Treatment group	Group	Number of individuals	Blotting intervals (min)	Blotting regions
Wet 1	1	10	10,20,30	Back, Stomach
Wet 2	2	10	20,40,60	Back, Stomach
Wet 3	3	10	30, 60, 90	Back, Stomach
Dry 1	4	10	20, 40, 60	Back, Stomach
Dry 2	5	10	40, 80, 120	Back, Stomach
Dry 3	6	10	20, 60, 100*, 140	Back, Stomach

Dermal secretion rate

To define the dermal secretion rate of *P. edulis* individuals, six experimental groups were formed (wet and dry groups, Tab.1). For the three wet groups, individuals were wiped clean using a wet cloth, placed into a plastic container to which water was added and left for ten minutes. This was to simulate environmental conditions where moisture was readily available prior to animal capture and sampling. Subsequently, individuals were allowed to air dry for ten minutes in a new container, before being patted down with a Kim wipe (Kimtech Science Brand, Kimberley-Clark Professional, USA) to ensure no moisture remained. For the dry groups (Dry 1-3), individuals were cleaned using a cloth, allowed to sit in a dry container for ten minutes, before any moisture from the skin's surface was removed with a Kim-wipe.

In all wet and dry groups, dermal secretion production was defined as absent if no sign of dermal secretion was seen on the Kim-Wipe after blotting; if a clear sign of dermal secretion on the Kim-wipe occurred from the blotting event, we defined dermal secretion as present. To determine whether moisture was present, or absent, at each defined interval thereafter (Tab. 1), a small section (1 cm x 1cm) of the ventral and dorsal region was blotted with a single-ply Kim-wipe. In the third dry group individuals were sprayed with water at 100 min to determine if this would increase dermal secretion production.

When an individual urinated in its container and came in contact with the urine, as to allow for moisture absorption or the false confirmation of dermal secretion present, this individual was removed from the treatment group. The period of optimum dermal secretion production was defined as any time interval where more than 80% of individuals had dermal secretion present.

Dermal secretion collection protocol

The collection of dermal secretion was conducted according to a predetermined sampling protocol for both the physiological and biological validation process. All animals were handled with clean, decontaminated equipment and fresh, disposable gloves. Individuals were held in one hand and gently but firmly swabbed (2mm-diameter plastic cotton swab without adhesive; Citoswab® transport swab, 2120-0015, Haimen City, China) three times on the dorsal surface (2.5cm). Directly following this we repeated the process on the ventral side of the individual with a new plastic cotton swab. In an attempt to reduce cross-contamination and the removal of dermal secretion, care was taken not to come in contact with the dorsal or ventral body region during the sampling event.

Sampled individuals were placed back into their respective containers until the next swabbing event. Individual swabs were placed into a 2ml Eppendorf tube containing 1ml 70% ethanol and sealed with parafilm to reduce potential leakage and evaporation.

All Eppendorf tubes were placed at -20˚C until extraction and analysis.

Dermal secretion extraction protocol

All dermal secretion samples were extracted at the Endocrine Laboratory, NZG, South Africa. Dermal secretion samples were kept at room temperature for 30 minutes prior to the start of the extraction process. The samples were then shaken on a water bath shaker at 70 rpm for 5 minutes before being briefly vortexed. Following a fifteen second centrifuge spin down (1500*g*), 500 µL of each sample was transferred into a new, pre-labelled 2ml Eppendorf tube. The extracts were then placed into an incubator oven at 60˚C until dry (~5 hours). Two to three glass beads were added to the dried extract of each tube prior to the addition of 500 µL assay buffer. Following the vortex of each sample for 15 second at maximum speed, samples were placed into a sonicator for 20 minutes. Finally, the samples were shaken on a water bath shaker for 30 minutes at 70 rpm.

Biological validation

To determine whether we could detect GC concentrations within dermal secretion samples from *P. edulis,* we conducted a biological validation on six individuals of unknown sex. Here we used animal handling and constraint as the biological stressor. Amphibian restraint and handling have been shown to act as a mild, acute stressor which leads to an increase in GC production which has been monitored in urine, blood and dermal secretion (Narayan 2013; Narayan et al. 2012; Santymire et al. 2018). The six individuals were divided into two group where only dorsal (n=3) or ventral (n=3) swabs were collected. We collected dermal secretion samples from each individual at time 0; this sample was defined as a baseline sample. Individuals were then handled continuously for three minutes before being placed back into their individual containers. Following this we collected dermal secretion samples at five minute intervals until minute 20, before taking a final sample at 60 minutes post-stressor. A total of six samples were collected per individual.

Physiological validation

The physiological validation was conducted on 16 female and 15 male *P. edulis*. The 31 *P. edulis* individuals, none of which formed part of the biological validation group, were randomly assigned to one of two groups, namely a control (8M, 8F) or ACTH group (7M, 8F).

Dermal secretion was collected from all 31 study animals at 07h00 daily for two consecutive days to determine baseline dGC concentrations. At 06h00 on the fourth day we injected each of the ACTH animals (7M,8F) intraperitoneally with synthetic ACTH (0.443mg/g of Synacthen®, Novartis, South Africa Pty Ltd) in 100 µL frog ringer solution (6.6 g NaCl, 0.15 g KCl, 0.15 g CaCl2, and 0.2 g NaHCO3 to 1 liter of distilled water). We chose this ACTH dose as it has been used successfully in a number of frog species to evoke a stress response (Graham et al. 2013; Kindermann et al. 2012; Narayan et al. 2010).

The control animals (8M/8F) were injected with 100 µL Ringer solution intraperitoneally.

Forty five minutes after the injection event we collected the first dorsal and ventral dermal secretion swabs from all 31 *P. edulis* individuals. Following this, dermal secretion samples were collected at three hour intervals over two days, before collecting daily samples for an additional two days to determine whether dermal secretion GC concentrations had returned to baseline levels.

The collection of urine samples, as an additional means of technique validation, was also conducted throughout the study period. As individuals could not be left on a dry surface for extended periods of time following the ACTH and Ringer injections, we aimed to collect urine only when dermal secretion was collected throughout the entire study. Here, dermal secretion collection was conducted over a clean, decontaminated container to allow for urine collection should an individual urinate. The collected urine would then be transferred to a new, labelled Eppendorf tube, parafilmed and stored at -20˚C until analysis.

Enzyme immunoassay analysis

Biological validation

The EIA analysis of samples collected from the biological and physiological validation events were analysed at the Endocrine Research Laboratory, University of Pretoria. Mucosal extracts collected during the biological validation event (*n=36*) were measure for immunoreactive dGCs using five EIAs detecting 11, 17 dioxoandrostanes (11, 17 DOA), 11, 17, 21-trihydroxy-20-one (Cortisol), 5β-3α-ol-11-one (3α-11oxo-CM), 5α-pregnane-3β, 11β, 21-triol-20-one (37e) and Corticosterone (CC). Details of the five EIAs, including cross-reactivities, are described by Palme and Mostl (1997) for 11,17 DOA and Cortisol, Möstl et al. (2002) for 3α-11oxo-CM and Touma et al. (2003) for 37e and CC. Assay sensitivity was 0.02 ng/ml for 11,17 DOA, Cortisol and 3α-11oxo-CM, while the CC and 37e EIAs had sensitivities of 0.04 ng/ml and 0.008 ng/ml respectively. Serial dilutions of extracted samples gave displacements curves that were parallel to the respective standard curves in all assays. Intra-assay coefficient of variation (CV), determined by repeated measurements of high- and low-value quality controls was 4.04 % for 11,17 DOA, 4.64 % for cortisol, 3.29 % for 3α-11oxo-CM, 6.62 % for 37e and 4.25 % for CC. Assays were performed on microtiter plates as described by (Ganswindt et al. 2012).

Physiological validation

A total of 18 samples per individual, from all ACTH and Control animals, were analysed (Dorsal: 3 pre- and 6 post-injection; Ventral: 3 pre- and 6 post-injection). As a result of the biological validation results (see Results section below), only the CC EIA was used during this analysis. The CC EIA had an assay sensitivity of 0.04 ng/ml. Serial dilutions of extracted samples gave displacements curves that were parallel to the respective standard curves in all assays. The intra-assay CV ranged from 4.15% to 5.41%, while the inter-assay CV ranged from 10.19% to 11.20%.

URINE EIA VALIDATION & CREATININE DETERMINATION

For further validation purposes, five urine samples (2 pre/3post injection) were analysed from two male and two female individuals in the ACTH group, as well as two male and two female individuals from the saline group, using four EIAs. The sensitivities of the respective EIAs were 40pg/mL for CC, 20 pg/mL for the 3α-11oxo-CM and 11,17DOA, and 80pg/mL for 37e. The intra-assay CV ranged from 3.83-6.70%, while the inter-assay CV ranged from 6.76-15.07% for all EIAs used.

The measurement of Creatinine in urine is a test of renal function in amphibians. This was done using the Jaffe method explained in detail by Narayan et al. (2010). The results were used to calculate the concentration of uGCMs in relation to urinary creatinine concentrations.

Data analysis

Analytical statistics were performed using R software (R 3.2.1, R Development Core Team 2013).To determine whether there was a correlation between dGC concentrations excreted by the dorsal and ventral region of all individuals, a linear mixed-effect model was performed with individual ID as a random factor to account for repeated measures (R package: nlme, MuMin). Additionally, to determine whether a significant difference in

time till peak dGC concentration (post-injection) occurred between body regions and experimental groups (ACTH, control) for each sex, a one-way ANOVA was conducted.

Table 2. The time of peak dGC sample collection, the percentage dGC response to the injection event and the normality of data from the respective ACTH and control groups. Values are given as median±SD.

Male				
Group	Region	Number of peak dGC response samples	Time to peak dGC sample post-injection (hours)	Median peak percentage dGC response (post-injection)
ACTH	Dorsal	7	5.50±3.37	139.13±18.47
ACTH	Ventral	7	5.50±2.13	133.33±19.29
Control	Dorsal	8	7.00±3.11	100.00±16.90
Control	Ventral	8	4.00±3.82	103.70±37.14
Female				
Group	Region	Number of peak dGC response samples	Time to peak dGC sample post-injection (hours)	Median percentage dGC response post-injection (ng/ml)
ACTH	Dorsal	8	5.50±3.37	139.47±18.24
ACTH	Ventral	8	4.00±2.94	152.38±25.93
Control	Dorsal	8	7.00±3.45	118.35±11.09
Control	Ventral	8	4.00±2.49	112±17.34

Baseline GC levels and the adrenal response to a stressor are not uniform between individuals and sexes (Dingemanse et al. 2010); as such, baseline and elevated dGC concentration from different individuals cannot be compared directly. To determine the effect of a stressor on the HPA axis, the absolute dGC change was determined, defined as percentage dGC response, by calculating the quotient of baseline and peak dGC samples (Santymire et al. 2018). Thus, a 100% (1-fold) increase was indicative of baseline value and no change in HPA activity. For statistical analyses, only individual *peak percentage dGC response* was used for comparison between experimental groups and sexes (Tab. 2). The normality of the peak percent dGC response data was calculated using a Shapiro-Wilk test for male and female ACTH and control groups. Depending on the normality of the respective data groups, a t-test or Wilcoxon Rank sum test was used to determine: (i) whether the *peak percentage dGC response* observed in ACTH groups were significantly higher than their respective control groups and (ii) whether a significant difference in *peak percentage dGC response* exists between different body regions within specific experimental groups (ACTH: dorsal vs ventral; Control: dorsal vs ventral) of both sexes. A one-way ANOVA was conducted to determine whether a significant

difference in dorsal and ventral *peak percentage dGC response* was present between (i) male and female ACTH animals and (ii) male and female control animals. The α-level of significance was set at 0.05. Values are given as median ± SD.

Results

Dermal secretion rate

The majority of individuals (>80%) in the wet and dry groups showed ventral dermal secretion production at their first blotting event (Wet group: 20 and 30 min; Dry group: 20, 40 min); the first wet group at 10 min being the only exception. Despite continuous blotting, all individuals continued to produce ventral dermal secretion at the given time intervals until 90 min, where less than 50% of individuals of the applicable group produced ventral dermal secretion.

In contrast to the ventral region, there was only one dorsal group producing dermal secretion at the first blotting event (wet group 3, time: 30 min). Dorsal dermal secretion production was found at 30 min and 60 min for the majority of individuals in wet group 1 and 2 respectively. For the dry groups, no blotting event at any time interval showed dermal secretion production in more than 60% of the tested individuals. However, after lightly spraying the individuals of group 3 with water at 100 min, eight of the ten individuals showed signs of dermal secretion production at 140 min.

Biological validation

Three of the five EIAs (Cortisol, 11,17 DOA, 3a,11oxo-CM) used during the biological validation process displayed GC concentrations below their respective linear ranges and were thus unable to successfully monitor adrenal activity within dermal secretion samples. Similarly, less than 50% of the dermal secretion samples analysed with the 37e EIA had dGC concentrations above the linear range. The CC EIA was the only assay able to successfully monitor dGC concentrations in all dermal secretion samples analysed. The time of peak dGC concentrations differed considerably between regions, with peak samples observed after 15±25.98 min and 20±8.66 min for the dorsal and ventral regions respectively. Dorsal samples collected from three individual showed a 106±4.14% increase in dGC concentrations, while ventral samples showed a considerably higher dGC concentration increase of 140±19.09% from baseline values.

Physiological validation

There was no significant correlation in the pattern of dGC secretion between body regions of all study individuals ($p=0.29$, $t=1.06$, $df=134$, $r^2=0.04$). Similarly, no significant difference in the time until peak dGC concentration (post-injection) in ACTH and control males ($F_{3,23}=0.711$, $p=0.555$) and females ($F_{3,26}=2.98$, $P=0.69$), across both body regions, were observed. Despite this, a considerable time range within groups, as to when peak dGC samples were collected, was found (range: 45 min – 10 hours, Tab. 2, Fig. 1).

For the study males, a significant difference in *peak percentage dGC response* levels were found between dorsal samples collected from ACTH and control individuals (t-test: $t=3.75$, $df=11$, $p=0.001$, Fig. 2).

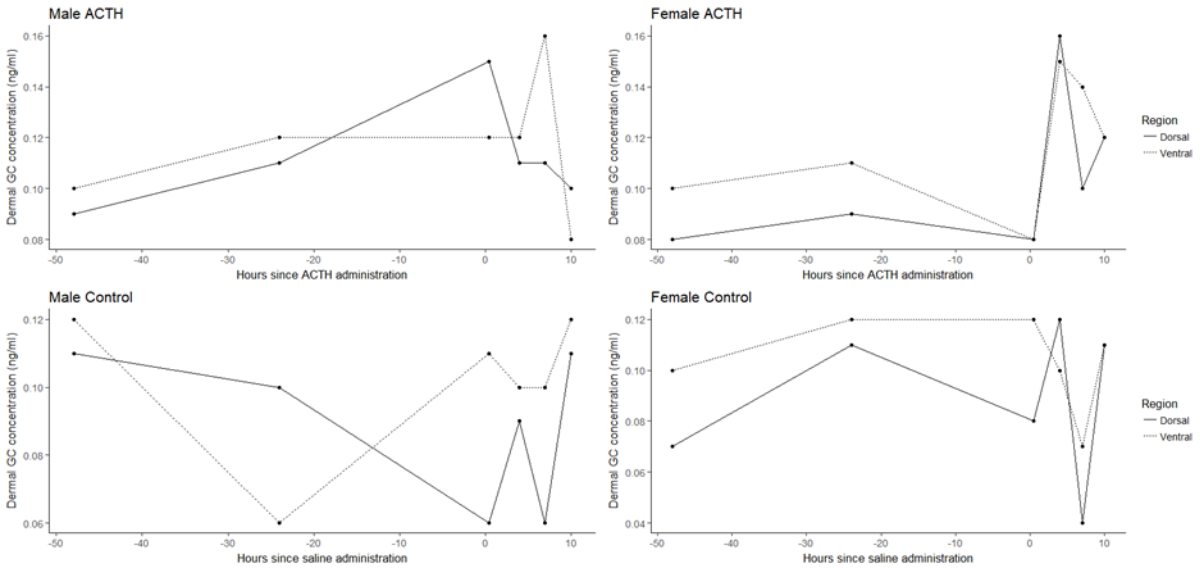

Figure 1. The dermal GC concentrations of saline and ACTH administrated male and female frogs.

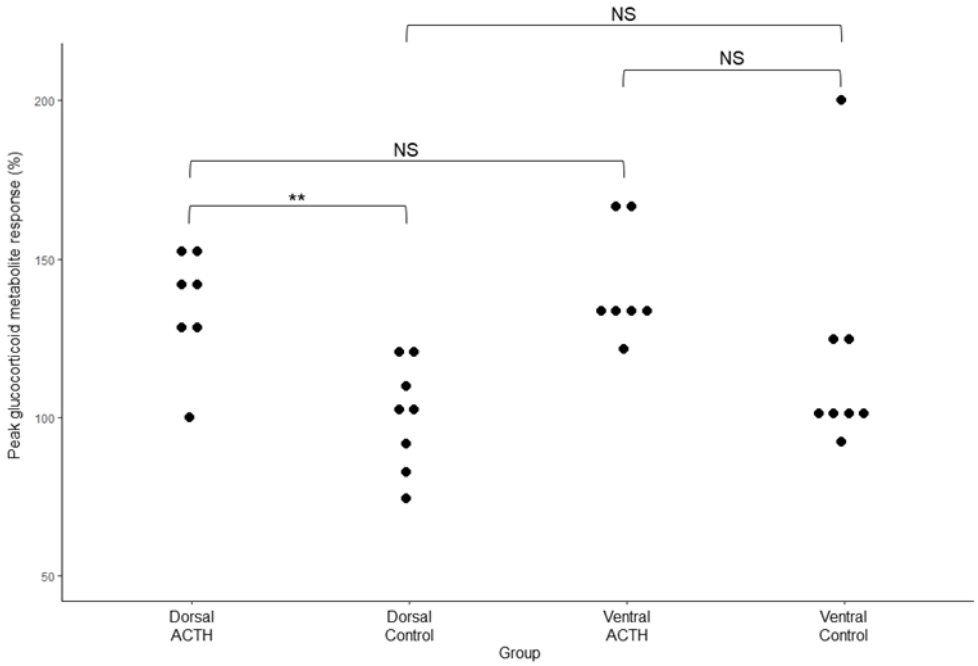

Figure 2. The difference in peak dermal glucocorticoid responses between ACTH and saline injected male frogs across both body regions monitored. Significance and non-significance levels are shown.

In contrast to this, there was no significant difference between male ACTH and control ventral samples (Wilcoxon rank sum test: W=34, p=0.06), despite a considerably higher ACTH ventral median peak percentage dGC response level (Tab. 2). To note is the presence of a single outlier in the control ventral group which had a 200% response, which may be responsible for the lack of a significance between the ACTH and control

group in this regard (Fig. 2). Finally, we found no significant difference in the *peak percentage dGC response* values of the ACTH dorsal and ventral samples (t-test: t=-0.70, df=11, p=0.50) or between the control dorsal and ventral samples (Wilcoxon rank sum test: W=18, p=0.25).

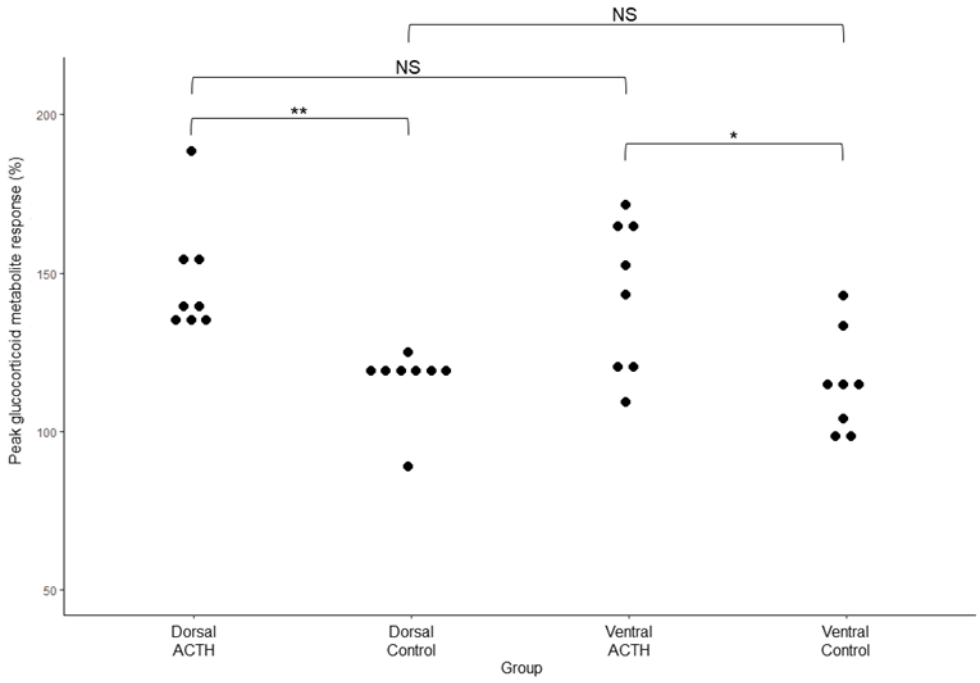

Figure 3. The difference in peak dermal glucocorticoid responses between ACTH and saline injected female frogs across both body regions monitored. Significance and non-significance levels are shown.

For the female study animals, a significant difference in *peak percentage dGC response* levels were found when comparing ACTH and control dorsal (Wilcoxon rank sum test: W=64, p=0.0009) and ventral samples (t-test: t=2.38, df=12, p=0.038). Similar to males, we found no significant difference in *peak percentage dGC response* values between ACTH dorsal and ventral samples (Wilcoxon rank sum test: W=30, p=0.86), as well as between control dorsal and ventral samples (W=35, p=0.45, Fig. 3).

A one-way ANOVA showed no significant difference in the dorsal and ventral *peak percentage dGC response* of ACTH males and females ($F_{3,26}$=0.52, p=0.67), as well as between control males and females ($F_{3,28}$=1.062, p=0.381).

Urine Results

For both male ACTH animals, the 37e and 3a-11oxo-CM EIAs showed marked increases in uGCM concentrations (200-550%) following the ACTH administration. In contrast to this both saline injected males showed a decrease in uGCM concentrations following saline administrations.

Similar to the ACTH injected males of the study, both the 37e and 3a-11oxo-CM EIAs were able to monitor an increase in uGCM concentrations; however, this was only observed in

one female (160-200% increase). Both saline injected females showed no change or a decrease in uGCM concentrations following the injection.

Unfortunately, the creatinine analysis was unable to determine creatinine quantities in the collected urine samples.

Discussion

This is the first study to validate the use of dermal secretions as a matrix for monitoring stress hormones in an African amphibian. Additionally, the study also determined the possibility of using various body regions for monitoring dGC concentrations in *P. edulis*. Finally, the study successfully described the time period required for dermal secretion recovery following a swabbing event. Although many questions remain regarding the technique, this study has provided an invaluable step forward. A final step yet to be completed is to conduct a Specific Gravity analysis on the collected urine samples to ensure the uGCM results are reliable and can be publish.

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