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Research Article

cyp21a2 Knockout Tadpoles Survive Metamorphosis Despite Low Corticosterone

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Abstract

Corticosteroids are so vital for organ maturation that reduced corticosteroid signaling during postembryonic development causes death in terrestrial vertebrates. Indeed, death occurs at metamorphosis in frogs lacking proopiomelanocortin (*pomc*) or the glucocorticoid receptor (GR; *nr3c1*). Some residual corticosteroids exist in *pomc* mutants to activate the wild-type (WT) GR and mineralocorticoid receptor (MR), and the elevated corticosteroids in GR mutants may activate MR. Thus, we expected a more severe developmental phenotype in tadpoles with inactivation of 21-hydroxylase, which should eliminate all interrenal corticosteroid biosynthesis. Using CRISPR/Cas9 in *Xenopus tropicalis*, we produced an 11-base pair deletion in *cyp21a2*, the gene encoding 21-hydroxylase. Growth and development were delayed in *cyp21a2* mutant tadpoles, but unlike the other frog models, they survived metamorphosis. Consistent with an absence of 21-hydroxylase, mutant tadpoles had a 95% reduction of aldosterone in tail tissue, but they retained some corticosterone (~40% of WT siblings), an amount, however, too low for survival in *pomc* mutants. Decreased corticosteroid signaling was evidenced by reduced expression of corticosteroid-response gene, *klf9*, and by impaired negative feedback in the hypothalamus-pituitary-interrenal axis with higher messenger RNA expression levels of *crh*, *pomc*, *star*, and *cyp11b2* and an approximately 30-fold increase in tail content of progesterone. In vitro tail-tip culture showed that progesterone can transactivate the frog GR. The inadequate activation of GR by corticosterone in *cyp21a2* mutants was likely compensated for by sufficient corticosteroid signaling from other GR ligands to allow survival through the developmental transition from aquatic to terrestrial life.

Key Words: corticosteroids, 21-hydroxylase, CRISPR, *Xenopus tropicalis*, metamorphosis

Abbreviations: ACTH, adrenocorticotropin; ALDO, aldosterone; CORT, corticosterone; *crh*, corticotropin-releasing hormone; *cyp11b2*, aldosterone synthase; GR, glucocorticoid receptor; HMA, heteroduplex mobility assay; HPA, hypothalamic-pituitary-adrenal; HPI, hypothalamic-pituitary-interrenal; *klf9*, Krüppel-like factor 9; KO, knockout; LC-MS/MS, liquid-chromatography tandem mass-spectrometry; MBS, modified Barth's solution; mRNA, messenger RNA; MR, mineralocorticoid receptor; NF, Nieuwkoop and Faber; PCR, polymerase chain reaction; *pomc*, proopiomelanocortin; *rpl8*, ribosomal protein L8; sgRNA, single-guide RNA; *star*, steroidogenic acute regulatory protein; TH, thyroid hormone; *ush1g*, Usher syndrome 1G; WT, wild-type.

Corticosteroids are critical for organ maturation and survival during the aquatic to terrestrial developmental transition that occurs in tetrapod vertebrates (1, 2). In amphibian development, metamorphosis is a hormone-coordinated process comparable to birth in mammals and hatching in birds (3). In frogs, the process of metamorphosis requires thyroid hormone (TH) (4), and thyroidectomy in tadpoles permanently prevents initiation of metamorphosis, and addition of exogenous TH to tadpoles before endogenous plasma TH is detectable by radioimmunoassay causes metamorphic events to occur early (5). Before gene disruption technology, it was accepted that corticosteroids lack an independent developmental role, aside from accelerating TH-dependent development. In particular, exogenous corticosteroids given to premetamorphic tadpoles do not cause an increased developmental rate, but when given in conjunction with exogenous TH, corticosteroids accelerate metamorphosis faster than TH alone (6). In addition, stressful rearing conditions cause increased corticosteroid levels and increased metamorphic rate in tadpoles that have already started metamorphosis (7). Corticosteroids act to boost TH signaling and thus accelerate TH-dependent development by increasing tissue sensitivity to TH and synergizing with TH to induce the TH-response gene Krüppel-like factor 9 (*klf9*) (2, 3, 5, 6, 8, 9).

The results of studies using exogenous hormone treatments suggested that corticosteroids may be required to mediate the effects of the environmental on metamorphic rate but may not be required for survival through metamorphosis. In contrast to those expectations, recent studies in tadpoles revealed that mutations in *nr3c1*, the gene encoding the glucocorticoid receptor (GR), and in proopiomelanocortin (*pomc*), the gene encoding adrenocorticotropin (ACTH and other peptides), are lethal during metamorphosis and thus demonstrated the indispensability of glucocorticoid signaling for development in tadpoles (5, 6). Further analysis of the cause of death in these mutant frog models showed that exogenous TH allowed survival through metamorphosis in *pomc* and GR mutants, indicating that augmentation of TH signaling is an essential action of corticosteroids during natural metamorphosis (9, 10). However, vital roles for corticosteroids in metamorphosis

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independent of TH signaling are not ruled out because corticosteroid signaling through the mineralocorticoid receptor and the ability of the interrenals to produce aldosterone were still possible in *nr3c1* and *pomc* knockout (KO) tadpoles.

cyp21a2 codes for the cytochrome P450 enzyme 21-hydroxylase, which catalyzes a key step in the biosynthesis of glucocorticoids and mineralocorticoids (11). In frogs, as in mice, the main glucocorticoid is corticosterone (CORT), whereas cortisol is the main glucocorticoid in humans. To make corticosteroids in frogs, 21-hydroxylase converts progesterone to 11-deoxycorticosterone. Then, the multifunctional enzyme aldosterone synthase (*cyp11b2*) converts 11-deoxycorticosterone to CORT then to 18-hydroxycorticosterone and then to aldosterone (12). Disruptive mutations in the *cyp21a2* gene renders humans unable to synthesize glucocorticoids and mineralocorticoids efficiently (1), and affected individuals die at birth from lung atelectasis, hypoglycemia, and salt-wasting, with girls exhibiting virilization of external genitalia (1, 13–17). Similarly, mice lacking 21-hydroxylase die at birth and can be rescued by prenatal treatment with dexamethasone (GR agonist) and/or restoration of 21-hydroxylase activity by a transgenic vector (18–20). On the other hand, 21-hydroxylase–mutant zebrafish survive to adulthood, though zebrafish, unlike humans, mice, and frogs, also survive in the absence of a functional GR (21, 22). Glucocorticoid deficiency in all these cases from fish to humans is accompanied by defective negative feedback in the hypothalamic-pituitary-adrenal (HPA) axis causing overproduction of ACTH, steroidogenic enzymes, and adrenal steroids (21–23).

To gain further insight into the importance of corticosteroid signaling in frog metamorphosis, we used CRISPR technology to create *cyp21a2* KO *X. tropicalis* frogs with the goal of completely blocking the production both of CORT and aldosterone and testing the hypothesis that lack of signaling by both corticosteroids would result in a more severe phenotype than observed in *nr3c1* and *pomc* KO tadpoles. We used wild-type (WT) tadpoles and tadpoles mutant for *cyp21a2* and analyzed growth and development throughout the metamorphic period, quantified steroid hormone content in tail tissue, and measured messenger RNA (mRNA) expression levels of CORT-response genes as well as key genes in the hypothalamic-pituitary-interrenal (HPI) axis (comparable to the HPA axis in mammals).

Materials and Methods

Animal Husbandry

WT and mutant laboratory-reared male and female adult *X. tropicalis* were mated by priming with 20 IU of ovine luteinizing hormone (National Hormone and Peptide Program) in the evening and boosted with 200 IU the next morning for breeding. Tadpoles were reared at 26 °C in reconstituted reverse-osmosis water with water changes occurring every 3 days and fed Sera Micron larval growth food twice daily. The use of animals was in accordance with the protocol approved by the University of Cincinnati Institutional Animal Care and Use Committee (protocol No. 06-10-03-01).

CRISPR-Cas9 Single-Guide RNA Design and RNA Synthesis

To create *cyp21a2* KO frogs, the CRISPR-Cas9 system was used to target exon 8 of the *cyp21a2* gene of *X. tropicalis*. The single-guide RNA (sgRNA) (5′-GTGGTTCCC CTGGCTGT) was designed and checked to have no predicted off-targets greater than 1-bp mismatch using ZiFiT (24) and UCSC genome browser (http://genome.ucsc.edu/) (25). Cas9 mRNA was prepared from NotI-linearized pCS2-Cas9 (gift from A. Zorn) using the MEGAscript SP6 in vitro transcription kit (Ambion). The sgRNA sequence was cloned into the plasmid DR274 (gift from I. Blitz), and sgRNA was prepared from DR274-21-OH using MEGAscript T7 in vitro transcription kit (26). mCherry mRNA from KpnI-linearized CS108-mCherry (gift from Dr M. Khokha) was also prepared using the MEGAscript T7 in vitro transcription kit.

CRISPR-Cas9 Microinjections

Following WT mating, zygotes were collected within 1 hour of being laid and then dejellied by transferring them to 3% L-cysteine (Sigma) in 0.1X modified Barth's solution (MBS) for approximately 10 minutes. Dejellied eggs were washed with 0.1× MBS then transferred to an injection dish containing 3% Ficoll in 0.1× MBS. One-cell-stage embryos were injected with a mixture containing 500 pg Cas9 mRNA, 150 pg sgRNA, and 400 pg mCherry in 2 nL using a Picospritzer III (Parker Hanaffin Corp) as previously described (27). After 4 hours, the surviving embryos were transferred to 0.01× MBS, which was replaced every day for 3 days. Hatched tadpoles were sorted based on mCherry fluorescence under a fluorescence dissection stereomicroscope to identify successfully injected eggs. Injected embryos positive for mCherry expression were founders and reared to adulthood and then crossed to obtain F1 and F2 generations.

Genetic Screening

The heteroduplex mobility assay (HMA) was used to genotype F1 and F2 tadpoles for *cyp21a2* mutations (10, 28, 29). Genomic DNA was prepared from tail tips by means of the HotSHOT protocol used in mice (30). Briefly, tail tips were excised using a razor blade and incubated in 50 µL of 25 mM NaOH/0.2 mM EDTA for 15 minutes at 95 °C, and then 50 µL of 40 mM Tris-HCl was added to neutralize the solution followed by vortexing. Polymerase chain reaction (PCR) (DreamTaq, Thermo Fisher) was then carried out using 1 µL of the neutralized solution containing genomic DNA using primers to amplify a 180-bp region surrounding the CRISPR target site. The forward and reverse primers used were 5′-AGGAGCAGTGGGAATGGAGA and 5′-AAATGCATTTCAGGTTTTTAGTAAATCTTACC. DNA strands were then separated and allowed to reanneal in the PCR machine (95 °C for 5 minutes, 16 °C for 10 minutes, and 25 °C for 5 minutes). The samples were then loaded on 8% polyacrylamide gels and run at 150 V. Gels stained with ethidium bromide were imaged using an ultraviolet transilluminator gel imager (Fisher). An HMA pattern observed in F1 individuals was chosen and found to have an 11-bp frameshift mutation identified by Sanger sequencing using F2 animals. F2 individuals were sorted into WT, heterozygous, and homozygous mutants based on HMA patterns and used in experiments. *nr3c1* (GR) KO tadpoles and *pomc* (ACTH) KO tadpoles from the respective heterozygous mutant F1 adults were from the laboratory colony and bred and genotyped as described (10, 31).

Growth and Development

At 20 days post fertilization, 56 F2 sibling tadpoles were genotyped by HMA and staged according to Nieuwkoop and Faber (NF) (32). The genotyped animals were then placed into 3 tanks, 1 tank per genotype, and 10 animals of each genotype were haphazardly selected from these tanks and reared individually in 2-L buckets starting day 21. Tadpoles were fed Sera micron larval growth food ad lib daily with water changes occurring every 3 days. All tadpoles were reared on the same shelf level and buckets were shifted to different positions randomly across the shelf each day to minimize effects of slight temperature variation across the shelf (< 2 °C). We recorded the number of days required by individual tadpoles of each genotype to reach (1) NF stage 58 (NF 58) (forelimb emergence, maximum tadpole size, midway through metamorphosis) starting from fertilization and (2) NF 66 (tail resorption, end of metamorphosis) starting from NF 58. Snout-vent length from anterior-most point of head not including tentacles to posterior part of abdomen was also recorded when individual tadpoles reached NF 58.

Steroid Hormone Measurements by Liquid-Chromatography Tandem Mass-Spectrometry

Steroid hormone content was measured from F2 tadpoles of each genotype at metamorphic climax (NF 62) using liquid-chromatography tandem mass-spectrometry (LC-MS/MS) as in Paul et al (33). Briefly, 8 to 10 tails (~50 mg each) per genotype were dissected from mutant and WT siblings from *cyp21a2* and *pomc* clutches, snap-frozen in liquid nitrogen, and stored at −80 °C. Each frozen 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. Briefly, tail homogenates were centrifuged, supernatant was extracted, and 2 µL of internal standard mixture (1 µM progesterone-d9, 1 µM corticosterone-d8, 1 µM aldosterone-d4, 1 µM hydrocortisone-d4) was added. The LC-MS/MS analysis of 4 steroid hormones—progesterone, corticosterone, hydrocortisone (cortisol), and aldosterone—along with 4 deuterated internal standard steroids, progesterone-d9, corticosterone-d8, hydrocortisone-d4, and aldosterone-d4 were performed using a Luna C18(2) column from Phenomenex (3 µm, 100 mm × 2 mm inner diameter) in an Exion LC system coupled with a Sciex X500B QTOF mass spectrometer. A high-resolution multiple-reaction monitoring method was created for quantitation of each steroid. Peak area ratios were used to construct steroid calibration curves, and all of them had a linear regression with R^2 greater than 0.96. The limit of detection was 0.02 to 0.1 ng/mL in methanol and 0.03 to 0.1 ng/mL in tadpole tail matrix across the 4 steroids. The limit of quantification threshold was 0.06 to 0.4 ng/mL and 0.1 to 0.7 ng/mL across the 4 steroids in methanol and tadpole tail matrix, respectively. The intra-assay variation ranged from 2.37% to 7.29% for the 4 assayed steroids. Absolute quantification of steroid content in tails was conducted using recovery and matrix effects of progesterone, corticosterone, hydrocortisone, and aldosterone from their respective internal standards. Recoveries for progesterone, corticosterone, hydrocortisone, and aldosterone were 96.9%, 88.8%, 85.3%, and 92.7%, respectively. Matrix (ion suppression) effects for progesterone, corticosterone, hydrocortisone, and aldosterone were 95.8%, 94.3%, 44.7%, and 99.5%, respectively.

Quantitative Polymerase Chain Reaction

To measure hormone response gene induction during natural metamorphosis, F2 tadpoles were genotyped at NF 50-53 (premetamorphosis), and tails from tadpoles of each genotype were harvested at NF 54 (beginning of metamorphosis, NF 58, and NF 62 (climax of metamorphosis), n = 10 per genotype. To characterize HPI feedback, brains (forebrain plus midbrain portion including pituitary) and kidneys (with interrenal tissue embedded within the kidney capsule) were harvested and snap-frozen on dry ice from tadpoles anesthetized in MS222 at NF 62 when HPI activity is highest, n = 10 (33–35) following the described procedure (36). RNA extraction was performed using TRI REAGENT RT following the manufacturer's instructions (Molecular Research Center Inc). Complementary DNA synthesis from 1 µg RNA for each sample was obtained using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR (qPCR) was carried out using SYBR Green or TaqMan master mix on a 7300 Real Time PCR System (Applied Biosystems) for *klf9* on tails and brains, Usher syndrome 1G (*ush1g*) on tails, corticotropin-releasing hormone (*crh*) and proopiomelanocortin (*pomc*) on brains, and steroidogenic acute regulatory protein (*star*) and aldosterone synthase (*cyp11b2*) on kidneys with gene-specific primers that spanned large introns to mitigate genomic DNA contamination (Table 1). The reference gene ribosomal protein L8 (*rpl8*) was used and showed no significant differences among genotypes or treatments (data not shown) (37, 38). The relative quantification method ΔΔCt was used to compare expression levels of target genes normalized to *rpl8* (39).

Tail-Tip Assay

To determine the ability of progesterone to activate the frog GR, WT and GR KO tadpoles (10) were used to measure induction of the GR-specific response gene *klf9* in an in vitro tail-tip assay as previously described (6, 40). Briefly, HMA-genotyped sibling wild-type and GR KO NF 54 tadpoles were incubated overnight in a 0.05% sulfadiazine (ICN) solution to help eliminate microorganisms. The next day, tadpoles were anesthetized in 0.01% tricaine (MS-222) and dipped in 70% ethanol to sterilize the epidermis. Tail tips were cut approximately 5 to 6 mm from the end of the tail and placed in 10 mL of 62% osmotic strength Leibovitz's-15 medium (L-15, Fisher) with 10× antibiotic–antimycotic solution (1000 U/mL penicillin G, 2.5 mg/mL amphotericin B, 1000 mg/mL streptomycin; Mediatech). After 2 to 3 hours, the tail tips were transferred to 62% L-15 with 1× antibiotic-antimycotic solution and incubated overnight at 26 °C in an airtight chamber with 95% O_2% and 5% CO_2 to allow tails tips to equilibrate to culture. After this incubation, vehicle (ethanol), corticosterone (100 nM), or progesterone (500 nM) were added to the culture medium containing tail tips and incubated for 24 hours, n = 6 per treatment per genotype. The dose of CORT (100 nM) was obtained from previous studies, where this dose gives a high level of induction at 24 hours, whereas higher doses give lower induction (U-shaped dose-response curve) (41, 42). The dose of progesterone was determined from a preliminary dose-response study using 500 nM, 1000 nM, and 1500 nM, where even

Table 1. Primer and probe sequences of genes studied using quantitative polymerase chain reaction

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Probe	Master mix
Krüppel-like factor 9 (*klf9*)	CCTTAAAGCCCATTACAGAGTCCAT	GCAGTCAGGCCACGTACA	ACAGGTGAACGCCCTTTT	TaqMan
Usher syndrome 1G (*usb1g*)	CTGTAGGACACGTATTTCATGATTAAGC	CAACATTAACAGGGTATGATAAAAT CAATATATCTTTATTACAAAAT	CCTGACGCATTTTGTG	TaqMan
Ribosomal protein L8 (*rpl8*)	GAAGGTCATCTCATCTGCAAACAG	CTTCAGGATGGGTTTGTCAATACGA	CAACCCCAACAATAGCT	TaqMan
Krüppel-like factor 9 (*klf9*)	TAAAGCCCATTACAGAGTCCAT	CACTCCTCATGAACCTCTTCTC	NA	SYBR Green
Corticotropin-releasing hormone (*crh*)	CTCCGTGAAGTCTTAGAAATGG	CAATGATGTCCATGAGTTTCCT	NA	SYBR Green
Proopiomelanocortin (*pomc*)	CCGATGTGCAGACCTCAGCAGT	TACTTTCCGACAGAGGCTGCAA	NA	SYBR Green
Steroidogenic acute regulatory protein (*star*)	GCAAATGGATAAATCAGGTTCG	CATCTCTCCTTCATTCAGTGTT	NA	SYBR Green
Aldosterone synthase (*cyp11b2*)	CAGTGGACCTTTATGCTGATCT	CTCGGATGAAACGAAGAGAATCC	NA	SYBR Green
Ribosomal protein L8 (*rpl8*)	CCACAATCCTGAAACAAAGAAA	CCTTGTATTTATGGTATGCACG	NA	SYBR Green

Abbreviation: NA, not applicable

the lowest dose tried (500 nM) showed significant induction (data not shown). After 24 hours, the tail tips were snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction for gene expression analysis.

Statistical Analysis

Data were checked for normal distribution using the Shapiro-Wilk test of normality. For normally distributed data, unpaired *t* tests were performed with base R (43). For data that did not follow normal distribution, nonparametric Kruskal-Wallis tests were conducted in R followed by pairwise comparisons using Wilcoxon rank sum exact tests.

Results

CRISPR Design and Breeding to Obtain *cyp21a2*-Mutant Tadpoles

Because several amino acids encoded by exons 8, 9, and 10 are involved in heme binding by the 21-hydroxylase enzyme critical for structural stability and catalytic activity, we targeted exon 8 of the *cyp21a2* gene so that a frameshift mutation would nullify enzymatic activity (1, 12, 14, 15–18) (Fig. 1A). RNAs for sgRNA, Cas9, and the red fluorescent protein mCherry were injected into single-celled embryos, and embryos expressing mCherry 3 days post injection were raised to adulthood as F0 founder animals. To produce homozygous mutants, founders were bred to WT *X. tropicalis* to produce F1 animals, which were screened using HMA to identify *cyp21a2*-heterozygous individuals. Animals sharing the same HMA pattern were selected and sequenced to reveal an 11-bp frameshift mutation resulting in an early stop codon at amino acid 382 (Fig. 1B). Heterozygous F1 individuals with this 11-bp frameshift mutation were crossed with each other to produce F2 offspring, where HMA screening of embryos (Fig. 1C) showed approximately 25% WT, 50% heterozygous, and 25% homozygous for the mutation (data not shown).

Reduced Growth and Development Rates in *cyp21a2* Mutants

We hypothesized that the *cyp21a2* mutation would result in slower growth and development followed by death at metamorphosis, as we had observed in *pomc* KO tadpoles (31). Thus, we recorded the stage of mutant, heterozygous, and WT F2 tadpoles at 21 days post fertilization and also recorded the days when they reached NF 58 (forelimb emergence, maximal tadpole size) and NF 66 (tail resorption, end of metamorphosis) (32). Tadpoles started diverging in developmental rate before the earliest stage that they can be genotyped using tail tips without high mortality (~NF 52, early premetamorphosis) so that at 21 days, the mutants averaged between NF 52 and 53 while the WT and heterozygous tadpoles averaged between NF 56 and 57 (prometamorphosis, plasma levels of TH increasing) (Fig. 2A). This difference in stage was also observed at forelimb emergence where the mutant animals took longer to reach NF 58 (Fig. 2B). In addition to the developmental delay in premetamorphosis, we found that developmental delay also occurred during metamorphosis, where the mutants took 2 to 3 days longer from forelimb emergence (NF 58) to tail resorption (NF 66) (Fig. 2C). No significant difference in size (snout-vent length) was observed at NF 58 across genotypes during the larval growth period (Fig. 2D). Because

the mutants took longer to achieve that size, the growth rate was reduced in mutants compared to WT. In contrast to expectations, all genotypes survived through metamorphosis and into adulthood (fertility of homozygous mutant adults was not evaluated).

Reduced Corticosterone Content in *cyp21a2* Mutants

Because *cyp21a2* mutants completed metamorphosis whereas *pomc* mutants did not, we wanted to know if *cyp21a2* mutants have CORT content as low as *pomc* mutants. Because 21-hydroxylase catalyzes a critical step in CORT and aldosterone (ALDO) biosynthesis from steroid hormone precursors in the interrenal glands, lack of functional 21-hydroxylase is expected to severely impair the ability of tadpoles to produce CORT and ALDO. We measured corticosteroids in the tails using LC-MS/MS in mutant and WT F2 tadpoles at metamorphic climax (NF 62) because CORT and ALDO levels peak then (44–47). We included measurements of CORT and ALDO in *pomc* mutants to enable a direct comparison with *cyp21a2* mutants because our previous study used enzyme-linked immunoassay to measure plasma CORT levels in *pomc* mutants (31). CORT content was significantly lower (~40% of WT amount) both in *cyp21a2* and *pomc* mutants compared to their respective WT siblings (Fig. 3A and C). ALDO content in tails was significantly lower in *cyp21a2* but not in *pomc* mutants compared to their respective WT siblings (Fig. 3B and D).

Reduced Corticosterone Signaling During Metamorphosis in *cyp21a2* Mutants

To test the hypothesis that the reduced CORT content had an effect on CORT-response gene induction, we measured mRNA expression of the CORT response genes *ush1g* (48) and *klf9* (5, 41, 42) in tail and brain. The expression of *ush1g* increased significantly in the tail at metamorphic climax (NF 62) but was not significantly different among genotypes (Fig. 4A). On the other hand, the *klf9* mRNA level in tail peaked at NF 62 in WT but was significantly reduced by approximately 35% in the mutants (Fig 4B). As in the tail, *klf9* expression was significantly lower in *cyp21a2* mutant brains at NF 62 (Fig. 4C). *Ush1g* brain levels were not tested because *ush1g* is not induced by CORT in brain (48).

Disrupted Negative Feedback in the Hypothalamic-Pituitary-Adrenal Axis in *cyp21a2* Mutants

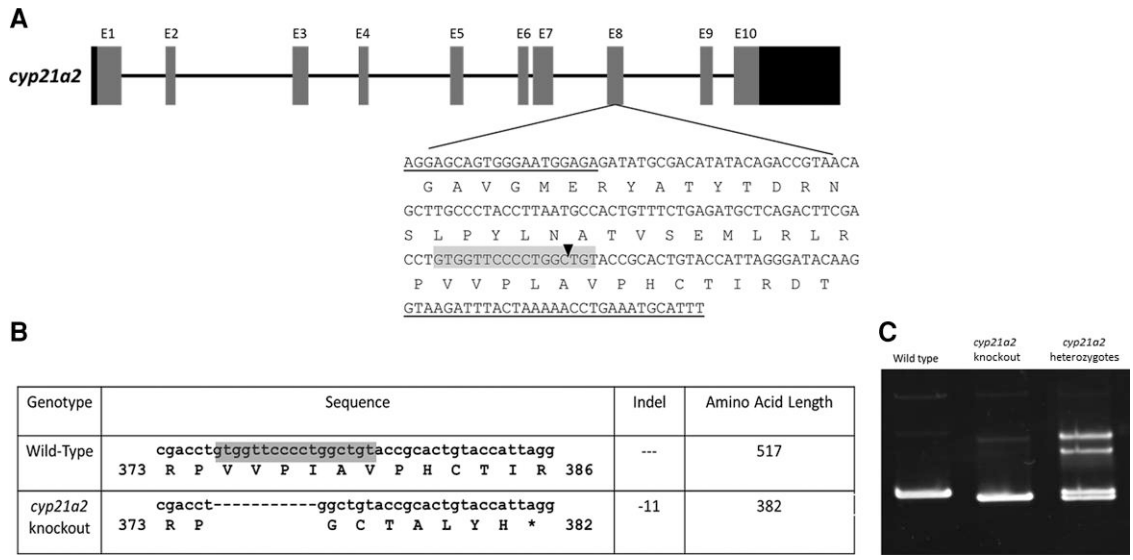
Following the unexpected observation of survival of *cyp21a2*-mutant tadpoles through metamorphosis despite having low CORT and ALDO content, we hypothesized the existence of compensatory glucocorticoid signaling. Impaired negative feedback due to reduced CORT content in 21-hydroxylase deficiency is expected to result in stimulation of HPI axis gene expression and excessive production of corticosteroid precursors. Indeed, we found significantly higher mRNA levels of *crh* and *pomc* in the brain of the mutants (Fig. 5A-5C). Because brain regions outside the hypothalamus express *crh* and *pomc*, our results are consistent with but cannot conclude that we measured disrupted negative feedback at the level of hypothalamus and pituitary. To further examine negative feedback, we also assayed the interrenals for ACTH stimulation and found higher mRNA expression levels of *star*, which accomplishes a rate-limiting step in steroidogenesis by transporting cholesterol within the mitochondria, and of aldosterone synthase (*cyp11b2)*, a steroidogenic enzyme catalyzing CORT and ALDO synthesis in steps subsequent to 21-hydroxylase (Fig. 5D). We also used LC-MS/MS to measure cortisol and progesterone content in tails in WT and mutant tadpoles at NF 62. Progesterone content was significantly higher in *cyp21a2* mutants (Fig. 6A) but significantly lower in *pomc* mutants (Fig. 6C). Most samples in the *pomc*-mutant group had progesterone content below the limit of detection. Cortisol was significantly lower in *pomc* mutants but not in *cyp21a2* mutants (Fig. 6B and 6D).

Corticosterone-Response Gene Induction by Corticosteroid Precursors

Signaling through the GR is indispensable for survival through metamorphosis (10), and CORT treatment before climax of metamorphosis can prevent death due to low CORT levels in *pomc*-KO tadpoles (31). To account for survival of *cyp21a2* KOs through metamorphosis, we hypothesized that the corticosteroid precursor progesterone, which is present in approximately 30-fold higher quantities in the 21-hydroxylase mutants, might be able to transactivate GR, as it can in humans though at lower binding affinity compared to cortisol (49). We did not test this by treating *cyp21a2*-mutant tadpoles with precursors in vivo because they already had high amounts of progesterone, and we did not use their tails in vitro because they were expected to be no different from WT, that is, 21-hydroxylase is not present in tadpole tails. An in vitro tail-tip assay was used to minimize potential steroid precursor conversion of exogenous steroids that may occur in interrenal cells in vivo. Further, we compared WT and GR-mutant tail tips to establish whether progesterone may be acting specifically through GR. We found that in vitro treatment of tails with CORT or progesterone resulted in significant induction of *klf9* in the WT tails but not in tails from GR-KO tadpoles, thereby showing that progesterone can activate the frog GR (Fig. 7).

Discussion

In this study we used CRISPR-Cas9 to target exon 8 of the *cyp21a2* gene in *X. tropicalis* and then isolated mutants with an 11-bp deletion resulting in a frameshift mutation and an early stop codon, which is expected to completely disrupt 21-hydroxylase enzyme activity (11, 13–17). Indeed, *cyp21a2*-mutant tadpoles had an approximately 60% reduction in CORT and an approximately 95% reduction in ALDO but no significant difference from WT in cortisol. These mutants also had reduced expression of the corticosteroid response gene *klf9* indicating impaired hormone signaling. As a consequence of low CORT signaling, the *cyp21a2* mutants had impaired negative feedback indicated by higher mRNA expression of *crh*, *pomc*, *star*, and aldosterone synthase (*cyp11b2)* and overproduction of the 21-hydroxylase substrate progesterone. Analysis of growth and development in *cyp21a2*-mutant tadpoles showed that development to metamorphosis as well as during metamorphosis were both delayed. Intriguingly, the mutants survived through metamorphosis to adulthood despite a low CORT content that was



Genotype	Sequence	Indel	Amino Acid Length
Wild-Type	cgacctgtggttcccctggctgtaccgcactgtaccattagg 373 R P V V P I A V P H C T I R 386	---	517
cyp21a2 knockout	cgacct----------ggctgtaccgcactgtaccattagg 373 R P G C T A L Y H * 382	-11	382

Figure 1. Genomic analysis of *cyp21a2* targeting by CRISPR. A, The CRISPR target site of *cyp21a2* is in the eighth exon. The region targeted has been expanded, showing the single-guide RNA binding site (shaded), the exact cut site (black triangle), and the forward and reverse polymerase chain reaction (PCR) primer binding sites (underlined) used to amplify the targeted region. Amino acids are listed below the messenger RNA sequence. E, exon. Black bars at the beginning and end of exon 1 and exon 10 represent the 5′ and 3′ untranslated regions, respectively. B, Sequence analysis of *cyp21a2* mutation shows an 11-bp deletion. The wild-type sequence is shown at the top of the panel with the highlighted portion representing the CRISPR target site, while *cyp21a2* mutant tadpoles demonstrate an 11-bp deletion at the target site. Amino acid translation is shown below each sequence, and the *cyp21a2* mutant sequence predicts a frameshift and early stop codon. The first 373 amino acids are in common followed by amino acids that were changed due to the frameshift mutation. C, Heteroduplex mobility assay (HMA). PCR amplification of wild-type (lane 1), *cyp21a2* knockout (lane 2), and *cyp21a2* heterozygous (lane 3) tadpole DNA followed by boiling and reannealing of the PCR products results in diagnostic banding patterns on an ethidium bromide-stained polyacrylamide gel electrophoresis gel. The wild-type (180 bp) and knockout (169 bp) bands run at the predicted PCR product sizes, and the addition of heteroduplex bands running more slowly through the gel were expected in the heterozygote sample.

insufficient to allow survival through metamorphosis in *pomc*-mutant tadpoles.

An explanation for the survival of *cyp21a2*-mutant tadpoles is expected to be consistent with results from previous studies showing that the only known role for CORT in inducing larval to adult transition events is through increasing TH signaling and that sufficient TH signaling is required to complete metamorphosis. In particular, CORT acts on cells to increase TH signaling by synergizing with TH to induce expression of the CORT and TH response gene *klf9*, which in turn induces TH receptor expression thereby making cells more sensitive and responsive to circulating TH (4–6, 50). Also, CORT increases TH conversion from the main circulating form thyroxine to the active form 3,5,3′-triiodothyronine and decreases degradation of thyroxine and 3,5,3′-triiodothyronine to inactive forms, thereby increasing the

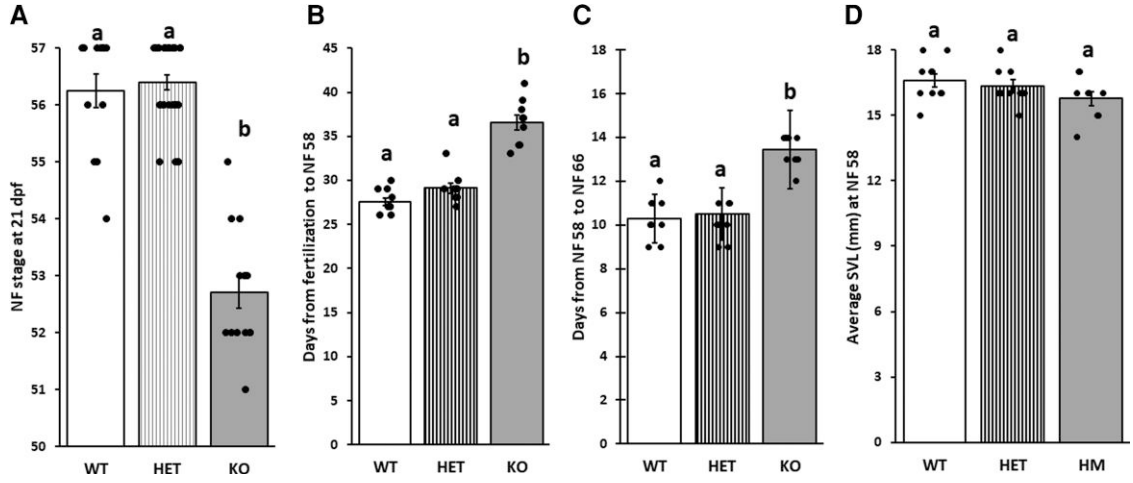


Figure 2. Delayed growth and development in *cyp21a2* mutant tadpoles. A, At 21 days post fertilization (dpf), 56 tadpoles from a *cyp21a2* F2 clutch were genotyped and staged according to Nieuwkoop and Faber (NF); there were 12 wild-type (WT, white bars), 30 heterozygous (HET, gray bars), and 14 *cyp21a2* mutant (KO, black bars) tadpoles. Sibling tadpoles of each genotype (WT, HET, KO) were reared individually starting at 21 dpf, n = 10 per genotype. B, The average number of days taken to reach NF 58 after fertilization; C, the average number of days taken to reach NF 66 starting from NF 58; and D, the average snout-vent-length (SVL) at NF 58 were recorded. Letters indicate statistically significant differences between bars determined for each genotype based on non-parametric Kruskal-Wallis test followed by pairwise comparisons using Wilcoxon rank sum exact test ($P < 0.05$). Error bars represent SE. KO, *cyp21a2* mutants.

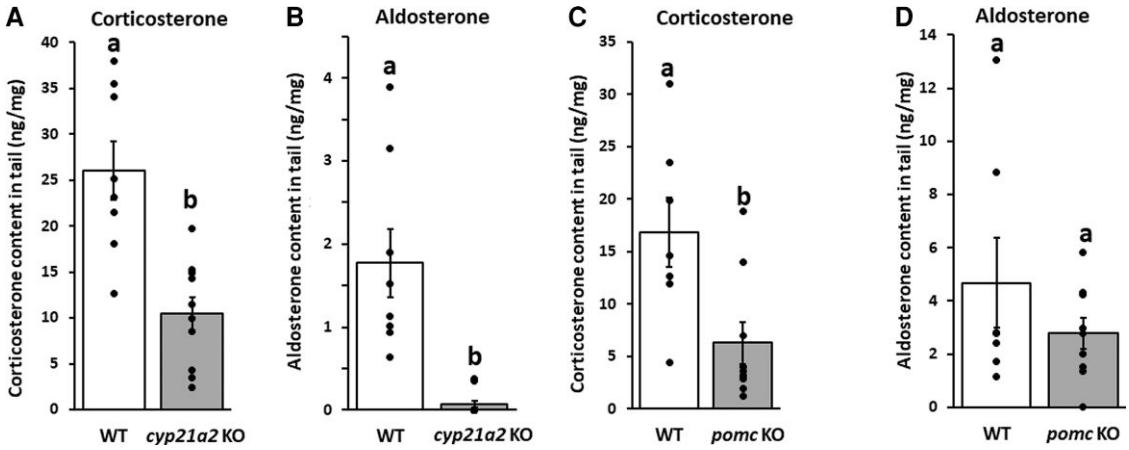


Figure 3. Reduced corticosterone and aldosterone quantities in *cyp21a2* mutants. Tails (~50 mg each) from genotyped Nieuwkoop and Faber (NF) stage 62 (metamorphic climax) tadpoles were harvested to measure A, corticosterone (CORT) and B, aldosterone (ALDO) from a *cyp21a2* clutch and C, CORT and D, ALDO from a *pomc* clutch via liquid-chromatography tandem mass-spectrometry. White bars, wild-type (WT); black bars, knockout (KO). n = 8-10 tails/genotype. Error bars represent SE. Letters indicate statistically significant groups based on A and C, unpaired *t* test and B and D, nonparametric Wilcoxon rank sum test; *P* less than .05.

intracellular concentration of active TH. Further, recent studies using frog KO models show that tadpoles with mutant TH receptors, GR, or *pomc* die at metamorphosis and that GR and *pomc*-mutant tadpoles can be rescued by exogenous TH alone, indicating that CORT signaling to boost TH signaling above the level achieved by endogenous TH alone is required for survival (9, 31, 50). Thus, these findings indicate that sufficient TH signaling must be achieved in *cyp21a2* mutants in the face of low CORT by (1) having higher plasma TH and/or (2) supplementing the corticosteroid signaling beyond the reduced augmentation of TH signaling afforded by the low CORT levels.

Our data on *ush1g* expression support the view that *cyp21a2* mutants have higher corticosteroid signaling than *pomc* mutants, despite similar low CORT levels. *Ush1g* is regulated only by CORT and not TH (48). In *pomc* mutants, *ush1g* expression is significantly lower than WT, but the expression of *ush1g* is not different from WT in *cyp21a2* mutants. Thus, *cyp21a2* mutants exhibit higher corticosteroid signaling than *pomc* mutants. We suggest the higher corticosteroid signaling in *cyp21a2* mutants compared to *pomc* mutants increases TH signaling enough for their survival. However, we cannot rule out the possibility that higher endogenous TH levels (from low CORT leading to reduced negative feedback leading to increased CRH leading to increased TSH leading to increased TH) may also contribute to increased TH signaling and survival in *cy21a2* mutants. Future studies to measure TH plasma levels in mutants will be required to address this possibility.

To account for the higher corticosteroid signaling in *cyp21a2* compared to *pomc*-mutant tadpoles, we hypothesized that the low CORT plus some combination of other corticosteroids and corticosteroid precursors activate the glucocorticoid receptor enough to increase TH signaling sufficient for survival through metamorphosis. Tadpole interrenals express *cyp17a1* at low levels and thus are presumably capable of converting progesterone to 17-alpha-hydroxyprogesterone potentially enabling interrenal production of cortisol and androstenedione (51, 52). However, reports differ on whether *Xenopus* interrenal tissue produces cortisol (53). Thus, we measured cortisol and found it present in WT tadpoles and it was not different in *cyp21a2* mutants, but *pomc* mutants had half the WT amount of cortisol. Thus, the amount cortisol may provide enough corticosteroid signaling in *cyp21a2* mutants, even when they have the same low CORT that disallows *pomc*-mutant survival. It is also possible that *cyp21a2* mutants have higher GR expression to increase the ability of the lower corticosteroid amounts to be effective. This possibility seems unlikely because, even though we did not measure GR expression, GR autoregulation by CORT does not occur in the tail and brain and is only transiently induced in the intestine (< 24 hours) (54). Another possible source of corticosteroid signaling involves progesterone, which was greatly increased (~30-fold) in *cyp21a2* mutants. Progesterone is known to transactivate the GR in mammalian systems though at 100- to 1000-fold higher EC_{50} (49). We performed an in vitro tail-tip assay to show that progesterone is capable of transactivating the frog GR. The in vitro dose of progesterone was approximately 10-fold lower than we measured in mutant tadpole tails, suggesting that progesterone may activate the GR in vivo in the mutants. We suggest that *cyp21a2*-mutant tadpoles live whereas *pomc*-mutant tadpoles die despite similarly low CORT content because *cyp21a2* mutants may experience the higher combined effect of normal cortisol levels and elevated progesterone and potentially other corticosteroid precursors on GR transactivation enabling sufficient glucocorticoid signaling for survival through metamorphosis. Future experiments with *cyp21a2* and *pomc*-double KOs may enable an in vivo test of the ability of cortisol or corticosteroid precursors to provide sufficient corticosteroid signaling for survival through metamorphosis.

The nearly undetectable amount of ALDO in *cyp21a2* mutants indicates that 21-hydroxylase is required for its production in frogs. *pomc*-mutant tadpoles were also expected to have reduced ALDO content due to lack of ACTH-dependent steroidogenesis. However, CORT but not ALDO amount was lower in *pomc* mutants, even though CORT and ALDO are made in the same interrenal cells by the same multifunctional enzyme (aldosterone synthase, CYP11a2). Glucocorticoid and mineralocorticoid production are distinctly regulated in mammals (55, 56), and thus these steroids may be partly

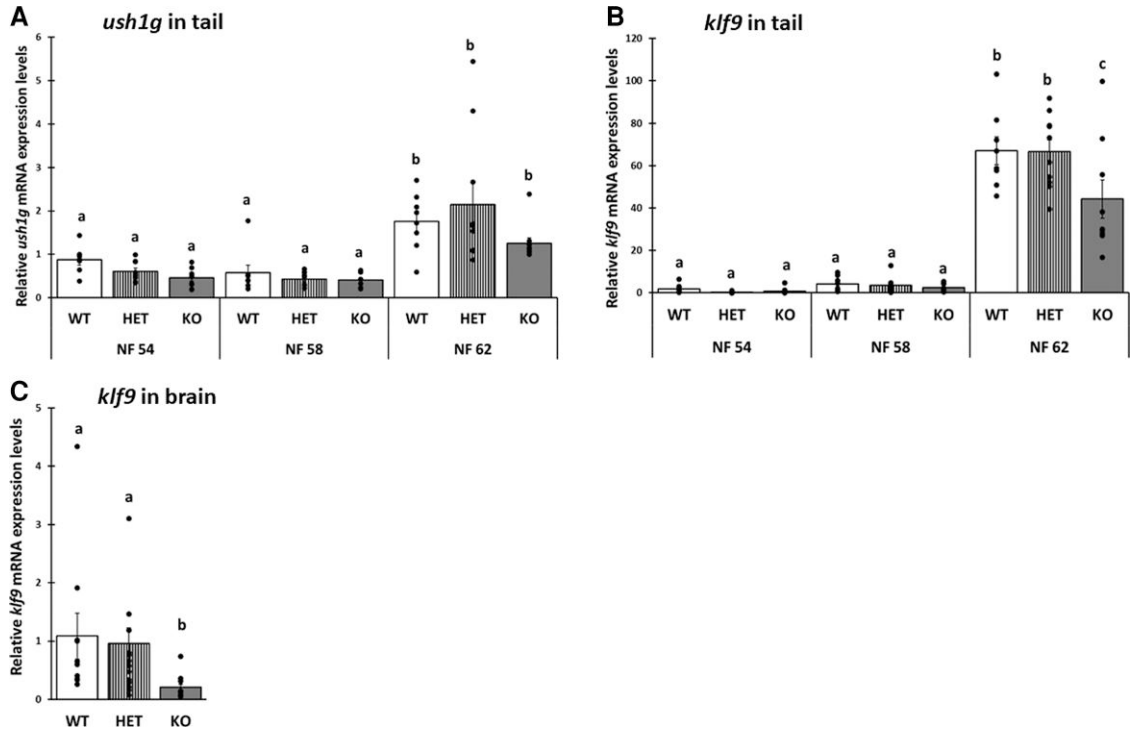


Figure 4. Impaired corticosterone (CORT) signaling in *cyp21a2* mutants. Total RNA was collected from tails of wild-type (WT, white bars), heterozygous (HET, gray bars), and *cyp21a2* mutant (KO, black bars) tadpoles at Nieuwkoop and Faber (NF) 54, 58, and 62 and from brains of tadpoles at NF 62 to analyze messenger RNA (mRNA) expression of A, the CORT-only response gene Usher syndrome 1G (*ush1g*) in tails; B, CORT and TH response gene Krüppel-like factor 9 *(klf9)* in tails; and C, *klf9* in brains at NF 62. Bars represent mean mRNA levels relative to the housekeeping gene *rpl8*. Error bars represent SE. Letters indicate statistically significant differences between bars determined for each genotype by nonparametric Kruskal-Wallis test followed by pairwise comparisons using Wilcoxon rank sum exact test for. *P* less than .05. n = 8-10 tails or brains per genotype per stage.

independently regulated in frogs as well. Indeed, tadpoles have a functioning renin-angiotensin-aldosterone system, exhibit salt/water homeostasis, and express atrial natriuretic peptide (57–59). Also, arginine vasopressin and ACTH stimulate interrenal ALDO and CORT secretion, but arginine vasopressin stimulates production of ALDO and CORT to a similar degree

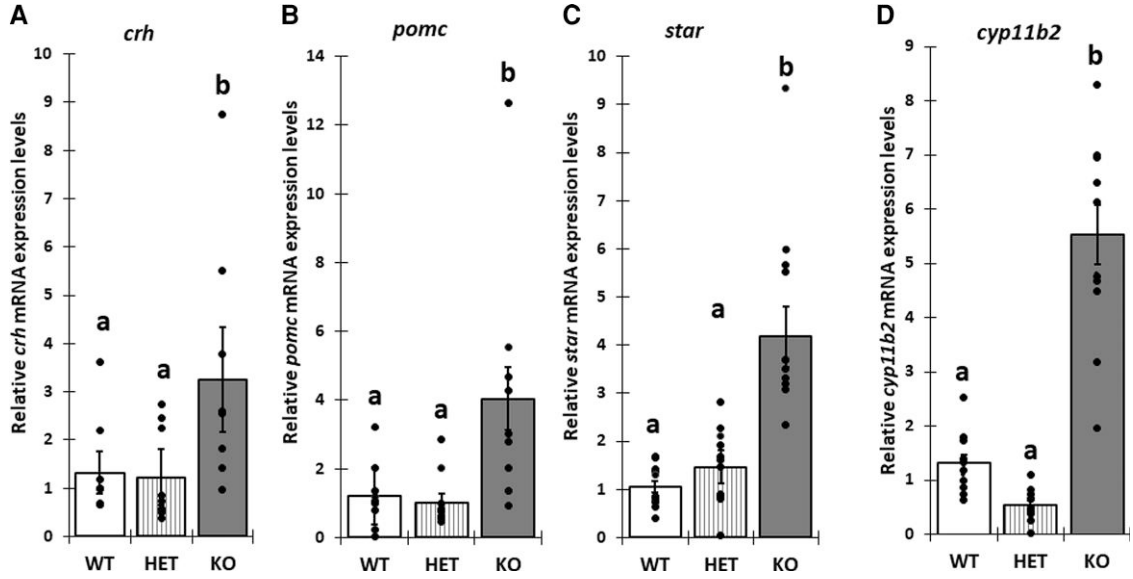


Figure 5. cyp21a2 mutants have a hyperactive hypothalamus-pituitary-interrenal (HPI) axis. Total RNA was collected from brain and kidney of wild-type (WT, white bars), heterozygous (HET, gray bars), and *cyp21a2* mutant (KO, black bars) tadpoles at Nieuwkoop and Faber (NF) 62 to analyze messenger RNA (mRNA) expression of A, corticotropin-releasing hormone (*crh*); B, proopiomelanocortin (*pomc*); C, steroidogenic acute regulatory protein (*star)*; and D, aldosterone synthase *(cyp11b2)*. Bars represent mean mRNA levels relative to the housekeeping gene *rpl8*. n = 8-10 brain and kidney samples per genotype. Error bars represent SE. Letters indicate statistically significant differences between bars determined for each genotype based on nonparametric Kruskal-Wallis test followed by pairwise comparisons using Wilcoxon rank sum exact test. *P* less than .05.

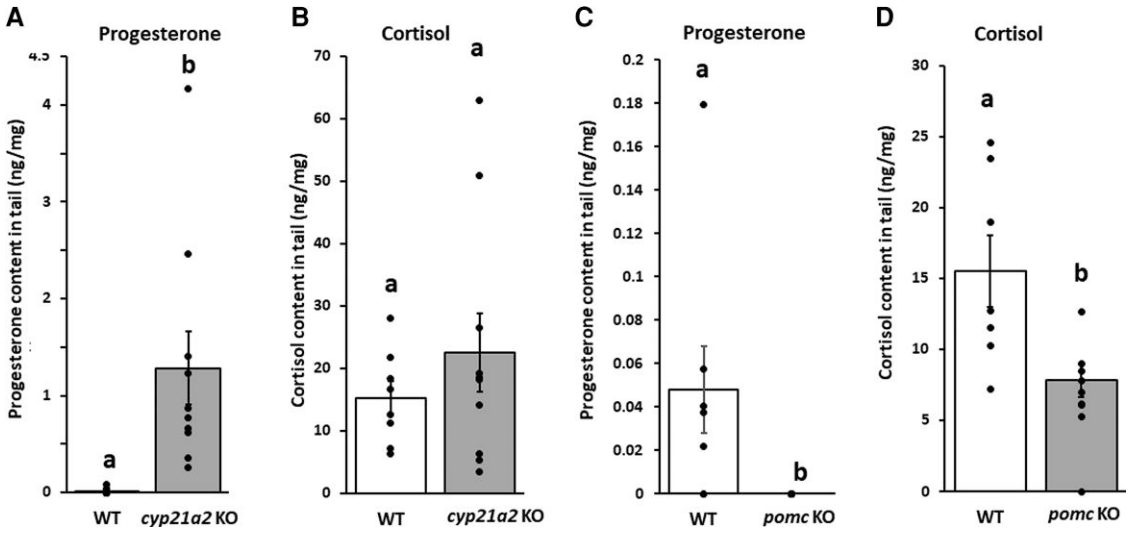


Figure 6. Quantification of progesterone and cortisol in *cyp21a2* mutant and *pomc* mutant tadpoles. Tails (~50 mg each) from genotyped Nieuwkoop and Faber (NF) stage 62 (metamorphic climax) tadpoles were harvested to measure A, progesterone and B, cortisol from a *cyp21a2* clutch and C, progesterone and D, cortisol from a *pomc* clutch via liquid-chromatography tandem mass-spectrometry. White bars, wild-type (WT); black bars, knockout (KO). n = 8-10 tails/genotype. Error bars represent SE. Letters indicate statistically significant groups based on B and D, unpaired *t* test and A and C, nonparametric Wilcoxon rank sum text; *P* less than .05.

whereas ACTH stimulates CORT 2-fold more than it stimulates ALDO (47, 60). Future work is required to explain the WT ALDO amounts in *pomc* mutants and the potential independent regulation of CORT and ALDO synthesis in tadpoles. In any case, the normal ALDO quantities in *pomc* mutants were not sufficient to allow survival through metamorphosis, and *cyp21a2* mutants survived with almost undetectable quantities of ALDO, indicating that ALDO per se is not required for survival through metamorphosis in tadpoles.

Both *cyp21a2*-KO and *pomc*-KO tadpoles retained some ability to synthesize corticosterone (~40% of WT), whereas the amount of cortisol was normal in *cyp21a2* mutants and 50% reduced in *pomc* mutants. This result suggests tadpole cortisol production may be at least partly under ACTH control. Comparison with mammalian systems regarding 21-hydroxylase-independent corticosteroid synthesis is challenging because newborn *cyp21a2* mutants are treated with glucocorticoids/dexamethasone to prevent mortality (18, 19, 61–66). Hence, we do not know whether untreated mammalian *cyp21a2* mutants produce residual glucocorticoids. *cyp21a2*-KO zebrafish also show some residual or basal corticosterone and cortisol levels (22). In zebrafish, CYP17a2 has the ability in vitro to convert 17-hydroxyprogesterone to 11-deoxycortisol, which may explain the measurable cortisol in *cyp21a2*-mutant fish and perhaps tadpoles as well (22). Extra-adrenal production of corticosteroids occurs independently from *cyp21a2* in the brain, heart, and skin in mammals (67, 68). In particular, the rat *cyp2d4* and human *cyp2d6* isoforms of the *cyp2d* family of enzymes carry out 21-hydroxylase activity in brain rather than *cyp21a2* (69). *cyp2d6* is present in the frog genome and is expressed at similar levels as *pck1* in tadpole tail (unpublished RNA-sequencing data), though interrenal expression of *cyp2d6* was not assayed. Future studies are required to determine the basis for the low CORT and normal cortisol in *cyp21a2* mutant tadpoles.

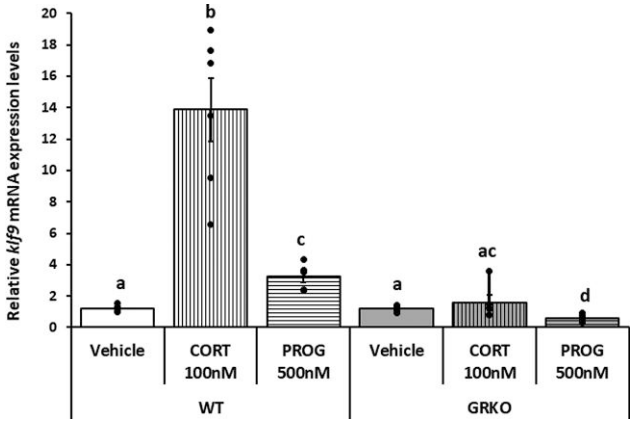


Figure 7. Transactivation of corticosterone (CORT) response gene by progesterone through glucocorticoid receptor (GR). GR knockout (GRKO) or wild-type (WT) Nieuwkoop and Faber (NF) 54 tadpole tails were treated in vitro with vehicle, 100 nM CORT, or 500 nM progesterone (PROG) for 24 hours, and tails were processed for RNA extraction and quantitative polymerase chain reaction. Messenger RNA (mRNA) expression levels of *klf9* were measured relative to the normalizing gene *rpl8*. n = 6 per genotype per treatment. Error bars represent SE. Expression levels were analyzed across genotypes and treatment groups. Letters above bars represent statistically significant groups based on nonparametric Kruskal-Wallis test followed by pairwise comparisons using Wilcoxon rank sum exact test; *P* less than .05.

All vertebrate examples with *cyp21a2* null mutations (fish, frog, mouse, and humans) exhibit low plasma levels or tissue content of glucocorticoids and mineralocorticoids, glucocorticoid signaling deficiency, and impaired negative feedback in the HPA/I axis (1, 22, 62, 65). However, vertebrates are divergent from each other with respect to the impact of altered corticosteroid signaling on development (70). No corticosteroid signaling mutation in zebrafish seems to affect development, as disrupted *pomc*, GR, MR, *cyp21a2* knockout fish survive to adulthood. On the other extreme, survival in mammals is compromised in all such mutations, and they die of different causes depending on the mutant gene. Frogs seem to be intermediate in that developmental progression and survival

can withstand some decrease in corticosteroid signaling, as in *cyp21a2* mutants, but too little corticosteroid signaling is lethal, as in GR and *pomc* mutants. Also, in frogs, death from low corticosteroid signaling is due to reduced TH signaling because TH can rescue GR and *pomc* mutants from death, whereas in mammals, death appears to be due to a direct action of corticosteroids on a developmental event or physiological function.

Conclusion

We have used a genetic engineering strategy to create *cyp21a2*-mutant *X. tropicalis*, a novel corticosteroid-deficient frog model. By using molecular and biochemical approaches, we have shown that *cyp21a2* is necessary for normal glucocorticoid and mineralocorticoid content with the consequent effects of impaired negative feedback on the HPI axis. In contrast to *pomc* and *nr3c1* (GR)-KO tadpoles and *cyp21a1*-KO mice, *cyp21a2* mutant tadpoles survive to adulthood. Our results suggest possible compensatory corticosteroid signaling from the combined effect of cortisol, the remaining CORT, and increased progesterone. Future work using *pomc-cyp21a2* double mutants (which should have minimal CORT precursors and should be unable to convert exogenous precursors to CORT) will examine corticosteroid precursors in detail and their potential ability to rescue the double mutants in vivo.

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Disclosures

The authors have nothing to disclose.

Data Availability

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

References

1. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Endocr Rev*. 2000;21(3):245-291.
2. Buchholz DR. Xenopus metamorphosis as a model to study thyroid hormone receptor function during vertebrate developmental transitions. *Mol Cell Endocrinol*. 2017;459:64-70.
3. Sachs LM, Buchholz DR. Frogs model man: in vivo thyroid hormone signaling during development. *Genesis*. 2017;55(1-2):e23000.
4. Paul B, Sterner ZR, Buchholz DR, Shi YB, Sachs LM. Thyroid and corticosteroid signaling in amphibian metamorphosis. *Cells*. 2022;11(10):1595.
5. Sachs LM, Buchholz DR. Insufficiency of thyroid hormone in frog metamorphosis and the role of glucocorticoids. *Front Endocrinol (Lausanne)*. 2019;10:287.
6. Bonett RM, Hoopfer ED, Denver RJ. Molecular mechanisms of corticosteroid synergy with thyroid hormone during tadpole metamorphosis. *Gen Comp Endocrinol*. 2010;168(2):209-219.
7. Lorenz C, Opitz R, Lutz I, Kloas W. Corticosteroids disrupt amphibian metamorphosis by complex modes of action including increased prolactin expression. *Comp Biochem Physiol C Toxicol Pharmacol*. 2009;150(2):314-321.
8. Bagamasbad PD, Bonett RM, Sachs L, *et al*. Deciphering the regulatory logic of an ancient, ultraconserved nuclear receptor enhancer module. *Mol Endocrinol*. 2015;29(6):856-872.
9. Das B, Matsuda H, Fujimoto K, Sun G, Matsuura K, Shi YB. 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*. 2010;168(2):174-180.
10. Sterner ZR, Shewade LH, Mertz KM, Sturgeon SM, Buchholz DR. Glucocorticoid receptor is required for survival through metamorphosis in the frog *Xenopus tropicalis*. *Gen Comp Endocrinol*. 2020;291:113419.
11. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev*. 2011;32(1):81-151.
12. Aragao-Santiago L, Gomez-Sanchez CE, Mulatero P, Spyroglou A, Reincke M, Williams TA. Mouse models of primary aldosteronism: from physiology to pathophysiology. *Endocrinology*. 2017;158(12);4129-4138.
13. Janner M, Pandey AV, Mullis PE, Flück CE. Clinical and biochemical description of a novel CYP21A2 gene mutation 962_963insA using a new 3D model for the P450c21 protein. *Eur J Endocrinol*. 2006;155(1):143-151.
14. Fujieda K, Mukai T. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Nippon Rinsho*. 2004;62(2):361-367
15. Bruque CD, Delea M, Fernández CS, *et al*. Structure-based activity prediction of CYP21A2 stability variants: a survey of available gene variations. *Sci Rep*. 2016;6:39082.
16. Neves Cruz J, da Costa KS, de Carvalho TAA, de Alencar NAN. Measuring the structural impact of mutations on cytochrome P450 21A2, the major steroid 21-hydroxylase related to congenital adrenal hyperplasia. *J Biomol Struct Dyn*. 2020;38(5):1425-1434.
17. Robins T, Carlsson J, Sunnerhagen M, Wedell A, Persson B. Molecular model of human CYP21 based on mammalian CYP2C5: structural features correlate with clinical severity of mutations causing congenital adrenal hyperplasia. *Mol Endocrinol*. 2006;20(11):2946-2964.
18. Tajima T, Okada T, Ma XM, Ramsey WJ, Bornstein SR, Aguilera G. Restoration of adrenal steroidogenesis by adenovirus-mediated transfer of human cytochrome P450 21-hydroxylase into the adrenal gland of 21-hydroxylase-deficient mice. *Gene Ther*. 1999;6(11):1898-1903.
19. Tajima T, Ma XM, Bornstein SR, Aguilera G. Prenatal dexamethasone treatment does not prevent alterations of the hypothalamic pituitary adrenal axis in steroid 21-hydroxylase deficient mice. *Endocrinology*. 1999;140(7):3354-3362.
20. Perdomini M, Dos Santos C, Goumeaux C, Blouin V, Bougnères P. An AAVrh10-CAG-CYP21-HA vector allows persistent correction of 21-hydroxylase deficiency in a Cyp21$^{-/-}$ mouse model. *Gene Ther*. 2017;24(5):275-281.
21. Facchinello N, Skobo T, Meneghetti G, *et al*. Nr3c1 null mutant zebrafish are viable and reveal DNA-binding-independent activities of the glucocorticoid receptor. *Sci Rep*. 2017;7(1):1-13.
22. Eachus H, Zaucker A, Oakes JA, *et al*. Genetic disruption of 21-hydroxylase in zebrafish causes interrenal hyperplasia. *Endocrinology*. 2017;158(12):4165-4173.
23. Hannah-Shmouni F, Chen W, Merke DP. Genetics of congenital adrenal hyperplasia. *Endocrinol Metab Clin North Am*. 2017;46(2):435-458.
24. Sander JD, Maeder ML, Reyon D, *et al*. ZiFiT (Zinc Finger Targeter): an updated zinc finger engineering tool. *Nucleic Acids Res*. 2010;38(suppl_2):W462-W468. doi:10.1093/nar/gkq319
25. Hwang WY, Fu Y, Reyon D, *et al*. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol*. 2013;31(3):227-229.

26. Blitz IL, Biesinger J, Xie X, Cho KWY. Biallelic genome modification in F0 Xenopus tropicalis embryos using the CRISPR/Cas system. *Genesis*. 2013;51(12):827-834.
27. Choi J, Suzuki KT, Sakuma T, Shewade L, Yamamoto T, Buchholz DR. Unliganded thyroid hormone receptor α regulates developmental timing via gene repression in *Xenopus tropicalis*. *Endocrinology*. 2015;156(2):735-744.
28. Ota S, Hisano Y, Muraki M, *et al.* Efficient identification of TALEN-mediated genome modifications using heteroduplex mobility assays. *Genes Cells*. 2013;18(6):450-458.
29. Foster SD, Glover SR, Turner AN, Chatti K, Challa AK. A mixing heteroduplex mobility assay (mHMA) to genotype homozygous mutants with small indels generated by CRISPR-Cas9 nucleases. *MethodsX*. 2019;6:1-5.
30. Truett GE, Heeger P, Mynatt RL, Truett AA, Walker JA, Warman ML. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques*. 2000;29(1):52-54.
31. Shewade LH, Schoephoerster JA, Patmann MD, Kulkarni SS, Buchholz DR. Corticosterone is essential for survival through frog metamorphosis. *Endocrinology*. 2020;161(12):bqaa193.
32. Nieuwkoop PD, Faber J. *Normal Table of Xenopus Laevis (Daudin)*. New York: Garland Publishing; 1956:162-203.
33. Paul B, Sterner ZR, Bhawal R, Anderson ET, Zhang S, Buchholz DR. Impaired negative feedback and death following acute stress in glucocorticoid receptor knockout *Xenopus tropicalis* tadpoles. *Gen Comp Endocrinol*. 2022;326:114072.
34. Huang H, Cai L, Remo BF, Brown DD. Timing of metamorphosis and the onset of the negative feedback loop between the thyroid gland and the pituitary is controlled by type II iodothyronine deiodinase in Xenopus laevis. *Proc Natl Acad Sci U S A*. 2001;98(13):7348-7353.
35. Manzon RG, Denver RJ. Regulation of pituitary thyrotropin gene expression during Xenopus metamorphosis: negative feedback is functional throughout metamorphosis. *J Endocrinol*. 2004;182(2):273-285.
36. Patmann MD, Shewade LH, Schneider KA, Buchholz DR. Xenopus tadpole tissue harvest. *Cold Spring Harb Protoc*. 2017;2017(11):pdb.prot097675
37. Mughal BB, Leemans M, Spirhanzlova P, Demeneix B, Fini JB. Reference gene identification and validation for quantitative real-time PCR studies in developing *Xenopus laevis*. *Sci Rep*. 2018;8(1):496.
38. Verbrugghe E, Martel A, Pasmans F. Reference gene validation for quantitative real-time PCR studies in amphibian kidney-derived A6 epithelial cells. *Altern Lab Anim*. 2019;47(2):63-70.
39. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*. 2001;25(4):402-408.
40. Buchholz DR, Hayes TB. Variation in thyroid hormone action and tissue content underlies species differences in the timing of metamorphosis in desert frogs. *Evol Dev*. 2005;7(5):458-467.
41. Bonett RM, Hu F, Bagamasbad P, Denver RJ. Stressor and glucocorticoid-dependent induction of the immediate early gene krüppel-like factor 9: implications for neural development and plasticity. *Endocrinology*. 2009;150(4):1757-1765.
42. Shewade LH, Schneider KA, Brown AC, Buchholz DR. In-vivo regulation of Krüppel-like factor 9 by corticosteroids and their receptors across tissues in tadpoles of *Xenopus tropicalis*. *Gen Comp Endocrinol*. 2017;248:79-86.
43. *R: A Language and Environment for Statistical Computing. Version 3.5.2*. R Foundation for Statistical Computing; 2018.
44. Wright ML, Guertin CJ, Duffy JL, Szatkowski MC, Visconti RF, Alves CD. Developmental and diel profiles of plasma corticosteroids in the bullfrog, *Rana catesbeiana*. *Comp Biochem Physiol A Mol Integr Physiol*. 2003;135(4):585-595.
45. Jolivet G, Hatey JL. Variations in aldosterone and corticosterone plasma levels during metamorphosis in *Xenopus laevis* tadpoles. *Gen Comp Endocrinol*. 1984;56(1):59-65.
46. Kulkarni SS, Buchholz DR. Corticosteroid signaling in frog metamorphosis. *Gen Comp Endocrinol*. 2014;203:225-231.
47. Kikuyama S, Suzuki MR, Iwamuro S. Elevation of plasma aldosterone levels of tadpoles at metamorphic climax. *Gen Comp Endocrinol*. 1986;63(2):186-190.
48. Schneider KA, Shewade LH, Buisine N, Sachs LM, Buchholz DR. A novel stress hormone response gene in tadpoles of *Xenopus tropicalis*. *Gen Comp Endocrinol*. 2018;260:107-114.
49. Pijnenburg-Kleizen KJ, Engels M, Mooij CF, *et al.* Adrenal steroid metabolites accumulating in congenital adrenal hyperplasia lead to transactivation of the glucocorticoid receptor. *Endocrinology*. 2015;156(10):3504-3510.
50. Sterner ZR, Buchholz DR. Glucocorticoid receptor mediates corticosterone-thyroid hormone synergy essential for metamorphosis in *Xenopus tropicalis* tadpoles. *Gen Comp Endocrinol*. 2022;315:113942.
51. Sakurai N, Maruo K, Haraguchi S, *et al.* Immunohistochemical detection and biological activities of CYP17 (P450c17) in the indifferent gonad of the frog *Rana rugosa*. *J Steroid Biochem Mol Biol*. 2008;112(1-3):5-12.
52. Navarro-Martín L, Velasco-Santamaría YM, Duarte-Guterman P, *et al.* Sexing frogs by real-time PCR: using aromatase (cyp19) as an early ovarian differentiation marker. *Sex Dev*. 2012;6(6):303-315.
53. Forsburg ZR, Goff CB, Perkins HR, Robicheaux JA, Almond GF, Gabor CR. Validation of water-borne cortisol and corticosterone in tadpoles: recovery rate from an acute stressor, repeatability, and evaluating rearing methods. *Gen Comp Endocrinol*. 2019;281:145-152.
54. Krain LP, Denver RJ. Developmental expression and hormonal regulation of glucocorticoid and thyroid hormone receptors during metamorphosis in *Xenopus laevis*. *J Endocrinol*. 2004;181(1):91-104.
55. El Ghorayeb N, Bourdeau I, Lacroix A. Role of ACTH and other hormones in the regulation of aldosterone production in primary aldosteronism. *Front Endocrinol (Lausanne)*. 2016;7:72.
56. Chong C, Hamid A, Yao T, *et al.* Regulation of aldosterone secretion by mineralocorticoid receptor-mediated signaling. *J Endocrinol*. 2017;232(3):525-534.
57. Taylor P, Scroop GC, Tyler MJ, Davies M. An ontogenetic and interspecific study of the renin-angiotensin system in Australian anuran amphibia. *Comp Biochem Physiol Part A Physiol*. 1982;73(2):187-191.
58. Tei S, Santarella B, Farnesi RM, Vagnetti D. Ultrastructural analysis and cytochemical localization of atrial natriuretic peptide-stimulated guanylate cyclase in internal gills of *Bufo bufo* tadpoles. *Eur J Morphol*. 2000;38(1):41-50.
59. Kloas W, Hanke W. Localization and quantification of angiotensin II (A II) binding sites in the kidney of *Xenopus laevis*—lack of A II receptors in the adrenal tissue. *Gen Comp Endocrinol*. 1992;86(2):173-183.
60. Okada R, Yamamoto K, Hasunuma I, Asahina J, Kikuyama S. Arginine vasotocin is the major adrenocorticotropic hormone-releasing factor in the bullfrog *Rana catesbeiana*. *Gen Comp Endocrinol*. 2016;237:121-130.
61. Gotoh H, Sagai T, Hata JI, Shiroishi T, Moriwaki K. Steroid 21-hydroxylase deficiency in mice. *Endocrinology*. 1988;123(4):1923-1927.
62. Gotoh H, Kusakabe M, Shiroishi T, Moriwaki K. Survival of steroid 21-hydroxylase-deficient mice without endogenous corticosteroids after neonatal treatment and genetic rescue by transgenesis as a model system for treatment of congenital adrenal hyperplasia in humans. *Endocrinology*. 1994;135(4):1470-1476.
63. Riepe FG, Tatzel S, Sippell WG, Pleiss J, Krone N. Congenital adrenal hyperplasia: the molecular basis of 21-hydroxylase deficiency in H-2(aw18) mice. *Endocrinology*. 2005;146(6):2563-2574.
64. Parker KL, Chaplin DD, Wong M, Seidman JG, Smith JA, Schimmer BP. Expression of murine 21-hydroxylase in mouse

adrenal glands and in transfected Y1 adrenocortical tumor cells. *Proc Natl Acad Sci U S A*. 1985;82(23):7860-7864.
65. Bornstein SR, Tajima T, Eisenhofer G, Haidan A, Aguilera G. Adrenomedullary function is severely impaired in 21-hydroxylase-deficient mice. *FASEB J*. 1999;13(10):1185-1194.
66. Shiroishi T, Sagai T, Natsuume-Sakai S, Moriwaki K. Lethal deletion of the complement component C4 and steroid 21-hydroxylase genes in the mouse H-2 class III region, caused by meiotic recombination. *Proc Natl Acad Sci U S A*. 1987;84(9):2819-2823.
67. Taves MD, Gomez-Sanchez CE, Soma KK. Extra-adrenal glucocorticoids and mineralocorticoids: evidence for local synthesis, regulation, and function. *Am J Physiol Endocrinol Metab*. 2011;301(1):E11-E24.
68. Mellon SH, Miller WL. Extraadrenal steroid 21-hydroxylation is not mediated by P450c21. *J Clin Invest*. 1989;84(5):1497-1502.
69. Kishimoto W, Hiroi T, Shiraishi M, *et al*. Cytochrome P450 2D catalyze steroid 21-hydroxylation in the brain. *Endocrinology*. 2004;145(2):699-705.
70. Buchholz DR. More similar than you think: frog metamorphosis as a model of human perinatal endocrinology. *Dev Biol*. 2015;408(2):188-195.