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Topic 4.6
Deformed frogs and environmental retinoids*

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Abstract: Since the early 1990s, a substantial number of deformed frogs have been observed in North America, particularly in the upper Midwest and Canada. Attempts to understand the etiology of the deformed frog problem have met with limited success to date with nearly as many proposed explanations as research groups working on the problem. Models for the mechanism underlying the development of deformed frogs include parasite/predation, ultraviolet radiation, and chemical exposure. Each model has its strengths and weaknesses. Despite contentious debate among researchers, there is an overall consensus that the increasing prevalence of deformed frogs is the result of a water-borne contaminant that has recently appeared, or reached a critical concentration. Our detailed analysis of malformed frogs collected in Minnesota ponds and lakes suggested that limb patterning was being modified by the disruption of a retinoid-sensitive developmental signaling pathway. Accordingly, we focused in the identification and characterization of bioactive retinoids from lake water and showed that retinoid treatment of frog embryos at sensitive times of development could recapitulate the full spectrum of limb abnormalities observed in field specimens in the laboratory. These data have led to the conclusion that inappropriate modulation of retinoid signaling by environmental contaminants is the mechanism underlying the increased incidence of frog malformations.

INTRODUCTION

Development of the embryo is a very sensitive and vulnerable part of the life cycle of all animals. The molecular mechanisms controlling embryonic development are highly conserved among vertebrates, and components of these pathways are frequently reused in regulating adult physiology [1–3]. Hence, agents that disrupt physiology or development in animals are likely candidates to similarly impact human physiology and development. The amphibian model system is especially suitable for identifying potential risks to human development. This results both from strong conservation of developmental pathways and because amphibian development is accessible to environmental contaminants in the water. Early warnings provided by monitoring wild populations of amphibians are especially important in cases of non-point source contamination, where it is not possible to identify appropriate sites for direct chemical monitoring.

There has been a dramatic increase in the numbers of deformed amphibians found in North America and Japan since the early 1990s. At the same time, a worldwide decline in the numbers of

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many amphibian species, particularly the highly aquatic ones, has also been observed. This suggests that environmental modification is negatively impacting amphibian populations. While the cause of amphibian declines in relatively pristine environments remains unknown, there is an emerging consensus that the increasing prevalence of deformed frogs is the result of a water-borne contaminant that has appeared, or reached a critical concentration, in recent years. This conclusion is based on several lines of evidence. When it first became evident that the incidence of amphibian malformation was significantly greater than the historical rate [4,5], it was noted that the incidence of malformation was correlated with the life history of the frogs. Thus, highly aquatic species such as green frogs and mink frogs have high rates of malformations (>>50 %); whereas, primarily terrestrial species such as wood frogs have malformation rates closer to the historical rate (<1 %). Species that spend intermediate periods of embryonic and larval development in the water, such as leopard frogs, exhibit intermediate rates of malformations (5–50 %).

A number of models have been proposed to explain the occurrence of malformed frogs in North America. These include UV radiation, parasites, and environmental contamination of various sorts. The topic has been recently reviewed although it should be noted that the reviews contain significant errors of fact and interpretation as well as assumptions that are not clearly stated [6–8]. As Stocum correctly points out [8], the number of hypotheses, opinions and viewpoints about the causes of amphibian malformations currently exceeds the amount of data available with which to analyze the problem.

In the brief review that follows, we summarize the current state of knowledge regarding the deformed frog investigation. In contrast with other investigators who believe that there may be nearly as many different causes as sites under study [6], we conclude that there is evidence to support an hypothesis that a single causal mechanism can account for the vast majority of the observed phenotypes in wild amphibian populations.

ULTRAVIOLET RADIATION

Exposure to UV radiation was originally postulated to be a potential source of amphibian malformations. This model can potentially explain why deformed frog reports are highest in the Northern Midwest where UV radiation is abundant presumably due to ozone depletion in the upper atmosphere. But, as has been noted elsewhere [6–8], there is little cause to suspect that UV radiation is a significant source of amphibian malformations. UV treatment of embryos should lead to destruction of the apical ectodermal ridge. Since this structure is required for limb outgrowth, the expected result is bilateral, blunt-ended truncations. The types of malformations observed in field specimens are inconsistent with these effects in that blunt truncations are relatively rare.

There are data supporting two other potential roles for UV in amphibian malformations and declines. Blaustein and colleagues have demonstrated a positive association between UV exposure and amphibian decline [9,10]. A second possibility is that UV interacts with environmental chemicals leading to the production of new metabolites [11–13]. In this scenario, high levels of UV (as found in the upper Midwest) would interact locally with ubiquitous and otherwise innocuous chemicals to produce teratogens. This possibility could account for the geographic distribution of malformed frogs.

PARASITES

The parasite model for frog malformations is interesting and potentially significant. Considering that frog malformations have appeared sporadically in the literature for hundreds of years [14], it is seductive to consider that what we now observe is merely an expansion of a historical phenomenon and therefore, no cause for alarm.

What is commonly referred to as the parasite model [14–17] is really two conjoined models. Supernumerary limbs are explained by larval trematodes (*Ribeiroia ondatrae*) burrowing into the developing limb as first speculated by Sessions and Ruth [16]. Missing and reduced limbs are explained

as predation by fishes and invertebrates [4,14,15,17]. Some authors believe that mechanical perturbations by the burrowing *Ribeiroia* larvae are responsible for limb duplications [14–16] although the available experimental evidence demonstrates only the production of duplicated digits by simulated mechanical perturbations in the laboratory [16]. Therefore, the conclusion that mechanical perturbation causes limb duplications is as yet unsupported by any published experimental data.

Johnson and colleagues performed laboratory experiments testing the association between *R. ondatrae* exposure and limb malformations [17,18]. These studies utilized *Ribeiroia* sp. metacercaria and wild-caught Pacific tree frog (*Hyla regilla*) [17] or Western toad (*Bufo boreas*) [18] tadpoles. Johnson and colleagues demonstrated that exposure to *Ribeiroia* metacercaria was associated with duplicated limbs, reduced limbs, missing limbs, and bony triangles in a dose-dependent manner [17,18]. The authors conclude that the exposure to *Ribeiroia* ondatrae metacercaria is associated with limb malformations and correctly observe that despite this correlation, no inferences were possible regarding the mechanism through which *Ribeiroia* act. One such mechanism could be mechanical perturbation, although one immediately wonders why only a single species of trematode appears to be responsible. It should be possible for many types of trematode and other parasite larvae to mechanically disrupt the limb field. Indeed, wild caught frogs are often heavily infested with parasites of all kinds with no correlation between parasite number or location and limb malformations. We consider it more likely that *R. ondatrae* either produce factors that mimic vertebrate signaling molecules or elicit the host to produce such factors at inappropriate times, locations, or levels during development. Other authors have reached similar conclusions [17,18], and this is a ripe area for future study.

It has been proposed that parasite infestation is the most parsimonious explanation for frog malformations [14]. However, proponents of this idea invoke two very different phenomena (parasites and predation) in a wholly nonparsimonious manner that explains only a subset of malformations. The majority of frog malformations in the field are limb reductions that are inconsistent with predation [5,7,19]. In addition, most affected animals die at metamorphosis with a variety of craniofacial and gastrointestinal defects in addition to limb deformities. Therefore, the parasite model, as currently articulated [14–16] is an unsatisfactory explanation for the majority of frog malformations found in the wild.

CHEMICALS

Studies by the NIEHS, based on an early amphibian development assay (FETAX: frog embryo teratogenesis assay xenopus) have detected agents in the water at sites with malformed frogs that induce developmental defects [20]. Field data regarding the occurrence of frog deformities indicate a correlation between intensive agriculture and deformed frogs [5]. Thus, agrochemical contamination of surface water is considered a source of the causative agent(s). In addition to malformations, the catastrophic declines and die-offs that have occurred globally appear in some cases to be a consequence of exposure to agrochemicals [21], although at this point the relationship between amphibian declines and malformations is unclear. It has been suggested that frog malformations are caused by different combinations of chemicals at each site through interactions between the chemical mixtures and components of the local water chemistry [20]. While it is unreasonable to exclude possible mechanisms, a priori, it seems equally unreasonable to speculate that different chemical mixtures at each site elicit the same or similar malformations.

RETINOIDS

Extensive research from many laboratories has established an essential role for retinoids in a large number of cellular processes (recently reviewed in [22–25]). Among these are development and patterning of limbs and the central nervous system. The precise quantities of retinoids present during early development are especially critical; serious developmental defects result from either too much or too little retinoid signaling. Retinoid signaling is primarily mediated through the activities of two classes of nu-

clear hormone receptors, the RARs and RXRs. The endogenous activators of RAR are thought to be all-trans retinoic acid (atRA) and 9-cis RA (9cRA). RXR is activated by 9cRA but not by atRA. RAR requires heterodimerization with RXR in order to bind DNA and activate transcription of RAR target genes. An interesting and significant feature of RXR is that, in addition to heterodimerizing with RAR, it is a common heterodimeric partner for ten other families of nonsteroidal nuclear receptors including the thyroid hormone and vitamin D3 receptors [26,27]. Therefore, inappropriate modulation of retinoid signaling would be expected to have pleiotropic effects on development and adult physiology.

We analyzed skeletal dysplasias observed in severely affected frogs from Minnesota and identified two classes of common limb abnormalities [19]. First, supernumerary or absent limbs were observed, suggesting that the process of limb initiation was being affected. Second, primary and supernumerary limbs both showed characteristic skeletal abnormalities, including truncated and phocomelic limbs, suggesting that limb growth and pattern formation are also being modified. In the phocomelic limbs, the skeletal elements within affected segments are folded back on themselves, such that the proximal and distal ends of the bone lie adjacent to one another and the mid-portion of the bone projects laterally, forming a "bony triangle" (BT) (Fig. 1).

Exposure of developing limb buds to teratogenic retinoids (e.g., retinoic acid) in frogs, mice, and chicks can induce the BT phenotype [28–31]. No analysis of the teratogenic mechanisms underlying this dysplasia has yet been carried out. We have not found evidence of any other teratogenic agent that induces this unique skeletal dysplasia, suggesting that BTs are diagnostic for retinoid exposure. It is possible that more than one agent is responsible for the whole spectrum of different observed abnormalities. However, we note that retinoic acid can induce supernumerary limbs in early mouse embryos [32,33] as well as developing limbs of chicks [31,34,35] and amphibians [36,37], and regenerating tails of amphibians [28,38,39]. Incomplete and missing limbs can also be induced in response to retinoid treatment, depending on the mode of exposure (reviewed in [40,41]). Thus, it is possible that a single agent, such as a retinoid, could be responsible for all of the observed malformation phenotypes.

The issue of the types of duplications induced by retinoid exposure has been raised previously [6,8,14,15]. In each case, the authors incorrectly state that retinoid exposure causes only proximodistal (PD) duplications in regenerating limbs and only limb reductions in developing limbs [42]. It has been shown that retinoid exposure at critical times in development can elicit complete limb duplications and even duplicated pelvic girdles in mammals [32] and frogs [36,37]. Retinoid exposure during tail regeneration also has the ability to reprogram the tails to regenerate into legs, which leads to the development of a cluster of satellite limbs in the metamorphosing frog [38,39]. Both duplicated pelvic girdles and satellite limbs have been observed in field specimens from deformed frog sites [43]. There are also significant and substantial differences between regeneration and development [40,41]. The observation that RA treatment causes PD duplications in regenerating limbs has no relevance when considering the ability of RA to induce complete limb duplications in developing animals. Lastly, it appears that con-

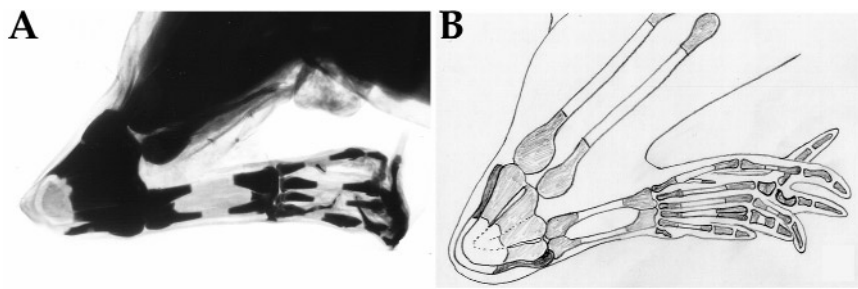

Fig. 1 Victoria blue stain to show a bony triangle in the zeugopodial (tibia/fibula) segment of the duplicated hindleg of a Minnesota deformed frog. A. Cartilage is darkly stained, the lighter area at apex of triangle is bone. Proximal and distal ends of the bones are next to one another. B. Diagram of skeletal elements in A.

clusions regarding mechanism may have been drawn without anyone actually doing the decisive experiments. A well-known hallmark of development is the existence of windows of sensitivity to perturbation. Treatments outside the window of sensitivity will not elicit effects. Therefore, conclusions regarding mechanisms of malformation drawn in the absence of a careful analysis of the windows of sensitivity to retinoid exposure are likely to be invalid.

We tested the effects of treating developing *Xenopus laevis* embryos with retinoids to evaluate the tenability of our hypothesis that altering retinoid signaling causes limb deformities. Pilot studies were undertaken to identify the stages of development where treatment with retinoids affected limb patterning [43]. A variety of effects on early development were observed, consistent with pervious studies in the literature on retinoid effects on amphibian development. The phenotypes include normal development, early embryonic lethality, duplicated limb buds, bony triangles and truncated limbs. These phenotypes could be elicited in response to treatment with retinol palmitate, atRA, and the synthetic RAR-specific activator TTNPB [43]. A notable result is that limb dysplasias were only observed when larvae were treated during stages of limb bud development (stages 48–55). Exposures at earlier developmental stages induced malformations in other organ systems (e.g., craniofacial, axial), but not in limbs [43]. In accord with our results (although they reach different conclusions) Degitz and colleagues have also shown that retinoid exposure causes multiple malformations ranging from bony triangles to limb reductions and lethality depending on the species and time of exposure [42].

The results from the pilot experiments led us to focus on treating limb bud stage tadpoles with TTNPB to ascertain the precise windows of sensitivity to retinoid exposure. We found that TTNPB induces all of the limb malformations observed in the field, including duplicated limbs, distal reductions, and bony triangles. TTNPB is very stable and induces malformations after relatively short exposures (3–24 h). Results from TTNPB treatment indicate that there are multiple developmental windows of sensitivity during limb bud development, and that these windows are remarkable short. Treatment at all stages is developmentally toxic at high doses and long exposures, although TTNPB treatment is not acutely toxic when embryos are treated after the gastrula stage. The surviving larvae typically die a few weeks after exposure.

Treatment at the very beginning of limb outgrowth (stages 49/50) with doses between 100–1000 nM TTNPB was lethal to virtually all of the tadpoles [43]. In contrast, less than 10 % mortality is observed at lower levels of TTNPB. No morphological changes were observed in limb buds 4 weeks after exposure at these lower doses.

Treatment of embryos beginning at stage 50 also led to very high mortality and nearly no malformations (Table 1). Treatment beginning one day later at stage 51 elicited a high incidence of defects (Table 1). Duplicated limbs were induced at low doses of TTNPB (40, 80 nM), whereas higher doses resulted in 100 % mortality. Treatment at stage 52 yielded much less mortality at low doses with substantial malformations at doses of 8 nM and higher. The predominant defects seen at this stage were bony triangles. Lastly, treatment at stages 53–55 induced hypomorphic limbs at high frequency [43].

Table 1 Treatment of *Xenopus laevis* tadpoles with TTNPB elicits stage-specific limb defects. *X. laevis* tadpoles were treated at the indicated stages with a concentration series of TTNPB for 24 h, then allowed to develop normally and scored for survival and the presence of malformed limbs.

	Stage 50		Stage 51		Stage 52	
TTNPB	% Mortality	% Malformed	% Mortality	% Malformed	% Mortality	% Malformed
None	12.5	0	40	0	10	0
8×10^{-9}	44.4	0	70	0	40	33.3
4×10^{-8}	25	0	41.6	50	10	66.6
8×10^{-8}	33.3	0	58.3	80	11.1	100
4×10^{-7}	50	0	100	NA	72.7	100
8×10^{-7}	50	0	100	NA	77	100

It is also notable that we observe both unilateral and bilateral defects in the treated animals, as has been observed in wild specimens from affected sites. Since TTNPB elicits these characteristic limb defects, we conclude that inappropriate regulation of RAR-mediated signaling during distinct developmental windows is the proximate cause of the limb defects observed in laboratory animals, and by inference those in the field. Interestingly, the developmental windows for the production of limb duplications, bony triangles and hypomorphic limbs appear to be separate and mostly nonoverlapping, within the precision of the experimental protocol.

Although *Xenopus* is a valuable laboratory model organism for experimental work, it is not a species that is native to North America and is not found in areas with high frequencies of amphibian malformations. For that reason, we and others conducted parallel experiments in native species. We showed that retinoids such as retinol palmitate, atRA and TTNBP can induce limb malformations, including BTs, in *Rana pipiens* (D. M. Gardiner, unpublished) and *Rana sylvatica* [43]. Degitz and colleagues have produced similar results in other native species [42].

The model that environmental retinoids is the cause of frog deformities predicts that retinoids will be found at sites where deformed frogs are found. Furthermore, it should be the case that the same compounds are not present, or are present at much lower levels at sites where deformed frogs do not occur. Therefore, we extracted hydrophobic substances from water samples, fractionated these by HPLC, and tested the fractions for their ability to activate RAR in transient transfection assays. A critical point and a fundamental difference between this approach and more commonly used approaches is that we are not making any assumptions about the nature of the compounds beyond that they are present in the water. Fractionation is guided simply by the ability of fractions to activate RAR. Active fractions were purified to homogeneity as judged by UV absorption spectra and then analyzed by electrospray (ES/MS) and electron impact mass spectroscopy (EI/GC/MS) for exact mass determination and fragmentation analysis to identify the causal agents.

We focused on two sites where deformed frogs are routinely found, the CWB site in Minnesota and a site in Mission Viejo, CA, almost 2000 miles away. We found fractions that activated RAR from both sites and showed that this activity could be recovered from the water samples by both solvent and solid phase extraction (Figs. 2, 3). The activity resolved into three closely spaced but distinct HPLC peaks, suggesting the presence of geometric isomers of the same compound. Notably, similar activity peaks were identified in the water samples from a vernal pond in Mission Viejo, CA as in the permanent lake in CWB Minnesota (Figs. 2, 3). These data suggest that the compound is unlikely to be natural in origin and provide an important confirmation of the laboratory results with retinoid exposure. Taken together, the laboratory and field observations strongly support our contention that an environmental retinoid(s) is present and responsible for frog deformities in the water samples from the sites tested. We infer that the same compounds will be found at other deformed frog sites and are currently establishing a testing program to test this hypothesis.

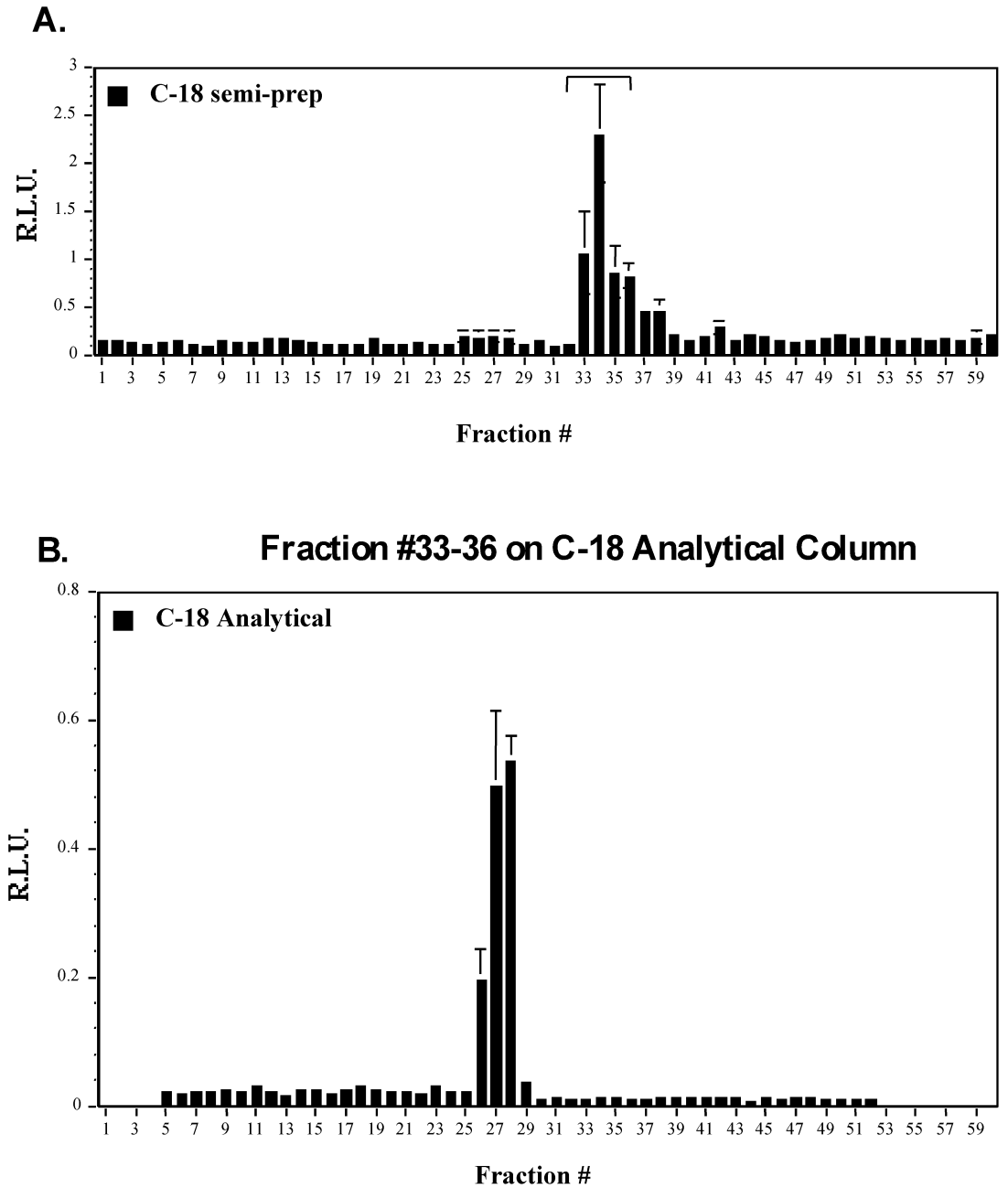

Fig. 2 Biologically active retinoids are found in a severely affected site. Solid phase extracts of lake water (2000 l) were fractionated by reversed-phase HPLC. Aliquots of column fractions were tested for their ability to activate RAR-dependent reporter gene transcription in transient transfection assays.

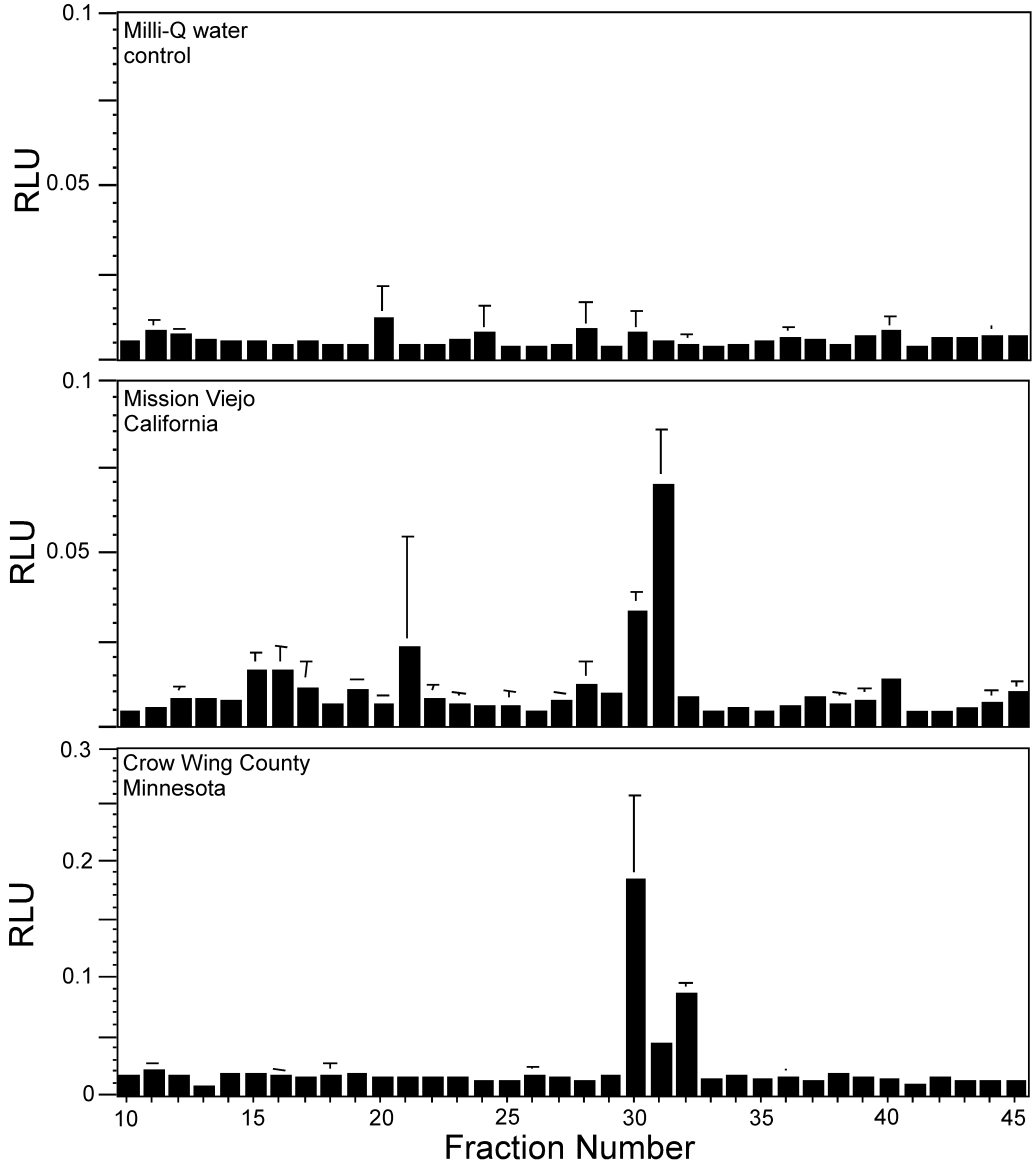

Fig. 3 Similar bioactive retinoid peaks are found in Mission Viejo, CA and Crow Wing County, MN. Organic extracts of lake water were prepared and fractionated by reversed-phase HPLC on an analytical C-18 column. Aliquots of column fractions were tested for their ability to activate RARα-dependent transcription of a luciferase reporter gene in transient transfection assays.

SUMMARY

The concept of parsimony dates back to the Middle Ages where its roots may be found in the writings of the English philosopher and theologian William of Ockham (1285–1349). His famous principle, often referred to as "Occam's razor", holds that in attributing an explanation to a set of observations, one should not make more assumptions than the minimum needed. The fundamental truth is that when considering a set of models to explain an observation, the **simplest** explanation that explains **all** of the observations is most often correct. Occam's razor metaphorically shaves off extraneous assumptions and variables that are not needed to explain the phenomenon in question. While it is formally possible that each and every site at which deformed frogs are found results from a different causative agent(s) or complex interactions between numerous agents and the local water chemistry, logic should tell us that the probability is exceedingly small. The most probable explanation for the vast majority of frog malformations will be the minimum set of causal agents that can explain all of the observed phenomena. As was described above, inappropriate modulation of retinoid signaling pathways during critical points in development can explain **ALL** of the observed frog deformities, even such bizarre phenotypes as an apparently normal frog with a cluster of ectopic limbs attached. Retinoids are capable of causing a wide variety of developmental abnormalities including craniofacial, gastrointestinal and neurological defects. Although little discussed, a common phenotype at affected sites is dead or dying frogs and retinoids can also account for these observations. We cannot completely exclude the possibility that frog malformations in the wild have multiple etiologies, but it is highly likely that the majority result from retinoid exposure.

Lastly, statements such as "With regard to an ecological enigma such as the malformed frogs, however, a focused light that illuminates the esoteric molecular or cellular processes of developmental biology poses the danger of blinding the investigator to the contextual clues shadowed in the surrounding environment" [6] should not be allowed to pass into the literature unchallenged. Although some authors may find the molecular and cellular processes of developmental biology to be "esoteric", cognoscenti who have followed the advances in the molecular genetics of development made in recent years will realize that the fundamental molecular and cellular processes underlying development cannot be ignored in the search for rational explanations. The vertebrate limb is not a black box that is acted on by a myriad of shadowy contextual clues in the environment, but rather the most intensively studied and well-understood model in vertebrate development. To treat it otherwise is reminiscent of the humoral theories of disease prevalent in past centuries.

RECOMMENDATIONS FOR FUTURE RESEARCH

Both parasites and retinoids can elicit frog malformations in the laboratory. Since only a single parasite species causes such malformations and it appears very unlikely that mechanical perturbation will be the cause, it is plausible that the parasites are providing or inducing a developmental signal in the affected animals. The application of Occam's razor leads to the inference that this agent will be a retinoid, alter the concentrations of retinoids in the animal, or otherwise modulate the expression of retinoid target genes. Thus, it would be interesting to identify the molecular mechanisms through which *Ribeiroia* act to cause malformations.

Since it seems beyond question that inappropriate retinoid signaling is playing an important role in the etiology of deformed frogs, the next step is to find out when and where such compounds are present in the environment. It is critical that a series of field surveys be undertaken that will reveal the presence, concentration, and stability of the candidate retinoids in bodies of water and determine whether drinking water supplies are affected. One would like to understand the route through which the compounds enter the animals (e.g., diet, transport across gills or skin through exposure to sediments or water) and whether human and animal populations are at risk from similar exposure. We believe that studying mechanisms can lead to important inferences about the nature and mode of action of environ-

mental contaminants. This will lead to a deeper understanding of how animals interact with the environment and a rational basis for future risk assessment.

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