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Research paper

Glucocorticoid receptor is required for survival through metamorphosis in the frog *Xenopus tropicalis*

Zachary R. Sterner, Leena H. Shewade[1], Kala M. Mertz, Savannah M. Sturgeon, Daniel R. Buchholz*

Department of Biological Sciences, University of Cincinnati, Cincinnati, OH, United States

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ABSTRACT

Stress hormones, also known as glucocorticoids, are critical for survival at birth in mammals due at least in part to their importance in lung maturation. However, because air breathing is not always required for amphibian survival and because stress hormones have no known developmental impact except to modulate the developmental actions of thyroid hormone (TH), the requirement for stress hormone signaling during metamorphosis is not well understoodi. Here, we produced a glucocorticoid receptor knockout (GRKO) *Xenopus* line with a frameshift mutation in the first exon of the glucocorticoid receptor. Induction by exogenous corticosterone (CORT, the frog stress hormone) of the CORT response genes, *klf9* (Krüppel-like factor 9, also regulated by TH) and *ush1g* (Usher's syndrome 1G), was completely abrogated in GRKO tadpoles. Surprisingly, GRKO tadpoles developed faster than wild-type tadpoles until forelimb emergence and then developed more slowly until their death at the climax of metamorphosis. Growth rate was not affected in GRKO tadpoles, but they achieved a smaller maximum size. Gene expression analysis of the TH response genes, *thrb* (TH receptor beta) and *klf9* showed reduced expression in the tail at metamorphic climax consistent with the reduced development rate. These results indicate that glucocorticoid receptor is required for survival through metamorphosis and support dual roles for GR signaling in control of developmental rate.

1. Introduction

Glucocorticoids, or stress hormones, are a class of steroid hormones vital for the developmental maturation of organs in preparation for birth, principally the lungs, but also brain, liver, and kidney (Bolt et al., 2001; de Kloet et al., 2008; Fowden and Forhead, 2015; Liggins, 1994; Wada, 2008). Importantly, infants born prematurely experience insufficient glucocorticoid signaling resulting in inadequate gas exchange in immature lungs, and death occurs in extreme hormone deficiency (Davis and Sandman, 2010). While glucocorticoid treatment saves lives through maturational effects of the neonate lungs, overexposure to glucocorticoids can lead to negative consequences later in life, such as an increased risk of heart disease, neurological disorders, type 2 diabetes, and obesity, via incompletely known mechanisms (Harris and Seckl, 2011; Liu et al., 1997; Miguel et al., 2019).

Glucocorticoid levels in the blood are regulated centrally by the hypothalamic–pituitary adrenal axis during development and during stressful situations (Aguilera, 2011; Davis and Sandman, 2010; Jaudet and Hatey, 1984; Tsigos and Chrousos, 2002). Specifically, the central nervous system signals the hypothalamus to release corticotropin releasing hormone, which stimulates the pituitary to release adrenocorticotropic hormone, which then activates the adrenal glands to produce glucocorticoids. Glucocorticoids exert negative feedback at the level of the pituitary and the hypothalamus, allowing for homeostatic control of plasma levels of glucocorticoids (Makino et al., 2002). The developmental and metabolic actions of glucocorticoids are potentially mediated through two nuclear receptors: the type I corticosteroid receptor, also known as the mineralocorticoid receptor (MR), and the type II corticosteroid receptor, also known as the glucocorticoid receptor (GR) (Arriza et al., 1987; Kulkarni and Buchholz, 2014; Thompson, 1987). These nuclear receptors act as ligand-activated transcription factors to regulate gene expression (Beato and Klug, 2000; Funder, 1997).

In this report, we focus on GR signaling because its tissue distribution is more widespread than the MR and thus is likely to play a greater role in developmental regulation across tissues. Mice and zebrafish knockout models lacking GR have provided significant insights into the developmental role(s) of glucocorticoid signaling (Sakamoto

* Corresponding author.
E-mail address: buchhodr@ucmail.uc.edu (D.R. Buchholz).
[1] Current Address: SRI International- 333 Ravenswood Avenue, Menlo Park, CA 94025, United States.

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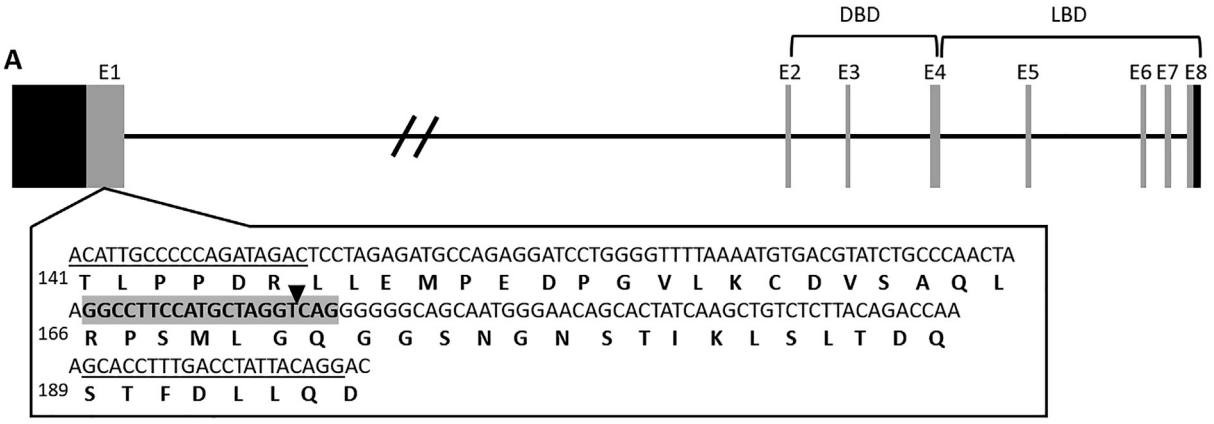

B

Genotype	Sequence	Indel
Wild-Type	TCTGCCCAACTAAGGCCTTCCATGCTAGGTCAGGGGGGCAGCAATGGGAACAGCACTATCAAGCTG 162 S A Q L R P S M L G Q G G S N G N S T I K L 183	---
GRKO	TCTGCCCAACTAAGGCCTTC----------CAGGGGGGCAGCAATGGGAACAGCACTATCAAGCTGT 162 S A Q L R P S R G A A M G T A L S S C 180	-10

Fig. 1. Genomic analysis of GR targeting by CRISPR A) The CRISPR target site of GR is in the first exon of 8 exons. Black bars at the start and end of the transcript represent 3′ and 5′ untranslated regions (UTRs). The region targeted has been expanded, showing the gRNA binding site (highlighted), the exact cut site (black triangle), and the forward and reverse PCR primer binding sites (underlined) used to amplify the targeted region. Amino acids are listed below the mRNA sequence. E = exon. DBD = DNA binding domain. LBD = ligand binding domain. B) Sequence analysis of GR mutation shows a 10-bp deletion. Computer generated translation is shown below each sequence. The wild-type sequence is shown at the top of the panel with the highlighted portion representing the CRISPR target site, while GRKO tadpoles demonstrate a 10-bp deletion within the target site. C) Computer translation of the wild-type and mutant GR sequences predicts a truncation mutation eliminating half of the N-terminal region and all of the the DNA-binding and the ligand-binding domains. The first 168 amino acids are in common followed by amino acids that were changed due to the frameshift mutation. NTD = N-terminal Domain. DBD = DNA-binding domain. H = Hinge Region. LBD = Ligand-binding domain. GRKO = Glucocorticoid receptor knockout.

and Sakamoto, 2019; Whirledge and DeFranco, 2018). GR knockout (GRKO) mice demonstrate a lethal phenotype shortly after birth because of impaired lung development, implicating the necessity of glucocorticoids in fetal lung maturation (Cole et al., 1995). In contrast, GRKO zebrafish are viable through adulthood (Facchinello et al., 2017). Like fish and unlike mammals, tadpoles of the biomedical model frog *Xenopus laevis* can survive metamorphosis and become fertile adults when completely denied access to air throughout life, though rearing in highly oxygenated water is required (Rose and James, 2013). Thus, glucocorticoid signaling for lung maturation, whether or not it occurs in frogs, may not be required for survival through metamorphosis. Similarly, glucocorticoid-dependent actions in other organs, as is known for mammals, are likely important in frogs, but such roles and requirements for glucocorticoids during metamorphosis are not well understood (Sachs and Buchholz, 2019). Also, because mammalian development occurs in a uterine environment in the presence of maternal hormones (Fowden and Forhead, 2015), developmental mechanisms of glucocorticoid action are challenging to isolate and analyze in mammals.

Tadpoles of *Xenopus* are free-living and free from maternal hormones, simplifying developmental analyses during the transition from aquatic to terrestrial living represented by birth in mammals and metamorphosis in amphibians (Buchholz, 2015). The current understanding for the role of corticosterone (CORT, the frog glucocorticoid) in amphibian development is to modulate the effects of thyroid hormone (TH), which is necessary and sufficient for initiating the changes that occur during metamorphosis (Kikuyama et al., 1993; Denver et al., 2002; Das et al., 2010; Kulkarni and Buchholz, 2014). In tadpoles, CORT levels, as well as TH levels, are low during premetamorphosis, slowly rise until they peak around metamorphic climax (NF 62, Nieuwkoop and Faber stage 62) (Nieuwkoop and Faber, 1967), and then decrease at the end of climax to the adult level (Leloup and Buscaglia, 1977; Jaudet and Hatey, 1984). Exogenous CORT treatment during early development inhibits growth and development (Glennemeier and Denver, 2002; Hayes et al., 1993), but CORT treatment in combination with TH accelerates developmental changes beyond those achieved by TH alone (Bonett et al., 2010). Similarly, stressful tadpole living conditions, such as crowding and low water, induce a stress response resulting in accelerated metamorphosis via increased CORT and TH plasma levels (Denver, 1998; Gomez-Mestre et al., 2013). Conversely, treatment with the GR antagonist, mifepristone (RU486) in the rearing water throughout larval development caused a 4-day delay to achieve tail resorption (Rollins-Smith et al., 1997). Gene expression studies identified genes induced by CORT,

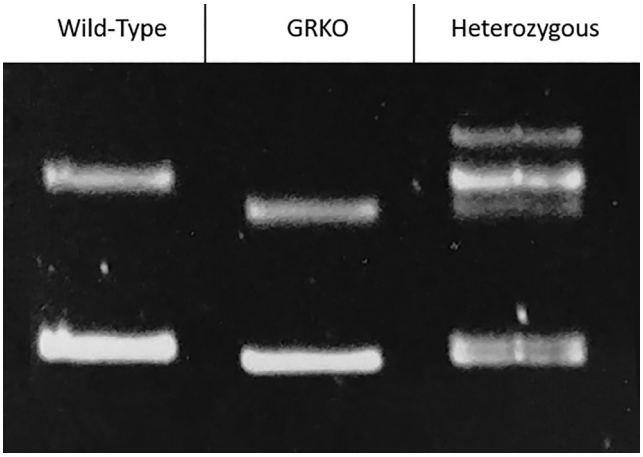

Fig. 2. Heteroduplex mobility assay (HMA). PCR amplification of wild-type (lane 1), GRKO (lane 2), and heterozygous (lane 3) tadpole DNA followed by boiling and reannealing of the PCR products results in diagnostic banding patterns on an ethidium bromide-stained gel. The lower bands run at the predicted PCR product sizes, and heteroduplex bands running more slowly through the gel were expected in the heterozygous sample. The identity of the upper bands in the wild-type and GRKO lanes are unknown.

including *klf9* (Krüppel-like factor 9) (Bagamasbad et al., 2012) and *ush1g* (Usher's syndrome 1G) (Schneider et al., 2018). With a single known exception (Miyakawa et al., 1984), CORT has no direct effect by itself to advance tadpole development except via modulation of TH signaling (Sachs and Buchholz, 2019).

In this study, our goal was to use CRISPR to knockout the frog GR to study the role of CORT/GR signaling in development. Whereas most embryological studies use the allotetraploid frog species *X. laevis*, gene knockout studies are greatly facilitated by use of the highly related *X. tropicalis* with its diploid genome and faster generation time (Kakebeen and Wills, 2019). Here, we characterized a GRKO line in *X. tropicalis* using genomic sequencing and CORT response gene induction, and then we used this line to examine the consequences for tadpole growth, development, and gene expression during metamorphosis.

2. Materials and methods

2.1. Animal husbandry

Wild-type outbred male and female adult *Xenopus tropicalis* from the lab colony were mated by priming with 20U of ovine luteinizing hormone (National Hormone and Peptide Program, http://www.humc.edu/hormones) in the evening and boosting with 200U the following morning. Tadpoles were reared at 26 °C and fed twice daily with powdered food (Sera Micron), and water was changed daily. Animal use in experiments was in accordance with the University of Cincinnati Institutional Animal Care and Use Committee (IACUC protocol # 06–10-03–01).

2.2. CRISPR-Cas9 microinjections

Xenopus tropicalis eggs were fertilized, de-jellied, and then zygotes or 2-cell stage embryos were injected with 500 pg Cas9 mRNA, 150 pg sgRNA, and 400 pg mCherry in 2 nL using a Picospritzer III (Parker Hanaffin Corp.) as previously described (Choi et al., 2015). Cas9 mRNA was prepared from NotI-linearized pCS2-Cas9 (gift from A. Zorn) using the MEGAscript SP6 *in-vitro* transcription kit (Ambion). The GR target sequence (5′ GGCCTTCCATGCTAGGTCAG) was cloned into the plasmid DR274 (gift from I. Blitz), and the sgRNA was prepared from DR274-GR using MEGAscript T7 (Blitz et al., 2013). mCherry mRNA from *Kpn*I-linearized CS108-mCherry (gift from Dr. M. Khokha) was also prepared using the MEGAscript T7 *in-vitro* transcription kit. Injected embryos positive for mCherry expression were founders and reared to adulthood and then crossed to obtain F1 and F2 generations.

2.3. Heteroduplex mobility assay and establishment of the GRKO line

The heteroduplex mobility assay (HMA) was utilized to genotype F1 and F2 tadpoles (Delwart et al., 1993; Ota et al., 2013). Genomic DNA was prepared from tail tips by means of the HotSHOT protocol used in mice (Truett et al., 2000). Briefly, tail tips were excised using a razor blade and incubated in 50 uL of 25 mM NaOH/0.2 mM EDTA for 15 min at 95C, and then 50 uL of 40 mM Tris-HCl was added to neutralize the solution followed by vortexing. Next, PCR reactions on 1uL genomic DNA were carried out using DreamTaq (Thermo) and the primers 5′-ACATTGCCCCCAGATAGAC and 5′-CCTGTAATAGGTCAAAGGTGC to amplify the CRISPR target region. Reactions were performed at 94C for 5 min, 32 cycles of (98C for 10 s, 52C for 15 s, and 72C for 30 s), and 72C for 5 min. DNA strands were then separated and allowed to re-anneal (95C for 5 min, 16C for 10 min, and 25C for 5 min). These reactions were loaded on 8% polyacrylamide gels and run at 150 V. Gels stained with ethidium bromide were imaged using a UV transilluminator (Fisher). Tadpoles were sorted into wild-type, heterozygous, and homozygous based on HMA patterns. One HMA pattern was kept from F1 individuals that had a single 10-bp frameshift mutation identified by Sanger sequencing using F2 animals. Genotyped animals from F2 individuals were used in experiments.

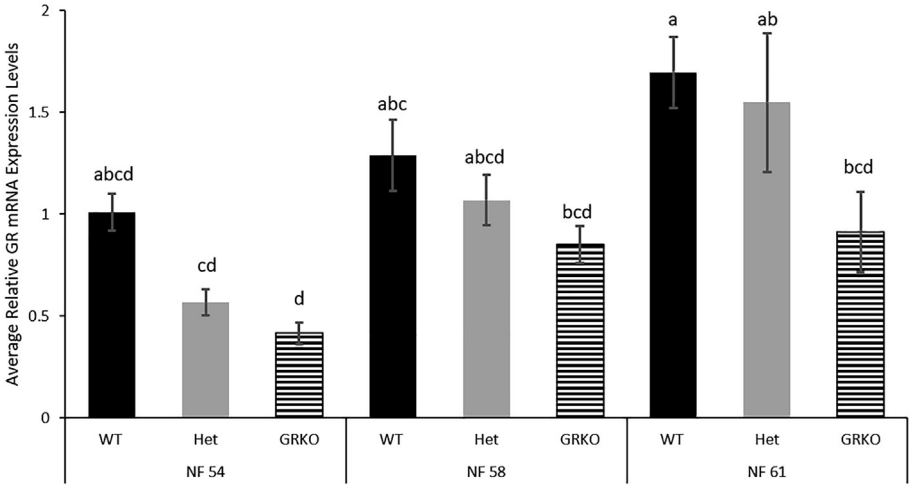

Fig. 3. GR expression during natural metamorphosis. Tails were harvested from wild-type, heterozygous, and GRKO individuals reared to NF 54, NF 58, and NF 61 for gene expression analysis of GR. GR expression levels were normalized to rpl8 and analyzed utilizing ANOVA. Letters above each bar represent significance groups based on Tukey's honest significant difference test ($p < 0.05$). n = 5 per genotype. Error bars represent standard error. WT = Wild-type. Het = Heterozygous. GRKO = Glucocorticoid receptor knockout.

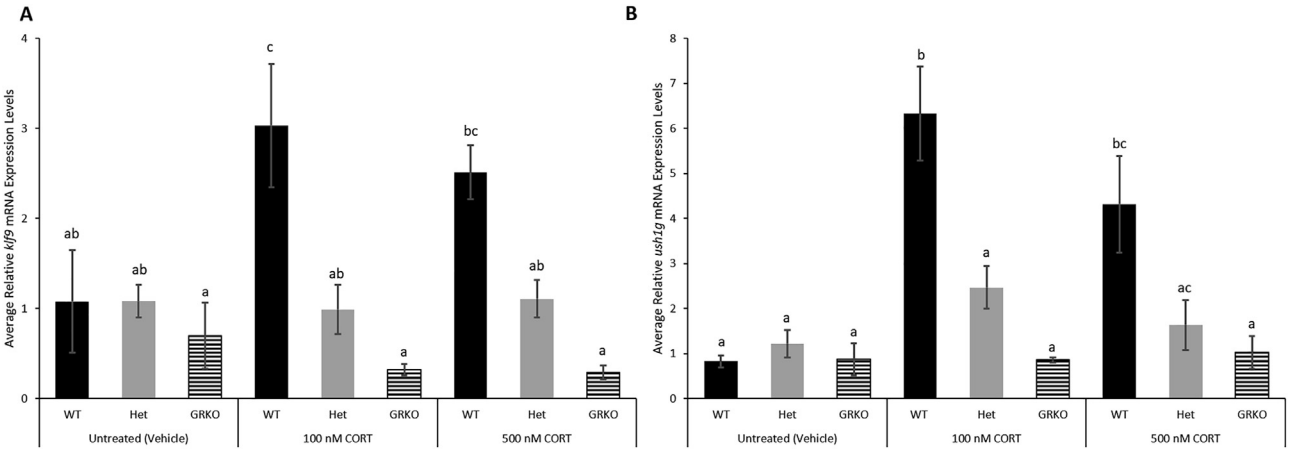

Fig. 4. Impaired CORT-response gene induction by exogenous CORT. Wild-type, heterozygous, and GRKO individuals at NF 54 were treated with vehicle, 100 nM CORT, or 500 nM CORT for 24 h, and tails were harvested for RNA extraction and quantitative PCR. mRNA expression levels relative to the normalizing gene *rpl8* of the CORT-response genes (A) *klf9* and (B) *ush1g* show lack of induction for both genes in both heterozygotes and GRKO animals. n = 5 per genotype per treatment. Expression levels were analyzed across genotypes and treatment groups. Letters above bars represent significance groups based on Tukey's honest significant difference test ($p < 0.05$). Error bars represent standard error. WT = Wild-type. Het = Heterozygous. GRKO = Glucocorticoid receptor knockout.

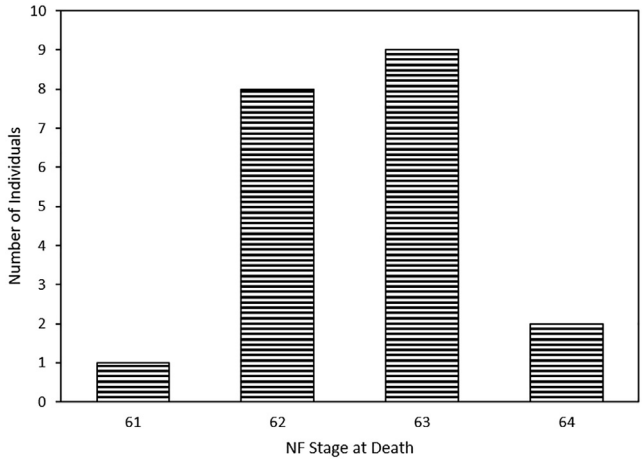

Fig. 5. Lethality of GR mutation. Twenty GRKO individuals were genotyped at NF 50-53 and individually reared through larval development. The histogram of NF stage at death of GRKO individuals reveals that mutant tadpoles all died, predominantly at metamorphic climax (NF 62-63) when TH and CORT levels are highest in wild-type individuals and gill resorption is occurring.

2.4. Growth and development

When F2 tadpoles reached NF 50-53 (foot paddle stage) (Nieuwkoop and Faber, 1967), they were sorted into genotypes (wild-type, heterozygous, GRKO) and individually reared in 2 L buckets. *Ad libitum* feeding occurred once daily with water changes occurring every 3 days. To reduce the chances of temperature bias, all tadpoles were reared on the same shelf level and were haphazardly shifted positions each water change. NF stage and snout-vent length (from anterior-most point of head not including tentacles to posterior part of cloacal opening) were measured every 5 days until each individual died or reached the completion of metamorphosis at NF 66 (tail resorption). Also, the number of days to NF 58 (forelimb emergence) and NF 61 (beginning of gill resorption) was recorded for each individual.

2.5. Hormone treatment and gene expression

To measure hormone response gene induction by exogenous CORT, F2 tadpoles genotyped at NF50-53 were treated at NF54 (just at beginning of metamorphosis) for 24 h. with vehicle (ethanol), 100 nM CORT, or 500 nM CORT. After 24 h of treatment, tails from MS222-anesthetized tadpoles were harvested and snap frozen on dry ice (n = 5 per genotype per treatment). For hormone response gene expression during natural metamorphosis, tails from tadpoles of each genotype were harvested at NF 54, NF 58, and NF 61 (n = 10). RNA extraction was performed using TRI REAGENT RT following the manufacturer's instructions (Molecular Research Center, Inc.). Complementary DNA synthesis from 1ug RNA for each sample was obtained using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). QPCR with 1uL cDNA was carried out using Luminaris Color Probe qPCR Master Mix High ROX (Thermo Scientific) and the TaqMan primer/probe sets (Applied Biosystems) on a 7300 Real Time PCR System (Applied Biosystems) for *klf9*, *ush1g*, *thrb*, and *rpl8* (Dhorne-Pollet et al., 2013). TaqMan primer probe sequences were the following: ribosomal protein L8 (*rpl8*) (forward: 5′-AGAAGGTCATCTCATCTGCAAACAG, reverse: 5′-CTTCAGGATGGGTTTGTCAATACG A, probe: 5′ CAACCCCAA CAATAGCT), Usher syndrome 1G (*ush1g*) (forward: 5′- GCGCTGCGGG TCATTG, reverse: 5′-GCGGCCACGTGCAG, probe: 5′-TCACCCCCCC GGCTGA), Krüppel-like factor 9 (*klf9*) (forward: 5′- CCTTAAAGCCCA TTACAGAGTCCAT, reverse: 5′-GCAGTCAGGCCACGTACA, probe: 5′-ACAGGTGAACGCCCTTTT), and thyroid hormone receptor β (*thrb*) (forward: 5′-CAAGAGTTGTTGATTTTGCCAAAAA, reverse: 5′-ACATGA TCTCCATACAACAGCCTTT, probe: 5′-CTGCCATGTGAAGACC).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) with genotypes, treatments, or stages as factors was performed with base R (R Core Team, 2018) and Tukey's honest significant difference test ($\alpha = 0.05$) as part of the agricolae package in R v3.4.4 (Mendiburu, 2017).

3. Results

3.1. CRISPR design and breeding to obtain GRKO tadpoles

To create glucocorticoid receptor knockout frogs, the CRISPR-Cas9 system was utilized to target the first exon of the GR gene of *X. tropicalis* to disrupt the DNA-binding domain (DBD) and the ligand binding domain (LBD) (Fig. 1A). To create null mutations in both alleles, founder *X. tropicalis* were first bred to wild-type *X. tropicalis* to produce F1 animals which were screened with HMA to identify GR heterozygous individuals. A single HMA pattern was kept and is represented in Fig. 2. Heterozygous individuals all harboring the same 10-base pair

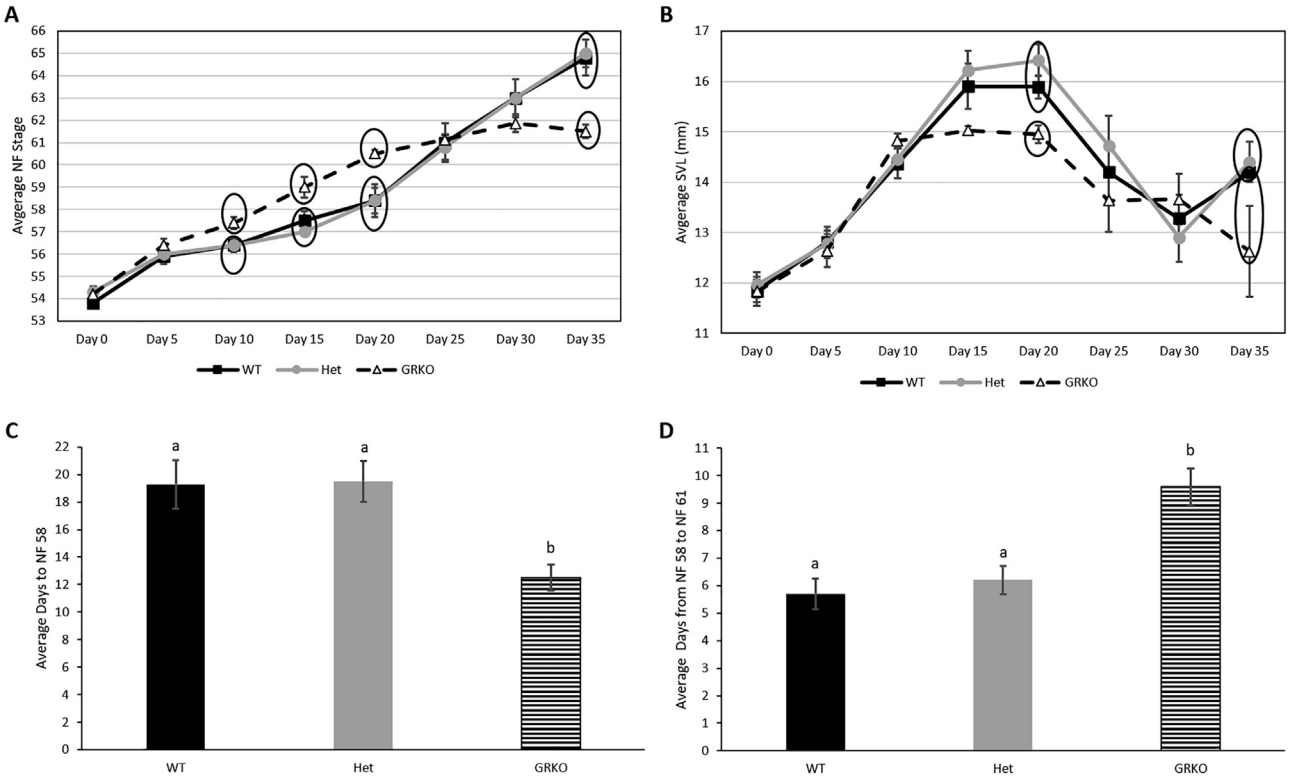

Fig. 6. Growth and development anomalies in GRKO tadpoles. Sibling tadpoles of each genotype, (wild-type, heterozygotes, GRKO) were reared individually starting at NF 54 (just before metamorphosis begins), and (A) developmental stage and (B) tadpole snout-vent length (SVL) were recorded every 5 days. Also, (C) day of forelimb emergence (NF 58) and (D) start of gill resorption (NF 61) was recorded for each tadpole. (A, B) Circled shapes represent significance groups based on Tukey's honest significant difference test ($p < 0.05$) assessed for each Day. (C, D) Letters above bars represent significance groups based on Tukey's honest significant difference test ($p < 0.05$). n = 10 per genotype. Error bars represent standard error. WT = Wild-type. Het = Heterozygous. GRKO = Glucocorticoid receptor knockout.

frameshift mutation (Fig. 1B, C) were crossed with each other to produce F2 offspring where approximately 25% were wild-type, 50% were heterozygous, and 25% were GRKO based on HMA screening.

3.2. Gene expression analysis of GR during natural metamorphosis

To determine if there was any evidence of nonsense-mediated decay, GR expression was analyzed via gene expression analysis in the tails of wild-type, heterozygous, and GRKO animals across natural metamorphosis (Fig. 3). At NF 54 and NF 58, there were no significant differences between the three genotypes. At NF 61, there was a significant decrease in GR expression in GRKO tadpoles compared to wild-type and heterozygous, suggesting nonsense-mediated decay.

3.3. Impaired CORT response-gene induction in GRKO tadpoles

To confirm the sequencing results predicting a non-functional receptor in GRKO tadpoles, CORT response gene induction was measured in wild-type, heterozygous, and GRKO tadpoles after 24 hrs of treatment with exogenous CORT at 0, 100, and 500 nM. We measured the induction of the CORT response gene *klf9* (Bagamasbad et al., 2012; Shewade et al., 2017) and the CORT-only response gene *ush1g* (Schneider et al., 2018) relative to the housekeeping gene *rpl8* (Dhorne-Pollet et al., 2013). As expected, GRKO tadpoles demonstrated no upregulation in either *klf9* or *ush1g* in response to CORT treatment, while both genes were induced by CORT in wild-type siblings (Fig. 4). As commonly observed in endocrine experiments, the upregulation of *klf9* in wild-type tadpoles showed an inverted U induction profile (Shewade et al., 2017; Vandenberg et al., 2012). Surprisingly, in GR heterozygous individuals, we found no significant upregulation in GR heterozygous individuals in either gene in response to CORT treatment at 100 nM or 500 nM.

3.4. Growth, Development, and Lethality

To examine the developmental consequences of impaired GR signaling, we attempted to rear GRKO tadpoles through metamorphosis and found that all perished between the start of gill resorption and mid-tail resorption (NF 61–64) (Fig. 5). Heterozygous individuals are viable and fertile as adults. To quantify the effects on growth and development prior to death, we individually raised 10 tadpoles of each genotype starting at NF 54. GRKO tadpoles developed at a faster rate compared to wild-type and heterozygous animals until NF 58, after which the developmental rate of the GRKO tadpoles dramatically decreased until death around NF 62–63 (Fig. 6A, C, D). Specifically, GRKO tadpoles reached forelimb emergence (NF 58) 7 days earlier than wild type but then reached beginning of gill resorption 4 days later then wild type, a difference of > 1 week in the ~4 week assay period (Fig. 6C, D). Tadpole size over time showed a different pattern among genotypes compared to development (Fig. 6.B). All genotypes grew similarly until Day 10, after which time the GRKO tadpoles reached their maximum size earlier than wild-type and heterozygous tadpoles.

3.5. Gene expression analysis across natural metamorphosis

Because of the developmental phenotypes observed in the GRKO tadpoles, we analyzed thyroid hormone (TH) and CORT response gene expression from tails of NF 54, NF 58, and NF 61 tadpoles from each genotype (Fig. 7). As development proceeded for each genotype, *thrb* and *klf9* increased, but *ush1g* increased only in wild type. At NF 61, GRKO tadpoles demonstrated significantly lower expression of *thrb* and *klf9* compared to wild-type individuals, consistent with the reduction in

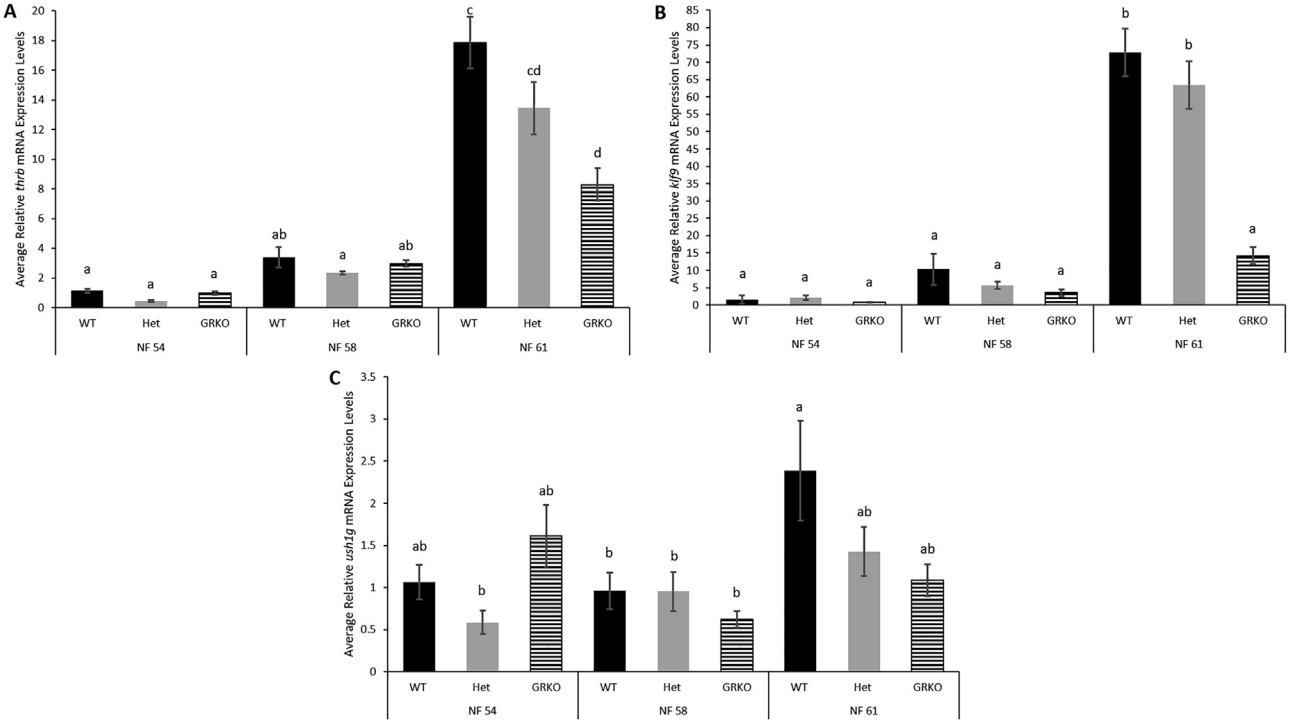

Fig. 7. Impaired TH-response gene expression during natural metamorphosis. Sibling wild-type, heterozygous, and GFKO tadpoles were reared to NF 54, NF 58, or NF 61, and their tails were harvested for RNA extraction and quantitative PCR. mRNA expression levels relative to the normalizing gene *rpl8* of (A) *thrb*, a TH-response gene (B) *klf9*, a gene induced by TH and/or CORT, and (C) *ush1g*, a CORT-response gene shows reduced TH-response gene expression at NF 61. For NF 54, n = 5 per genotype. For NF 58 and NF 61, n = 10 per genotype. Letters above each bar represent significance groups based on Tukey's honest significant difference test ($p < 0.05$). Error bars represent standard error. WT = Wild-type. Het = Heterozygous. GRKO = Glucocorticoid receptor knockout.

developmental rate at this stage. The converse was not seen, namely despite the increased developmental rate to achieve NF58 in GRKO animals, no difference in hormone response genes were seen at this stage.

4. Discussion

Here, we produced and characterized the growth and development of GR knockout *X. tropicalis*. We used CRISPR-Cas9 to target the first exon of GR and isolated a 10-bp mutant predicted to produce a truncated protein lacking the DNA- and ligand-binding domains and about half of the activation function domain 1 in the N-terminus, which interacts with coregulatory proteins (Bamberger et al., 1996; Kumar and Thompson, 2005). Loss of ligand-dependent gene induction was confirmed by showing that GRKO tadpoles failed to induce the CORT response genes, *klf9* and *ush1g*, in response to exogenous CORT in premetamorphic tadpoles. Surprisingly, we observed that gene expression of heterozygous individuals showed no significant differences from GRKO tadpoles, even at high doses of CORT (500 nM). It is possible that the expression level of GR in premetamorphosis is very low, potentially below a threshold in heterozygotes required to observe gene induction. We also observed significant evidence of nonsense-mediated decay (NMD) (Brogna and Wen, 2009) at late but not early stage tadpoles, suggesting that at least some mutant GR mRNA is being removed by this mechanism. It is possible that NMD occurs at earlier stages, but GR expression is lower at those stages such that any difference may be undetectable.

The most striking observation in GRKO tadpoles is that they do not survive through metamorphosis. GRKO tadpoles progress through most of the metamorphic changes but then die between metamorphic climax and nearly complete tail resorption. Thus, signaling through GR is required for metamorphic completion. This result contrasts with the expectation from prior studies that CORT signaling has no known effect on metamorphosis except to modify the rate of development controlled by TH (Kikuyama et al., 1993; Denver, 2009). On the other hand, death at metamorphosis is comparable to death at birth in GR knockout mice, both occurring at the aquatic to terrestrial developmental transition (Buchholz, 2015). The cause of death in GRKO mice is from lung atelectasis, but the cause of death in tadpoles is not yet known. Most GRKO tadpoles die around the time of gill resorption (NF 62 and NF 63), correlating with switching from using gills to lungs. Thus, tadpole suffocation is a possible cause of death, because under normal rearing conditions access to air is required for survival in tadpoles and adults (Rose and James, 2013). However, the GRKO tadpole lungs appeared normal at the gross morphology level (not shown), but neither histology nor gas exchange function was examined. Alternatively, tissue-specific GR knockout mice have shown that death within two days of birth occurs in 50% of mice lacking liver GR expression, presumably due to impaired regulation of glucose metabolism (Opherk et al., 2004). Further studies are required to identify the cause of death in GRKO tadpoles.

GRKO phenotypes were also observed prior to the death that occurs at the end of metamorphosis. In two independent growth and development experiments, GRKO tadpoles developed significantly faster to achieve forelimb emergence but from then took significantly longer to achieve metamorphic climax. As for growth, tadpole size was similar among genotypes until Day 15 when GRKO tadpoles achieved the stage at which maximum tadpole size occurs (forelimb emergence, NF 58). Because GRKO tadpoles achieved NF 58 earlier, they had less overall time to grow to that stage and thus achieved a smaller size compared to wild-type and heterozygotes that had longer time to grow until NF 58. These results complement previous studies showing that GR signaling induced early by exogenous CORT treatment inhibits development (Lorenz, Opitz, Lutz, & Kloas, 2009; Kobayashi, 1958), and later in metamorphosis GR signaling with exogenous CORT or stressful rearing conditions can accelerate metamorphic events (Bonett et al., 2010;

Kulkarni et al., 2011). These data suggest that GR signaling impedes development before forelimb emergence and then accelerates development in subsequent developmental stages.

To gain insight into the mechanisms underlying these divergent effects of GR signaling on developmental rate, we analyzed the expression of hormone response genes during natural metamorphosis, namely a TH response gene (*thrb*), a TH and CORT response gene (*klf9*), and a CORT response gene (*ush1g*) (Bagamasbad et al., 2012; Choi et al., 2015; Schneider et al., 2018). In wild-type tadpoles, these genes all reach a maximum expression level at climax of metamorphosis, corresponding to peak hormone titers that occur at that stage compared to minimal or non-detectable titers in premetamorphic tadpoles just beginning tissue transformations (Buchholz, 2015; Jaudet and Hatey, 1984). As expected, the CORT-response gene *klf9* was lower in GRKO tadpoles at climax, consistent with the requirement of GR for *klf9* induction (Shewade et al., 2017). The other CORT response gene had reduced expression at climax but was not significantly lower than in wild-type tadpoles. Importantly, we observed a significant decreased expression of *thrb* in the GRKO tadpoles compared to wild-type individuals. This decreased expression in *thrb* in GRKO tadpoles suggests a reduction of TH signaling as a consequence of lack of CORT signaling. This conclusion is consistent with the known synergistic effect CORT has on TH signaling, which can accelerate TH-dependent metamorphic events via co-regulation of response genes and alteration of intracellular TH metabolism (Bonett et al., 2010). The reduced *klf9* induction may also be due in part to reduced TH and CORT synergy as well as reduced direct CORT signaling. Because TH is necessary and sufficient to initiate metamorphic changes (Kikuyama et al., 1993; Denver and Glennemeier, 2009; Kulkarni and Buchholz, 2014), the reduced TH response gene expression in GRKO tadpoles suggests a lack of synergy with TH and may explain the impaired rate of development between forelimb emergence and death just before tail resorption.

Interestingly, at a time (forelimb emergence) when GRKO tadpoles were developing faster than WT individuals, there were no significant differences between *thrb* expression levels of any of the three genotypes. These findings demonstrate that CORT may have a developmental action independent of modulating TH signaling or that the hormone induced levels of *thrb* and *klf9* are still too low to detect a difference. Future hormone rescue studies with frogs lacking endogenous CORT synthesis are required to address these questions. Also, we observed normal growth and development and gene expression profiles in heterozygotes despite the lack of induction of CORT-response genes by exogenous CORT. It is possible that the amount of GR is insufficient in heterozygotes to enable a response to CORT treatment in premetamorphic tadpoles, but GR expression levels in heterozygotes may be sufficient for gene induction later in development when GR levels are higher.

In summary, we have created the first glucocorticoid receptor knockout organisms in *Xenopus*. In these GRKO tadpoles, we have demonstrated a lethal phenotype at metamorphosis, a phenotype seen in GR knockout mice but not zebrafish. We also observed an accelerated development to forelimb emergence followed by a delay in development after that until death at metamorphic climax. Even though growth rate was not impaired, overall size was reduced, apparently as a consequence of accelerated development to forelimb emergence, the stage at which maximum tadpole size is achieved. Reduced expression of TH response genes at metamorphic climax in GRKO tadpoles correlates with the delayed rate of development and implicates a role for GR in regulatory interactions with TH signaling. Future studies are required to examine mechanisms underlying how GR may affect rate of development throughout the larval period and how some CORT signaling may be independent of TH signaling.

Author Contributions

ZRS and DRB conceived and designed the study, DRB supervised experimental implementation, LHS designed and implemented CRISPR-Cas9 to create F_0 and F_1 individuals, ZRS performed all sample collection and data analysis, KMM and SMS performed HMA analyses and assisted with the growth and development assay, DRB and ZRS decided on the direction of the manuscript, ZRS wrote the manuscript, and all authors critically revised the manuscript and gave final approval of the version to be published.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2020.113419.

References

Aguilera, G., 2011. HPA axis responsiveness to stress: implications for healthy aging. Exp. Gerontol. 46, 90–95. https://doi.org/10.1016/j.exger.2010.08.023.

Arriza, J.L., Weinberger, C., Cerelli, G., Glaser, T.M., Handelin, B.L., Housman, D.E., Evans, R.M., 1987. Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science (80-) 237, 268–275. https://doi.org/10.1126/science.3037703.

Bagamasbad, P., Ziera, T., Borden, S.A., Bonett, R.M., Rozeboom, A.M., Seasholtz, A., Denver, R.J., 2012. Molecular basis for glucocorticoid induction of the krüppel-like factor 9 gene in hippocampal neurons. Endocrinology 153, 5334–5345. https://doi.org/10.1210/en.2012-1303.

Bamberger, C.M., Schulte, H.M., Chrousos, G.P., 1996. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocr. Rev. 17, 245–261. https://doi.org/10.1210/edrv-17-3-245.

Beato, M., Klug, J., 2000. Steroid hormone receptors: an update. Hum. Reprod. Update 6, 225–236. https://doi.org/10.1093/humupd/6.3.225.

Blitz, Ira L., Biesinger, Jacob, Xie, Xiaohui, Cho, K.W.Y., 2013. Biallelic genome modification in F0 Xenopus tropicalis embryos using the CRISPR/Cas system. Genesis 51, 827–834. https://doi.org/10.1002/dvg.22719.

Bolt, R.J., Weissenbruch, M.M. Van, Lafeber, H.N., 2001. State of the art glucocorticoids and lung development in the fetus and preterm infant. Pediatr. Pulmonol. 32, 76–91. https://doi.org/10.1002/ppul.1092.

Bonett, R.M., Hoopfer, E.D., Denver, R.J., 2010. Molecular mechanisms of corticosteroid synergy with thyroid hormone during tadpole metamorphosis. Gen. Comp. Endocrinol. 168, 209–219. https://doi.org/10.1016/j.ygcen.2010.03.014.

Brogna, S., Wen, J., 2009. Nonsense-mediated mRNA decay (NMD) mechanisms. Nat. Struct. Mol. Biol. 16, 107–113. https://doi.org/10.1038/nsmb.1550.

Buchholz, D.R., 2015. More similar than you think: Frog metamorphosis as a model of human perinatal endocrinology. Dev. Biol. 408, 188–195. https://doi.org/10.1016/j.ydbio. 2015.02.018.

Choi, J., Suzuki, K.I.T., Sakuma, T., Shewade, L., Yamamoto, T., Buchholz, D.R., 2015. Unliganded thyroid hormone receptor-regulates developmental timing via gene repression in Xenopus tropicalis. Endocrinology 156, 735–744. https://doi.org/10.1210/en.2014-1554.

Cole, T.J., Blendy, J.A., Monaghan, A.P., Krieglstein, K., Schmid, W., Aguzzi, A., Fantuzzi, G., Hummler, E., Unsicker, K., Schutz, G., 1995. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev. 9, 1608–1621. https://doi.org/10.1101/gad.9.13.1608.

Das, B., Matsuda, H., Fujimoto, K., Sun, G., Matsuura, K., Shi, Y.B., 2010. Molecular and genetic studies suggest that thyroid hormone receptor is both necessary and sufficient to mediate the developmental effects of thyroid hormone. Gen. Comp. Endocrinol. 168, 174–180. https://doi.org/10.1016/j.ygcen.2010.01.019.

Davis, E.P., Sandman, C.A., 2010. The timing of prenatal exposure to maternal cortisol and psychosocial stress is associated with human infant cognitive development. Child Dev. 81, 131–148. https://doi.org/10.1111/j.1467-8624.2009.01385.x.

de Kloet, E.R., Karst, H., Joëls, M., 2008. Corticosteroid hormones in the central stress

response: quick-and-slow. Front. Neuroendocrinol. 29, 268–272. https://doi.org/10.1016/j.yfrne. 2007.10.002.
Delwart, E.L., Shpaer, E.G., Louwagie, J., McCutchan, F.E., Grez, M., Rübsamen-Waigmann, H., Mullins, J.I., 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. Science 262, 1257–1261. https://doi.org/10.1126/science.8235655.
Denver RJ, Glennemeier KA, Boorse GC. Endocrinology of complex life cycles: amphibians. In: Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Ruben RT, editors. Hormones, Brain and Behavior, 2nd ed. San Diego,CA: Academic Press (2009) p. 707–44. doi: 10.1016/B978-008088783-8.00021-8.
Denver, R.J., 2009. Stress hormones mediate environment-genotype interactions during amphibian development. Gen. Comp. Endocrinol. 164, 20–31. https://doi.org/10.1016/j.ygcen. 2009.04.016.
Denver, R.J., Glennemeier, K.A., Boorse, G.C., 2002. Endocrinology of Complex Life Cycles: Amphibians. Horm. Brain Behav. 2, 469–XI. https://doi.org/10.1016/B978-012532104-4/50030-5.
Denver, R.J., 1998. Hormonal correlates of environmentally induced metamorphosis in the Western spadefoot toad, Scaphiopus hammondii. Gen. Comp. Endocrinol. 110, 326–336. https://doi.org/10.1006/gcen.1998.7082.
Dhorne-Pollet, S., Thélie, A., Pollet, N., 2013. Validation of novel reference genes for RT-qPCR studies of gene expression in Xenopus tropicalis during embryonic and post-embryonic development. Dev. Dyn. 242, 709–717. https://doi.org/10.1002/dvdy.23972.
Facchinello, N., Skobo, T., Meneghetti, G., Colletti, E., Dinarello, A., Tiso, N., Costa, R., Gioacchini, G., Carnevali, O., Argenton, F., Colombo, L., Dalla Valle, L., 2017. Nr3c1 null mutant zebrafish are viable and reveal DNA-binding-independent activities of the glucocorticoid receptor. Sci. Rep. 7, 1–13. https://doi.org/10.1038/s41598-017-04535-6.
Fowden, A.L., Forhead, A.J., 2015. Glucocorticoids as regulatory signals during intrauterine development. Exp. Physiol. 100, 1477–1487. https://doi.org/10.1113/EP085212.
Funder, J.W., 1997. Glucocorticoid and mineralocorticoid receptors: biology and clinical relevance. Annu. Rev. Med. 48, 231–240. https://doi.org/10.1146/annurev.med.48.1.231.
Glennemeier, K.A., Denver, R.J., 2002. Small changes in whole-body corticosterone content affect larval Rana pipiens fitness components. Gen. Comp. Endocrinol. 127, 16–25. https://doi.org/10.1016/S0016-6480(02)00015-1.
Gomez-Mestre, I., Kulkarni, S., Buchholz, D.R., 2013. Mechanisms and consequences of developmental acceleration in tadpoles responding to pond drying. PLoS One 8, 1–12. https://doi.org/10.1371/journal.pone.0084266.
Harris, A., Seckl, J., 2011. Glucocorticoids, prenatal stress and the programming of disease. Horm. Behav. 59, 279–289. https://doi.org/10.1016/j.yhbeh.2010.06.007.
Hayes, T., Chan, R., Licht, P., 1993. Interactions of temperature and steroids on larval growth, development, and metamorphosis in a toad (Bufo boreas). J. Exp. Zool. 266, 206–215. https://doi.org/10.1002/jez.1402660306.
Jaudet, G.J., Hatey, J.L., 1984. Variations in aldosterone and corticosterone plasma levels during metamorphosis in Xenopus laevis tadpoles. Gen. Comp. Endocrinol. 56, 59–65. https://doi.org/10.1016/0016-6480(84)90061-3.
Kakebeen, A., Wills, A., 2019. Advancing genetic and genomic technologies deepen the pool for discovery in Xenopus tropicalis. Dev. Dyn. 248, 620–625. https://doi.org/10.1002/dvdy.80.
Kikuyama, S., Kawamura, K., Tanaka, S., Yamamoto, K., 1993. Aspects of amphibian metamorphosis: hormonal control. Int. Rev. Cytol. 145, 105–148.
Kobayashi, H., 1958. Effect of Desoxycorticosterone acetate in metamorphosis induced by thyroxine in anuran tadpoles. Endocrinology 62 (4), 371–377. https://doi.org/10.1210/endo-62-4-371.
Kulkarni, S.S., Buchholz, D.R., 2014. Corticosteroid signaling in frog metamorphosis. Gen. Comp. Endocrinol. 203, 225–231. https://doi.org/10.1016/j.ygcen.2014.03.036.
Kulkarni, S.S., Gomez-Mestre, I., Moskalik, C.L., Storz, B.L., Buchholz, D.R., 2011. Evolutionary reduction of developmental plasticity in desert spadefoot toads. J. Evol. Biol. 24, 2445–2455. https://doi.org/10.1111/j.1420-9101.2011.02370.x.
Kumar, R., Thompson, E.B., 2005. Gene regulation by the glucocorticoid receptor: Structure:function relationship. J. Steroid Biochem. Mol. Biol. 94, 383–394. https://doi.org/10.1016/j.jsbmb.2004.12.046.
Leloup, J., Buscaglia, M., 1977. La triiodothyronine, hormone de la metamorphose des Amphibies. C. R. Acad. Sci. Paris, Ser. D. 284, 2261-2263.
Liggins, G.C., 1994. The role of cortisol in preparing the fetus for birth. Reprod. Fertil. Dev. 6, 141–150.
Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., Sharma, S., Pearson, D., Plotsky, P., Meaney, M., 1997. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science 277 (5332), 1659–1662. https://doi.org/10.1126/science.277.5332.1659.
Lorenz, C., Opitz, R., Lutz, I., Kloas, W., 2009. Corticosteroids disrupt amphibian metamorphosis by complex modes of action including increased prolactin expression. Comp. Biochem. Physiol.-C Toxicol. Pharmacol. 150, 314–321. https://doi.org/10.1016/j.cbpc.2009.05.013.
Makino, S., Hashimoto, K., Gold, P.W., 2002. Multiple feedback mechanisms activating corticotropin-releasing hormone system in the brain during stress. Pharmacol. Biochem. Behav. 73, 147–158. https://doi.org/10.1016/S0091-3057(02)00791-8.
Mendiburu, F. ,2017. agricolae: Statistical Procedures for Agricultural Research. R package version 1.2-8.
Miguel, P.M., Pereira, L.O., Silveira, P.P., Meaney, M.J., 2019. Early environmental influences on the development of children's brain structure and function. Dev. Med. Child Neurol. 61, 1127–1133. https://doi.org/10.1111/dmcn.14182.
Miyakawa, M., Arai, Y., Kikuyama, S., 1984. Corticosterone stimulates the development of preoptic catecholamine neurons in tadpoles Bufo bufo japonicus. Anat. Embryol. (Berl) 170, 113–115. https://doi.org/10.1007/BF00318994.
Nieuwkoop, P.D., Faber, J., 1967. Normal table of Xenopus laevis (Daudin). North-Holland Publishing Company, Amsterdam.
Opherk, C., Tronche, F., Kellendonk, C., Kohlmüller, D., Schulze, A., Schmid, W., Schütz, G., 2004. Inactivation of the glucocorticoid receptor in hepatocytes leads to fasting hypoglycemia and ameliorates hyperglycemia in streptozotocin-induced diabetes mellitus. Mol. Endocrinol. 18, 1346–1353. https://doi.org/10.1210/me.2003-0283.
Ota, S., Hisano, Y., Muraki, M., Hoshijima, K., Dahlem, T.J., Grunwald, D.J., Okada, Y., Kawahara, A., 2013. Efficient identification of TALEN-mediated genome modifications using heteroduplex mobility assays. Genes to Cells 18, 450–458. https://doi.org/10.1111/gtc.12050.
R Core Team, 2018. R: A language and environment for statistical computing.
Rollins-Smith, L.A., Barker, K.S., Davis, A.T., 1997. Involvement of glucocorticoids in the reorganization of the amphibian immune system at metamorphosis. Dev. Immunol. 5, 145–152. https://doi.org/10.1155/1997/84841.
Rose, C.S., James, B., 2013. Plasticity of lung development in the amphibian, Xenopus laevis. Biol. Open 2, 1324–1335. https://doi.org/10.1242/bio.20133772.
Sachs, L.M., Buchholz, D.R., 2019. Insufficiency of thyroid hormone in frog metamorphosis and the role of glucocorticoids. Front. Endocrinol. 10, 17–20. https://doi.org/10.3389/fendo.2019.00287.
Sakamoto, T., Sakamoto, H., 2019. 'Central' actions of corticosteroid signaling suggested by constitutive knockout of corticosteroid receptors in small fish. Nutrients 11, 1–9. https://doi.org/10.3390/nu11030611.
Schneider, K.A., Shewade, L.H., Buisine, N., Sachs, L.M., Buchholz, D.R., 2018. A novel stress hormone response gene in tadpoles of Xenopus tropicalis. Gen. Comp. Endocrinol. 260, 107–114. https://doi.org/10.1016/J.YGCEN.2018.01.006.
Shewade, Leena H., Schneider, Katelin A., Brown, Audrey C., Buchholz, Daniel R., 2017. In-vivo regulation of Krüppel-like factor 9 by corticosteroids and their receptors across tissues in tadpoles of Xenopus tropicalis. Gen. Compar. Endocrinol. 248, 79–86. https://doi.org/10.1016/j.ygcen.2017.02.007.
Thompson, E.B., 1987. The structure of the human glucocorticoid receptor and its gene. J. Steroid Biochem. 27, 105–108. https://doi.org/10.1016/0022-4731(87)90300-1.
Truett, G.E., Heefer, P., Mynatt, R.L., Truett, A.A., Walker, J.A., W. M.,, 2000. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and Tris (HotSHOT). BioTechniques 29, 52–54. https://doi.org/10.2144/00291bm09.
Tsigos, C., Chrousos, G.P., 2002. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J. Psychosom. Res. 53, 865–871. https://doi.org/10.1016/S0022-3999(02)00429-4.
Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee, D.H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W.V., Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455. https://doi.org/10.1210/er.2011-1050.
Wada, H., 2008. Glucocorticoids: mediators of vertebrate ontogenetic transitions. Gen. Comp. Endocrinol. 156, 441–453. https://doi.org/10.1016/j.ygcen.2008.02.004.
Whirledge, S., DeFranco, D.B., 2018. Glucocorticoid signaling in health and disease: Insights from tissue-Specific GR knockout mice. Endocrinology 159, 46–61. https://doi.org/10.1210/en.2017-00728.