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Research paper

Impaired negative feedback and death following acute stress in glucocorticoid receptor knockout *Xenopus tropicalis* tadpoles

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ABSTRACT

Blood glucocorticoid levels are regulated by the hypothalamo-pituitary-adrenal/interrenal axis (HPA axis in mammals, HPI axis in amphibians), and negative feedback by glucocorticoid signaling is a key player in that regulation. Glucocorticoid and mineralocorticoid receptors (GR and MR) mediate negative feedback in mammals, but little is known about nuclear receptor-mediated feedback in amphibians. Because amphibians have only one corticosteroidogenic cell type responsible for glucocorticoid and mineralocorticoid production, we hypothesized that GR knockout (GRKO) tadpoles have elevated levels of glucocorticoids and mineralocorticoids as well as axis components regulating their production. We also examined the response to stress and potential for increased aldosterone signaling in GRKO tadpoles. We found that GRKO tadpoles have severe hyperactivity of the HPI axis, namely high mRNA expression levels of *pomc, cyp17a1, cyp21a2, cyp11b2*, and *star*, and high tissue content of corticosterone, aldosterone, 17-hydroxyprogesterone, 21-deoxycortisol, and progesterone. Such aberrant HPI activity was accompanied by reduced survival after acute temperature shock and shaking stress. Like mammalian models of HPA hyperactivity, GRKO tadpoles have high MR mRNA expression levels in brain, kidney, heart, and skin and high levels of the inflammatory cytokine *tnf-α* and the profibrotic factor *tgf-β* in kidneys. This study showed GR is critical for negative feedback to the amphibian HPI axis and for survival from acute stressors. This study also showed GRKO tadpoles exhibit altered expression/overproduction of regulators of salt-water homeostasis and associated biomarkers of kidney disease.

1. Introduction

Glucocorticoids (GCs) negatively regulate their own production by the hypothalamic–pituitary-adrenal (HPA) axis through the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) in mammals. MR and not GR occupancy by GCs is high under basal (non-stressful) conditions due to the high affinity of GCs to MR compared to GR. In contrast, full GR occupancy is only reached when cortisol levels are sufficiently high, for example in a stress response (ter Heegde et al., 2015). Nevertheless, reduced GR and/or MR signaling results in HPA hyperactivity due to lack of negative feedback characterized by overproduction of corticotropin releasing factor (CRF), adrenocorticotropic hormone (ACTH), and GCs (Gjerstad et al., 2018; Harris et al., 2013; Keller-Wood, 2015; Ladd et al., 2004). However, which receptor, GR or MR or both, involved in negative feedback regulation of the hypothalamic-pituitary-interrenal (HPI) axis in amphibians, is not established. Electrophoretic mobility shift assay showed frog GR can bind proximal promoters of frog *crf* genes, but MR can bind the same hormone response elements (Yao and Denver, 2007). Treatment of juvenile frogs with corticosterone (CORT; the main glucocorticoid in frogs) reduced *crf* mRNA expression in the anterior preoptic area (homolog of the mammalian paraventricular nucleus), but the receptor involved, i.e., GR and/or MR, was not determined (Yao et al., 2008). Treatment of premetamorphic tadpoles with dexamethasone (GR agonist) reduced plasma CORT, but the dexamethasone dose that can

Abbreviations: nr3c1, Glucocorticoid receptor GR; *crh*, corticotropin releasing hormone; *avp*, arginine-vasopressin; *pomc*, proopiomelanocortin; *star*, steroidogenic acute regulatory protein; *cyp17a1*, 17-alpha-monooxygenase; *cyp21a2*, 21-hydroxylase; *cyp11b2*, aldosterone synthase; *nr3c2*, mineralocorticoid receptor MR; *tnf-α*, tumor necrosis factor alpha; *edn-1*, endothelin-1; *fn-1*, fibronectin-1; *ctgf*, connective tissue growth factor; *pai-1*, plasminogen activator inhibitor type 1; *tgf-β*, transforming growth factor beta; *rpl8*, ribosomal protein L8; TH, thyroid hormone.

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activate only GR and not MR is unknown for tadpoles. Hence, while previous studies may suggest a larger role of GR compared to MR in HPI negative feedback, they do not necessarily preclude a contribution of MR. Also, the nonapeptide arginine vasopressin (AVP) acts synergistically with CRH to induce ACTH secretion in mammals (DeBold et al., 1984; Gibbs, 1986; Itoi et al., 1999; Scott and Dinan, 1998; Wotjak et al., 1996), and AVP appears to be the principal ACTH releasing factor in amphibians (Kikuyama et al., 2019; Okada et al., 2016). However, it is not known whether AVP is subject to GR-mediated negative feedback.

Glucocorticoid signaling in vertebrates plays a crucial role in adaptation to stressful environments (Degani and Nevo, 1986; Denver and Crespi, 2006; Nicolaides et al., 2015; Srinivasan et al., 2013). Prolonged exposure of tadpoles to stressors such as altered pH, temperature, salinity, predators, and short term exposure to shaking/confinement result in elevated CORT levels (Bókony et al., 2021; Burraco and Gomez-Mestre, 2016; Egea-Serrano et al., 2014; Fraker et al., 2021; Middlemis Maher et al., 2013). However, there is no clear evidence as to which receptor (GR or MR or both) is necessary for regulation of CORT levels before or during stress.

Lack of GR signaling is almost invariably associated with high glucocorticoid levels as evidenced from studies in both constitutive and tissue-specific GR knockout (GRKO) mouse models (Whirledge and DeFranco, 2018). Interestingly, because both GCs and mineralocorticoids bind to MR with the same affinity, excessive MR signaling in GRKO models has been implicated to cause vascular injury and inflammation underlying heart disease, renal disease, and stroke (Kubzansky and Adler, 2010). For example, cardiomyocyte-specific GRKO mice demonstrate severe heart histopathology and reduced survival as opposed GR-MR double knockout (Oakley et al. 2019). Transgenic mice overexpressing MR also demonstrated pathophysiological conditions in the heart and kidneys (Le Menuet et al., 2001; Ouvrard-Pascaud et al., 2005). However, it is not known whether lack of GR signaling alters MR signaling in amphibians.

To examine these issues, we used GRKO *X. tropicalis* tadpoles previously created in our lab using CRISPR technology to further our understanding of the role of GR in mediating negative feedback to the HPI axis and in response to stressors in tadpoles. We predicted GRKO tadpoles would demonstrate lack of negative feedback and therefore HPI hyperactivity, as indicated by increased mRNA expression of *crh*, *avp*, and *pomc* (gene coding for ACTH) in the brain and *star, cyp11b2*, and *cyp21a2* in the interrenals and high tissue content of corticosterone and aldosterone. We also predicted that GRKO tadpoles would have reduced recovery from shaking stress and temperature shock stress. Because no MR response gene nor physiological action of MR has so far been characterized in *X. tropicalis* tadpoles, we were able to address altered MR signaling in GRKO tadpoles only by measuring MR mRNA expression levels in the heart, brain, kidney, skin, and tails. We also quantified the mRNA expression levels of various profibrotic and inflammatory markers in the heart and kidney, which had altered expression levels in mammalian GRKO models.

2. Materials and methods

2.1. Animal husbandry

Lab-reared, F1 male and female adult *Xenopus tropicalis* heterozygous for the GR mutation were mated by priming with 20U of ovine luteinizing hormone (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) in the evening and boosted with 200U the next morning. The resulting F2 tadpoles were reared at 26 degrees Celsius in reverse osmosis water reconstituted to 800 μS with Crystal Sea Marine Mix (Marine Enterprises International, Baltimore, MD). Water changes occurred every 3 days, and tadpoles were fed powdered fry food (Sera Micron Nature) twice daily. The use of animals was in accordance with the guidelines outlined by the University of Cincinnati Institutional Animal Care and Use Committee (IACUC protocol # 21–06-21–01).

2.2. Genetic screening

Premetamorphic F2 tadpoles were genotyped using the heteroduplex mobility assay (HMA) (Foster et al., 2019; Ota et al., 2013; Sterner et al., 2020). Briefly, HMA involved obtaining genomic DNA template by incubating tail tips in 50 μL of 25 mM NaOH/0.2 mM EDTA for 15 min at 95 degrees Celsius followed by neutralization with 50 μL of 40 mM Tris-HCl. Next, PCR reactions using 1 μL of the genomic DNA template were carried out using DreamTaq (ThermoFisher) and the primers 5′-ACATTGCCCCCAGATAGAC and 5′-CCTGTAATAGGTCAAAGGTGC to amplify the CRISPR target region. The PCR products were then boiled, allowed to reanneal, run on an 8% polyacrylamide gel, stained with ethidium bromide, and imaged. F2 individuals were identified as wild-type, heterozygous, and homozygous mutants based on HMA patterns and then used in experiments.

2.3. Quantitative PCR

To measure gene expression during metamorphic climax at Nieuwkoop and Faber stage 61 (NF 61) (Bender et al., 2018; Manzon and Denver, 2004; Nieuwkoop and Faber, 1994) when HPI activity is highest, tails, brains (fore- plus midbrain portions), skin, heart, and kidneys from tadpoles of each genotype anesthetized with buffered 0.1% tricaine methane sulfate (Sigma-Aldrich) were harvested and snap frozen following described tissue harvest procedures (Patmann et al., 2017). RNA extraction was performed using TRI REAGENT RT following the manufacturer's instructions (Molecular Research Center, Inc., Cincinnati, OH). Complementary DNA synthesis from 1 μg total RNA for each sample was obtained using the High- Capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies, Thermo Fisher Scientific). Quantitative PCR (qPCR) was carried out using SYBR green master mix on a 7300 Real Time PCR System (Applied Biosystems) with gene-specific primers (Table 1). The relative quantification method ΔΔCt was used to compare expression levels of target genes normalized to the reference gene ribosomal protein L8 (*rpl8*) (Dhorne-Pollet et al., 2013; Livak and Schmittgen, 2001).

2.4. Steroid hormone measurements by LC-MS/MS

Steroid hormone content was measured from F2 tadpoles of each genotype at metamorphic climax (NF61) using liquid-chromatography tandem mass-spectrometry (LC-MS/MS). Ten tails (~100 mg each) per genotype were dissected from anesthetized, wild-type and GRKO tadpoles, snap frozen in liquid nitrogen, and stored at −80 degrees Celsius. Each tail was homogenized in 1 mL 100% methanol and sent on dry ice to the Cornell Proteomics and Metabolomics Facility where steroids were extracted and quantified. Tail homogenates were centrifuged, and supernatant was removed and saved. Water and ethyl acetate were added to the pellet to extract remaining steroids, and 2 μL internal standard mixture (1 μM progesterone-d9, 1 μM corticosterone-d8) was added. The organic phase was pooled with the previous methanol supernatant, dried using a speed vacuum SC110 (Thermo Savant, Milford, MA), and reconstituted in 100% methanol for LC-MS/MS analysis with a final concentration of the internal standards of 0.01 μM each.

The LC-MS/MS analysis of 5 steroid hormones, namely progesterone, 17-hydroxyprogesterone, corticosterone, 21-deoxycortisol, and aldosterone along with two deuterated internal standard (IS) steroids, progesterone-d9 and corticosterone-d8, was performed using a Luna C18(2) column from Phenomenex (3 μm, 100 mm × 2 mm i.d.) in Exion LC system coupled with Sciex X500B QTOF mass spectrometer (Framingham, MA). The analytes were separated using a mobile phase A containing 1 mM ammonium formate with 0.1% formic acid and mobile phase B having 1 mM ammonium formate in methanol with 0.1% formic acid with a flow rate of 300 μL/min and a column temperature of 30 degrees Celsius. The LC gradient used for separation of all those steroid hormones with baseline resolutions was initiated with 0–10% mobile

Table 1
SYBR primer sequences used to amplify target genes by quantitative PCR.

Gene	Forward primer (5′-3′)	Reverse Primer (5′-3′)
Corticotropin releasing hormone (*crh*)	CTCCGTGAAGTCTTAGAAATGG	CAATGATGTCCATGAGTTTCCT
Arginine-vasotocin (*avp*)	TGTGTAGAAGAGAACTTCGTGC	GAGTCCAAACTGCAACTCTCG
Proopiomelanocortin (*pomc*)	CCGATGTGCAGACCTCAGCAGT	TACTTTCCGACAGAGGCTGCAA
Steroidogenic acute regulatory protein (*star*)	GCAAATGGATAAATCAGGTTCG	CATCTCTCCTTCATTCAGTGTT
17-alpha-monooxygenase (*cyp17a1*)	GAAGTTTCACCGCAAGTTAGTG	CACAGAGCACAAACAACATTGG
21-hydroxylase (*cyp21a2*)	CTGGAAGACCCAAGAGTTACATA	GATCAGCACATTCTCTAGGTCAT
Aldosterone synthase (*cyp11b2*)	CAGTGGACCTTTATGCTGATCT	CTCGGATGAAACGAAGAGAATCC
Mineralocorticoid receptor (*nr3c2*)	TGAGGGTGAATTACATCAAAGAACT	CGTGTAGAAGCAGAATTCCAAC
Tumor necrosis factor alpha (*tnf-α*)	ACAAGGATGAGAGTAAGATGCC	CAGCAATAGATGAATCAGGGTC
Endothelin-1 (*edn-1*)	ATTCTTTCTCTGCTGGTTGTCC	GAACTTGTCTGTTGTGCTGTAG
Fibronectin-1 (*fn-1*)	GAATGTGTCTGCCTTGGAAATG	GCATGTACAGTCCACCATCATC
Connective tissue growth factor (*ctgf*)	CCTCAAGAGAGAACCTTAGTCGG	TCAGTAGTCTGTACTAGGCAGTTG
Plasminogen activator inhibitor type 1 (*pai-1*)	ATCACAAACATCCTGACACCTG	GTGGCTTCTTCAGATCAACTTC
Transforming growth factor beta (*tgf-β*)	CCTTACATCTGGAGCACAGATAC	GAACACAGCAGGGAGAGATAGAT
Ribosomal protein L8 (*rpl8*)	GAAGGTCATCTCATCTGCAAACAG	CTTCAGGATGGGTTTGTCAATACGA

phase B (MPB) from 0 to 3 min, 10– 70% MPB from 3 to 7 min, and then increased to 90% MPB in 11 min and 90–10% MPB from 18 to 19 min, and 10% MPB for equilibration of the column for 5 min. prior to the next run. The injection volume was 10 μL for the standards and 10 μL for samples. The autosampler temperature was kept constant at 15 degrees Celsius.

The Sciex X500B QTOF mass spectrometer with an ESI source was operated in the positive ion mode for this analysis. The instrument was calibrated before and during sample running with positive calibrant solution using calibrate delivery system (CDS). The electrospray voltage was set at 5.5 kV, and the temperature of the heated capillary was set at 300C. It was operated under the Ion Source gas1 and 2 at 20 psi, Curtain gas at 25 (arbitrary unit), CAD gas at 7 (arbitrary unit). The declustering potential (DP) was set to 120 eV with accumulation time of 0.10 s. The MS full scan measurement was done from *m/z* 100 to *m/z* 500 in profile mode followed by MRM HR scan acquired from 0 min to 24 min at three different collision energies for each analyte. The ion transitions were monitored in multiple reaction monitor (MRM) HR mode. Collision energy values were optimized to 35–70% for these transitions. The data were acquired using Sciex OS 2.0 software and the quantitation ratio between analyte/IS was calculated for each tail sample by integrating the peak areas by MQ4 Integration Algorithm of each analyte using MRM HR transitions in the same software.

2.5. Stress assay

Premetamorphic tadpoles (NF stage 54) of different genotypes were subjected to shaking stress and high temperature shock stress. For shaking stress, GRKO and wild-type tadpoles were placed in tanks (two tanks with 3 tadpoles per tank and one tank with 4 tadpoles adding up to 10 tadpoles per genotype) with just enough water to cover the tadpoles while preventing any vertical movement through the water column. The tanks were placed in an orbital shaker and agitated continuously at 100 rpm for 2 h. The shaking intensity was just enough to require constant spatial adjustment by the tadpoles but not enough to cause physical damage or unavoidable bumping into the sides of the tank (Glennemeier and Denver, 2002a; Glennemeier and Denver, 2002b). For temperature shock, 3–4 tadpoles from each genotype were held in separate nets and dipped into 40 degrees Celsius rearing water for 30 s then immediately returned to rearing water at 26 degrees Celsius. 10 tadpoles per genotype were used in total. At the end of two-hour shaking stress and 30-second temperature shock test, all tadpoles were allowed to recover in their rearing tanks for 24 h, at which point they were scored for survival. Both experiments (two-hour shaking stress and 30-second temperature shock test) were done twice with similar results, and data from one of the two experiments have been presented.

2.6. Statistical analysis

Differences in gene expression levels and steroid hormone levels between wild-type and GRKO tadpoles were analyzed using Student's *t*-test (parametric) or Mann-Whitney test (non-parametric) using R statistical software (R Core Team, 2018). A p value of <0.05 was considered statistically significant.

3. Results

3.1. Hyperactive HPA axis in GRKO tadpoles

To assess the effects on negative feedback from lack of signaling through GR, we assayed *crh*, *avp*, and *pomc* in brain and *star*, *cyp17a1*, *cyp21a2*, and *cyp11b2* in kidney/gonad complex from climax stage (NF 61) GRKO and wild-type tadpoles. In brain, we found significantly higher mRNA levels of *pomc* in GRKO tadpoles as expected but significantly lower *crh* and *avp* (Fig. 1A-C). Hypothalamus-specific expression levels were not assayed. In the kidney/gonad complex, expression of *star* and all three steroidogenic enzymes assayed was higher in the mutants (Fig. 1D-G). We also quantified steroid hormone levels in tail homogenates by LC-MS/MS. Deuterated progesterone-d9 and corticosterone-d8 were used as internal standards to assess recoveries and matrix effects, which ranged from 88 to 95% and 72–98%, respectively, among the five steroids assayed. An MRM-HR method was created for quantitation of each steroid, namely progesterone, 17-hydroxyprogesterone, corticosterone, 21deoxycortisol, and aldosterone. Peak area ratios were used to construct steroid calibration curves, and all of them had a linear regression with $R^2 > 0.97$. The limit of detection (LOD) was 0.02–0.2 ng/mL in methanol buffer and 0.03–0.1 ng/mL in tadpole tail matrix across the five steroids. The limit of quantification (LOQ) threshold was 0.06–0.7 ng/mL and 0.1–0.7 ng/mL across the five steroids in methanol buffer and tadpole tail matrix, respectively. Tail content for all five steroids was significantly higher in the GRKO tadpoles (Fig. 1H-L).

3.2. GRKO tadpole response to stress

To investigate if signaling through GR is necessary for stress recovery, we exposed tadpoles to previously established shaking stress for 2 h and temperature shock stress for 30 s (Glennemeier and Denver, 2002a; Glennemeier and Denver, 2002b). We tested NF 54 (Table 2) tadpoles to shaking stress for 2 h and then allowed them to recover for 24 h. At the end of the recovery period, none of the GRKO tadpoles survived. Similarly for the temperature shock stress, wild-type and GRKO tadpoles were allowed to recover for 24 h after a 30-second temperature shock stress, and after the recovery period, 90% of the wild-type but only 10% of the GRKO tadpoles were alive. The surviving tadpoles were observed

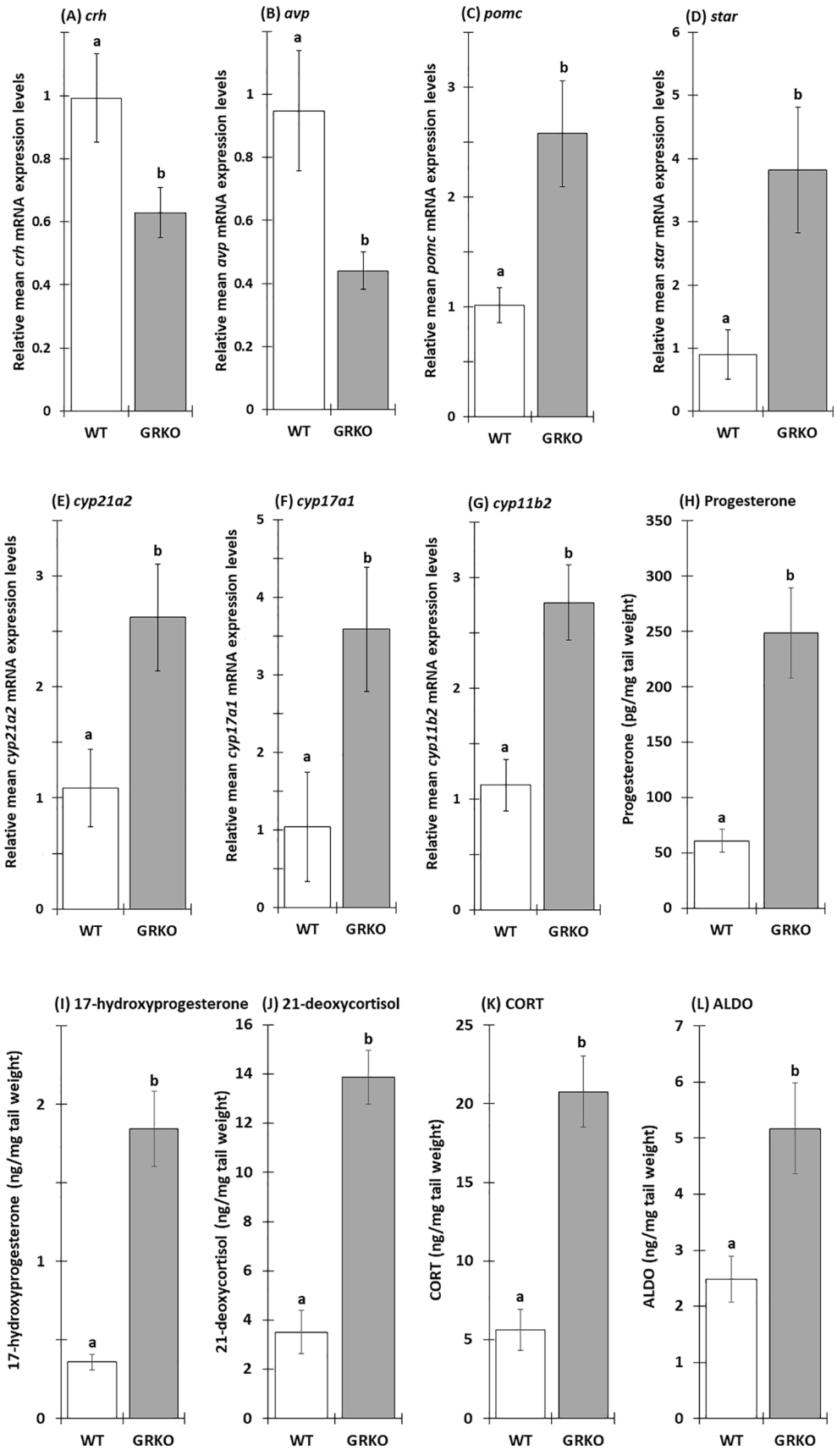

Fig. 1. Dysregulated HPI axis in GRKO tadpoles. Total RNA was collected from brains and kidneys of wild-type and GRKO metamorphic tadpoles to analyze mRNA expression of A) corticotropin releasing hormone (*crh*), B) arginine-vasopressin (*avp*), C) proopiomelanocortin (*pomc*), D) steroidogenic acute regulatory protein (*star*), E) 21-hydroxylase (*cyp21a2*), F) 17-hydroxylase (*cyp17a1*), and G) aldosterone synthase (*cyp11b2*). Bars represent mean mRNA levels relative to a wild-type sample and normalized by the housekeeping gene *rpl8*. n = 10–12 brain and kidney samples per genotype. Tadpole tails (~100 mg each) were harvested to measure H) progesterone, I) 17-hydroxyprogesterone (17-OHP), J) 21-deoxycortisol, K) CORT, and L) ALDO via LC-MS/MS in wild-type (WT, white bars), and glucocorticoid receptor knockout (GRKO, black bars) F2 tadpoles at Nieuwkoop and Faber (NF) stage 61 (metamorphic climax). n = 10 tails/genotype. Error bars represent SE. Letters indicate significant groups, $p < 0.05$.

Table 2
Death of GRKO tadpoles in response to shaking and temperature shock stress. Wild-type and GRKO NF 54 tadpoles were exposed to shaking stress for 2 h or temperature shock at 40 degrees Celsius for 30 s. Tadpoles were then allowed to recover under normal rearing conditions for 24 h. n = 10 / genotype / treatment. WT = wild-type tadpole, GRKO = glucocorticoid receptor knockout tadpole. Numbers represent number of tadpoles that survived after 24-hour recovery period. One experiment is shown out of two total that were performed, both had similar results.

Shaking stress		Temperature stress	
Genotype	**Survival**	**Genotype**	**Survival**
WT	10	**WT**	9
GRKO	0	**GRKO**	1

for another 24 h to check for possible late lethal phenotypes, but no further mortality occurred, and all survivors demonstrated normal feeding and swimming.

3.3. Expression profile of the mineralocorticoid receptor

Several studies in mammalian models but few using non-mammalian models have reported increased MR signaling during HPA hyperactivity or in the absence of GR (Cruz-Topete et al., 2019; Manwani et al., 2010; Tronche et al., 2004). No MR-response gene is known for *Xenopus*, so we investigated possible contributions to increased MR signaling in GRKO tadpoles by measuring MR mRNA expression levels in the brain, kidneys, tail, skin, and heart (Fig. 2). MR expression levels were significantly higher in the brain, kidneys, skin, and heart and significantly lower in tails of GRKO tadpoles.

3.4. Expression profile of pro-fibrotic and inflammatory markers

Lack of GR and increased MR signaling have been shown to cause fibrosis and inflammation in the kidneys and hearts in GRKO mice (Belden et al., 2017; Capelli et al., 2019; Joffe and Adler, 2005; Nakamura et al., 2022; Rickard et al., 2009; Wilson et al., 2009; Young, 2008; Young and Rickard, 2012). Here, we analyzed mRNA expression levels in tadpoles of known induced genes from mammals, namely tumor necrosis factor alpha (*tnf-α*) in kidney, endothelin-1 (*edn-1*), fibronectin-1 (*fn1*), and cyclin d2 (*ccnd2*) in the heart, and connective tissue growth factor (*ctgf*), plasminogen activator inhibitor type 1 (*pai-1*), and transforming growth factor beta 1 (tgf*β-1*) in heart and kidney (Bauersachs et al., 2015; Francis et al., 2003; Grossmann and Gekle, 2012; Juknevicius et al., 2004; Li et al., 2014; Lowe et al., 2018; Pacurari and Tchounwou, 2015; Rickard et al., 2009; Yuan et al., 2007). *tnf-α* and *tgfβ-1* were significantly upregulated in GRKO tadpole kidneys, while *ctfg* was significantly downregulated in the GRKO tadpole kidneys (Fig. 3). There was no significant difference in expression levels of the other genes in heart or kidney between wild-type and GRKO tadpoles (data not shown).

4. Discussion

We used GRKO tadpoles to investigate the role of GR in negative feedback to the HPI axis in amphibians. Similar to our prediction and consistent with previous vertebrate GRKO models, we found increased mRNA expression levels of the pituitary hormone *pomc* in the brain and, presumably due to the higher ACTH stimulation, increased *star, cyp17a, cyp21a2*, and *cyp11b2* in kidneys at NF 61 in GRKO tadpoles compared to wild-type (Facchinello et al., 2017; Ridder et al., 2005; Tajima et al., 1999; Ziv et al., 2013). Opposed to our predictions, *crh* and *avp* mRNA levels in the brain were significantly lower in the GRKO tadpoles. *Crh* and *avp* synergistically stimulate ACTH production which is crucial for interrenal steroid production. Several possible reasons could account for decreased *crh* and *avp* yet increased *pomc*. We dissected fore- and hindbrain, where *crh* levels vary widely among regions, rather than just the hypothalamus for measuring *crh* mRNA expression levels. In frog brain, CORT decreased *crh* mRNA levels in preoptic area (mammalian homolog of the hypothalamus) but increased *crh* mRNA levels in medial amygdala and bed nucleus of stria terminalis (Yao et al., 2008). A related issue was observed in zebrafish, where no detectable difference in *crh* mRNA expression was observed despite high cortisol levels likely because whole embryos were used rather than brain or hypothalamus (Faught and Vijayan, 2018). Similar to *crh, avp* is expressed in multiple brain regions, where AVP and CRH from the paraventricular nucleus stimulate ACTH production and are subject to negative feedback (Arnett et al., 2016). However, AVP produced by the magnocellular cells of the paraventricular nucleus and by the suprachiasmatic nucleus remains unaffected by direct glucocorticoid negative feedback in mammals (Davis et al., 1986; Fulford and Harbuz, 2005; Gallo-Payet et al., 2009; Kovács et al., 2000; Kuwahara et al., 2003; Sonneville et al., 2010; Wu and Childs, 1990). Rodent models have shown a correlation between MR overexpression in the hippocampus and low *crh* levels in the

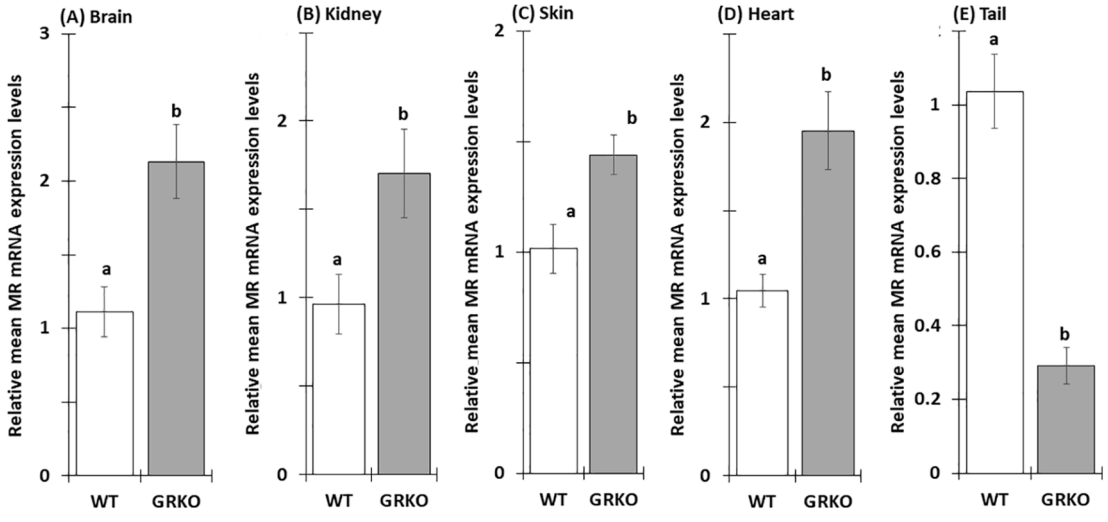

Fig. 2. GRKO tadpoles have altered MR expression levels. Total RNA was collected from A) brain, B) kidney, C) skin, D) heart, and E) tail of wild-type (WT, white bars) and glucocorticoid receptor knockout (GRKO, black bars) F2 tadpoles at Nieuwkoop and Faber (NF) stage 61 to analyze mRNA expression of mineralocorticoid receptor (MR). Bars represent mean mRNA levels relative to a wild-type sample and normalized by the housekeeping gene *rpl8*. n = 10–12 brain, kidney, and skin samples per genotype; n = 7 hearts/genotype; n = 10 tails/genotype. Error bars represent SE. Letters indicate significant groups, $p < 0.05$.

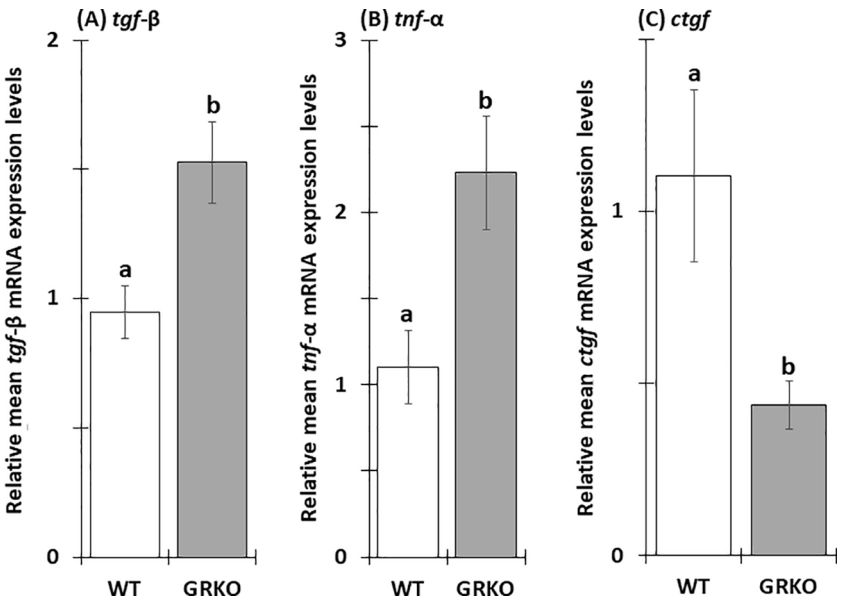

Fig. 3. GRKO tadpole kidneys have altered kidney expression levels of profibrotic and inflammatory factors. Total RNA was collected from kidneys of wild-type (WT, white bars) and glucocorticoid receptor knockout (GRKO, black bars) F2 tadpoles at Nieuwkoop and Faber (NF) stage 61 to analyze mRNA expression of A) transforming growth factor beta (*tnf-β*), B) tumor necrosis factor alpha (*tnf-α*), and C) connective tissue growth factor (ctgf). Bars represent mean mRNA levels relative to a wild-type sample and normalized by the housekeeping gene *rpl8*. n = 10–12 kidney samples per genotype. Error bars represent SE. Letters indicate significant groups, $p < 0.05$.

paraventricular nucleus (Welberg et al., 2001), which mimics the situation we observed, namely high MR and low *crh* expression in the brain in GRKO tadpoles. Other studies in mammalian models have shown that progesterone can reduce *avp* levels (Crofton et al., 1985; Watanabe et al., 1997), which again is consistent with the high progesterone levels and low *avp* expression in GRKO tadpoles. More investigation is needed to explain our *crh* and *avp* observations in GRKO tadpoles.

Consistent with increased *pomc*, *star*, *cyp21a2*, and *cyp11b2* mRNAs, we found high amounts of CORT and aldosterone (ALDO) content in tails. Previous studies showed that ACTH injections in tadpoles increased not only CORT but also ALDO, which is also consistent with increased production of both steroids seen here in GRKO (Green et al., 2016; Krug et al., 1983; Kumai et al., 1987; Perdomini et al., 2017; Thurmond et al., 1986). Progesterone is a precursor to CORT and ALDO and increased *star* supports the observation of higher tail content of progesterone in GRKO tadpoles. Our results match findings from mammalian studies where HPA hyperactivity and ACTH stimulation in rodent models increases production of progesterone, CORT, and ALDO, although changes in ALDO levels depended on the dose and time of ACTH administration (Hattangady et al., 2012; Haun and Haltmeyer, 1975; MacNiven et al., 1992; Thorpe et al., 2014). Another potential mechanism underlying increased ALDO production, shown in mammals, is that high progesterone antagonizes MR signaling and leads to increased Cyp11b2 activity and ALDO production in order to maintain sufficient MR signaling (Baker & Katsu, 2020; Braley et al., 1996; Michell & Noakes, 1985; Szmuilowicz et al., 2006; Wingo & Greenlee, 2011). Thus, progesterone's anti-mineralocorticoid activity may add to the HPI hyperactivity to explain higher mRNA expression of *cyp11b2* and high ALDO levels in GRKO tadpoles.

Although CORT is the main glucocorticoid in frogs, Cyp17a1 enzyme activity and cortisol has been detected in low but measurable quantities in several frog species, and hence accumulation of 17-hydroxyprogesterone (17-OHP; which is a cortisol precursor) was not unexpected (Do Rego et al., 2007; Forsburg et al., 2019; Gobbetti and Zerani, 1993; Navarro-Martín et al., 2012; Sakurai et al., 2008). We found high levels of 17-hydroxyprogesterone and *cyp11b2* expression, which explains the high levels of 21-deoxycortisol in the GRKO tadpoles. Thus, we show that GR not only regulates negative feedback to the HPI axis in tadpoles but also that negative feedback in frogs involves steroids such as progesterone, 17-hydroxyprogesterone, 21-deoxycortisol, CORT and ALDO, and steroidogenic enzymes, such as *star, cyp17a1, cyp21a2* and *cyp11b2*, which were previously unknown to be under HPI influence in amphibians.

Exposure to stressful conditions (altered pH, temperature, salinity, predators) has been found to elevate CORT levels in several frog species, and this increased glucocorticoid signaling plays a crucial role in response to and recovery from short-term stressors as well as adaptation to prolonged stressful environments (Adelizzi et al., 2019; Burraco and Gomez-Mestre, 2016; Chambers, 2011; Chambers et al., 2013; Florencio et al., 2020). CORT signaling antagonism by use of metyrapone (Cyp11b2 inhibitor) partially prevents elevation of CORT levels and impairs the tadpole response to the stressor. For example, metyrapone lessened the elevation in CORT levels and resulted in increased mortality upon predator exposure (Fraker et al., 2021). However, the receptors (GR and/or MR) involved in mediating CORT signaling upon perception of a stressful stimuli were not determined. To address this issue, we exposed GRKO tadpoles to a previously established shaking stress paradigm and a novel high temperature shock stress (Bonett et al., 2009; Glennemeier and Denver, 2002a; Glennemeier and Denver, 2002b). Strikingly, all GRKO tadpoles died by 24 h after a 2-hour gentle shaking stress previously shown to increase CORT levels in tadpoles (Glennemeier and Denver, 2002a; Glennemeier and Denver, 2002b). This result comports with our experience that GRKO tadpoles are very sensitive to slight laboratory mishandling / stress frequently resulting in death of mutant tadpoles. Our results also corroborate a recent study showing that RU486 (GR antagonist) reduces survival in tadpoles exposed to high salinity (Tornabene et al., 2021). Further, we exposed GRKO tadpoles to a 30-second temperature shock stress where only 10% GRKO tadpoles survived high temperature shock stress after 24 h as opposed to 90% survival in wild-type tadpoles. Though catecholamines are the first responders to vertebrate stress, previous studies have highlighted that lack of GR results in chromaffin cell apoptosis and inhibits adrenaline synthesis in mice (Davies and Lefkowitz, 1984; Hodel, 2001; Parlato et al., 2009; Sharara-Chami et al., 2010). Hence, while stress induced CORT levels might not be required for direct response to an acute stressor, basal CORT signaling dependent on GR might be critical for proper functioning of the chromaffin cells and catecholamine production.

In addition to the direct negative consequences of lack of signaling through GR, excessive signaling of CORT and ALDO through MR may also result in pathological conditions (Cruz-Topete et al., 2019; Manwani et al., 2010; Tronche et al., 2004). We found that GRKO tadpoles have the potential for excessive MR signaling through increased ligand and receptor. In particular, GRKO had not only higher CORT and ALDO to activate MR but also higher MR mRNA expression in the heart,

kidney, brain, and skin. MR is a thyroid hormone direct response gene in the tail, such that the low MR expression in GRKO tails may be a consequence of the reduced thyroid hormone signaling previously observed in GRKO tails (Bonett et al., 2010; Sterner et al., 2020; Sterner and Buchholz, 2022). Because lack of GR signaling and MR overexpression has been previously associated with pathologies in the heart and kidney (Bauersachs et al., 2015; Buonafine et al., 2018; Richardson et al., 2017; Rog-Zielinska et al., 2013), we examined mRNA expression levels of certain previously established biomarkers of heart and kidney disease in mammals. Tumor necrosis factor alpha (TNF-α) is an inflammatory cytokine produced by kidneys during acute inflammation. TGF-β is a cytokine which stimulates deposition of extracellular matrix on a site of tissue injury, excess of which in the kidneys leads to fibrosis and renal disease (Border and Noble, 1997). CTGF is a matricellular protein which can cooperate with TGF-β to exacerbate extracellular matrix production leading to sustained fibrosis in the heart and kidneys. ALDO increases TNF-α, TGF-β, and CTGF protein levels in the kidneys which can be reversed by spironolactone (a MR antagonist) treatment (Guney et al., 2009; Han et al., 2006; Martín-Fernández et al., 2016; Schreier et al., 2011). We found significant upregulation of *tnf-α* and *tgf-β* but downregulation of *ctfg* in GRKO tadpole kidneys. *ctfg* expression in the mesangial cells of the kidney depends on GR, as RU486 (GR and PR antagonist) was successful in inhibition of ALDO-induced *ctfg* expression in the mesangial cells of the kidney (Gauer et al., 2007). We did not find altered expression levels of profibrotic and inflammatory genes in the heart possibly because heart phenotype becomes prominent only in two month old cardiomyocyte GRKO mice and not during development (Oakley et al., 2019). Eight-month old GRKO zebrafish demonstrated a heart phenotype indicating weaker propulsive force in blood circulation and exhibited reduced heart rate (Facchinello et al., 2017). Elevated kidney expression of *tnf-α* and *tgf-β* in GRKO tadpoles in our study suggests that lack of GR signaling and/or increased MR signaling during development may cause inflammation and fibrosis in frog kidney.

5. Conclusions

In summary, the current study provides evidence that glucocorticoid receptor is necessary for negative feedback to the HPI axis in amphibians. Lack of negative feedback causes overproduction of steroid hormones CORT and ALDO and their precursor progesterone. We also report for the first time the steroid biosynthesis enzyme *cyp17a1* to be under HPI axis influence in tadpoles consequently leading to overproduction of 17-hydroxyprogesterone and 21-deoxycortisol. GR is also necessary for tadpole recovery from acute stressors, and lack of GR significantly reduces survival when premetamorphic tadpoles are exposed to shaking stress and high temperature shock stress. Finally, our study provides a preliminary insight into high levels of an important modulator of osmoregulation in vertebrates, i.e., MR, which together with lack of GR might be responsible for elevated expression of profibrotic and inflammatory biomarkers in the GRKO kidneys during development. Future studies should focus on investigating salt-water homeostasis and possible kidney disease phenotypes in GRKO tadpoles.

CRediT authorship contribution statement

Bidisha Paul: Conceptualization, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Zachary R. Sterner:** Investigation. **Ruchika Bhawal:** Investigation, Methodology, Formal analysis, Writing – review & editing. **Elizabeth T. Anderson:** Investigation, Writing – review & editing. **Sheng Zhang:** Methodology, Supervision, Writing – review & editing. **Daniel R. Buchholz:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data sets generated during and analyzed during the present study are not publicly available but are available from the corresponding author on reasonable request.

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