Sustained effects of the developmental environment on glucocorticoid physiology and body size in the delicate skink

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Abstract

The conditions animals experience during development can have sustained effects on morphology and physiology. For example, exposure to elevated levels of glucocorticoid (‘stress’) hormones during development can have programmatic effects on the neuroendocrine pathway the hypothalamic pituitary adrenal (HPA) axis. The HPA axis regulates the secretion of glucocorticoids at baseline levels (to maintain energy balance) and elevated levels (in response to disturbances or threats).

Introduction

The environment animals experience during development can have powerful effects on morphology, physiology, and behavior. In some cases, developmental effects can be sustained across life history stages and influence behavioral strategies, reproductive success, and survival. Developmental effects can also drive population level responses by promoting adaptive phenotypic responses and, thus, local adaptation, or by constraining phenotypic responses which can drive populations to extinction. For example, developmental effects are predicted to be important drivers of individual and population level responses to global climate change.

Developmental effects have been studied extensively in terms of glucocorticoid physiology. Glucocorticoids are steroid hormones that play important roles in vertebrate metabolism and stress responses. In responses to stressors or disturbances, vertebrates activate the neuroendocrine pathway the hypothalamic-pituitary-adrenal (HPA) axis that regulates the release of glucocorticoid hormones from the adrenal gland. Glucocorticoids promote physiological and behavioural responses that allow animals to cope with disturbances and stressors. Developing animals can be exposed to glucocorticoids prenatally (during gestation or *in ovo*) and postnatally via breastmilk (in mammals) and from their own endogenous production at baseline and elevated levels in responses to disturbances (e.g., food restriction, environmental conditions, parental interactions). Exposure to elevated glucocorticoids during development can decrease growth and development, body condition, immune function, and recruitment (CITE). Additionally, exposure to glucocorticoids during development can have sustained effects on HPA axis function such that animals exposed to short periods of elevated glucocorticoids during development secrete higher levels of glucocorticoids throughout their lifetime. Such sustained or programmatic effects on glucocorticoid physiology have been linked to changes in cellular metabolism, reproductive strategies, longevity, and individual fitness.

In oviparous lizards, developmental effects have also been studied extensively in relation to incubation temperatures. The temperatures eggs experience during development can affect a plethora of phenotypic traits in lizards such as body size, whole body metabolism, and even sex determination in some species. In general, lizards that develop in colder temperatures hatch later and are larger than lizards that develop in warm temperatures. Changes in body size due to incubation temperature are often sustained for a period before smaller hatched lizards ‘catch up’ in body size via increased growth rates. Likely, BLAH mechanisms plays a role in this. Link to glucocorticoids and mitochondria here.

Mitochondria paragraph:

Hook paragraph: Exposure to glucocorticoids during development do X, Y, and Z and are thought to be an important physiological mechanism that will drive population responses to global climate. Understanding the effects of exposure to glucocorticoids during development on growth, body condition, and survival is important for understanding how they will drive population level responses. Additionally, need to understand how glucocorticoid exposure during development may interact with other developmental conditions such as exposure do different temperatures.

Here, we tested the long-term effects of exposure to glucocorticoids and incubation temperatures on growth, hormone responses, mitochondrial function, and survival in the delicate stink (*Lampropholis delicata*). Delicate skinks are native Australian skinks that bladdy bladdy bladdy blah. Delicate skinks are oviparous and females lay clutches of x-y eggs. Incubation temperature range of this species is x – Y. They are easily housed and bred in captivity and are a highly tractable species for studies that seek to understand the long-term effects of developmental conditions.

We exposed skinks to one of three corticosterone treatments *in ovo* (high corticosterone, low corticosterone, or control) and incubated eggs at either high (28º C) or low (23º C) incubation temperatures (representing the minimum and maximum natural distribution of incubation temperatures). We measured body size in response to developmental treatments at four time points and survival over a ~1.5-year period. After 1.5 years, we measured hormone levels (corticosterone, testosterone, and thyroxine) in response to developmental treatments and mitochondrial bioenergetics from liver tissue. We predicted that lizards treated with corticosterone during development would be smaller compared control lizards. We predicted that lizards treated with corticosterone would have higher levels of baseline corticosterone and less efficient mitochondria as adults. We predicted that lizards incubated at colder temperatures would be larger at hatching compared to lizards incubated at warm temperatures but that differences in body size would not be present later in life. We predicted that high incubation temperatures would interact with corticosterone treatment such that lizards exposed to both these treatments would be smaller than lizards from all other treatments. Our research builds from recent publications that examine X and Y and does Z.

Methods

*Lizard husbandry and housing*

We collected gravid *Lampropholis delicata* from semi-urban parks in Sydney (Australia) and transported them back to the Australian National University. Females were housed communally with males in groups of 5-6 (3-4 females – width x length: 40x55 cm). These lizards formed the breeding colony from which all eggs were subsequently collected. Terraria contained non-stick matts as substrate, refuge (eucalyptus bark and half cut PVC pipe), a water container, and a container full of moist vermiculite for egg laying. Terraria were heated by heat chords and had UV lamps for UVA/UVB exposure. Lights were set to a photoperiod of 12:12 h (light/dark). Animals were provided with water every day (both spraying and when filling the water container). Crickets were provided every second day and were dusted in calcium and multivitamin once a week.

*Experimental timeline*

Lizard enclosures were checked for eggs three days a week. We measured the width and length of eggs to the nearest 0.XX cm using digital calipers and measured mass to the nearest 0.0X g using a digital balance. Eggs were treated with hormone solutions the day they were found with three days maximum between the day the eggs were laid and exposed to treatments. Following treatments, eggs were incubated until hatching was recorded. Eggs were also checked three days a week. On the day hatching was recorded, lizards were measured for snout vent length (SVL), tail length (the distance between the vent and the tip of the tail), and body mass. Lizards were then moved to solitary enclosures (see below). We opportunistically collected body size measurements at four additional time points (~X, Y, and Z). Lizards were euthanized at ~1.5 years of age (avg = X days) at which point they were sexed by manual examination, a blood sample was collected for hormone analyses, and liver tissue was collected to measure mitochondrial bioenergetics.

*Experimental treatments*

We exposed eggs to one of six corticosterone/temperature treatments in a full factorial design. For hormone treatments eggs were treated with either a high corticosterone (10 pg/mg), low corticosterone (5 pg/mg), or a control (vehicle) treatment and then incubated until hatch (avg = ) at either 23 or 28º C (representing the lower and upper range of natural incubation temperatures in this species). Corticosterone doses were selected based on published yolk corticosterone concentrations in other oviparous species and estimates of the percentage of steroids that are incorporated into the yolk following topical treatment (CITE). Corticosterone treatments were made by dissolving crystalline corticosterone (Sigma, Cat. No. XXX) in 100% ethanol. To dose eggs, 5µl of solutions was applied to eggshells using a micropipette. Control eggs were treated with 5µl of 100% ethanol. Following treatment with corticosterone solutions, eggs were incubated in covered plastics cups filled with damp vermiculate at either 23 or 28º C.

*Measurements of yolk corticosterone levels*

We measured corticosterone levels in a separate group of eggs to ensure that topical treatments increased corticosterone levels within a biological relevant range. Eggs dosed with treatments as above. We allowed them to incubate for 24 ± 2 hours prior to removing the egg yolk. Prior to dissection, eggs were weighed to the nearest 0.001 mg and length and width measurements were collection to the nearest 0.001 cm using dial calipers. To remove the egg yolk, we made a lateral incision with a razor blade on the eggshell. We used dissection scissors to widen the incision and removed the egg yolk using a small spatula. We used KimWipes to remove any albumin that surrounded the egg yolk. Egg yolks were weighed to the nearest in 0.001 mg, diluted with 1 mL of doubly distilled water, vortexed thoroughly, and stored at -20º C until assayed.

We used solid phase extraction (SPE) with silica bonded vacuum columns (Company, Cat. No. XXX) to extract corticosterone from yolk samples. We used a protocol modified from published protocols (CITE). Briefly, we prepared columns by washing them twice with 5 mL of doubly deionized water. Diluted yolk samples were run through the columns, followed by a wash with 5 mL of 40% methanol to remove lipids. Columns were then soaked with 5 mL of 100% methanol before samples were eluted with vacuum filtration. Samples were dried under nitrogen at 35º C and then stored at -20º C until assayed. To determine extraction efficiency, we pooled yolk samples and used SPE to extract corticosterone from an aliquot that was spiked with X ng/mL of corticosterone and an aliquot that was not spiked. We determined one value of extraction efficiency for each group of samples that was assayed on a hormone plate (see below).

We measured yolk corticosterone levels using Arbor Assay Enzyme Immunoassay (EIA) kits (Cat. No. K014). Samples were reconstituted in XXX µl of assay buffer for a dilution of [1:X], vortexed thoroughly, and mixed on a test tube shaker for 20 minutes. An external standard of XX pg/ml was run on every plate and used to calculate inter-plate variation. All samples and standard were run in triplicate. All plates were read on FLUOstar Omega microplate readers at 450 nm (but see below). Corticosterone levels were calculated from a 4 parameter nine-point standard curve ranging from 39.063 to 10,000 pg/mL. Intra- and inter-plate variation was X and X% respectively. Assay parallelism was assessed by comparing a six-point dilution of a pooled yolk sample to the standard curve (p > 0.XX indicating no significant difference between the slopes of the lines). Yolk corticosterone levels were measured from two sets of eggs from separate years. Due to logistical constraints, different plate readers were used for each set of eggs. We used ‘egg set’ as a factor in analyses (see below) to account for variation from using different plate readers.

*Mitochondrial bioenergetics*

Lizards were fasted for 72 ± 4 hours prior to euthanasia. Lizards were euthanized via an injection of DRUG (DOSE) followed by rapid decapitation. Immediately following decapitation, trunk blood was collecting using heparinized microcapillary tubes and kept on ice until processing (>1 hour). Blood was centrifuged at XXXX rpm for X minutes. The isolated plasma was stored at -20ºC. Following blood collections, whole livers were removed, rinsed twice in 1mL of 1M PBS, and stored in XXX solution prior to further processing (> 1 hour).

*Plasma hormone levels*

We measured corticosterone, thyroxine, and testosterone (males only) from 5 µl of plasma. We were unable to collect 5 µl of plasma from n = X individuals and instead assayed samples of X -Y µl of plasma. Although these samples were assayed, they were excluded prior to statistical analyses because they had higher levels of hormones despite being assayed at the same concentration of samples of 5 µl of plasma.

All hormones were measured from raw plasma diluted to [1:X] using EIA kits. All samples and standards were run in triplicate and all plates were read on a FLUOstar Omega microplate reader at 450 nm. Plasma corticosterone levels were quantified using methods described above for measuring yolk corticosterone levels. Intra- and inter-plate variation for plasma corticosterone assays was X and X% respectively. Thyroxine (T4) levels were quantified with Arbor Assay kits (Cat. No. K050). An external standard of 1,000 ng/mL was run on every plate and used to calculate inter-plate variation. Thyroxine levels were calculated from a 4 parameter six-point standard curve ranging from 0.625 to 20 ng/mL. Intra- and inter-plate variation for thyroxine assays was X and X% respectively. Testosterone levels in males were quantified with Arbor Assay kits (Cat. No. K032). An external standard of XX pg/ml was run on every plate and used to calculate inter-plate variation. Testosterone levels were calculated from a linear six-point standard curve ranging from X to x pg/mL. A linear fit was used for the standard curve to calculate high values that were not captured with a 4-parameter fit. We used rank order statistics to analyze testosterone data (see below) because linear standard curves may underestimate high hormone values. Intra- and inter-plate variation for testosterone assays was X and X% respectively.

*Statistical analysis*

Data

* Yolk CORT levels
* Hatching success
* Body size/mass at hatching
* Body size/mass at three other time points
* Baseline CORT
* Death rate in colony