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UMI
CRYPTIC METAMORPHOSIS IN THE EVOLUTION OF ANURAN DIRECT DEVELOPMENT

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Anatomy and Cell Biology
University of Toronto

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Abstract

The direct developer Eleutherodactylus coqui lacks a free-living tadpole, hatching as a frog. The role of the metamorphic trigger thyroid hormone (TH) in direct development is unknown. Expression patterns of the TH receptors, TRα and TRβ, which are thought to mediate metamorphosis, were examined to investigate the possibility of their involvement in direct development. Both TR transcripts were present in full-grown oocytes. TR levels in whole embryos were measured using RT-PCR. TRα expression was fairly constant throughout embryogenesis. TRβ mRNA was barely detectable in early embryogenesis, but levels increased substantially by thyroid gland maturation, and transcripts were most abundant during late pre-hatching and early post-hatching stages. These expression profiles are similar to those of Xenopus TRs. As in metamorphosing frogs, precocious TRβ upregulation was induced by TH treatment, indicating that the molecular signals implicated in the control of metamorphosis have been retained in direct development.

The necessity of TH for completion of direct development is demonstrated. Embryos treated with methimazole, a TH inhibitor, were developmentally arrested in a manner reminiscent of metamorphic inhibition in other frogs. Morphological effects were pleiotropic: remodelling of the jaw, limbs, tail, cartilage, musculature and skin were all affected. These remodelling events were contemporaneous with the period of high TRβ expression. Development was rescued by co-treatment with TH; therefore, endogenous TH is necessary for production of the adult morphology even in direct developers.

Recapitulation of the opercular fold, a larval structure previously thought absent from direct developers, is noted. Observation of the development and degeneration of this structure indicates the developmental stage during which the tadpole was likely excised from the ancestral life-history. Despite radical alterations to early embryogenesis, direct developers still undergo a metamorphic period. A new model for direct development is proposed, hypothesizing that the embryonic and metamorphic phases of the ancestral ontogeny are juxtaposed.
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In my Ph.D. toils through the years
For advice, I bent plenty of ears.
So to my mentors, my friends and my peers
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CHAPTER ONE
GENERAL INTRODUCTION

This thesis concerns the developmental mechanisms underlying direct development in the frog, *Eleutherodactylus coqui*, with particular reference to the role of thyroid hormone in this derived ontogeny. Direct development occurs when embryogenesis produces the adult morphology directly, through deletion of the free-living larval period from the life-history of the organism. The term “direct development” has also been used in a different context in the “evolution and development” literature (Peterson et al., 1997). In this thesis, unless explicitly stated, I shall use the term as defined above. In this context, direct development has relatively shallow evolutionary origins, being secondarily derived from a metamorphosing ancestry. Examples of direct development are found in both protostome and deuterostome phyla (Peterson et al., 1997).

One of the best-studied examples of direct development occurs in the sea-urchin genus, *Heliocidaris*. *H. tuberculata* is separated from the congeneric *H. erythrogramma* by only 10 million years; yet the evolution of direct development within the latter species has led to a remarkable degree of ontogenetic divergence from the ancestral mode utilized by *H. tuberculata* and other indirect developing sea urchins (Wray and Raff, 1990; Raff, 1996). Studies on direct-developing ascidians have also identified developmental modifications in the embryogenesis of such anuran species (Swalla et al., 1991). Profound developmental modifications, which occur within a relatively short time frame in evolutionary terms, may be permissible because the obviation of a larval phase rids the organism of any developmental constraints imposed by the existence of this phase (Wake and Roth, 1989). This argument has been used to explain reorganization of the hyolingual apparatus in plethodontid salamanders (Roth and Wake, 1989). In all the above examples, direct developers evolved from metamorphosing ancestors. I shall review metamorphosis, with emphasis on the Amphibia, before examining anuran life-history strategies and direct development.

**Metamorphosis: phylogenetic considerations.**
Metamorphosis is a radical change in morphology coincident with a change in the life-history of an organism. Metamorphosis occurs in biphasic organisms, which have both a larval and an adult phase. Such a dualistic mode of existence is advantageous because the larva and adult can
occupy different ecological niches and hence do not have to compete for the same resources. The distribution of metamorphosing animals throughout the Metazoa attests to the success of this life-history mode, as biphasic animals are found within many protostome and deuterostome phyla (Arthur, 1997).

There is no universally accepted definition of a larva. In fact, Hickman (1999) identifies three conflicting definitions: structural, ecological and morphogenetic regulatory. Each of these definitions is rather extensive, but the structural definition is essentially equivalent to the morphological criterion of a larva proposed by previous authors (cited in Arthur, 1997). This author defines a larva as “a morphological state that is eliminated by the metamorphic transition to the juvenile” (p.271), and I too shall employ this definition, since it is the one most suited to my developmental and morphological approach.

The radical transformation in morphology which occurs during metamorphosis is orchestrated in a precisely controlled manner by specific hormonal signals. The molecular identity of such metamorphic effectors varies between phyla. In insects, ecdysone induces metamorphosis (Gilbert et al., 1996), whereas in amphibia the signal is thyroid hormone (TH) (Kaltenbach, 1996). Intriguingly, while the ligands inducing such radical body plan changes vary, many evolutionarily divergent organisms utilize nuclear hormone receptor signalling to effect metamorphic changes at a molecular level. These pathways are well-described in both insects and amphibians (Gilbert et al., 1996; Chatterjee and Tata, 1992).

While the ability of TH to induce radical changes in conformation is most dramatically evidenced in the Amphibia, several other chordates can be induced to metamorphose by TH. Metamorphosis in the teleost Epinephelus coioides can be precociously induced by TH (de Jesus et al., 1998), manifested by dorsal spine resorption, scale and adult pigment development, and a switch from planktonic to benthic feeding. More subtle metamorphic changes were induced by TH in the zebrafish (Brown, 1997). The ability of TH to induce metamorphosis is not limited to teleosts. The most dramatic changes noted in fish metamorphosis occur in the flounder, where the initial bilateral symmetry of the face transforms so that both eyes are on one side. Flounder metamorphosis can be induced by TH and inhibited by the goitrogen thiourea (Inui and Miwa, 1985). Upregulation of both the thyroid hormone receptor TRα and TRβ mRNAs, and also of
thyroxine (T4) levels occurs around the time of metamorphic climax in the flounder, a situation similar to anurans (Yamano and Miwa, 1998).

The role of TH is not identical in all fish. Metamorphosis in the agnathan Petromyzon is inhibited by TH and induced by the antithyroid agent potassium perchlorate (Manzon et al., 1998). Given the paucity of knowledge regarding vertebrate phylogeny (Carroll, 1988), which of the agnathan or the gnathostome mechanisms of TH deployment is ancestral in fishes cannot be inferred without outgroup comparison. Examination of the urochordates provides some insight into this question. Their tailed pelagic larva clearly indicates their chordate heritage, possessing a dorsal nerve cord and notochord (Romer and Parsons, 1977). During metamorphosis, the tail, notochord and nerve cord degenerate, as the organism adopts a radically different morphology and becomes sessile (Romer and Parsons, 1977). Treatment of Ascidia malaca with TH resulted in precocious tail resorption and body reorganization, implicating TH in urochordate metamorphosis (Patricolo et al., 1981). The metamorphosis-promoting ability of TH in both urochordates and gnathostomes suggests that lampreys have been evolutionarily innovative in utilizing TH to modulate their life-history. Regressing further into the evolutionary past, the sand-dollar Peronella can be induced to metamorphose precociously by TH treatment, and normal metamorphosis in this echinoderm is inhibited by thiourea and potassium perchlorate (Saito et al., 1998). TH was also found to accelerate the formation of the adult skeleton in three other sea-urchin genera (Chino et al. 1994), indicating widespread deployment of TH-mediated metamorphosis throughout the deuterostome phyla.

TH is also important in the development of animals that do not undergo metamorphosis, such as birds and mammals. In both these groups, development can be divided into three periods with respect to TH activity (McNabb and King, 1993). The first period occurs in the absence of zygotic TH synthesis, before the thyroid gland develops. In the second phase, the circulating levels are initially low, and rise as development progresses. During the third phase, adult TH levels are attained. In both mammals and birds, development of the thyroid gland depends on whether the developmental mode is altricial or precocial. Altricial animals hatch or are born at a relatively immature stage relative to precocious developers. The thyroid gland of altricial animals matures later in embryogenesis and adult thyroid levels are only attained after hatching or birth. In precocial animals, which hatch or are born at a more advanced state, the thyroid gland matures relatively early in gestation (McNabb, 1992). Perturbation of thyroid signalling in
mammals, either through TH deficiency or by mutation of the cognate receptor leads to endocrinological and developmental defects (Usala and Weintraub, 1991, Gauthier et al., 1999), including cretinism (Rajatanavin et al., 1997). Normal differentiation of the central nervous system, skeleton, heart and body musculature, lungs and intestine is also affected (McNabb, 1993).

Metamorphosis in the Amphibia.
The class Amphibia consists of three orders: Gymnophiona (caecilians), Caudata (urodeles) and Anura (frogs). All three orders contain members exhibiting a biphasic life history, which is presumed primitive in this class (Duellman and Trueb, 1986). The morphology of caecilians is extremely derived: they lack limbs and have a greatly elongated body with reduced eyes that are covered with skin or bone (Duellman and Trueb, 1986). The larvae, if present, do not look remarkably different from the adults. During metamorphosis, the most notable change is degeneration of the tail fin; closure of the gill slits also occurs at this time (Duellman and Trueb, 1986). In urodeles, the change in morphology from larva to adult is more pronounced, with remodelling of the jaw, tail, gills and skin occurring (Duellman and Trueb, 1986). In anurans, specialization of the larva and the adult is the most extreme; hence metamorphosis in this order results in the most radical changes within the Amphibia. Metamorphic changes in frogs are pleiotropic. Such extensive remodelling facilitates the transition from the aquatic environment inhabited by the tadpole to the terrestrial environment inhabited by the adult frog. Larval mouthparts specialised for herbivory degenerate, and cartilages transform into the adult jaw needed for a carnivorous diet. The gut shrinks, also to accommodate the new feeding mode. Excretion, which is ammonotelic in larvae, becomes ureotelic in adults. This change, due to activation of urea-cycle enzymes in the liver, reduces water-loss, which would otherwise mitigate against terrestriality. The simple epithelium that covers the larva transforms into a thick and specialized epidermis, which affords protection from dessication and may additionally display complex pigmentation patterns. Changes in the kidney and pancreas occur, the axial musculature remodels, the larval tail degenerates, and the limbs extend. These changes are summarized by Duellman and Trueb (1986).

The morphologies of anuran larvae and adults appear dissimilar, presumably because each has been moulded by natural selection to a form which optimizes reproductive success in disparate environments. Additionally, natural selection acts upon the ontogeny of different species, so that
larvae inhabiting one environment may possess modifications from the basic larval body plan. Such ontogenetic selection has resulted in both adaptive radiation and convergent evolution. Tadpoles living in bromeliads are thin and flattened, carnivorous larvae have enlarged mouthparts, and surface-feeders have upturned mouthparts (Orton, 1953). Convergent evolution of body forms is exemplified by the occurrence of similar modifications in different species of mountain-brook larvae, which have evolved independently in at least six anuran families (Orton, 1953). Direct development is another example of such convergent evolution, having evolved at least twelve times within the Anura (Duellman and Trueb, 1986).

Anuran life-history strategies.
Reproductive strategies in the Anura are highly diverse. Duellman and Trueb (1986) have identified 29 different reproductive modes, based on the site of egg development. Included in this developmental smorgasbord are species in which the larvae develop inside a foam nest (Chiromantis); those which brood live embryos in their stomach (Rheobatrachus); “marsupial frogs” in which the tadpoles develop within a parental pouch (Gastrotheca); and the most extreme modification, direct development, where the free-living larva has been deleted from the life-history (Eleutherodactylus). While larval morphologies are highly divergent, the body plan of adult frogs is similar between different species. One possible explanation for this similarity is that the niches occupied by adult frogs vary less than the niches inhabited by their larval precursors. An alternative explanation is that the production of the adult morphology is developmentally constrained. One possible constraint would be the process of metamorphosis. If the transition from larva to adult is contingent on activation of a highly orchestrated signalling cascade controlled by thyroid hormone, then initially divergent ontogenies will converge at the onset of metamorphosis, resulting in the production of morphologically similar adults. Direct developers do not exhibit a free-living larva, and are thought to have eliminated the metamorphic phase from their ontogeny. However, since they produce a perfectly normal frog, the role of thyroid hormone in their development is of particular interest.

Direct development in the Amphibia.
Direct development occurs in all three amphibian orders. In caecilians, which do not undergo many morphological changes, the classification of direct development is associated with the evolution of oviductal gestation (Wake, 1993). In salamanders, ontogenetic modification has allowed the animals to escape from constraints imposed by structural coupling of breathing and
feeding apparati (Roth and Wake, 1989). Its success as a strategy is indicated by the massive radiation of direct developing plethodontids (Wake and Hanken, 1996; Duellman and Trueb, 1986). In frogs, multiple independent occurrences of direct development indicate that developmental mechanisms are evolutionarily labile. Duellman and Trueb (1986) noted that the adoption of a terrestrial life-style correlates with the trend towards direct development. The term “direct development”, the absence of a free-living larval stage, has been employed in slightly different contexts in the literature. Some authors include in this definition animals which hatch as non-feeding tadpoles and undergo metamorphosis after utilizing their endogenous yolk supply. An anuran example of this type of development is *Nectophrynoides malcomi* (Wake, 1980). This author considers *N. malcomi* a direct developer since although a morphologically evident tadpole forms, it does not forage for food and is relatively non-motile. This classification is ecological rather than morphological. Whilst one approach is not inherently better than the other, my thesis is concerned with the mechanistic basis of direct development, so I am using the morphological approach espoused by Arthur (1997). He considers a larva to be “a morphological state that is eliminated by the metamorphic transition to the juvenile” (p271). Using this definition, I class *N. malcomi, Arthroleptella*, which has non-feeding aquatic tadpoles, *Rheobatrachus*, the gastric brooder, and *Pipa*, where tadpoles develop in specialised cysts in the female’s back, as indirect developers. However, as Duellman and Trueb (1986) note, there is tremendous variety in anuran reproductive modes, with a trend in terrestrial frogs towards direct development. Hence, it is difficult to draw a discrete division between direct and indirect developers.

**Direct development in Eleutherodactylus.**

The most extreme form of direct development is exemplified by the genus *Eleutherodactylus*. Embryogenesis in these frogs is radically modified, so that little hint of their metamorphic ancestry can be gleaned by observing their development (Townsend and Stewart, 1985). The extraordinary success of this developmental modification is demonstrated by the abundance of species within the genus. With over 450 species, *Eleutherodactylus* is the most speciose vertebrate genus (Hanken, 1992). Eleutherodactylids appear to be K-strategists, with high parental investment per clutch. In *E. coqui*, eggs are fertilized internally and brooded by the male until hatching (Elinson et al., 1990). About 40 eggs are laid per clutch. The first indication of their unusual development is apparent upon egg-laying. The diameter of an *E. coqui* egg is 3.5mm, compared to 1.3mm in *Xenopus*, which means the volume ratio between the two species’
eggs is greater than 20:1 (Elinson. 1987). These large maternal nutrient stores allow prolonged intra-oval development and direct production of the adult. Little is known about early pattern formation in *E. coqui*, but as in all frogs, cleavage is holoblastic (Elinson. 1987). Development appears fairly unremarkable until late neural plate stage, when both fore- and hind-limb buds appear (Townsend and Stewart. 1985). While similar to amniote limb formation, this contrasts with the situation in metamorphosing anurans, where limb buds are not discernible until some time after hatching (Nieuwkoop and Faber. 1994; Taylor and Kollros. 1946).

Many larval characteristics are lacking in *E. coqui*, including cement gland, lateral line organs, coiled gut, larval mouthparts, certain larval jaw muscles and cartilages (Elinson. 1990). Other jaw muscles and cartilages, which form late in development, initially assume a mid-metamorphic configuration and remodel to produce the adult morphology (Hanken et al., 1992, 1997b). Detailed studies of cranial muscle and cartilage formation in *E. coqui* reveal ontogenetic innovations, which indicate that direct development is more than simply the deletion of the larval phase from the life-history, as the formation of embryonic structures has been modified. During early embryogenesis, some cranial cartilages typical of larval anurans are absent, as is the case of the suprarostrals. Others assume a mid-metamorphic morphology from their inception, examples being the jaw suspensorium and hyobranchial skeleton (Hanken et al., 1992). A similar situation exists in the jaw musculature, where the larval-specific suspensorioangularis does not form, and the initial configurations of the other four muscles normally present in larvae are mid-metamorphic (Hanken et al., 1997b).

Some features reminiscent of larvae have been retained in *Eleutherodactylus*, including several events of jaw ontogenesis. One such example is the insertion of the orbitohyoideus muscle on the ceratohyal cartilage. This novel insertion point evolved in amphibian tadpoles as a feeding adaptation, and at metamorphosis, the insertion reverts to the ancestral vertebrate site on the lower jaw. Although *E. coqui* embryos do not feed, the initial insertion point of the orbitohyoideus is still on the ceratohyal, and later shifts to the lower jaw (Hanken et al., 1997b). Another larval character in *Eleutherodactylus* is the presence of gills, although these are transient in *E. coqui* and have been eradicated from the ontogeny of *E. mubicola* (Lynn. 1942). The most notable larval-type feature is the tail. In *E. coqui*, the tail is large, thin and heavily vascularized (Townsend and Stewart, 1985). It begins to shrink during late embryogenesis, and regresses completely within a few days of hatching. There are no other clearly discernible larval features,
except perhaps the keratinized egg-tooth, which has been proposed to be homologous with the larval mouthparts of tadpoles (Elison. 1990). The egg-tooth is used to allow the tiny froglets to hop out of the jelly capsule: hatching occurs after nearly three weeks of intra-oval development (Townsend and Stewart, 1985).

**Thyroid hormone and metamorphosis.**

The role of the thyroid in amphibian metamorphosis was implicated in 1912 when Gudernatsch fed extracts of various horse tissues to tadpoles and found that larvae exposed to thyroid extracts underwent precocious metamorphosis. Kendall (1915) identified the active component of the thyroid, when he isolated \( T_4 \) from thyroglobulin. Activation of the thyroid axis is necessary and sufficient for induction of metamorphosis. This has been empirically demonstrated by precocious induction of metamorphosis by treatment with TH (Kendall, 1919), and by inhibition of metamorphosis by thyroidectomy (Allen, 1916) or goitrogen treatment (Gordon et al., 1943; Hughes and Astwood, 1944). Additionally, biochemical measurements of circulating TH levels demonstrate a massive rise in the plasma levels of 3,3',5-triiodothyronine (\( T_3 \)) and thyroxine (\( T_4 \)) immediately before the onset of metamorphic climax (Leloup and Buscaglia, 1977). Levels of TH available to different tissues can be spatiotemporally regulated by the action of deiodinases (Becker et al., 1997; Berry et al., 1998a, 1998b), thereby allowing exquisite modulation of the TH-response. These enzymes catalyse removal of iodine groups from specific positions on \( T_3 \) and \( T_4 \). Type II deiodinase converts \( T_4 \) to the biologically potent \( T_3 \), whereas Type III deiodinase converts both \( T_3 \) and \( T_4 \) to inactive derivatives (Becker et al., 1997). The biological relevance of this activity was recently demonstrated by the construction of a transgenic *Xenopus* which overexpresses Type III deiodinase (Huang et al. 1999). The later events of metamorphosis, most notably tail regression, were inhibited. Tail regression requires high levels of TH, and it has been suggested that these elevated levels are required to overcome the activity of endogenous deiodinases, which are particularly active in the tail until very late in metamorphic climax (St. Germain et al., 1994).

That TH can precisely orchestrate specific gene transcription cascades during metamorphic morphogenesis was demonstrated by the finding that TH treatment can induce at least two distinct classes of response genes, early response and delayed response genes (Wang and Brown, 1991, 1993). In detailed spatial expression studies, Berry et al. (1998a, 1998b) have shown that
members of these different classes are expressed in precise domains of the metamorphosing tadpole, in metamorphosing and resorbing tissues.

A molecule which has been proposed to exert an opposite effect to the metamorphic actions of TH is prolactin. Treatment of premetamorphic Rana tadpoles with prolactin prevents tail resorption, although other aspects of metamorphosis, such as forelimb emergence and head remodelling, are not affected (Dodd and Dodd, 1976). Prolactin also prevents T3-mediated tail regression in vitro, with concomitant inhibition of TR autoinduction (Baker and Tata, 1992). These findings have led to the suggestion that prolactin may be the amphibian equivalent of insect juvenile hormone (Chatterjee and Tata, 1992). However, prolactin mRNA increases at the time of metamorphic climax, which is precisely the opposite of what would be expected if prolactin is an endogenous juvenilizing agent (Buckbinder and Brown, 1993). Therefore, the role of endogenous prolactin in metamorphosis remains to be elucidated.

**Thyroid hormone receptors: structure and function.**

Thyroid hormone receptors (TRs), members of the nuclear hormone receptor superfamily, are the molecular effectors of TH-mediated signalling (Chatterjee and Tata, 1992). Evidence for their importance comes from several sources:

i) demonstration of TH-binding ability (Sap et al. 1986; Weinberger et al. 1986).

ii) isolation of naturally occurring mutations in human TRβ, which act as dominant negatives and confer a specific pathology (Usala and Weintraub, 1991).

iii) isolation of thyroid response elements (TREs) in the promoters of thyroid-responsive genes to which TRs bind (Glass et al. 1988).

iv) demonstration of their ability to act as TH-dependent signal transducers. Cells transformed with TR isoforms can respond to ligand by the induction of target gene expression (Koenig et al. 1988).

v) creation of mouse TRα and TRβ mutants (Gauthier et al., 1999). These mutants show specific pathological and developmental defects, including bone, gut and auditory abnormalities.

Like other nuclear hormone receptors, TRs can be divided into four structural domains, called A/B, C, D and E (Fig. 1). As reviewed by Laudet (1997), the A/B domain is involved in transactivation, the C domain in DNA binding, the D domain is a flexible hinge containing
Like other nuclear hormone receptors, TRs can be divided into four functional domains (Laudet, 1997):

1) A/B domain. involved in transactivation
2) C domain. involved in DNA binding
3) D domain. a flexible hinge containing nuclear localization signals
4) E domain. involved in ligand binding.

The particular receptor shown here is *Rana catesbeiana* TRα (Schneider et al., 1993).
nuclear localization signals, and the E domain is responsible for ligand binding, dimerization and transactivation. There are two subfamilies of TR gene, known as TRα and TRβ (Chatterjee and Tata, 1992).

Regulation of thyroid hormone receptors and their molecular interactions appears very complex. In *Xenopus*, alternative splicing of TRβ mRNA in the 5' region can potentially produce multiple different N-terminal isoforms (Yaoita et al., 1990). Additionally, there are two transcription initiation sites, only one of which is TH-inducible (Shi et al., 1992; Kanamori and Brown, 1992). An additional level of complexity is afforded by dimerisation partnerships. While TRs can bind DNA as monomers or homodimers, they also heterodimerize with other nuclear hormone receptors (Laudet, 1997). One known heterodimeric partner family is the retinoic acid receptor (RXR) group. In the absence of TH, TR-RXR dimers repress transcription of target genes, whereas in the presence of TH, these dimers activate high levels of transcription (Wong et al., 1995b). TR and RXR genes are co-ordinately expressed in *Xenopus* metamorphosis, their spatiotemporal expression pattern correlating with remodelling in various tissues (Wong et al., 1995a). It has been hypothesized that TR/RXR heterodimers may mediate TH-signalling in metamorphosis (Shi et al. 1996).

Intriguingly, a number of nuclear receptors, which had no known ligand at their time of discovery, have been identified. Among these so-called "orphan" receptors is the gene TRα2, a TRα variant gene that cannot bind ligand. It is highly expressed in the mammalian brain (Cook and Koenig, 1990), and acts as a dominant negative (Koening et al., 1989). Naturally occurring dominant-negative variants of both TRα and TRβ have also been isolated from hepatocellular carcinoma cells (Lin et al., 1996). Such antimorphs may regulate activity of the receptor in vivo. Other unliganded orphan receptors could potentially modulate TR signalling. However, the approach of "reverse endocrinology" has identified novel ligands for several of the orphan receptors (Kliewer et al., 1999), leading to the possibility of cross-talk between signalling pathways. The complexity of TR signalling means that there is potential for transduction of much information through a precisely organised signalling cascade, which would allow tissue- and temporal-specific responses to TH.
Thyroid hormone receptors in metamorphosis.

Extensive metamorphic remodelling occurs during metamorphic climax (Taylor and Kollros, 1946; Nieuwkoop and Faber, 1994). These events are driven by zygotic hormone synthesis from the thyroid gland, following its maturation at the end of pre-metamorphosis (Dodd and Dodd, 1976). TRα and TRβ are hypothesized to transduce signals from the thyroid axis during anuran metamorphosis (Chatterjee and Tata, 1992; Shi et al., 1996). Both receptors have been cloned from both *Xenopus laevis* and *Rana catesbeiana*, two species of metamorphosing frogs (Brooks et al., 1990; Yaoita et al., 1990; Schneider and Galton, 1991; Helbing et al., 1992). In *Xenopus*, two copies of each receptor have been found (Yaoita et al., 1990). Numerous studies on *Xenopus* and *Rana* have documented expression of TRα and TRβ (Table 1, and references therein). The mRNA of TRα is initially more abundant than that of TRβ. Pre-metamorphic TRα mRNA levels are in 50-fold excess over TRβ mRNAs (Kawahara et al., 1991). TRα mRNA is first detected in early larval stages, and reaches high levels by pro-metamorphosis, well before metamorphic climax (Yaoita and Brown, 1990; Atkinson et al., 1994; Schneider and Galton, 1991). TRβ mRNA levels are very low until late in pro-metamorphosis (Yaoita and Brown, 1990; Davey et al., 1994). A massive upregulation of TRβ mRNA occurs at the onset of metamorphic climax, so that the number of both transcripts is nearly equal (Yaoita and Brown, 1990; Kawahara et al., 1991; Davey et al., 1994). Treatment of pre-metamorphic tadpoles with exogenous TH induces precocious metamorphosis (Etkin, 1950; Tata, 1968). Preceding any morphological change is the upregulation of TRβ expression, which is detectable within four hours of treatment (Baker and Tata, 1992). TRβ is the fastest known TH-response gene (Baker and Tata, 1992), and a significant degree of upregulation occurs in the absence of protein synthesis (Kanamori and Brown, 1992). The rapidity of this induction indicates that TRβ is a sensitive marker for thyroid signalling.

Until recently, studies of thyroid receptor activity in frogs were confined to mRNA level analyses, due to the lack of antibodies recognizing the frog proteins. The production of *Xenopus* TR antibodies allowed the developmental distribution of receptor proteins to be analysed (Eliceiri and Brown, 1994; Fairclough and Tata, 1997). An *in situ*-based analysis found that both TRα and TRβ proteins increased during metamorphosis in the small intestinal epithelium, liver
### Table 1: Comparison of thyroid-related developmental features in *Xenopus laevis* and *Rana (pipiens and catesbeiana).*

<table>
<thead>
<tr>
<th>Stage</th>
<th><em>Xenopus laevis</em></th>
<th><em>Rana</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-metamorphosis (pre)</td>
<td>Nieuwkoop-Faber(^1) (NF) I-54(^2)</td>
<td>Taylor-Kollros(^2) (TK) I-X(^3)</td>
</tr>
<tr>
<td>pro-metamorphosis (pro)</td>
<td>NF 55-57(^2)</td>
<td>TK X-XIX(^3)</td>
</tr>
<tr>
<td>metamorphic climax</td>
<td>NF 58-66(^3)</td>
<td>TK XX-XXV(^3)</td>
</tr>
<tr>
<td>thyroid follicles first visible</td>
<td>NF 49-50(^3)</td>
<td>TK III(^3)</td>
</tr>
<tr>
<td>thyroid gland mature</td>
<td>NF 54(^3)</td>
<td>TK IX(^3)</td>
</tr>
<tr>
<td>TRα mRNA- pre levels</td>
<td>low initially, increase(^4,5)</td>
<td>low (in liver)(^5)</td>
</tr>
<tr>
<td>TRα mRNA- pro levels</td>
<td>peak(^4,5)</td>
<td>increase towards peak(^5,7)</td>
</tr>
<tr>
<td>TRα mRNA- climax levels</td>
<td>high(^4,5)</td>
<td>high, decrease at late stages(^6,7)</td>
</tr>
<tr>
<td>TRβ mRNA- pre levels</td>
<td>low(^4,5)</td>
<td>none (in liver)(^6)</td>
</tr>
<tr>
<td>TRβ mRNA- pro levels</td>
<td>low, slight increase(^4,5,8)</td>
<td>low(^9)</td>
</tr>
<tr>
<td>TRβ mRNA- climax levels</td>
<td>peak(^4,5)</td>
<td>peak(^9)</td>
</tr>
<tr>
<td>T3-induction of TRα mRNA</td>
<td>2-4 fold (in tadpole)(^4,10)</td>
<td>none (in liver)(^7)</td>
</tr>
<tr>
<td>T3-induction of TRβ mRNA</td>
<td>4 to 15-fold (in tadpole)(^4,8,10)</td>
<td>4-fold (in red blood cells)(^7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11-fold (in tadpole)(^11)</td>
</tr>
</tbody>
</table>

**Data from:**

and hind-limb bud (Fairclough and Tata, 1997). T₃ treatment caused upregulation of TRβ protein in these tissues. A quantitative study showed broadly similar results (Eliceiri and Brown, 1994). TRα protein was found at low levels throughout embryogenesis and increased through pre-metamorphosis. This investigation found evidence supporting the post-transcriptional regulation of TRα, because while the amount of this mRNA in head and tail extracts steadily increased between NF52-62 (Nieuwkoop and Faber, 1994), the protein levels remained constant. TRβ protein upregulation closely mirrored the increasing levels of the mRNA. By late metamorphosis, the TRβ protein levels exceeded those of TRα, although the TRα mRNA amount always exceeded that of TRβ mRNA. Essentially, the temporal expression profiles of the TR mRNAs is reflected by the distribution of their proteins. Hence, TR mRNAs are suitable markers for thyroid activity during metamorphosis.

**Thyroid hormone and neoteny.**

The phylogeny of the amphibia is unresolved at present, but many authors argue that lissamphibians are monophyletic (Parsons and Williams, 1963; Laurin and Reisz, 1997; Feller and Hedges, 1998). If the Lissamphibia form a clade, the ancestral lissamphibian was likely metamorphic since in the three extant orders of amphibia metamorphosis is ancestral (Duellman and Trueb, 1986; Wake, 1993). In certain amphibian groups, this ancestral biphasic life-history has undergone dramatic alterations (Roth and Wake, 1989; Hanken, 1992). This thesis is concerned with one such alteration, direct development, which occurs in all three amphibian orders (Wake, 1993). However, another notable change in life-history, neoteny, is evident in some urodeles. Neoteny is a form of paedomorphosis, the acquisition of reproductive capability in a somatically larval form (Gould, 1977). Neotenous urodeles become sexually mature without undergoing metamorphosis, and they remain aquatic, keeping their large gills, gill slits, tail fin and larval skin (Dodd and Dodd, 1976).

Neoteny has evolved independently several times within the urodeles (Rose, 1999). While the developmental mechanisms which facilitate neoteny have not been precisely elucidated, it is evident that more than one mechanism is involved. Some species exhibit facultative neoteny, whereas others are obligatorily neotenic (Dodd and Dodd, 1976). Facultative neotenes, such as *Ambystoma gracile*, have both metamorphic and neotenic populations. The neotenic populations can be induced to metamorphose by treatment with T₄ (Eagleson and McKeown, 1980).
Neotenous ambystomatids undergo some degree of metamorphic remodelling in certain tissues, including remodelling of the skeleton to a late-larval form, transformation from monocuspid to bicuspid teeth, and haemoglobin switching (reviewed by Rose, 1999). Treatment of the axolotl *Ambystoma mexicanum* accelerated development of the adult axolotl form, as evidenced by precocious limb growth and skin transformation (Brown, 1997). These studies are consistent with the possibility that early metamorphic events still occur and are under the control of TH in *Ambystoma*.

Injection of T₄ into the hypothalamus induces metamorphosis in *A. tigrinum* and *A. mexicanum*, indicating that there is a hypothalamic block to metamorphosis in these species (Norris and Gern, 1976; Rosenkilde and Ussing, 1996). Additional aberrations in the thyroid axis of *A. mexicanum* include no Type II deiodinase activity (Galton, 1992), and possibly a decrease in TH activity between mid- to late-larval stages (Rose, 1999). The exact lesion responsible for neoteny in this species is unclear, since it is difficult to dissociate cause from effect with regard to these abnormalities in the thyroid axis (Rose, 1999). Treatment of *Ambystoma* with T₃ caused induction of TRs (Yaoita and Brown, 1990). Hence, it can be concluded that the tissues of *Ambystoma* have retained metamorphic competence.

The tissues of perennibranchiate amphibians are relatively TH-insensitive when compared with ambystomatids. This is true for all cryptobranchids, sirens, proteids, and amphiumids, plus two plethodontid genera (Rose, 1999). These are not sister families (Larson and Dimmick, 1993), so the loss of TH sensitivity must have arisen independently. In the plethodontid *Typhlonectes*, the thyroid is either rudimentary or absent (Uhlenhuth, 1923). This finding explains neoteny in *Typhlonectes* but not in other perennibranchiates, where the endocrine complex is functional (reviewed by Lynn and Wachowski, 1951). Evidence for loss of tissue sensitivity in perennibranchiates derived from reports that they did not respond to thyroid material or iodine compounds (Lynn and Wachowski, 1951). However, some metamorphic transformations have been induced in sirens, cryptobranchids and proteids upon TH treatment (reviewed by Lynn and Wachowski, 1951).

TRα and TRβ have been cloned from the proteid *Necturus*, and conceptual translation of their sequences does not indicate disruptive mutations (Safi et al., 1997). Only TRα expression was detectable (Safi et al., 1997), though it is not specified if larval stages were examined. It has
been suggested that the lack of response to TH in *Necturus* is because it is incapable of TR autoinduction in response to ligand stimulation (Tata, 1996). However, as Rose (1999) has emphasized, while most investigations of perennibranchiate neoteny concentrate on adults, the sparse reports on larval stages indicate that they are capable of undergoing some TH-induced remodelling. Hence, more studies are necessary on the larvae of perennibranchiates before it can be concluded that their life-history has been emancipated from TH control. Comparative investigations on different families may elucidate the exact nature of the lesion(s) in the thyroid axis which cause neoteny in perennibranchiates.

**Thyroid hormone in direct development.**

Several laboratories have investigated the role of TH in *Eleutherodactylus* (Lynn and Peadon, 1955; Hughes, 1966; Elinson, 1994; Jennings and Hanken 1998). Much of this work was performed in the 1950’s and 1960’s, before the advent of molecular techniques, and with a limited supply of embryos. Despite such practical limitations, these early investigations provided some insight into the role of TH in direct development. Hughes (1966) performed both hypophysectomy and thyroidectomy on *E. martinicensis*. When embryos were thyroidectomized before a stage equivalent to Townsend Stewart (TS) 10, tail regression was prevented. When the operation was performed after TS12-equivalent, no effect was noted, leading Hughes to conclude that there was a brief period, equivalent to TS11, when embryos required TH for tail reduction. No effect of thyroidectomy on limb length was observed. Hypophysectomy prevents production of thyroid stimulating hormone (TSH), resulting in thyroid inactivity (Dodd and Dodd, 1976). *E. martinicensis* embryos hypophysectomized between TS9-12 equivalents did not undergo tail regression, but this could be induced by treatment with T4 (Hughes, 1966). Hypophysectomy also slowed degeneration of the pronephros, a metamorphic event in other frogs (Hughes, 1966).

Chemical ablation of the thyroid axis was achieved by treatment with various goitrogens. *E. martinicensis* embryos treated with phenylthiourea (PTU) at TS9-equivalent did not undergo tail regression or pronephric degeneration, events which occurred in embryos treated after TS12-equivalent (Hughes, 1966). These inhibitory effects of PTU were also documented by Lynn, who worked with *E. martinicensis* and *E. ricordii* (Lynn, 1948; Lynn and Peadon, 1955). Treatment of *E. ricordii* embryos with prolactin also inhibited tail regression (Hughes and Reier, 1972). Treatment with T4 precociously induced tail regression and pronephric degeneration (Lynn and Peadon, 1955), but other organ systems appeared unaffected. Hughes and Lynn noted
that only two degenerative changes (tail and pronephric loss) demonstrated thyroid-responsiveness. While these authors noted the similarity between such degenerative changes in *Eleutherodactylus* and those occurring in metamorphosis, the specificity of the effects caused by drug treatments was questioned (Lynn, 1955). It was thought that direct development was likely independent of thyroid functioning (Lynn and Peadon, 1955; Hughes, 1966).

More recently, TH-responsiveness at a biochemical level was demonstrated in *E. coqui*. Treatment of embryos with T\(_3\) resulted in the precocious induction of the urea-cycle enzyme arginase in the liver (Callery and Elinson, 1996). This parallels the metamorphic response of the liver in other frogs (Cohen, 1970). Outgrowth of limb explants did not appear to be stimulated by T\(_3\) (Elinson, 1994). Studies of thyroid gland histology were initially performed in *E. nubicola* by Lynn (1936), and more recently in *E. coqui* by Jennings and Hanken (1998). The latter work showed that the thyroid gland first appears at TS10, and appears to be active during the latter third of development, as judged by colloid production and other morphometric analyses.

**Models of direct development**

Various models have been proposed to explain direct development. Lynn, having observed only a limited response to modulation of the thyroid axis, suggested one possible explanation: emancipation from TH control (Lynn and Peadon, 1955). In this scenario, the embryo becomes freed from the constraints of the thyroid axis and proceeds directly to the formation of the adult. An alternative possibility suggested by these authors and Hanken et al. (1997a) is that the thyroid axis is deployed precociously in embryogenesis. A possible mechanism for such a heterochronic acceleration of the thyroid axis is the provision of maternal stores of hormone in the egg. Jennings (1994, 1997) investigated this possibility, and found both T\(_3\) and T\(_4\) in the egg. However, the relevance of these maternal stores to direct development is unclear, since maternal provisioning of these hormones occurs in both fish and metamorphosing frogs (Weber et al., 1992, 1994).

Jennings and Hanken (1998) noted that thyroid axis activation occurs in late embryogenesis (TS11-15), and is temporally coincident with certain remodelling changes that occur in other frogs during metamorphosis. Such transformations include cranial jaw and cartilage remodelling, and tail regression (Hanken et al. 1992, 1997b; Townsend and Stewart, 1985). Since these events occur before hatching in *E. coqui*, yet after hatching in metamorphosing frogs,
Hanken et al. (1997a) concluded that direct development involves the heterochronic acceleration of the thyroid axis in *Eleutherodactylus*.

**Current investigation**

Direct development involves deletion of the larval phase, but the mechanisms involved in constructing this derived life-history are unclear. In this thesis, I investigate the role of thyroid hormone signalling in *E. coqui* embryogenesis, to determine how the metamorphic phase found in the life-history of biphasic anurans has been modified in direct developers. I employ two methods to examine the role of thyroid signalling. In Chapter Two, I provide correlative evidence of thyroid-mediated development by examination of TRα and TRβ mRNA levels during embryogenesis. In Chapter Three, I document the effects of thyroid inhibition on direct development. In addition, in Chapter Four, I describe the embryological formation and disappearance of a larval structure, the opercular fold, previously thought absent from direct developers. Based upon these investigations, I suggest the developmental stage at which the tadpole may have been excised from the ancestral life-history. Finally, I synthesize my experimental findings into a novel model for direct development.
CHAPTER TWO
ANALYSIS OF THYROID HORMONE RECEPTOR EXPRESSION

Introduction

Thyroid hormone (TH) is instrumental in orchestrating amphibian metamorphosis (Dodd and Dodd, 1976; Shi et al., 1996). As described in the General Introduction, numerous studies on Xenopus laevis, Rana pipiens and R. catesbeiana have documented metamorphic changes, development of the thyroid gland, and expression of the thyroid hormone receptors, TRα and TRβ. Most of the studies concentrating on TR expression and distribution have been performed on Xenopus. In this frog, TRα mRNA expression is detected shortly after hatching and reaches high levels well before the onset of metamorphic climax (Yaoita and Brown, 1990). TRβ mRNA expression is tightly correlated with metamorphosis. First detected at NF40 (Eliceiri and Brown, 1994), it is expressed at very low levels until late in pro-metamorphosis, when it becomes dramatically upregulated. Its expression remains elevated until metamorphosis is complete (Yaoita and Brown, 1990). The protein distribution of the TRs is broadly reflective of the RNA expression pattern (Eliceiri and Brown, 1994; Fairclough and Tata, 1997), suggesting that expression analysis is a good indicator of protein distribution for these genes. TRβ mRNA is induced within a few hours of TH treatment (Baker and Tata, 1992). The speed of this upregulation means that TRβ is a suitable marker for the onset of thyroid axis activity.

The role of thyroid hormone in direct developers is unclear, despite decades of interest (Lynn, 1936; Hughes, 1966; Elinson, 1990). Jennings and Hanken (1998) showed that histologically, the thyroid gland is visible from TS10 in E. coqui, and appears active during the last third of embryogenesis. As TRs are implicated in metamorphosis and TRβ is the earliest inducible metamorphic response gene (Baker and Tata, 1992), I analysed the expression patterns of E. coqui TRs to gain correlative evidence about the temporal regulation of thyroid-dependent signalling during embryogenesis. Since TRβ mRNA is the more developmentally regulated and ligand-inducible of the receptors (Table 1, General Introduction), I constructed a detailed developmental expression profile for this receptor. I examined expression of both receptors in the oocyte, to investigate the possibility that maternal stores of TRs may facilitate direct development. Additionally, I measured the ability of TH to induce receptor expression, to
determine whether ligand-sensitivity has been retained in *E. coqui*. This question is of interest since treatment of early embryos with exogenous 3,3',5-triiodothyronine (T₃) elicits markedly few morphological responses (Callery, 1994).
Materials and Methods

**Embryo culture and drug treatment**

Adult *Eleutherodactylus coqui* were collected from the El Verde Field Station in the Luquillo Forest in Puerto Rico under permits issued by the Departamento de Recursos Naturales, Puerto Rico. Mating pairs were housed at 26°C in a 12 hr light/dark cycle. Embryos were produced from spontaneous matings, staged according to Townsend and Stewart (1985), and cultured in 20% Steinberg's solution (12 mM NaCl; 130 μM KCl; 70 μM Ca(NO₃)₂; 170 μM MgSO₄; 920 μM Tris pH 7.4). 3,3',5-triiodothyronine (T₃) was prepared according to Callery and Elinson (1996). Before any invasive procedures were performed, embryos were anaesthetized in 0.2% 3-aminobenzoic acid ethyl ester (MS222).

**RNA isolation**

RNA was isolated using the Trizol method (BRL). Three to six embryos were homogenized per mL of Trizol, depending on the embryonic stage. A preliminary purification step was inserted after homogenization to remove any solid matter. Extracts were centrifuged at 13,000g for 2 minutes and any pelleted material was discarded. Thereafter, the manufacturer’s protocol was followed. RNA pellets were dissolved in 20-30 μL of diethylpyrocarbonate (DEPC)-treated water and digested with three units RQ1 DNase I (Promega) at 37°C for 15 minutes. Samples were purified by extraction with an equal volume of a 1:1 phenol:chloroform mixture, followed by extraction with one volume of chloroform. RNA was further purified by lithium precipitation. One volume of 8M LiCl solution was added to the samples and precipitation was facilitated by incubation at -70°C for 30 minutes. After a 13,000g centrifugation for 20 minutes at 4°C, RNA pellets were washed in 70% ethanol, air-dried and dissolved in 15μL DEPC-treated water. RNA concentration was quantified using a GeneQuant spectrophotometer (Pharmacia).

**Reverse transcription**

One to five μg of RNA template was denatured at 70°C for 10 minutes in the presence of either 1μM of a gene-specific primer (for PCR cloning) or 40ng random hexamers (for the semi-quantitative PCR assay). Samples were then cooled to 0°C for 5 minutes. Reverse transcription (RT) was performed for 50 minutes at 42°C in the presence of 1X first strand synthesis buffer (BRL). 0.5mM each of dATP, dCTP, dGTP and dTTP, 0.1M DTT, 20 units RNaseguard.
Pharmacia); and 200 units MMLV (BRL). The reaction was stopped by heating to 70°C for 15 minutes and samples were stored at -20°C.

**Cloning**

For cloning the thyroid hormone receptors TRα and TRβ, RNA was derived from embryos which had been treated with 1.5μM T3 for three days commencing at TS11. Five μg RNA template was used in the RT reaction. Gene-specific oligos (α1, β1; see Table 2 for sequences) were used to prime the reaction. These oligos were designed by Schneider et al. (1993), and used in the cloning of the *Rana catesbeiana* thyroid hormone receptors. They are designed to amplify a region extending from the 3' end of the DNA-binding domain to the 5' end of the ligand binding domain. The PCR reactions contained: the cDNA equivalent of 500ng RNA template from the RT reaction; 3mM MgCl2; 1X PCR buffer (BRL); 0.2mM of each dNTP; 2.5 units of Taq (BRL); and 0.5μM of both the appropriate upstream and downstream primers. All primers had restriction enzyme sites added to their 5' ends to facilitate subcloning. The TRα and TRβ primer pairs (α2 and α3, β2 and β3; Table 2) were designed by Schneider et al. (1993). PCR cloning conditions were 94°C for 5 minutes, at which point the Taq was added, followed by 35 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute, with a 10 second extension per cycle. PCR products were separated on a low melting-point TA-0.8% agarose gel. TA is 40mM Tris pH8: 0.1% glacial acetic acid. Bands of the appropriate molecular weight were identified (TRα=254 base pairs: TRβ=258 b.p.). Two microlitre aliquots were sucked out of the gel bands using a pipette and used as template for a further 25 cycles of PCR, to yield sufficient product for subcloning.

Ribosomal protein L8 was amplified from TS12 liver cDNA which had been primed with random hexamers. PCR primers L81 and L82 were designed using PC/GENE. The sequences of the selected primers were highly conserved between *Homo sapiens* and *Xenopus laevis*. Restriction sites were also added to the 5' ends of these primers. Amplification conditions were: 26 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, with a 10 second extension per cycle.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Function</th>
<th>Sequence (5'-3')</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1$</td>
<td>RT primer</td>
<td>ATCACTATCTGGCATGGATG</td>
<td>RCCERBAA 649-668</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>+ primer for cloning</td>
<td>CAGGAATTCAAGTACGATGGTTGTTGTA</td>
<td>RCCERBAA 318-336</td>
</tr>
<tr>
<td>$\alpha_3$</td>
<td>- primer for cloning</td>
<td>GCCAAAGCTTCGCTGGTACCTTCCTGCACCT</td>
<td>RCCERBAA 554-571</td>
</tr>
<tr>
<td>$\alpha_4$</td>
<td>+ primer for PCR assay</td>
<td>CACCCGAAAATCAGTGCCAGC</td>
<td>RCCERBAA 350-369</td>
</tr>
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<td>$\alpha_5$</td>
<td>- primer for PCR assay</td>
<td>GATCAATCTCTCCTCCGAGCC</td>
<td>RCCERBAA 476-497</td>
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<tr>
<td>$\alpha_6$</td>
<td>oligo for probing blots</td>
<td>CCTGGAATGATTCACAGCGGGTACC</td>
<td>RCCERBAA 419-443</td>
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<td>AACTGCGCTAAGGCTTCTAA</td>
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<td>CGTGAATCCCTACAGGGTTAAAG</td>
<td>XL920A 203-226</td>
</tr>
</tbody>
</table>

**Table 2:** Oligodeoxynucleotide sequences used.

Underlined regions of sequence indicate either the EcoRI site (GAATTC) present in the + primers or the Hind III site (AAGCTT) present in the - primers, which were used to create cohesive ends for subcloning. The position of these primers on the orthologous frog genes is indicated. RCCERBAA is *Rana catesbeiana* c-erbA, RCCERBAB is *R. catesbeiana* c-erbAB, and XL920A is *Xenopus laevis* ribosomal protein L8. The position of *E. coqui* primers is given relative to the *Rana* and *Xenopus* orthologues, since the *E. coqui* clones are only partial. See also Figures 3-5 for positions of the primers relative to the sequences of the corresponding genes.
Subcloning and sequencing

PCR products were separated on a TA-agarose gel and bands corresponding to the appropriate molecular weight were excised (TRα=254 b.p.; TRβ=258 b.p.; L8=616 b.p.). DNA was purified using the GENECLEAN II Kit (Bio 101). Both vector (pBluescript SKII+; Stratagene) and PCR products were independently digested with EcoR1 and HindIII, the enzyme sites engineered onto the 5' ends of the primers. Ligation was performed using T4 DNA ligase (BRL) according to the conditions recommended by the supplier for plasmid cloning with cohesive ends. DH5α cells (BRL) were transformed with the ligation products according to the supplier’s protocol. Transformed colonies were identified by blue-white selection after growth on LB-Ampicillin plates spread with isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3 indolyl β-D-galactopyranoside (XGal) according to Sambrook et al. (1989). Positive colonies were amplified and their plasmid DNA was isolated by the Wizard mini-prep method (Promega). Putative clones were sequenced manually by the dideoxynucleotide chain termination method, using the Sequenase version 2.0 protocol (USB). Sequences were identified by BLAST (NIH). The identified TRα, TRβ and L8 clones were named pECTRA, pECTRB and pECL8, respectively.

Northern Blotting

L8 expression was analysed by Northern blotting to verify its suitability as an mRNA level comparator between different developmental stages in E. coqui, as is the case in Xenopus laevis (Shi and Liang, 1994). Four μg RNA isolated by the Trizol method was separated on a formaldehyde gel as described by Sambrook et al. (1989) and blotted onto a Hybond N+ membrane (Amersham) overnight, according to the manufacturer’s protocol. Blots were baked at 80°C for 1.5 hours and pre-hybridized for 30 minutes at 65°C in a hybridization oven in Rapid Hyb buffer (Amersham). The L8 probe was obtained by isolation of the restricted insert from the plasmid and subsequent purification of the insert from a low melting-point agarose gel. Random priming with 32P dCTP was performed using the NEBlot labelling kit (NEB). Probe was purified through a MicroSpin G-50 column (Pharmacia) and incorporation was monitored by a scintillation counter. Overnight hybridization at 65°C was followed by 2X 15 minute low stringency washes at 30°C and 2X 15 minute high stringency washes, also at 30°C. The low stringency washes were in 2X SSC, 0.1% SDS, and the high stringency washes were in 0.1% SSC, 0.1% SDS. 20X SSC is 3M NaCl; 0.3M Na citrate; pH 7. Blots were exposed to autoradiographic film (BioMax MS, Kodak).
**RT-PCR assay**

The design of the RT-PCR assay was based on the method devised for the quantitation of the multidrug resistance gene, *mdr-1* (Murphy et al., 1990). RT was performed as described previously, using random hexamers. One µg of RNA was used as the template. PCR conditions were 94°C for 3 minutes, 80°C for 2 minutes (during which time the Taq was added), followed by 26 cycles of 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute. PCR reaction mixtures were the same as those described previously, except that the primer pairs used were: α4 and α5; β4 and β5; L83 and L84 (Table 2). Amplifications from different primer sets were performed in separate reactions. The molecular weights of the predicted products were verified by comparison with a 100 b.p. DNA ladder (BRL). The molecular weights of the amplified products of TRα, TRβ and L8 were 148, 122 and 448 b.p. respectively.

Since the *E. coqui* TR paralogues share 74% nucleotide identity (see Results), it was necessary to verify that the primers were gene-specific. Specificity of amplification was confirmed by performing PCR using 0.1ng of either ECTRA or ECTRβ vector as template, with either α4 and α5, or β4 and β5 primer pairs.

The linear amplification range for each primer set was determined by amplification of a dilution series of cDNA. In subsequent assays, the input cDNA amount was within the linear range. Input cDNA amounts (in RNA equivalents) for the different genes analysed were: ≤25ng for TRα; ≤75ng for TRβ; and ≤25ng for L8. Controls included minus RT reactions to confirm that DNase I digestion was complete, and water blanks to check for contamination of solutions with template DNA.

PCR products were analysed on a 5% acrylamide/1X TBE gel and electroblotted onto Hybond N+ membrane for 2 hours at 400mA using an Xcell II blotting module (Novex) with 0.5X TBE as the buffer. TBE is 90mM Tris pH 8; 90mM boric acid; 2mM EDTA pH 8. Blots were denatured for 10 minutes by placing them with the DNA side facing upwards on filter paper which had been pre-soaked in 0.4M NaOH. After washing in 2X SSC for 15 minutes, blots were baked at 80°C for 1.5 hours. PCR products were detected by hybridization with gene-specific oligos (α6, β6, L85; Table 2). Oligos were end-labelled with 32P ATP using T4 polynucleotide kinase (BRL) according to the manufacturer’s protocol. Probe was purified through a spin-
column (Pharmacia) and incorporation was checked by a scintillation counter. Blots were pre-hybridized in Rapid Hyb for 30 minutes at 40°C before probe was added. Overnight hybridization at 40°C was followed by 1X low stringency wash and 2X high stringency washes, each for 15 minutes at 40°C. The low stringency wash was in 5X SSC, 0.1% SDS. The α and β high stringency washes were in 0.2X SSC, 0.1% SDS; the L8 low stringency wash was in 1X SSC, 0.1% SDS. Signals were quantitated using the Storm 860 Phosphorimager (Molecular Dynamics) with ImageQuant software.

Within each experimental series, the results for each mRNA were calculated as a percentage of the maximum signal (% max) achieved within that series. In most cases, the cDNA samples were amplified at least twice. When multiple runs were performed the average of the % max values was taken for each sample. The input cDNA quantity/quality between different samples within a series was controlled for by expressing the TRα and TRβ signals relative to the L8 signal from that sample. L8 is expressed at constant levels throughout development (Figure 2). The values quoted in the Results for TRα and TRβ levels are normalized values representing the TR:L8 ratios of the samples.

**Assay strengths and limitations**

Northern blotting of frog TRs has not proven very successful, so other researchers have used alternative methods for measuring the expression patterns of these genes, including a modified primer extension assay (Yaoita and Brown, 1990) and RNase protection (Kawahara et al., 1991). I devised an RT-PCR assay to measure TR expression in *E. coqui*. RT-PCR is advantageous since it allows the detection of rare mRNAs, and is thus a sensitive assay. However, the extreme sensitivity of PCR can lead to variability in amplification, as was noted in some of my experimental runs. Most notably, amplification of the control gene, L8, from early stage samples (oocyte to TS4) proved more difficult than amplification from later stage samples, perhaps because of inhibitory contaminants derived from the yolky cytoplasm. Data points used in the Results were confined to those where there was not a marked discrepancy in L8 amounts between samples being compared.

Since TRα, TRβ and L8 were amplified in separate tubes, the assay cannot account for intra-tube variations. Hence, it is not suitable for detecting subtle changes in gene expression. Consequently, data have been tabulated as “fold-differences”. To design a more sensitive assay,
Figure 2: Northern blot probed with ECL8.

This blot shows that RNA levels of *E. coqui* ribosomal protein L8 remain reasonably constant throughout development. Hence, this gene is a suitable control for use in the comparison of RNA levels between samples of different developmental stages. Four µg total RNA were loaded in each lane. Numbers represent the Townsend Stewart (TS) stage of the embryos from which the RNA was isolated. htc= hatching stage, and 2wk p.h.=2 weeks post-hatching.
TS stage

4  5  6  7  8  10  11  12  13  htc 2wk p.h.
an internal standard would need to be employed (Horikoshi et al., 1993). This technique involves making recombinant constructs of the genes of interest to change the length of the amplified region, so that these exogenous constructs can be differentiated from the endogenous message during amplification. The advantage of this technique is that it is quantitative. By utilizing a known amount of an internal standard, one can determine the number of molecules of the target mRNA present in a sample. While such an assay is more informative than the one employed in this thesis, it is also more complex to employ. Since I wished to document large changes in TR levels, I deemed that a simpler but less precise assay was sufficient. My assay is termed “relative-quantitative”, as it compares the relative amounts of RNA present between different samples. A “quantitative” assay reveals the absolute number of molecules of a mRNA present in a sample. While my assay has limitations, it should be stressed that the data derived from independent experimental series show consistent trends, demonstrating the robustness of the assay.
Results

**Sequence analysis of cloned cDNAs**

Nucleotide sequence analysis confirmed that the two thyroid hormone receptors, TRα and TRβ, and ribosomal protein L8 had been cloned from *E. coqui*. Nucleotide comparisons of the three gene fragments with their closest homologues in the GENBANK database are shown in Figures 3-5. *R. catesbeiana* and *E. coqui* TRα and TRβ share 94% and 91% identity respectively, while the *E. coqui* paralogues share 74% identity. The L8 clone was partially sequenced from both ends. The 5' end has 91% identity with its *Xenopus laevis* orthologue (over nucleotides 175-260 on the *Xenopus* sequence), and the 3' end shares 94% identity over nucleotides 605-745.

**Establishment of RT-PCR assay conditions**

To confirm that the RT-PCR amplification conditions were gene-specific, two levels of control were employed. Firstly, gene-specific primers were designed. Neither the α nor the β primer pairs cross-amplified the paralogous gene, demonstrating the specificity of the amplification reactions (Figure 6A). Secondly, gene-specific oligos were used during end-labelling. To ensure the oligos were gene-specific, cDNA from TS12 embryos was amplified in separate reactions with both the α and β primer sets, and probed with both the α6 and β6 oligos. The oligos did not detect the paralogous gene product, which provides further evidence of the specificity of the assay (Figure 6B). PCR linearity curves are shown in Figure 7. The maximal input cDNA amounts (in RNA equivalents) used were 25ng for TRα; 75ng for TRβ; and 25ng for L8.

**Developmental expression of TR mRNAs**

The developmental expression patterns of TRα and TRβ mRNAs are shown in Figure 8. TRβ mRNA levels changed dramatically as embryogenesis progressed. In this developmental series, TRβ mRNA was barely detectable at TS5 and no band was visible in the TS7 sample. By TS10, when the thyroid gland becomes histologically apparent (Jennings and Hanken, 1998), the message was clearly detectable, and levels continued to rise during the last third of embryogenesis. TRα mRNA expression in the same embryos was much more constant. While some variation was observed between individual samples, no clearly discernible developmental trends were evident. A secondary band of slightly higher molecular weight was detected in some
**Figure 3: TRα sequence comparison.**

Nucleotide sequence comparison between *R. catesbeiana* c-erbAα (RCCERBAA) and its *E. coqui* homologue, thyroid hormone receptor alpha (ECTRA). Sequences of the oligodeoxynucleotides used in experiments (outlined in Table 2) are marked in bold.
Nucleotide sequence comparison of *Rana catesbeiana* and *E. coqui* TRα

| RCCERBAA | TCTGGGATGAGAATTACGTGTGAAATGACGACGAGATCTGGACTGAC 50 |
| RCCERBAA | TCTGGGATGAGAATTACGTGTGAAATGACGACGAGATCTGGACTGAC 50 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 100 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 100 |
| RCCERBAA | CATGCCACAGCCAGCTCTGATGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 150 |
| RCCERBAA | CATGCCACAGCCAGCTCTGATGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 150 |
| RCCERBAA | AGCTGATGTTGAGGAATACTCGGAAGGGAGGAGATGATGTAAGAATGTACTGCTGTGG 200 |
| RCCERBAA | AGCTGATGTTGAGGAATACTCGGAAGGGAGGAGATGATGTAAGAATGTACTGCTGTGG 200 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 300 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 300 |
| RCCERBAA | AGCTGATGTTGAGGAATACTCGGAAGGGAGGAGATGATGTAAGAATGTACTGCTGTGG 350 |
| RCCERBAA | AGCTGATGTTGAGGAATACTCGGAAGGGAGGAGATGATGTAAGAATGTACTGCTGTGG 350 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 400 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 400 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 450 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 450 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 500 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 500 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 550 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 550 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 600 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 600 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 650 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 650 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 700 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 700 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 750 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 750 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 800 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 800 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 850 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 850 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 900 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 900 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 950 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 950 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1000 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1000 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1050 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1050 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1100 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1100 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1150 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1150 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1200 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1200 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1250 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1250 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1300 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1300 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1350 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1350 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1400 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1400 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1450 |
| RCCERBAA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1450 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1500 |
| ECCTRA | ACCCGGAATCAGTGTTCAGCTGCTCCTGAGATCCACATCTGATGAGATGTAAGAATGTACTGCTGTGG 1500 |

RCCERBAA: Rana catesbeiana; ECTR: E. coqui TRα.
Figure 4: TRβ sequence comparison.

Nucleotide sequence comparison between *R. catesbeiana* c-erbAβ (RCCERBAB) and its *E. coqui* homologue, thyroid hormone receptor beta (ECTRB). Sequences of the oligodeoxynucleotides used in experiments (outlined in Table 2) are marked in bold.
Nucleotide sequence comparison of *Rana catesbeiana* and *E. coqui* TRβ

| RCCERBAB | ATGCCTAGCAGCATGCAGG/TACATACCCAGCTATTTGACAAAAAGATGATGA 50 |
| ECTRLB  | AGTAACAGGAAGACGTGCAAGAATATGCTGTCCTTCAAAAGATGCATCGCTG 250 |
| RCCERBAB | AGTAAACAAGCAGACAGAGCTGCAAGAATATGCTGTCCTTCAAAAGATGACCTACA 350 |
| ECTRLB  | AGTAAACAAGCAGACAGAGCTGCAAGAATATGCTGTCCTTCAAAAGATGACCTACA 350 |
| RCCERBAB | AGTAAACAAGCAGACAGAGCTGCAAGAATATGCTGTCCTTCAAAAGATGACCTACA 350 |
| ECTRLB  | AGTAAACAAGCAGACAGAGCTGCAAGAATATGCTGTCCTTCAAAAGATGACCTACA 350 |
| RCCERBAB | AGTAAACAAGCAGACAGAGCTGCAAGAATATGCTGTCCTTCAAAAGATGACCTACA 350 |
| ECTRLB  | AGTAAACAAGCAGACAGAGCTGCAAGAATATGCTGTCCTTCAAAAGATGACCTACA 350 |
| RCCERBAB | AGTAAACAAGCAGACAGAGCTGCAAGAATATGCTGTCCTTCAAAAGATGACCTACA 350 |
| ECTRLB  | AGTAAACAAGCAGACAGAGCTGCAAGAATATGCTGTCCTTCAAAAGATGACCTACA 350 |
| RCCERBAB | AGTAAACAAGCAGACAGAGCTGCAAGAATATGCTGTCCTTCAAAAGATGACCTACA 350 |
| ECTRLB  | AGTAAACAAGCAGACAGAGCTGCAAGAATATGCTGTCCTTCAAAAGATGACCTACA 350 |
Figure 5: L8 sequence comparison.

Nucleotide sequence comparison between *X. laevis* ribosomal protein L8 (XL920A) and its *E. coqui* homologue, L8 (ECL8). Sequences of the oligodeoxynucleotides used in experiments (outlined in Table 2) are marked in bold.
## Nucleotide sequence comparison of *Xenopus laevis* and *E. coqui* ribosomal protein L8

|        | GGCCTAGTCACAAATGGGACGATGTGATCAGGGGACAGAGA | GCTCTGTTTTGAAAGGAGGACAGGAGTGGTCTGCTCAAGCTT | CGGGCTACCTCCTTGCCAAAGGTTGCCT | AGACATTATCCCAGTCCAGGCGGCTGGTCTCCCTTGCCAAAGGTTGCCT | TCCGTGATCTCTTACAGGTTTAPAPAGGACAGAGTTGTTCGTTGCAGCT | TCCGTGATCTCTTACAGGTTTAPAPAGGACAGAGTTGTTCGTTGCAGCT | GAGGGAATCCCATAACCCGAGACGTTTTGTAAGTTGTAGGCAAGAAAGCTCAGCT | GAGGGAATCC....Not sequenced | GAGGGAATCCGTTGCTCCGTGGTGTGGCTATGAATCCTGTTGAACATCCCTTCCGCTG | GCCACGTGTCCCGTGGTGTGGCTATGAATCCTGTTGAACATCCCTTCCGCTG | GTGGTAACCACACACACATTTGCTAGCCCTCACACCAGGAGGATGCC | CCAGGTGGTGCGAAGGCTTTTATTGTGCTGCTGCTGCTGACTGGTGCTGCT | GCCCGGTAACAAGACTGTCRAGGAAAAAGGAGAACCTAAACTTGCTATCTC | GCTAAATACACAAATATTGTAARAAAAACAAAAAA 50 | 100 | 150 | 200 | 250 | 299 | 300 | 350 | 400 | 450 | 500 | 550 | 600 | 650 | 700 | 750 | 800 | 839 |
Figure 6: Specificity controls.

A. Primer specificity

The TRα and TRβ-specific primers do not amplify the paralogous receptors. PCR was performed under the conditions described in the Materials and Methods, except that 1 μCi dCTP was added per reaction, and the products were analysed directly by exposing the gel to film. The control is a water blank.

B. Oligo specificity

The TRα and TRβ-specific oligos (α6 and β6) do not cross-hybridize with the paralogous receptor PCR products. Lanes 1-3 were amplified using the TRα-specific primers (α4 and α5) and lanes 4-6 were amplified using the TRβ-specific primers (β4 and β5). Lanes 1 and 4 are water blanks, lanes 2 and 5 are -RT controls, lanes 3 and 6 contain TS12 cDNA.
**Figure 7: PCR linearity assays**
cDNAs from embryos in the last third of embryogenesis were used as templates for the linearity assays. The linear range of amplification is shown by a straight line. In the case of TRα, the solid curved line represents the actual signals detected, whereas the dotted straight line represents the predicted linear plot. The maximum cDNA levels (in terms of the RNA equivalents derived from the cDNA reaction) used in subsequent assays were: 25ng for TRα, 75ng for TRβ, and 25ng for L8.
PCR Linearity Curves

TRα

TRβ

L8
Figure 8: Developmental expression of TRs.

Expression of TRs throughout development, as determined by RT-PCR. Expression of the receptors in the various stages is compared with the expression of ribosomal protein L8, a gene which is not developmentally regulated.

Embryos were staged according to Townsend and Stewart (TS) (1985). 2wk p.h.=2 weeks post hatching.
Developmental expression of TRs

blank  TS5  TS7  TS10  TS12  TS13  hatch  2wk p.h.

TRα

TRβ

L8 (con)
TRβ amplifications (Figure 8). Given the specificity of the TRβ oligo probe, it is likely that this is recognising TRβ. One possibility is that an alternatively spliced isoform of TRβ is being recognised. Alternatively, a small proportion of the TRβ amplification product may have been retarded in the gel (perhaps due to conformational change), resulting in a secondary band.

To confirm the differences in developmental induction of the two genes during embryogenesis, TRα and TRβ mRNAs in early and late stage embryo batches were measured using the RT-PCR assay described in the Materials and Methods (Table 3). In two out of three cases. a modest increase in TRα message was detected in the later stage relative to the earlier one. However, in the same samples, there was a massive increase in TRβ mRNA. In three out of four cases, the TRβ mRNA levels at the later stage were over an order of magnitude greater than those found in the early stage embryos.

Since profound changes in TRβ mRNA levels were detected, and upregulation of this gene is temporally coincident with the onset of metamorphosis in both *Xenopus laevis* and *Rana catesbeiana* (Yaoita and Brown, 1990; Davey et al. 1994), its expression profile during embryogenesis was examined using the RT-PCR assay (Figure 9). All values were normalized to L8. The three series shown here were then integrated by adjusting TS15/hatching levels of TRβ mRNA to 100, and plotting the other points relative to this standard. Hatching occurs at the end of TS15, and so embryos in this group may vary in age by approximately three days. While one series (green) shows comparatively high early levels, in two other series. TRβ mRNA levels in embryos TS7 or younger were less than 10% of the TS15 values. These results are in agreement with the previous results in Table 3; thus, in seven independent experiments (Table 3, Figure 9). TRβ mRNA levels show a substantial increase between early (TS4 to TS7) and late stages (TS14 to two weeks post-hatching). TRβ expression was detected as faint bands in nine pre-TS9 embryo samples, demonstrating that TRβ mRNA is present in embryos before the development of the thyroid gland, although at very low levels.

At TS10, the thyroid gland is developing but does not yet contain colloid (Jennings and Hanken, 1998). The TRβ mRNA level has begun to increase by this point and is between approximately 30% and 50% of its hatching level in the TS10 samples analysed. A four-fold increase in TRβ mRNA was observed between TS4 and TS8 in a single amplification round from another set of
Table 3: Upregulation of TR mRNAs during development.

mRNA levels of each receptor were measured in late-stage embryos relative to the levels present in early-stage embryos. All values were normalized relative to L8 expression levels TRβ is upregulated much more than TRα in the same embryos. One of the sample sets was not measured for TRα (n.d.).
Figure 9: Quantitation of TRβ mRNA levels during embryogenesis.

Scattergraph of TRβ mRNA levels in various developmental stages as quantitated by RT-PCR and phosphorimager analysis. As in all other experiments, the values shown have been normalized to L8 RNA levels. Three developmental series are represented on the graph. In each case values are expressed relative to the TS15/hatching value, which was arbitrarily set at 100. Post-hatching stages are abbreviated as p.h. TRβ mRNA levels, low in early stages, increase as embryogenesis proceeds, reaching maximal levels after hatching.
embryos (not included in Figure 9). This finding provides a preliminary indication that TRβ mRNA upregulation begins before the thyroid gland contains follicles. This appears to be the case in *Xenopus* too, where a sensitive RT-PCR assay demonstrated that TRβ mRNA levels increase in slight but consistent increments from shortly after NF40 (Kanamori and Brown, 1992). In *E. coqui*, the TS13 TRβ mRNA levels vary quite widely, from 44% to 108% of the hatching levels within these experimental samples. TS13 can be longer than two days in duration, which may account for some of the variability. Additionally, the thyroid appears to be most active at this point, since its follicle volume and number peak at TS13 (Jennings and Hanken, 1998). The developmental state of thyroid, which is changing throughout TS13, may influence the receptor message levels. Nevertheless, in all cases there was an increase in the TRβ mRNA level at TS13 in comparison with the previously assayed stage. By TS15, when the froglets look morphologically adult, TRβ mRNA levels are still increasing, and reach their maximum detected values some time after hatching. The maximum developmental variation in TRβ mRNA levels observed was a 35-fold increase between TS6 and 1 week post-hatching. Even at the last point examined, two weeks after hatching, TRβ mRNA was higher than it had been at any other stage of development, 20% higher than the hatching value. In *X. laevis*, TRβ mRNA levels drop rapidly upon the completion of tail resorption (Yaoita and Brown, 1990). In contrast, TRβ mRNA levels in *E. coqui* remain high long after completion of tail resorption, which occurs within a few days of hatching.

**Presence of maternal TR mRNAs in the oocyte**

It has been hypothesized that direct development may result from heterochronic acceleration of the thyroid axis into early embryogenesis (Hanken et al., 1997a), possibly facilitated by maternal TR stores (Rose, 1999). To address this possibility, the *E. coqui* oocyte was assayed for the presence of TRα and TRβ mRNAs. Full-grown oocytes were isolated from the ovary, hence they were not matured. The amount of each mRNA present was measured relative to TS12 levels, a stage at which both receptors are easily detectable. Each experiment was duplicated, and the mean value was tabulated. Oocytes contained both TR mRNAs (Figure 10 and Table 4). Interestingly, they were loaded with TRβ mRNA, at a level which exceeds the maximal embryonically produced amount, being four and ten-fold higher than at TS12 in the two samples examined.
Figure 10: Maternal and TH-induced TR mRNA levels.

A. Maternal TR mRNA levels. TRα mRNA levels are at least as high as at TS12. TRβ levels are even higher than at TS12, when upregulation of embryonic expression is already underway, indicating that there are maternal TR stores in the oocyte.

B. TH-induction of TR mRNAs. TRα mRNA is not induced by T₃ to a visually detectable extent. TRβ mRNA induction, as visualized on an autoradiogram, is depicted here.
Table 4: Oocyte TR mRNA levels.

TRα and TRβ mRNA levels were measured in both oocytes and TS12 embryos. The amount in the oocyte is expressed relative to the amount found in the TS12 embryo. Two different series are shown: each set of cDNA was analysed twice and the average value of each relative quantitation was recorded. All values were normalized relative to L8 expression levels.

<table>
<thead>
<tr>
<th>Series</th>
<th>Relative mRNA levels (oocyte:TS12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>TRα: 1</td>
</tr>
<tr>
<td>II</td>
<td>TRα: 3</td>
</tr>
</tbody>
</table>

Table 5: TH-induced upregulation of TR mRNAs.

Early stage embryos (TS5-6) were treated for 2-3 days with 1.5μM T3. RNA was extracted from both control and hormone-treated samples at TS7-8 and TR mRNA levels were measured. The relative quantitation was performed twice on each cDNA and the average induction recorded. All values were normalized relative to L8 expression levels.

<table>
<thead>
<tr>
<th>Start stage</th>
<th>End stage</th>
<th>Days treatment</th>
<th>Fold increase</th>
</tr>
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<td>TRα: 1</td>
</tr>
<tr>
<td>TS5</td>
<td>TS7</td>
<td>3</td>
<td>TRα: 1</td>
</tr>
<tr>
<td>TS6</td>
<td>TS8</td>
<td>3</td>
<td>TRα: 2</td>
</tr>
</tbody>
</table>
**TH-mediated induction of TRs**

To determine whether TH-mediated signalling has been conserved at the molecular level in *E. coqui*, early stage embryos were treated for two to three days with 1.5μM T₃, which was previously shown to induce tail resorption (Callery, 1994). When the controls reached TS7/8 their TR mRNA levels were measured. Each experiment was duplicated, and the mean value was tabulated. TRβ mRNA was upregulated three to six-fold by TH treatment, whereas in only one out of three cases was even a doubling of TRα mRNA observed (Figure 10 and Table 5). This result is similar to the situation in *Xenopus* where TRα mRNA showed only a two- to four-fold induction, whereas a four- to fifteen-fold induction was observed for TRβ mRNA (Yaoita and Brown, 1990; Kanamori and Brown, 1992; Baker and Tata, 1992).
Discussion

Examination of TRα and TRβ mRNA levels during *E. coqui* embryogenesis indicates that temporally, the expression patterns of these genes are similar to those of metamorphosing frogs. TRα levels remained relatively constant throughout embryogenesis, with the maximal detected increase being three-fold between TS7 and hatching stages. Even at the earliest stage examined, TS4, which is only a day after the neurula stage, TRα mRNA was clearly detectable. The expression of TRα mRNA at fairly high levels early in *E. coqui* development and well before the onset of thyroid maturation mirrors the situation in *Xenopus*. In the latter frog, zygotic TRα mRNA is detectable within a few days of fertilization (NF35), and reaches substantial levels shortly afterwards (Yaoita and Brown, 1990), well before maturation of the thyroid gland at NF54 (Nieuwkoop and Faber, 1994).

The TRβ mRNA expression profile much more accurately predicts metamorphosis in *Xenopus*. TRβ mRNA levels are low during the pre-metamorphic stages but increase dramatically around NF54, just before the onset of metamorphic remodelling (Yaoita and Brown, 1990, Kanamori and Brown, 1992). Similarly, in *E. coqui*, there is a massive upregulation of TRβ mRNA between early embryogenesis and late embryogenesis or early post-hatching stages. As much as a 25-fold increase was detected between TS7 and hatching. Low levels of TRβ mRNA were detectable in early-stage embryos, demonstrating that some TRβ message is present before activation of the thyroid gland. TRβ mRNA levels in early embryogenesis (TS4-7) were generally lower than those found in slightly older embryos (TS8-10), indicating that TRβ mRNA upregulation initiates before thyroid gland maturation (Jennings and Hanken, 1998). While the dramatic increase in *Xenopus* TRβ mRNA levels occurs after thyroid maturation, a sensitive quantitative PCR assay indicates that there is a slight increase in TRβ mRNA levels during late premetamorphosis (Kanamori and Brown, 1992), before the thyroid is mature.

In *E. coqui*, TRβ mRNA levels increase further as the thyroid gland becomes active during the later phases of development. During this period (TS12-15), some remodelling events reminiscent of metamorphosis in other frogs have been reported. These include tail regression (Townsend and Stewart, 1985), limb elongation (Elinson, 1994), and jaw muscle and cartilage remodelling (Hanken et al., 1992, 1997b). While direct involvement of TH in these events has not been demonstrated.
(with the exception of the tail), the temporal correlation of their occurrence with the time of peak TRβ mRNA expression provides a tempting parallel with the metamorphic period in other frogs.

The highest TRβ mRNA levels detected were in the post-hatching froglets. This is surprising, since by this time, the animals have generated the adult morphology. In *Xenopus*, after completion of tail resorption at NF66, TRβ mRNA levels drop to very low levels (Yaoita and Brown, 1990). In *E. coqui*, tail resorption can continue for up to a week post-hatching, but by two weeks post-hatching there is no tail left, so remnants of this organ cannot be responsible for the elevated TRβ mRNA levels at two weeks post-hatching. One possibility is that internal remodelling has not yet finished, hence TH-mediated signalling is still occurring. TH is involved in neural development in mammals (Iglesias et al., 1996), and iodine deficiency causes cretinism in humans (Morreale de Escobar et al., 1987). Thyroid hormone is also involved in neural remodelling in anurans (Denver, 1998). TRβ mRNA expression is particularly enriched in the brain during *Xenopus* metamorphosis (Kawahara et al., 1991). Newly hatched *E. coqui* froglets are incapable of co-ordinating feeding movements until a couple of weeks after hatching (Schosser and Roth, 1997), so perhaps some neural rewiring is still occurring. Also, upregulation of the liver-enzyme arginase continues well into post-hatching development (Callery and Elinson, 1996); this enzyme is TH-responsive in both *E. coqui* and metamorphosing frogs. The gut is not yet mature, and production of the adult digestive tract is controlled by TH in biphasic anurans (Shi and Ishizuya-Oka, 1996).

Tail regeneration is the last metamorphic event in *Xenopus* (Nieuwkoop and Faber, 1994). If the above events in *E. coqui* are homologous to TH-mediated metamorphic remodelling, then they must have been heterochronically retarded relative to external remodelling events. This could be due to changes in tissue sensitivity to TH. Investigation of the spatial distribution in *Eleutherodactylus* tissues of TRβ and Type III deiodinase, which inactivates TH (Becker et al., 1997), may indicate whether this hypothesis is likely. Another possibility is that the controls governing downregulation of TRβ mRNA after generation of the adult morphology have been lost in *Eleutherodactylus*.

It has been suggested that direct development results from early activation of the thyroid axis (Lynn and Peadon, 1955; Hanken et al., 1997a). One hypothesis is that such precocious activity could be mediated by maternal stores of TH and/or TRs (Hanken et al. 1997a; Rose, 1999). To investigate this possibility, TR levels in primary oocytes were quantitated relative to levels at TS12, when
significant amounts of both receptor mRNAs are detectable. High levels of both receptors were found in the oocytes. Receptor abundance of TRβ mRNA appeared at least equivalent to the maximal levels detected in post-hatching froglets. This finding is consistent with the hypothesis that maternal receptor stores mediate precocious formation of adult structures in *Eleutherodactylus*. However, TRα mRNA is abundant in *Xenopus* oogenesis (Kawahara et al., 1991), and mRNA and protein of both receptors are detected in the primary oocytes of *Xenopus* (Eliceiri and Brown, 1994). Quantitation of the levels found in *Xenopus* oocytes relative to metamorphic stages is not recorded in the literature. TRα and TRβ mRNAs and TRβ protein are reported to disappear after oocyte maturation in *Xenopus* (Eliceiri and Brown, 1994), although TRα protein is still present.

The function of these maternal stores is not clear even in the model system *Xenopus*. although the TRα protein is quite stable (Eliceiri and Brown, 1994). Chatterjee and Tata (1992) suggested that upon production of ligand, maternal receptor stores in *Xenopus* may be responsible for the initial triggering of the metamorphic cascade. Before it is possible to assess whether maternal TR mRNA stores have been deployed in a unique fashion during direct development, the role of these maternal stores in metamorphosing frogs must be elucidated. However, if *Xenopus* TR mRNA levels are low relative to metamorphic levels, this would suggest that the accumulation of high levels in *E. coqui* oocytes is autapomorphic (unique) to *Eleutherodactylus* and hence may be a derived feature important in direct development.

TH can cause autoinduction of TRs (Kawahara et al, 1991; Kanamori and Brown, 1992). Such autoinduction has been suggested to be responsible for the rapid upregulation of TRβ mRNA during metamorphosis (Baker and Tata, 1992). In *Xenopus*, TRβ mRNA is highly inducible by T3 (Yaoita and Brown, 1990). Induction is rapid, the amount of RNA present increasing by 10% within 3 hours of treatment (Tata, 1996). A significant amount of upregulation can occur even in the absence of protein synthesis (Kanamori and Brown, 1992), hence TRβ is classed as an “immediate early” response gene (Chatterjee and Tata, 1992). TRα is less ligand-inducible, showing less upregulation when premetamorphic *Xenopus* tadpoles are treated with T3 (Yaoita and Brown, 1990). It does show some inducibility, with the amount of mRNA increasing by 10% within six hours of T3 treatment (Tata, 1996). When premetamorphic *Xenopus* tadpoles are treated with T3, they undergo a form of precocious metamorphosis, the most obvious changes being the elongation

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of Meckel’s cartilage and tail regression (Tata, 1968). Such morphological changes are preceded by precocious upregulation of TRβ mRNA (Yaoita and Brown, 1990).

In *Eleutherodactylus*, development appears refractive to TH-induced acceleration, as only tail regression, pronephric degeneration and arginase induction have been induced precociously by T₃ (Lynn and Peardon, 1955; Callery and Elinson, 1996). To determine whether *E. coqui* TRs are capable of autoinduction, early stage embryos were treated with T₃. TRα mRNA was not induced, which is unsurprising given its limited responsiveness in *Xenopus* and its substantial presence early in *E. coqui* embryogenesis. TRβ mRNA was ligand-inducible, like its *Xenopus* orthologue, increasing up to 6 fold upon T₃ treatment. It should be mentioned that a 1.5µM T₃ concentration was used in these induction experiments; this is significantly higher than the 100nM concentration used in many *Xenopus* experiments (e.g. Tata, 1996; Table II). Previously, 200nM T₃ had only a slight effect on tail regression (Elinson, 1994). 1.5µM was used here as it elicits massive tail regression. Induction of TRβ mRNA by lower doses of ligand was not investigated. However, since the circulating levels of T₃ caused by exogenous treatment are unknown, it would be difficult to compare directly the relative responsiveness of the *Xenopus* and *E. coqui* TRβ genes to a specific dose of T₃ in vivo. The auto-induction of TRβ mRNA by T₃ indicates that TH-responsiveness has been conserved at a molecular level in *E. coqui*.

This experimental investigation has been confined to RNA level-analyses. While the expression patterns of the *Xenopus* TRs broadly correlate with the protein distribution, there is some evidence for post-translational regulation of TRs in this organism (Eliceiri and Brown, 1994). Since protein distribution is more reflective of the biological activity of a gene than is RNA distribution, analysis of TR protein distribution in *Eleutherodactylus* would be a fruitful avenue of future study. Such analyses will be contingent upon the availability of suitable antibodies, whether they be cross-reactive *Xenopus* antibodies or novel *E. coqui* TR antibodies.

In summary, the developmental expression of TR α and TRβ mRNAs in *E. coqui* is similar to that found in metamorphosing frogs. TRα mRNA is detected early in development, and its levels remain fairly constant, even after thyroid gland activation. TRβ mRNA is very low in early embryogenesis, but levels increase substantially around the time of thyroid maturation and remain high during the last third of embryogenesis, when the thyroid gland appears active (Jennings and
Hanken. 1998) and remodelling events reminiscent of metamorphosis occur. The temporal coincidence of high TRβ mRNA expression, thyroid activity and morphological remodelling suggests that E. coqui undergoes a cryptic metamorphosis in ovo. The possibility that precocious embryonic accumulation of TRβ mRNA may mediate the early events of direct development is not supported by these data, since high levels of TRβ mRNA are not found before development of the thyroid gland. Maternal TR mRNAs in primary oocytes could conceivably mediate direct development if they are translated during maturation, but such arguments remain speculative until more is known about the role of maternal TRs in metamorphic frogs. E. coqui, while appearing morphologically refractive to TH, has retained its responsiveness at a molecular level, as demonstrated by the ligand induction of TRβ.
CHAPTER THREE
DEVELOPMENTAL EFFECTS OF THYROID INHIBITION

Introduction

The necessity of thyroid signalling during metamorphosis was demonstrated by the inability of thyroidectomized tadpoles to metamorphose (Allen, 1916). Such surgical perturbations were complemented by chemical perturbations of thyroid signalling, whereby metamorphosis was prevented by treatment with goitrogens including phenylthiourea (PTU), perchlorate and methimazole (Lynn and Wachowski, 1951; Brown, 1997). In the genus *Eleutherodactylus*, inhibition of the thyroid axis has also been attempted both embryologically and chemically. The hypophysis indirectly regulates the thyroid through thyroid stimulating hormone, TSH. Either hypophysectomy or thyroidectomy before TS12 prevents tail and pronephric degeneration (Hughes, 1966). This effect is also produced by treatment with the goitrogen phenylthiourea (PTU) (Lynn and Peadon, 1955; Hughes, 1966). While these authors noted the similarity between such degenerative changes in *Eleutherodactylus* and those occurring in metamorphosis, they were unconvinced of the specificity of the effects caused by drug treatments. Hence, no consensus on the role of thyroid hormone in direct development was attained (Lynn, 1948; Lynn and Peadon, 1955; Hughes, 1966).

In this chapter, I describe the effects of methimazole inhibition of the thyroid axis. Methimazole is a goitrogen which is used to inhibit metamorphosis in frogs (Buckbinder and Brown, 1993; Becker et al., 1997). It acts by inhibiting oxidation of iodide in the thyroid, so that iodine cannot be incorporated into organic form (Nakashima et al., 1978). I provide detailed descriptions of the effects of methimazole on direct development, together with evidence for the specificity and reversibility of its actions. I report similarities between remodelling events in *E. coqui* and metamorphic remodelling events.
Materials and methods

Embryo culture and drug treatment
Embryos were procured and cultured as described in the previous chapter. A 1M methimazole stock (Sigma) was shielded from the light and used to create a working solution of 1mM methimazole in 20% Steinberg’s solution. For the rescue experiments, embryos were immersed in a solution of 1mM methimazole and 50nM 3,3',5-triiodothyronine (T3) in 20% Steinberg’s solution. Embryos were anaesthetized in 0.2% 3-aminobenzoic acid ethyl ester (MS222) before any manipulations were performed.

Morphometry
Embryos were anaesthetized in 0.2% MS222 and their dimensions were measured using an ocular micrometer in a Nikon dissecting microscope. Statistical analysis was performed using the KwikStat 4 program (TexaSoft). The non-parametric Kruskal-Wallis test was used to compare the different treatment groups, and the significance of the differences between means was assessed by the Tukey multiple comparisons test, also using the KwikStat 4 package.

Whole-mount immunochemistry
Embryos were fixed in Dent’s fixative (80% methanol; 20% DMSO) and stored at 4°C. Before processing, embryos were bleached in one part H2O2: two parts Dent’s fixative, and their skin was dissected away, leaving the abdomen intact. Embryos were rehydrated through a methanol series into TBS (150mM NaCl; 10mM Tris pH8). Samples were washed in TBT (0.1% Triton-X-100 in TBS) for four 30 minutes and blocked for 20 minutes. Blocking solution is TBT plus 10mg/ml bovine serum albumin (Fraction V. Sigma). Primary antibody incubation occurred overnight at 4°C. The 12/101 monoclonal mouse antibody, which stains skeletal muscle, was obtained as a hybridoma culture supernatant from the Developmental Studies Hybridoma Bank, Iowa, and used at a 1:20 dilution in blocking solution. Following three 2 hour TBT washes and a 20 minute block, a goat anti-mouse alkaline phosphatase-conjugated secondary antibody was added at a 1:500 dilution in blocking solution and samples were incubated overnight at 4°C. Three 2 hour TBT washes were followed by two 10 minute rinses in alkaline phosphatase (AP) buffer (100mM NaCl, 50mM MgCl2, 0.1% Tween 20, 5mM levamisole, 100mM Tris pH 9.5). Muscle staining was visualised by addition of 660µg of 4-Nitroblue tetrazolium chloride (NBT, Boehringer Mannheim) and 165µg of 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP, Boehringer
Mannheim per mL of AP buffer. The colour reaction was stopped by a brief rinse in water, followed by fixation in 10% buffered formalin (3.7% formaldehyde, 100mM phosphate buffer, pH 7.4).

*Cartilage staining.*

Embryos were processed for cartilage staining with Alcian Blue according to Klymkowsky and Hanken (1991).
Results

**Thyroid-dependency of direct development.**

TS8/9 embryos (n>50) were treated with 1mM methimazole, an inhibitor of thyroid hormone synthesis which prevents metamorphosis in *Xenopus* (Buckbinder and Brown, 1993). Development was normal until TS12, at which stage embryogenesis arrested (Figure 11). The ontogenetic effects of methimazole were pleiotropic: limb growth was stunted, the tail failed to resorb, adult skin patterning was absent, the head and body shape remained embryonic in character, and ventral suturing of the head and body skin did not occur. Treatment of early embryos (TS4-6, n=24) did not enhance the effect of methimazole, presumably because zygotic hormone synthesis had not yet begun.

While development was arrested, the embryos appeared quite healthy, indicating that the developmental effects induced by 1mM methimazole were not caused by drug toxicity. Treatment of embryos with 2.5mM methimazole did not result in an obvious enhancement of phenotype, whereas 5mM and 10mM concentrations were toxic. Therefore, 1mM was the concentration used. Methimazole-treated embryos developed toepads and eyelids, which normally appear after TS13, indicating that the effects of methimazole were due to specific inhibition of the embryonic thyroid axis, and not merely to general developmental retardation. This was conclusively demonstrated by rescuing ontogenesis with exogenous thyroid hormone. Embryos (n>50) treated between TS9 with 1mM methimazole, were co-treated with 50nM T₃ for 8 days, starting at TS13. Development was substantially rescued (Figure 11). Some rescued embryos were morphologically indistinguishable from controls, although in many cases lower jaw remodelling was incomplete. Whereas methimazole-arrested embryos remained aquatic, the rescued embryos adopted a terrestrial habitat, often hopping out of the buffer and suspending themselves from the petri-dish lid, as did their sibling controls.

Morphometric measurements were made to determine quantitatively the extent and significance of differences between control, methimazole-inhibited and T₃-rescued embryos (Figure 12). Characters selected for morphometric analysis were hindlimb and tail length, jaw shape, and ventral body width. In all cases, the methimazole-treated embryos were significantly different from their untreated siblings. With the exception of thigh length, rescue appeared complete, as no significant differences were found between control and T₃-rescued embryos.
**Figure 11**: Methimazole inhibition of *E. coqui* development.

A. The control embryo is early post-hatching. Its morphology appears adult except for the presence of a tail, which has regressed significantly. Limbs have elongated, and the snout has narrowed. The skin appears adult, with leg bands (black arrowhead), tubercles (white arrowhead) and raised dorsolateral ridges (white arrow).

B. This embryo was treated with 1mM methimazole continuously from TS 9. Development has been inhibited; the tail is large and the limbs are stunted. The skin is devoid of the adult characteristics present in the control froglet. A prominent snout has not formed.

C. This methimazole-treated embryo was rescued by co-treatment with T₃ for 8 days, starting at TS13. The froglet appears similar to the control. Its tail is regressing, its snout is pointed and its skin is adult. Limbs appear shorter than in the control case.

Magnification: x5.
control  methimazole  methimazole and T3
Figure 12: Morphometry of methimazole-treated and T3-rescued embryos.

Quantitative differences exist between control, methimazole-treated and T3-rescued embryos, with respect to various body dimensions. Measurements were made under a dissecting scope using an ocular micrometer. Data were subjected to non-parametric statistical analysis (Kruskal-Wallis test, followed by Tukey multiple comparisons test). Statistically significant differences between treatment groups are indicated by different superscripts. $n=22$ for control (C) and T3-rescued groups (M/T3), and $n=21$ for the methimazole-treated group (M). Error bars represent 2 standard errors of the mean (S.E.M.). The lower jaw-suture line (D) and snout-upper jaw (E) measurement positions are indicated on Figure 14D.
Morphometric measurements of methimazole-treated and rescued embryos
To confirm that methimazole was not acting by retarding development, embryos were cultured in methimazole for four weeks. These embryos had similar body dimensions to their methimazole-treated siblings which had been measured two weeks earlier. Their body proportions had not reached those of post-hatching controls (Figure 13). The development of embryos cultured in methimazole for extended periods remains inhibited.

_Effects of methimazole on specific structures._

**Skin:** During normal development, the _E. coqui_ epidermis is transparent until TS6, when the first melanophores appear on the trunk (Townsend and Stewart, 1985). As development proceeds, the concentration of melanophores increases, so that the underlying yolk becomes progressively less visible. By the end of TS13, the yolk can no longer be seen through the dorsal skin, as it is completely covered by pigment cells. During the last stages of embryogenesis, the skin transforms from a thin, smooth epidermis with simple pigmentation into a tougher, thicker layer, with tubercular protrusions and more complex pigmentation patterns. Raised ridges form dorsolaterally on either side of the midline. The adult skin can be seen in Figures 11 and 14. Note the banding patterns on the limbs which form at TS14. The pigment patterns on the skin of adult _E. coqui_ can be found in a variety of forms, which appear to be genetically inherited.

In contrast to the highly differentiated control skin, which was that of an early post-hatching embryo, the skin of the methimazole-treated embryo had a uniform appearance (Figure 11; Figure 14A-C). Tubercles and other protrusions did not form. The predominant pigment cell was the melanophore, but the concentration of this cell type was insufficient to obscure the underlying yolk, which was visible through the dorsal surface. Xanthophores were observed in some methimazole-treated embryos, but at low concentrations. Adult-specific pigment patterns were absent. The leg bands characteristic of adult frogs did not form. The skin had the appearance of a TS12 embryo. In contrast, the skin of the T₃-treated embryo was indistinguishable from the hatchling frog; it possessed a tough epidermis possessing tubercles, dorsal lateral ridges, complex pigmentation and adult banding patterns.

The ventral epidermis of control embryos also underwent TH-dependent remodelling (Figure 14 D-F). The most caudal skin of the head, here termed the ventrolateral head fold, was initially raised relative to the body epidermis and appeared quite distinct both ventrally and laterally to the body and limb skin. During TS13, it fused with the body and limb epidermis ventrally and
Figure 13: Efficacy of long-term methimazole treatment.
Embryos kept in methimazole for extended periods do not reach control proportions. The control and methimazole embryos (CON) and (METH) were measured at one week-post hatching. These embryos were then fixed, but a second batch of “methimazole long-term” sibling embryos (MLT) were kept in methimazole for a further two weeks, and then measured. These embryos do not appear to have changed in proportion relative to their methimazole-treated sibs measured two weeks previously. Hence, the methimazole effect is not due merely to developmental retardation. For CON and METH, n=7, and for MLT, n=5. Another batch of embryos was subjected to similar conditions, with the same result. Error bars represent 1 S.E.M.
Long-term methimazole treatment

- Lower leg length:width ratio
- Thigh length:width ratio
- Tail length (mm)
- Lower jaw-suture line (mm)
- Snout-upper jaw length (mm)
- Ventral width (mm)
**Figure 14: Thyroid-dependency of skin remodelling.**

A-C. Dorsal view of skin. The control case (A) has a highly patterned epidermis. No yolk can be seen through the skin, which has adult pigmentation. The methimazole-treated embryo (B) has a simply pigmented epidermis. The prominent pigment cell is the melanophore, and a few xanthophores are present. Adult patterning is absent, and the underlying yolk is clearly visible. The T3-rescued froglet (C) has skin which is indistinguishable from the control. Magnification: x20.

D-F. Ventral view of head. The control embryo (D) has undergone suturing between the ventral head and body epidermis. The suture mark is indicated by a white arrowhead. The black and white lines drawn on this figure indicate the regions measured for morphometric analysis (Figure 12). The black line represents the “snout-upper jaw” length, and the white line represents the “lower jaw-suture line” length. The methimazole-treated embryo has not undergone suturing. The methimazole-treated embryo has not undergone suturing and its head and body epidermis are quite distinct. The unfused ventrolateral skin-fold is indicated by a black arrowhead. The T3-treated embryo has undergone suturing. Magnification: x8.

G. The ventral surface of a newly-metamorphosed *Rana pipiens* froglet displays suture marks similar to those seen in *E. coqui* (white arrowhead). Magnification: x4.
methimazole and T3

Dorsal

control  methimazole  methimazole and T3

A  B  C

Ventral

D  E  F

Rana pipiens

G
laterally to produce the contiguous epidermis of the adult frog. Initially, suture marks indicated the site of this epidermal remodelling (Figure 14D), but these disappeared within a week of hatching, leaving no trace of the embryological fusion events. Fusion of the ventrolateral head fold with the body and limb epidermis was completely inhibited by methimazole (Figure 14E): T₃ treatment rescued these suturing events (Figure 14F). Such extensive skin remodelling has an intriguing parallel in metamorphosing frogs (Figure 14G), where ventral suturing occurs in the corresponding region after the limbs burst through the skin window during metamorphic climax (Helff, 1926; Taylor and Kollros, 1946).

**Limbs:** Limb buds appear during TS4 of *E. coqui* development, shortly after neural fold closure. While limb bud formation in *E. coqui* is temporally comparable with other vertebrates, it is heterochronically accelerated relative to metamorphosing anurans, where limb development is not discernible until after hatching (Gosner, 1960). Methimazole treatment, even from early stages (TS4-6, n=24) had no effect on the differentiation of the limb bud into a foot paddle (TS7), and digits formed normally at TS9. During TS12, limbs normally undergo rapid elongation. This growth was inhibited by methimazole, and the hindlimbs appeared short and stumpy in appearance. To determine the extent of such inhibition, the length:width ratios of both the lower leg and thigh regions of the hindlimb were measured and differences between the treatment groups were statistically analysed (Figure 12A, B). Both lower leg and thigh growth were significantly stunted by methimazole treatment. T₃ co-treatment completely rescued the dimensions of the lower leg segment. Thigh remodelling remained somewhat refractive to rescue, as the its length:width ratio was intermediate between, and significantly different from, the methimazole-treated and control frogs.

**Tail:** In biphasic anurans, tail regression is the final event of metamorphosis. *E. coqui* possess a tail with a highly modified morphology. In comparison with a tadpole’s tail, it is thin and well vascularized, with a high surface area: volume ratio. It is thought to function as a respiratory structure, aiding gas exchange through the jelly (Townsend and Stewart, 1985). The tail becomes visible at TS4 and reaches its maximal length by TS9. It starts to shrink at TS13, and disappears completely within a week of hatching (Townsend and Stewart, 1985). The tail is one of the few organs in which TH has been developmentally implicated in *Eleutherodactylus*. TH treatment of TS8 embryos caused precocious tail regression in *E. coqui* (Callery, 1994), and treatment of *E. martinicensis* with the goitrogen phenylthiourea (PTU) inhibited tail resorption
Methimazole, like PTU, inhibited tail regression. Co-treatment with exogenous T₃ restored tail resorption to control levels (Figure 12C).

**Head:** In metamorphosing anurans, the larval head is radically remodelled during metamorphosis, as the tadpole switches from a herbivorous to a carnivorous diet. These ontogenetic changes in the jaws and cranium have been extensively studied, and the development of the cranial cartilages and musculature have been described in detail in *Eleutherodactylus* (Lynn, 1942; Hanken et al., 1992, 1997b). Many larval-specific structures never form. Instead, the cranial muscles and cartilages adopt a mid-metamorphic configuration, which remodels during late embryogenesis (TS12-TS15) into the adult morphology (Hanken et al., 1992, 1997b). While this remodelling recapitulates metamorphic events, thyroid hormone has not been directly implicated in this transition. Observation of the external morphology of methimazole-treated embryos indicated that head remodelling was affected (Figure 14). The mouthparts of control and treated embryos were indistinguishable until TS12, but elongation of the lower jaw during TS13 was inhibited. The prominent snout typical of froglets did not form; instead the methimazole-treated embryos had a rounded head which is characteristic of the embryonic head shape. To quantitate the changes in head shape, two measurements were made, indicated on Figure 14D. When viewed ventrally, the distance between the anterior tip of the head and the upper jaw shrinks gradually during development, as the upper part of the head narrows into a snout (Figure 14D). The distance between the anterior part of the lower jaw and the most posterior part of the head fold/suture line increases during development, as the lower jaw elongates (Figure 14D). Since the suture marks do not form in methimazole-treated embryos, the most posterior point on the ventrolateral head fold was used as the equivalent reference point. In both these cases, remodelling was inhibited by methimazole treatment and restored to control levels by co-treatment with T₃ (Figure 12D,E).

To examine in more detail the head structures being affected by methimazole, cartilage and musculature were examined by alcian blue staining and immunochemistry with the 12/101 antibody for skeletal muscle, respectively. The most dramatic effect on the cartilaginous skeleton was inhibition of elongation of Meckel's cartilage (Figure 15A-C, n=14 per treatment group). Co-treatment with T₃ led to partial elongation of Meckel's cartilage (Fig. 15C). The morphology of the ceratohyal cartilage also seemed altered by methimazole. Remodelling of the cranial musculature was affected (Figure 15D-F, n=15 per treatment group). Normally, the
Figure 15: Inhibition of head remodelling by methimazole.

Ventral view of head cartilages visualised by Alcian Blue staining (A-C, x13). Anterior to the left.

A. Control embryo, early post-hatching stage. The lower jaw is greatly enlarged due to elongation of Meckel’s cartilage (arrow). The ceratohyal cartilage (arrowhead) has adopted the post-hatching morphology.

B. Embryo treated with methimazole from TS9 onwards. The lower jaw appears small as Meckel’s cartilage has not elongated, and the ceratohyal cartilage has a different appearance from that of the control.

C. Embryo rescued with T3 from TS13 for 7 days. Meckel’s cartilage has partially elongated, but the ceratohyal cartilage looks more similar to the methimazole-treated embryo than the control.

Lateral view of head musculature (D-F, x19), visualised by immunochemistry with 12/101 antibody to skeletal muscle. Anterior to the left.

D. Control embryo, early post-hatching stage. The M.depressor mandibulae (Mdm) has enlarged and appears adult in shape (arrow). Its posterior portion (*) has extended backwards, and partially obscures some of the muscles behind it, including the M. cucullaris (Mcuc), marked by a white arrowhead.

E. Embryo treated with methimazole from TS10 onwards. The Mdm remains small and undifferentiated. No posterior extension is evident, and the Mcuc is not obscured.

F. Embryo rescued with T3 from TS13 for 8 days. The Mdm is enlarged and has begun to extend in a posterior direction, partially obscuring the Mcuc.
Musculus depressor mandibulae (Mdm) greatly enlarges and extends posteriorly as it differentiates, to the extent that it eventually overlies the M. cucullaris, which initially appears quite posterior to the Mdm (Schlosser and Roth, 1997). In methimazole-treated embryos, the Mdm condensation did not differentiate fully, but appeared equivalent to that of a TS12 embryo; leaving the Mcuc clearly visible (Figure 15E). Co-treatment with T3 led to complete rescue of the cranial musculature. The embryo depicted in Figure 15F was in the process of remodelling, and had caught up with the control embryo. Its Mdm was more like the control embryo than the methimazole-treated one.

Axial musculature: Morphometric analysis of the ventral width of embryos indicated that the methimazole-treated embryos were significantly wider than their control and T3-rescued counterparts (Figure 12). During normal development, embryonic ventral width decreases. This is coincident with the repositioning of the rectus abdominis muscle bands from their initial lateral position to a more medial position, converging on the ventral midline (Elinson and Fang, 1998). Since in tadpoles this ventral convergence of the rectus abdominis occurs during metamorphosis (Ryke 1953, Lynch 1984), the axial musculature was examined to determine if this event is thyroid hormone-dependent in E. coqui. Significant differences were noted between the control and methimazole-treated embryos (Figure 16). Movement of the rectus abdominis towards the ventral midline was inhibited, and a large amount of yolk was visible on the ventrum. Additionally, the posterior portion of the pectoral muscle did not extend posteriorly, but remained small and similar in appearance to a TS12-13 embryo (Figure 16A.B). The lateral muscle bands, composed of the M. obliquus externus and the M. transversus, normally extend both dorsally and ventrally to cover the yolk between the dorsal axial musculature and the rectus abdominis. In methimazole-treated embryos, these lateral muscles remained as a relatively narrow band, so a significant amount of yolk was visible when the embryos were viewed from a lateral aspect (Figure 16D,E). Co-treatment with T3 re-initiated remodelling of all affected muscle groups (Figure 16C.F). Embryos treated with T3 for 8 days were indistinguishable from their hatchling control counterparts. Treatment with T3 for longer periods generally resulted in death.

Temporal sensitivity to methimazole
Since methimazole affects the synthesis of thyroid hormone, once zygotic hormone synthesis commences, the effects of methimazole should diminish. This was tested by commencing
**Figure 16:** Axial musculature

Axial musculature stained with 12/101 antibody. Ventral view, anterior to the top (A-C, x14); lateral view (D-F, x10), anterior to the right.

A. D. Control animal, hatching stage. The segmented rectus abdominis muscles are converging on the ventral midline, and the intervening linea alba (black arrowhead) is thin. The pectoral muscles (white arrow) have extended posteriorly, and are level with the fourth segment of the rectus abdominis. The lateral muscles (white arrowhead) have expanded, so that little yolk is visible between them and the dorsal axial musculature.

B. E. Embryo treated with methimazole form TS9 onwards. The rectus abdominis muscles are still widely separated by a thick linea alba. The posterior projection of the pectoral muscle is inhibited. This muscle extends only as far back as the second segment of the rectus abdominis.

C. F. $T_3$-rescued animal. rescued from TS13 for 8 days. The linea alba separating the rectus abdominis bands is narrowing. The pectoral muscles have extended posteriorly, level with the fourth segment of the rectus abdominis. The lateral muscles have begun to expand.
control
methimazole
methimazole and T3

Ventral
A B C

Lateral
D E F
methimazole treatment at progressively later stages. Five developmental series were performed (Table 6: Figure 17). No reduction in the efficacy of methimazole was noted if treatment commenced at TS10. Some embryos treated at TS11 showed varying degrees of further development, including partial limb growth and partial skin transformation. All embryos treated at TS12 and TS13 exhibited some remodelling. Many of the TS13-treated embryos appeared like normal froglets, except that their tail did not regress fully.

Morphometric measurements were performed on Series I and II embryos, and the data sets were combined and subjected to statistical analysis (Figure 18). As in the previous experiments, hindlimb growth, jaw remodelling, tail resorption and narrowing of the body width were all inhibited relative to controls when methimazole treatment started at TS9. No statistically significant difference was found between the embryos treated at TS9 and the embryos treated at TS11. By TS13, with the exception of the tail, all other characters were significantly different from their earlier-treated siblings, and did not differ significantly from the control embryos. Tail resorption was the most refractive character: the tail length of TS13-treated embryos was not statistically different from the earlier-treated groups. These developmental observations and morphometric measurements indicate that the period of thyroid hormone dependence is from late TS11-TS13 for most of the remodelling events, with tail resorption requiring hormone even after this time.

**Methimazole-arrested embryos can re-enter ontogenesis.**

Embryos kept in methimazole for extended periods appeared developmentally arrested, and most closely resembled TS12 in stage. Embryos that had been kept in methimazole for 11-18 days were transferred into 20% Steinberg's solution for 8-29 days (n=9). Upon relief from methimazole inhibition, embryos re-entered the ontogenetic program, and took on a frog-like morphology (Figure 19). Skin suturing, jaw remodelling, skin differentiation and tail regression occurred to varying extents in the 20% Steinberg's-cultured embryos, while those embryos kept in methimazole remained developmentally static.
Table 6: Temporal sensitivity to methimazole.

Methimazole treatment after TS10 had progressively diminishing effects. Morphologies were observed 7-10 days after the controls reached TS15. Some TS11-treated embryos seemed fully inhibited (series II); whereas others underwent significant transformation (Series III). If embryos reached TS13 before methimazole treatment commenced, they appeared frog-like, except for their substantial tail.

*a froglet morphology. TH-dependent remodelling complete: adult skin with tubercles, full pigmentation and leg bands; limbs elongated; jaws adult in appearance; ventral skin sutured; tail gone.

*bembryonic morphology. TH-dependent remodelling not initiated: uniform epidermis lacking secondary differentiations (tubercles, leg bands, adult pigmentation patterns); limbs small; jaws embryonic; no suturing of ventral skin; full tail.

<table>
<thead>
<tr>
<th>Series</th>
<th>Stage of methimazole commencement and observed effects on development</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>control (n=5) froglet; tail stumpy/gone.</td>
</tr>
<tr>
<td></td>
<td>TS9 (n=4) embryonic</td>
</tr>
<tr>
<td></td>
<td>TS11 (n=5) embryonic skin; no suturing; slight limb growth; full tail</td>
</tr>
<tr>
<td></td>
<td>TS13 (n=5) froglet; tail substantial.</td>
</tr>
<tr>
<td>II</td>
<td>control (n=6) froglet; tail stump present.</td>
</tr>
<tr>
<td></td>
<td>TS9 (n=6) embryonic</td>
</tr>
<tr>
<td></td>
<td>TS11 (n=6) no limb growth; full tail</td>
</tr>
<tr>
<td></td>
<td>TS13 (n=6) elongated limbs; partial tail</td>
</tr>
<tr>
<td>III</td>
<td>control (n=4) froglet</td>
</tr>
<tr>
<td></td>
<td>TS9 (n=4) embryonic</td>
</tr>
<tr>
<td></td>
<td>TS11 (n=4) skin transforming (tubercles but no leg bands); suturing; some limb growth; full tail</td>
</tr>
<tr>
<td></td>
<td>TS13 (n=4) froglet; partial tail.</td>
</tr>
<tr>
<td>IV</td>
<td>control (n=2) froglet</td>
</tr>
<tr>
<td></td>
<td>TS8 (n=5) embryonic</td>
</tr>
<tr>
<td></td>
<td>TS10 (n=5) no suturing; slight limb growth? (thigh length same as TS8-treated embryos; lower leg slightly longer); embryonic jaw; full tail</td>
</tr>
<tr>
<td></td>
<td>TS12 (n=5) suturing; limbs slightly shorter than controls; lower jaw partially extended; tail full.</td>
</tr>
<tr>
<td>V</td>
<td>control (n=5) froglet; tail stumpy/gone.</td>
</tr>
<tr>
<td></td>
<td>TS9 (n=5) embryonic</td>
</tr>
<tr>
<td></td>
<td>TS11 (n=5) partial limb growth; partial lower jaw extension; full tail; egg tooth present</td>
</tr>
<tr>
<td></td>
<td>TS14 (n=5) limbs virtually control length; partial tail.</td>
</tr>
</tbody>
</table>
Figure 17: Temporal sensitivity to methimazole inhibition.

Dorsal view (x4) of embryos treated with methimazole commencing from progressively later stages of development. The post-hatching control looks like a tiny adult, except for the tiny tail remnant. The embryo treated from TS9 (B) shows a great degree of inhibition. The TS11-treated embryo (C) also is inhibited to a large extent, although its hindlegs appear slightly longer than the embryo in (B). By TS13 (D), methimazole treatment has little effect, except on the tail, which still appears large.
Figure 18: Morphometry of temporal sensitivity to methimazole.

Statistical differences exist between the body dimensions of embryos treated at progressively later stages with methimazole. Measurements were made under a dissecting scope using an ocular micrometer. Data were subjected to non-parametric statistical analysis (Kruskal-Wallis test, followed by Tukey multiple comparisons test). Statistically significant differences between treatment groups are indicated by different superscripts. n=11 per treatment group, except for TS9-treated embryos, where n=10. Error bars represent 2 S.E.M.
Temporal sensitivity to methimazole inhibition

A. Lower leg length/width ratio

B. Thigh length/width ratio

C. Tail length (mm)

D. Lower jaw-suture line (mm)

E. Snout-upper jaw (mm)

F. Ventrail width (mm)

Treatment stage (TS)
**Figure 19: Resumption of remodelling after removal from methimazole.**

Embryos removed from methimazole are capable of undergoing remodelling, even after extended periods of inhibition. Dorsal (A, B; x5) and ventral (C, D; x12) views.

A. C. Embryo which had been cultured in methimazole for 24 days, starting at TS9.

This embryo still has a prominent tail and its skin has not sutured ventrally (arrowhead).

B. D. Embryo which had been cultured in methimazole for 16 days, and then transferred to 20% Steinberg's solution for a further 8 days. Its tail has regressed substantially and it has developed a prominent snout. Pigmentation appears more adult-like than in the methimazole treated embryo. Ventrally, the skin has sutured, and the suture mark is barely visible (arrowhead).
Discussion

Previously, it was found that chemical or surgical ablation of the *Eleutherodactylus* thyroid before a stage equivalent to TS12 prevented tail and pronephric degeneration (Lynn and Peadon, 1955; Hughes, 1966). Thyroxine treatment induced precocious degeneration of these organs (Lynn and Peadon, 1955). The lack of other developmental effects caused by modulation of the thyroid axis led to the speculation that embryogenesis in *Eleutherodactylus* is largely thyroid independent (Lynn and Peadon, 1955; Hughes, 1966). In my investigation, treatment of *E. coqui* embryos with the TH-synthesis inhibitor methimazole resulted in developmental arrest at around TS12. The effects were pleiotropic, and resembled metamorphic arrest in other frogs. Affected organ systems included the skin, limbs, jaw musculature and cartilages, tail, and axial musculature. The specificity of this effect was demonstrated by rescue of the frog morphology by co-treatment with T₃.

Efficacy of methimazole inhibition was greatly decreased if treatment commenced after TS11. This stage correlates with the histologically mature appearance of the thyroid gland in *E. coqui* (Jennings and Hanken, 1998). Methimazole prevents thyroid hormone synthesis but cannot interfere with the action of pre-existing TH. Consequently, if treatment starts after activation of the thyroid gland, one would expect such late methimazole treatment to be ineffective. Interestingly, tail regression is inhibited by methimazole even if treatment starts at TS12 or TS13. This is reminiscent of the metamorphic sequence in *Xenopus*, where tail regression is the terminal event of metamorphosis. Type III deiodinase is upregulated in the pre-metamorphic *Xenopus* tail upon T₃ treatment (St. Germain et al., 1994). Endogenous levels of this enzyme, which inactivates T₃, are particularly high in the tail of *Rana catesbeiana* tadpoles during the early stages of metamorphosis (Becker et al., 1997). Thus, tail regression is prevented until high levels of TH are circulating. *E. coqui* tail regression is the last externally discernible remodelling event of embryogenesis, and it would be interesting to determine whether the refractiveness of tail regression in this direct developer is also due to elevated Type III deiodinase in this tissue.

The requirement of high TH levels to induce tail regression may explain why other workers saw inhibition of this remodelling event, yet no inhibition of other remodelling events. Methimazole is reported to be an efficacious goitrogen (Brown, 1997), so it may reduce endogenous thyroid activity to lower levels than do the thyroid inhibitors phenylthiourea (PTU) or prolactin, and result in more severe developmental effects. Treatment of *E. coqui* embryos with these goitrogens was not
performed in this investigation. However, figures showing PTU-treated *Eleutherodactylus* embryos in previous papers appear to show some inhibitory events similar to those reported here (Lynn, 1948; Lynn and Peadon, 1955). A dorsal view of a PTU-treated *E. ricordii* embryo (Lynn, 1948; Plate I, Figure 4) appears to have stunted limbs and a wider body than the control. The effects of PTU on limb inhibition were noted in this paper. However, in a later manuscript, the author noted that while limb growth and differentiation were inhibited by PTU, they did eventually reach control levels. and so the role of TH in limb development was not clear (Lynn and Peadon, 1955). Skin patterning also appears undeveloped in the PTU-treated embryos depicted (Lynn, 1948; Plate II, Figures 6 and 8). This effect was attributed to the depigmentation caused by PTU-inhibition of tyrosinase, the enzyme responsible for melanin production, and was not considered to be a thyroid-dependent event. A ventral view of a PTU-treated *E. martinicensis* embryo has a shortened lower jaw relative to the control (Lynn and Peadon, 1955; Plate II, Figure 12). Additionally, the ventral suturing of the head and body skin has not occurred as the head fold appears quite distinct from the body.

Since the embryology of *Eleutherodactylus* is so derived relative to metamorphosing frogs, it is hard to recognise homologous events, such as ventral suturing and jaw remodelling, which is why these events may have been overlooked in previous investigations. Additionally, the question of drug toxicity causing non-specific effects precluded previous researchers from making definitive statements about the role of TH in *Eleutherodactylus* embryogenesis. The relative non-toxicity of methimazole, as evidenced by the absence of mortality using a 1mM concentration, coupled with the reversal of its actions by T₃ makes it much easier to address the role of the thyroid in direct development. The apparently smooth and continuous development of *E. coqui* embryos into the adult morphology is revealed to be a biphasic process upon methimazole treatment: the first phase occurs independently of zygotic thyroid production, and the second phase requires embryonic hormone synthesis. The division between these two phases occurs around TS11, when a slight reduction in the efficacy of methimazole is detected.

Co-treatment of methimazole-inhibited embryos with T₃ allows them to reinitiate ontogenesis and attain the frog morphology. The remodelling events that occur during this time parallel those occurring in metamorphosis. This inhibition and rescue experiment demonstrates that the late phases of limb extension (TS12-TS14) are clearly TH-dependent. Previous attempts to demonstrate a role for TH in *E. coqui* limb development were unsuccessful (Elinson, 1994). Limb
differentiation was not affected by methimazole, presumably because it was already quite advanced even before thyroid gland maturation. Completion of limb development in anurans is thyroid-dependent, but a reasonable degree of differentiation, including some cartilage development, can occur in the absence of a thyroid gland (reviewed by Elinson, 1994). Elinson (1994) concluded that the degree of development of Rana limbs in the absence of a thyroid is equivalent to a TS9/10 E. coqui leg. This suggests that even though early limb development in Eleutherodactylus is highly derived relative to metamorphosing frogs, mechanistically, both morphogenetic processes converge at a similar developmental stage, requiring TH to complete their growth. While the lower limb length of the rescued embryos appeared the same as in the controls, the thigh length remained somewhat stunted. These differences could possibly reflect differential TH-sensitivities of the tibiofibular and femoral bones and cartilages. Osteoblastic cells from different skeletal sites have been demonstrated to respond differentially to TH in culture (Milne et al., 1998).

Development of cranial cartilages was examined in detail in both E. nubicola (Lynn, 1942) and E. coqui (Hanken et al., 1992). Early chondrogenic events are highly derived in Eleutherodactylus, with many larval cartilages, such as the suprarostrals and cornua trabeculae being absent (Hanken et al., 1992). However, by TS10 the existing cartilages assume a mid-metamorphic configuration and then remodel during TS12-14 into the adult morphology (Hanken et al., 1992). A similar situation occurs in the cranial musculature (Hanken et al., 1997b). Some larval muscles, such as the suspensorioangularis, are entirely absent, but by TS12, a mid-metamorphic configuration of the cranial muscles has formed and again undergoes remodelling events highly reminiscent of metamorphosis (Hanken et al., 1997b). Previously, no definitive proof that these events are thyroid-dependent existed. This investigation shows that as in metamorphosing anurans, elongation of Meckel’s cartilage and differentiation of the M. depressor mandibulae are thyroid-dependent events.

The hyobranchial skeleton was not examined as it did not stain well with Alcian Blue. Utilization of a sensitive Type II collagen antibody might provide additional evidence for the dependence of the cranial cartilages upon TH, as the hyobranchial skeleton does remodel extensively between TS12 and TS15 in Eleutherodactylus and can be visualized with this antibody (Lynn, 1942; Hanken, 1992). The role of TH in hyobranchial development merits further investigation, since an initial wave of remodelling occurs between TS9, when the hyobranchial skeleton generally resembles that of larval anurans, and TS10, when it has assumed a mid-metamorphic configuration (Hanken et al., 1992). It would be interesting to determine whether early methimazole treatment could inhibit the
initial remodelling wave which occurs in the ceratohyal cartilages, as this would demonstrate TH involvement in E. coqui remodelling one stage earlier than is shown in the present investigation.

Development of the hypaxial musculature during E. coqui embryogenesis shows some similarities with metamorphosing frogs. The rectus abdominis arises laterally in E. coqui, Xenopus and Rana (Elinson and Fang, 1998). During metamorphosis in the latter two species, the bands converge on the ventral midline (Ryke 1953, Lynch, 1984). The assumption of a ventral position by the rectus abdominis also occurs around the time of hatching in E. coqui (Elinson and Fang, 1998). Although the remodelling occurs during metamorphosis, there is no direct evidence in the literature for the role of thyroid hormone in this event in any frog. In my investigation, methimazole prevented the ventral convergence of the rectus abdominis in E. coqui. It also inhibited the posterior extension of the pectoral muscles and the extension of the lateral muscle bands, comprised of the M. obliquus externus and M. transversus. All three events were rescued by T3, indicating that thyroid-signalling is responsible for the extensive remodelling of the hypaxial musculature that occurs in the later phases (TS12-15) of E. coqui embryogenesis.

Development of the skin has not been studied in Eleutherodactylus. This study was confined to changes in the external morphology during embryogenesis. A detailed histological examination of epidermal differentiation in E. coqui would be useful since it would provide a whole new suite of characters against which to compare the development of metamorphosing versus direct developing frogs. Nevertheless, observation of the skin under a dissecting scope reveals that the skin of E. coqui transforms by the time of hatching from the thin, uniform, sparsely pigmented epidermis found in TS12 embryos to a thick, highly pigmented, undulating, tubercular and highly differentiated epidermis. Much of this transformation appears thyroid-dependent, as it is inhibited by methimazole and rescued by T3. The transformation of larval skin to adult skin in metamorphosing frogs has been well documented (Fox, 1985; Furlow et al., 1997). Specific changes include death and shedding of the larval apical and skein cells, and proliferation of the basal cell population into the adult epidermis (Furlow et al., 1997). Externally the adult epidermis of frogs is tough and keratinized, and adult pigmentation is adopted. Thus, the transformation of E. coqui skin appears to recapitulate the transition found in other frogs, and can be regarded as another metamorphic remodelling event that has been retained in direct developers.
Another novel character documented here in *Eleutherodactylus* is the ventral suturing of the head and body epidermis. This suturing occurs in normal embryogenesis during TS13. Its TH-dependence was demonstrated by methimazole inhibition and T₃-rescue. In metamorphosing frogs, the formation of a contiguous ventral epidermis also appears TH-dependent, since hypophysectomised tadpoles treated with low doses of thyroxine do not undergo proper epidermal remodelling in the opercular region (Kollros, 1959). Despite the radically different modes of early limb development, and the highly modified opercular morphogenesis in *E. coqui* (described in Chapter Four), this direct developer still recapitulates the ventral suturing seen in metamorphosing frogs. Again, initially divergent early embryogenesis converges on a common morphogenetic pathway utilising TH-signalling.

When embryos which had been cultured in methimazole for extended periods were removed from the influence of the goitrogen, they were able to re-enter the ontogenetic program. Completion of metamorphosis is also attained when partially metamorphosed *Xenopus* tadpoles are removed from methimazole inhibition after long-term culture (Elinson, pers. comm.). These results show that the competence to respond to thyroid hormone is retained for long periods. Such flexibility is advantageous in an indirect life-history such as *Xenopus*, since it allows the onset of metamorphosis to be varied temporally. The time from fertilization to metamorphic onset varies both between species and within species, the latter variation depending on environmental conditions. The retention of tissue-level responsiveness to TH over long periods is an essential pre-requisite to such developmental lability. The mechanism involved in the long-term memory of these tissues is unknown but one may speculate that unliganded TRs are retained in these tissues and may even be transcriptionally silencing adult genes in the absence of TH. Upon ligand binding, these receptors may activate the metamorphic cascade. The rapidity of development in *E. coqui* means that there is not an extended period of stasis in their life history, such as the larval period in other frogs. However, the tissues in *Eleutherodactylus* have, like those in their metamorphic ancestors, retained the ability to respond to TH after extended periods of thyroid inactivity.

Methimazole will not interfere with the action of pre-existing, maternally-derived hormone, and TH has been found in fish eggs and frog gastrulae (Weber et al., 1992, 1994). Therefore, a potential role for TH in early embryogenesis cannot be discounted. What can be concluded from these results is that *Eleutherodactylus* development, while appearing smooth and continuous, is actually biphasic, like its metamorphosing ancestors. The separation between the two discrete phases of its
embryogenesis occurs at the time of zygotic hormone synthesis. The last third of embryogenesis (TS12-15) is largely under the control of TH. Many remodelling events occur during this time which parallel those seen in metamorphic frogs. This period of TH-dependent remodelling is probably effected by TR-mediated signalling, given that TRβ mRNA is massively upregulated during this time. Hence, *Eleutherodactylus*, while considered an extreme direct developer (Townsend and Stewart, 1985) still undergoes an extensive metamorphosis.
CHAPTER FOUR
RECAPITULATION OF THE OPERCULAR FOLD

Introduction

Direct developing frogs have a radically modified ontogeny which lacks a free-living larval stage, the embryos hatching as miniature frogs. Members of the genus *Eleutherodactylus* exhibit the most extreme form of direct development and omit several larval characters, including lateral line organs, cement gland, a coiled gut and larval mouthparts (Sampson, 1904; Lynn, 1942; Elinson, 1990). Another larval structure believed to be absent is the operculum (Lynn, 1942; Elinson, 1990; Duellman and Trueb, 1986). In tadpoles, this structure originates during embryogenesis as paired folds of skin in the branchial region, termed opercular folds. These folds move caudal to cover the gills and the presumptive forelimb buds, and seal across the ventrum of the tadpole (Duellman and Trueb, 1986). The opercular cavity so formed protects the gills and encases the developing forelimbs, thus streamlining the aquatic tadpole. During metamorphosis, the opercular epithelium thins and a perforation forms through which the forelimbs emerge (Helff, 1926; Sasaki et al., 1983).

Earlier workers documented “dermal folds” in *Eleutherodactyus* at stages roughly equivalent to TS9 and TS10 (Sampson, 1904 p496; Lynn, 1942). The origin and fate of these folds were unknown, but their possible homology to the opercular folds of tadpoles was deemed “extremely unlikely” (Lynn, 1942, p33). Here, I show that the dermal folds in *Eleutherodactylus* are indeed homologous with the opercular folds of metamorphosing frogs. The development of these structures is recapitulated despite the presence of precocious limb buds, vestigial gills and the lack of a free-living larval stage, all factors which should mitigate against its evolutionary retention. Identification of the opercular fold indicates the stage from which the tadpole has likely been excised from *E. coqui* development.
**Materials and Methods**

*Embryo culture and manipulation*

Embryo culture and drug treatments were performed as described previously. For limb ablation experiments, operations were performed in petri-dishes that had a 2% agarose bed. Embryos were cultured in 20% Steinberg's solution containing 2mg/mL BSA and 100μg/mL gentamycin until wounds had healed.

**Results**

*Recapitulation of the opercular fold.*

At first glance, an early *E. coqui* embryo appears more avian than amphibian, developing atop a large yolk sac, although cleavage is holoblastic (Elinson, 1987) (Figure 20A). The most striking feature is the early appearance at TS4 of large limb buds reminiscent of amniote embryos, which in other frogs develop much later. At TS8, a fold of skin emanates from the region dorsoanterior to the gills and moves ventrad during TS9 in a characteristic-shaped curve on either side of the embryo to cover the gills. The leading edge of this fold forms the beginning of a shelf over the forelimbs. The most caudal part of the shelf extends to a point just anterior to the front of pigment cells, indicating that the posterior edge of the fold is contiguous with the body wall which is gradually covering the yolk sac (Elinson and Fang, 1998). The fold subsequently migrates over the precociously developing limb bud, encompassing approximately half the limb bud by the end of TS9 (Figures 20B.C). At the greatest extent of coverage during early TS10, about 2/3 of the limb is obscured and only the digits are visible. During TS10, shortly after maximal coverage of the forelimbs occurs, the epithelium covering the limb thins and rapidly perforates (Figure 20D). This hole always has a characteristic D-shape, indicating that its formation is unlikely to be merely the result of pressure from the underlying limb. The edge of the perforation never encompasses the leading edge of the epithelium, resulting in the formation of a thin strap. By TS11, the forelimbs have burst through the hole, resulting in the appearance of embryos with "overall straps" (Figure 20F). These straps, which are attached anteriorly to the head, and posteriorly to the body wall that is migrating over the yolk sac, are completely separate from the underlying yolk epithelium. Within a couple of days, the overall straps degenerate.
Figure 20: Development and perforation of the opercular fold.

A. Dorsal view of TS6 embryo freed from its jelly capsule (x14).
B. Lateral view of late TS9 embryo, with half of forelimb covered by the fold (x18).
C. Close-up of forelimb in B, with the opercular fold indicated by black arrows (x54).
D. Lateral view of late TS10 embryo. The fold has perforated, leaving an epithelial strap (x18).
E. Lateral view of TS10 limb-ablated embryo. The opercular perforation still forms in the characteristic D-shape, again leaving an epithelial strap (x18).
F. TS11 embryo, in which the limbs have burst through the perforation, and the epithelial straps appear as "overall straps" (black arrows) in ventral view (x22).
To confirm that the appearance of the hole was not merely due to pressure from the large forelimb, the developing limb-bud was ablated by Elinson (pers. comm.). In *Rana*, a hole still forms in the absence of the forelimb (Helff. 1926). Even when *E. coqui* forelimb buds were ablated, a normal, D-shaped hole appeared in the opercular fold leading to the formation of a strap (Figure 20E). These experiments show that the formation of a hole in the opercular fold of *E. coqui* is similar to opercular perforation in tadpoles, as neither is due to pressure from the forelimb.

*Opercular perforation appears thyroid hormone-insensitive.*

Little is known about the mechanism of opercular perforation during metamorphosis. Local implantation of thyroxine pellets under the opercular epithelium on one side of a *Rana* tadpole precociously induced perforation unilaterally, suggesting that thyroid hormone signalling, which occurs during metamorphic climax, may be responsible for opercular perforation (Kaltenbach, 1953). Zygotic thyroid hormone levels were modulated to determine whether they could affect opercular perforation in *E. coqui*. TS5-7 embryos were treated with 1mM methimazole, a metamorphic inhibitor which prevents thyroid hormone synthesis (Nakashima et al., 1978; Buckbinder and Brown, 1993). This treatment inhibits thyroid-dependent events that occur later in *E. coqui* embryogenesis, including jaw remodelling, limb elongation, tail reduction and skin changes (Chapter Three). However, opercular perforation was not inhibited in 4 experiments (n=19 embryos). Since methimazole prevents only thyroid hormone synthesis, and thyroid hormone has been detected in frog gastrulae (Weber et al., 1994), it is possible that perforation is initiated by maternal stores of hormone. Exogenous T3 was added to try to accelerate hole formation by overstimulating the thyroid axis. Treatment of TS5-7 embryos with 50nM T3 did not accelerate hole formation in 4 experiments (n=20 embryos), although this concentration is able to rescue developmental events inhibitable by methimazole in *E. coqui* (as described in Chapter Three), and is therefore capable of inducing tissue remodelling in this frog.
Discussion

In metamorphosing species, the formation of the opercular fold marks the end of embryonic life (Shumway, 1940), and its perforation marks the onset of metamorphic climax (Etkin, 1932; Taylor and Kollros, 1946). These events bracket the life of the tadpole, occurring weeks or months apart. The dramatic condensation of development in *E. coqui* means that the entire opercular development and perforation takes only a few days. Detection of the opercular fold and perforation allows precise definition of the *E. coqui* stage equivalents of the start- and end-points of the feeding larval phase in metamorphosing frogs.

Reliance upon one character for comparative staging is unsatisfactory since heterochronic shifts can distort interpretation (Schlosser and Roth, 1997). To ensure that opercular morphogenesis has not been drastically shifted with respect to other developmental characters, a heterochrony plot of developmental features between *E. coqui* and generalized anuran development was constructed (Figure 21). The generalized anuran description came from the Gosner staging table (Gosner, 1960; Duellman and Trueb, 1986). Most *E. coqui* features were taken from the Townsend-Stewart staging table (Townsend and Stewart, 1985), with the exception of the positioning of jaw articulation, which was observed directly. Opercular morphogenetic events were added to this plot. This heterochrony plot clearly indicates that there is a dissociation between developmental events occurring pre- and post-TS10 in *E. coqui* with respect to generalized anuran development. Opercular formation and perforation straddle this break-point. There has evidently been a heterochronic truncation between early and late TS10 in *E. coqui*, relative to Go25 and Go41 in biphasic anurans. Despite the occurrence of this heterochronic truncation, two co-variant suites of general developmental characters are detected in this plot: the pre-TS10 suite and the post-TS10 suite. Opercular formation and maximal extension fall into their expected position in the first suite, just as opercular thinning and perforation do in the second suite. The events of opercular morphogenesis themselves have not been shifted relative to other general developmental characters; hence they are suitable reference points from which to determine the *E. coqui* stage-equivalent of the feeding tadpole. TS8 (opercular fold formation) is equivalent to Go22, and early TS10 (maximal opercular fold extension) is equivalent to Go24. Opercular perforation (Go41) and limb emergence (Go42) occur sequentially during TS10. Thus, I conclude that the developmental break-point at which the tadpole was deleted from the ancestral metamorphic life-history maps to TS10 in *E. coqui*.
Figure 21: Heterochrony plot of general developmental characters.

Sixteen morphological characters are included on this heterochrony plot between *E. coqui* and the standard anuran staging table (Gosner, 1960; Duellman and Trueb, 1986). For *E. coqui*, characters 7-11, 13 & 14 were direct observations. Optic vesicle formation was determined by Schlosser and Roth (1997) in *E. coqui* and Shumway (1942) in *Rana pipiens*. The remaining *E. coqui* points were taken from the Townsend-Stewart staging table (Townsend and Stewart, 1985).

Characters are:

1. Neural fold formation.
2. Optic vesicle.
3. Neural groove closed.
4. Heart-beat and gill formation.
5. Gill circulation.
6. Tail fin circulation.
7. Operculum covers gill base.
8. Operculum at maximal coverage.
10. Operculum perforates.
11. Mouth angle between nostril and mid-point of eye.
12. Tail resorption starts.
13. Mouth angle between midpoint and posterior margin of eye.
14. Mouth angle at posterior margin of eye.
15. Tail resorption complete.

The plot reveals the existence of two co-variant character suites, each surrounded by an ellipse. The second suite, containing metamorphic characters, has been pre-displaced relative to the first suite of general embryonic characters in *E. coqui*. This heterochrony reflects the condensation of development in *E. coqui*, as previously documented by Schlosser and Roth (1997).

The pink dots represent the opercular characters, which straddle the break-point in the life-history. This break-point occurs at TS10. The green dot represents hatching, which has been post-displaced in *E. coqui*. 
Heterochrony plot of developmental characters

E. coqui (Townsend-Stewart Stage)
In *Rana*, opercular perforation is induced by thyroid hormone (TH). (Kaltenbach, 1953). No effect of TH inhibition or exogenous hormone treatment was noted in *E. coqui*. Three possible explanations exist. Firstly, opercular perforation may be exquisitely sensitive to TH. In *Rana*, it is the first event of metamorphic climax, and therefore presumably requires low levels of thyroid hormone. If the situation holds true for *E. coqui*, then perhaps methimazole treatment does not render the embryos totally functionally athyroid, and exceedingly low circulating TH levels effect opercular perforation. Trace amounts of maternal hormone could possibly induce perforation. Analysis of TR of expression in the opercular region by *in situ* hybridization might provide some clue as to whether TH-mediation of opercular perforation is likely. If very low levels of TH can induce opercular perforation in *E. coqui*, then there might be high levels of TR expression immediately prior to opercular perforation. Alternatively, opercular perforation may have become free from TH-control in direct developers. Thirdly, it is conceivable that the opercular fold in *Eleutherodactylus* is not in fact homologous to the opercular fold of metamorphosing frogs, but has evolved convergently. I view this last possibility as exceedingly improbable, since convergent evolution occurs under selective pressure, and there does not appear to be any selective advantage for a direct developer to produce such a transient structure. Coupled with its harmonious temporal appearance with respect to other structures. I conclude that this is indeed the *Eleutherodactylus* homologue of the opercular fold in metamorphosing anurans.

Recapitulation, the retention of ancestral ontogenetic characters as a consequence of phylogenetic history, occurs even in the highly derived ontogeny of *E. coqui* (Schlosser and Roth, 1997; Hanken et al., 1992, 1997a). Recapitulated characters include the tail, external gills, Rohon-Beard neurons (Schosser and Roth, 1997), and as documented here, the opercular fold. Why should such a structure, which appears redundant in a frog with intraoval development, be recapitulated during its embryogenesis? One possible reason for recapitulation of the opercular fold is that its movement over the limb is connected with the movement of the body wall over the yolk sac (Elinson and Fang, 1998). In this scenario, the fold has lost its original protective function, but has been co-opted into a new role, namely coverage of the yolk sac. Opercular perforation is then required to free the trapped forelimbs. Alternatively, the fold may be a vestigial structure, and could eventually be eliminated from the ontogenetic program, in a manner similar to the loss of gills in some species of *Eleutherodactylus* (Lynn, 1942).
Early development in *E. coqui* involves much morphological novelty, such as precocious limb development and lack of tadpole mouthparts (Sampson, 1904; Lynn, 1942; Elinson, 1990).

Although *E. coqui* recapitulates both larval (Schlosser and Roth, 1997) and metamorphic (Hanken et al., 1992) traits, ontogeny has been accelerated by excision of the feeding larval period present in metamorphosing frogs, as defined by the period from maximal opercular extension until forelimb perforation. Truncation of this period from weeks or months to a few days indicates that the excision occurred during TS10. Late embryogenesis (TS12-15) appears akin to the metamorphic period in other frogs, as methimazole inhibition demonstrates thyroid-dependence even in this direct developer.
CHAPTER FIVE
GENERAL DISCUSSION

Existing hypotheses suggest that direct development results from:

I. Emancipation of ontogeny from thyroid hormone (TH) control (Lynn and Peadon, 1955; Hughes, 1966).

II. Heterochronic acceleration of the thyroid axis (Hanken et al., 1997a; Rose, 1999).

Hypothesis I: Emancipation

The results that I have documented in Chapter Three demonstrate that this hypothesis is not substantiated, since inhibition of the thyroid axis causes pleiotropic effects on development, which are reminiscent of metamorphic arrest in other frogs. Development can be rescued by co-treatment with exogenous TH, conclusively demonstrating that direct development in *Eleutherodactylus* is TH-dependent.

Hypothesis II: Heterochronic acceleration

The heterochronic acceleration hypothesis has been proposed to act by two possible mechanisms, which are not necessarily mutually exclusive:

A: Maternal stores of TH or TR may mediate direct development before the thyroid develops (Hanken et al., 1997a; Rose, 1999). The role of maternal TH in direct development is not discernible from the experimental approach employed in this thesis. Evidence in support of this possibility comes from the finding of T₃ and T₄ in *E. coqui* eggs (Jennings, 1997). Implicit in hypotheses that invoke maternal TH in direct development is the idea that it is being deployed in a unique (i.e. autapomorphic) manner in direct developers. However, given the presence of maternal TH in metamorphosing frogs (Fujikara and Suzuki, 1991; Weber et al., 1994; Jennings, 1997), maternal TH stores may be a primitive feature (symplesiomorphy) of the Anura (Hanken et al., 1997a). In this latter case, analysis of maternally-mediated signalling may not tell us anything significant about novelties arising in direct development.

If maternal stores of TH or TRs are involved in direct development, then they could act very early in embryogenesis before the thyroid axis is active, to allow the precocious development of adult features (Hanken et al., 1997a). In mammals, TRs have been implicated in early neural
development. before the thyroid gland is active (Forrest et al., 1991; Xiao et al., 1998; Gauthier et al., 1999). However, if maternal stores are hypothesized to play an early role in tissue patterning in direct developers, then some explanation regarding how such stores become spatially localized during cleavage of the large egg is required.

Maternally-derived TR transcripts have been proposed as possible activators of the thyroid axis in *Xenopus* (Chatterjee and Tata, 1992). These authors suggest that the early competence of *Xenopus* tadpoles to TH at NF 42-44 may be through TR proteins synthesized from trace amounts of maternal mRNAs. While intriguing, hypotheses invoking maternal TRs remain speculative in the absence of any empirical evidence. To date, such empirical evidence has not been forthcoming because there has been no method to perturb signalling through TRs in *vivo*. The recent development of transgenesis in *Xenopus* (Kroll and Amaya, 1996), however, promises to aid in the elucidation of the roles of various genes during metamorphosis. The transgenic Type III deiodinase frog did not undergo tail resorption, but other aspects of its metamorphosis proceeded normally (Huang et al., 1999). This result indicates that the level of deiodinase expression may not be high enough to remove all endogenous TH, as one would expect to see at least as severe a phenotype as in a thyroidectomized tadpole if TH signalling had been abolished. The production of transgenic tadpoles expressing dominant negative TRs may indicate more clearly whether these genes are utilized in development before maturation of the thyroid gland in *Xenopus*. Once these data are accumulated for metamorphosing frogs, direct developing frogs can be examined for deviations from the ancestral condition.

**B:** *The thyroid axis has been heterochronically accelerated into embryogenesis* (Hanken et al., 1997a; Jennings and Hanken, 1998). Such precocious activation could potentially be mediated by maternal TH or TRs, which is why possibilities A and B are not mutually exclusive. Alternatively, the thyroid axis could be activated by another, unknown mechanism in *Eleutherodactylus*. Hanken et al. (1997a) postulate that the thyroid axis has been heterochronically accelerated because it becomes active before hatching and the onset of feeding in *Eleutherodactylus*, but after these events occur in *Xenopus*. As noted by these authors, however, the time periods between fertilization and hatching vary dramatically between the two species. In *Xenopus*, hatching occurs on the third day of development, whereas hatching in *E. coqui* occurs after almost three weeks. It is my contention that hatching has been heterochronically delayed in direct developers. This is supported by the anomalous positioning...
of hatching as a character on the heterochrony plot shown in Chapter Four (Figure 21). Whereas the other characters plotted fall within one of two co-variant character suites, hatching has evidently been delayed in *Eleutherodactylus* relative to its occurrence in metamorphosing anurans. Therefore, hatching is not a suitable reference point against which to judge developmental heterochronies.

*A new model for direct development.*

An existing model for direct development is shown in Figure 22 (Hanken, 1992). Figure 22A shows the ancestral life-history, with the embryogenic and metamorphic periods temporally separated by the developmentally static larval phase. In Figure 22B, direct development is viewed as proceeding directly to the adult morphology during embryogenesis, the metamorphic phase of the life-history having been deleted. I propose that this metamorphic phase was retained during the evolution of direct development, but was directly juxtaposed with the embryonic phase. The intervening larval phase was deleted (Figure 22C). This model is supported empirically by my observation that TRβ mRNA is low in the embryonic period, and upregulates during the metamorphic period (Chapter Two, Figures 8 and 9). The existence of this cryptic metamorphosis in *Eleutherodactylus* is proven by the inhibition of metamorphic-type remodelling when TH-signalling is inhibited. The point of juxtaposition of the embryonic and metamorphic phases of the ancestral ontogeny is predicted to have occurred at TS10 from analysis of opercular development, which indicates the dissociation of two co-variant general developmental character suites around the time of thyroid activation. Independent support for this stage as a break-point in *Eleutherodactylus* comes from the finding that *E. coqui* limbs transplanted onto *R. pipiens* embryos will develop until TS10 before arresting (Elinson, 1994). Thus, while the thyroid axis and concomitant metamorphic events can be viewed as heterochronically accelerated relative to the development of early embryonic characters, this can be explained by deletion of the larval period.

My model, shown in Figure 22C, schematically depicts the rearrangement of the anuran life-history to produce a direct developer. This model indicates that the embryonic and metamorphic periods formed a smooth junction in the ancestral direct developer. It is evident that this is not the case in the modern species, *E. coqui*, where the boundary would be more aptly represented by a jagged line indicating heterochronic shifts in various developmental characters. During the evolution of modern direct developers, development of some characters has been temporally
Figure 22: Models of direct development.

A. Ancestral development: metamorphosis. In biphasic anurans, the embryonic (E) and metamorphic (M) periods are separated by an intervening, developmentally static, larval phase.

B. Direct development: existing model. In this model, direct developers have eliminated both the larval and metamorphic phases from their life-history, and embryogenesis results in the direct production of the adult.

C. Direct development: new model. Here, the metamorphic phase has been retained in the ontogeny of direct developers, but it has been juxtaposed with the embryonic period after deletion of the intervening larval period. The juxtaposition of the E and M periods is hypothesized to have occurred at TS10 of the ancestral ontogeny.
Models of direct development

E = embryogenesis
M = metamorphosis

dissociated relative to other characters (Hanken et al., 1992, 1997a,b; Schlosser and Roth, 1997). It should be noted, however, that some of these studies used hatching as a reference point. Hatching has itself been post-displaced, as demonstrated by the heterochrony plot in Chapter Four (Figure 21). One example of a heterochronic shift is limb development, which is pre-displaced relative to early cranial development: yet after TS9 is post-displaced relative to cranial development (Schlosser and Roth, 1997). For my model to be validated in its entirety, I would predict that events that are metamorphic in other anurans, that is, under thyroid control, will also be under thyroid control in *Eleutherodactylus*. If opercular perforation is truly independent of TH in *E. coqui*, then my model would have to be modified to acknowledge that at least one metamorphic event in *Eleutherodactylus* has been emancipated from TH control.

**Can a direct developer be considered metamorphic?**

Previously, *E. coqui* has been described as a “non-metamorphosing frog” (Jennings, 1994). Additionally, Hanken et al. (1992) have stated that it is “inappropriate and misleading to describe development in this group [*Eleutherodactylus*] as involving a ‘metamorphosis within the egg’” (p115). Therefore, I shall defend my use of the term metamorphosis in the context of direct development. To proceed with such a defence, one needs first to procure a definition of metamorphosis. The difficulty of constructing a comprehensive yet specific definition of metamorphosis has been considered by Rose and Reiss (1993): no such perfect definition exists.

The term metamorphosis, derived from Greek, translates as “change in form”. Such a morphological definition is employed by Webster’s Dictionary, which defines metamorphosis as “a marked and more or less abrupt change in the form or structure of an animal during postembryonic development”. This definition has been utilized by several biologists working on metamorphosis (Sehnal et al., 1996, Sanderson and Kupferberg, 1999). While such a morphological definition is useful to biologists working within a developmental perspective, according this usage, any “more or less abrupt change” in postembryonic morphology should be classed as metamorphic. However, changes such as secondary sex differentiations, including antler growth in deer, and puberty in humans, which fall under this definition, are clearly not embodied in the idea of metamorphosis. I view that a definition encompassing both morphology and ecology may most closely reflect the concept of metamorphosis, which I consider a radical change in morphology coincident with a change in the life-history of an organism.
Direct developers hatch out of the aquatic environment of the jelly capsule to assume a terrestrial existence. However, since the eggs are terrestrial, it might be argued that *Eleutherodactylus* does not undergo a change in life-history and thus should not be considered metamorphic. I view that the use of the term "metamorphosis" is appropriate here, as direct developers are secondarily derived from metamorphosing ancestors. The morphological changes in *Eleutherodactylus* are clearly homologous with the morphological changes that occur in the biphasic ancestral pattern. These biphasic ancestors would have occupied ecologically distinct larval and adult niches. Mechanistically, both the ancestral (biphasic) and derived (direct developing) modes of ontogeny are controlled by the metamorphic effector, TH. Therefore, I think that any change in amphibians should be deemed metamorphic if it is TH-dependent, and if this TH-dependency correlates either currently or ancestrally with an ecological life-history transition.

**Modifications to early embryogenesis.**

Development of both cranial cartilages and muscles has been extensively studied in *E. coqui* (Hanken et al., 1992, 1997b). In both these organ systems, many larval-specific structures have either been radically modified or are entirely absent. However, around TS12, the existing cartilages and muscles assume a mid-metamorphic position and are subsequently remodelled into the adult morphology. My investigation reveals that this cranial remodelling which occurs during the late phases of *Eleutherodactylus* embryogenesis is mediated by TH. The question still remains as to how the early events of cranial development, and indeed the many other derived aspects of early *Eleutherodactylus* ontogeny, have arisen. As mentioned in the Introduction, the basic larval body plan can be extensively modified. The adaptive radiation and convergence of larval forms are testimony to the developmental lability of anuran embryogenesis in response to selective pressure. Certain other direct developers, such as the New Zealand anuran *Leiopelma*, bear an uncanny similarity to *Eleutherodactylus*, demonstrating the power of convergent evolution (Stephenson, 1955). However, there are differences between direct developers, even within the same genus. For instance, in *Leiopelma hochstetteri*, the gular fold, a likely homologue of the opercular fold in *E. coqui*, completely covers the forelimbs. In the congeneric *L. archeyi*, the gular fold never covers more than the forelimb base (Stephenson, 1955). It is possible that modifications to early ontogenesis in *Eleutherodactylus*, such as loss of cement gland, precocious limb development, novel cranial morphogenesis and lack of both coiled gut and lateral line organs (Elinson, 1990) are multiple, independently occurring adaptations to a novel reproductive mode.
**Thyroid hormone as a developmental constraint.**

Within the plethodontid lineage of salamanders, there are two tribes of direct developers, the Bolitoglossini and the Plethodontini (Wake and Hanken, 1996). Adults of the former tribe show some morphological innovation, such as reorganization of the hyobranchial skeleton (Roth and Wake, 1989). The discovery of morphological novelty in the bolitoglossines provided support for the idea that ontogenetic repatterning, such as direct development, could allow emancipation from constraints imposed by the larval morphology (Roth and Wake, 1989). Such morphological innovation has not been seen in the direct developing plethodontines, however, indicating that ontogenetic repatterning may be a necessary but not sufficient pre-condition for changes to the adult form (Roth and Wake, 1989; Wake and Hanken, 1996).

This paradox, namely the lack of adult morphological innovation in species that show ontogenetic repatterning, can be applied to the Anura (Hanken et al., 1992). I hypothesize that the key developmental constraint in anuran life-history is metamorphosis. In this scenario, early frog development is relatively free from developmental constraints and can thus respond to selection by producing highly specialised and divergent embryonic/larval morphologies. However, remodelling of embryonic/larval tissues into their adult counterparts is TH-dependent and must occur in a precisely orchestrated fashion if a functional frog is to result.

Metamorphosis acts like a funnel: divergent body-plans enter at one end and are remodelled through a common signalling pathway (the thyroid axis) to produce a relatively uniform adult morphology when they emerge from the other end of the ontogenetic funnel (Figure 23).

TH has previously been proposed to act as a developmental constraint in anuran evolution, but in quite a different manner. Wassersug (1974) noting that no anuran paedomorphs exist, suggested that morphological constraints on the reproductive system might prevent larval reproduction and thus make metamorphosis obligatory. Hayes (1997) suggests that neoteny has not evolved within the Anura because TH may be required for sex differentiation and reproduction in frogs. Evidence supporting this hypothesis includes the finding that thiourea treatment caused sex-reversal to 100% female in *Xenopus laevis*. The same treatment applied to *Hyperolius viridiflavus* resulted in 100% males (Hayes, 1997). However, this theory must be reconciled with the finding that hypophysectomized and thyroidectomized tadpoles undergo normal sex differentiation (Hsu et al., 1974).
**Figure 23:** Metamorphosis: an ontogenetic funnel.

The leading tadpole in this group has a generalized larval morphology. However, larvae of many frog species possess specialized forms, hence there is a wide diversity of larval morphologies within the Anura (Orton, 1953). This tremendous diversity at the larval stage of anuran life-history is not replicated in the adults, where there is much less morphological divergence (Hanken et al., 1992). Metamorphosis acts as an ontogenetic funnel, wiping away divergent larval morphologies and sculpting a uniform adult shape. The complex and highly co-ordinated TH-signalling cascade which orchestrates metamorphosis may constrain the adult morphology. Its pleiotropic effects on many tissues may result in regulatory couplings, since the tissues must respond holistically to generate a viable adult. The importance of a co-ordinated response is evident from the effects of precocious TH treatment: individual tissues transform, but not in an integrated manner, and the organism is not viable.

Images after Orton (1953).
Metamorphosis: An ontogenetic funnel
Compartmentalization and “set-aside” cells.

Metamorphosis in anurans entails major restructuring, with extensive cell death and proliferation. One mechanism postulated to facilitate the orchestration of this ontogenetic resculpting is compartmentalization. Compartmentalization refers to the presence of two cell populations within an organism, with discrete larval and adult ontogenetic programs (Alberch, 1987). During metamorphosis, larval cells die and the adult population, initially a few isolated nests of cells, proliferates to produce adult structures. In salamanders, there is evidence that compartmentalized cells give rise to the adult epibranchial cartilage (Alberch, 1987). In anurans, the larval intestinal epithelium apoptoses at metamorphosis, and repopulation with adult epithelial cells is thought to arise from isolated nests of stem cells within the larval epithelium (Dauca and Hourdry, 1983). Similar changes occur in the stomach (Ishizuya-Oka et al., 1998). The jaw musculature of amphibians also undergoes extensive remodelling at metamorphosis, with apoptosis of larval myofibres and proliferation of satellite muscle cell populations (De Jongh, 1968; Alley, 1989). The extent of compartmentalized tissues in amphibian larvae is unknown. However, Berry et al. (1998a, 1998b) have identified TR response programs involved in both proliferation and resorption in multiple tissues, indicating that there may be widespread compartmentalization within anuran larvae. Mechanistically, compartmentalized cells can be viewed as those altering their behaviour in response to TH by either apoptosing (larval compartment) or proliferating (adult compartment). If two different cell populations, larval and adult, exist within the same organ, then utilization of a common signal such as TH to effect the destruction of the former and proliferation of the latter would allow a smooth and co-ordinated transition into the adult structure. Abolition of this signalling could result in the loss of co-ordination between larval and adult cell lineages and would be deleterious to the organism.

Compartmentalization is reminiscent of Type I embryogenesis (Davidson et al., 1995; Peterson et al. 1997). In many marine invertebrate phyla, the egg develops into a simple ciliated, motile feeding trochophore larva, with a limited number of cells. During embryogenesis, two cell lineages are specified: the larval lineage and the “set aside” lineage. At metamorphosis, the cells which comprise the larval structures are lost, and the adult body is generated from a separate population of cells, termed “set-aside” cells (Davidson et al., 1995). Set-aside cells are presumed by Peterson et al. (1997) to have evolved once within the Metazoa, at a time before the protostome-deuterostome divergence. This common ancestor is thought to have possessed both a trochophore larva and set-aside cells. A modern example of this developmental mode is
exhibited by many echinoderms, where larval structures degenerate completely, and the adult forms from the localized rudiment at the onset of metamorphosis. This extreme type of development is termed “maximum indirect development” by Davidson et al. (1995).

Maximum indirect development is contrasted with direct development (Peterson et al., 1997), as seen in the chordates and arthropods. These authors use the term “direct development” in a different sense to the way I have used it in this thesis. Direct development, sensu Peterson et al. (1997), occurs when “the post embryonic forms immediately display specific and definitive adult body plan characters of their respective groups” (p.628). Chordates are considered direct developers because during embryogenesis, adult structures such as the notochord and dorsal CNS form directly, and there is no morphologically evident transition, as occurs in maximum indirect development. In organisms with secondarily derived larvae, the larval cells contribute to structures that are definitive of the adult phyyletic body plan (such as the notochord and dorsal CNS of chordates). In contrast, the larval cells of maximally indirect developing organisms never contribute to the adult body plan.

Peterson et al. (1997) view that “maximum indirect development and direct development, as seen in modern invertebrates, are two extremes of a continuum in which the product of embryogenesis consists of increasing proportions of set-aside cells and decreasing proportions of larva-specific cells” (p.625). Therefore, it appears that the difference between maximum indirect development and direct development is qualitative rather than quantitative, being dependent upon both the relative proportions of larval and set-aside or compartmentalized cells, and the developmental time at which this set-aside cell population is activated. Presumably, pre-displacement (Gould, 1977) of set-aside cell proliferation could allow the rapid production of an adult morphology, with a tendency towards direct development. This may account for the evolution of direct development in arthropods, chordates and ecdysozoans (Aguinaldo et al. 1997). Similarly, post-displacement or inhibition of such proliferation could result in the appearance of “neotenous” phyla, as appears to have occurred in the Rotifera, where the adult form is similar to a trochophore larva (Peterson et al., 1997).

Peterson et al. (1997) consider that stem cells of adult vertebrates and imaginal discs of holometabolous insects are set-aside cells. I suggest that the adult compartment of cells activated during metamorphosis in amphibians should also be considered as set-aside cells, even
though the amphibian larva, like that of insects, is secondarily derived (Elinson, 1990), and therefore is not homologous to the larvae of marine invertebrates.

Davidson and Peterson (1995) propose that the trochophore ancestor of protostomes and deuterostomes possessed gene regulatory networks to specify set-aside cells. I think that a further requirement for such a maximally indirect developing ancestor would be the presence of a regulatory pathway to control the switch from the larval to set-aside compartments. I hypothesize that this ancestral regulatory pathway may have been governed by nuclear hormone receptors (NRs), most likely involving the retinoic acid receptor RXR subclass. While I do not have any direct evidence to support this theory, several empirical studies provide indirect support. As mentioned in the General Introduction, TH is involved in the metamorphosis of both chordates and echinoderms. This finding implicates NR-signalling in life-history transitions within the deuterostomes. RXR is implicated in anuran metamorphosis. Its upregulation correlates with metamorphic transitions in various tissues, and it facilitates the binding of TRs to TREs (Shi et al., 1996). RXRs, therefore, may be involved in the compartmentalized/set-aside cells of anurans. RXRs are also necessary for insect metamorphosis. The Drosophila RXR is an essential component of the ecdysone receptor, which transmits the metamorphic signal (Hall and Thummel, 1998). Again, the Drosophila larva is secondarily derived, but its imaginal discs are considered as set-aside cells by Peterson et al. (1997). Proliferation of these discs requires RXR signalling (Hall and Thummel, 1998). TH and iodoscompounds can induce metamorphosis in the cnidarian, Aurelia, and metamorphosis is inhibited in the absence of iodine (Spangenberg, 1967, 1971). This implicates NR signalling in the life-history transition of a diploblast. An RXR has been cloned from a cnidarian (Kostrouch et al., 1998), indicating the antiquity of this gene family. Finally, arachidonic acid metabolites have been implicated in metamorphosis of the cnidarian, Hydra (Leitz et al., 1994). These compounds are structurally similar to retinoic acid. Arachidonic acid metabolites can activate chick RXR-γ (Eager et al., 1992).

Peterson et al. (1997) class vertebrate stem cells as set-aside cells. Evidence for the role of NRs in compartment switching in direct developing chordates includes their effects on defined cell populations. Several amniote cell populations respond to TH by apoptosis. These include lymphocytes (Mihara et al., 1999), osteoblasts (Varga et al., 1999), and muscle cells (Nakashima et al., 1998). TH appears to prevent apoptosis of some cerebellar cells (Xiao et al., 1998). RXRs can also induce terminal differentiation and apoptosis (Crowe and Shuler, 1998).
Viewing phyletic life-histories as maximally indirect or direct may be creating an artificial dichotomy from a natural spectrum. The important mechanisms to consider are those that originally gave rise to set-aside cells, those which allow the transition from larval to set-aside cells, and those which allow the pre-displacement or post-displacement of set-aside cell activation. It is my contention that the middle mechanism, involving the activation of the set aside cells, is orchestrated through NR/RXR signalling.

This hypothesis can be tested by determining whether RXR-mediated signalling is involved in the metamorphosis of the many maximally-indirect developing phyla. A simple method would be to treat embryos with either RXR agonists or antagonists. 9-cis retinoic acid is a readily available RXR agonist but it also activates RARs. RXR-selective ligands have recently been developed (LeMotte et al., 1996), and when commercially available, it would be interesting to see if they could induce metamorphosis in invertebrate phyla. An alternative method would be to try dsRNA interference (Fire et al., 1998), which has been shown to specifically deplete genes in both invertebrates and plants. dsRNA encoding for the conserved RXR DNA-binding domain could be introduced into maximally indirect developing embryos, to determine whether this gene is required for metamorphosis.

**Summary of current investigation**

In this investigation, I have demonstrated that direct development in *Eleutherodactylus* is extensively dependent on thyroid hormone signalling. Direct evidence for the involvement of TH comes from the developmental arrest of embryos treated with the thyroid inhibitor methimazole. Correlative evidence for the molecular mechanism by which this signalling is mediated is provided by the expression pattern of thyroid hormone receptors. The temporal expression profiles of TRα and TRβ mRNAs are similar to those of metamorphic frogs. TRα appears very early in embryogenesis, whereas TRβ upregulates dramatically around the time of thyroid differentiation and just prior to the commencement of remodelling events which are methimazole-inhibitable. Recapitulation of a larval character, the opercular fold, previously thought absent from direct developers, was observed. Comparison of the temporal appearance and perforation of this structure in *Eleutherodactylus* relative to metamorphosing anurans indicates that the tadpole was excised from the ancestral ontogeny during TS10.
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