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Research Article

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Corticosterone Is Essential for Survival Through Frog Metamorphosis

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Abbreviations: ACTH, adrenocorticotropic hormone; cDNA, complementary DNA; CLIP, corticotropin-like intermediate peptide; CORT, corticosterone; CRH, corticotropin-releasing hormone; GRKO, glucocorticoid receptor knockout; klf9, Krüppel-like factor 9; MBS, modified Barth's solution; mRNA, messenger RNA; MSH, melanocyte-stimulating hormone; NF, Nieuwkoop and Faber; PCR, polymerase chain reaction; pomc, proopiomelanocortin; SE, standard error of the mean; T3, tri-iodothyronine; TALEN, transcription activator-like effector nuclease; TH, thyroid hormone; thrb, TH receptor β; ush1g, Usher syndrome type-1G

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Abstract

Thyroid hormone (TH) is required for frog metamorphosis, and corticosterone (CORT) increases TH signaling to accelerate metamorphic progression. However, a requirement for CORT in metamorphosis has been difficult to assess prior to the recent development of gene-editing technologies. We addressed this long-standing question using transcription activator-like effector nuclease (TALEN) gene disruption to knock out *proopiomelanocortin* (*pomc*) and disrupt CORT production in *Xenopus tropicalis*. As expected, mutant tadpoles had a reduced peak of plasma CORT at metamorphosis with correspondingly reduced expression of the CORT-response gene *Usher syndrome type-1G* (*ush1g*). Mutants had reduced rates of growth and development and exhibited lower expression levels of 2 TH response genes, Krüppel-like factor 9 (*klf9*) and TH receptor β (*thrb*). In response to exogenous TH, mutants had reduced TH response gene induction and slower morphological change. Importantly, death invariably occurred during tail resorption, unless rescued by exogenous CORT and, remarkably, by exogenous TH. The ability of exogenous TH by itself to overcome death in *pomc* mutants indicates that the CORT-dependent increase in TH signaling may ensure functional organ transformation required for survival through metamorphosis and/or may shorten the nonfeeding metamorphic transition to avoid lethal inanition.

Key Words: ACTH, *pomc*, TALENs, thyroid hormone, *Xenopus*

The transition from an aquatic environment to a terrestrial lifestyle in vertebrates is a hormone-regulated process that shows conserved endocrine mechanisms across birth, hatching, and metamorphosis (1-3). The 2 main hormones

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regulating this transition are thyroid hormone (TH) and a glucocorticoid hormone (either cortisol or corticosterone depending on the species, hereafter referred to as *CORT*) (4-8). These hormones exhibit a peak in plasma levels at the developmental transition and act during development largely via their nuclear receptors (6, 7, 9-11). These receptors are widely expressed among tissues and regulate development through alteration of gene expression (12, 13). Metamorphosis is an established model for studying the developmental actions of TH and CORT because it occurs in a free-living organism and is exquisitely and dramatically controlled by these hormones (14). Recent advances in genetic manipulations further advance the utility of *Xenopus tropicalis* as a model to study hormone actions and interactions during development (15).

In frogs, TH is necessary and sufficient to initiate metamorphic changes in all tissues, and higher doses of TH result in more rapid changes (12, 16). Exogenous CORT treatment by itself does not induce metamorphic effects but rather inhibits growth and development (17-19). However, when exogenous CORT is given to tadpoles in combination with exogenous TH, development is accelerated compared to tadpoles treated with TH alone (20-22). In stressful aquatic conditions, plasma levels of TH and CORT increase, leading to increased TH signaling and accelerated metamorphosis (6, 23). Thus, the role of CORT is known to be significant in shortening the larval period in stressful conditions, for example, as observed in accelerated metamorphosis in spadefoot toads in response to pond drying (24).

Two main mechanisms explain how CORT action increases TH signaling and accelerates metamorphosis (25). First, the gene *Krüppel-like factor 9* (*klf9*) contains an enhancer containing hormone response elements both for TH and CORT and is thereby independently and synergistically upregulated by these hormones (26). Other response genes may have this property (27, 28), but importantly, *klf9* is a transcription factor that can make tissues more responsive to circulating TH by enhancing TH-dependent TH receptor induction (29). Second, CORT modulates tissue deiodinases to increase TH activation and decrease degradation activities, which increases intracellular TH levels and thus increases tissue responsivity (21, 25, 30). These effects of CORT on *klf9*, TH receptor, and deiodinases increase the amount of TH signaling and accelerate TH-dependent change.

We recently found that tadpoles with disrupted glucocorticoid receptor (GR) die around gill and tail resorption (31). This lethality may be due to an essential developmental action of CORT independent of TH signaling, as is apparently the case in mammalian lung development (32). Alternatively, lack of CORT signaling through the GR may yield a level of TH responsivity too low to support sufficient TH signaling to finish metamorphosis. Prior studies provided equivocal evidence for a required role of CORT in metamorphic development. Specifically, hypophysectomized and hypothalectomized tadpoles showed inhibited metamorphosis that could or could not be rescued from death as tadpoles by exogenous TH combined with adrenocorticotropic hormone (ACTH) treatment (16, 33). Here, to gain insight into this issue and further our understanding of the role of CORT in metamorphosis, we have created *proopiomelanocortin* (*pomc*) mutant frogs affecting the coding region for ACTH. ACTH is a peptide hormone released from the pituitary gland in response to corticotropin-releasing hormone and stimulates the interrenal glands to synthesize CORT (6). We analyzed growth, development, gene expression, and hormone replacement in *pomc* mutants to examine the role of CORT-regulated TH signaling in amphibian metamorphosis.

Materials and Methods

Transcription activator-like effector nuclease construction and messenger RNA synthesis

Left and right transcription activator-like effector nuclease (TALEN) arms were designed using the ZiFit program (34) to target the coding region of α-melanocyte stimulating hormone within ACTH in the third exon of *pomc* (Fig. 1A). Golden Gate TALEN assembly kit (catalog No. 1000000016, AddGene) was used to construct both TALEN arms (35). Plasmids encoding the TALEN arms were linearized using NotI, and mRNA synthesis was performed using the T7 mMessage mMachine kit (Invitrogen). Additionally, mCherry mRNA was transcribed from KpnI-linearized CS108-mCherry vector (gift from Dr Mustafa Khokha).

Animals and microinjection

Lab-reared wild-type adult male and female *X tropicalis* were primed with 20 U of human chorionic gonadotrophin (Sigma) or ovine luteinizing hormone (National Hormone and Peptide Program) the evening prior to boosting with 200 U the morning of breeding. Eggs were collected and de-jellied by transferring to 3% L-cysteine (Sigma) in 0.1X modified Barth's solution (MBS) for approximately 10 minutes. De-jellied eggs were then transferred to an injection dish containing 3% Ficoll in 0.1X MBS. One-cell–staged embryos were coinjected with a mixture containing 400 pg of each TALEN arm mRNA and 25 pg of mCherry mRNA. After 4 hours, the surviving embryos were transferred to 0.01X MBS, which was replaced every day for

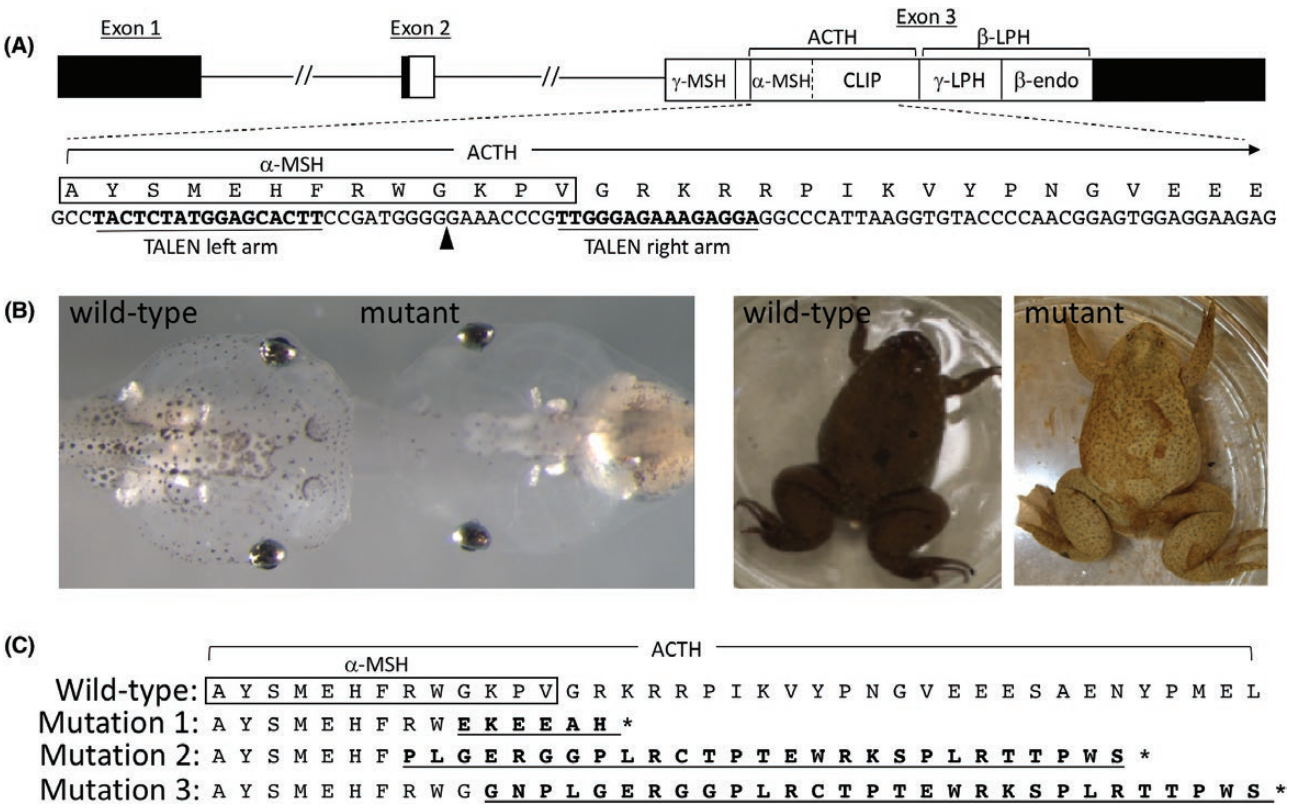


Figure 1. Adrenocorticotropic hormone (ACTH) transcription activator-like effector nuclease (TALEN) design, external phenotypes, and mutant peptide sequences. A, The *pomc* locus in *Xenopus tropicalis* is shown with annotations for intron-exon structure, 5′ and 3′ untranslated regions (black portion of exons), and peptide processing products. The α-MSH peptide sequence (boxed) and a portion of the ACTH peptide sequence (bracketed) are shown above the corresponding DNA sequence. The left and right TALEN arm recognition sites (bold and underlined) flank a spacer region, which is cleaved at the center (arrowhead) by dimerized FokI nucleases fused to the TALEN arms. B, TALEN-injected founders have a lighter pigmentation as tadpoles and a lighter pigmentation with abnormal skin shedding as adults. C, Amino-acid sequence alignment at the TALEN target site is shown for wild-type α-MSH and ACTH peptides as well as the predicted amino acid sequences for 3 different insertion-deletion mutations identified. Amino acid residues in bold are different from the wild-type sequence because of frame-shift mutations, and asterisks represent STOP codons. α-MSH, α-melanocyte-stimulating hormone; endo, endorphin; CLIP, corticotropin-like intermediate peptide; LPH, lipotropic hormone; *pomc*, proopiomelanocortin.

3 days. Hatched tadpoles were sorted based on mCherry fluorescence under a fluorescence dissection stereomicroscope to identify successfully injected eggs. The sorted embryos were reared at 26°C in reconstituted reverse-osmosis water and fed Sera Micron food twice daily with daily water changes. Tadpoles were staged according to the Nieuwkoop and Faber (NF) staging table (36). All animal maintenance and experimentation were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the Endocrine Society Ethical Guidelines, and the University of Cincinnati Institutional Animal Care and Use Committee (protocol 06-10-03-01).

Genetic screening

Genomic DNA was extracted from tail tips of mCherry-expressing tadpoles using the Quick gDNA Miniprep kit (Zymo Research) and used for polymerase chain reaction (PCR) amplification (DreamTaq, Thermo Fisher) of a 500-bp region surrounding the TALEN target site. The forward primer (5′-GACCCATTTCCGGTGGAATAA) and reverse primer (5′-GTCATTAGAGGTGTCTGGCTC) were at a concentration of 0.2 μM each, and the reaction conditions were: 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds followed by 72°C for 5 minutes. Founders with a *pomc* disruption were identified based on the Direct Sequencing of the Product genotyping assay (37) and were reared to sexual maturity as the F0 generation and were subsequently crossed with each other to obtain an F1 population. To identify F1 heterozygous mutants, the F1 population was screened via the heteroduplex mobility assay (38) followed by TOPO cloning (Zero Blunt PCR TOPO cloning kit, Thermo Fisher) and sequencing. F2 compound mutant heterozygous (biallelic knockout) individuals were obtained either by crossing 2 F1 heterozygous mutant parents or back-crossing an F1 mutant parent with an F0 individual with an adult shedding phenotype (see "Results"). After showing a 100% correspondence between the tadpole mutant pigmentation phenotype (see "Results") and biallelic

pomc mutations, biallelic knockout tadpoles were identified using the pigmentation phenotype and compared with sibling F2 tadpoles with wild-type pigmentation and with tadpoles from wild-type parents.

Plasma extraction and enzyme immunoassay analysis

Mutant and sibling tadpoles and tadpoles from wild-type parents at NF 56 and NF 62 were anesthetized using Tricaine (MS-222), and blood was extracted with heparinized capillary tubes (microhematocrit, 0.5-mm i.d., 75-mm length, Kimble-Chase, catalog No. 505) following the described procedure (39). The blood was centrifuged at 5000 rpm for 20 minutes to collect supernatant plasma, which was stored at –80°C until use. Blood from 3 to 4 tadpoles was pooled per sample to obtain 3 pooled plasma samples per stage per genotype. Enzyme immunoassay for corticosterone was performed using an enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Cayman Chemicals, cross-reactivity with cortisol < 2.5%, aldosterone < 0.5%) (40).

Fat body weight measurement

Mutant and sibling tadpoles at NF 64 were anesthetized using MS222 (tricaine methanesulfonate) and measured for snout-to-vent length, and fat bodies were harvested as described (39). To measure fat weight, 1.5-mL Eppendorf tubes containing fat bodies in 1ml of 60% phosphate-buffered saline were weighed, and blank values obtained from the phosphate-buffered saline–containing 1.5-mL Eppendorfs without fat bodies were subtracted to get the fat weight. The sample size was n = 5.

Growth and development study

Two weeks post fertilization, 20 mutant and 20 sibling tadpoles at the same size and stage were placed in groups of 4 each in 3-L tanks. Tadpoles were fed a fixed amount of Sera Micron food twice daily, before and after daily water changes. Tank positions were changed haphazardly with every water change to avoid bias from slight temperature variation (< 2°C) across the shelf. Snout-to-vent length and developmental stage were recorded every 5 days until NF 64. Time to NF 64 was also recorded. Six mutant and 2 wild-type tadpoles died during the course of the experiment. The entire experiment was repeated with an unrelated clutch.

Hormone treatments

For gene expression, mutant and sibling tadpoles just before metamorphosis (NF 54) were treated with 0 nM, 2 nM, or 10 nM tri-iodothyronine (T3) in 1-L beakers for 24 hours. For CORT rescue, mutant tadpoles at metamorphic stages (NF 58, 60, and 62) were treated with different doses of exogenous CORT (Acros Organics) in 1-L beakers with water and hormones replaced daily. Survival or not to tail resorption (NF 66) was recorded. For TH rescue, mutant and sibling tadpoles at NF 60 were treated with 0 or 5 nM T3 in 1-L beakers with water and hormone changed daily. Digital images were taken of each tadpole daily, and day to achieve NF 62, 64, and 66 was recorded.

Quantitative real-time polymerase chain reaction

Tails from tadpoles at NF 54, 58, and 62, and tails and brains after treatment with TH were harvested and immediately snap-frozen and stored at –80°C. Each brain sample was a pool of 2 brains. Total RNA from frozen tissues was extracted using Tri-Reagent RT (Molecular Research Centre Inc) following the manufacturer's instructions. One µg of RNA was used per sample for cDNA synthesis using the All-in-One cDNA synthesis super-mix (BioMake) following the manufacturer's protocol. A total of 1 µL of complementary DNA (cDNA) was used in each 20-µL quantitative PCR reaction with 2X TaqMan Universal master mix (Applied Biosystems). The FAM-labeled primer-probe sets used were *ush1g* (forward: 5′-CTGTAGGACACGTATTTCATGATTAAGC, reverse: 5′-CAACATTAACAGGGTATGATAAAATCAATATATCTTTATTACAAAAT, probe: 5′-CCTGACGCATTTTGTG); *klf9* (forward: 5′-CCTTAAAGCCCATTACAGAGTCCAT, reverse: 5'-GCAGTCAGGCCACGTACA, probe: 5′-ACAGGTGAACGCCCTTTT); TH receptor β (*thrb*) (forward: 5′-CAAGAGTTGTTGATTTTGCCAAAAA, reverse: 5′-ACATGATCTCCATACAACAGCCTTT, probe: 5′-CTGCCATGTGAAGACC); and *rpl8* (forward: 5′-CACAATCCTGAAACCAAGAAAACCA, reverse: 5′-CCACACCACGGACACGT, probe: 5′-AAGGCCAAGAGAAACT). The reactions were run on a 7300 Real-Time PCR system (Applied Biosystems) with the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 10 seconds and 60°C for 1 minute). The relative quantification method ΔΔCt (41) was used to measure expression levels of target genes normalized to the reference gene *rpl8* at different developmental time points for each sample group with a sample size of n = 5 samples per stage per genotype.

Statistical analysis

Data analysis was performed using JMP Pro 12 statistical analysis software. Analysis of variance followed by

pairwise comparisons using the Tukey-Kramer post hoc test were performed to identify significant differences among samples for plasma CORT measurements and for time to reach NF 62, 64, and 66 after TH treatments. All other statistical comparisons were performed between mutant and sibling tadpoles within time, stage, or hormone treatment using *t* test. A *P* value of less than .05 was considered statistically significant for post hoc tests and *t* tests.

Results

Transcription activator-like effector nuclease design

To create knockout *X tropicalis* devoid of functional ACTH peptide, we targeted the gene *pomc*, which consists of 3 exons encoding a large precursor polypeptide that is processed into several biologically active peptides including ACTH (Fig. 1A) (42). The 39-amino acid ACTH peptide can be further processed to produce the 13-amino acid α-MSH (α-melanocyte–stimulating hormone) and the 22-amino acid CLIP (corticotropin-like intermediate peptide). α-MSH acts to darken the skin by upregulating melanin synthesis and inducing pigment dispersal in melanocytes (43). We designed TALENs to target the α-MSH coding region at Gly-10 to disrupt ACTH and produce a pigmentation phenotype for visualization of mutants. Function studies show that the amino acid residues Gly-10 and Pro-12 of α-MSH are important for its biological activity (44). Similarly, alanine substitutions at positions 6 to9 and 15 to 19 abolish ACTH stimulation of melanocortin 2 receptor expressed on CORT-producing cells (45). Thus, most if not all mutations are expected to functionally disrupt α-MSH and ACTH. Frame-shift mutations would also eliminate the downstream *pomc* peptides, CLIP, and β-lipotropin (containing γ-lipotropin, β-MSH, and β-endorphin) (Fig. 1A). The γ-MSH coding region upstream of α-MSH would not be disrupted, but we did not test for the possibility of reduced mRNA levels from non-sense–mediated decay (46).

Production of *proopiomelanocortin* mosaic founders

mRNAs for *pomc* TALENs and the red fluorescent protein mCherry were injected into single-celled embryos to obtain mosaic F0 animals. Injected embryos were sorted based on mCherry expression 3 days post injection. The observation of a blanched skin color starting 1-week post fertilization in some injected individuals suggested functional disruption of α-MSH (Fig. 1B). The blanched founder tadpoles were raised to adulthood, during which the lighter color persisted (see Fig. 1B). We realized later that these founders likely had mosaicism in the pituitary to allow survival through metamorphosis. The adult founders also displayed an abnormality in the molting process wherein the replaced stratum corneum remained attached to the epithelium and had the appearance of incomplete sloughing (see Fig. 1B), as seen in previous studies on CORT, ACTH, and molting in frogs (47). Some pale-skinned adults also appeared larger in body size compared to wild-type, consistent with human and mouse *pomc* deficiency, which exhibit pale skin, hyperphagia, and obesity phenotypes (48, 49), but we did not undertake a quantitative study to characterize these adult phenotypes.

Sequencing analysis of founder offspring

We initially obtained 3 F1 clutches by crossing 2 founder females and 3 founder males, all with the shedding phenotype. Each of these clutches had blanched tadpoles as early as 1 week post hatching. The nonmendelian frequency of these blanched tadpoles, namely 25% to 90%, demonstrated mosaicism among germ cells of founders. We PCR-amplified a 500-bp region surrounding the TALEN target site followed by Topo cloning to enable sequencing of PCR products representing both alleles in each tadpole. Four representative F1 tadpoles of each skin pigmentation type from 2 clutches and 2 representatives of each type from a third clutch were used in the sequencing analysis. We recovered 3 mutant alleles in total among 19 individuals from 3 F1 clutches, namely 2 distinct 14-bp deletions and 1 2-bp insertion at the TALEN target site (Table 1, Fig. 1C). Each of these mutations indicates a frame-shift mutation in α-MSH/ACTH at position 8, 10, or 11 followed by a premature STOP codon after 6, 27, or 29 mutant amino acids (see Fig. 1C). All tadpoles with wild-type pigmentation had at least 1 allele with a wild-type sequence, and all blanched tadpoles showed the presence of 2 frame-shifted mutant alleles, except 1 individual from clutch 3 that had a single mutant allele in each of 10 sequencing runs (see Table 1). Several of these heterozygous F1s were raised to sexual maturity and bred to obtain F2 tadpoles by crossing siblings or back-crossing with F0 parents. We could thus reliably identify biallelic mutant tadpoles by their dark vs light skin pigmentation. For subsequent experiments, we designated the F2 tadpoles with blanched pigmentation as "mutant" and F2 tadpoles with wild-type pigmentation as "sibling," recognizing that "sibling" denotes a

Table 1. Genomic sequencing of tadpoles with a wild-type or blanched pigmentation phenotype

Cl	Phen	Ind	Al	Nucleotide sequence	Indels	No.
1	WT	1	1	AAAGAGGGTTGCCCAAAGGGGGTAGCCTTCACGAGGTATCTCAT	0	×1
1	WT	2	1	AAAGAGGGTTGCCCAAAGGGGGTAGCCTTCACGAGGTATCTCAT	0	×1
1	WT	3	1	AAAGAGGGTTGCCCAAAGGGGGTAGCCTTCACGAGGTATCTCAT	0	×2
1	WT	3	2	AAAGAGGGT----------AGCCTTCACGAGGTATCTCAT	−14	×1
1	WT	4	1	AAAGAGGGTTGCCCAAAGGGGGTAGCCTTCACGAGGTATCTCAT	0	×2
1	WT	4	2	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	−14	×2
1	MT	1	1	AAAGAGGGT----------AGCCTTCACGAGGTATCTCAT	−14	×1
1	MT	1	2	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	−14	×1
1	MT	2	1	AAAGAGGGT----------AGCCTTCACGAGGTATCTCAT	−14	×1
1	MT	2	2	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	−14	×2
1	MT	3	1	AAAGAGGGT----------AGCCTTCACGAGGTATCTCAT	−14	×4
1	MT	3	2	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	−14	×1
1	MT	4	1	AAAGAGGGT----------AGCCTTCACGAGGTATCTCAT	−14	×1
1	MT	4	2	AAAGAGGGTTGCCCAAAGGGGGggTAGCCTTCACGAGGTATCTCAT	+2	×3
2	WT	1	1	AAAGAGGGTTGCCCAAAGGGGGTAGCCTTCACGAGGTATCTCAT	0	×2
2	WT	2	1	AAAGAGGGTTGCCCAAAGGGGGTAGCCTTCACGAGGTATCTCAT	0	×3
2	WT	2	2	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	−14	×1
2	WT	3	1	AAAGAGGGTTGCCCAAAGGGGGTAGCCTTCACGAGGTATCTCAT	0	×2
2	WT	3	2	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	−14	×1
2	WT	4	1	AAAGAGGGTTGCCCAAAGGGGGTAGCCTTCACGAGGTATCTCAT	0	×1
2	MT	1	1	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	−14	×1
2	MT	1	2	AAAGAGGGT----------AGCCTTCACGAGGTATCTCAT	−14	×2
2	MT	2	1	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	−14	×3
2	MT	2	2	AAAGAGGGT----------AGCCTTCACGAGGTATCTCAT	−14	v1
2	MT	3	1	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	−14	×1
2	MT	3	2	AAAGAGGGT----------AGCCTTCACGAGGTATCTCAT	−14	×1
3	WT	1	1	AAAGAGGGTTGCCCAAAGGGGGTAGCCTTCACGAGGTATCTCAT	0	×2
3	WT	1	2	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	−14	×1
3	WT	2	1	AAAGAGGGTTGCCCAAAGGGGGTAGCCTTCACGAGGTATCTCAT	0	×1
3	WT	2	2	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	−14	×2
3	MT	1	1	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	−14	×10
3	MT	2	1	AAAGAGGGTTGCC----------TTCACGAGGTATCTCAT	v14	×2
3	MT	2	2	AAAGAGGGT----------AGCCTTCACGAGGTATCTCAT	−14	×2

Abbreviations: Al, allele (upper and lower case letters in the nucleotide sequence are wild-type and insertions, respectively, and dashes represent deletions); Cl, clutch; Ind, individual; indels, size of insertion or deletion in base pairs; MT, blanched pigmentation; No., number of sequences obtained per allele per individual; Phen, pigmentation phenotype observed; WT, wild-type pigmentation.

mixture of homozygous wild-type tadpoles and tadpoles heterozygous for a *pomc* mutation.

Impaired plasma corticosterone peak at metamorphic climax

Because ACTH is primarily responsible for inducing the interrenal glands to produce CORT, the lack of functional ACTH is expected to severely impair the ability of tadpoles to produce CORT. Previous studies showed that during metamorphosis, CORT levels are minimal around the beginning of metamorphosis (NF 56) and reach a peak at metamorphic climax (NF 62) (50). We therefore compared CORT production using enzyme-linked immunoassay analysis in mutant and sibling F2 tadpoles and tadpoles from wild-type parents (Fig. 2A). We detected low levels of CORT in all 3 sample groups at NF 56. CORT levels peaked at NF 62 both in wild-type and sibling sample groups. There was a slight increase in CORT production in mutant tadpoles at NF 62, but the

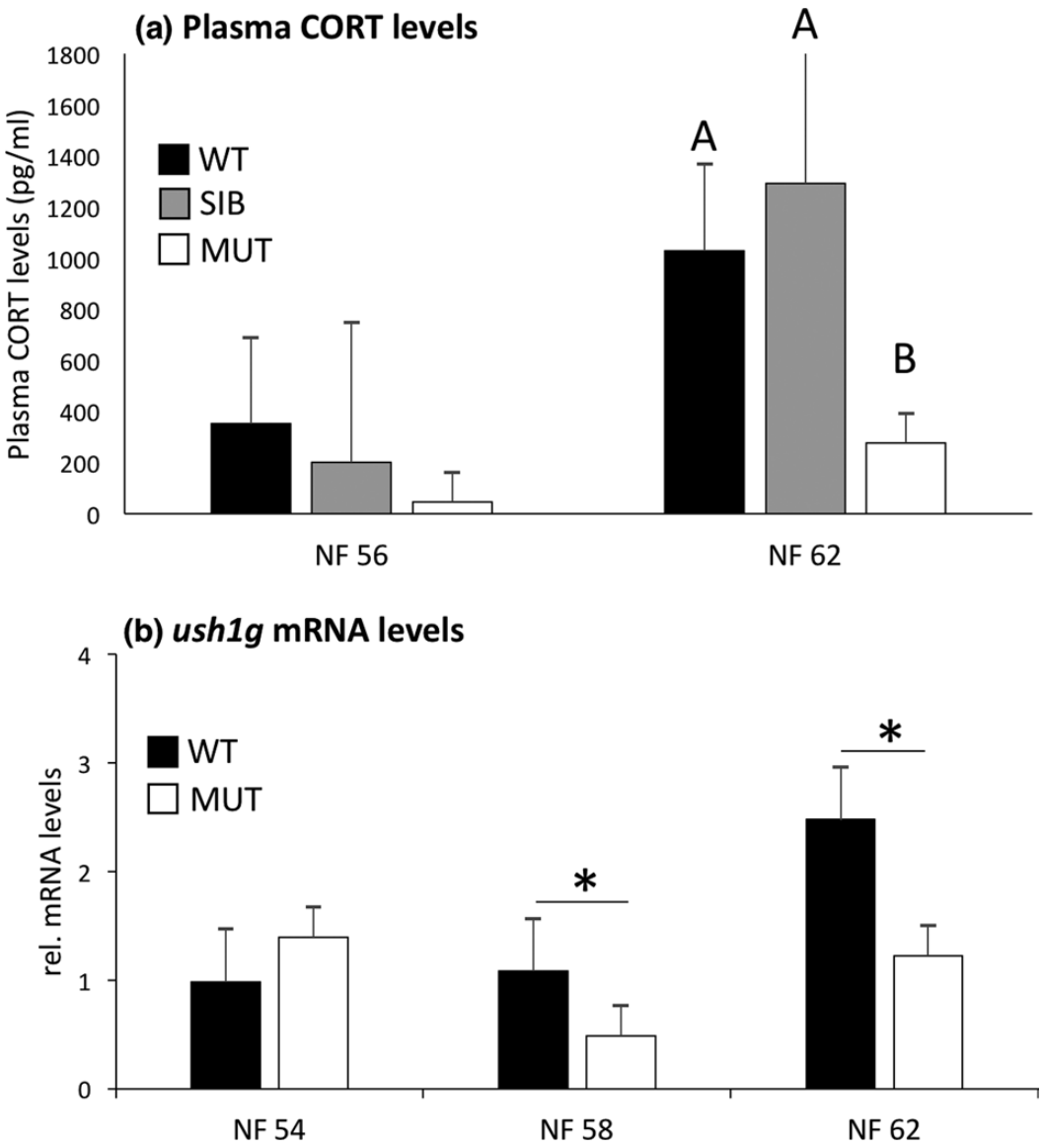


Figure 2. Endocrine and molecular confirmation of impaired corticosterone (CORT) production. A, Blood was collected to measure plasma CORT levels via enzyme-linked immunoassay from tadpoles at Nieuwkoop and Faber (NF) stage 56 (early metamorphosis) and NF 62 (metamorphic climax) from offspring of wild-type parents (WT, black bars) and from *pomc* mutant F2 offspring that were pigmented (sibling, SIB, gray bars) or blanched (mutant, MUT, white bars). Error bars indicate SE. Letters denote significant differences between bars at the same stage (Tukey pairwise post hoc test, $P < .05$, n = 3 plasma samples, each sample contained blood from 1-3 individuals). This experiment was repeated with a different clutch, and similar results were obtained. B, Total RNA was collected from tails of WT and MUT tadpoles at NF 54, 58, and 62 to analyze messenger RNA (mRNA) expression for the CORT-response gene *Usher syndrome 1G* (*ush1g*). Bars represent mean mRNA levels relative to the housekeeping gene *rpl8*. Error bars represent SE. Asterisks denote significant differences between bars determined for each stage (*t* test, $P < .05$, n = 5 tail samples per bar).

levels were about 4 times lower than in the other groups and were similar to the levels observed at NF 56. This result was repeated with a second clutch from different parents and confirmed the inability to achieve a normal CORT peak in *pomc*-mutant animals.

Reduced corticosterone signaling during metamorphosis

To confirm that the reduced CORT levels had an effect on CORT-response gene induction, we measured mRNA expression of the CORT-only response gene *ush1g* (51). This gene is induced by CORT, and thyroid hormone has no influence on its expression. The *ush1g* levels in wild-type tadpoles followed the same developmental expression profile as seen previously, peaking at NF 62. However, no increase in *ush1g* mRNA was observed in mutant tadpoles (Fig. 2B), suggesting that the *pomc* mutants did not have sufficient plasma CORT to induce CORT response genes at metamorphosis.

Fat mobilization in *pomc* mutants

CORT has lipolytic effects in tadpoles, and exogenous CORT treatment reduces fat body weight (52). We tested whether *pomc*-mutant animals had an increase in lipid content by measuring fat bodies at the end of metamorphosis. We found increased fat body weight in mutant tadpoles after metamorphic climax (NF 64) compared to sibling F2 tadpoles (Fig. 3).

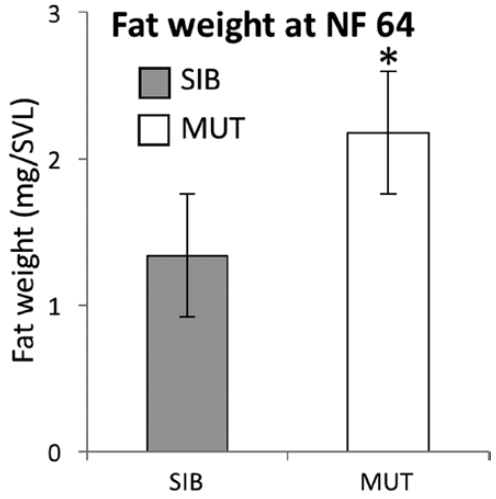


Figure 3. Increased fat body size in *pomc* mutants. Fat bodies were harvested from mutant (MUT) and sibling (SIB) tadpoles just before death near midtail resorption Nieuwkoop and Faber (NF) stage 64) to measure fat weight relative to body size (snout-vent length, SVL). Error bars indicate SE. Asterisk denotes significant difference between bars (*t* test, $P < .05$, n = 10 samples per bar).

Growth and development profile

To determine the role of *pomc* in regulation of growth and development in tadpoles, we recorded the size and stage of mutant and sibling F2 tadpoles every 5 days starting 2 weeks post fertilization (NF 48) until midtail resorption (NF 64), when all mutants die (Fig. 4A and 4B). On day 1 (premetamorphosis), all the tadpoles were at the same size and stage, but after 5 days when the TH-dependent developmental events of metamorphosis were just beginning, mutant tadpoles showed a significant delay in growth and development. Subsequently, at any given time point throughout the larval period and metamorphosis, the mutants were significantly smaller in size and behind in development. Mutant tadpoles reached NF 64 approximately 2 weeks after sibling F2 tadpoles (Fig. 4C).

Effect of exogenous corticosterone on survival through metamorphosis

Mutant tadpoles achieve NF 64, at which point they arrest for 3 to 4 days then die. This lethal phenotype can be reversed by daily treatment of mutant tadpoles with exogenous CORT (25 nM and 50 nM) starting at stages before gill resorption, NF 58, 60, and 62 (Fig. 5). This requirement for CORT treatments persisted through adulthood, and discontinuation of CORT treatments in mutant juveniles and adults invariably led to death within 1 to 2 weeks (n = 16). Continuously CORT-treated adults were kept alive and healthy for more than 13 months, and a mutant male (females were not tried) was fertile, producing 50% mutant offspring, based on pigmentation phenotype, when crossed to a *pomc* F1 heterozygous female.

Expression profile of thyroid hormone response genes

The reduced development rate observed in *pomc*-mutant tadpoles may be accompanied by reduced expression levels of the TH response genes important for developmental progression. The gene *klf9* is a transcription factor induced directly by TH or CORT and synergistically by hormone co-treatment (26). The gene *thrb* is induced directly by TH and indirectly through TH and CORT induction of *klf9*, which contributes to *thrb* upregulation (25, 29). We measured the expression of *kfl9* and *thrb* in brain and tail at key developmental time points, namely beginning of metamorphosis (NF 54), midpoint of blood TH and CORT levels (NF 58), and peak of TH and CORT levels at metamorphic climax (NF 62)(Fig. 6). For both *klf9* and *thrb*, we found

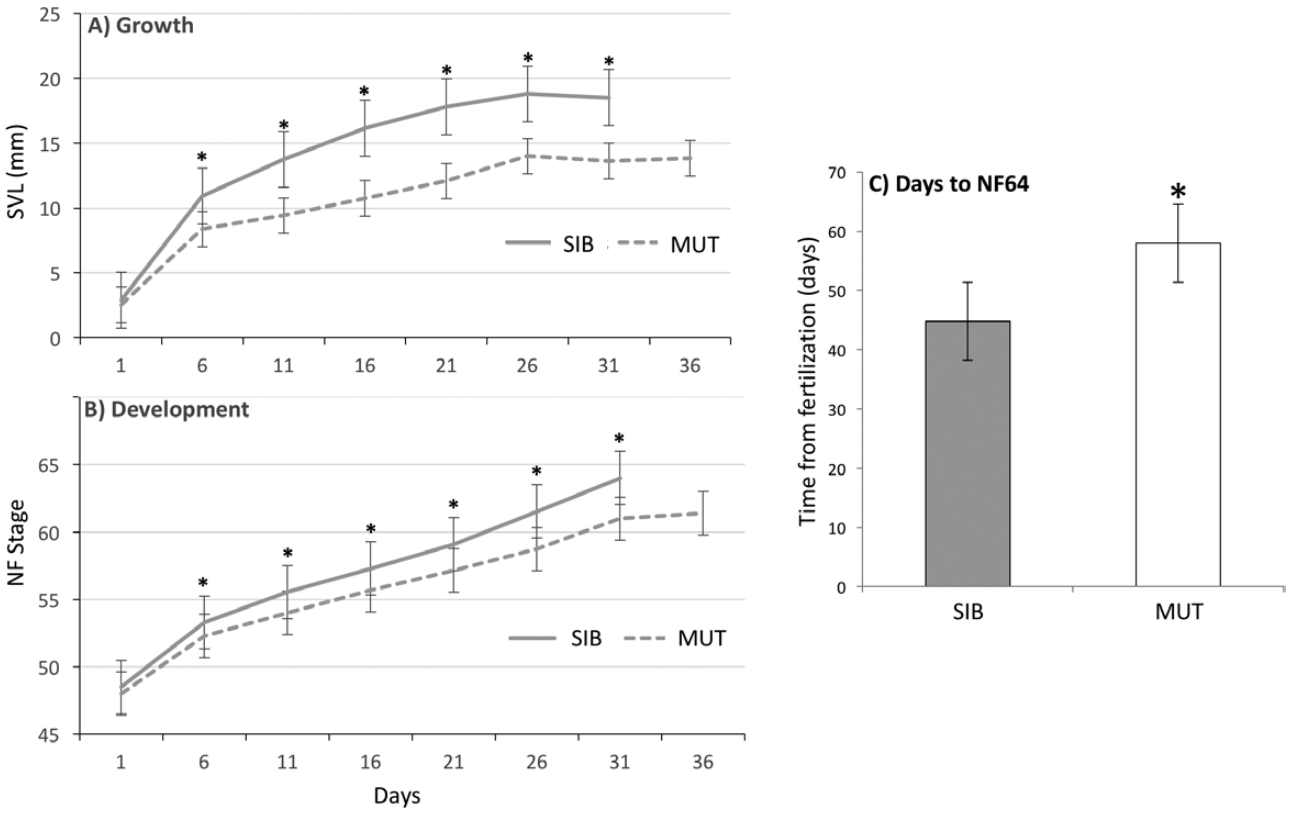
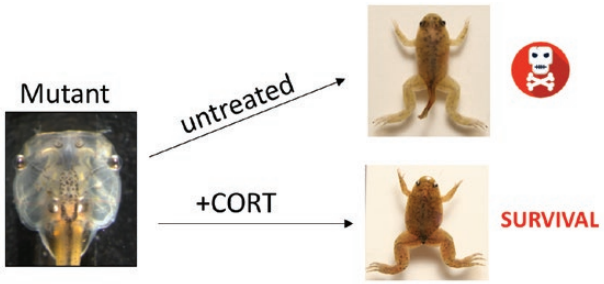


Figure 4. Delayed growth and development and death in mutant tadpoles. Mutant (MUT) and sibling (SIB) tadpoles were sorted based on head pigmentation 2 weeks after fertilization and were reared in 5 groups of 4 tadpoles each throughout the larval period. A, Tadpole size (snout-vent length, SVL) and B, Nieuwkoop and Faber (NF) stage were measured every 5 days. Death occurred at NF 64 in mutant tadpoles, so the experiment was stopped at this stage for SIB tadpoles as well. Asterisks denote significant differences at each time point (*t* test, $P < .05$, n = 5 samples of 4 tadpoles each). C, Progress to NF 64 was monitored daily and recorded for each tadpole. Asterisk denotes significant difference to NF 64 (*t* test, $P < .05$, n = 5 samples of 4 tadpoles each). Error bars indicate SE. This experiment was repeated with similar results.



NF STAGE	SAMPLE SIZE	CORT TREATMENT	SURVIVAL
NF 58	2	25nM	2/2
NF 60	3	25nM	3/3
NF 60	4	50nM	4/4
NF 62	2	25nM	1/4
NF 62	3	50nM	3/3

Figure 5. Rescue from death by exogenous corticosterone (CORT). A, Diagram depicts mutant tadpoles die at approximately Nieuwkoop and Faber (NF) stage 64 unless treated with exogenous CORT. B, Table of results shows survival of mutant tadpoles treated with 25 or 50 nM CORT starting at different developmental stages. Numbers represent survivors and total sample size for each treatment.

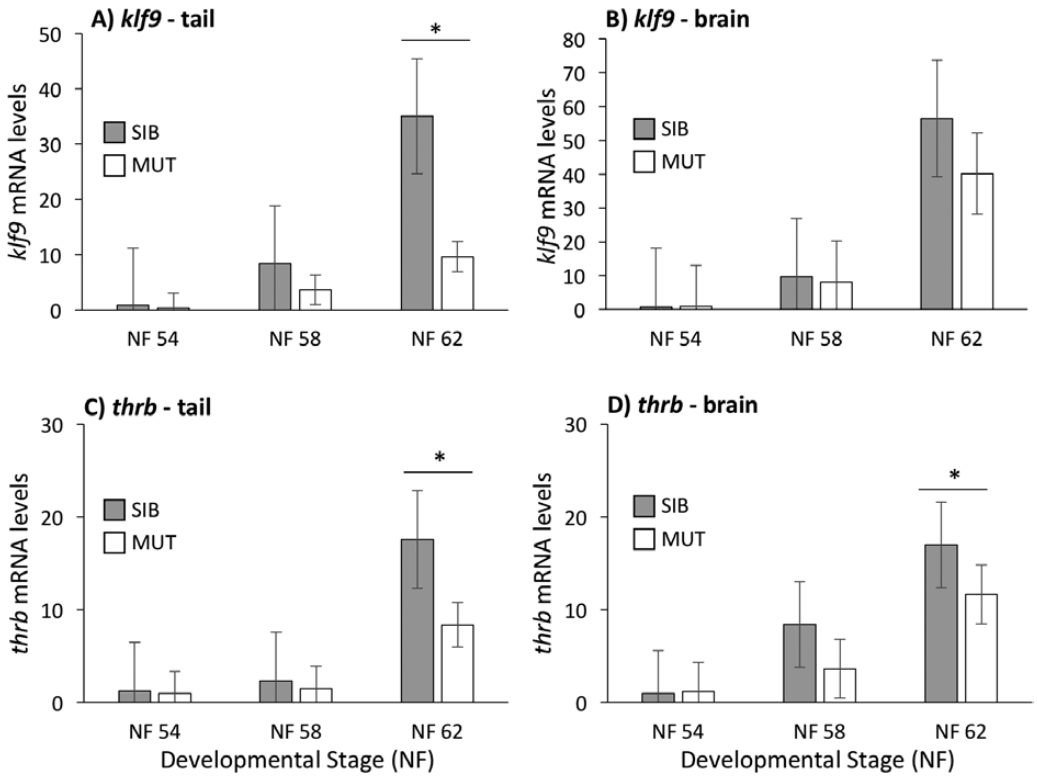


Figure 6. Reduced expression of thyroid hormone (TH) response genes during metamorphosis in mutants. A-D, Total RNA was collected from tails and brains of mutant (MUT) and sibling (SIB) tadpoles at Nieuwkoop and Faber (NF) stage 54, 58, and 62 (beginning, middle, and climax of metamorphosis) and analyzed for expression of *Krüppel-like factor 9* (*klf9*) and *TH receptor β* (*thrb*) messenger RNA (mRNA) expression. Bars represent mean mRNA levels relative to housekeeping gene *rpl8*. Error bars represent SE. Asterisks denote significant differences between bars at each stage (*t* test, $P < .05$, n = 5, each brain sample is a pool of 2 brains).

the expression differences were not significantly different until metamorphic climax, at which point mutants had reduced expression in tail and brain, except the lower expression level of *klf9* in brain was not statistically significant.

Effect of exogenous thyroid hormone on target gene induction

To compare TH tissue responsivity at the level of gene expression, we measured expression levels of *klf9* and *thrb* in tail tissues of pre-metamorphic (NF 54) mutant and sibling F2 tadpoles after 0, 2, or 10nM of T3 treatment for 24 hours (Fig. 7). Gene induction was significantly higher in control vs T3 treatment for both mutants and siblings, but the level of induction was significantly less in mutants at both T3 doses, indicating lower tissue responsivity in the *pomc* mutants.

Effect of exogenous thyroid hormone on morphology and survival through metamorphosis

To examine TH tissue responsivity at the morphological level, we treated NF 60 mutant and sibling F2 tadpoles individually and daily with 5 nM TH. Exogenous TH did not significantly accelerate development in sibling F2 tadpoles, but for mutant tadpoles, time to NF 62 and NF 64 decreased by 6 days (Fig. 8A and 8B). Importantly, exogenous T3 treatment was sufficient to enable survival through metamorphosis in mutant tadpoles. With continued T3 treatment, sibling F2 tadpoles survived for at least 2 weeks beyond complete tail resorption when the experiment ended, but mutant tadpoles died between 1 and 2 weeks after tail resorption.

Discussion

In the present study, we created and characterized *pomc*-mutant *X tropicalis* and revealed a vital role for CORT during amphibian metamorphosis. Specifically, we observed death during metamorphic climax just before complete tail resorption in F2 mutant tadpoles. Coincident with death, mutant tadpoles exhibited severely reduced CORT production and CORT response gene expression (*ush1g*) at metamorphic climax. Before metamorphic climax, the low CORT at NF 56 and the low *ush1g* expression at NF 54 both in wild-type and mutant tadpoles is consistent with previous studies showing similar CORT plasma levels from NF 54 to 58 and thus consistent with a relationship

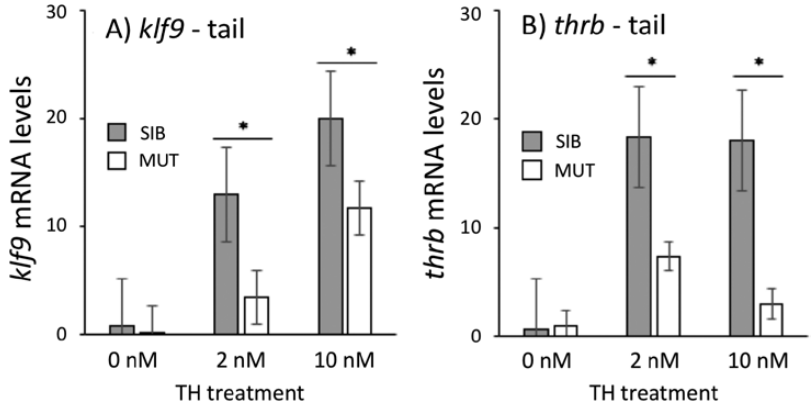


Figure 7. Impaired induction of thyroid hormone (TH) response genes by TH in mutants (MUTs). A and B, Total RNA was collected from tails of MUT and sibling (SIB) tadpoles at Nieuwkoop and Faber (NF) stage 54 (beginning of metamorphosis), treated with 0, 2, and 10 nM tri-iodothyronine for 24 hours, and analyzed for expression for *Krüppel-like factor 9* (*klf9*) and *TH receptor β* (*thrb*) messenger RNA (mRNA) expression. Bars represent mean mRNA levels relative to the housekeeping gene *rpl8*. Error bars represent SE. Asterisks denote significant differences between bars for each hormone treatment (*t* test, $P < 0.05$, n = 5).

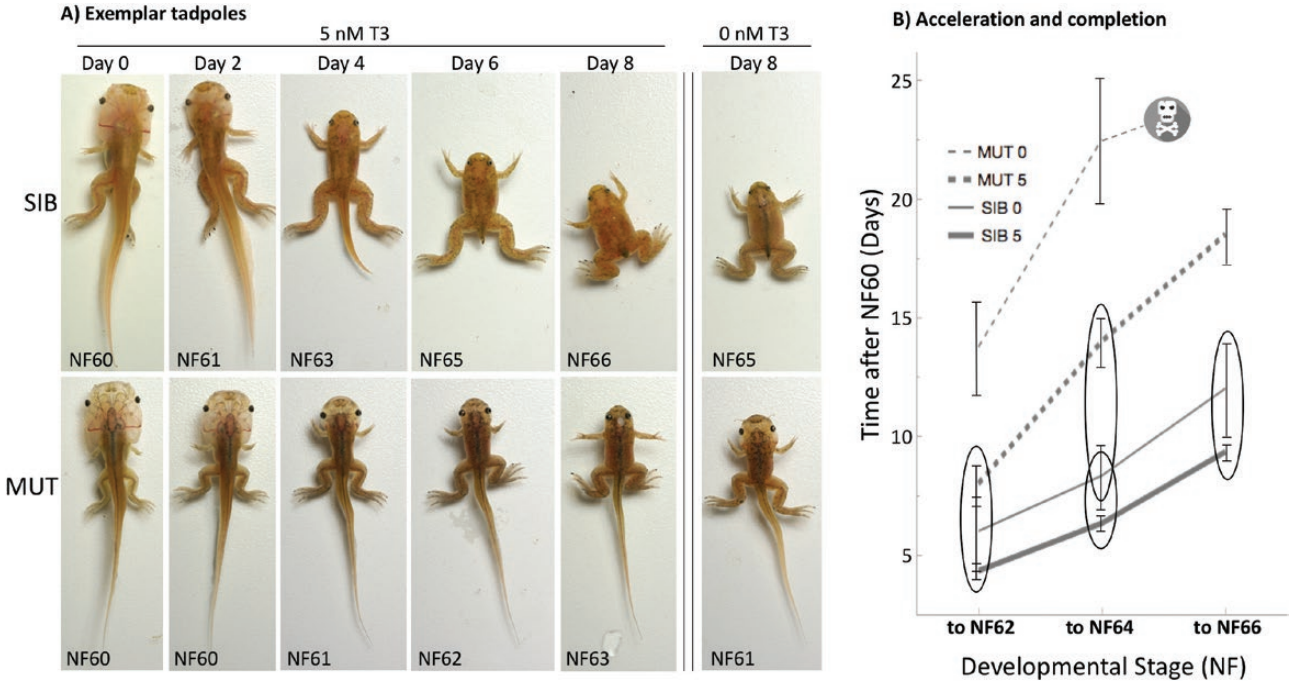


Figure 8. Developmental acceleration and survival through metamorphosis in mutant (MUT) tadpoles from exogenous thyroid hormone treatment. MUT and sibling (SIB) tadpoles were treated with 0 or 5 nM tri-iodothyronine (T3) beginning at Nieuwkoop and Faber (NF) stage 60. A, Exemplar tadpoles are shown after 0 to 8 days of treatment. NF stages are indicated. B, The number of days from NF 60 to NF 62, 64, and 66 was recorded for MUT (n = 5) and SIB (n = 3) tadpoles. Ovals surrounding data points indicate significance groups within a stage (Tukey pairwise post hoc test, $P < .05$). This experiment was repeated twice with similar results.

between low CORT levels and low *ush1g* expression. The basis for the lower *ush1g* levels in mutants at NF 58 is not clear because we did not measure CORT in mutants at NF 58. Even though ACTH, a pituitary peptide within *pomc*, is the primary stimulator of interrenal glands to produce CORT (6), the presence of CORT in *pomc* mutants may be explained by a baseline level of CORT production by the interrenals in the absence of ACTH stimulation and/or by the existence of other possible CORT secretogogues (53, 54). In any case, rescue from death using exogenous CORT showed that the greatly reduced level of CORT signaling was insufficient for survival through metamorphosis and was responsible for death rather than an effect from loss of one or more other *pomc* peptides.

Our experiments mainly compared blanched individuals vs their pigmented siblings (containing a mixture of tadpoles heterozygous and wild-type for *pomc* disruption). Sequencing analysis indicated a 100% correspondence

between the blanched phenotype and biallelic frame-shift mutations and between the pigmented phenotype and at least one wild-type allele. The potential problems with this approach are unlikely to affect the conclusions from our results. First, it is possible that heterozygotes differ from wild-type individuals. However, in our studies on CORT levels, we found pigmented F2 tadpoles from *pomc*-mutant founders and tadpoles from wild-type parents had the same CORT levels at climax, about 4 times higher than the climax CORT levels seen in blanched mutants. For this and other phenotypes, the expected effect of using a mixture of heterozygous and wild-type tadpoles would be an increase in the variation manifest as bigger error bars in the sibling measurement, which did not preclude the observed significant differences between mutant and sibling tadpoles. Second, our use of founders opens the possibility of an in-frame mutation allowing a blanched individual to have ACTH activity or a pigmented individual to lack ACTH activity. This possibility is unlikely because peptide structure/function studies suggest that any out-of-frame or in-frame mutation would unlikely affect ACTH and not α-MSH or vice versa, though not all amino acid substitutions in α-MSH and ACTH have been functionally evaluated (44, 45). In any case, we found only out-of-frame mutations after sequencing many alleles in offspring from 5 founders and these same 5 founders were used to obtain tadpoles for all experiments. Also, we did not observe a measurement from a blanched individual, suggesting it may have functional ACTH activity or vice versa.

The observed increased fat body weight in *pomc* mutants is consistent with previous studies. During normal development in tadpoles, the amount of whole-body lipids is low during premetamorphosis, rises during prometamorphosis, and declines at metamorphic climax (55-57). Experimental treatment with ACTH and CORT showed catabolic effects on whole-body lipid content (52, 58). However, we cannot rule out that altered CORT levels by themselves or through altered feedback on corticotropin-releasing hormone (CRH) may have affected fat content indirectly via effects on feeding and appetite. CRH has an inhibitory effect on foraging in tadpoles, whereas short-term exogenous CORT treatment stimulates feeding (59). The *pomc* mutants have low CORT levels and are expected to have high CRH levels, resulting in inconclusive expectations for an effect on appetite in relation to lipid content in the mutant tadpoles.

For metamorphic development, we found that TH-dependent development occurred more slowly in *pomc* mutants. CORT signaling is known to increase tissue responsivity to TH by regulating deiodinases to increase the active form of TH inside the cell and by inducing *klf9*, which helps induce TH receptor expression (21, 25). Thus, the low CORT in *pomc* mutants is expected to result in lower TH responsivity in peripheral tissues and thus slower metamorphic development when TH is available. In support for this effect of CORT on TH signaling, we observed reduced responsivity to TH, wherein mutants had impaired TH response gene induction and slower TH-induced morphological development in response to exogenous TH compared to sibling F2 tadpoles. Interestingly, it is well known that high CORT levels, from stressful rearing conditions, can also retard TH-dependent development in young tadpoles (17). The mechanism is not clear but may involve negative feedback on the hypothalamus and pituitary to reduce TH production rather than through some effect on peripheral tissue responsivity to TH. Importantly, this effect of high CORT to slow development in young tadpoles changes to an acceleratory effect in metamorphosing tadpoles through CORT's actions to increase TH signaling (17). The observations in *pomc* mutants with low CORT to slow the rate of metamorphic progression is complementary to the previously known effects of high CORT levels to increase the rate of development after the initiation of metamorphosis.

The most striking finding in *pomc* mutants was death at midtail resorption just after metamorphic climax. Some sufficient amount of TH signaling is required for completion of metamorphic development, as indicated by recent studies on TH receptor–mutant tadpoles (60-63). In *pomc* mutants, if reduced TH signaling was somehow related to death at metamorphosis, then restoring sufficient TH signaling with exogenous TH should allow metamorphic completion. Indeed, *pomc* mutants treated with exogenous TH achieved complete tail resorption, suggesting lack of CORT action to increase TH responsivity prevented completion of metamorphosis.

The ability to rescue *pomc* mutants with just TH does not rule out a vital CORT-dependent action independent of TH. The baseline level of plasma CORT in *pomc* mutants could be sufficient for some unknown vital CORT action, for example, lung maturation, but not enough CORT signaling to provide sufficient TH responsivity. Interestingly, mutant tadpoles lacking the GR (GRKO) die around metamorphic climax (NF 61-62) (31). The GRKO tadpoles may also lack sufficient TH signaling because of decreased TH responsivity as suggested here for *pomc* mutants, or the lethality in GRKO may be due to an essential developmental action of CORT independent of TH. A definitive demonstration of a vital requirement for CORT not related to TH during metamorphosis will require further study.

Some distinct differences between *pomc* mutants and GRKO tadpoles are not currently understood. First, *pomc* mutants die a couple stages later than GRKO (NF 64 vs NF 61-62) perhaps from the baseline amount of CORT

present at metamorphic climax in *pomc* mutants allowing slightly more TH and/or CORT signaling and thus more metamorphic progress. Second, *pomc* mutants have impaired growth whereas GRKO do not. The effects of CORT on growth may be relayed by the wild-type mineralocorticoid receptor in GRKO mutants, but the low CORT levels in *pomc* mutants would have low signaling both through GRKO and mineralocorticoid receptors. Third, *pomc* mutants develop more slowly than wild-type throughout the larval period, but GRKO tadpoles initially develop faster and then slow down close to metamorphic climax compared to wild-type. This delayed development in *pomc* mutants may be due to impaired lipid metabolism impairing growth, which may alter developmental timing, but mechanistic relationships between metabolism, growth, and development are not well understood (but see [64, 65]).

A consensus from *pomc*, GR, and TH receptor knockout mutants is that insufficient TH signaling disallows completion of metamorphosis, but it remains to be determined what specifically leads to death. It is important to point out that all TH-dependent developmental events prior to gill resorption occur in all these mutants. That is, enough TH signaling, either by derepression (as in TH receptor knockouts) or by TH response gene induction in tissues highly responsive to TH (not requiring CORT-induced TH responsivity) (as in *pomc* and possibly GRKOs), allows limbs, skin, and skeletal remodeling to occur. Other tissues fail to undergo transformation, for example, gill and tail resorption. Some vital tissue(s) may rely on a sufficiently high amount of TH signaling to transform, allowing metamorphosis to complete before TH levels return to juvenile baseline, or some disharmonious coexistence of larval and adult organs may be incompatible with life. Alternatively, a delay or stall in metamorphosis is unlikely by itself the cause of death, because, as seen in TH receptor knockout tadpoles, they stall in development at NF 61 for 1 to 2 weeks before dying. Similarly, *pomc* mutants stall for 3 to 4 days at NF 64 before dying. In both cases, because all tadpoles stop eating around NF 60, total time after cessation of feeding until death would be significant and may explain death. In the case of *pomc* mutants, low CORT is the cause of delayed development and some possible developmental dysfunction and thus is related to the cause of death. Thus, we conclude that CORT is essential for survival through metamorphosis. Further study will be required to understand the basis of lethality in mutant tadpoles blocked from completing metamorphosis.

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

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