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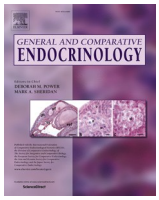

Research paper

Glucocorticoid receptor mediates corticosterone-thyroid hormone synergy essential for metamorphosis in *Xenopus tropicalis* tadpoles

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ABSTRACT

In all vertebrates, thyroid hormone (TH) is critical for normal growth and development. In amphibians, corticosterone (CORT) has no action to advance development by itself but can accelerate development induced by TH. CORT accomplishes this acceleration by increasing tissue sensitivity and responsivity to TH. However, the receptor through which CORT acts to affect TH signaling is not known. To examine the role of the glucocorticoid receptor (GR), GR knockout tadpoles and wild-type tadpoles treated with the GR antagonist, RU486, were exposed to exogenous TH and/or CORT then assayed for gene expression and morphology. We found that levels of the response genes *klf9* and *thrb* induced by TH and associated changes in morphology were decreased in GR knockout tadpoles compared to wild-type tadpoles, suggesting that GR signaling contributes to tissue responsivity to TH. To directly examine the role of GR in TH signaling, we co-treated tadpoles with TH and CORT and found that the TH response gene, *thrb*, was induced significantly beyond the level induced by TH alone in wild-type tadpoles but not in GR knockout tadpoles or wild-type tadpoles treated with RU486. Similarly, tail and gill resorption was greater in tadpoles treated with CORT plus TH compared to TH alone in wild-type tadpoles but not in tadpoles with impaired GR signaling. Surprisingly, even though GR knockout tadpoles die at metamorphosis, treatment with TH alone enabled their survival. These results demonstrate that signaling through GR is responsible for enhancing TH signaling and is essential for the completion of metamorphosis.

1. Introduction

Two hormones predominantly control the timing of growth and development in amphibians, thyroid hormone (TH) and corticosterone (CORT) (Denver, Glennemeier, and Boorse, 2002; Sachs and Buchholz, 2019). These hormones act through nuclear receptors, acting as ligand-activated transcription factors, to accomplish their specific actions. For TH, those nuclear receptors are TH receptors alpha and beta (Denver, Glennemeier, and Boorse, 2002). CORT can act also through two receptors, Type-I (mineralocorticoid receptor-MR) and Type-II (glucocorticoid receptor-GR) (Sapolsky et al., 2000). Certain chemicals/pesticides have been shown to have antagonistic actions on MR and GR, which might affect progression of amphibian metamorphosis (Heimeier and Shi, 2010; Carr and Patiño, 2011; Zhang, et al., 2019; Sterner et al., 2020).

Our current understanding of the role of TH in amphibian metamorphosis is that TH is necessary and sufficient to initiate the developmental events of metamorphosis and the level of TH signaling regulates the rate of developmental progression (Allen, 1938; Dodd and Dodd, 1976; Shi, 1999), whereas our current understanding for the role of CORT in metamorphosis is to modulate the level of TH signaling (Kikuyama et al., 1993;Denver, Glennemeier, and Boorse, 2002; Das et al., 2010; Kulkarni and Buchholz, 2014). In tadpoles, CORT and TH levels are low during premetamorphosis, rise until they peak at metamorphic climax (NF 62, Nieuwkoop and Faber stage 62) (Nieuwkoop and Faber, 1994), and then decrease to the adult level (Leloup and Buscaglia, 1977; Jaudet and Hatey, 1984). Exogenous CORT treatment during early development inhibits growth and development (Hayes et al., 1993; Glennemeier and Denver, 2002), while exogenous TH treatment induces precocious metamorphosis and accelerated

Abbreviations: ACTH, adrenocorticotropic hormone; ACTHKO, adrenocorticotropic hormone knockout; cDNA, complementary DNA; CORT, corticosterone; GR, glucocorticoid receptor; GRKO, glucocorticoid receptor knockout; klf9, Krüppel-like factor 9; MR, mineralocorticoid receptor; NF, Nieuwkoop and Faber; PCR, polymerase chain reaction; qPCR, quantitative PCR; rpl8, ribosomal protein L8; RU486, mifepristone-GR antagonist; T3, triiodothyronine; TH, thyroid hormone; thrb, TH receptor β; WT, wild-type.

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development (Etkin, 1935; Etkin, 1965). Conversely, treatment with the GR antagonist, mifepristone (RU486) in the rearing water throughout larval development caused a 4-day delay to achieve tail resorption (Rollins-Smith et al., 1997). CORT treatment in combination with TH accelerates developmental changes beyond the increased rate achieved by TH alone, which has been observed via gene expression and accelerated tail resorption (Bonett et al., 2010). Similarly, stressful tadpole living conditions, such as crowding or low water, induce a stress response resulting in accelerated metamorphosis via increased CORT and TH plasma levels (Denver, 1997; Gomez-Mestre et al., 2013). This acceleration in developmental processes, such as tail resorption, are examples of synergy, where the two hormones induce changes together greater than the sum of changes when present alone.

This CORT / TH synergy is due to CORT increasing tissue sensitivity and responsivity to TH. CORT increases the activity of deiodinase type 2 and decreases activity of deiodinase type 3, thereby increasing the intracellular concentration of the active form of TH (Galton, 1990). Also, the transcription factor KLF9 is regulated via an enhancer element called the "synergy module", where CORT and TH hormone response elements allow each hormone to induce *klf9* individually and also synergistically when both hormones are present (Bagamasbad et al., 2015). Among the genes regulated by KLF9 is TH receptor beta, whose expression levels govern tissue sensitivity / responsivity to TH. Together, these effects of CORT increase intracellular levels of active TH and expression levels of TH receptor to result in increased tissue sensitivity and responsivity of tissues to TH levels in the blood.

To examine the mechanism of synergy further, we used two models of impaired signaling through GR to determine its role in CORT synergy with TH. Signaling through GR was blocked by using glucocorticoid receptor knockout (GRKO) tadpoles and tadpoles treated with a GR antagonist, RU486. GR is likely a main player in the synergy of CORT and TH because CORT acts through GR and not MR in *klf9* induction (Shewade et al., 2017) and GR knockout animals die at metamorphosis because of a possible reduction in TH signaling from reduced CORT/ TH synergy (Sterner et al., 2020).

2. Materials and methods

2.1. Animal husbandry

Glucocorticoid receptor heterozygous mutants (Sterner et al., 2020) and wild-type (WT) male and female adult *Xenopus tropicalis* from the lab colony were mated by priming with 20U of ovine luteinizing hormone (National Hormone and Peptide Program, Torrance, CA) in the evening and boosting with 200U the following morning. Tadpoles were reared at 26 degrees C, fed twice daily with powdered food (Sera Micron), and water was changed daily. Genotypes of tadpoles from GR heterozygous crosses were determined by heteroduplex mobility assay as described (Sterner et al., 2020). Animal use in experiments was in accordance with the University of Cincinnati Institutional Animal Care and Use Committee (IACUC protocol # 06-10-03-01).

2.2. Thyroid hormone survival treatments

Beginning at NF 60, WT and GRKO tadpoles were treated with 0 nM tri-iodothyronine (T3) (Sigma St. Louis, MO), 10 nM T3, or 20 nM T3, n = 6 for each genotype and treatment. This stage was chosen because GRKO tadpoles achieve this stage without assistance but die one or two stage later. Treatment before this stage may dysregulate development, and treatment after this stage may be too late to prevent death. Water and hormone changes occurred daily until 2 weeks after reaching NF 66. The number of days required to reach Nieuwkoop and Faber (NF) stage 62 (gill resorption), NF 64 (tail ~ 5–10 mm), and NF 66 (completion of tail resorption) were recorded. Survival to NF 66, 1-week past NF 66, and 2-weeks past NF 66 were also recorded.

2.3. Corticosterone and thyroid hormone treatments for gene expression

At NF 54, WT, GRKO, and WT treated with 150 nM RU486 (Sigma, St. Louis, MO) were treated with vehicle (ethanol), 100 nM CORT (Sigma, St. Louis, MO), 2 nM T3, and 100 nM CORT plus 2 nM T3. After 24 h, tails and livers from MS222-anesthetized tadpoles were harvested and snap frozen on dry ice. n = 12 per genotype and treatment.

2.4. Gene expression

RNA extraction was performed using TRI REAGENT RT following the manufacturer's instructions (Molecular Research Center, Inc.). Complementary DNA synthesis was performed from 1 μg RNA for each sample using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). qPCR with 1 μL cDNA in 20 μL reaction volume was carried out using Luminaris Color HiGreen qPCR Master Mix high ROX (Thermo Scientific) and SYBR primer sets on the 7300 Real Time PCR System (Applied Biosystems) for *klf9*, *thrb*, and *rpl8* (Dhorne-Pollet et al., 2013). SYBR primer sequences were the following: ribosomal protein L8 (*rpl8*) (forward: 5′-CCACAATCCTGAAACAAAGAAA, reverse: 5′-CCTTGTATTTATGGTATGCACG), Krüppel-like factor 9 (*klf9*) (forward: 5′-TAAAGCCCATTACAGAGTCCAT, reverse: 5′-CACTCCTCATGAACCTCTTCTC), and thyroid hormone receptor β (*thrb*) (forward: 5′-CATTGGACAAGCTCCCATAGTTA, reverse: 5′-AGCCTTTAAGAAGGATGATCTGG). Gene expression was normalized to *rpl8* using the △△Ct method (Livak and Schmittgen, 2001).

2.5. In-vivo corticosterone and thyroid hormone treatments

WT, GRKO, or WT treated with 150 nM RU486 were treated with vehicle (ethanol), 100 nM CORT, 2 nM T3, and 100 nM CORT plus 2 nM T3 for 6 days. Water and hormone changes occurred daily. Measurements of NF stage, head width, tail length, and limb length occurred on days 0 and 6. Animals were visualized utilizing the Leica S4E stereomicroscope. Pictures of animals were taken by placing a Samsung Galaxy S9 up to the ocular lens. Tail and gill images were taken at 0.63x. Hindlimb images were taken at 2.5x for WT and GRKO and 1.25x for WT and WT + RU486 experiment. Pictures were imported into Microsoft PowerPoint and adjusted to 4.5″ (Height) × 6″ (Width) and then cropped. Tails were uploaded to GIMP (v 2.10.24), background was removed to only include the tail (beginning at the base of the tail to the filamentous tip), and then tails were exported as PNG files. Change in head width (gill resorption) and change in tail length (tail resorption) were calculated using values from day 0 and subtracting day 6 values from those starting values. Change in hindlimb length (hindlimb growth) and change in NF stage were calculated using values from day 0 and subtracting values from day 6

2.6. Statistical analysis

Two-way analysis of variance (ANOVA) with genotype or RU486 treatment, CORT or T3 treatments, or stages as factors was performed with base R (R Core Team, 2018) and Tukey's honest significant difference test (α = 0.05). Two-way ANOVA was also performed using genotype or RU486 treatment and morphological measurements (head width, limb length, tail length, and stage) as factors. Following this, Tukey's honest significant difference test was done (α = 0.05).

3. Results

3.1. Dose response to thyroid hormone

To identify a submaximal dose of TH that may distinguish between WT and GRKO animals in proceeding synergy experiments, WT and GRKO animals were treated with 0, 2, and 10 nM T3 and tails were harvested. GRKO demonstrated no significant response to 2 nM or 10

nM T3 for *klf9*, and the higher dose was required for significant induction of *thrb* (Fig. 1). WT individuals exhibited significant upregulation above vehicle after 2 nM for both genes, and 10 nM treatment demonstrated significant upregulation above the 2 nM T3 treatment for *thrb* (Fig. 1A,B).

3.2. Thyroid hormone treatments enable survival in GRKO

During natural metamorphic progression, GRKO tadpoles have reduced expression of TH response genes (*thrb* and *klf9*), observed in the tail, indicating reduced TH signaling, and die around metamorphic climax (NF 60–62) (Sterner et al., 2020). Even though 10 nM T3 was not sufficient to increase response gene induction in GRKO to match WT (Fig. 1A,B), 10 nM T3 is the maximum endogenous plasma TH levels during climax of metamorphosis (Leloup and Buscaglia, 1977). Therefore, we tested 10 and 20 nM TH to augment the endogenous amount of TH signaling present in GRKO to determine if insufficient TH signaling may account for death of GRKO tadpoles during metamorphosis. Treatments with TH were followed by recording time to easily identifiable developmental time points (NF stage) and survival 2-weeks past NF 66. All WT individuals treated with and without TH reached NF 66 and survived 2 weeks after NF 66 with no significant effect on developmental timing (Fig. 1C). Exogenous TH treatment (10 nM and 20 nM T3) in GRKO increased the rate of development, though still slower than WT for the later developmental time points and allowed for completion of metamorphosis. However, 50% mortality occurred within the first week after tail resorption and about 70% during the second week in TH-treated GRKO tadpoles (Table 1).

Table 1
Metamorphic Survival After Thyroid Hormone Treatments in WT and GRKO Tadpoles. WT and GRKO tadpoles at NF 60 were treated with different concentrations of T3 (10 nM, 20 nM). Survival to NF 66, 1-week past NF 66, and 2-weeks past NF 66 were recorded. Numbers represent number of tadpoles that reached certain time points. n = 6. WT = wild-type, GRKO = glucocorticoid receptor knockout, TH = thyroid hormone.

Genotype	T3 Treatment	NF 66	1-week past NF 66	2-weeks past NF 66
WT	Untreated	6/6	6/6	6/6
WT	10 mM	6/6	6/6	6/6
WT	20 mM	5/6	5/6	5/6
GRKO	Untreated	0/6	0/6	0/6
GRKO	10 mM	5/6	4/6	3/6
GRKO	20 mM	4/6	2/6	1/6

3.3. Expression of thyroid hormone response genes after corticosterone and thyroid hormone treatment in the tail of WT and GRKO tadpoles

The survival of GRKO tadpoles with T3 treatment indicated that the level of TH signaling in GRKO tadpoles was not sufficient for completion of metamorphosis, suggesting that CORT-dependent synergy with TH requires GR. To directly establish a role for GR in CORT and T3 synergy at the level of gene expression, WT and GRKO tadpoles were treated with vehicle, CORT, T3, and CORT + T3. Expression of two TH response genes (*klf9* and *thrb*) was examined in the tail (Fig. 2 A,B). For *klf9*, WT individuals demonstrated a significant increase in expression after 100 nM CORT, 2 nM T3, and 100 nM CORT and 2 nM T3, where the combined treatment also had significant induction above the single hormone treatments (Fig. 2A). Similar results were observed for *thrb* in WT, except CORT alone did not induce *thrb* (Fig. 2B). To distinguish between additive and synergistic components for co-hormone inductions that were significantly higher than the corresponding single hormone inductions, the portion of the induction in co-hormone treatment that was above the

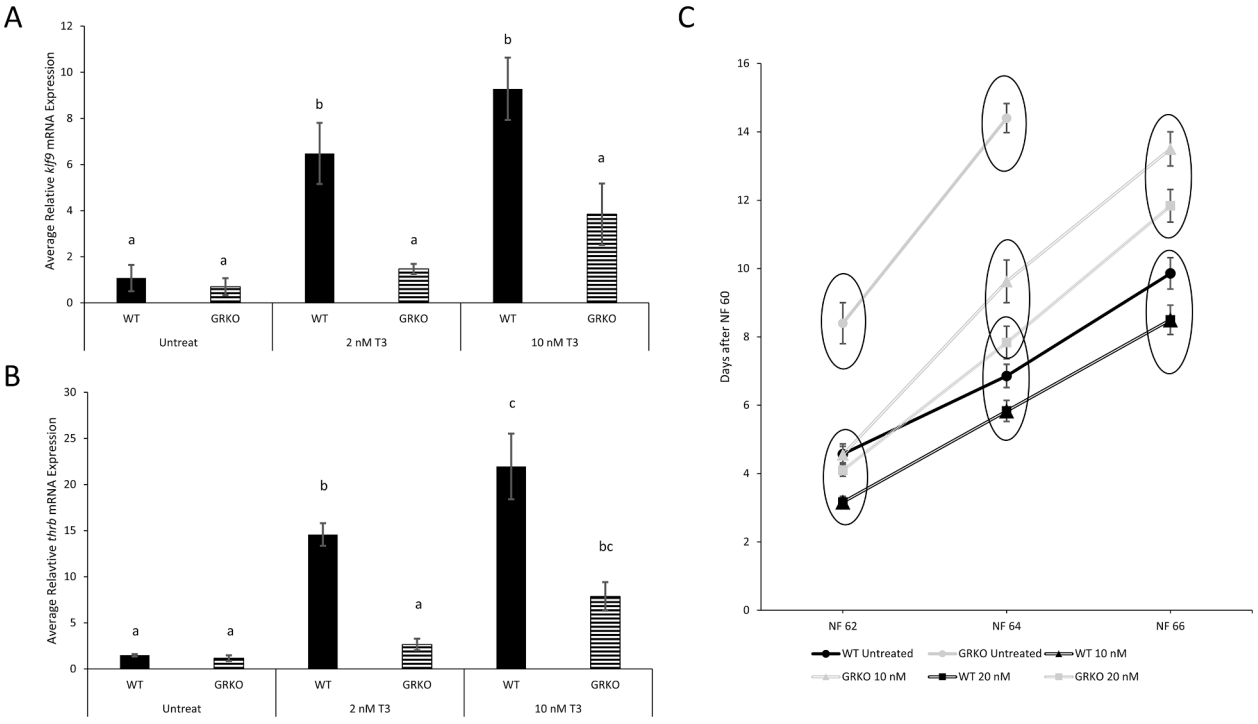

Fig. 1. GRKO tadpoles have reduced TH tissue responsivity and survive metamorphosis if provided T3. T3 induction levels of (A) *klf9* and (B) *thrb* expression in tail are reduced in GRKO tadpoles. WT and GRKO tadpoles at NF 54 were treated for 24 h, and then tails were harvested to perform gene expression analysis, n = 5. (C) Exogenous T3 increased the rate of metamorphosis in GRKO and allowed them to survive through tail resorption. WT and GRKO tadpoles at NF 60 were treated daily with 10 nM and 20 nM T3, and days to NF 62, 64, and 66 were recorded. n = 6. Letters represent significance groups among all treatments within a panel, and circles represent significance groups within each treatment. Error bars represent standard error of the mean. WT = wild-type, GRKO = glucocorticoid receptor knockout, T3 = active form of TH.

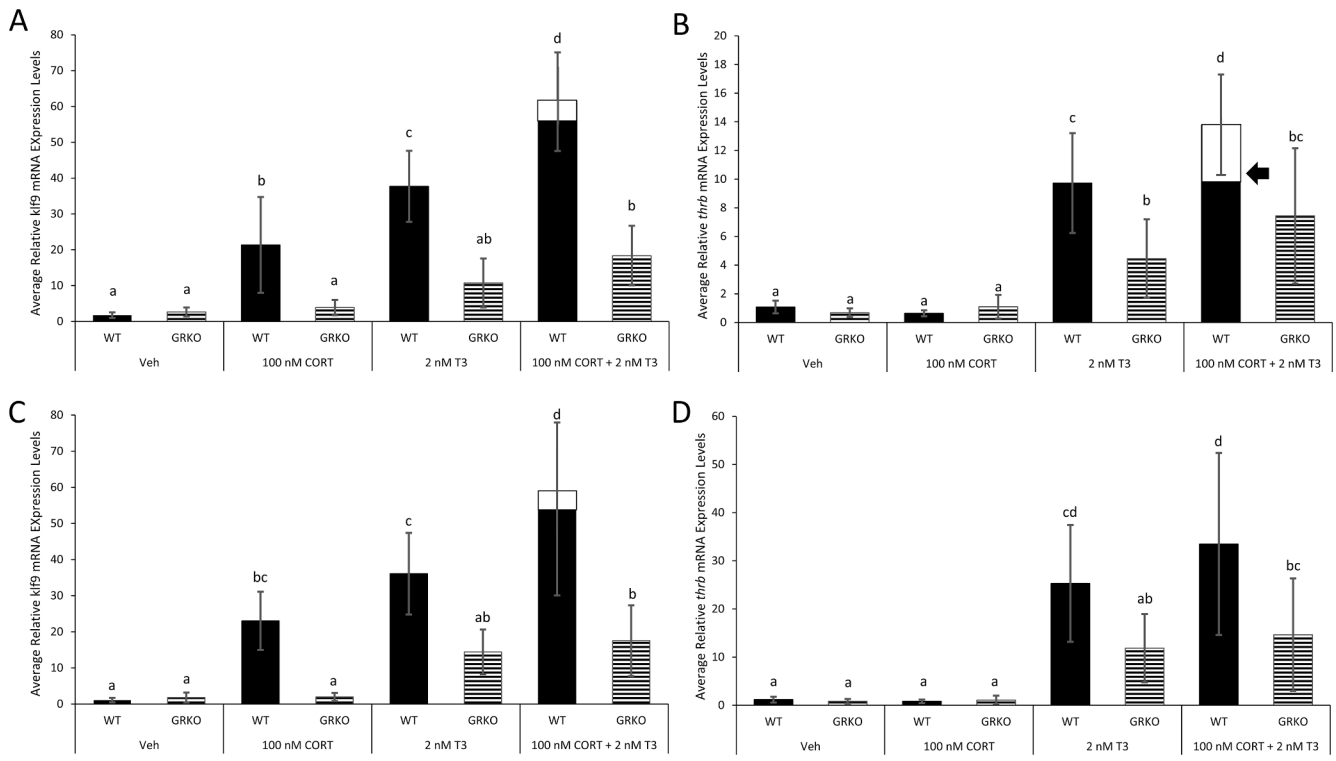

Fig. 2. GRKO tadpoles exhibit lack of CORT/TH synergy for *thrb* expression in the tail. WT and GRKO tadpoles at NF 54 were treated for 24 h with vehicle, 100 nM CORT, 2 nM T3, and 100 nM CORT plus 2 nM T3, and then gene expression was examined for (A) *klf9* in tail, (B) *thrb* in tail, (C) *klf9* in liver, and (D) *thrb* in liver. White portions of treatment bars for hormone co-treatments significantly higher than in T3 alone represent expression above additive response estimated from T3 and CORT responses singly. Black arrow indicates a synergistic response, defined as greater than one standard deviation above the estimated additive portion of the response. Error bars represent standard deviation (SD). Letters represent significance groups among all treatments within each panel. n = 12, WT = wild-type, GRKO = glucocorticoid receptor knockout, Veh = vehicle (ethanol treatment), CORT = corticosterone, T3 = active form of TH.

sum of the inductions above vehicle baseline observed in the single hormone treatments is shown by the white portions of the bars. We reasoned, as previously (Bonett et al., 2010), that the co-treatment induction level represented synergy when it was more than one standard deviation greater than the estimated additive response (i.e., the error bar, which equals one standard deviation, is completely contained within the white portions of the bar, see Fig. 2B, black arrow). For GRKO, the induction levels of *klf9* and *thrb* were always less than those observed in WT, and CORT had no effect by itself as seen previously (Fig. 2A,B) (Sterner et al., 2020). Importantly, the level of induction after T3 + CORT was not significantly different from T3 alone, indicating no synergy was observed in the absence GR for either gene (Fig. 2B).

3.4. Expression of thyroid hormone response genes after corticosterone and thyroid hormone in the liver of WT and GRKO tadpoles

To determine if we could observe synergy at the gene expression level in liver as seen in tail, livers were harvested from the same tadpoles and analyzed similarly (Fig. 2 C,D). For *klf9* and *thrb*, the level of inductions in WT after single and co-hormone treatments followed nearly the same pattern as seen in tail, except there was no synergy as defined herein, namely there lacked an increase in one standard deviation of the co-treatment above the estimated additive level. The results were similar for GRKO livers as was found in for GRKO in the tail, i.e., reduced induction levels compared to WT and no synergy.

3.5. Expression of thyroid hormone response genes after corticosterone and thyroid hormone treatment in the tail and liver of WT and RU486-treated WT tadpoles

To validate previous findings from GRKO individuals, WT individuals were treated with RU486, a GR antagonist, and gene expression of two TH response genes (*thrb* and *klf9*) was analyzed (Fig. 3). The results for both genes in both tail and liver in WT vs RU486-treated animals are nearly parallel with results from WT vs GRKO animals. CORT induced *kfl9* only in WT controls showing that the RU486 dose was effective at blocking signaling through GR (Fig. 3A, B) (Shewade et al., 2017). TH induced *kfl9* in tail and liver, but unlike in GRKO, TH-induced *kfl9* levels were not significantly different between WT and RU486, perhaps indicating a difference in TH sensitivity between chronic loss of GR signaling in GRKO and acute loss in RU486-treated tadpoles. Liver co-treatment showed high variability in WT co-treatment, and only in WT tail was expression from the co-hormone treatment significantly higher than the individual hormones (Fig. 3A, C) As before, the increase from co-treatment over single hormones in tail was not one standard deviation above the estimated additive level of induction for *klf9*, and thus we conclude that we did not observe synergy for this gene (Fig. 3A, error bar larger than white portion of bar). For *thrb*, TH and not CORT induced *thrb* in tail and liver to a similar level for WT and RU486-treated tadpoles, and only in tail was expression from co-treatment higher than the individual hormones (Fig. 3B, D), which did meet our criterion for synergy (Fig. 3B, black arrow).

3.6. Morphological change after in vivo corticosterone and thyroid hormone treatments

To examine the role of GR in synergy between CORT and TH at the

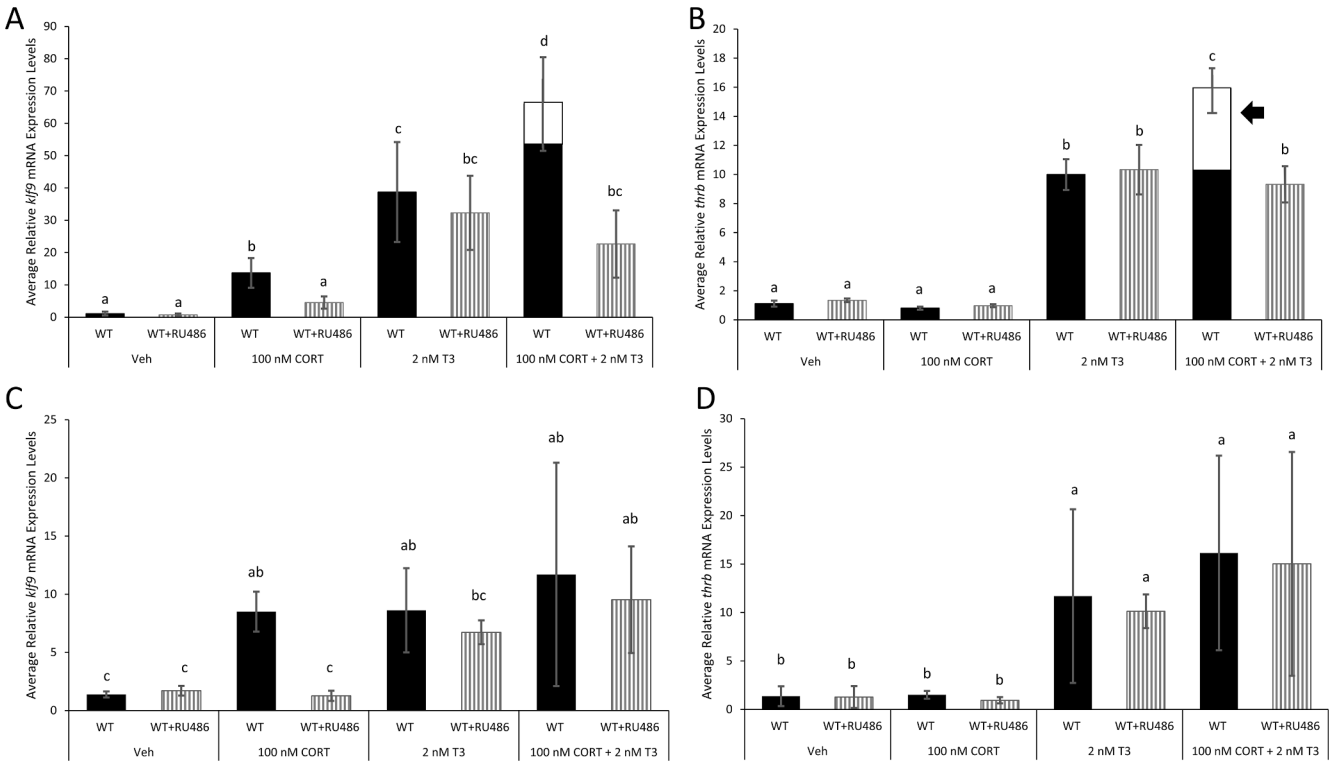

Fig. 3. Wild-type tadpoles treated with RU486 exhibit lack of CORT/TH synergy for *thrb* expression in tail. WT and RU486-treated tadpoles at NF 54 were treated with vehicle, 100 nM CORT, 2 nM T3, and 100 nM CORT plus 2 nM T3, and then gene expression was examined for (A) *klf9* in tail, (B) *thrb* in tail, (C) *klf9* in liver, and (D) *thrb* in liver. White portions of treatment bars for hormone co-treatments significantly higher than in T3 alone represent expression above additive response estimated from T3 and CORT responses singly. Black arrow indicates a synergistic response, defined as greater than one standard deviation above the estimated additive portion of the response. Error bars represent standard deviation (SD). Letters represent significance groups among all treatments within each panel. n = 8, WT = wild-type, GRKO = glucocorticoid receptor knockout, Veh = vehicle (ethanol treatment), CORT = corticosterone, T3 = active form of TH.

level of morphology, tadpoles were subjected to the same hormone treatments as for gene expression but were examined after 6 days (Fig. 4 with exemplar images and Fig. 5 with quantification). As expected, vehicle and CORT treatment were not different in tail length, gill size, NF stage, or hindlimb length when comparing day 0 and day 6 within and across these two treatments for both WT and GRKO (not shown and

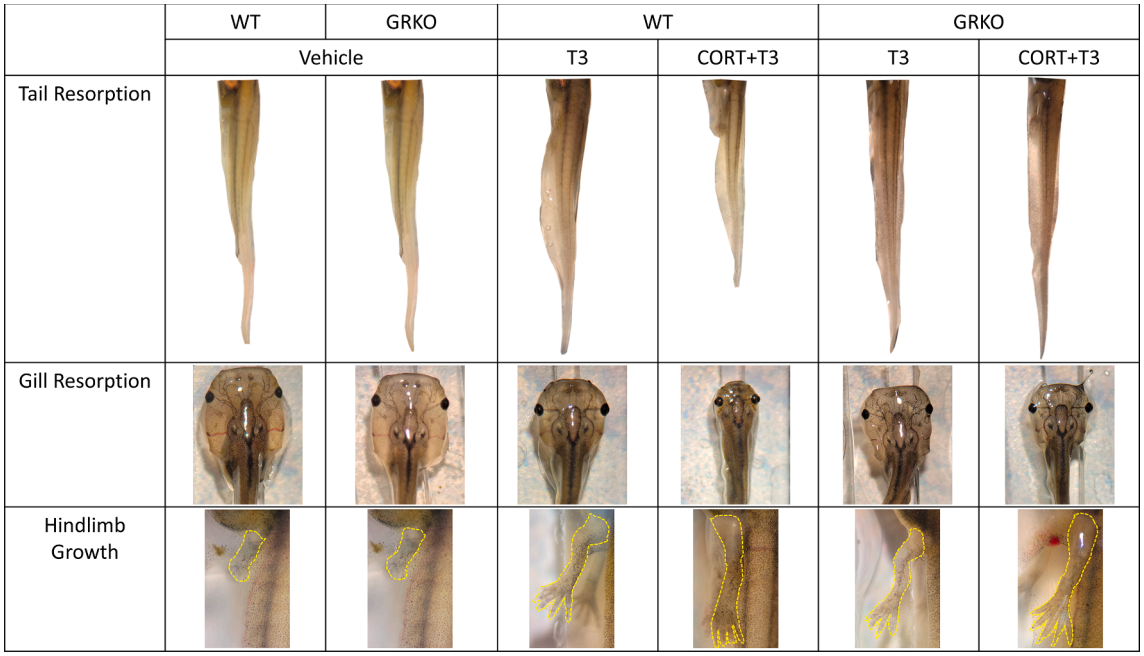

Fig. 4. Exemplar images of WT and GRKO tadpoles treated with or without T3 and/or CORT. Representative images of WT and GRKO tadpoles treated for 6 days with vehicle, 2 nM T3, and 100 nM CORT plus 2 nM T3 are shown. Note the stronger effect of T3 + CORT compared to T3 alone in tail and gill in wild-type (WT) and not GRKO tadpoles. n = 5, Veh = vehicle (ethanol treatment), T3 = active form of TH.

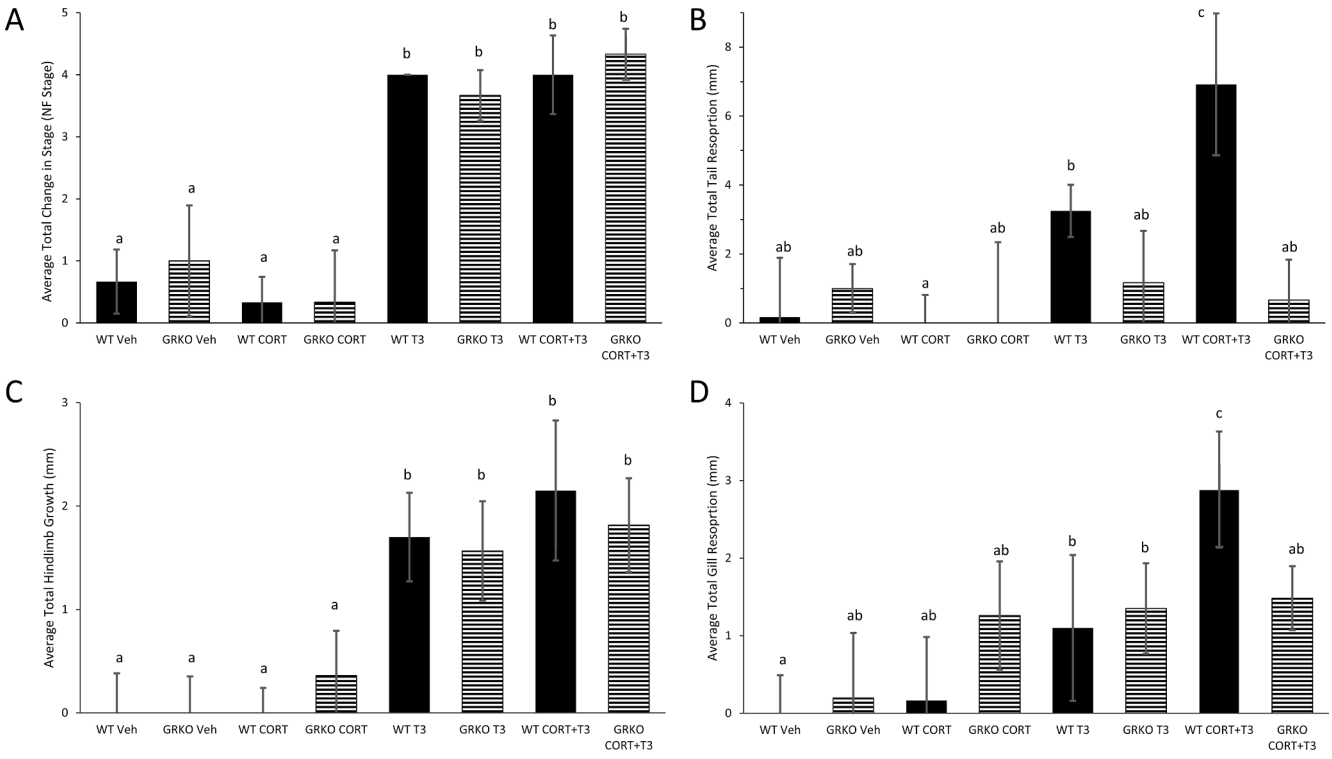

Fig. 5. GRKO tadpoles exhibit lack of CORT/TH synergy for tail and gill resorption. WT and GRKO were measured before and after treatment for 6 days with vehicle, 100 nM CORT, 2 nM T3, and 100 nM CORT plus 2 nM T3. Bars represent average total change from Day 0 to Day 6 in (A) NF stage, (B) tail length, (C) hindlimb length, and (D) gill width. Letters represent significance groups among all treatments within each panel. Error bars represent standard deviation. n = 5, WT = wild-type, GRKO = glucocorticoid receptor knockout, Veh = vehicle (ethanol treatment), CORT = corticosterone, T3 = active form of TH.

Fig. 5). After treatment with T3, there was a significant change for both genotypes in NF stage and hindlimb length, but weak responses were seen in tail or gill resorption which are less responsive to T3 than hind limb at this stage (Fig. 4, Fig. 5). After CORT and T3 combined treatment, we observed no significant change above that seen in T3 alone for NF stage and hindlimb growth (Fig. 5 B, D) for both genotypes, but we did observe significant increase above that in T3 alone for both tail resorption and gill resorption in WT only (Fig. 5 A,B). In GRKO, this

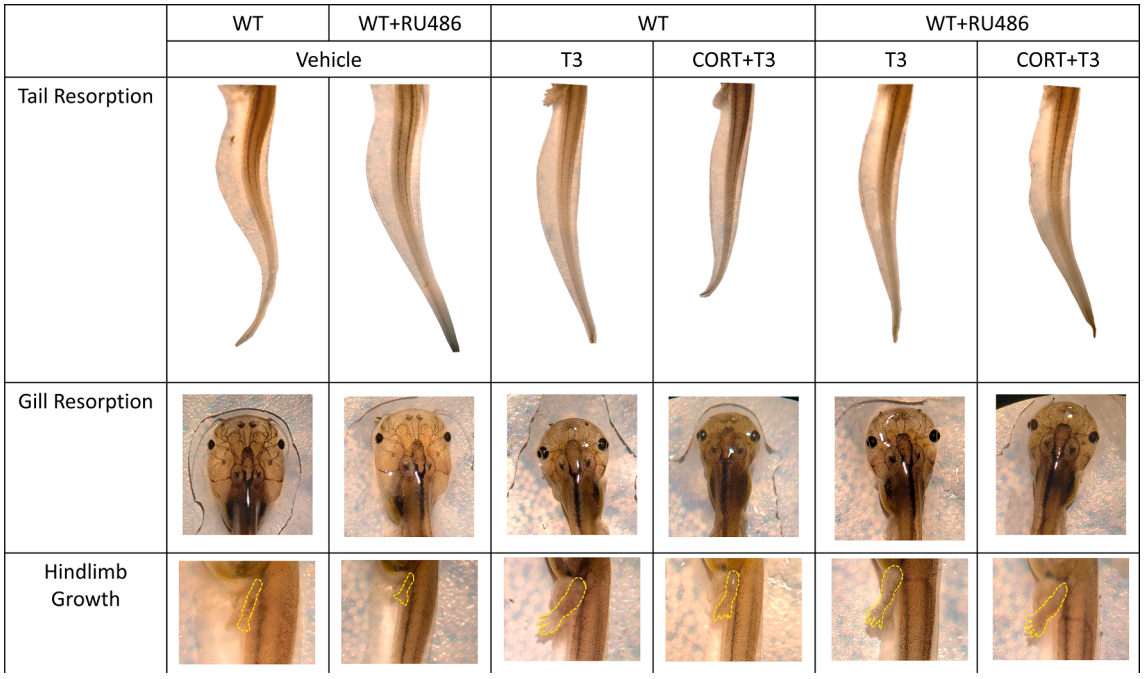

Fig. 6. Exemplar images of WT and RU486-treated tadpoles treated with or without T3 and/or CORT. Representative images of WT and RU486-treated tadpoles treated for 6 days with vehicle, 2 nM T3, and 100 nM CORT plus 2 nM T3 are shown. Note the stronger effect of T3 + CORT compared to T3 alone in tail and gill in wild-type (WT) and not RU486-treated tadpoles. n = 5, Veh = vehicle (ethanol treatment), RU486 = GR antagonist, T3 = active form of TH.

lack of a difference between T3 alone and hormone co-treatment for tail and gill resorption indicated a lack of synergy. These morphological results observed in GRKO tadpoles were compared with WT tadpoles treated with RU486, a GR antagonist, and essentially the same effects described above were also observed (Fig. 6 with exemplar images, Fig. 7 with quantification).

4. Discussion

Glucocorticoid receptor knockout (GRKO) tadpoles die during the climax of metamorphosis, but the cause of death is not known. CORT signals though GR and/or MR and has virtually no known developmental effect in tadpoles apart from increasing tissue sensitivity and responsivity to TH (Kikuyama et al., 1993; Denver, 2009; Kulkarni and Buchholz, 2014; Sachs and Buchholz, 2019). Because TH is necessary and sufficient to initiate frog metamorphosis (Allen, 1938; Dodd and Dodd, 1976; Shi, 1999) and because CORT/TH synergy is essential for metamorphic completion (Shewade et al., 2020), we hypothesized that GR is the CORT receptor responsible for most or all endocrine interaction with TH, thereby explaining death at metamorphosis in GRKO tadpoles. We used GRKO mutants and RU486-treated tadpoles (in which GR signaling is antagonized) treated with T3 and/or CORT to examine the role of GR in mediating hormone interactions between CORT and TH during frog metamorphosis.

We found previously that TH response gene expression during natural metamorphosis was lower in GRKO (Sterner et al., 2020), which could be due to lower plasma TH levels and/or reduced tissue TH sensitivity / responsivity. Here, our results using TH-induced metamorphosis point to reduced tissue responses to TH. Unlike WT tadpoles, GRKO individuals demonstrated little to no significant upregulation of the TH response genes *klf9* and *thrb*. Also, as seen for gene expression, GRKO mutants had an impaired morphological response to exogenous TH. These results establish that the lower TH responses in gene expression during natural development are, at least in part, a consequence of reduced TH sensitivity due to lack of signaling through GR, but we do not rule out that altered TH levels may contribute to lower responses to endogenous TH during natural development in GRKO tadpoles.

Untreated GRKO individuals perish before completion of metamorphosis as previously described (Sterner et al, 2020), but here we found that TH supplementation beginning at NF 60 enables a majority of GRKO individuals to survive to the completion of metamorphosis, as previously observed in adrenocorticotropic hormone knockout (ACTHKO) tadpoles (Shewade et al., 2020). These results show that there is insufficient TH signaling in both these mutants to allow for the completion of metamorphosis. Supplementation of TH signaling through exogenous T3 treatment presumably overcame the decreased endogenous TH signaling and allowed for the survival through metamorphosis. These results strongly suggest TH synergy with CORT signaling occurs via GR and is necessary for metamorphic completion. These results, however, do not rule out a possible vital role of CORT signaling independent of TH. CORT and MR are still present in GRKO mutants and may carry out potential vital roles of CORT signaling during metamorphosis. Also, GRKO animals die at NF 60-61, such that GR may carry out a vital developmental role after that stage, i.e., NF63-66. Also, ACTHKO animals have wild-type GR and MR, such that the lower CORT levels may be sufficient to carry out some but not all potential vital CORT-dependent roles. Future studies in mutant tadpoles lacking both GR and MR would provide key information about potential vital roles of CORT signaling.

In contrast to T3 treatments in GRKO tadpoles, the responses to T3 alone were not significantly different between WT and WT treated with RU486 at the gene and morphological levels. Previous work established that CORT synergy with TH is due in part to CORT regulation of deiodinases that act on available TH to increase the intracellular amount of the high affinity form of TH, i.e., T3 (Bonett et al., 2010). Thus, at time

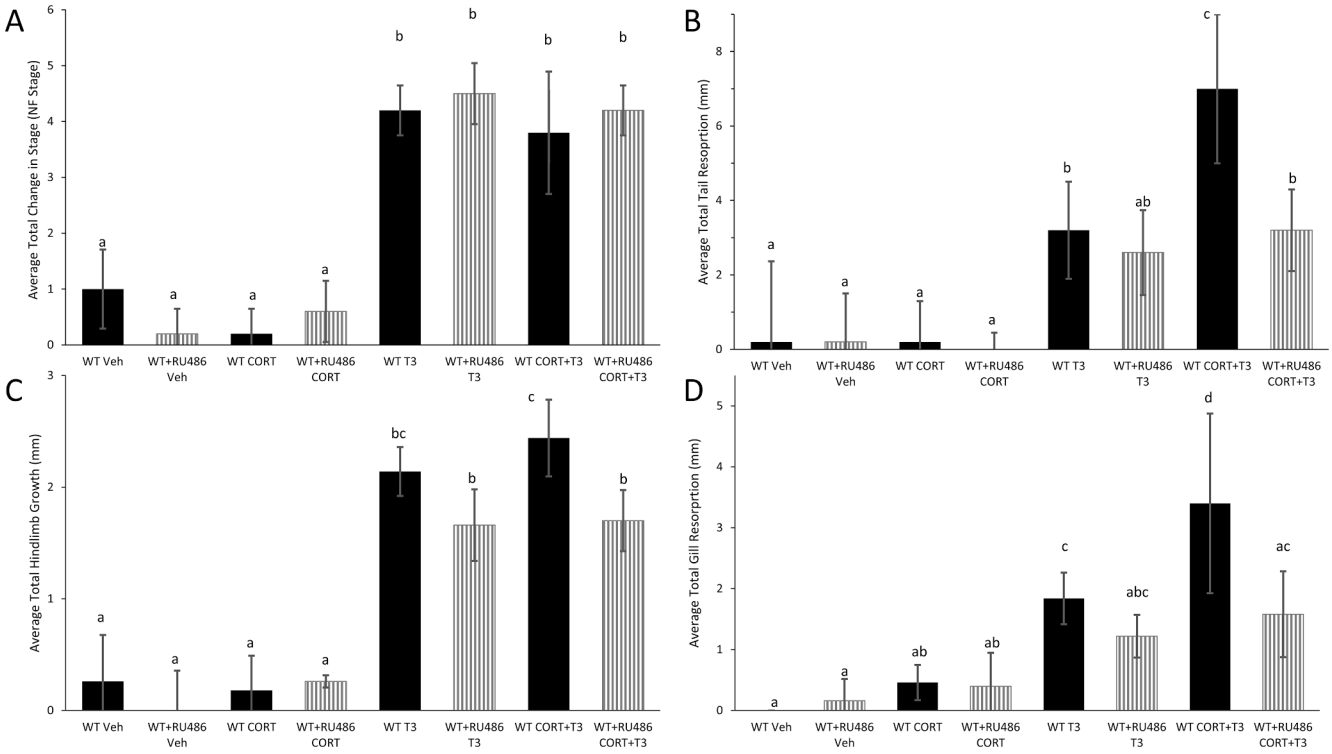

Fig. 7. WT tadpoles treated with RU486 exhibit lack of CORT/TH synergy for tail and gill resorption. WT and RU486-treated tadpoles were measured before and after treatment for 6 days with vehicle, 100 nM CORT, 2 nM T3, and 100 nM CORT plus 2 nM T3. Bars represent average total change from Day 0 to Day 6 in (A) NF stage, (B) tail length, (C) hindlimb length, and (D) gill width. Letters represent significance groups among all treatments within each panel. Error bars represent standard deviation. n = 5, WT = wild-type, RU486 = GR antagonist, CORT = corticosterone, T3 = active form of TH.

zero, WT and WT treated with RU486 will have identical TH responsivity, whereas GRKO tadpoles have lower TH responsivity. As a consequence of the lower TH responsivity in GRKO, GRKO responds less to T3 alone than RU486 animals respond to T3 alone. However, an increase in TH responsiveness (i.e., *thrb* induction) by virtue of CORT/GR signaling during the 24-hr co-hormone treatment did not occur in both GRKO and RU486 approaches, indicating that signaling through GR allows increased TH responsivity, i.e., GR contributes to synergy.

To test the role of GR in CORT/TH synergy directly, we compared gene expression and morphological responses to hormone co-treatments in WT tadpoles and tadpoles with impaired signaling through GR. At the gene expression level, treatment with CORT and TH demonstrates synergy in *thrb* in the tail in WT tadpoles (Fig. 2B, 3B). Synergy was not observed in GRKO or RU486-treated individuals, demonstrating that GR is required for synergy between CORT and TH for *thrb* expression in tail. We did not observe evidence of synergy from *klf9* responses in the tail (Fig. 2A, 3A), perhaps because 2 nM TH was sufficient to maximize *klf9* expression (Fig. 1B) and thus augmenting T3 responses via synergy with CORT would not increase *klf9* expression further. As in the tail, we did not observe synergy for *klf9* in liver, but unlike in tail, we also did not detect synergy in the liver for *thrb*. Perhaps, as suggested for the tail *klf9* response, a lower dose of T3 that produces a lower gene induction would allow an increase above that level to enable CORT signaling to show an effect of increasing the response to T3 and thus show evidence of synergy in liver.

At a morphological level, consistent with gene expression data, we observed synergy in the tail (Figs. 4, 5B, 6, 7B). This was observed as a significant CORT-induced doubling in resorption above that induced by T3 alone in WT, but not in GRKO or WT animals treated with RU486. Similarly, we observed synergy in the gills, measured via gill resorption (Figs. 4, 5D, 6, 7D). The amount of gill resorption twice above T3 treatment alone in the WT, but not in the GRKO or RU486-treated WT, indicated that GR is mediating CORT and TH synergy in the gills and tail.

Surprisingly, there was no evidence of synergy in hindlimb length or when measuring developmental stage progression (NF stage) (Fig. 4; 5A, C; 6; 7 A,C). At early stages in development (NF 48–57), NF stage is based on hindlimb morphology (Nieuwkoop and Faber, 1994), so the consistency between these two measures is expected. The hindlimb is more sensitive than other tissues (Brown et al., 2005; Yaoita, 2019), such that lack of an effect of co-hormone treatment above T3 alone could be due to lack of synergy. Alternatively, as suggested for *klf9* and for liver responses, the 2 nM T3 treatment may have produced a maximum effect that could not be increased via CORT action to increase TH tissue sensitivity / responsivity.

5. Conclusions

In summary, we show that GR has a major role in TH and CORT synergy for gene expression in *thrb* in the tail and for morphology of the tail and gill. GR-dependent synergy has not been ruled out for the other gene and tissues examined, and future experiments are needed to identify involvement of CORT/TH synergy in these and other genes and tissues. We also confirmed that TH – CORT synergy is necessary for the completion of metamorphosis, as shown by GRKO survival through metamorphosis via supplementation with T3 alone. These data demonstrating GR involvement in synergy with TH were reproduced utilizing a GR antagonist (RU486). Potential roles of GR independent of TH and any role for MR in metamorphosis await further experimentation.

CRediT authorship contribution statement

Zachary R. Sterner: Conceptualization, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Daniel R. Buchholz:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

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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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