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Effects of Perchlorate in Amphibians

James A Carr, Christopher Theodorakis

Ecological Importance of Amphibians

There are 3 taxonomic orders of extant amphibians within the class amphibia:

1) order Anura, which includes all tailless amphibians (frogs and toads);
2) order Caudata (urodeles), which includes the newts and salamanders; and
3) order Gymnophiona (Apoda), which is composed of the caecilians.

These 3 orders make up more than 6000 species distributed worldwide (UNEP–WCMC Species Database 2004 **[Not in References]**). Approximately 283 species can be found in the United States, of which 152 species are endemic (World Resources Institute, Earthtrends 2004).

Amphibians live in a diverse array of habitats, from equatorial rainforests to Alaska, and not all species spend part of their life history on both water and land. For example, some frogs (family Pipidae) and salamanders (family Cryptobranchidae) are highly adapted for an aquatic existence and spend little if any time on land. In contrast, other frog (family Leptodactylidae) and salamander (family Plethodontidae, the lungless salamanders) species do not normally visit water as part of their life history strategies (Feder 1992). Likewise, amphibian modes of development and reproduction are diverse. Although many frog species go through metamorphosis as part of their postembryonic development, there are frog species (*Eleutherodactylus coqui*) that exhibit direct development with no larval stage. Although external fertilization takes place in most amphibian species, caecilian species employ

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internal fertilization, and three-fourths of the species within the order are viviparous or live-bearing. Given this diversity of life history patterns, it is important to use care when attempting to extrapolate from data collected on a single species to all amphibians.

Potential Role of Contaminants in Amphibian Declines

Several lines of evidence support anecdotal observations that frog populations are declining globally. Recent data have quantified large-scale declines in amphibian populations inhabiting North America as well as, in some cases, population declines in genetically isolated species with an extremely limited geographic distribution (Houlahan et al. 2000). Urban encroachment and consequent loss of ecological corridors for migration have resulted in the reduction of some North American species to a single natural population, such as the case with the Wyoming (*Bufo baxteri*) and Houston (*Bufo houstonensis*) toads (USFWS 1984, 2001). In other cases, species with historically wide distribution within the Sierra Nevada, such as the California red-legged frog (CRLF; *Rana aurora draytonii*) or the Cascades frog (*Rana cascadae*), have incurred significant loss (70% in the case of the CRLF) in their geographic distribution (Fellers and Drost 1993). In the most dramatic cases, entire species have become extinct. Two species of *Rheobatrachus*, the gastric brooding frog, have not been observed since the 1980s (Ingram and Donald 1993; Hines et al. 1999).

Before we can consider the potential effects of perchlorate exposure on amphibian declines, it is important to look at the relative role that contaminant exposure may play in this phenomenon. Several potential causes have been forwarded for amphibian population declines; the leading hypotheses have been formalized by Collins and Storfer (2003). Class I hypotheses include factors such as loss of habitat, introduction of alien species, and over-exploitation and collection of amphibians. There is accumulating evidence that stochastic variation in breeding success, coupled with isolation of certain populations by reduction in ecological corridors, may be a leading cause of extinctions at the population level (Richter et al. 2003). In the Western United States, habitat loss and fragmentation have been implicated; more than 90% of aquatic habitats have been destroyed in Southern California and Arizona. Given the rapid human population increases in California and the limited natural water sources, especially in Southern California, it is not surprising that 3 of the 10 anuran species currently listed as endangered or threatened inhabit California (USFWS 2003). In the case of the CRLF, population declines in the 20th century

were preceded by the conversion of Sacramento and San Joaquin Valley wetlands and riparian habitats to agricultural land (Federal Register 2000). Streams were stripped of natural vegetation and canalized, and livestock grazing removed vegetation cover and undercut the banks along streams, causing increased water temperatures and lack of cover. These changes in riparian habitat reduced historical wetlands by more than 90%, with most habitat loss having occurred before 1939, and CRLF populations were entirely absent from the Central Valley floor by 1960. Those populations that remained in the Sierra Nevada foothills were separated from other breeding populations, further increasing the genetic isolation of remaining populations. The introduction of bullfrogs also has been linked empirically to adverse effects on survival in the CRLF. Less than 5% of CRLF tadpoles survive in ponds containing bullfrog tadpoles, whereas 30% to 40% of the tadpoles survive in the absence of bullfrog tadpoles (Lawler et al. 1999; Federal Register 2000). In a long-term study, Vredenburg et al. **[only Vredenburg is listed in References; no co-authors. Which is correct?]**(2004) demonstrated that introduction of trout into mountain ponds in the Sierras was responsible for the decline of this mountain frog (*Rana mucosa*) due to predation on tadpoles.

Class II hypotheses include global changes in climate (UV radiation, global warming), emerging diseases (such as the opportunistic chytridomycete fungus), and contaminants such as pesticides and industrial waste products. In the case of *Rheobatrachus*, there is compelling evidence for a role of the opportunistic chytridomycete fungus as a proximate cause for mass mortality in southern and northern populations of this genus (Berger et al. 1998). The temporal and geographic decline of *Rheobatrachus* and sympatric species inhabiting riparian areas of eastern Australia is consistent with an epidemic outbreak of chytrid fungus, and several recent studies confirm the association of this fungus with mass mortality events in anuran species (Berger et al. 1998; Morehouse et al. 2003). Chytrid fungus also has been associated with declines in North American populations of the boreal toad and with declines in the single remaining population of the Wyoming toad (*Bufo baxteri*) (USFWS 2001). Recently, chytrid fungus has been implicated in declining populations of the mountain yellow-legged frog (*Rana mucosa*, Fellers et al. 2001), another species inhabiting the Sierra Nevada range.

The fact that amphibians have a relatively permeable integument and generally deposit eggs with little protection from contaminants has led to speculation that they may be sensitive indicators of contaminant exposure (see Blaustein and Johnson 2003), in a way serving as "canaries in the coal mine." However, the role of contaminants in amphibian declines

has been hotly debated, especially because many reports of declining species have occurred in areas that should be protected, at least in theory, from widespread agricultural or industrial contamination (USGS 2004). There are data linking airborne drift of contaminants (organophosphorus insecticides) with amphibian declines in California. Sparling et al. (2001) reported that surface waters in Sequoia National Park at an elevation (2000+ m) that had been associated with declining frog populations contained greater than 100 $ng \cdot L^{-1}$ chlorpyrifos and greater than 65 $ng \cdot L^{-1}$ diazinon. Furthermore, treefrog (*Hyla regilla*) tadpoles collected from populations in Sequoia and Yosemite National Parks, and located downwind from agricultural areas in the Sacramento and San Joaquin valleys, had body burdens of chlorinated pesticide residues 2 to 3 times greater than tadpoles from coastal areas of California (Sparling et al. 2001).

Although there have been no studies directed at examining a potential link between geographical and historical use of perchlorate and amphibian population declines, there are some data on overlap between amphibian populations and perchlorate-contaminated watersheds located in designated habitats for endangered or threatened amphibian species. As a result of cleanup efforts associated with ground water contamination in an Aerojet General Corporation's facility in Sacramento County, perchlorate was detected in ground water at concentrations as great as 8 $mg \cdot L^{-1}$. As of March 2004, perchlorate had been detected in wells and other drinking water supplies in 10 counties, with the highest concentrations reported as of March 2004 in San Bernardino and Sacramento counties, where perchlorate concentrations as high as 820 $\mu g \cdot L^{-1}$ and 400 $\mu g \cdot L^{-1}$, respectively, were reported. Perchlorate concentrations greater than 100 $\mu g \cdot L^{-1}$ have been detected in more than 139 wells in Los Angeles, Sacramento, and San Bernardino counties (California EPA 2003). Los Angeles County is listed in the proposed critical habitat designation for the CRLF (USFWS 2000). Other counties designated as containing critical habitat for the CRLF (Federal Register 2000) and for which perchlorate data are available (California EPA 2003) are Riverside (4.0 to 56 $\mu g \cdot L^{-1}$), Santa Clara (4.0 to 8.5 $\mu g \cdot L^{-1}$), San Diego (4.0 to 4.7 $\mu g \cdot L^{-1}$), and Ventura (5.7 to 20 $\mu g \cdot L^{-1}$, all measured on the U.S.N.**[is this abbreviation for US Navy?]**–operated Saint Nicolas island, 34 of 34 samples containing perchlorate >4 $\mu g \cdot L^{-1}$). Thus, it appears that concentrations of perchlorate in water bodies in California measured since 1997 can be quite substantial (greater than 100 $\mu g \cdot L^{-1}$ in many cases). It is important to note, however, that the large majority of these data reflect measurements made on groundwater, and the degree to which surface waters inhabited by frogs

may come in contact with contaminated ground water is an additional uncertainty.

In summary, although no studies to date have been designed to examine explicitly an association between the geographical and historical use of perchlorate and amphibian declines, data on the existing overlap between perchlorate ground water and historical species distribution can be taken into account when critical habitat for relocation and reestablishment of amphibians is being set aside.

Routes of Exposure

Perchlorate may enter surface waters from runoff or via ground water contamination and may be present in vegetation (Yu et al. 2004) eaten by larval amphibians. Another potential route of exposure is via airborne drift; sodium chlorate (ClO_3^-), which disrupts thyroid function by a mechanism similar to that of perchlorate (Hooth et al. 2001; Van Sande et al. 2004 **[References show 2003; which is correct?]**), is applied as an agricultural defoliant on cotton in West Texas (West Texas IPM update, 2002 **[Not in References]**) and possibly elsewhere.

The sodium-dependent iodide symporter (NIS) is the only known mechanism for transporting perchlorate across epithelial membranes. This protein has been identified in epithelia from many extra-thyroidal tissues (see Chapter 3). Thus, the possibility exists for several possible routes of perchlorate uptake in amphibians. Theoretically, perchlorate may enter the body across epithelia in the skin or gills (in amphibian larvae) which are in direct contact with surface water or in herbivorous tadpoles via dietary intake by ingesting vegetation that has accumulated perchlorate (Yu et al. 2004) (see **Figure** 6-1). Preliminary studies have provided empirical evidence for perchlorate sensitive $^{125}I^-$ uptake by the gastrointestinal (GI) tract in frogs (Harrison et al. 2002; Carr et al. 2003). The elimination rate of perchlorate in frogs is not known, although based on studies in mammals, perchlorate should be eliminated relatively rapidly. In *Xenopus laevis* larvae, the effects of a 70-d perchlorate exposure are reversed within 28 d of non-treatment (Goleman et al. 2002b **[throughout this chapter, Goleman 2002a and 200b are cited, but both Goleman 2002 references are listed as "b"; please correct in References]**). A conceptual model illustrating environmental effects and fate of perchlorate in amphibians is in **Figure** 6-2.

Amphibians must obtain and sequester iodide from their environment in order to synthesize thyroid hormones (THs). Because iodide and perchlo-

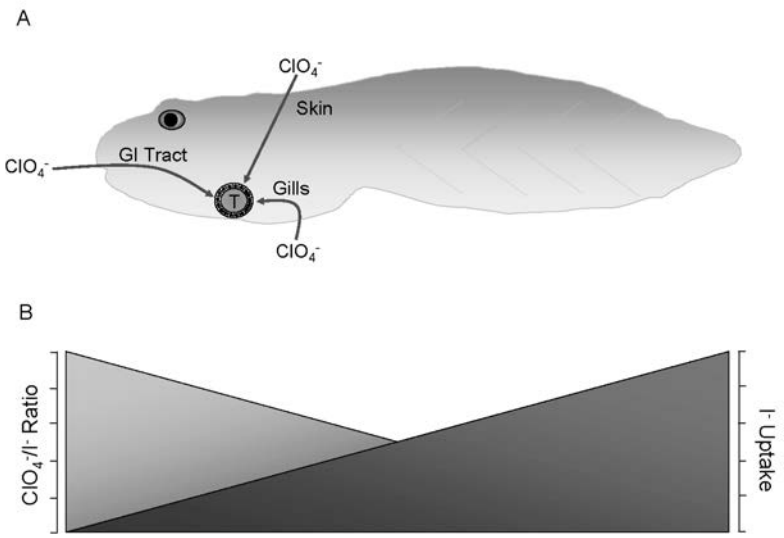

Figure 6-1 A) Potential sites for perchlorate uptake in larval frogs. Perchlorate may be taken up by a sodium-dependent iodide symporter (NIS) in a number of epithelia including skin, GI tract, and respiratory epithelium. Once in the bloodstream, perchlorate travels to the thyroid gland (T) to block iodide uptake (also see Chapter 3). B) The efficiency of iodide uptake by a larval frog will depend upon the relative amounts of perchlorate and iodide in the environment. As perchlorate in the environment increases, the ability to take up iodide decreases proportionately.

rate are transported by the same protein, it is logical that the degree to which either anion is taken up will depend upon the relative abundance of the other (**Figure** 6-1B). Simply stated, the effect of perchlorate will depend upon the availability of iodide to the organism. There is empirical evidence to support this hypothesis. Sparling et al. (2003) found that simultaneous exposure to iodide reduced the antimetamorphic effects of perchlorate exposure in tree frogs. Hu et al. (2003) found that increasing iodide availability eliminated the inhibitory effects of perchlorate on forelimb emergence, tail absorption, and hindlimb growth in *X. laevis* (**Table** 6-1).

Hypothalamus–Pituitary–Thyroid Axis in Amphibians

In amphibians, there are paired thyroid glands attached to the hyoid cartilage, and TH synthesis is thought to occur much as it does in mammals. For example, organification of iodide is blocked by propylthiouracil (PTU) (Burd 1992) and methimazole (Becker et al. 1997) in amphibians

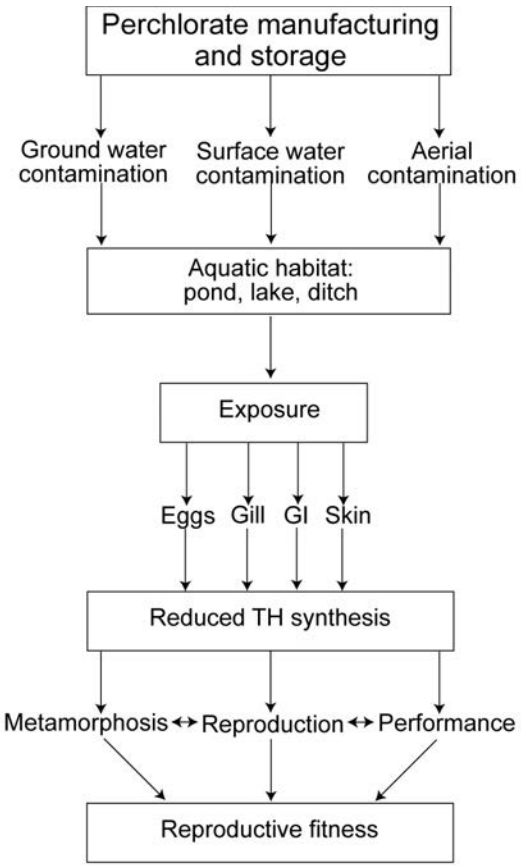

Figure 6-2 Conceptual model illustrating potential sources of perchlorate and potential outcomes of perchlorate exposure in amphibians

just as in mammals. In addition, thyroglobulin (Tg) structure appears to have been phylogenetically conserved in those amphibians in which the protein has been purified or the gene for TG **[should "TG" be "Tg"?]** cloned. Amphibian and reptilian THs are structurally identical to those in mammals and in all other vertebrate classes, and techniques (such as radioimmunoassay, high-performance liquid chromatography) for measuring THs in mammalian species can be applied successfully to amphibians and reptiles. Taken together, these findings indicate that pathways for TH synthesis in amphibians and reptiles are similar if not identical to the pathways in mammals.

It is generally believed that THs are transported in the blood attached to transport proteins in amphibians and reptiles, similar to the situation in mammals. Transthyretin mRNAs and proteins have been sequenced or purified in amphibian (Prapunpoj et al. 2000; Yamauchi et al. 2002) and reptilian (Prapunpoj et al. 2002) species, suggesting that a transthyretin-like binding protein has been conserved evolutionarily. Both T_3 and T_4 are present in the circulation of amphibians and reptiles, and deiodination is presumed to be an important means of hormone activation and deactivation, as in mammals. Homologs of the mammalian 5'DI, 5'DII, and 5D are present in amphibians (Becker et al. 1997).

Table 6-1 Mean incidence of forelimb emergence, complete tail absorption, and hindlimb length in *Xenopus laevis* tadpoles exposed to FETAX[a] medium or ammonium perchlorate in the presence or absence of sodium iodide for 70 d

Treatment	Forelimb emergence (FLE)[b] (% ± SEM)	Complete tail absorption[c] (% ± SEM)	Hindlimb length (mm)
Frog embryo teratogenesis assay (FETAX) control	91.6 ± 1.83	91.1 ± 2.44	19.1 ± 0.19 (n = 158)
14 mg·L^{-1} ammonium perchlorate (AP)	0.00 ± 0.00[d]	0.00 ± 0.00[d]	2.45 ± 0.07[d] (n = 167)
14 mg·L^{-1} sodium iodide (NaI)	88.3 ± 3.61	84.5 ± 5.68	20.1 ± 0.22 (n = 147)
14 mg·L^{-1} NaI + 14 mg·L^{-1} AP	94.3 ± 1.21	92.1 ± 0.66	19.0 ± 0.19 (n = 162)

[a] Controls were reared in untreated FETAX (*Xenopus*, Dawson and Bantle 1987). Exposures began within 24 h of fertilization and continued for 70 d. Sample size of 4 tank replicates for incidence data. Sample size for hindlimb length measurements is indicated in parentheses. Mortality during the 70-d exposure was <6% for all groups except the NaI treatment, in which mortality was 12%.

[b] Forelimb emergence, number with forelimbs divided by number hatching during the 70-d exposure. Hatching exceeded 87% in all groups.

[c] Number with complete tail absorption divided by number hatchings.

[d] Asterisks indicate significantly different from FETAX medium control ($p < 0.05$).

The function of the hypothalamo–hypophysial–thyroid axis has been essentially conserved in amphibians in the sense that thyroid gland function is controlled primarily by thyroid-stimulating hormone (TSH; Dodd and Dodd 1976). mRNAs encoding both the alpha and the beta subunits of TSH have been sequenced in *X. laevis* (Buckbinder and Brown 1993). Recently, Okada et al. (2004) developed a homologous RIA **[spell RIA, please]** for TSH in bullfrogs, facilitating the ability to accurately measure plasma TSH in anurans. Given the abundant evidence that perchlorate elevates blood TSH levels in other organisms (due to removal of feedback inhibition by thyroxine), measurement of blood TSH in amphibians will be an important tool for assessing perchlorate exposure in future studies.

In adult amphibians, thyrotropin-releasing hormone (TRH) acts on the anterior pituitary gland to stimulate TSH secretion (Denver 1988; Denver and Licht 1989). Hypothalamic corticotropin-releasing factor (CRF) also appears to be an important stimulator of pituitary TSH secretion in amphibians. Treatment of frog and hatchling turtle pituitaries with CRF elicits TSH secretion (Denver and Licht 1989; Boorse and Denver 2004; Okada et al. 2004). In larval amphibians, TSH secretion is not stimulated by TRH (Denver and Licht 1989), and CRH is the principal

hypothalamic regulator of the pituitary–thyroid axis during metamorphosis (Denver et al. 2002).

Role of Thyroid Hormones in Reproduction and Development in Amphibians

Thyroid hormones and amphibian metamorphosis

A unique and dramatic feature of normal life history for many amphibian species is a postembryonic development phase called "metamorphosis," in which the organism transforms from an aquatic larval form to a terrestrial or semiterrestrial form. This requires normal TH production, which is why many studies have focused on the impact of perchlorate exposure on metamorphosis. Thyroid hormones are required for the massive reprogramming of gene expression that is required for the morphological, biochemical, and physiological changes that take place during metamorphosis, including resorption of the larval tail, reorganization of the GI tract, development of limbs, restructuring of the skeleton, and a switch in nitrogen metabolism from ammonia to urea production (Shi 2000).

A consequence of perchlorate disruption of TH synthesis is that less TH is available for interaction with TH receptors. Amphibians possess TH receptors that are similar in structure and function to their mammalian counterparts, and receptor-mediated gene transcription is the major form of TH action during metamorphosis. It is generally believed that THs interact with their receptors to control metamorphosis by directly influencing the expression of immediate early-response genes whose products, in turn, affect the expression of late-response genes, resulting in a gene regulation cascade (Shi 2000). Many of the early TH response genes encode transcription factors (including the TRß gene) that, in turn, repress or activate the expression of late response genes (Shi 2000) during metamorphosis.

There are many hormones that can act to modify TH action during metamorphosis. Corticosteroids (corticosterone, aldosterone) secreted from the larval interrenal tissue have been reported to accelerate TH-induced metamorphosis, although the effects are stage and tissue dependent. Corticosteroid levels in blood are elevated during metamorphosis, and receptors for these steroids are present in a number of larval organs, including tail and liver (see Krain and Denver 2004 for a recent review). Prolactin (PRL), a pituitary hormone, has been reported to inhibit TH-induced metamorphosis. A physiological role for PRL in metamorphosis remains a matter of debate, however, because the production of PRL and

its receptor are elevated during normal metamorphosis (Buckbinder and Brown 1993).

Not all amphibians must complete metamorphosis in order to reproduce. For example, some urodele species reach reproductive maturity and breed in a larval form, a condition generally known as "neoteny." Some species (the tiger salamander, *Ambystoma tigrinum* and the Mexican axolotl, *Ambystoma mexicanum*, e.g.) undergo metamorphosis only under favorable environmental conditions. This phenomenon, called "facultative neoteny," cannot be explained by a lack of functional TH receptors because the axolotl has functional alpha and beta forms of the receptor (Safi et al. 2004). Other species never undergo metamorphosis, a condition referred to as "obligate neoteny." Unlike facultative neotenes, obligate neotenes (such as the mudpuppy *Necturus maculosus*) do not respond to TH treatment by initiating metamorphosis. Neoteny has not been reported to date for any anuran species.

Thyroid hormone production and secretion increases during metamorphosis, coinciding with dramatic morphological changes such as emergence of the forelimbs and tail resorption. Expression and activity of a 5' deiodinase (Type II or D2) increases during metamorphosis (Becker et al. 1997; Manzon and Denver 2004) in a tissue-specific manner, presumably resulting in more circulating T_3, although some there are some data to indicate tissue specific increases during metamorphic climax in the activity of type III deiodinase (Manzon and Denver 2004), which inactivates T_4 and T_3 (see Chapter 3). Early attempts to induce TH secretion and metamorphosis with TRH were unsuccessful, suggesting that control of the pituitary–thyroid axis in larval amphibians was very different than what was known to occur in mammals. There is now substantial evidence that CRF exerts dual control over the pituitary–thyroid and pituitary–adrenal axes during metamorphosis. Administration of CRF to tadpoles elevates plasma thyroxine and corticosterone levels (Gancedo et al. 1992; Denver 1993; Boorse and Denver 2004) and accelerates metamorphosis in a number of anuran and urodeles species (Miranda et al. 2000; Boorse and Denver 2002, 2004). Levels of immunoreactive CRF in the median eminence increase during the latter stages of metamorphosis (Carr and Norris 1990), further implicating this peptide in the hypothalamic regulation of thyroid function.

As described later in this chapter, disruption of thyroid function by perchlorate or other contaminants can affect the rate of metamorphosis or limb growth. There are important ecological consequences to interrupting or delaying metamorphosis. The length of the larval period can vary tre-

mendously within amphibians, taking up to 3 years in some species such as the American bullfrog (*Rana catesbeiana*) or lasting just a few weeks in some desert-adapted species inhabiting ephemeral ponds, such as in species of spadefoot toads that inhabit the southwestern United States (genera *Scaphiopus* and *Spea*, family Pelobatidae). Delaying or disrupting the timing of metamorphosis can have potentially dramatic effects on organism performance and may ultimately affect fitness by making larvae more susceptible to aquatic predators. In desert-dwelling species, a delay in the timing of metamorphosis may decrease the likelihood that the animals will complete metamorphosis before the pond dries up. The potential ecological consequences of delaying metamorphosis are illustrated in **Figure** 6-3.

Thyroid hormones and reproduction in amphibians

Normal TH secretion is required for masculinization in anurans based upon data collected primarily in *X. laevis*. Thyroid hormones appear to be necessary for expression of androgen receptors in *X. laevis* (Cohen and Kelley 1996; Robertson and Kelley 1996), and blocking normal TH synthesis can cause feminization of offspring (Hayes 1997; Goleman et al. 2002b). The link between THs and reproductive development is less clear for urodeles because neotenic salamanders breed successfully year after year in some locales. No clear link has been established between seasonal changes in thyroid activity and sex steroid levels or gonadal function in anurans (Vandorpe et al. 1990), although some (Tasaki et al. 1986; Gancedo et al. 1995) have reported sex differences in seasonal cycles of plasma T_3 and T_4 in frogs.

Other roles for thyroid hormones in amphibians

In mammals and birds, THs increase oxygen consumption in virtually every organ in the body by influencing cellular respiration via a number of long- and short-term actions on mitochondrial energy transfer. Amphibians do not produce significant amounts of heat, and data suggesting a link between THs and oxygen consumption in amphibians are equivocal, with some studies reporting an effect (May et al. 1976; Gupta and Deka-Borah 1995) and others reporting no effect (Gupta and Chakrabarty 1990) of THs on respiration in amphibians.

Thyroid hormones play an important role in brain development in amphibians as they do in mammals. Thyroid hormones are required for proper development of the median eminence and hypothalamus, and specifically play a role in the development of catecholaminergic cells groups in the amphibian brain (Kikuyama et al. 1979; Carr et al. 1991; Norris

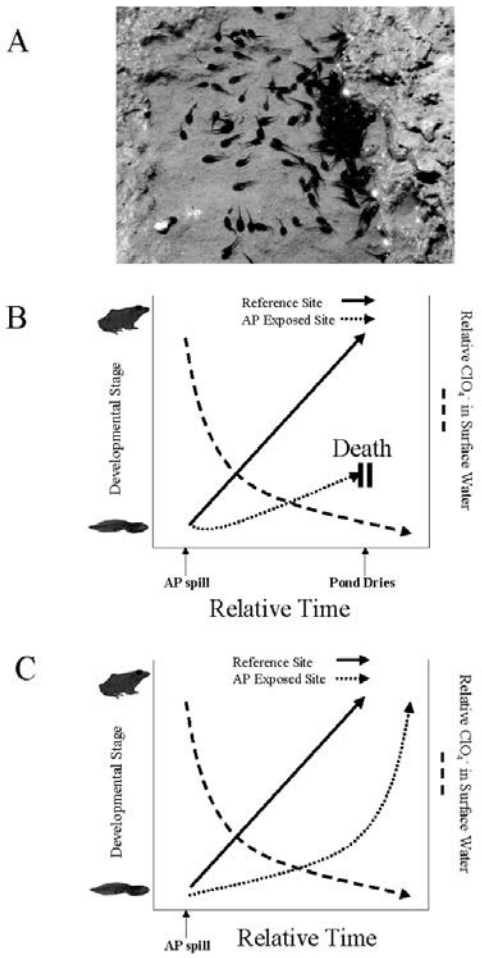

Figure 6-3 Perchlorate inhibition of metamorphosis may have different consequences for tadpoles with short and long larval periods. This model is based on data indicating that perchlorate disappears gradually from pond water over time (compare perchlorate values for the INF pond at Longhorn Army Ammunition Plant (LHAAP) in Smith et al. 2002 v. Carr et al. 2003) provided that the source of perchlorate contamination is eliminated. A) Tadpoles inhabiting ephemeral ponds must develop rapidly before the pond dries up. B) Perchlorate effects on metamorphosis in a species with a short larval period. After perchlorate exposure, the rate of metamorphosis depends on the amount of perchlorate remaining in the water body. Because the effects of perchlorate are reversible, the rate of development would be expected to increase as perchlorate concentrations in the water decrease. In tadpoles with a short larval period, perchlorate inhibition of metamorphosis may have serious consequences because any delay in the timing of metamorphosis may prevent the animals from leaving the pond before it dries up. C) Perchlorate effects on metamorphosis in a species with a long larval period. In animals with a multi-year larval period, perchlorate exposure earlier in development may delay, but not prevent, metamorphosis because the animals would recover from perchlorate exposure over time. **[Please reduce this caption to short phrases that describe its components and move the remainder to the chapter text. Please also provide an editable version of this graphic, or a 300-dpi version so that it will be readable in print.]**

et al. 1992), very similar to the role they play in the development of catecholaminergic pathways in the mammalian brain. Thyroid hormones also are required for development of the cerebellum (Hauser and Gona 1983, 1984; Hauser et al. 1986) and visual pathways in frogs (Hoskins and Grobstein 1985).

Perchlorate Effects on Amphibians

Mechanism of perchlorate action in amphibians

Although a full-length NIS has yet to be cloned from any amphibian or reptilian species, indirect evidence indicates that perchlorate acts to blocks iodide transport across the basaolateral plasma membrane of thyroid follicle cells just as has been shown in mammals. Partial cDNA fragments encoding putative NIS proteins have been sequenced from whole *X. laevis* tadpoles (Carr et al. 2003) and isolated bullfrog tadpole thyroid glands (unpublished results). Administration of excess iodide can prevent the inhibition of perchlorate-induced inhibition of TH synthesis in amphibians, presumably by competing with perchlorate for the NIS on thyroid follicle cells (Hu et al. 2003; Sparling et al. 2003).

Acute toxicity of perchlorate in amphibians

Perchlorate is not overtly toxic to adult or larval frogs at concentrations that are likely to be encountered in the environment. Studies on the lethality of ammonium perchlorate (AP) in embryonic and larval *X. laevis* have reported LC50s ranging from 496 $mg \cdot L^{-1}$ to 510 $mg \cdot L^{-1}$ (Bantle et al. 1999; Goleman et al. 2002a). In a more recent study, Dean et al. (2004) examined the acute toxicity of sodium perchlorate in premetamorphic green frog tadpoles. The LC50 for 96 h mortality for sodium perchlorate in green frog tadpoles was 5500 $mg \cdot L^{-1}$ (Dean et al. 2004).

Perchlorate effects on amphibian metamorphosis

The ability of perchlorate salts to inhibit metamorphosis has been known and exploited as a research tool for decades. Given the known mode of action for perchlorate, it is not surprising that perchlorate exposure affects thyroid follicle cell structure. Miranda et al. (1996) reported that exposure of *Bufo arenarum* larvae to perchlorate caused hypertrophy of thyroid follicle cells and an increase in thyroid gland volume. Consistent with the reported effects of perchlorate on thyroid follicle cell structure, high concentrations of perchlorate salts block metamorphosis and prevent normal development of the immune system in *X. laevis* (Rollins-Smith

et al. 1990, 1993, 1997). Interestingly, perchlorate also interferes with the development of hardwired connections between the central nervous system and the immune system by disrupting the normal development of catecholaminergic innervation to immune organs such as the spleen (Kinney et al. 1996). The effects of perchlorate on development of the neuroendocrine system are complex, as some processes such as development of thyrotropes and lactotropes are altered whereas the development of other systems, such as the CRF pathway, does not appear to be affected (Miranda et al. 1997 **[is this Miranda and Dezi 1997?]**). However, this latter finding (or lack of response) has not yet been confirmed by other laboratories and is not consistent with the observed effects of thyroid inhibition on the development of hypophysiotropic pathways. For example, Shi et al. (1994) reported that normal, circulating THs are required for development of the CRF and pro-opiomelanocortin neuronal systems in rats. Given the well-known role of THs in controlling the development of the central nervous system (CNS), more work needs to be done on the effects of perchlorate on CNS development in amphibians.

The majority of studies conducted on perchlorate and amphibian metamorphosis have employed large concentrations of perchlorate as a tool for inhibiting metamorphosis, but have not addressed the exposures were environmentally relevant. This is understandable, as it wasn't until the late 1990s that the extent to which water supplies were contaminated with perchlorate became known (see Chapter 1). Some early studies reported that perchlorate could be detected in drinking water supplies across the country (Urbansky 2002). These findings spawned a more intensive look at perchlorate in surface and ground waters associated with military and aerospace facilities. Subsequent studies revealed that surface waters that serve as breeding ponds for amphibians may have relatively high perchlorate concentrations, in some cases as great as 30 $mg \cdot L^{-1}$ (Smith et al. 2001; USACE 2004). These studies prompted two new questions. First, do environmentally relevant concentrations of perchlorate interfere with metamorphosis? Second, can disruption of thyroid function in amphibians serve as a diagnostic tool predicting exposure consequences in other wildlife and humans? Recent research by independent laboratories indicates that environmentally relevant concentrations of perchlorate alter thyroid function, metamorphosis, limb growth and reproductive development in the laboratory. Exposure to AP in the 5 to 100 $\mu g \cdot L^{-1}$ range for 70 d can delay metamorphosis, while concentrations greater than 147 $\mu g \cdot L^{-1}$ completely inhibit metamorphosis (Goleman et al. 2002a). Using a 14-d exposure (compared to the 70-d exposure used by Goleman et al. 2002a, 2002b), Tietge et al. (2005) demonstrated that exposure to 250 µg

perchlorate·L^{-1} inhibited metamorphosis in *X. laevis*. Perchlorate has a potent inhibitory effect on hindlimb growth within a wide range of environmental concentrations (Goleman et al. 2002a, 2002b) but does not influence overall organismal growth at concentrations up to 14 mg·L^{-1} (Goleman et al. 2002b). Because of the differential effects of perchlorate on hindlimb growth and somatic growth, the ratio of hindlimb length to snout-vent length has been used as a non-invasive measure of perchlorate exposure in larval frogs (Carr et al. 2003).

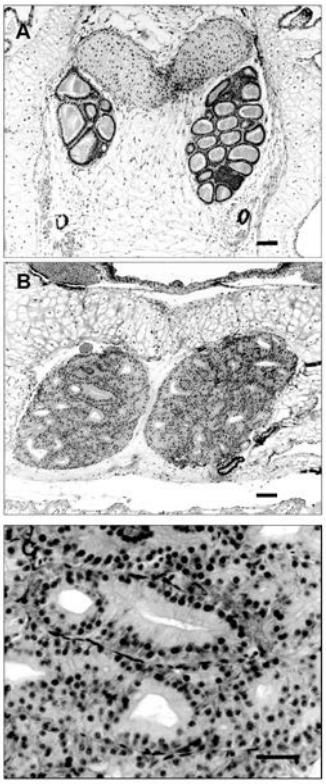

Figure 6-4 Changes in thyroid histology in a larval tadpole exposed to a high (14 mg·L^{-1}) but environmentally relevant concentration of ammonium perchlorate. A) Appearance of thyroid gland in a control animal. Calibration bar = 100 µm. B) Appearance of thyroid gland in a perchlorate-exposed tadpole. Calibration bar = 100 µm. C) Higher-magnification photomicrograph of tissue in B. Calibration bar = 50 µm. Note pronounced colloid depletion and hypertrophy of thyroid follicle cells. Similar changes have been observed in wild-caught tadpoles exposed to perchlorate under natural conditions (Carr et al. 2003).

Among the most sensitive measures of perchlorate exposure in developing frogs are changes in thyroid histology, specifically follicle cell height and colloid depletion, both of which occur not as a direct result of perchlorate action but because of secondary or indirect effects on TSH secretion due to interference with normal negative feedback mechanisms. Exposure of developing frogs to AP results in colloid depletion and follicle cell hypertrophy (Goleman et al. 2002b; Carr et al. 2003) (**Figure** 6-4). Exposure of larval *X. laevis* to sodium perchlorate as low as 16 µg·L^{-1} beginning at stage 51 or 54 for just 8 d is sufficient to cause colloid depletion, while exposure for 14 d to 16 µg perchlorate·L^{-1} results in follicle cell hypertrophy as well as colloid depletion (Tietge et al. 2005). The effects of perchlorate on thyroid follicle cell hypertrophy and colloid depletion are linearly related within the concentration range of 16 to 4000 µg·L^{-1} after either 8- or 14-d exposure beginning at stages 51 or 54 (Tietge et al. 2005). Collectively, these findings show that environmentally relevant concentrations of perchlorate can inhibit metamorphosis in the

surrogate species *X. laevis*. Furthermore, these findings demonstrate that thyroid histopathological indicators of perchlorate exposure are extremely sensitive, with responses occurring at the lowest perchlorate concentrations that have been tested to date. Whether concentrations less than 16 µg perchlorate·L^{-1} also produced thyroid changes remains to be tested.

As with other endocrine-disrupting chemicals, the response of frogs to perchlorate depends upon timing, both with respect to exposure duration and the development stage during exposure. As mentioned previously, the antimetamorphic effects of perchlorate are reversible in *X. laevis*, suggesting that amphibians may recover from perchlorate exposure, although certain developmental processes such as hindlimb growth and completion of metamorphosis may be delayed. In *X. laevis*, perchlorate exposure late in metamorphosis is not as effective at inhibiting metamorphosis or hindlimb growth exposure early in development (Hu et al. 2003; **Table** 6-2). Furthermore, the Tier I Frog Metamorphosis Assay for thyroid disruption as originally proposed by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC; Federal Register 1998) is relatively insensitive for detecting perchlorate effects because the assay test exposure occurs in a very late stage (Nieuwkoop-Faber stage 60) *X. laevis* (Goleman et al. 2002a). It is presumed that late-stage tadpoles are relatively insensitive to perchlorate and other contaminants that act directly upon the thyroid gland because blood levels of T_4 are already elevated by stage 60 and genetic reprogramming of T_4-sensitive tissues has already begun by the time forelimb emergence begins (Shi 2000). Other evidence indicates that adult frogs may not be as sensitive as larval frogs to perchlorate. Exposure of adult female *X. laevis* for 10 weeks to perchlorate concentrations as high as 14 mg·L^{-1} failed to cause thyroid follicle cell hypertrophy or colloid depletion (Goleman et al. 2004). Exposure of larval *X. laevis* to these conditions produces profound alterations in thyroid histopathology (see **Figure** 6-4).

Perchlorate effects on reproduction in amphibians

Thyroid hormones are required for masculinization in some anurans. Specifically, THs appear to be important for gonadal and secondary sex determination, possibly by inducing androgen receptor expression in androgen sensitive tissues. Exposure to perchlorate (Goleman et al. 2002a) or other goitrogenic chemicals such as PTU (Hayes 1997) can cause feminization of male offspring, producing ovaries in genetically male individuals.

Table 6-2 Mean hindlimb length in larval *X. laevis* exposed to ammonium perchlorate beginning at different developmental stages and continuing for 68-d post-hatch

	Hindlimb length (mm ± SEM)	
Nieuwkoop-Faber stage[a]	Control[b]	Treatment (14 mg . L^{-1} AP)
NF 1-10	19.2 ± 0.50	3.60 ± 0.33
NF 49	13.2 ± 0.51	4.97 ± 0.38
NF 55	17.9 ± 0.29	13.8 ± 0.48

[a] Stage at which exposure began (Nieuwkoop and Faber 1994). Exposures continued for 68-d post-hatch.

[b] Controls were reared in untreated Frog Embryo Teratogenesis Assay (FETAX)–*Xenopus* (Dawson and Bantle 1987)

[Please note there is no callout to Table 6-3, shown on the last page, following the References. If the table should be included in the chapter, please indicate where we should place the callout.]

Oxidative stress and susceptibility to UV radiation

The interaction between chemical exposure and UV has been the subject of recent study, and one of the proposed mechanisms of the decline of amphibians and increased incidence of deformities is an interaction between UV and chemical exposures (Schmidt 1997; Ankley et al. 1998). Thus, an increase in UV exposure is not necessarily a prerequisite of increased incidence of malformations or a decrease in population levels because it could be that an increased exposure to chemicals makes the organisms more susceptible to the effects of UV by interfering with their ability to cope with solar radiation. In fact, an in situ study of groundwater toxicity in amphibians (Bruner et al. 1998) found that the acute toxicity and magnitude of developmental effects depended on the amount of ambient solar radiation (i.e., the water was more toxic on sunny days than on cloudy days). Thus, ecological risk assessments that rely solely on measurement of environmental concentrations of chemicals and exposure of laboratory organisms to these concentrations may be biased if it does not take into account interactions between chemicals and natural stressors such as UV. For example, it has been suggested that, because development of the amphibian skin and melanocytes is mediated by THs, and because one function of melanocytes is to protect the animals against UV exposure, then exposure of organisms to thyroid-disrupting chemicals may increase an amphibian's susceptibility to UV (Burkhart et al. 1999). Indeed, recent evidence has found that exposure of *Xenopus* larvae to perchlorate

dramatically enhanced developmental deformities in UV-exposed individuals (Burkhart et al. 2000).

In order to test this hypothesis, *X. laevis* tadpoles were exposed to 2 doses of sodium perchlorate and UV radiation. The doses of perchlorate used were 0.05 and 10 $mg \cdot L^{-1}$, concentrations that were found in previous studies to inhibit and arrest metamorphosis in this species, respectively (Goleman et al. 2002b). The doses of UV were similar to those found in natural sunlight. The endpoints were levels of DNA strand breakage, oxidative DNA damage, and UV-induced photodimers. It was found that both perchlorate and UV exposure induced oxidative damage in the DNA, and that this may have been influenced by the levels of ammonia that accumulated as waste products in the exposure beakers. In addition, mortality was strongly correlated with the amount of oxidative DNA damage present. Furthermore, the amount of UV dimers in the DNA was enhanced by treatment with perchlorate, at least in some treatments (McDaniel 2004).

Field studies

An initial assessment of perchlorate in water, soil, and animal tissue samples at Longhorn Army Ammunition Plant (LHAAP) in East Texas revealed that perchlorate concentrations in tissues from frogs and tadpoles ranged from nondetectable to >500 µg perchlorate$\cdot kg^{-1}$ in animals collected from 1 pond where water concentrations of the contaminant were as great as 30 $mg \cdot L^{-1}$ (Smith et al. 2002). American bullfrog tadpoles collected from this site had significantly shorter hindlimbs but identical body length compared to tadpoles collected from a reference site with no detectable perchlorate contamination (Carr et al. 2003). In anuran tadpoles, hindlimb growth requires normal TH secretion, and Goleman et al. (2002a) demonstrated that perchlorate exposure causes a concentration-dependent reduction in hindlimb growth in *X. laevis*, presumably due to reduced TH synthesis (Goleman et al. 2002b). Animals from the contaminated site at LHAAP were at an earlier stage in metamorphosis than animals from the control site (Carr et al. 2003). Histological analysis of thyroid glands from tadpoles collected from the contaminated site, however, revealed smaller thyroid glands, a finding that is not consistent with the increase in thyroid gland size that one would expect to occur after perchlorate exposure. However, the comparison may have been confounded by the fact that the thyroid gland increases in size normally during metamorphosis, and as a result, animals from the reference site may have had larger thyroid glands because they were, on average, at more advanced stages of metamorphosis (Carr et al. 2003). To avoid a confound-

ing effect of developmental stage on the analysis of thyroid tissue response to perchlorate, Carr et al. (2003) examined thyroid histology in stage-matched chorus frog tadpoles inhabiting reference and contaminated sites at LHAAP. Comparison of stage-matched chorus frog tadpoles from a perchlorate and contaminated reference site indicated gross histological changes in the thyroid gland that were consistent with perchlorate exposure. Tadpoles from the contaminated site had significantly greater colloid depletion and greater hypertrophy of thyroid follicle cells compared to tadpoles from the reference site (Carr et al. 2003). The follicle cell hypertrophy and colloid depletion observed in the tadpoles from the contaminated site are consistent with histopathological changes reported by others (Goleman et al. 2002b; Tietge et al. 2005).

There is only one report of potential thyroid disruption in response to perchlorate exposure under field conditions. Carr et al. (USACE 2004 [**This USACE website is no longer available; please replace or update the reference.**]) reported a correlation between perchlorate concentrations and thyroid follicle cell height, but not colloid depletion, in adult male cricket frogs (*Acris crepitans*) collected from the Lake Waco and Lake Belton watersheds. Cricket frogs inhabiting streams contaminated with 10 to 30 µg perchlorate·L^{-1} exhibited follicle cell hypertrophy when compared to frogs inhabiting streams with 5 µg perchlorate·L^{-1} or less (USACE 2004).

Research Recommendations

In order to understand the mechanism of perchlorate action in amphibians, the amphibian NIS must be characterized in more detail, both with respect to molecular structure and the 1st-order rate kinetics for iodide and perchlorate transport by this protein. Establishment of kinetic parameters will allow researchers to determine how exposure endpoints such as blood levels of perchlorate might interfere with iodide transport. Moreover, the role of the NIS in extrathyroidal tissues needs to be better understood in order to characterize the most likely routes of perchlorate exposure in amphibians. Furthermore, the role that environmental iodide plays in determining the response of amphibians to perchlorate needs to be better elucidated. At the very least, the relative availability of iodide to the organism must be considered when perchlorate effects are examined. Although our research indicates that perchlorate may interfere with normal gonadal differentiation (Goleman et al. 2002b), the implications of this result for reproductive success and population stability are unknown. Although population modeling holds promise for testing the popula-

tion-level effect of perchlorate, multigenerational exposure studies should also be conducted to gather empirical data on the biological significance of perchlorate-induced changes in sex ratio. Finally, recent data indicate effects of perchlorate on thyroid histology at concentrations as low as 16 $\mu g \cdot L^{-1}$ (Tietge et al. 2005). However, as with other studies (Goleman et al. 2002b), histological changes in the thyroid occur at the lowest concentrations of perchlorate tested, and a true lowest-observable-effect concentration (LOEC) has yet to be determined, although it appears to be less than 16 μg perchlorate $\cdot L^{-1}$. Future studies should include perchlorate concentrations sufficiently low to bracket an LOEC for perchlorate on thyroid histology in tadpoles.

Conclusions

Amphibian metamorphosis is a unique model for examining the role of THs in development and growth. Amphibians require TH for normal growth, development, and, at least in anurans, normal reproductive development. At present, there are no empirical data linking perchlorate exposure to amphibian declines. However, given the large database on perchlorate levels in surface waters, potential exposure can and may be considered when determining critical habitat designations for threatened or endangered amphibian species. Environmentally relevant concentrations of perchlorate alter the rate of metamorphosis in a reversible fashion. The antimetamorphic effects of perchlorate may have important ecological effects, especially in those species inhabiting ephemeral ponds and having short developmental rates. Many of the same endpoints used to gauge perchlorate exposure in other vertebrates have been used successful to assess perchlorate exposure in amphibians, with the most sensitive being histopathological endpoints. These endpoints have been used successfully to assess perchlorate exposure under both laboratory and field conditions. Recent data also indicate that the response to perchlorate depends upon the timing of exposure, both in the length of exposure and the period during development when exposure occurs. Adult frogs are not as sensitive to perchlorate exposure as are developing frogs, and tadpoles at later stages of development are not as sensitive to the antimetamorphic effects of perchlorate as are tadpoles at earlier stages of development. Amphibians represent a unique and powerful model for examining the organismal response to perchlorate exposure and the potential effects of this contaminant on thyroid homeostasis, reproduction, and epithelial iodide transport.

References

Ankley GT, Tietge JE, DeFoe DL, Jensen KM, Holcombe GW, Durhan EJ, Diamond SA. 1998. Effects of ultraviolet light and methoprene on survival and development of *Rana pipiens*. Environ Toxicol Chem 17:2530–2542.

Bantle JA, Dumont JN, Harvey JG, Mattie DR. 1999. FETAX assay of ammonium perchlorate. Toxicologist 48:528.

Becker KB, Stephens KC, Davey JC, Schneider MJ, Galton VA. 1997. The type 2 and type 3 iodothyronine deiodinases play important roles in coordinating development in *Rana catesbeiana* tadpoles. Endocrinology 138:2989–2997.

Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, Slocombe R, Ragan MA, Hyatt AD, McDonald KR, Hines HB, Lips KR, Marantelli G, Parkes H. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc Natl Acad Sci 95:9031–9036.

Blaustein AR, Johnson PTJ. 2003. The complexity of deformed amphibians. Front Ecol Environ 1:87–94.

[Not cited; delete?] Blaustein AR, Romansic JM, Kiesecker JM, Hatch AC. 2003. Ultraviolet radiation, toxic chemicals and amphibian population declines. Diversity Distributions 9:123–140.

Boorse GC, Denver RJ. 2002. Acceleration of *Ambystoma tigrinum* metamorphosis by corticotropin-releasing hormone. J Exp Zool 293: 94–98.

Boorse GC, Denver RJ. 2004. Expression and hypophysiotropic actions of corticotropin-releasing factor in *Xenopus laevis*. Gen Comp Endocrinol 137:272–282.

Bruner MA, Rao M, Dumont JN, Hull M, Jones T, Bantle JA. 1998. Ground and surface water developmental toxicity at a municipal landfill: Description and weather-related variation. Ecotoxicol Environ Saf 39:215–226.

Buckbinder L, Brown DD. 1993. Expression of the *Xenopus laevis* prolactin and thyrotropin genes during metamorphosis. Proc Natl Acad Sci 90:3820–3824.

Burd GD. 1992. Development of the olfactory nerve in the clawed frog, *Xenopus laevis*: II. Effects of hypothyroidism. J Comp Neurol 315:255–263.

Burkhart JG, Cline JE, Harmon G, Fort DJ. 2000. Possible involvement of thyroid and retinoid receptor disruption in amphibian malformation. Society of Environmental Toxicology and Chemistry 21st Annual Meeting; 2001 Nov 11–16; Nashville, TN.

Burkhart JG, Cline JE. 1999. Interaction among environmental factors that contribute to malformation in frogs. Society of Environmental Toxicology and Chemistry 20th Annual Meeting; 1999 Nov 14–18; Philadelphia, PA.

[California EPA] alifornia Environmental Protection Agency, Department of Health Services. March 2004. Perchlorate database. Available from http://www.dhs.ca.gov/ps/ddwem/chemicals/perchl/perchlindex.htm. Accessed 6 Apr 2006.

Carr DL, Laharrague F, Kahn B, Pressley TA, Carr JA. 2003. Molecular characterization of a putative sodium/iodide symporter in the South African clawed frog, *Xenopus laevis*. Ann NY Acad Sci 986:711–712.

Carr JA, Norris DO. 1990. Immunohistochemical localization of corticotropin releasing factor and arginine vasotocin like immunoreactivities in the brain and pituitary of the American Bullfrog (*Rana catesbeiana*) during development and metamorphosis. Gen Comp Endocrinol 78:180–188.

Carr JA, Norris DO, Samora A. 1991. Organization of tyrosine hydroxylase-immunoreactive neurons in the di- and mesencephalon of the American bullfrog (*Rana catesbeiana*) during metamorphosis. Cell Tissue Res 263:155–163.

Carr JA, Urquidi LJ, Goleman WL, Hu F, Smith PN, Theodorakis CW. 2003. Ammonium perchlorate disruption of thyroid function in natural amphibian populations: Assessment and potential impact, In Linder G, ed, Multiple Stressor Effects in Relation to Declining Amphibian Populations, ASTM STP 1443, ASTM International, West Conshohocken, PA, USA, pp 131–142.

Cohen MA, Kelley DB. 1996. Androgen-induced proliferation in the developing larynx of *Xenopus laevis* is regulated by thyroid hormone. Dev Biol 178:113–123.

Collins JP, Storfer A. 2003. Global amphibian declines: sorting the hypotheses. Diversity Distributions 9: 89–98.

Dawson DA, Bantle JA. 1987. Development of a reconstituted water medium and preliminary validation of the frog embryo teratogenesis assay—Xenopus (FETAX). J Appl Toxicol 7:237–244.

Dean KE, Palacheck RM, Noel JM, Warbritton R, Aufderheide J, Wireman J. 2004. Development of freshwater water-quality criteria for perchlorate. Environ Toxicol Chem 23:1441–1451.

Denver RJ. 1988. Several hypothalamic peptides stimulate in vitro thyrotropin secretion by pituitaries of anuran amphibians. Gen Comp Endocrinol 72:383–393.

Denver RJ. 1993. Acceleration of anuran amphibian metamorphosis by corticotropin-releasing hormone-like peptides. Gen Comp Endocrinol 91:38–51.

Denver RJ, Boorse GC, Glennemeier KA. 2002. Endocrinology of complex life cycles: amphibians. In: Pfaff D, Arnold A, Etgen A, Fahrbach S, Moss R, Rubin R, editors. Hormones, brain and behavior. San Diego (CA): Academic. Volume 2, p 469–513.

Denver RJ, Licht P. 1989. Neuropeptide stimulation of thyrotropin secretion in the larval bullfrog: evidence for a common neuroregulator of thyroid and interrenal activity during metamorphosis. J Exp Zool 252:101–104.

Dodd MHI, Dodd JM. 1976. The biology of metamorphosis. In: Moore JA, editor. Physiology of the amphibia. New York (NY): Academic. Volume 3, p 467–599.

Feder ME. 1992. A perspective on environmental physiology of the amphibians. In: Feder ME, Burggren WW, editors. Environmental physiology of the amphibians. Chicago (IL): Univ Chicago Pr. p 1–6.

Federal Register. 1998. Endocrine disruptor screening program:Statement of policy and priority-setting workshop notice. Federal Register 63:71542–71568.

Federal Register. 2000. Endangered and threatened wildlife and plants; Proposed designation of critical habitat for the California Red-legged Frog (*Rana aurora draytonii*). Federal Register 65:54892

Fellers GM, Drost CA. 1993. Disappearence of the Cascades frog *Rana cascadae* at the southern end of its range, California, USA. Biol Conserv 65:177–181.

Fellers GM, Green DE, Longcore JE. 2001. Oral chytridiomycosis in Mountain Yellow-Legged Frogs (*Rana muscosa*). Copeia 2001:945–953.

Gancedo B. Alonso-Gomez AL, de Pedro N, Corpas I, Delgado MJ, Alonso-Bedate M. 1995. Seasonal changes in thyroid activity in male and female frog, *Rana perezi*. Gen Comp Endocrinol 97:66–75.

Gancedo B, Corpas I, Alonso-Gomez AL, Delgado MJ, Morreale de Escobar G, Alonso-Bedate M. 1992. Corticotropin-releasing factor stimulates metamorphosis and increases thyroid hormone concentration in prometamorphic *Rana perezi* larvae. Gen Comp Endocrinol 87:6–13.

Goleman WL, Carr JA, Anderson TA, 2002b **[Both 2002 references are shown as "b"; please correct]**. Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. Environ Toxicol Chem 21:590–597.

Goleman WL, Gentles BA, Hu F, Carr JA. 2004. Evidence that adult frogs are less responsive to the thyroid disrupting effects of ammonium perchlorate. Integ Comp Biol 43:1022.

Goleman WL, Urquidi LJ, Anderson TA, Kendall RJ, Smith EE, Carr JA. 2002b **[Both 2002 references are shown as "b"; please correct]**. Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis*. Environ Toxicol Chem 21:424–430.

[Not cited; delete?] Gray RH. 2000. Historical occurrence of malformations in the Cricket Frog, *Acris crepitans*, in Illinois. Trans Illinois State Acad Sci 93:279–284.

Gupta BB, Chakrabarty P. 1990. Effects of thyroidal, gonadal and adrenal hormones on tissue respiration of streaked frog, *Rana limnocharis*, at low temperature. Indian J Exp Biol 28:23–26.

Gupta BB, Deka-Borah H. 1995. In vivo and in vitro effects of metabolic hormones on tissue respiration in toad, *Bufo melanostictus* during hibernation and active phase. Indian J Exp Biol 33:604–607.

Harrison KD, Zozzaro PE, Collie NL, Carr JA. 2002. Iodide transport in *Xenopus laevis* gut and skin. Amer Zool 41:1467.

Hauser KF, Gona AG. 1983. Effects of thyroidectomy and season on the external granular layer of the cerebellum in metamorphosing bullfrog tadpoles (*Rana catesbeiana*). Exp Neurol 79:265–277.

Hauser KF, Gona AG. 1984. Purkinje cell maturation in the frog cerebellum during thyroxine-induced metamorphosis. Neuroscience 11:139–155.

Hauser KF, Uray NJ, Gona AG. 1986. Granule cell development in the frog cerebellum during spontaneous and thyroxine-induced metamorphosis. J Comp Neurol 253:185–196.

Hayes TB. 1997. Hormonal mechanisms as developmental constraints on evolution: examples from the anura. Am Zool 37:482–490.

Hines H, Mahony M, McDonald K. 1999. An assessment of frog declines in wet subtropical Australia. In: Campbell A, editor. Declines and disappearances of Australian frogs. Canberra (AU): Environment Australia. p 44–63.

Hooth MJ, Deangelo AB, George MH, Gaillard ET, Travlos GS, Boorman GA, Wolf DC. 2001. Subchronic sodium chlorate exposure in drinking water results in a concentration-dependent increase in rat thyroid follicular cell hyperplasia. Toxicol Pathol 29:250–259.

Hoskins SG, Grobstein P. 1985. Development of the ipsilateral retinothalamic projection in the frog *Xenopus laevis*. III. The role of thyroxine. J Neurosci 5: 930–940.

Houlahan JE, Findlay CS, Schmidt BR, Meyer AH, Kuzmin SL. 2000. Quantitative evidence for global amphibian population declines. Nature 404:752–755.

Hu F, Gentles A, Goleman WL, Carr JA. 2003. Developmental stage and environmental iodide influence the antimetamorphic effects of perchlorate. Society of Environmental Toxicology and Chemistry 24th Annual Meeting; 2003 Nov 9–13; Austin, TX.

Ingram GJ, McDonald KR. 1993. An update on the decline of Queenslands frogs. In: Lunney D, Ayers D, editors. Herpetology in Australia: a diverse discipline. Mosman (AU): Royal Zoological Soc of NSW. p 297–303.

Kikuyama S, Miyakawa M, Arai Y. 1979. Influence of thyroid hormone on the development of preoptic-hypothalamic monoaminergic neurons in tadpoles of *Bufo bufo japonicus*. Cell Tissue Res 198:27–33.

Kinney KS, Felten SY, Cohen N. 1996. Sympathetic innervation of the amphibian spleen: developmental studies in *Xenopus laevis*. Dev Comp Immunol 20:l 51–59.

Krain LP, Denver RJ. 2004. Developmental expression and hormonal regulation of glucocorticoid and thyroid hormone receptors during metamorphosis in *Xenopus laevis*. J Endocrinol 181:91–104.

Lawler SP, Dritz D, Strange T, Holyoak M. 1999. Effects of introduced mosquitofish and bullfrogs on the threatened California red-legged frog. Cons Biol 13:613–622.

[Not cited; delete?] LeNoir JS, McConnell LL, Fellers GM, Cahill TM, Seiber JN. 1999. Summertime transport of current-use pesticides from California's central valley to the Sierra Nevada mountain range, USA. Environ Toxicol Chem 18:2715–2722.

Manzon RG, Denver RJ. 2004. Regulation of pituitary thyrotropin gene expression during *Xenopus* metamorphosis: negative feedback is functional throughout metamorphosis. J Endocrinol 182:273–285.

May TW, Packer RK. 1976. Thyroid hormones stimulate in vivo oxygen consumption of adult *Rana pipiens berlandieri* at high environmental temperatures. Gen Comp Endocrinol 30: 525–527.

McDaniel LN. 2004. Ultraviolet radiation-induced genotoxicity in *Xenopus laevis* exposed to sodium perchlorate [MS thesis]. Lubbock (TX): Texas Tech Univ.

Miranda LA, Affanni JM, Paz DA. 2000. Corticotropin-releasing factor accelerates metamorphosis in *Bufo arenarum*: effect on pituitary ACTH and TSH cells. J Exp Zool 286:473–480.

Miranda LA, Dezi RE. 1997. Immunocytochemical distribution of corticotropin-releasing factor in the brain and hypophysis of larval *Bufo arenarum*; effect of KClO4 during early development. Tissue Cell 29:643–649.

Miranda LA, Paz DA, Dezi RE, Pisano A. 1995. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in *Bufo arenarum* larvae with inhibited thyroid function. Gen Comp Endocrinol 98:166–176.

Miranda LA, Pisano A, Casco V. 1996. Ultrastructural study on thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. *Biocell* 20:147–153.

Morehouse EA, James TY, Ganley AR, Vilgalys R, Berger L, Murphy PJ, Longcore JE. 2003. Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. Mol Ecol 12:395–403.

Nieuwkoop PD, Faber J. 1994. Normal Table of *Xenopus laevis* (Daudin): A Systematical and Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis, 3rd ed. Garland Publishing, New York, NY, USA.

[Not cited; delete?] Norman MF, Carr JA, Norris DO. 1987. Adenohypophysial-thyroid activity of the tiger salamander, *Ambystoma tigrinum*, as a function of metamorphosis and captivity. J Exp Zool 242:55–66.

Norris DO, Carr JA, Desan PH, Smock TK, Norman MF. 1992. Monoamines and their metabolites in the amphibian (*Ambystoma tigrinum*) brain: quantitative changes during metamorphosis and captivity. Comp Biochem Physiol 103A:279–283.

Okada R, Yamamoto K, Koda A, Ito Y, Hayashi H, Tanaka S, Hanaoka Y, Kikuyama S. 2004. Development of radioimmunoassay for bullfrog thyroid-

stimulating hormone (TSH): effects of hypothalamic releasing hormones on the release of TSH from the pituitary in vitro. Gen Comp Endocrinol 135:42–50.

Prapunpoj P, Richardson SJ, Schreiber G. 2002. Crocodile transthyretin: structure, function, and evolution. Am J Physiol Regul Integr Comp Physiol 283:R885–896.

Prapunpoj P, Yamauchi K, Nishiyama N, Richardson SJ, Schreiber G. 2000. Evolution of structure, ontogeny of gene expression, and function of *Xenopus laevis* transthyretin. Am J Physiol Regul Integr Comp Physiol 279:R2026–2041.

Richter SC, Young JE, Johnson GN, Seigel RA. 2003. Stochastic variation in reproductive success of a rare frog, *Rana sevosa*: implications for conservation and for monitoring amphibian populations. Biol Conserv 111:171–177.

Robertson JC, Kelley DB. 1996. Thyroid hormone controls the onset of androgen sensitivity in the developing larynx of *Xenopus laevis*. Dev Biol 176:108–123.

Rollins-Smith LA, Blair P. 1990. Expression of class II major histocompatibility complex antigens on adult T cells in *Xenopus* is metamorphosis-dependent. Dev Immunol 1:97–104.

Rollins-Smith LA, Davis AT, Blair PJ. 1993. Effects of thyroid hormone deprivation on immunity in postmetamorphic frogs. Dev Comp Immunol 17:157–164.

Rollins-Smith LA, Flajnik MF, Blair PJ, Davis AT, Green WF. 1997. Involvement of thyroid hormones in the expression of MHC class I antigens during ontogeny in *Xenopus*. Dev Immunol 5:133–144.

Safi R, Bertrand S, Marchand O, Duffraisse M, de Luze A, Vanacker JM, Maraninchi M, Margotat A, Demeneix B, Laudet V. 2004. The axolotl (*Ambystoma mexicanum*), a neotenic amphibian, expresses functional thyroid hormone receptors. Endocrinology 145:760–772.

Schmidt CW. 1997. Amphibian deformities continue to puzzle researchers. Environ Sci Technol 31:324A–326A.

Shi YB. 2000. Amphibian metamorphosis: from morphology to molecular biology. New York (NY): Wiley-Liss.

Shi ZX, Levy A, Lightman SL. 1994. Thyroid hormone-mediated regulation of corticotropin-releasing hormone messenger ribonucleic acid in the rat. Endocrinology 134:1577–1580.

Smith PN, Theodorakis CW, Anderson TA, Kendall RJ. 2002. Preliminary assessment of perchlorate in ecological receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. Ecotoxicology 10:305–313.

Sparling DW, Fellers GM, McConnell LL. 2001. Pesticides and amphibian population declines in California, USA. Environ Toxicol Chem 20:1591–1595.

Sparling DW, Harvey G, Nzengung V. 2003. Interaction between perchlorate and iodine in the metamorphosis of *Hyla versicolor*. In: Linder G, editor. Multiple stressor effects in relation to declining amphibian populations. West Conshohocken (PA): ASTM International. p 131–142. ASTM STP 1443.

[Not cited; delete?] Storfer A. 2003. Amphibian declines: future directions. Diversity Distributions 9:151–163.

Tasaki Y, Inoue M, Ishii S. 1986. Annual cycle of plasma thyroid hormone levels in the toad, *Bufo japonicus*. Gen Comp Endocrinol 62:404–410.

Tietge JE, Holcombe GW, Flynn KM, Kosian PA, Korte JJ, Anderson LE, Wolf DC, Degitz SJ. 2005. Metamorphic inhibition of *Xenopus laevis* by sodium perchlorate: effects on development and thyroid histology. Environ Toxicol Chem 24:926–933.

[USACE] US Army Corps of Engineers. 2004. Final report: Bosque and Leon River watersheds study. Available from http://www.swf.usace.army.mil/ppmd/Perchlorate/index.html. Accessed _____**[This site is Not Found; please provide an updated reference or delete the citation.]**

[USFWS] US Fish and Wildlife Service. 1984. Houston toad recovery plan. Albuquerque (NM): USFWS. p 72.

[USFWS] US Fish and Wildlife Service. 2001. Wyoming toad (*Bufo baxteri*) population and habitat viability assessment (PHVA). **[Please provide city and state of publication.]** p 109.

[USFWS] US Fish and Wildlife Service. 2003. Available from http://endangered.fws.gov/. Accessed May 2003. **[is this meant to cite a specific article? If so, please provide title and verify current URL.]**

[Not cited; delete?] [USGS] US Geological Survey. 2003. NAWQA database. http://orxddwimdn.er.usgs.gov/servlet/. Accessed 30 Jan 2003. **[We are unable to access this site; please provide an updated URL.]**

[USGS] US Geological Survey. 2004. Available from http://www.usgs.gov/amphibian_faq.html. Accessed 5 Apr 2006.

Urbansky ET. 2002. Perchlorate as an environmental contaminant. Environ Sci Pollut Res Int 9:187–192.

Vandorpe G, Kuhn ER, Gevaerts H. 1990. Failure to relate thyroid hormones and in vitro 5'-monodeiodination activity to oocyte development and sex steroids in the giant swamp frog *Dicroglossus occipitalis* at the equator. Gen Comp Endocrinol 79:469–476.

Van Sande J, Massart C, Beauwens R, Schoutens A, Costagliola S, Dumont JE, Wolff J. 2003 **[citation shows 2004; which is correct?]**. Anion selectivity by the sodium iodide symporter. Endocrinology 144:247–252.

Volk H, Charlemagne J, Tournefier A, Ferrone S, Jost R, Parisot R, Kaufman J. 1998. Wide tissue distribution of axolotl class II molecules occurs independently of thyroxin. Immunogenetics 47:339–349.

Vredenburg VT. 2004. Reversing introduced species effects: experimental removal of introduced fish leads to rapid recovery of a declining frog. Proc Natl Acad Sci 101:7646–7650.

West Plains IPM Update. 2002. Vol. 7, No. 16. Available from: http://www.tpma.org/news_letters/_wplains_hockley/WPU09102002.htm. Accessed 5 Apr 2006.

World Resources Institute. 2004. Earthtrends. Available from: http://earthtrends.wri.org/. Accessed 6 Apr 2004. **[Does this reference intend to refer to a particular issue or article? If so, is it still accessible?]**

Yamauchi K, Eguchi R, Shimada N, Ishihara A. 2002. The effects of endocrine-disrupting chemicals on thyroid hormone binding to *Xenopus laevis* transthyretin and thyroid hormone receptor. Clin Chem Lab Med 40:1250–1256.

Yu L, Canas JE, Cobb GP, Jackson WA, Anderson TA. 2004. Uptake of perchlorate in terrestrial plants. Ecotoxicol Environ Saf 58:44–49.

Table 6-3 Effects of perchlorate on endpoints in amphibians and reptiles

Endpoint	Species	Response	Concentration ($\mu g \cdot L^{-1}$)	Reference
Metamorphosis				
Forelimb emergence	*X. laevis,* larvae	Inhibits	5	Goleman et al. 2002a
Tail resorption	*X. laevis,* larvae	Inhibits	18	Goleman et al. 2002a
Hindlimb growth	*X. laevis,* larvae	Inhibits	18	Goleman et al. 2002a
Somatic growth	*X. laevis,* larvae	No effect	5–133 000	Goleman et al. 2002a
Reproduction				
Gonad differentiation	*X. laevis,* larvae	Feminization	59–14 000	Goleman et al. 2002b
Immune system				
MHC class I antigens	*X. laevis,* larvae	No effect	1 000 000	Rollins-Smith et al. 1997
MHC class II antigens	*X. laevis,* larvae	Inhibition	1 000 000	Rollins-Smith et al. 1990
	A. mexicanum	No effect	100 000 000	Volk et al. 1998
Splenic innervation	*X. laevis,* larvae	Reduced	1 000 000	Kinney et al. 1996
Nr of thymocytes and splenocytes	*X. laevis*, juveniles	Reduced	1,000,000	Rollins-Smith et al. 1993
Neuroendocrine				
Development of CRF neurons	*B. arenarum*	No effect	340 000	Miranda and Dezi 1997
Pituitary cell development	*B. arenarum*	Enlarged lactotropes and thyrotropes	340 000	Miranda et al. 1995
Thyroid histopathology				
Colloid	*X. laevis*	Depletion	16	Tietge et al. 2005
Follicle cell	*X. laevis*	Hypertrophy	16–14 000	Goleman et al. 2002b; Teitge et al. 2005
Thyroid gland volume	*B. arenarum*	Increase	340 000	Miranda et al. 1996
	X. laevis	No significant effect	59–14 000	Goleman et al. 2002b

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