Developmental origins of a novel gut morphology in frogs

Stephanie Bloom,[a] Cris Ledon-Rettig,[a] Carlos Infante,[b] Anne Everly,[b] James Hanken,[b] and Nanette Nascone-Yoder[a],*

[a] Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606, USA

[b] Department of Organismic and Evolutionary Biology, and Museum of Comparative Zoology, Harvard University, Cambridge, MA 02138, USA

*Author for correspondence (e-mail: nmnascon@ncsu.edu)

Stephanie Blooma and Cris Ledon-Rettiga have contributed equally to this work.

SUMMARY Phenotypic variation is a prerequisite for evolution by natural selection, yet the processes that give rise to the novel morphologies upon which selection acts are poorly understood. We employed a chemical genetic screen to identify developmental changes capable of generating ecologically relevant morphological variation as observed among extant species. Specifically, we assayed for exogenously applied small molecules capable of transforming the ancestral larval foregut of the herbivorous *Xenopus laevis* to resemble the derived larval foregut of the carnivorous *Lepidobatrachus laevis*. Appropriately, the small molecules that demonstrate this capacity modulate conserved morphogenetic pathways involved in gut development, including downregulation of retinoic acid (RA) signaling. Identical manipulation of RA signaling in a species that is more closely related to *Lepidobatrachus*, *Ceratophrys cranwelli*, yielded even more similar transformations, corroborating the relevance of RA signaling variation in interspecific morphological change. Finally, we were able to recover the ancestral gut phenotype in *Lepidobatrachus* by performing a reverse chemical manipulation to upregulate RA signaling, providing strong evidence that modifications to this specific pathway promoted the emergence of a lineage-specific phenotypic novelty. Interestingly, our screen also revealed pathways that have not yet been implicated in early gut morphogenesis, such as thyroid hormone signaling. In general, the chemical genetic screen may be a valuable tool for identifying developmental mechanisms that underlie ecologically and evolutionarily relevant phenotypic variation.

INTRODUCTION

The emergence of new phenotypic variation is generally assumed to arise from novel mutations (or equivalent environmental perturbations; West-Eberhard 2003; Moczek et al. 2011) or the recombination of standing genetic variation. Yet even with genomic sequence information from hundreds of species, we understand little about the origins of morphological variants that ultimately result in adaptive diversification. To understand the relationship between genetic variants and phenotypic innovation, we must first understand how endogenous and environmental signals are modified in quantity, time or space to produce novel phenotypic variation that can be integrated with preexisting developmental programs (Gilbert 2003). This is currently a formidable task to achieve using non-model systems, and evolutionary innovations are rare among model systems (Kopp 2011).

Several elegant studies have identified developmental underpinnings of novel trait variation by perturbing pathways known to influence the morphogenesis of those traits (Kopp et al. 2000), or of similar traits, in model organisms (Abzhanov et al. 2004; Moczek et al. 2007). These studies disrupted the function of relevant genes through, for instance, RNAi or viral-induced gene silencing, to mimic the form of morphological variation observed in extant species. One limitation of such candidate approaches is that they may preclude the discovery of novel or alternate genetic or epigenetic variants that might also give rise to the same phenotype. Although forward approaches such as quantitative trait locus analysis (e.g., Shapiro et al. 2004) or classical mutagenesis screens have the potential to expand a repertoire of candidate pathways underlying novel traits, they are often expensive and time-consuming, and few non-model systems meet the criteria necessary for such analyses. Recently, screening embryos against a library of chemicals has provided an alternative and powerful approach to revealing the role of specific signaling pathways in development (Wheeler and Brandli 2009). Although typically used to produce phenotypes that mimic developmental abnormalities, we propose that chemical genetic screens can also be used to produce phenotypes that mimic naturally occurring interspecific variation.

Chemical genetic screens work by targeting, in vivo, various modulators of the conserved signaling pathways that control metazoan development. This is achieved by the action of cell permeable "small molecule" reagents (i.e., low molecular

weight organic compounds) that inhibit or activate specific pathway components. Small molecules thus act as "mutational equivalents," inducing loss- or gain-of-function phenotypes that often mimic genetic mutants (Stockwell 2000; Wheeler and Brandli 2009). This approach has at least two key advantages when used in an evolutionary context. First, because small molecules are cell permeant and bind to the conserved functional domains of their target proteins, they are largely species-independent and thus can be employed in non-model organisms. Second, embryos can be exposed to small molecules during specific developmental windows, thus avoiding pleiotropic and lethal effects that are commonly encountered in traditional genetic screens. In effect, small molecule exposure might be able to mimic a regulatory change that arose during evolution and resulted in altered gene expression during a particular stage of development; such regulatory changes are seen as an important source of evolutionary novelty (reviewed in Wray 2007; Carroll 2008; Kirschner and Gerhart 2010).

In this study, we focus on identifying evolutionary changes in development that may have resulted in adaptive variation in gut phenotypes among anuran larvae. Digestive physiology and morphology is a critical aspect of any organism's ecological niche (Karasov and Diamond 1988), and evolutionary diversification often goes hand in hand with a change in food resources (Schluter 2000; Grant and Grant 2008). In particular, an individual's nutritional intake is influenced by the configuration and length of its digestive tract. Generally, carnivores have a capacious, proteolytic stomach and short intestine, while herbivores and omnivores have a long intestine and a stomach that is enzymatically simple (Stevens and Hume 1995). This range of dietary variation and corresponding gut phenotypes can be observed among larvae of the South American anuran clade, Ceratophryinae (e.g., *Chacophrys*, *Ceratophrys*, and *Lepidobatrachus*). Like most anuran larvae, *Chacophrys* larvae are mainly herbivorous (Duellman and Trueb 1986; Wild 2000; Rossa-Feres et al. 2004). In contrast, *Ceratophrys* larvae are microphagous carnivores that process their tadpole or invertebrate prey orally before swallowing it, whereas *Lepidobatrachus* larvae are megalophagous carnivores that consume their animal prey whole (Ruibal and Thomas 1988). Both *Ceratophrys* and *Lepidobatrachus* have derived stomach and intestine morphologies to accommodate their unique diets (Fig. 1H and I; Fabrezi 2011), although the molecular and developmental origins of these adaptive traits remain elusive.

To address the mechanisms underlying the development and evolution of the novel gut phenotype found specifically in *Lepidobatrachus*, we used a chemical genetic screen to identify developmental changes that are capable of modifying ancestral gut morphogenesis to yield the derived gut morphology. This screen identified modulators of pathways that have been previously implicated in gut development (thereby providing validation for the approach), as well as unexpected pathways. Our results suggest that evolutionary modifications to these developmental pathways might have led to ecologically and evolutionarily important phenotypic variation among anuran larvae. Chemical genetic screening may be a valuable tool for uncovering developmental mechanisms that generate selectable morphological variation.

MATERIALS AND METHODS

Characterizing gut morphogenesis

Xenopus laevis embryos were obtained by in vitro fertilization using established procedures and reared at 16°C in 0.1× Marc's Modified Ringers (Sive et al. 1998). The developmental stages of *Xenopus* embryos were determined using the morphological criteria of Nieuwkoop and Faber (NF; Nieuwkoop and Faber 1994). *Lepidobatrachus laevis* embryos were obtained through natural matings of adults that were collected in Salta, Argentina, and maintained in a colony at Harvard University. To induce spawning, male and female adults were injected with luteinizing hormone-releasing hormone (Sigma-Aldrich, St. Louis, MO, USA) at a dosage of 0.1 mg/kg body weight. Embryos were reared at 22°C in 10% Holtfreter solution (Holtfreter 1931). *Ceratophrys cranwelli* embryos were obtained from K. Thomas (The Frog Ranch, Granite Bay, CA), and reared at 28°C in 10% Holtfreter solution. The developmental stages of *Lepidobatrachus* and *Ceratophrys* embryos were determined using the morphological criteria of Gosner (GS; Gosner 1960). For all species, the morphogenesis of the stomach, gastroduodenal loop (an anatomical feature that occurs between the foregut and midgut; Fig. 1A), intestine, liver, and pancreas was characterized at end stage (approximately NF46 in *Xenopus*, GS23–25 in *Ceratophrys* and *Lepidobatrachus*).

Chemical genetic screen

To identify molecular signaling changes that might underlie the differential morphogenesis of the carnivorous gut, we screened for small molecules capable of transforming the ancestral tadpole foregut to the derived morphology found in *Lepidobatrachus*. *X. laevis* was chosen as the representative model of the ancestral character state because *Xenopus* larvae possess an ancestral-type digestive tract common to most extant tadpoles (including tadpoles of *Chacophrys pierottii*, an omnivorous species that is closely related to *Ceratophrys* and *Lepidobatrachus*: Maxson and Ruibal 1998; Fabrezi and Quinzio 2008). It is therefore likely that, on the molecular level, *Xenopus* possess a developmental program similar to that possessed by the ceratophryine ancestor (i.e., of *Chacophrys*, *Ceratophrys*, and *Lepidobatrachus*).

Approximately 200 small molecules were applied to *Xenopus* embryos from late neurula stages (NF18-22) through the end of gut morphogenesis (NF46). This panel included 143 custom-
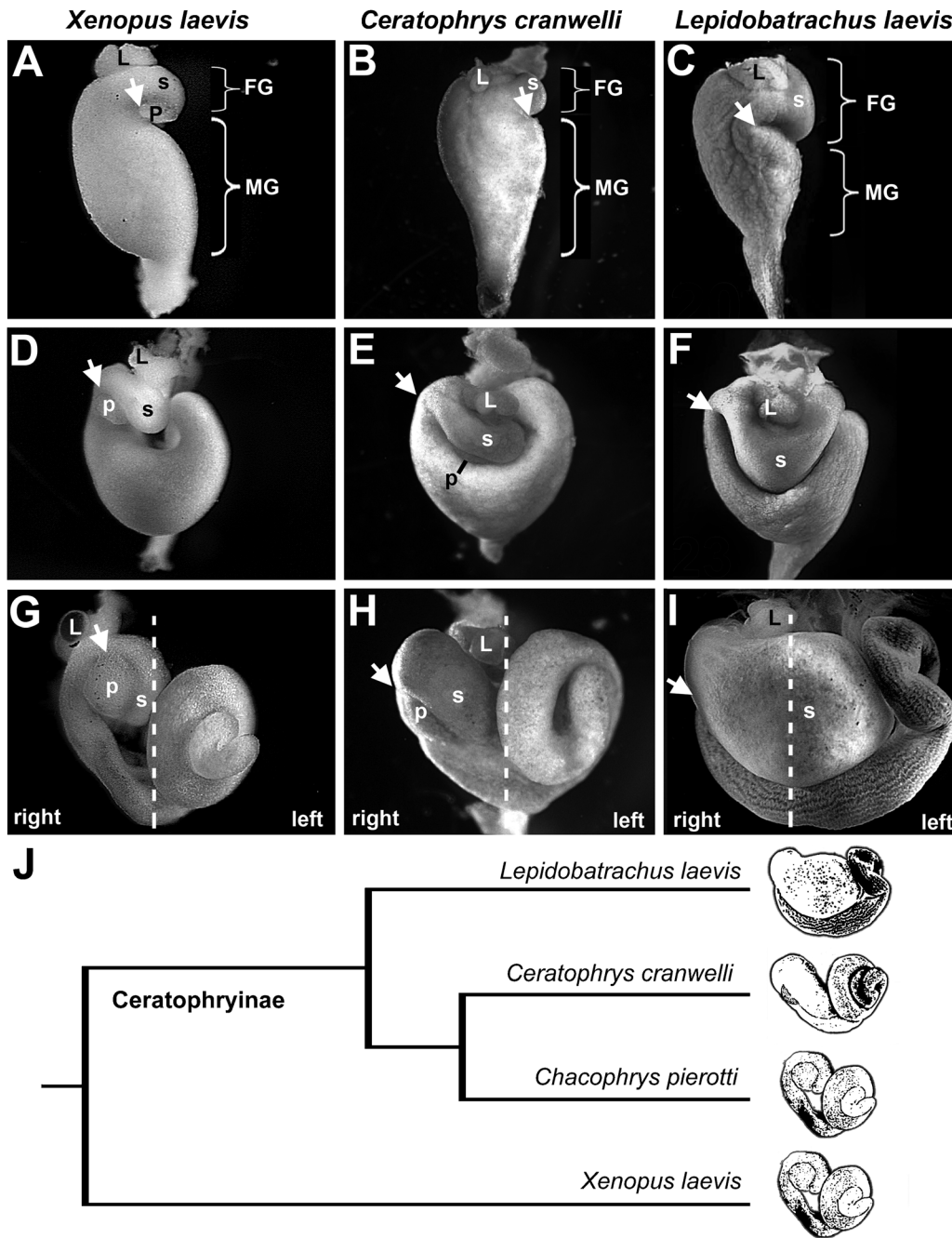
Fig. 1. Gut development in omnivorous and carnivorous anuran larvae. Ventral views of the developing gut of an omnivorous tadpole (*Xenopus laevis*) at Nieuwkoop and Faber (NF) stages 41 (A), 43 (D), and 46 (G) are compared with the developing guts of carnivorous *Ceratophrys cranwellii* and *Lepidobatrachus laevis* tadpoles at comparable Gosner stages (GS) 21 (B and C), 23 (E and F), and 25 (H and I). In *Xenopus* (A), the GD loop (arrow) is located in a proximal position along the length of the gut tube, the foregut (FG) is small relative to the midgut (MG), and the pancreas is located within the GD concavity. The GD loop is similarly positioned in *Ceratophrys* (B), although the pancreas is not visible early in development. In *Lepidobatrachus*, the GD loop forms more distally, which leaves the portion of the gut tube proximal to the GD loop of more equal proportion to the prospective midgut (C). The relative positions of the developing stomach (s), liver (L), and pancreas (p) are indicated, where visible. (The pancreas remains dorsal in *Lepidobatrachus* and is not visible in these ventral views.) Dashed lines in G, H, and I indicate the approximate position of the embryonic midline and the left and right sides of each embryo. Images are not to scale. The cladogram (J) illustrates the relationships among *Xenopus* and three ceratophryine genera, including *Ceratophrys* and *Lepidobatrachus*.

synthesized novel compounds (Dush et al. 2011) and 59 commercially available reagents that target pathways known to be involved in morphogenesis (Table S1). The concentrations of each compound were based on published IC$_{50}$ values in tissue culture or aquatic embryo assays; novel compounds were assayed at 100 μM. At least four embryos were assayed per compound and any compounds that yielded the *Lepidobatrachus*-like gastroduodenal loop (as described in Results) were repeated with embryos from different clutches.

Modulation of retinoic acid (RA) signaling

All-trans RA (a diffusible cell signaling molecule) and diethylaminobenzaldehyde (DEAB; an RA signaling antagonist) were prepared in ethanol, and Ro-41-5253 (an RA signaling antagonist) was prepared in dimethyl sulfoxide. Stock solutions were diluted to working concentrations in either 0.1× MMR (*X. laevis*) or 10% Holtfreter (*L. laevis* and *C. cranwelli*) media. In each experiment, sibling controls were cultured in an appropriate dilution of ethanol or DMSO to control for any effects of the solvent.

Groups of 10 or more *X. laevis* embryos (stage NF22) were each cultured in 10 ml of DEAB (0.4, 0.5, 0.6, or 0.7 mM) or Ro-41-5253 (1 or 1.5 μM) solution at 16°C. After 24 h, embryos were washed five times in 0.1× MMR and maintained in 0.1× MMR until stage NF45/46 to evaluate gut phenotypes.

Groups of five to seven *C. cranwellii* embryos were cultured in 30 ml of DEAB solution (0.4, 0.5, 0.6, or 0.7 mM; *Ceratophrys* and *Lepidobatrachus* embryos and tadpoles are significantly larger than *Xenopus* and require larger culture volumes) at stages GS16, GS17, or GS18. At stage GS19, embryos were washed five times in 10% Holtfreter and maintained in 10% Holtfreter until stage GS23–25 (equivalent to NF44–46; McDiarmid and Altig 1999) to evaluate gut phenotypes.

Groups of 10 *Lepidobatrachus* embryos at stages GS16, GS17, or GS18 were each cultured in 100 ml of RA solution (0.05, 0.1, 0.5, or 1 μM) at 22°C. At stage GS19, embryos were washed five times in 10% Holtfreter then maintained in 10% Holtfreter until stage GS23–25 (equivalent to NF44–46; McDiarmid and Altig 1999) to evaluate gut phenotypes.

In situ hybridization

Some embryos of all species were fixed in MEMFA (Sive et al. 1998) at various stages and processed for in situ hybridization. *Pitx2* and *Nkx2.5* were detected in *X. laevis* and *L. laevis* using *X. laevis* riboprobes as previously described (Smith et al. 2000; Muller et al. 2003; Lipscomb et al. 2006) with the following modifications for large embryos: rehydration washes were carried out for 10 min each, additional washes were performed before and after refixation, and a 1/3000 dilution of alkaline phosphatase-conjugated, anti-digoxygenin anti-FAB

fragments was used. The *X. laevis Pitx2* riboprobe has the potential to recognize all three *Pitx2* isoforms, but only isoform c is expressed asymmetrically in the left lateral plate mesoderm of vertebrate embryos (Schweickert et al. 2000). *Pitx2c* was detected in *Ceratophrys* using a *Ceratophrys Pitx2c* riboprobe. To create the *Pitx2c* probe, *Pitx2c* was amplified from complementary DNA synthesized from total RNA collected from stage GS19 *Ceratophrys* using degenerate primers. The amplified fragment was cloned into the pCR2-TOPO cloning vector (Invitrogen), transfected into chemically competent cells, cultured, purified, and used for riboprobe synthesis.

RESULTS

Interspecific variation in anuran larval gut morphogenesis

In this study, the *Xenopus* larval digestive system developed as previously described (Fig. 1A, D, and G; Chalmers and Slack 1998, 2000). As in other vertebrates, the digestive organs arise from a primitive gut tube that undergoes looping and rotation events to shape the final anatomical configuration. For example, the gastroduodenal loop begins as a left-side concavity that rotates rightward as the foregut elongates, ultimately fixing the stomach, liver, and pancreas on the embryo's right side, with the pancreas closely apposed to the concavity of the loop (arrows, Fig. 1A, D, and G; Muller et al. 2003). As in most anuras, larval *X. laevis* form a rudimentary stomach (Griffiths 1961) that does not develop a pyloric sphincter or produce pepsinogen, a proteolytic enzyme (Ishizuya-Oka et al. 1998). Simultaneously with foregut morphogenesis, the midgut elongates extensively (up to nine times the snout-to-vent length of the larva), forming several concentric intestinal coils that occupy the left side of the visceral cavity (Fig. 1G; Chalmers and Slack 1998). This long intestine facilitates nutrient absorption and bacterial fermentation (Pryor and Bjorndal 2005).

Gut morphogenesis proceeds differently in the carnivorous larvae of *Ceratophrys* and *Lepidobatrachus*. As observed in *X. laevis*, the *Ceratophrys* embryonic foregut exhibits a left-side concavity, that eventually loops rightward (Fig. 1B, E, and H). However, in contrast to the anteroposteriorly oriented foregut of *Xenopus*, *Ceratophrys*' foregut remains oriented transversely before moving rightward and becomes substantially larger than *Xenopus*. Further, the appearance of the dorsal pancreatic rudiment in the concavity of the loop is delayed in *Ceratophrys* relative to *Xenopus* (cf. Fig. 1D and E), although the organ ultimately forms and resides in the same position (Fig. 1G and H).

The *Lepidobatrachus* embryonic foregut exhibits an even more extreme phenotype. Most notably, the left-side concavity forms more posteriorly along the gut tube (Fig. 1C). Consequently, a larger segment of the gut tube is situated anterior to the loop, the stomach becomes fixed in a transverse orientation and the

liver remains closer to the midline (Fig. 1I). As in *Ceratophrys*, the stomach is enlarged; however, the expansion is dramatic in *Lepidobatrachus*, filling most of the anterior visceral cavity (Fig. 1I). Although the dorsal pancreas eventually becomes visible in the concavity of the *Ceratophrys* gastroduodenal loop, the pancreas is greatly reduced in *Lepidobatrachus* (a small group of insulin-positive cells was detected dorsal to the stomach; data not shown) and invisible in ventral view as it is displaced from the concavity of the GD loop. Finally, midgut elongation in *Lepidobatrachus* is uniquely attenuated, and the intestine remains practically uncoiled (Fig. 1I; Fabrezi 2011).

Modulating specific signaling pathways produces interspecific variation in *X. laevis*

In a chemical genetic screen of approximately 200 small molecules applied to *Xenopus* embryos, five structurally distinct compounds resulted in the formation of a more carnivore-like foregut that resembles that of *Lepidobatrachus*: cyclopamine (Fig. S1), DEAB (Fig. 2), latrunculin (Fig. S1), Ro-41-5253 (Fig. 2), and triiodothyronine (T3; Fig. S1). Cyclopamine is an alkaloid that inhibits signaling via hedgehogs, a highly conserved family of secreted morphogens that regulates key features of development and patterning (Ryan and Chiang 2012). DEAB inhibits retinaldehyde dehydrogenase, which catalyzes the conversion of retinal, a Vitamin A derivative, to RA, a diffusible signaling molecule that plays essential roles in cell growth, differentiation and patterning during development (Blomhoff and Blomhoff 2006). Latrunculin is a compound that inhibits the polymerization of actin, a process essential for cytoskeletal dynamics during cell migration (Ridley 2011). Ro-41-5253 is a soluble antagonist of a RA receptor, RARα (Keidel et al. 1994), which binds RA and transduces the RA signal to the nucleus to control gene expression. Finally, T3 is the metabolically active form of thyroid hormone (TH), which controls numerous physiological processes, including anuran metamorphosis (Laudet 2011).

In this study, we chose to investigate more closely the effects of DEAB and Ro-41-4253 because they both target different components of RA signaling, thus corroborating the potential evolutionary relevance of this pathway for the novel trait of interest. In embryos exposed to DEAB and Ro-41-5253, the gastroduodenal loop is displaced posteriorly with respect to the stomach, liver, and pancreas, a phenotype that is strikingly reminiscent of what is found in the *Lepidobatrachus* embryo (Fig. 2A–C). Moreover, the stomach is oriented transversely and the liver and pancreas become fixed more medially, rather than adopting their normal right-sided orientations (cf. Fig. 2A and B). Finally, the pancreas is often reduced and positioned more dorsally and medially; that is it is displaced from the concavity of the GD loop (cf. Fig. 2A and B). Among the treated *X. laevis* individuals there was some variation in the degree of this displacement, but any displacement always co-occurred with a

transverse orientation of the stomach, a condition not observed in untreated *X. laevis*. This carnivore-like phenotypic profile is induced by small molecules in a concentration-dependent manner (Fig. 2D).

We reasoned that the same signaling modulations would not only elicit similar morphological changes in *Ceratophrys* but also that they could actually produce a more accurate form of the carnivore gut morphology in these animals, as *Ceratophrys* might possess any requisite changes in developmental programming that may have occurred, evolutionarily, after *Xenopus* and ceratophryine ancestors diverged. Indeed, we observe the same profile of foregut changes in *Ceratophrys* embryos exposed to DEAB, including the transversely oriented stomach, medial accessory organ orientation, and reduced pancreas (Fig. 2E and F), but, additionally, the attenuated midgut elongation of *Lepidobatrachus* is also induced by DEAB in *Ceratophrys* (cf. Fig. 2C and F).

Increased RA signaling produces interspecific variation in *L. laevis*

The above results show that multiple components of the RA pathway produce similar phenotypic variation when perturbed, implicating decreased RA availability or RARα-mediated activity in the development of the altered gastroduodenal loop found in *L. laevis*. If decreased RA signaling underlies the development of a carnivore foregut, then increasing RA signaling in *Lepidobatrachus* embryos might restore the foregut to a more herbivorous, that is ancestral, state. To test this hypothesis, we exposed *Lepidobatrachus* embryos to exogenous all-trans RA, an agonist for RARα. As predicted, RA-treated *Lepidobatrachus* tadpoles exhibit several anatomical features that are reminiscent of an herbivorous larval foregut, including a more anterior gastroduodenal loop, with the liver-oriented slightly posterior to the loop (Fig. 3A and B). In addition, the pancreas, which normally is hidden dorsally, became visible in ventral view, closely apposed to the concavity of the gastroduodenal loop, as in *Xenopus* (cf. Fig. 3B and C). These effects were induced at high frequency over multiple doses and stages (Fig. 3D).

Molecular markers further indicate the authenticity of these small-molecule induced phenocopies. In *Xenopus*, as in other vertebrates, gastroduodenal looping occurs adjacent to the expression domain of *Nkx2.5*, a conserved marker for the boundary between the stomach and duodenum (Fig. 4A; Smith et al. 2000). In *Lepidobatrachus* embryos and DEAB-treated *Xenopus* embryos, *Nkx-2.5* is still expressed at the stomach-duodenal boundary, but the loop forms more posteriorly relative to the *Nxk-2.5* expression domain (cf. Fig. 4A–C). In contrast, in RA-treated *Lepidobatrachus* embryos, as in *Xenopus*, the loop forms adjacent to the expression of *Nkx2.5* (Fig. 4D). Variation in RA signaling levels can generate evolutionarily relevant variation in the topography of the foregut loop.
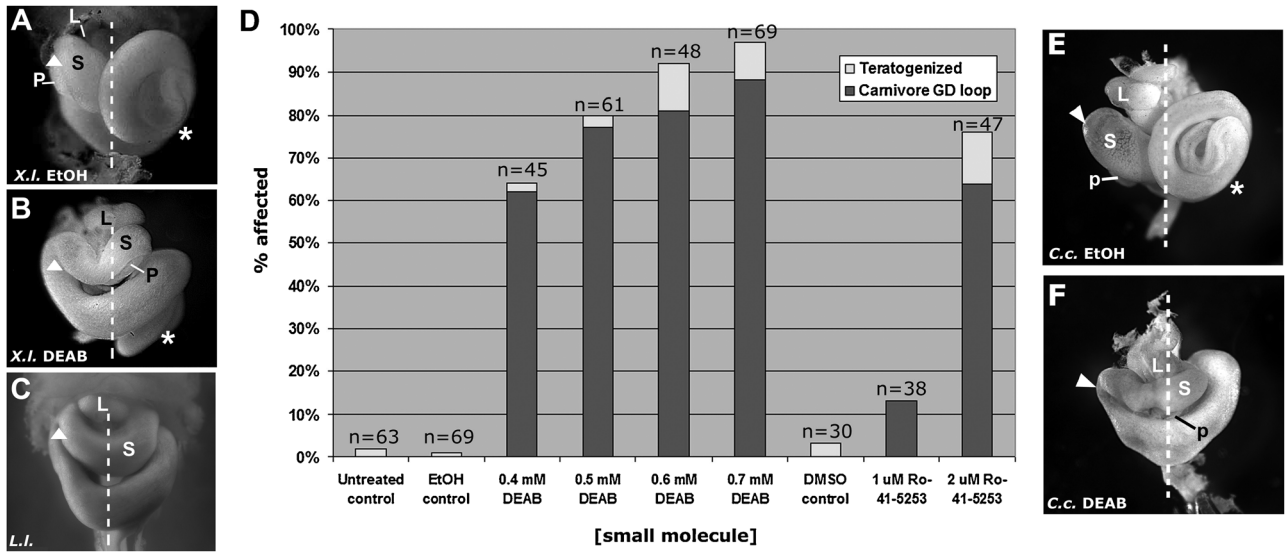
Fig. 2. Treatment of anuran embryos with a retinoic acid (RA) synthesis inhibitor results in the formation of a more derived/carnivore-like GD loop morphology. *X. laevis* embryos were subjected to an acute chemical treatment with solvent control (ethanol, EtOH; A) or an RA synthesis inhibitor, DEAB (0.4 mM; B). The *Xenopus* GD loop (arrowhead; NF46) shifts posteriorly upon exposure to DEAB (B) and the final foregut anatomy appears similar to the normal morphology of *L. laevis* (C; GS23; Although *Xenopus* NF46 is most equivalent to GS25, the relative anatomical topology of the foregut organs is already established by GS23 and is more easily visualized at this stage that is before stomach expansion.). (D) Effects on gut morphogenesis after treatment with small molecule inhibitors of retinoic acid synthesis (DEAB) or signaling (Ro-41-5253) are concentration dependent. The percentage of embryos with the derived/carnivore-like GD loop and organ placement (NF46) is indicated for different concentrations of each molecule. Embryos that exhibit severely disrupted development (e.g., massive edema, tail curvature) or abnormal, uninterpretable phenotypes not resembling either species are classified as "teratogenized." Results are pooled from five different experiments. DMSO was used as the solvent control for Ro-41-5253. *C. cranwellii* embryos were subjected to an acute chemical treatment with solvent control (EtOH; E) or an RA synthesis inhibitor, DEAB (0.5 mM; F). As observed in *Xenopus*, the *Ceratophrys* GD loop (arrowhead; GS25) develops in a more posterior position upon exposure to DEAB (F) and, further, the intestine (*) does not elongate, phenotypes remarkably similar to the morphological features found in *Lepidobatrachus* (C; GS23). The relative positions of the developing stomach (S), liver (L) and pancreas (p) are indicated, where visible.

Chemically modulating RA signaling shifts *Pitx2* expression

To investigate the possibility that left-right asymmetric development has diverged between species possessing herbivore and carnivore gut phenotypes, we compared the embryonic expression of the gene *Pitx2*. *Pitx2* is a homeobox transcription factor that is expressed exclusively on the left side in vertebrate embryos, where it serves as a key effector in the formation of left-right asymmetries in internal organs (Ryan et al. 1998; Campione et al. 1999). RA is known to influence *Pitx2* expression patterns during development (Chazaud et al. 1999; Matt et al. 2005, 2008). Moreover, in *Xenopus* embryos, asymmetric *Pitx2* expression correlates with the segment of the prospective gut tube that will form the concavity of the gastroduodenal loop (Fig. 5A; Muller et al. 2003).

As in *Xenopus* and other vertebrates, we found that *Pitx2* is expressed exclusively on the left side of *Ceratophrys* and *Lepidobatrachus* embryos (Fig. S2) at a stage immediately preceding gut morphogenesis (equivalent stages NF32 and GS20 in *Xenopus* and Ceratophryines, respectively; McDiarmid and Altig 1999); however, in *Lepidobatrachus*, the expression domain is positioned considerably more posteriorly and extends farther ventrally (cf. Fig. S2C with A, B and Fig. 5B with A). According to published fate maps of amphibian embryos, this region is fated to give rise to a more posterior region of the gut tube, consistent with the more posterior origin of the carnivorous gut loop in *Lepidobatrachus* (Tahara and Nakamura 1961; Chalmers and Slack 2000; Muller et al. 2003).

To determine if a shift in *Pitx2* expression also accompanies the shifted gut looping observed in *Xenopus* treated with small molecule RA inhibitors, *Pitx2* expression was evaluated in the context of DEAB. Exposure of *Xenopus* embryos to DEAB resulted in a posterior and ventral expansion of the *Pitx2* expression domain (Fig. 5C). This new domain correlates with a more posterior position along the gut tube and is consistent with the more posterior gastroduodenal loop induced by inhibition of RA signaling. Conversely, treatment of *Lepidobatrachus* embryos with RA resulted in an anterior and dorsal shift in the expression domain of *Pitx2* (Fig. 5D). This new domain is fated to give rise to a more anterior position along the gut tube, consistent with the more anterior gastroduodenal loop observed in RA-treated *Lepidobatrachus* tadpoles.
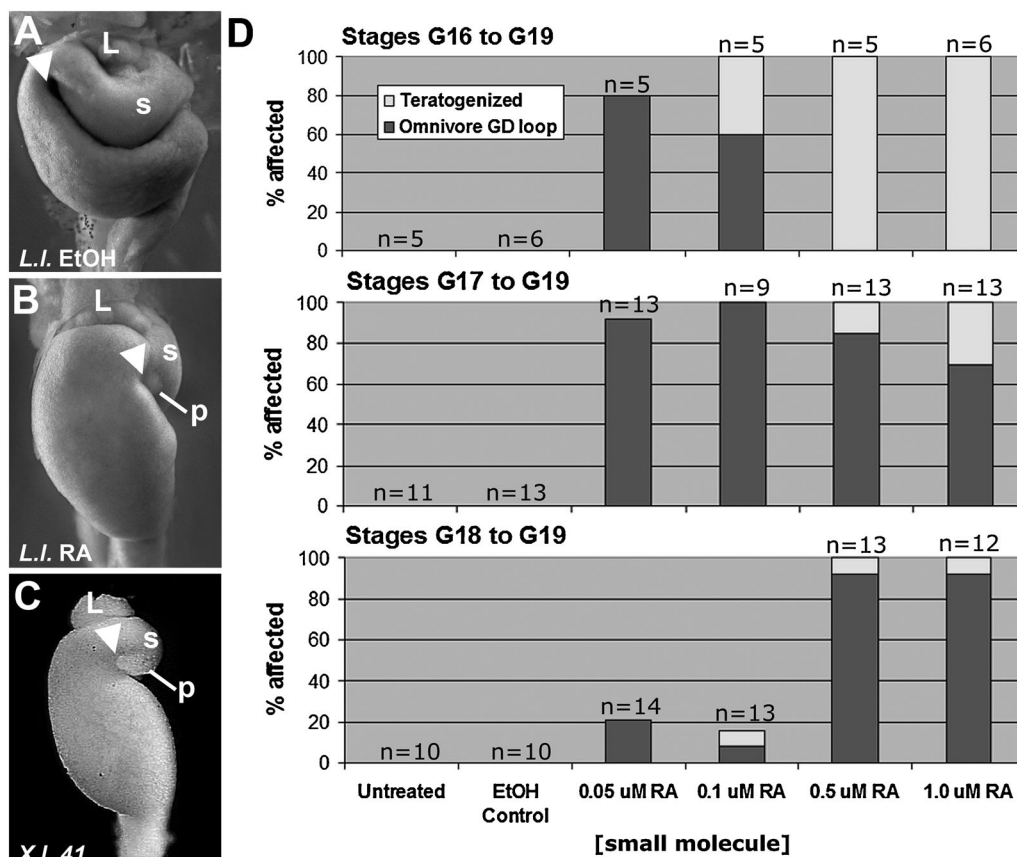
Fig. 3. Treatment of *L. laevis* with RA results in a more ancestral/omnivore-like GD loop morphology. *Lepidobatrachus* embryos were exposed to solvent control (ethanol, EtOH; A) or RA (B). RA-treated *Lepidobatrachus* embryos (B; GS23) exhibit a lack of midgut elongation, a known teratogenic effect of excess RA in vertebrates. In this context, the GD loop (arrowhead) shifts anteriorly upon exposure to ectopic RA (B) and foregut morphology appears remarkably similar to that of untreated *X. laevis* at a comparable degree of midgut elongation (C; NF41, reproduced from Fig. 1A). (D) Effects on gut morphogenesis after treatment with ectopic RA at successively later developmental stages are both concentration- and stage-dependent. Results are pooled from two different breedings. "Teratogenized" classification is as in Fig. 2. The relative positions of the developing stomach (s), liver (L), and pancreas (p) are indicated, where visible.

DISCUSSION

Selection on phenotypic variation may yield novel adaptive traits, but little is known about the proximate developmental mechanisms by which the requisite phenotypic diversity arises. In this study, we performed a small molecule screen to interrogate these mechanisms, reasoning that small molecule inhibitors or activators could alter development in an extant species with the ancestral character state and "reproduce" the morphogenetic context that generated a novel phenotype in a derived lineage. Our results implicate specific and possibly interdependent developmental pathways in the generation of novel gut morphologies.

The molecules identified by our screen have the potential to modify developmental processes as diverse as actin polymerization (via latrunculin; Yarmola et al. 2000), cell differentiation (via T3) and the many morphogenetic processes controlled by hedgehog signaling (via cyclopamine). In particular, our screen identified two structurally distinct compounds, DEAB and Ro-41-5253, that inhibit different steps in RA signaling, thus strongly implicating this pathway in the generation of the novel larval gut morphologies possessed by *Lepidobatrachus*. Treating *Xenopus* embryos with these molecules promotes the development of a foregut phenotype that is remarkably similar to that of *Lepidobatrachus*, including the exaggerated gastroduodenal loop, transversely oriented foregut, medially positioned liver and reduced, dorsally situated pancreas. Importantly, this transformation is even more accurate when the embryos of *Ceratophrys*—a lineage more closely related to *Lepidobatrachus* that maintains an ancestral type gastro-duodenal loop—are treated with DEAB. Along with the expected conversions of foregut morphology obtained with *Xenopus*, the same treatment in *Ceratophrys* prevents the midgut from extending and coiling, a phenotypic profile that is, overall, remarkably similar to that possessed by *Lepidobatrachus*.
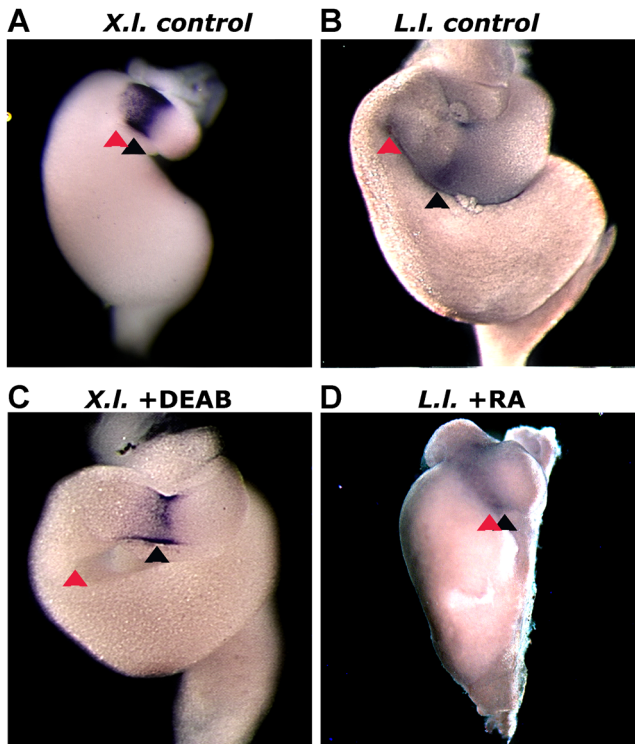
Fig. 4. Chemically modulating RA signaling in anuran embryos shifts the location of the GD loop along the anterioposterior axis of the gut tube. The stomach-duodenal boundary in each species is indicated by expression of the homeobox transcription factor *Nkx2.5*. The GD loop (red arrowhead) is located adjacent to the *Nkx2.5* expression domain (black arrowhead) in *Xenopus* controls (A; NF42), but it is shifted posteriorly following DEAB treatment (C; NF45). Conversely, the GD loop is located posterior to *Nkx2.5* expression in *Lepidobatrachus* controls (B; GS23), but develops in a more anterior position following RA treatment (D, GS23).

Fig. 5. Chemically modulating RA synthesis and signaling shifts the expression of *Pitx2*, a left-side determinant of asymmetric gut looping. Compared to the domain of *Pitx2* expression (purple) revealed by in situ hybridization in *Xenopus* embryos (A), the *Pitx2* domain is shifted posteriorly and ventrally in the *Lepidobatrachus* embryo (B). (C and D) *Pitx2* expression is shifted posteriorly and ventrally in *Xenopus* embryos exposed to DEAB (C), and anteriorly and dorsally in *Lepidobatrachus* embryos exposed to RA (D). Arrows in (A) and (C) indicate the posterior limit of *Pitx2* expression; those in (B) and (D) indicate the anterior limit.

Molecular evidence from this study corroborates our hypothesis that altered RA signaling may have promoted the morphological evolution of the larval foregut that accompanied the transition to larval carnivory in *Lepidobatrachus*. An RA-inhibitory small molecule treatment (DEAB) that produces a carnivore-like gut in *Xenopus* also produces a heterotopic shift in *Pitx2* expression which mimics the normal pattern of *Pitx2* found in *Lepidobatrachus*. The more posterior domain of *Pitx2* found in *Lepidobatrachus* and DEAB-treated *Xenopus* is correlated with a more posterior position of the gastroduodenal loop. Conversely, increasing RA signaling in *Lepidobatrachus* embryos results in an anteriorly restricted *Pitx2* domain, which is similar to that observed in unmanipulated *Xenopus*. As RA receptor complexes have been shown to bind to cis-regulatory regions of the *Pitx2* gene (Kumar and Duester 2010), these heterotopic shifts, together with evidence that *Pitx2* regulates the looping topography of the vertebrate gut tube (e.g., Logan et al. 1998; Muller et al. 2003; Latacha et al. 2005), suggest that
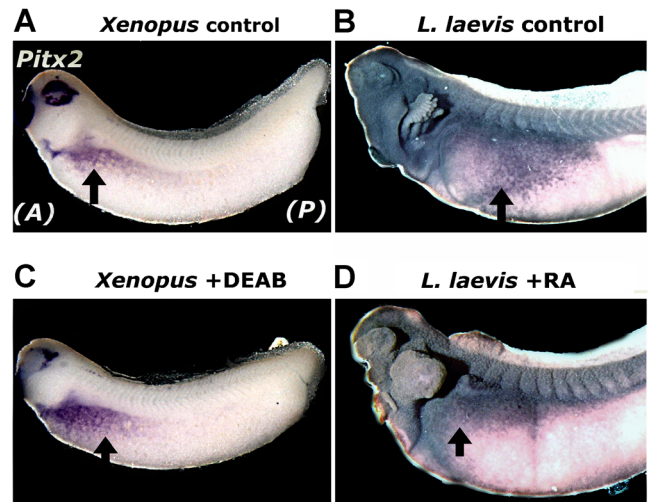
simple RA-mediated shifts in asymmetric gene expression might have facilitated the emergence of a novel gut phenotype without compromising basic digestive function.

In addition to RA, our chemical genetic screen also implicated increased TH signaling in the development of the carnivore gut morphology. TH mediates the formation of adult morphology during amphibian metamorphosis (Ishizuya-Oka et al. 1998; Schreiber et al. 2005), and many craniofacial and digestive features that represent metamorphic changes in most frogs develop precociously in larval *Lepidobatrachus* (Hanken 1992; Fabrezi and Quinzio 2008). For example, early *Lepidobatrachus* tadpoles have adult-like, pepsinogen-producing stomach glands (Carroll et al. 1991) and apparent pancreas hypoplasia (a prerequisite to metamorphic remodeling; Mukhi et al. 2008), suggesting that embryos or larvae have higher TH levels, increased TH receptor (TR) availability or increased TR sensitivity.

It is plausible that the pathways identified by our screen may interact to produce the derived carnivore phenotype. Both TRs and RARs require retinoid X receptor (RXR) as a heterodimerizing partner in order to promote transcription of target genes (Rowe 1997), and elevated TR expression decreases RA responsiveness in anuran embryos (Banker and Eisenman 1993). Increased TH activity in *Lepidobatrachus* could diminish the available pool of RXR heterodimerizing partners and thereby reduce RA signaling. Furthermore, as
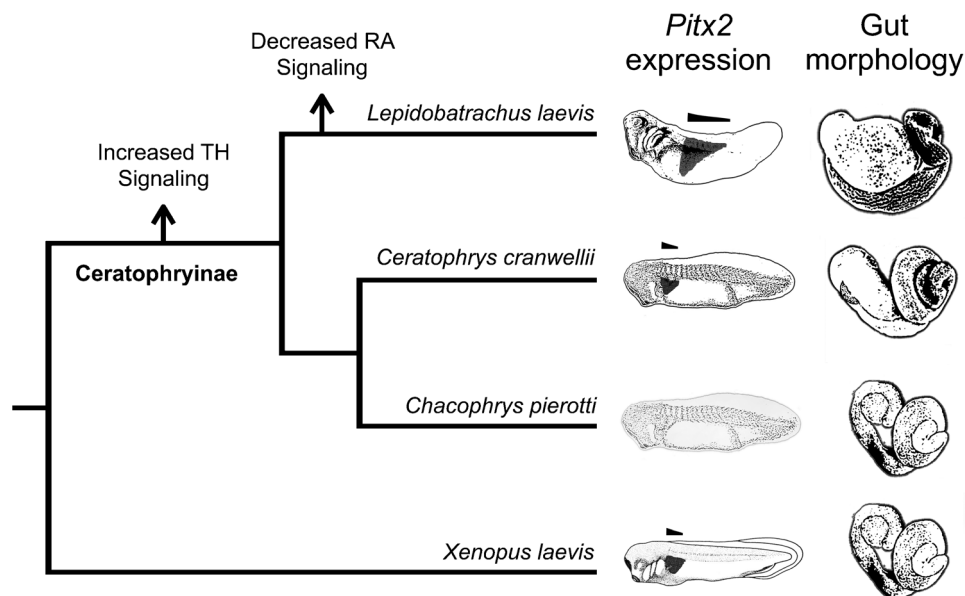
Fig. 6. An evolutionary hypothesis regarding the sequence in which increased thyroid hormone (TH) or decreased RA signaling arose in ceratophryine lineages. The domain of *Pitx2* expression (depicted with dark shading on the embryo diagrams) is anteriorly restricted in *Xenopus* and *Ceratophrys*, but begins and extends more posteriorly in *Lepidobatrachus* (as highlighted by the horizontal bars over each diagram). These domains are correlated with the position of the gastroduodenal loop, which is positioned more dorsally in *Lepidobatrachus*. *Lepidobatrachus* possesses the most extreme larval carnivore morphology, with an enlarged and transversely oriented stomach and a severely reduced, dorsally positioned pancreas. *Lepidobatrachus* and *Ceratophrys* share several TH dependent traits, such as rapid development and precocious pepsinogen production (see Discussion Section; data not shown), which suggests that increased TH signaling occurred in the ancestors of all ceratophryine lineages. In contrast, only *Lepidobatrachus* larvae possess a posteriorly shifted *Pitx2* domain (a pattern that can be reproduced in *Xenopus* using an RA synthesis inhibitor), and *Ceratophrys* respond to inhibited RA synthesis by developing the more derived, carnivore phenotype, which suggests that decreased RA signaling occurred only in *Lepidobatrachus*. The domain of *Pitx2* expression is unknown for *Chacophrys*.

hedgehog signaling is controlled by TH in the metamorphosing anuran digestive tract (Ishizuya-Oka et al. 2001; Hasebe et al. 2008), increased TH activity may upregulate hedgehog signaling, which antagonizes RA activity during gut morphogenesis (Tehrani and Lin 2011). Future studies are needed to determine if any of these pathways were targeted by natural selection during the evolution of the carnivore phenotype, and how they might interact.

The pathways identified in this study exemplify the benefits of using a chemical genetic screen as a tool for discovering developmental routes to evolutionary change. The fact that RA signaling was repeatedly the target of small molecules capable of transforming the ancestral, herbivorous gut phenotype into the derived, carnivorous gut phenotype provides confirmation that the chemical genetic approach works. RA signaling has been implicated in both gut and pancreas development (Lipscomb et al. 2006; Pearl et al. 2009), and we might expect molecules affecting this pathway to be identified by our screen. Likewise, cyclopamine disrupts hedgehog signaling, which plays numerous roles in foregut and pancreas morphogenesis (Tsukui et al. 1999); and actin polymerization, which is inhibited by latrunculin, has been implicated in organ looping (Itasaki et al. 1989, 1991; Ramasubramanian et al. 2006). Yet,

the fact that TH also produced the carnivore phenotype reveals the ability of a chemical genetic screen to identify potential novel pathways. While TH is known to be involved with gut remodeling later during metamorphosis (Stolow and Shi 1995; Ishizuya-Oka et al. 2001; Hasebe et al. 2008), it has not yet been implicated in early gut development (Wheeler and Liu 2012). Thus, a chemical genetic screen has the ability to both corroborate existing candidate pathways and mechanisms and suggest new ones.

Traditional or chemical genetic screens are a springboard for asking evolutionary questions, but fully addressing a hypothesis regarding the origins of a novel trait requires ancillary lines of evidence. First, a screen may identify equivalent mechanisms for producing a particular phenotype, but one or more might actually underlie phenotypic variation in natural populations (True and Haag 2001). Thus, while forward approaches are capable of phenocopying novel traits, demonstrating that specific developmental pathways are relevant to the derived lineage in question (e.g., by performing a reciprocal manipulation in the derived species as in Fig. 3) will considerably strengthen an evolutionary hypothesis. Second, if several pathways are implicated in the emergence of a novel trait, one must consider whether evolutionary changes in these pathways

occurred simultaneously or sequentially. Future investigations will further illuminate the evolutionary sequence in which increased TH or decreased RA signaling arose in ceratophryine lineages. The fact that all ceratophryine lineages including *Chacophrys* (the herbivorous outgroup) exhibit accelerated larval development (a process that is typically TH dependent; Fabrezi 2011), and that *Ceratophrys* respond to inhibited RA synthesis (via DEAB) by developing the more derived, carnivore phenotype, suggests that increased TH signaling arose in the common ancestor of all three ceratophryine lineages but decreased RA signaling arose only in *Lepidobatrachus* (Fig. 6).

In our study, a panel of small molecules generated phenotypes that mimic interspecific morphological variation. Applying chemical genetic screens to embryos of other amenable non-model species with limited genetic tools or molecular resources may reveal the role of specific regulatory networks and their target genes in the development of new adaptive forms, providing a novel route to uncovering general principles of morphological evolution.

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Supplementary Material

The following supplementary material is available online:

Table S1. Small molecules used in *Xenopus* omnivore-to-carnivore screen

Fig. S1. *Xenopus* embryos form a more derived/carnivore-like GD loop morphology upon exposure to small molecules. *Xenopus laevis* embryos were subjected to an acute chemical treatment with cyclopamine (A; a hedgehog signaling inhibitor; 10–40 μM; $P < 0.001$), latrunculin (B; an actin polymerization inhibitor; 60–100 μM; $P < 0.01$) or triiodothyronine (C; thyroid hormone; 0.25–1 mM; $P < 0.05$). The percentage of embryos with the derived/carnivore-like GD loop and organ placement (NF46) is indicated for each molecule. Embryos that exhibit severely disrupted development (e.g., massive edema, tail curvature) or abnormal, uninterpretable phenotypes not resembling either species are classified as "teratogenized." Controls for cyclopamine and latrunculin included DMSO (solvent).

Fig. S2. The expression domain of *Pitx2* in anuran embryos. In situ hybridization patterns (purple stain) indicate the expression of *Pitx2* mRNA in the left lateral plate mesoderm of (A) *X. laevis* (NF 34), (B) *Ceratophrys cranwellii* (GS 21), and (C) *Lepidobatrachus laevis* (GS 21) embryos. Arrows delimit expression domains, which reveal the heterotopic shift in *Lepidobatrachus*. Expression was not detected in the right side (not shown).