Experimental Manipulation of Corticosterone Does Not Affect Venom Composition or Functional Activity in Free-Ranging Rattlesnakes

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ABSTRACT

Venom is an integral feeding trait in many animal species. Although venom often varies ontogenetically, little is known about the proximate physiological mediators of venom variation within individuals. The glucocorticoid hormone corticosterone (CORT) can alter the transcription and activation of proteins, including homologues of snake venom components such as snake venom metalloproteinases (SVMPs) and phospholipase A_2 (PLA_2). CORT is endogenously produced by snakes, varies seasonally and also in response to stress, and is a candidate endogenous mediator of changes in venom composition and functional activity. Here, we tested the hypothesis that CORT induces changes in snake venom by sampling the venom of wild adult rattlesnakes before and after they were treated with either empty (control) or CORT-filled (treatment) Silastic implants. We measured longitudinal changes in whole-venom composition, whole-venom total protein content, and enzymatic activity of SVMP and PLA_2 components of venom. We also assessed the within-individual repeatability of venom components. Despite successfully elevating plasma CORT in the treatment group, we found no effect of CORT treatment or average plasma CORT level on any venom variables measured. Except for total protein content, venom components were highly repeatable within individuals ($R > 0.9$). Our results indicate that the effects of CORT, a hormone commonly associated with stress and metabolic functions, in adult rattlesnake venom are negligible. Our findings bode well for venom researchers and biomedical applications that rely on the consistency of venoms produced from potentially stressed individuals and provide an experimental framework for future studies of proximate mediators of venom variation across an individual's life span.

Keywords: hormone, rattlesnake, venom, stress, glucocorticoid.

Introduction

Venom is used in defense or prey capture by a wide array of animal groups and can vary intra- as well as interspecifically. For example, venom variation is well documented within and among populations of snakes (Minton and Weinstein 1986; Alape-Girón et al. 2008; Calvete et al. 2010, 2011; Currier et al. 2010; Massey et al. 2012; Sunagar et al. 2014; Holding et al. 2018; Zancolli et al. 2019), wasps (Perez-Riverol et al. 2017; Mathé-Hubert et al. 2019), spiders (Binford 2001), snails (Remigio and Duda 2008; Abdel-Rahman et al. 2011), and scorpions (Abdel-Rahman et al. 2009; Rodríguez-Ravelo et al. 2013). Venom can also vary within individual animals seasonally (Keegan et al. 1960; Gubensek et al. 1974; Williams and White 1992) and as they age (i.e., ontogenetic shifts; Gutiérrez et al. 1990; Andrade and Abe 1999; Mackessy et al. 2006; Underwood and Seymour 2007; Zelanis et al. 2007; Alape-Girón et al. 2008; Barlow et al. 2009; Madrigal et al. 2012; Durban et al. 2013, 2017; Jackson et al. 2016; Cipriani et al. 2017; McElroy et al. 2017; Santana et al. 2017; Borja et al. 2018), suggesting the existence of gene regulatory mechanisms that induce plasticity.

Venom plasticity and longitudinal variation in venom are best documented from ontogenetic shifts in crotaline snakes. Rattlesnake venoms exhibit shifts in the expression of specific proteins such as snake venom metalloproteinase (SVMP) and phospholipase A_2 (PLA_2) classes, which are considered adaptive in that they often mirror size-associated changes in diet (Mackessy 1996; Alape-Girón et al. 2008; Gibbs et al. 2011) and can produce changes in the killing action of venom (Mackessy 1988; Alape-Girón et al. 2008; Saviola et al. 2015). Additionally, variation in venom carries consequences for humans, as envenomated patients express varied symptoms (Bush et al. 2002; Wasserberger et al. 2006; Calvete et al.

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2011; Massey et al. 2012; Casewell et al. 2014) and varied responses to standard treatment protocols when bitten by a single species (Fry et al. 2003; Calvete et al. 2009; Núñez et al. 2009; Casewell et al. 2014; Saviola et al. 2015). The accessibility of venom as a functional trait combined with the ecological and biomedical significance of venom variation has made venom systems an emerging model for studies of adaptive variation and the genomic origins of novel function (reviewed in Casewell et al. 2020). Yet little is known about the proximate physiological mediators of venom variation, limiting studies of venom gene regulation and its evolution. To inform future studies of variation in venom, it is important to understand the proximal physiological mediators and drivers of variation in snake venoms.

A candidate mediator of venom variation is the glucocorticoid hormone corticosterone (CORT), a metabolic hormone that can exert both direct and genomic effects on many aspects of development and function in vertebrates. Circulating CORT changes in response to acute and chronic stressors (e.g., Berger et al. 2005; Lutterschmidt et al. 2009; Baugh et al. 2014; Taff and Vitousek 2016) such as drought or extensive fasting, triggering an emergency life-history stage to mobilize energy stores and downregulate nonessential processes like reproduction (Wingfield et al. 1998; Busch and Hayward 2009). Rattlesnakes are known to survive prolonged drought conditions with low prey availability (McCue 2007; McCue et al. 2012); thus, their CORT levels may be affected by prolonged fasting. CORT is a proposed mediator of ontogenetic shifts in behavior, morphology, and physiology across vertebrate groups (reviewed in Wada 2008), as well as a proposed mediator of behavioral and physiological plasticity in response to changes in environment (reviewed in Hau et al. 2016; Taff and Vitousek 2016). Size-associated changes in CORT have been documented in snakes, suggesting that circulating CORT may vary with ontogeny in some species (Gangloff et al. 2017). At the molecular level, CORT can decrease or inhibit protein synthesis by affecting transcription factor recruitment (Sapolsky et al. 2000; DesRochers et al. 2009; Duan et al. 2014) and can also influence enzyme activity through upstream actions on protein kinase activation pathways (e.g., Li et al. 2001; Lorenz et al. 2009; Kang et al. 2010; Liu et al. 2010). In the context of venom, some snake venom proteins, such as matrix metalloproteases and PLA_2 (Kini 2003; Fry and Wüster 2004; Fry 2005), are homologous with proteins that exhibit decreases in both protein activity and abundance in response to high levels of CORT (e.g., Nakano et al. 1990; Vishwanath et al. 1993; Wick et al. 1993; Chuang et al. 2015). CORT was implicated as an in vitro high-affinity inhibitor of PLA_2 type II in Russell's viper venom (Shukla et al. 2015), and plasma CORT concentrations were associated with small but detectable whole-venom compositional changes in newly captive northern Pacific rattlesnakes (Claunch et al. 2017*b*). Additionally, glucocorticoid receptor (GR) is one of the primary endocrine receptors of CORT, and GR binding sites are located near at least one three-finger toxin gene in cobras (Ma et al. 2001). When the local GRs are bound, expression of this toxin is inhibited. Furthermore, GRs can interact with transcription factor NF-κB (Ray and Prefontaine 1994), which is directly involved in the stimulation of venom production by sympathetic release of noradrenaline (Luna et al. 2009). Overall, this evidence provides strong support for the hypothesis that CORT acts as a possible proximate mediator of snake venom variation.

To explore the possibility that CORT affects venom synthesis, composition, and activity, we conducted a repeated-measures experimental study with free-ranging southern Pacific rattlesnakes (*Crotalus helleri*, Meek 1906). Individual snakes were treated either with CORT implants or with blank implants, and their venoms were compared using a before-after treatment study design. Whole-venom changes in overall composition and protein content were assessed for effects of the CORT treatment. Additionally, venom proteins with known homologues affected by CORT (PLA_2, SVMP) were assessed for changes in enzymatic activity associated with treatment. Plasma CORT levels were quantified across the study to assess the relationship between total circulating plasma CORT and venom activity. If CORT mediates significant changes in protein activity and/or concentration in snake venom, we predicted that relative to control snakes, rattlesnakes implanted with CORT would exhibit decreased PLA_2 and SVMP activity and display a greater magnitude of whole-venom compositional change between samplings. If venom is affected by CORT, the associated changes may influence the effectiveness of the venom in subduing prey (Mackessy 1988; Alape-Girón et al. 2008; Saviola et al. 2015) or the efficacy of antivenom in treating snakebite (Calvete et al. 2009; Núñez et al. 2009; Casewell et al. 2014; Saviola et al. 2015). Overall, our experimental approach will inform our understanding of the extent to which CORT mediates venom plasticity, a relationship that is important from both an ecological and a biomedical perspective.

Methods

Study Animal and Hormone Manipulation

Snake capture, blood sampling, hormone manipulation, and hormone measurement were reported previously in Claunch et al. (2017*a*). Briefly, we captured 30 adult male *Crotalus helleri* in Santa Ynez, California, from mid-April to early May 2015 and implanted them with radio transmitters in the lab (SI2-2T, Holohil Systems, Carp, Ontario). Snakes recovered for 1 or 2 d before release at the site of capture and then recovered in the field for at least 2 wk after surgery and before hormone implantation. We divided snakes into two weight classes; those greater than 800 g received two 15-mm (large) implants, and snakes less than 800 g received two 7.5-mm (small) implants. We randomly assigned snakes within each weight class to either a treatment group or a control group. We injected treatment snakes with implants filled with crystalline CORT (500 mg, Sigma C2505, lot SLBJ5337V) and injected control snakes with empty implants. Within each pair of treatment implants, one was fast release (small hole in the tubing), and one was slow release (intact). We injected implants intracoelomically on each side of the lower third of the body via a sterilized 12-g passive integrated transponder tag injexctor. The maximum total dosage for large CORT-treated snakes was 11.91 $\pm$ 1.94 SD mg/kg, while for small CORT-treated snakes it was 14.71 $\pm$ 4.23 SD mg/kg. Rattlesnakes were collected under California Department of Fish and Wildlife Scientific Collecting Permit SC-13134, and experimental procedures were

preapproved by the California Polytechnic State University Institutional Animal Care and Use Committee (protocol 1416) and the University of California at Santa Barbara Institutional Animal Care and Use Committee (protocol Taylor 1415, animal activity 027).

Sampling Frequency

On the day of hormone implantation, we sampled each snake for blood and venom immediately after capture and before implants were administered (preimplant sample). We sampled snakes again 15.05 ± 1.20 SD d later, also immediately after capture (postimplant sample). Because of depredation, hiding in burrows, or insufficient venom samples, our final sample size for analyses was reduced to four small and seven large snakes in the treatment group and six small and four large snakes in the control group. We recaptured snakes in October and November 2015 for radio transmitter removal and released them at their sites of capture after recovery.

Blood Collection

We separated plasma from whole blood via centrifugation at 10,000 rpm for 3 min and stored it at −20°C until radioimmunoassay, performed as described in Lind et al. (2010). Briefly, we extracted samples in dichloromethane and then dried them in a 40°C water bath under nitrogen gas, followed by incubation overnight in 100 μL of antiserum (Esoterix Endocrinology, Calabasas Hills, CA) and 100 μL of tritiated steroid. We separated unbound steroid from bound steroid using dextran-coated charcoal and then counted bound steroid in samples with a liquid scintillation counter. We corrected final concentrations for individual extraction efficiency. Mean recovery for CORT was 64%. We performed serial dilutions for the standard curve in triplicate (CORT curve range, 2,000–4 pg). The limit of detection was 2.5 ng/mL. We calculated the intra-assay coefficient of variation (CV) from six pooled standards run within the assay, resulting in a CV of 7.3%.

Venom Sampling

We did not extract venom from these snakes before the current study. We attempted to deplete venom stores at sampling for consistency and to induce complete regeneration at the first sample. We allowed approximately 15 d between sampling, the range reported for complete replenishment of venom proteins in other viperid species (Luna et al. 2009; Currier 2012). We collected venom via the "hands off the head" technique described in Claunch et al. (2017*b*). After collection, we immediately froze venom samples on dry ice and then transferred them to a −80°C freezer until analysis.

Snakes with a recent meal (determined via palpation) gave visibly smaller venom samples, likely having recently spent venom on the meal. However, we observed food bulges in snakes only during the preimplant sampling (i.e., meals are unlikely to affect the interpretation of the CORT implants). After we collected venom, we released the snakes at the site of capture. The preimplant venom sample from one small treatment snake was too small to quantify, and one large control snake had only a preimplant sample, so we excluded these snakes from venom analysis because of the repeated-measures design.

Venom Composition

Reversed-phase high-performance liquid chromatography (HPLC) at 220 nm was used to assay changes in protein composition. First, we resuspended venom samples in liquid chromatography–mass spectrometry water and diluted them to a concentration of 0.18 mg/mL. We determined venom protein concentration from the A280 reading from a NanoDrop spectrophotometer (ThermoFisher Scientific). We then loaded 15 μg of venom protein onto a Prominence HPLC system (Shimadzu). We ran samples through a Phenomenex Aeris WIDEPORE column (3.6 μm, C18, part 00G-4482-AN) maintained at 25°C and then eluted samples with solution A as 0.1 mM trifluoroacetic acid (TFA) in water and solution B as 0.06 mM TFA in acetonitrile. We measured absorbance at 220 nm over 135 min with the following run parameters: 5 min at 10% B, 110 min increasing from 10% to 55% B, 5 min increasing from 55% to 75% B, 5 min at 75% B, 5 min decreasing from 75% to 10% B, and 5 min at 10% B. We quantified venom peak areas using Shimadzu LabSolutions 2 software, combining default peak calls by the i-PeakFinder algorithm with manual curation of the peak baseline and filtering of peaks making up less than 0.1% of the total area as noise. We aligned the control and treatment HPLC profiles from each individual and called peaks that were present. As such, within-individual peak identities may be unique to each individual. To secondarily measure changes in particular protein classes known to elute at certain times on the HPLC, we divided HPLC profiles into four separate regions known to contain different venom protein classes for analysis: (1) myotoxin, (2) bradykinin-potentiating peptide (BPP), (3) snake venom serine protease (SVSP)/PLA_2, and (4) SVMP (Holding et al. 2018).

Venom Total Protein Concentration

We determined the protein concentration of each venom sample using the microassay protocol of the Quick Start Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA) with a bovine serum albumin standard. Whole venom was diluted 1 : 200 in phosphate-buffered saline (PBS) before the protein concentration was determined.

Venom Activity Levels

We determined the PLA_2 and SVMP activity of each venom sample as measures of venom function. We measured the SVMP activity of each venom sample using the standard product protocol in the EnzChek gelatinase/collagenase kit (Life Technologies, Carlsbad, CA), as this kit has been previously shown to measure the metalloproteinase activity of rattlesnake venoms (Biardi et al. 2011). We diluted the fluorescently labeled gelatin substrate to a concentration of 1 : 50 and then placed 0.5 μg of venom diluted in PBS in each reaction well on a 96-well microplate (Corning 3912, Corning, Tewksbury, MA). We measured fluorescence intensity in relative fluorescence units (RFUs) every 1 min after the addition

to the gelatin substrate using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany), subtracting the background fluorescence of blank (substrate-only) wells from each measurement. From these data, we calculated the slope (RFU/min) from the linear part of the reaction (0–9 min after substrate addition), which we used as our measure of SVMP activity. We measured each venom in triplicate and used the average slope of the three reactions in analyses. Larger slopes indicate that the venom is degrading the substrate more quickly, which indicates higher SVMP enzymatic activity in the focal sample.

We assayed venom PLA_2 activity like we assayed SVMP activity, using the EnzChek PLA_2 assay kit (Life Technologies, Waltham, MA) following standard product protocols. Again, we pipetted 0.5 μg of venom into each well and measured fluorescence intensity every minute. We used the linear part of the reaction (1–15 min) to calculate the slope of each reaction. We ran samples in duplicate and used the average of the two reactions for further analyses.

Statistical Analyses

Statistical analyses and results for circulating plasma CORT are reported in Claunch et al. (2017*a*). All statistical analyses were conducted in R (ver. 3.4.0 patched; R Core Team 2017). We assessed changes in venom composition between treatments in several ways. First, we conducted multivariate analyses of isometric log ratio (ilr)–converted regionalized venom composition data using the compositions package (van den Boogart et al. 2018). The ilr transformation takes the data from the simplex to real space and removes the sum constraint at the cost of one dimension of the data set. We conducted permutational multivariate ANOVA (perMANOVA) using the Euclidean distance with 10,000 permutations via the adonis function in the vegan package (Oksanen et al. 2018) in R. The first model includes venom composition as the response and treatment group (CORT or blank implant), time (before or after implantation), and an interaction between the two as predictors. If our CORT treatment affects venom composition, we would expect a significant treatment by time interaction where CORT-treated snakes differ in venom composition between pre- and postimplant samples. To assess potential influences of fine-scale fluctuation in CORT on venom, we also ran a model including plasma CORT and time as predictors. We calculated the partial η^2 (η_p^2) to report effect sizes (Lakens 2013). Second, we subjected regionalized venom composition data to centered log ratio transformation, which preserves the relationships among original variables, for univariate analysis (described in detail below). Finally, we calculated the Aitchison (1986) compositional distance between the pre- and postimplant samples from each snake using the aDist function in the zCompositions package (Palarea-Albaladejo and Martin-Fernandez 2015) on nonregionalized ilr-transformed data (Templ et al. 2011) as a robust measure of the change in compositional distance between the paired samples. We describe univariate analysis of Aitchison distance below.

We conducted univariate linear models to assess the changes in protein-grouped composition, whole-venom composition (Aitchison distance), protein activities, and whole-venom protein content associated with treatment. Response variables tested in separate models included Aitchison distance between paired samples, changes in the composition of regions 1–4, total protein concentration of venom, PLA_2 activity, and SVMP activity. For all but Aitchison distance (which is already a measure of multivariate compositional change), the model responses were calculated by subtracting the values of the postimplant sample from those of the preimplant sample for each individual. All univariate models were assessed using ANCOVA in R (ver. 3.4.0 patched). To satisfy model assumptions of the normal distribution of residuals, Aitchison distance was log transformed. For each response above, two models were run to assess coarse and fine-scale effects of the treatment. The first model included treatment as a factor, and the second included the measured change in plasma CORT concentration regardless of treatment as a continuous variable; these predictors could not be included in the same model because of collinearity. Covariates included size class (to account for differences in implant size) and time between extractions (in days). SVMP activity naturally separated into two distinct groups unrelated to treatment assignment or implant type. To improve SVMP model fits, we included a grouping variable of SVMP expression (high/low). Linear models were also constructed to assess the potential relationship between the changes in regional composition and activity for PLA_2 and SVMP in regions 3 and 4, respectively. Interactions between size class and either treatment or change in CORT were included only if their inclusion visually improved model fit in residuals versus predicted plots compared with the model without the interaction. To account for false detection rate (FDR) with multiple hypothesis testing (Benjamini and Hochberg 1995), we grouped models with the same response variable and corrected all the *P* values reported within each group using the p.adjust function in R with method BH (R Core Team 2017). We calculated η_p^2 to report effect sizes (Lakens 2013). Univariate final models, results, FDR corrections, and effect sizes are reported in tables 2–4. If our CORT treatment affected any of our response variables, we would expect treatment to be a significant predictor of the change in the response variable.

Finally, we conducted a repeatability analysis to determine the degree to which venom components were repeatable across samples within individual snakes. We used the package rptR to assess the degree of within-group repeatability in the measurement of interest and conduct a likelihood ratio test to assess the input model against a null model excluding the grouping factor (Nakagawa and Schielzeth 2010; Stoffel et al. 2017). We assessed the repeatability of activity, total protein, and regional protein composition of venom with snake ID as the grouping variable. Values of *R* approaching 1 indicate that venom composition and activity were highly repeatable within individuals (i.e., most variation is among rather than within individuals), while *R* values approaching 0 indicate little repeatability (i.e., most variation is within rather than among individuals).

Results

Effects of CORT Treatment on Plasma CORT

Results were reported previously in detail in Claunch et al. (2017*a*). Plasma CORT levels did not differ initially between

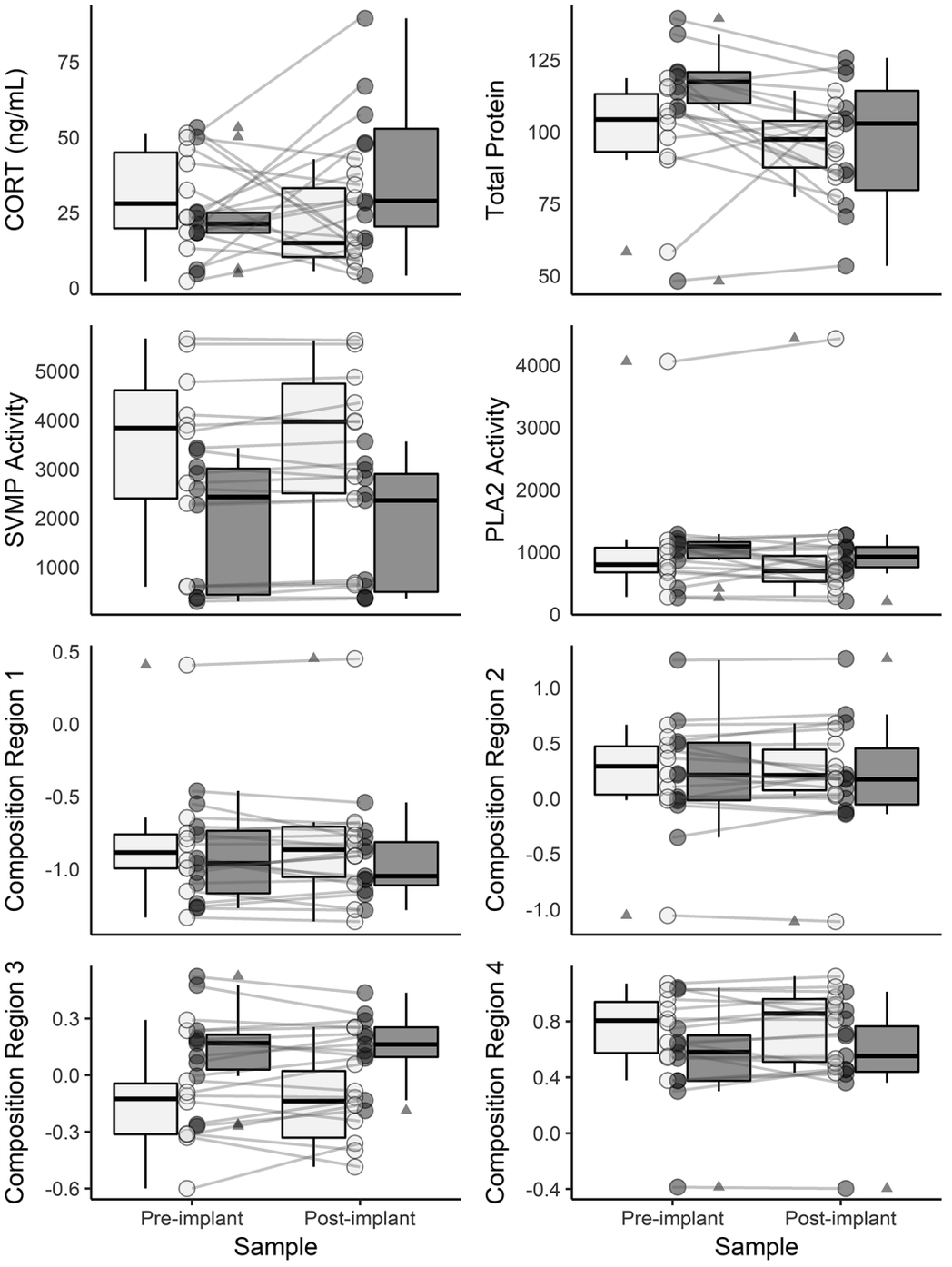

Figure 1. Effects of repeated sampling and corticosterone (CORT) implant treatment on *Crotalus helleri* plasma CORT and venom variables. White indicates snakes with blank (control) implants ($n = 10$), while gray indicates snakes treated with CORT implants ($n = 11$). Triangles indicate outliers of the box and whisker plots. Circles indicate samples from individual snakes and are shaded semitransparently so that overlapping data points are more easily visualized. Lines connect samples from individual snakes; steeper slopes indicate greater within-individual changes. Despite increases in plasma CORT associated with the CORT implant treatment, all venom variables except for total protein content were highly repeatable within individuals from pre- to postimplant samples. Venom composition regions 1–4 are primarily composed of myotoxin, bradykinin-potentiating peptide, snake venom serine protease/phospholipase A_2 (PLA_2), and snake venom metalloproteinase (SVMP), respectively.

treatment groups but increased by 60% in snakes treated with CORT implants relative to controls 15 d after implantation (fig. 1).

Effects of CORT Treatment on Venom Composition

There was no detectable effect of CORT treatment on whole-venom composition, as confirmed by a lack of treatment by time interaction in the perMANOVA and the fact that the Aitchison distance of complete (nonregionalized) venom composition between before and after implantation was not affected by treatment group, plasma CORT, or any covariates (table 1; figs. 1–5). Despite random assignment, CORT and blank groups may have differed slightly in venom composition both before and after implantation because of random partitioning of individual variation within the population, as indicated by a significant main effect of treatment group in the perMANOVA before FDR correction. Despite this, there were also no detectable differences in the magnitude of the change of composition in regions 1–4 before and after implantation among treatment groups after FDR correction (fig. 1; table A1), indicating no distinguishable effects of CORT treatment on myotoxin, BPP, SVSP/PLA_2, or SVMP composition.

Effects of CORT Treatment on Venom Protein Concentration and Activity

After FDR correction, changes in total protein, SVMP activity, and PLA_2 activity between before and after implantation were unrelated to treatment group, plasma CORT, or covariates (table 2). Changes in PLA_2 activity were weakly negatively correlated with changes in the relative composition of region 3 (primarily SVSP/PLA_2), such that increases in relative composition were marginally correlated with increases in PLA_2 activity ($P = 0.021$; table 3). Changes in the relative composition of region 4 (primarily SVMP) were unrelated to changes in SVMP activity (table 3).

Repeatability of Venom Components within Individual Snakes

All measured venom activity and grouped venom compositions were highly repeatable within individual snakes (all $R > 0.9$; all $P < 0.001$), except for total protein content, which was relatively less repeatable ($R = 0.42$; $P = 0.03$; table 4; figs. 1–5).

Discussion

To our knowledge, this is the first study to assess repeated venom samples after exogenous manipulation of hormones in free-ranging snakes. Though CORT-treated snakes exhibited 60% higher plasma CORT than controls (Claunch et al. 2017*a*), CORT treatment did not affect whole or regional venom composition, venom activity of SVMP or PLA_2 groups, or total protein concentration. Our lack of an observed effect could be interpreted in a few ways. First, our results may be related to threshold effects. Increased CORT is implicated in many ontogenetic shifts, but the magnitude and duration of the CORT increase required to induce these changes likely differ across species and individuals (Wada 2008). As such, threshold levels of CORT required to induce fitness

Table 1: Effects of corticosterone (CORT) and blank (control) implants on the venom composition of *Crotalus helleri*

Measurement, model, variable	df	*F*	*P*	Adjusted *P*	η_p^2
Whole-venom composition (perMANOVA):					
Model 1:					
Treatment	1, 38	3.115	**.042**	.210	.076
Time	1, 38	.012	.998	.999	.0003
Treatment × time	1, 38	.005	.999	.999	.0001
Model 2:					
Log CORT	1, 39	.851	.439	.999	.021
Time	1, 39	.011	.998	.999	.0003
Log Aitchison distance:					
Model 1:					
Treatment	1, 16	.073	.791	.952	.004
Size class	1, 16	.273	.608	.952	.017
Time between extractions	1, 16	.006	.939	.952	.0004
Treatment × size class	1, 16	.177	.679	.952	.107
Model 2:					
Change in CORT	1, 16	.037	.851	.952	.002
Size class	1, 16	.265	.614	.952	.016
Time between extractions	1, 16	.004	.952	.952	.0002
Change in CORT × size class	1, 16	.713	.411	.952	.042

Note. Whole-venom composition before and after implant treatment was assessed via permutational multivariate ANOVA (perMANOVA), and the change in composition between pre- and postimplant samples (log Aitchison distance) was assessed via ANOVA. Adjusted *P* values represent false detection rate corrections for multiple hypothesis testing applied to all *P* values within each measurement. Partial η^2 (η_p^2) effect sizes are reported. A value of *P* below the threshold of $\alpha = 0.05$ is in bold.

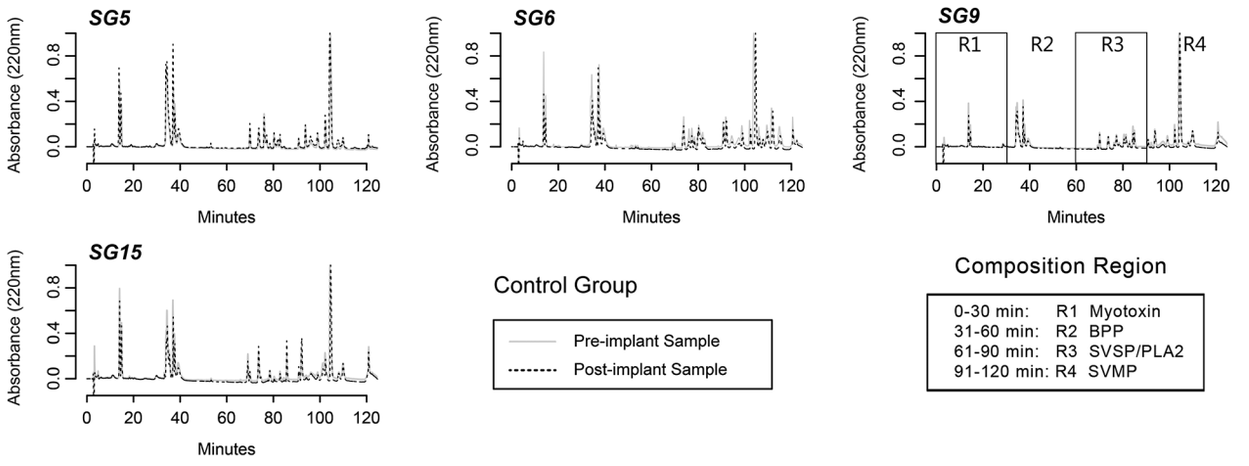

Figure 2. Chromatograms from high-performance liquid chromatography of venom from four individual *Crotalus helleri* treated with blank implants (control group; $n = 10$) indicating venom composition from pre- and postimplant samples, which are overlaid on the same plot for each individual. Preimplant samples are indicated by solid light gray lines, while postimplant samples are indicated by dotted black lines. Notice the nearly complete overlap of each sample, indicating very little change in relative venom composition between samples. Representative venom composition regions (R1–R4) are demarcated on SG9 to indicate regions used in analyses. BPP = bradykinin-potentiating peptide; SVSP = snake venom serine protease; PLA_2 = phospholipase A_2; SVMP = snake venom metalloproteinase.

effects are often not known (Jessop et al. 2013). It is possible that our manipulation of circulating CORT in a biological system did not reach the necessary thresholds to induce changes like the in vitro inhibition of venom PLA_2 activity demonstrated by Shukla et al. (2015). If venom composition in crotalids is influenced by ultimate and irreversible ontogenetic changes, as with metamorphosis among many amphibians (Wada 2008), it is also possible that no level of CORT would induce a change in adult snake venom.

On the other hand, components of venom may respond to CORT differently or in opposition, such that effects are not detectable. For example, although we predicted that CORT would cause decreases in venom proteins, we did not see an effect of implant treatment on total venom protein concentration. Protein concentration is a coarse measure of the amount of protein per unit of fluid. The liquid components of venom appear to be regulated at a different timescale than protein production and may be influenced by external factors. Protein concentration of venom is high early in the replenishment

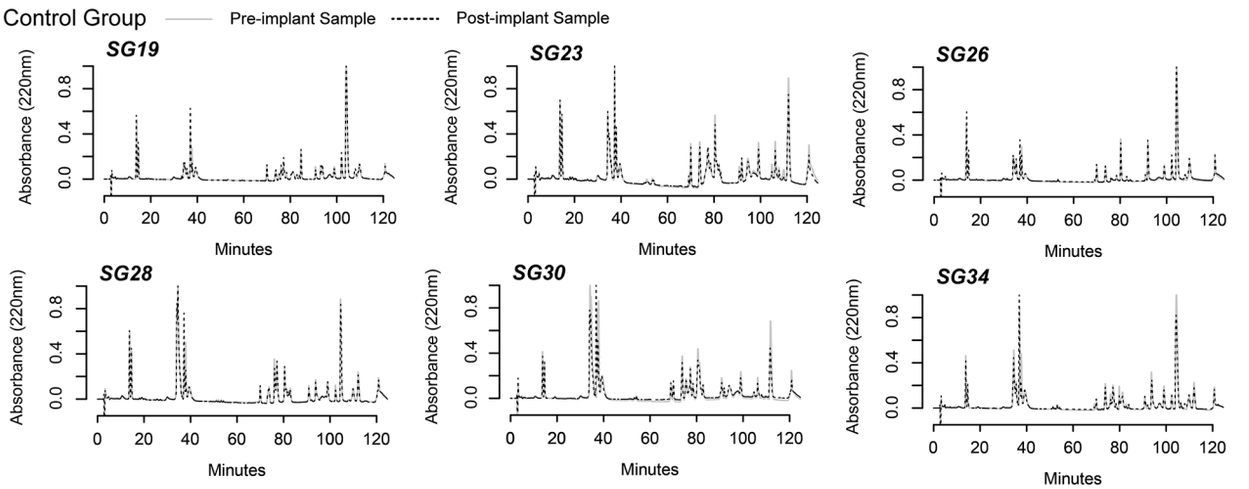

Figure 3. Chromatograms from high-performance liquid chromatography of venom from the remaining six individual *Crotalus helleri* treated with blank implants (control group; $n = 10$) indicating venom composition from pre- and postimplant samples, which are overlaid on the same plot for each individual. Preimplant samples are indicated by solid light gray lines, while postimplant samples are indicated by dotted black lines. Notice the nearly complete overlap of each sample, indicating very little change in relative venom composition between samples. See figure 2 for a visual representation of the venom composition regions used in the analyses.

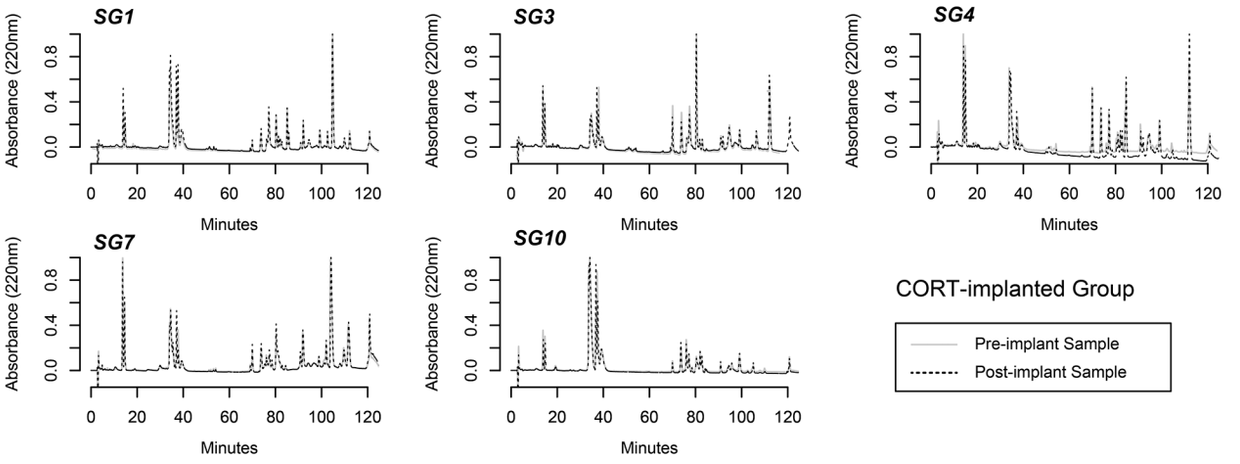

Figure 4. Chromatograms from high-performance liquid chromatography of venom from five individual *Crotalus helleri* treated with corticosterone (CORT) implants (treatment group; $n = 11$) indicating venom composition from pre- and postimplant samples, which are overlaid on the same plot for each individual. Preimplant samples are indicated by solid light gray lines, while postimplant samples are indicated by dotted black lines. Notice the nearly complete overlap of each sample, indicating very little change in relative venom composition between samples. See figure 2 for a visual representation of the venom composition regions used in the analyses.

phase compared with several days later (Willemse et al. 1979; Currier 2012), which is more likely due to liquid components of venom increasing over time than to destruction of just-synthesized proteins (Rokyta et al. 2015). CORT can cross-react with mineralocorticoid receptors such as aldosterone receptors, which leads to water reabsorption into the bloodstream (Gaeggeler 2005; Thunhorst et al. 2007; Morris et al. 2009). If water is withheld from venom fluid when CORT is high, this could lead to an apparent increase in the protein concentration of venom. However, if CORT also decreases relative protein abundance in venom, no effect on total protein concentration would be detected because both fluid and proteins would decrease. This type of response to elevated CORT could be adaptive, as it may ensure similar venom potency despite decreased overall venom yield during periods of starvation. While this makes intuitive sense, we observed no changes in relative protein abundances when assessing the regional composition of venom. One consideration, however, is that the ability to observe change in venom compositional data is dependent on the change of the abundance of proteins in one or more groups at differing magnitudes. We could observe the scenario described above only if

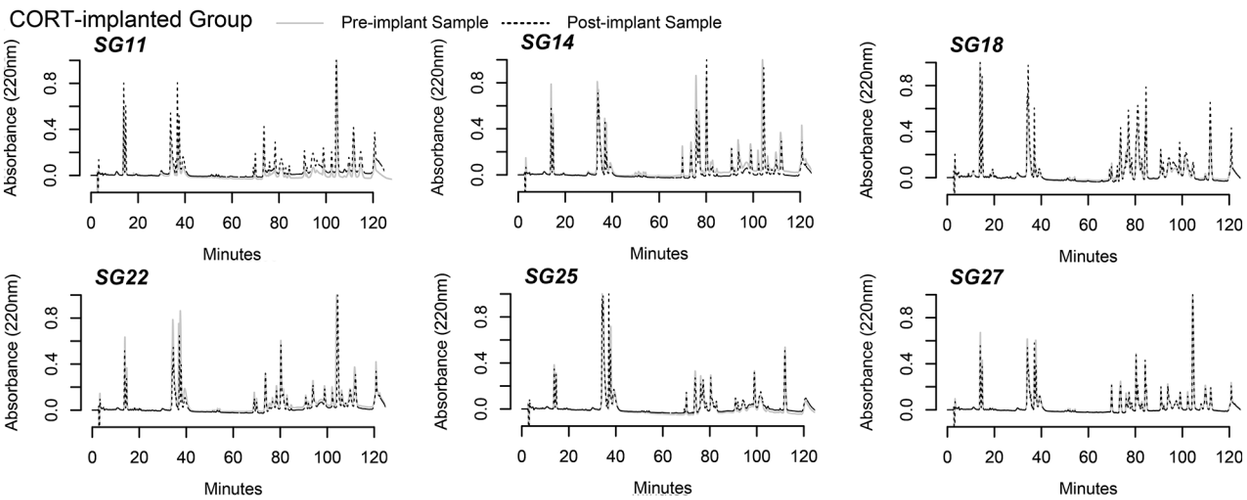

Figure 5. Chromatograms from high-performance liquid chromatography of venom from the remaining six individual *Crotalus helleri* treated with corticosterone (CORT) implants (treatment group; $n = 11$) indicating venom composition from pre- and postimplant samples, which are overlaid on the same plot for each individual. Preimplant samples are indicated by solid light gray lines, while postimplant samples are indicated by dotted black lines. Notice the nearly complete overlap of each sample, indicating very little change in relative venom composition between samples. See figure 2 for a visual representation of the venom composition regions used in the analyses.

Table 2: Results from an ANOVA assessing changes in venom and hormone levels in *Crotalus helleri* after corticosterone (CORT) implant treatment

Measurement, model, variable	df	F	P	Adjusted P	η_p^2
Change in total protein:					
Model 1:					
Treatment	1, 16	1.721	.208	.712	.093
Size class	1, 16	.032	.860	.943	.002
Time between extractions	1, 16	.726	.407	.712	.039
Model 2:					
Change in CORT	1, 15	.005	.943	.943	.0003
Size class	1, 15	.066	.900	.943	.0038
Time between extractions	1, 15	.808	.383	.712	.047
Change in CORT × size class	1, 15	1.434	.250	.712	.083
Change in PLA_2 activity:					
Model 1:					
Treatment	1, 16	.0035	.953	.953	.0002
Size class	1, 16	6.381	**.022**	.132	**.278**
Time between extractions	1, 16	.536	.475	.57	.023
Model 2:					
Change in CORT	1, 16	1.558	.230	.46	.069
Size class	1, 16	4.261	.056	.168	**.190**
Time between extractions	1, 16	.602	.449	.57	.027
Change in SVMP activity:					
Model 1:					
Treatment	1, 14	.740	.404	.955	.043
Size class	1, 14	.009	.927	.955	.0005
Time between extractions	1, 14	.012	.916	.955	.0007
SVMP expression group	1, 14	.568	.464	.955	.033
Treatment × size class	1, 14	1.981	.181	.955	.114
Model 2:					
Change in CORT	1, 14	.012	.914	.955	.0008
Size class	1, 14	.092	.766	.955	.006
Time between extractions	1, 14	.003	.955	.955	.0002
SVMP expression group	1, 14	.308	.588	.955	.021
Change in CORT × size class	1, 14	.421	.527	.955	.028

Note. Measurement indicates a response variable. Adjusted P values represent false detection rate corrections for multiple hypothesis testing applied to all P values within each measurement. Partial η^2 (η_p^2) effect sizes are reported. A value of P below the threshold of $\alpha = 0.05$ and η_p^2 values above 0.14 are in bold. PLA_2 = phospholipase A_2; SVMP = snake venom metalloproteinase.

CORT decreased fluid retention and also caused proportional decreases in all protein regions, such that relative composition did not change. Applying the principle of parsimony, it is highly unlikely that CORT could suppress transcription of each protein region equally and more likely that CORT does not exert measurable effects on venom protein concentration in rattlesnakes.

In a related vein, protein expression and abundance in venom do not necessarily correlate to activity (Currier et al. 2010), which is confirmed by a lack of correlation between composition and activity in both SVMP and PLA_2 in our data (after adjusting P values). In any given biological system, the proportion of active to inactive isoforms of a given protein may change to compensate for a change in protein abundance, and changes in protein abundance and in proportions of active proteins can be independent of one another (Glanemann et al. 2003; Currier et al. 2010), such that CORT may affect each differently. Activation of proteins is often achieved through phosphorylation by various protein kinases (Alberts et al. 2015). While CORT is documented to exhibit rapid nongenomic effects, particularly in the activation of protein kinase pathways (Li et al. 2001; Liu et al. 2010), we found no evidence of CORT influencing either the activity or the composition of the protein groups. Again, it is more parsimonious that CORT does not exert effects on protein composition and activity than the alternative scenario, in which CORT causes equal decreases in all protein regions, preserving relative composition, and also increases the proportion of active isoforms of both PLA_2 and SVMP to maintain activity levels similar to those observed in pretreatment and control samples.

A likely possibility is that protein synthesis and existing proteins within the venom gland of *Crotalus helleri* are protected from or not sensitive to circulating CORT. Although the transcription of proteins is known to be affected by CORT (Sapolsky et al. 2000;

Table 3: Results from an ANCOVA assessing relationships between compositional and activity changes in *Crotalus helleri* venoms after corticosterone (CORT) implant treatment

Measurement, variable	df	F	P	Adjusted P	η_p^2
Change in composition of region 3:					
Change in PLA_2 activity	1, 15	6.691	**.021**	.084	**.283**
Treatment	1, 15	.042	.840	.840	.002
Size class	1, 15	.586	.456	.608	.025
Change in PLA_2 activity × treatment	1, 15	1.324	.268	.536	.056
Change in composition of region 4:					
Change in SVMP activity	1, 15	1.855	.193	.386	.093
Treatment	1, 15	.000	.996	.996	.000001
Size class	1, 15	2.786	.116	.386	**.141**
Change in SVMP activity × treatment	1, 15	.173	.684	.912	.009

Note. Compositional regions 3 and 4 are primarily composed of snake venom serine protease/phospholipase A_2 (PLA_2) and snake venom metalloproteinase (SVMP), respectively. Adjusted P values represent false detection rate corrections for multiple hypothesis testing applied to all P values within each measurement. Partial η^2 (η_p^2) effect sizes are reported. A value of P below the threshold of $\alpha = 0.05$ and η_p^2 values above 0.14 are in bold.

DesRochers et al. 2009; Duan et al. 2014), it is unknown whether the venom gland has GRs. Indeed, in other animals, tissue-specific expression of GRs is documented (e.g., Butts et al. 2011; Hoffman et al. 2015), with tissues lacking GRs showing functional insensitivity to CORT. If tissue-specific GR expression is heritable and if there is a benefit to CORT resistance in the transcription of venom proteins, it is possible that rattlesnakes may be largely evolutionarily insensitive to chronic CORT elevation (Claunch et al. 2017*a*; Stepanek et al. 2019). Rattlesnakes are adapted to withstand long periods without food, and resistance to CORT during these periods may ensure survival, such that venom remains potent for a chance encounter with prey. This hypothesis warrants investigation of GR expression and the effects of CORT in drought-tolerant venomous species compared with those with more consistent prey availability (e.g., *Agkistrodon* spp.).

Finally, the current best evidence for glucocorticoids affecting snake venom is limited to a correlative lab study (Claunch et al. 2017*b*) and two in vitro studies investigating the effects on specific protein isoforms in transfected mammalian cells (Ma et al. 2001) or already-expelled venom (Shukla et al. 2015). Although conducted on a similar timescale, the lab study did not effectively manipulate CORT in snakes as in the current study. The reported decreases in SVSP and SVMP and increases in PLA_2 with higher plasma CORT may be purely correlative. While dexamethasone (a synthetic glucocorticoid) decreased promoter activity of cobra cardiotoxin in transfected mammalian cells (Ma et al. 2001), this toxin is not present in rattlesnake venoms, so we are unable to draw direct comparisons to that study. The other in vitro study may be limited in scope to either the isoform of PLA_2 tested or the effects of already-expelled venom. Our measure of activity was coarse (i.e., not restricted to different isoforms), and we did not find an effect on overall PLA_2 activity. There are a variety of PLA_2 proteins present in venoms (Sunagar et al. 2014), and it is possible that CORT inhibition may be limited to specific protein isoforms, such as the type IIA isoform of PLA_2 (Shukla et al. 2015; see also Nakano et al. 1990; Wick et al. 1993), but other isoforms remain to be tested. It is also possible that the effects of CORT are detectable only after the venom is expelled, as the abovementioned studies investigated in

Table 4: Repeatability analysis of venom components in *Crotalus helleri*

Measurement	R	D	df	P	CI
Total protein composition	.42	3.56	1, 42	.0296	.004–.718
SVMP activity	**.994**	**85.9**	**1, 41**	**<.001**	**.985–.997**
PLA_2 activity	**.929**	**39.6**	**1, 41**	**<.001**	**.835–.969**
Composition region 1	**.967**	**56.1**	**1, 42**	**<.001**	**.921–.986**
Composition region 2	**.962**	**53.7**	**1, 42**	**<.001**	**.911–.962**
Composition region 3	**.921**	**38.5**	**1, 42**	**<.001**	**.820–.963**
Composition region 4	**.955**	**50.0**	**1, 42**	**<.001**	**.895–.981**

Note. Values of R indicate the repeatability statistic; values approaching 1 indicate that the variable was highly repeatable within individuals, whereas values approaching 0 indicate low repeatability. Values in bold were found to be highly repeatable. The D statistic, df, 95% confidence interval (CI), and P value for the likelihood ratio test of a model including snake ID as a grouping variable against a null model are also given. Venom composition regions 1–4 are primarily composed of myotoxin, bradykinin-potentiating peptide, snake venom serine protease/phospholipase A_2 (PLA_2), and snake venom metalloproteinase (SVMP), respectively.

vitro effects of CORT on specific proteins. Protein components within the venom gland may appear to be inhibited but change structure and become activated when expelled from the gland (Mackessy and Baxter 2006). Thus, CORT may have different effects on venom components in vitro and within the venom gland itself as a result of differences in the structural availability of inhibition sites. Overall, we found no evidence of in vivo effects from experimental CORT elevation on *C. helleri* venom components, which is most likely due to resistance of the venom gland to the genomic and nongenomic effects of CORT.

Ultimately, despite elevation of CORT, we found that venom activity, composition, and protein content were highly repeatable within individual free-ranging snakes. This finding is in concordance with the conclusions of Gregory-Dwyer et al. (1986) and Claunch et al. (2017*b*), which bodes well for researchers who rely on the consistency of multiple samples from the same individuals for research or antivenom production (Chippaux et al. 1991; Salazar et al. 2009; Currier 2012). The high degree of among-individual differences in venom traits corroborates studies reporting substantial among-individual variation within *C. helleri* as an important factor to consider in antivenom production (e.g., Sunagar et al. 2014). Specifically, these findings underscore the importance of maintaining multiple individuals with varied venoms for the purpose of antivenom production. Although most metrics were highly repeatable, some variation was evident within individuals, especially in total protein (fig. 1). This variation, while minimal, could be due to factors beyond control in a field study. Repeated sampling within a span of days can affect the overall protein concentration of venom (Willemse et al. 1979), but an effect of time between extractions was not evident in our analyses. Gibbs et al. (2011) reported substantial changes in PLA_2 composition in rattlesnake venom in response to a change in diet. Snakes in our study were not observed with food bulges between sampling periods, so all snakes likely fasted between samplings. Therefore, while inherent differences in diet may still explain variation among snakes, consumed prey likely did not affect the repeatability of the samples during the course of this experiment. We cannot rule out the possibility that snakes expended venom in defense or during unsuccessful predatory attempts, which may account for the observed variation in protein concentration. Overall, even with the uncertainty present in a field experiment, venom composition and activity were highly repeatable within individual snakes.

Our elevation of baseline CORT in the treatment snakes is within reported CORT levels from several species of free-ranging rattlesnakes, including the sister species *Crotalus oreganus* (Holbrook 1840; Schuett et al. 2004; Lutterschmidt et al. 2009; Lind et al. 2010; Holding et al. 2014), suggesting that the CORT implants elevated circulating CORT to within physiologically relevant levels. There is also a strong likelihood that CORT-implanted snakes experienced higher levels of CORT early in the study as an initial spike after implantation than when we took blood samples after 15 d (Romero et al. 2005; DesRochers et al. 2009; Müller et al. 2009). Despite experimental elevation of CORT during venom replenishment, we show that SVMP, PLA_2 activity, protein concentration, and total and regional venom composition of *C. helleri* venom were largely unaffected. Venom was highly repeatable, even under the influence of increased CORT. Although our study was limited to the duration of time over which venom is replenished (15 d), we doubt that a change in venom at longer timescales would be apparent without substantial changes to the endocrine signaling pathways associated with CORT. In the context of venom research for biomedical purposes, this result is reassuring, as it suggests that highly consistent venom samples can be collected repeatedly from the same adult individuals over a short timescale. In an ecological context, the resistance of venom to a common physiological mediator of plasticity suggests that individual venom consistency is integral to survival. We believe that studies similar to ours should be conducted on young individuals and with other hormones that vary during ontogeny, such as testosterone (King et al. 2000; Taylor et al. 2004; but see Schonour et al. 2020). Much about the potential physiological mediators of snake venom and the contexts in which these potential mediators are expressed in wild venomous snakes remains to be investigated. Despite previous suggestions that glucocorticoids could be important for venom regulation in adult snakes, our study provides experimental evidence suggesting otherwise and therefore calls for studies of other candidate mechanisms as the key proximate mediators of venom phenotypic plasticity.

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APPENDIX

Table A1: Results from an ANOVA assessing changes in venom and hormone levels in *Crotalus helleri* after corticosterone (CORT) implant treatment

Measurement, model, variable	df	F	P	Adjusted P	η_p^2
Change in composition of region 1:					
Model 1:					
Treatment	1, 17	.418	.526	.807	.018
Size class	1, 17	.279	.604	.807	.012
Time between extractions	1, 17	5.982	**.026**	.09	**.253**
Model 2:					
Change in CORT	1, 17	.061	.807	.807	.003
Size class	1, 17	.185	.673	.807	.008
Time between extractions	1, 17	5.583	**.030**	.09	**.245**
Change in composition of region 2:					
Model 1:					
Treatment	1, 16	.059	.812	.812	.003
Size class	1, 16	.487	.495	.685	.026
Time between extractions	1, 16	.310	.586	.685	.017
Treatment × size class	1, 16	1.827	.195	.685	.098
Model 2:					
Change in CORT	1, 16	.815	.379	.685	.043
Size class	1, 16	1.005	.330	.685	.053
Time between extractions	1, 16	.307	.587	.685	.016
Change in composition of region 3:					
Model 1:					
Treatment	1, 16	.044	.836	.836	.002
Size class	1, 16	.405	.534	.760	.017
Time between extractions	1, 16	2.578	.128	.470	.109
Treatment × size class	1, 16	4.544	**.048**	.470	**.193**
Model 2:					
Change in CORT	1, 17	.740	.402	.760	.037
Size class	1, 17	.171	.684	.760	.009
Time between extractions	1, 17	2.20	.157	.470	.109
Change in composition of region 4:					
Model 1:					
Treatment	1, 14	.069	.796	.947	.004
Size class	1, 14	2.431	.141	.517	**.146**
Time between extractions	1, 14	.032	.861	.947	.002
SVMP expression group	1, 14	.092	.767	.947	.005
Treatment × size class	1, 14	.057	.815	.947	.003
Model 2:					
Change in CORT	1, 14	.004	.950	.950	.0002
Size class	1, 14	3.571	.080	.517	**.170**
Time between extractions	1, 14	.043	.839	.947	.002
SVMP expression group	1, 14	.153	.702	.947	.007
Change in CORT × size class	1, 14	3.235	.094	.517	**.154**

Note. Measurement indicates the response variable in the analysis, with regions 1–4 primarily composed of myotoxin, bradykinin-potentiating peptide, snake venom serine protease/phospholipase A_2, and snake venom metalloproteinase (SVMP), respectively. Adjusted P values represent false detection rate corrections for multiple hypothesis testing applied to all P values within each measurement. Partial η^2 (η_p^2) effect sizes are reported. Values of P below the threshold of $\alpha = 0.05$ and η_p^2 values above 0.14 are in bold.

Literature Cited

Abdel-Rahman M.A., I.M. Abdel-Nabi, M.S. El-Naggar, O.A. Abbas, and P.N. Strong. 2011. Intraspecific variation in the venom of the vermivorous cone snail *Conus vexillum*. Comp Biochem Physiol C 154:318–325. https://doi.org/10.1016/j.cbpc.2011.06.019.

Abdel-Rahman M.A., M.A. Omran, I.M. Abdel-Nabi, H. Ueda, and A. McVean. 2009. Intraspecific variation in the Egyptian scorpion *Scorpio maurus palmatus* venom collected from different biotopes. Toxicon 53:349–359. https://doi.org/10.1016/j.toxicon.2008.12.007.

Aitchison J. 1986. The statistical analysis of compositional data. Chapman & Hall, London.

Alape-Girón A., L. Sanz, M. Flores-Díaz, M. Madrigal, M. Sasa, and J. Calvete. 2008. Snake venomics of the lancehead pitviper *Bothrops asper*: geographic, individual, and ontogenetic variations. J Proteome Res 7:3556–3571.

Alberts B., A. Johnson, J. Lewis, D. Morgan, M. Raff, K. Roberts, and P. Walter. 2015. Molecular biology of the cell. 6th ed. Taylor & Francis, New York.

Andrade D.V. and A.S. Abe. 1999. Relationship of venom ontogeny and diet in *Bothrops*. Herpetologica 55:200–204.

Barlow A., C.E. Pook, R.A. Harrison, and W. Wüster. 2009. Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. Proc R Soc B 276:2443–2449. https://doi.org/10.1098/rspb.2009.0048.

Baugh A.T., K. van Oers, N.J. Dingemanse, and M. Hau. 2014. Baseline and stress-induced glucocorticoid concentrations are not repeatable but covary within individual great tits (*Parus major*). Gen Comp Endocrinol 208:154–163. https://doi.org/10.1016/j.ygcen.2014.08.014.

Benjamini Y. and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B 57:289–300.

Berger S., L.B. Martin, M. Wikelski, L.M. Romero, E.K. Kalko, M.N. Vitousek, and T. Rödl. 2005. Corticosterone suppresses immune activity in territorial Galápagos marine iguanas during reproduction. Horm Behav 47:419–429.

Biardi J.E., K.T. Nguyen, S. Lander, M. Whitley, and K.P. Nambiar. 2011. A rapid and sensitive fluorometric method for the quantitative analysis of snake venom metalloproteases and their inhibitors. Toxicon 57:342–347.

Binford G.J. 2001. An analysis of geographic and intersexual chemical variation in venoms of the spider *Tegenaria agrestis* (Agelenidae). Toxicon 39:955–968.

Borja M., E. Neri-Castro, R. Pérez-Morales, J. Strickland, R. Ponce-López, C. Parkinson, J. Espinosa-Fematt, et al. 2018. Ontogenetic change in the venom of Mexican black-tailed rattlesnakes (*Crotalus molossus nigrescens*). Toxins 10:501.

Busch D.S. and L.S. Hayward. 2009. Stress in a conservation context: a discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biol Conserv 142:2844–2853.

Bush S., S.M. Green, J.A. Moynihan, W.K. Hayes, and M.D. Cardwell. 2002. Crotalidae polyvalent immune fab (ovine) antivenom is efficacious for envenomations by southern Pacific rattlesnakes (*Crotalus helleri*). Ann Emerg Med 409:619–624.

Butts C.L., Y.L. Jones, J.K. Lim, C.E. Salter, E. Belyavskaya, and E.M. Sternberg. 2011. Tissue expression of steroid hormone receptors is associated with differential immune responsiveness. Brain Behav Immun 25:1000–1007.

Calvete J.J., L. Sanz, Y. Angulo, B. Lomonte, and J.M. Gutiérrez. 2009. Venoms, venomics, antivenomics. FEBS Lett 583:1736–1743.

Calvete J.J., L. Sanz, P. Cid, P. De La Torre, M. Flores-Díaz, M.C. Dos Santos, and A. Borges. 2010. Snake venomics of the Central American rattlesnake *Crotalus simus* and the South American *Crotalus durissus* complex points to neurotoxicity as an adaptive paedomorphic trend along *Crotalus* dispersal in South America. J Proteome Res 9:528–544.

Calvete J.J., L. Sanz, A. Pérez, A. Borges, A.M. Vargas, B. Lomonte, Y. Angulo, et al. 2011. Snake population venomics and antivenomics of *Bothrops atrox*: paedomorphism along its transamazonian dispersal and implications of geographic venom variability on snakebite management. J Proteom 74:510–527.

Casewell N.R., N.W.T. Jackson, A. Laustsen, and K. Sunagar. 2020. Causes and consequences of snake venom variation. Trends Pharmacol Sci 41:570–581.

Casewell N.R., S. Wagstaff, W. Wüster, D. Cook, F. Bolton, S. King, D. Pla, L. Sanz, J.J. Calvete, and R.A. Harrison. 2014. Medically important differences in snake venom composition are dictated by distinct postgenomic mechanisms. Proc Natl Acad Sci USA 111:9205–9210.

Chippaux J.-P., V. Williams, and J. White. 1991. Snake venom variability: methods of study, results and interpretation. Toxicon 29:1279–1303.

Chuang T.-D., W.J. Pearce, and O. Khorram. 2015. miR-29c induction contributes to downregulation of vascular extracellular matrix proteins by glucocorticoids. Am J Physiol Cell Physiol 309:C117–C125.

Cipriani V., J. Debono, J. Goldenberg, T.N.W. Jackson, K. Arbuckle, J. Dobson, I. Koludarov, et al. 2017. Correlation between ontogenetic dietary shifts and venom variation in Australian brown snakes (*Pseudonaja*). Comp Biochem Physiol C 197:53–60.

Claunch N.M., J.A. Frazier, C. Escallón, B.J. Vernasco, I.T. Moore, and E.N. Taylor. 2017*a*. Physiological and behavioral effects of exogenous corticosterone in a free-ranging ectotherm. Gen Comp Endocrinol 248:87–96.

Claunch N.M., M.L. Holding, C. Escallon, B. Vernasco, I.T. Moore, and E.N. Taylor. 2017*b*. Good vibrations: assessing the stability of snake venom composition after researcher-induced disturbance in the laboratory. Toxicon 133:127–135.

Currier R. 2012. Investigating venom synthesis: exploring the composition, variation and gene expression dynamics of *Bitis arietans* venom. PhD diss. University of Liverpool.

Currier R.B., R.A. Harrison, P.D. Rowley, G.D. Laing, and S.C. Wagstaff. 2010. Intra-specific variation in venom of the African puff adder (*Bitis arietans*): differential expression and activity of snake venom metalloproteinases (SVMPs). Toxicon 55:864–873.

DesRochers D.W., J.M. Reed, J. Awerman, J.A. Kluge, J. Wilkinson, L.I. van Griethuijsen, J. Aman, and L.M. Romero. 2009.

Exogenous and endogenous corticosterone alter feather quality. Comp Biochem Physiol A 152:46–52.

Duan Y., W. Fu, S. Wang, Y. Ni, and R. Zhao. 2014. Effects of tonic immobility (TI) and corticosterone (CORT) on energy status and protein metabolism in pectoralis major muscle of broiler chickens. Comp Biochem Physiol A 169:90–95.

Durban J., A. Pérez, L. Sanz, A. Gómez, F. Bonilla, S. Rodríguez, D. Chacón, et al. 2013. Integrated "omics" profiling indicates that miRNAs are modulators of the ontogenetic venom composition shift in the Central American rattlesnake, *Crotalus simus simus*. BMC Genom 14:1–17.

Durban J., L. Sanz, D. Trevisan-Silva, E. Neri-Castro, A. Alagón, and J.J. Calvete. 2017. Integrated venomics and venom gland transcriptome analysis of juvenile and adult Mexican rattlesnakes *Crotalus simus*, *C. tzabcan*, and *C. culminatus* revealed miRNA-modulated ontogenetic shifts. J Proteome Res 16:3370–3390.

Fry B.G. 2005. From genome to "venome": molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. Genome Res 15:403–420.

Fry B.G., K.D. Winkel, J.C. Wickramaratna, W.C. Hodgson, and W. Wuster. 2003. Effectiveness of snake antivenom: species and regional venom variation and its clinical impact. J Toxicol Toxin Rev 22:23–34.

Fry B.G. and W. Wüster. 2004. Assembling an arsenal: origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences. Mol Biol Evol 21:870–883.

Gaeggeler H.-P. 2005. Mineralocorticoid versus glucocorticoid receptor occupancy mediating aldosterone-stimulated sodium transport in a novel renal cell line. J Am Soc Nephrol 16:878–891.

Gangloff E.J., A.M. Sparkman, K.G. Holden, C.J. Corwin, M. Topf, and A.M. Bronikowski. 2017. Geographic variation and within-individual correlations of physiological stress markers in a widespread reptile, the common garter snake (*Thamnophis sirtalis*). Comp Biochem Physiol A 205:68–76.

Gibbs H.L., L. Sanz, J.E. Chiucchi, T.M. Farrell, and J.J. Calvete. 2011. Proteomic analysis of ontogenetic and diet-related changes in venom composition of juvenile and adult dusky pigmy rattlesnakes (*Sistrurus miliarius barbouri*). J Proteom 74:2169–2179.

Glanemann C., A. Loos, N. Gorret, L.B. Willis, X.M. O'Brien, P.A. Lessard, and A.J. Sinskey. 2003. Disparity between changes in mRNA abundance and enzyme activity in *Corynebacterium glutamicum*: implications for DNA microarray analysis. Appl Microbiol Biotechnol 61:61–68.

Gregory-Dwyer V., N. Egen, A. Bianchi Bosisio, P. Righetti, and F. Russell. 1986. An isoelectric focusing study of seasonal variation in rattlesnake venom proteins. Toxicon 24:995–1000.

Gubensek F., D. Sket, V. Turk, and D. Lebez. 1974. Fractionation of *Vipera ammodytes* venom and seasonal variation of its composition. Toxicon 12:167–171.

Gutiérrez J., C. Avila, Z. Camacho, and B. Lomonte. 1990. Ontogenetic changes in the venom of the snake *Lachesis muta stenophrys* (bushmaster) from Costa Rica. Toxicon 28:419–426.

Hau M., S. Casagrande, J.Q. Ouyang, and A.T. Baugh. 2016. Glucocorticoid-mediated phenotypes in vertebrates: multilevel variation and evolution. Pp. 41–115 in M. Naguib, J. Mitani, L.W. Simmons, L. Barrett, S. Healy, and M. Zuk, eds. Advances in the study of behavior. Elsevier, Cambridge, MA.

Holding M.L., J.A. Frazier, S.W. Dorr, S.N. Henningsen, I.T. Moore, and E.N. Taylor. 2014. Physiological and behavioral effects of repeated handling and short-distance translocations on free-ranging northern Pacific rattlesnakes (*Crotalus oreganus oreganus*). J Herpetol 48:233–239.

Holding M.L., M.J. Margres, D.R. Rokyta, and H.L. Gibbs. 2018. Local prey community composition and genetic distance predict venom divergence among populations of the northern Pacific rattlesnake (*Crotalus oreganus*). J Evol Biol 31:1513–1528.

Hoffman C.J., H.C. McKenzie III, M.O. Furr, and A. Desrochers. 2015. Glucocorticoid receptor density and binding affinity in healthy horses and horses with systemic inflammatory response syndrome. J Vet Intern Med 29:626–635.

Jackson T.N.W., I. Koludarov, S.A. Ali, J. Dobson, C.N. Zdenek, D. Dashevsky, B. Op Den Brouw, et al. 2016. Rapid radiations and the race to redundancy: an investigation of the evolution of Australian elapid snake venoms. Toxins 8:309.

Jessop T.S., M. Letnic, J.K. Webb, and T. Dempster. 2013. Adrenocortical stress responses influence an invasive vertebrate's fitness in an extreme environment. Proc R Soc B 280:20131444.

Kang M., J. Ryu, J.H. Kim, H. Na, Z. Zuo, and S.H. Do. 2010. Corticosterone decreases the activity of rat glutamate transporter type 3 expressed in *Xenopus* oocytes. Steroids 75:1113–1118.

Keegan H.L., R.A. Hedeen, and F.W. Whittemore Jr. 1960. Seasonal variation in venom of black widow spiders. Am J Trop Med Hyg 9:477–479.

King R.B., J.H. Cline, and C.J. Hubbard. 2000. Age and litter effects on testosterone levels in young water snakes. Copeia 2000:593–596.

Kini R.M. 2003. Excitement ahead: structure, function and mechanism of snake venom phospholipase A_2 enzymes. Toxicon 42:827–840.

Lakens D. 2013. Calculating and reporting effect sizes to facilitate cumulative science: a practical primer for *t*-tests and ANOVAs. Front Psychol 4:863.

Li X., J. Qiu, J. Wang, Y. Zhong, J. Zhu, and Y. Chen. 2001. Corticosterone-induced rapid phosphorylation of p38 and JNK mitogen-activated protein kinases in PC12 cells. FEBS Lett 492: 210–214.

Lind C.M., J.F. Husak, C. Eikenaar, I.T. Moore, and E.N. Taylor. 2010. The relationship between plasma steroid hormone concentrations and the reproductive cycle in the northern Pacific rattlesnake, *Crotalus oreganus*. Gen Comp Endocrinol 166:590–599.

Liu W., E.Y. Yuen, and Z. Yan. 2010. The stress hormone corticosterone increases synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors via serum- and glucocorticoid-inducible kinase (SGK) regulation of the GDI-Rab4 complex. J Biol Chem 285:6101–6108.

Lorenz C., R. Opitz, I. Lutz, and W. Kloas. 2009. Corticosteroids disrupt amphibian metamorphosis by complex modes of action including increased prolactin expression. Comp Biochem Physiol C 150:314–321.

Luna M.S.A., T.M.A. Hortencio, Z.S. Ferreira, and N. Yamanouye. 2009. Sympathetic outflow activates the venom gland of the snake *Bothrops jararaca* by regulating the activation of transcription factors and the synthesis of venom gland. J Exp Biol 212:1535–1543.

Lutterschmidt W.I., D.I. Lutterschmidt, R.T. Mason, and H.K. Reinert. 2009. Seasonal variation in hormonal responses of timber rattlesnakes (*Crotalus horridus*) to reproductive and environmental stressors. J Comp Physiol B 179:747–757.

Ma D., A. Armugam, and K. Jeyaseelan. 2001. Expression of cardiotoxin-2 gene: cloning, characterization and deletion analysis of the promoter. Eur J Biochem 268:1844–1850.

Mackessy S.P. 1988. Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *C. v. oreganus*. Copeia 1988:92. https://doi.org/10.2307/1445927.

———. 1996. Characterization of the major metalloprotease isolated from the venom of the northern Pacific rattlesnake, *Crotalus viridis oreganus*. Toxicon 34:1277–1285.

Mackessy S.P. and L.M. Baxter. 2006. Bioweapons synthesis and storage: the venom gland of front-fanged snakes. Zool Anz 245:147–159.

Mackessy S.P., N.M. Sixberry, W.H. Heyborne, and T. Fritts. 2006. Venom of the brown treesnake, *Boiga irregularis*: ontogenetic shifts and taxa-specific toxicity. Toxicon 47:537–548.

Madrigal M., L. Sanz, M. Flores-Díaz, M. Sasa, V. Núñez, A. Alape-Girón, and J.J. Calvete. 2012. Snake venomics across genus *Lachesis*: ontogenetic changes in the venom composition of *Lachesis stenophrys* and comparative proteomics of the venoms of adult *Lachesis melanocephala* and *Lachesis acrochorda*. J Proteom 77:280–297.

Massey D.J., J.J. Calvete, E.E. Sánchez, L. Sanz, K. Richards, R. Curtis, and K. Boesen. 2012. Venom variability and envenoming severity outcomes of the *Crotalus scutulatus scutulatus* (Mojave rattlesnake) from southern Arizona. J Proteom 75:2576–2587.

Mathé-Hubert H., L. Kremmer, D. Colinet, J.-L. Gatti, J. Van Baaran, É. Delava, and M. Poirié. 2019. Variation in the venom of parasitic wasps, drift, or selection? insights from a multivariate Q_{ST} analysis. Front Ecol Evol 7:156.

McCue M.D. 2007. Western diamondback rattlesnakes demonstrate physiological and biochemical strategies for tolerating prolonged starvation. Physiol Biochem Zool 80:25–34.

McCue M.D., H.B. Lillywhite, and S.J. Beaupre. 2012. Physiological responses to starvation in snakes: low energy specialists. Pp. 103–131 in M.D. McCue, ed. Comparative physiology of fasting, starvation, and food limitation. Springer, Heidelberg, Germany.

McElroy T., C.N. McReynolds, A. Gulledge, K.R. Knight, W.E. Smith, and E.A. Albrecht. 2017. Differential toxicity and venom gland gene expression in *Centruroides vittatus*. PLoS ONE 12: e0184695.

Minton S.A. and S.A. Weinstein. 1986. Geographic and ontogenic variation in venom of the western diamondback rattlesnake (*Crotalus atrox*). Toxicon 24:71–80.

Morris D.J., S.A. Latif, and A.S. Brem. 2009. Interactions of mineralocorticoids and glucocorticoids in epithelial target tissues revisited. Steroids 74:1–6.

Müller C., B. Almasi, A. Roulin, C.W. Breuner, S. Jenni-Eiermann, and L. Jenni. 2009. Effects of corticosterone pellets on baseline and stress-induced corticosterone and corticosteroid-binding-globulin. Gen Comp Endocrinol 160:59–66.

Nakagawa S. and H. Schielzeth. 2010. Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. Biol Rev 85:935–956.

Nakano T., O. Ohara, H. Teraoka, and H. Arita. 1990. Glucocorticoids suppress group II phospholipase A_2 production by blocking mRNA synthesis and post-transcriptional expression. J Biol Chem 65:12745–12748.

Núñez V., P. Cid, L. Sanz, P. De La Torre, Y. Angulo, B. Lomonte, J. Gutiérrez, and J.J. Calvete. 2009. Snake venomics and antivenomics of *Bothrops atrox* venoms from Colombia and the Amazon regions of Brazil, Perú and Ecuador suggest the occurrence of geographic variation of venom phenotype by a trend towards paedomorphism. J Proteom 73:57–78.

Oksanen J., F.G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P.R. Minchin, et al. 2018. vegan: community ecology package. https://CRAN.R-project.org/package=vegan.

Palarea-Albaladejo J. and J.A. Martin-Fernandez. 2015. zCompositions: R package for multivariate imputation of left-censored data under a compositional approach. Chemom Intell Lab Syst 143:85–96.

Perez-Riverol A., J. Roberto, A. Musacchio, M. Sergio, and M.R. Brochetto-Braga. 2017. Wasp venomics: unravelling the toxins arsenal of *Polybia paulista* venom and its potential pharmaceutical applications. J Proteom 161:88–103. https://doi.org/10.1016/j.jprot.2017.04.016.

Ray A. and K.E. Prefontaine. 1994. Physical association and functional antagonism between the p65 subunit of transcription factor NF-κB and the glucocorticoid receptor. Proc Natl Acad Sci USA 91:752–756.

R Core Team. 2017. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/.

Remigio E.A. and J.R. Duda. 2008. Evolution of ecological specialization and venom of a predatory marine gastropod. Mol Ecol 17:1156–1162.

Rodríguez-Ravelo R., F.I.V. Coronas, F.Z. Zamudio, L. González-Morales, G.E. López, A.R. Urquiola, and L.D. Possani. 2013. The Cuban scorpion *Rhopalurus junceus* (Scorpiones, Buthidae): component variations in venom samples collected in different geographical areas. J Venom Anim Toxins Incl Trop Dis 19:1–10.

Rokyta D.R., M.J. Margres, and K. Calvin. 2015. Post-transcriptional mechanisms contribute little to phenotypic variation in snake venoms. G3 Genes Genomes Genet 5:2375–2382.

Romero L.M., D. Strochlic, and J.C. Wingfield. 2005. Corticosterone inhibits feather growth: potential mechanism explaining seasonal down regulation of corticosterone during molt. Comp Biochem Physiol A 142:65–73.

Salazar A.M., B. Guerrero, B. Cantu, E. Cantu, A. Rodríguez-Acosta, J.C. Pérez, J.A. Galán, A. Tao, and E.E. Sánchez. 2009. Venom variation in hemostasis of the southern Pacific rattlesnake (*Crotalus oreganus helleri*): isolation of hellerase. Comp Biochem Physiol C 149:307–316.

Santana R.C., D. Perez, J. Dobson, N. Panagides, R.J. Raven, A. Nouwens, A. Jones, G.F. King, and B.G. Fry. 2017. Venom profiling of a population of the theraphosid spider *Phogius crassipes* reveals continuous ontogenetic changes from juveniles through adulthood. Toxins 9:116.

Sapolsky R.M., L.M. Romero, and A. Munck. 2000. How do glucocorticoids influence stress responses? integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21:55–89.

Saviola A.J., D. Pla, L. Sanz, T.A. Castoe, J.J. Calvete, and S.P. Mackessy. 2015. Comparative venomics of the prairie rattlesnake (*Crotalus viridis viridis*) from Colorado: identification of a novel pattern of ontogenetic changes in venom composition and assessment of the immunoreactivity of the commercial antivenom CroFab®. J Proteom 121:28–43.

Schonour R.B., E.M Huff, M.L. Holding, N.M. Claunch, S.A. Ellsworth, M.P. Hogan, K. Wray, et al. 2020. Gradual and discrete ontogenetic shifts in rattlesnake venom composition and assessment of hormonal and ecological correlates. Toxins 12:659.

Schuett G.W., E.N. Taylor, E.A. Van Kirk, and W.J. Murdoch. 2004. Handling stress and plasma corticosterone levels in captive male western diamond-backed rattlesnakes (*Crotalus atrox*). Herpetol Rev 35:229–233.

Shukla P.K., L. Gautam, M. Sinha, P. Kaur, S. Sharma, and T.P. Singh. 2015. Structures and binding studies of the complexes of phospholipase A2 with five inhibitors. Biochim Biophys Acta Proteins Proteom 1854:269–277.

Stepanek J., N.M. Claunch, J.A. Frazier, I.T. Moore, B.J. Vernasco, C. Escallon, and E.N. Taylor. 2019. Corticosterone and color change in southern Pacific rattlesnakes (*Crotalus helleri*). Herpetologica 72:143–152.

Stoffel M., S. Nakagawa, and H. Schielzeth. 2017. rptR: repeatability estimation and variance decomposition by generalized linear mixed-effects models. Methods Ecol Evol 8:1639–1644.

Sunagar K., E.A.B. Undheim, H. Scheib, E.C.K. Gren, C. Cochran, C.E. Person, I. Koludarov, et al. 2014. Intraspecific venom variation in the medically significant southern Pacific rattlesnake (*Crotalus oreganus helleri*): biodiscovery, clinical and evolutionary implications. J Proteom 99:68–83.

Taff C.C. and M.N. Vitousek. 2016. Endocrine flexibility: optimizing phenotypes in a dynamic world? Trends Ecol Evol 31:476–488.

Taylor E.N., D.F. Denardo, and D.H. Jennings. 2004. Seasonal steroid hormone levels and their relation to reproduction in the western diamond-backed rattlesnake, *Crotalus atrox* (Serpentes: Viperidae). Gen Comp Endocrinol 136:328–337.

Templ M., P. Filzmoser, and K. Hron. 2011. Robust imputation of missing values in compositional data using the R-package robCompositions. New Techniques and Technologies for Statistics, Brussels.

Thunhorst R.L., T.F. Beltz, and A.K. Johnson. 2007. Glucocorticoids increase salt appetite by promoting water and sodium excretion. Am J Physiol Regul Integr Comp Physiol 293: R1444–R1451.

Underwood A.H. and J.E. Seymour. 2007. Venom ontogeny, diet and morphology in *Carukia barnesi*, a species of Australian box jellyfish that causes Irukandji syndrome. Toxicon 49:1073–1082.

van den Boogart K.G., R. Tolosana-Delgado, and M. Bren. 2018. compositions: compositional data analysis. https://CRAN.R-project.org/package=compositions.

Vishwanath B.S., F.J. Frey, M.J. Bradbury, M.F. Dallman, and B.M. Frey. 1993. Glucocorticoid deficiency increases phospholipase A2 activity in rats. J Clin Investig 92:1974–1980.

Wada H. 2008. Glucocorticoids: mediators of vertebrate ontogenetic transitions. Gen Comp Endocrinol 156:441–453.

Wasserberger J., G. Ordog, and T. Merkin. 2006. Southern Pacific rattlesnake bite: a unique clinical challenge. J Emerg Med 31: 263–266.

Wick F., Y. Hu, S. Schwarz, and G. Kroemer. 1993. Immunoendocrine communication via the hypothalamo-pitutitary-adrenal axis in autoimmune diseases. Endocr Rev 14:539–563.

Willemse G., J. Hattingh, R.M. Karlsson, S. Levy, and C. Parker. 1979. Changes in composition and protein concentration of puff adder (*Bitis arietans*) venom due to frequent milking. Toxicon 17:37–42.

Williams V. and J. White. 1992. Variation in the composition of the venom from a single specimen of *Pseudonaja textilis* (common brown snake) over one year. Toxicon 30:202–206.

Wingfield J.C., D.L. Maney, C.W. Breuner, J.D. Jacobs, M. Ramenofsky, and R.D. Richardson. 1998. Ecological bases of hormone-behavior interactions: the "emergency life history stage." Am Zool 38:191–206.

Zancolli G., J.J. Calvete, M.D. Cardwell, H.W. Greene, W.K. Hayes, M.J. Hegarty, H. Herrmann, et al. 2019. When one phenotype is not enough: divergent evolutionary trajectories govern venom variation in widespread rattlesnake species. Proc R Soc B 286:20182735.

Zelanis A., J. de Souza Ventura, A.M. Chudzinski-Tavassi, and M. de Fátima Domingues Furtado. 2007. Variability in expression of *Bothrops insularis* snake venom proteases: an ontogenetic approach. Comp Biochem Physiol C 145:601–609.