Amphibian deformities and *Ribeiroia* infection: an emerging helminthiasis

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Since their widespread appearance in the mid-1990s, malformed amphibians have evoked fear, as well as fascination within the scientific and public communities. Recent evidence from field and laboratory studies has implicated infection by a digenetic trematode – *Ribeiroia ondatrae* – as an important cause of such deformities. *Ribeiroia* spp. have a complex life cycle involving planorbid snails, amphibians and water birds. Under natural conditions, malformations might promote parasite transmission by increasing the susceptibility of infected amphibians to predation by definitive hosts. However, with respect to the recent outbreak of deformities, we suggest that exogenous agents (e.g. pesticides, nutrient run-off, introduced fishes) might be interacting with *Ribeiroia*, resulting in elevated infection levels, and we highlight the need for studies incorporating multiple stressor dynamics to further explore this problem.

Few environmental issues of the late 20th century captured as much attention and caused as much alarm as the widespread reports of malformed frogs in North America [1,2]. The frenzy originated near Henderson, MN, USA, in 1995, when a group of middle-school children stumbled upon a pond in which 50% of the emerging leopard frogs (*Rana pipiens*) exhibited severe deformities. Concern grew as numerous malformed amphibians were observed in many parts of North America. To date, grotesque malformations have been recorded in 60 species of frogs, toads and salamanders from 46 states in USA, with concentrations ('hotspots') in the west, midwest and northeast ([3], North American Reporting Center for Amphibian Malformations, http://www.npwrc.usgs.gov/narcam). The deformities primarily afflict the hind limbs, including extra, missing and grotesquely misshapen limbs (Fig. 1), and can affect >50% of a population. Public concern over possible threats to human health fueled investigations; countless news articles, television segments and even a recent book [2] have chronicled the enigmatic controversy. Since 1995, government agencies, such as the Environmental Protection Agency (http://www.epa.gov), National Institutes of Health (http://www.nih.gov/), National Science Foundation (http://www.nsf.gov) and US Fish and Wildlife Service (http://www.fws.gov), have collectively allocated millions of research dollars toward uncovering the agent(s) responsible. And, while no single cause can explain the rash of deformities, recent evidence from numerous hotspots implicates infection by a trematode parasite – *Ribeiroia ondatrae*.

A teratogenic trematode

Trematodes in the genus *Ribeiroia* (Digenea, Cathaemasiidae) have an indirect life cycle involving three hosts. The first intermediate hosts are planorbid snails in the genera *Biomphalaria, Helisoma* and *Planorbella* [4]. Although similar to echinostomes, *Ribeiroia* spp. lack

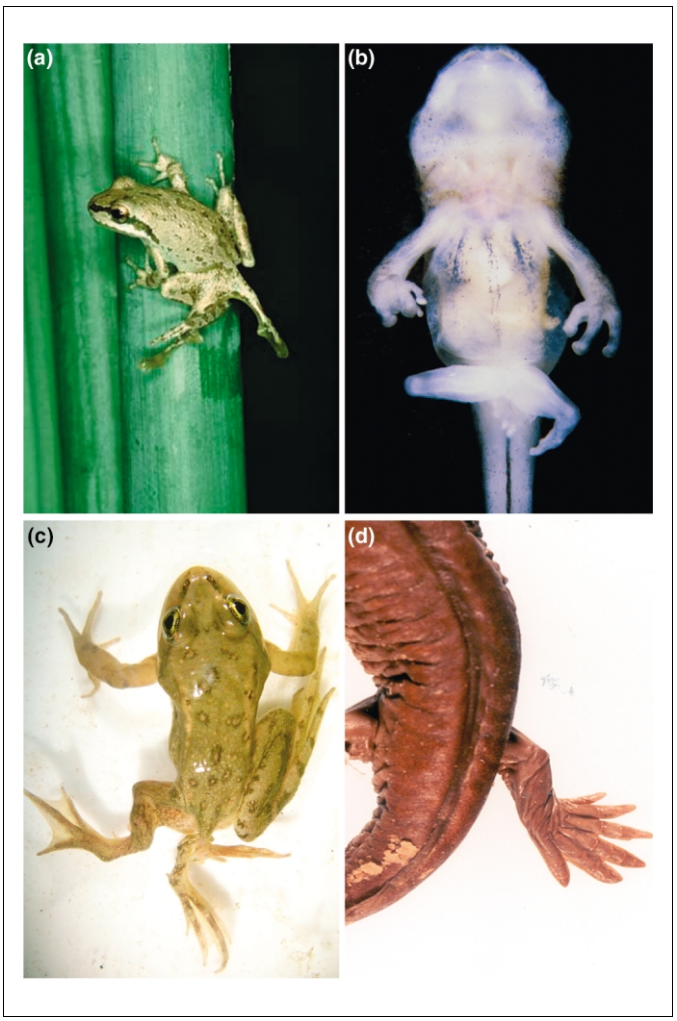

Fig. 1. Representative amphibian malformations associated with *Ribeiroia* infection. (a) Pacific treefrog (*Hyla regilla*) with two extra hind limbs. Reproduced, with permission, from Steven Holt (http://www.stockpix.com). (b) Partially missing hind limbs in an experimentally infected treefrog. (c) Oregon spotted frog (*Rana pretiosa*) with a bony triangle and missing digits in the left limb and two poorly developed extra limbs. (d) Tiger salamander (*Ambystoma tigrinum*) with an extra toe.

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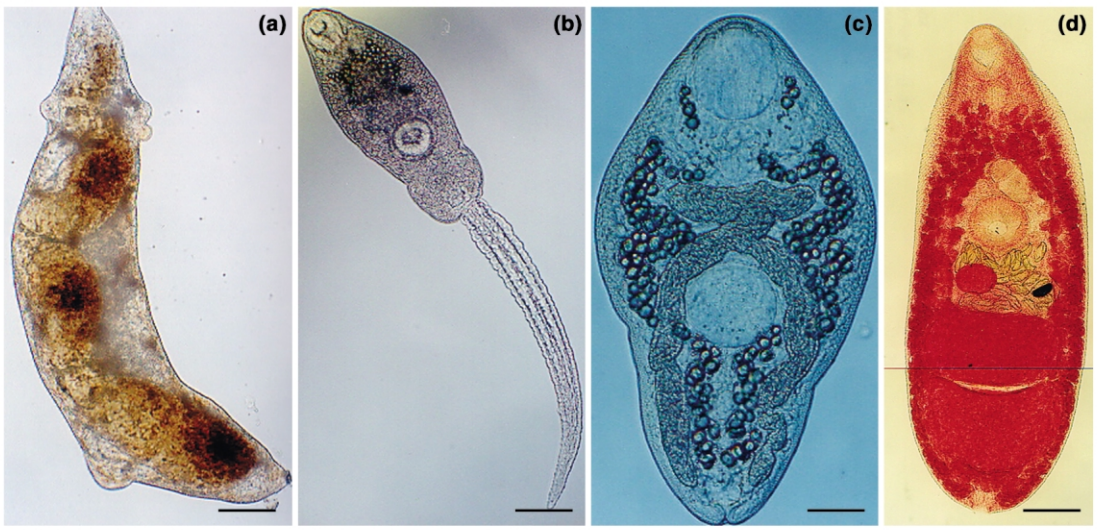

Fig. 2. Developmental stages of *Ribeiroia ondatrae*. Rediae (a) develop inside freshwater snails (e.g. *Planorbella* spp.), where they reproduce asexually to produce free-swimming cercariae (b). Cercariae encyst in a suitable second intermediate host, such as a fish or larval amphibian, and then transform into metacercariae (c). When the second intermediate host is consumed by a bird or mammal, metacercariae mature into adult parasites (d) within the definitive host and begin sexual reproduction. Scale bars = 165 μm (a); 150 μm (b); 82 μm (c); 241 μm (d).

collar spines and do not form metacercariae in snails. Instead, cercariae of *Ribeiroia* encyst either in larval amphibians or freshwater fishes (Fig. 2). These second intermediate hosts, in turn, are consumed by a wide variety of avian and mammalian definitive hosts. Most host records involve birds, particularly raptors and herons, but rats, muskrats and badgers also represent natural definitive hosts. Although the phylogeny of this group is still under investigation, three species are traditionally recognized: *R. ondatrae* in the Americas; *Ribeiroia congolensis* in Africa; and *Ribeiroia marini* in the Caribbean [4]. All species exhibit the distinctive esophageal diverticula characteristic of the genus (Fig. 2).

Historically, *Ribeiroia* spp. have been studied infrequently. However, *Ribeiroia* infection causes complete castration of the snail hosts and actively antagonizes schistosome sporocysts. Indeed, on the French island of Guadeloupe, mass introduction of *Ribeiroia* eggs was employed as a biological control agent to reduce the abundance of *Biomphalaria glabrata*, the primary snail host for *Schistosoma mansoni* [5]. In birds, infection is generally asymptomatic, but *Ribeiroia* has also been implicated in several unusual outbreaks of avian proventriculitis [4].

A connection between trematode infection and amphibian limb malformations was first hypothesized by Sessions and Ruth [6] when they investigated an outbreak of deformities among populations of Pacific treefrogs (*Hyla regilla*) and long-toed salamanders (*Ambystoma macrodactylum croceum*). Upon clearing and staining the unusual animals, they discovered numerous metacercariae concentrated around the deformed limbs. Experimental implantation of metacercariae-sized resin beads into the developing limb buds of *Xenopus laevis* induced some malformations, lending further support for the authors' hypothesis [6].

Subsequent field studies [7] identified a positive correlation between sites with malformations and the trematode *R. ondatrae* in northern California. Experimental exposure of larval Pacific treefrogs and western toads (*Bufo boreas*) to biologically relevant levels of *R. ondatrae* cercariae (12–48) induced high frequencies (30–100%) of severe limb malformations identical to those observed at field sites [7,8]. These included multiple extra limbs, skin webbings, bony triangles, and missing or partially missing limbs (Fig. 1). Some frogs were missing both hind limbs; others developed as many as six supernumerary limbs. Comparable malformation results have been reported for Northern leopard frogs (*Rana pipiens*) and wood frogs (*Rana sylvatica*) [9].

Field studies corroborate results from the laboratory. Larval wood frogs maintained in enclosures with fine mesh, which prevented entry of trematode cercariae, developed normally. In enclosures with larger mesh, however, larval wood frogs became infected with *Ribeiroia* cercariae and developed malformations [10]. Moreover, in a broad-scale field survey of malformation sites throughout the western USA, Johnson *et al.* [11] found that *Ribeiroia* infection was the single-best predictor of higher-than-baseline frequencies of malformations among 11 species of frogs, toads and salamanders. The average intensity of infection in a population was functionally related to the frequency of malformations – more *Ribeiroia* means more deformities. At some field sites, three or more species exhibited severe malformations, which could affect 90% of the population [11]. More recently, *Ribeiroia* infection has also been recorded at malformation hotspots in New York, Pennsylvania, Wisconsin, Illinois and Minnesota, including many of the sites that first galvanized the attention of the media and scientists ([12] P.T.J. Johnson and D.R. Sutherland, unpublished).

Toward a mechanistic understanding

Two hypotheses have been advanced to explain how invading trematode cercariae induce improper limb

development. In the first, the effect is mechanical – the parasites disturb the arrangement of growing limb cells, leading to abnormal limb formation or even duplication. In the second, *Ribeiroia* actively produces a compound that interferes with a retinoid-sensitive signaling pathway, thereby stimulating or inhibiting continued limb growth [7,13]. Although the effects of these mechanisms are difficult to distinguish and might even operate in concert, the deformities induced by *Ribeiroia* are similar to those produced when a developing amphibian's limb is damaged mechanically (via surgical rotation), suggesting that cellular rearrangement might be a sufficient mechanism [9].

Whether or not an amphibian becomes malformed following infection depends crucially on both the number of parasites to which it is exposed and the timing of exposure. Metacercariae of *Ribeiroia* are almost invariably concentrated around the base of an infected amphibian's limbs [11], and observational studies confirm that cercariae target this region [9]. However, to induce malformations, *Ribeiroia* cercariae must encyst during the window of early limb development. Amphibians exposed after limb development is completed are unlikely to develop malformations. Thus, differences in habitat use, relative size, activity levels and the timing of limb development help to explain why, even within a single pond, some amphibian species exhibit severe malformations, whereas others are apparently unaffected. If a frog does become malformed, however, it rarely survives to sexual maturity, succumbing to starvation or predation [6]. This observation has led some to suggest that malformations might benefit the parasite, enhancing its transmission between infected intermediate hosts (amphibians) and predatory definitive hosts (birds or mammals) [6,7]. However, experimental testing is necessary to determine whether malformations promote transmission to suitable predators.

A new phenomenon?

What we still fail to understand, however, is why these deformities are occurring now. Parasites removed from long-preserved specimens of malformed frogs indicate that *Ribeiroia*-induced malformations have occurred in North America since at least the 1940s [14]. They are, however, uncommon historically. An exhaustive literature review yielded <15 cases of 'mass malformations' in the USA. By contrast, >50 malformation sites associated with *Ribeiroia* have been discovered in the past five years. Whereas the importance of increased awareness and surveillance cannot be discounted, examinations of museum specimens in conjunction with re-surveys of historical field sites offer empirical evidence that the frequency and severity of malformations has increased, at least in some areas [15].

As a possible explanation for this apparent increase in malformations, some researchers have suggested that *Ribeiroia* have emerged as a consequence of changes in the ecology of its hosts. Malformation hotspots and *Ribeiroia* were associated with highly productive, artificial habitats such as farm ponds used to water crops and cattle [11]. Such habitats might have an important role in the suspected increase of *Ribeiroia* and amphibian malformations for three reasons: (1) these systems are often highly productive because of fertilizer and manure run-off, leading to increased algal production and denser populations of snail hosts; (2) the number of artificial impoundments has skyrocketed over the past 60 years, even as natural wetlands are continually destroyed; and (3) the other necessary hosts – birds and amphibians – are frequently found in such systems [14].

Additional stressors might interact with *Ribeiroia* to elevate the frequency of amphibian deformities. Kiesecker [10], for example, reported a synergistic interaction between trematode infection and pesticide exposure. Amphibian larvae exposed to both *Ribeiroia* cercariae and low levels of pesticides showed increased infection and a depressed immune response relative to amphibians exposed only to *Ribeiroia*. The presence of fish predators can indirectly increase the level of trematode infection in larval amphibians [16]. In response to effluent from caged-fish predators, tadpoles reduced their activity, which apparently left them more exposed to cercarial penetration. The introduction of fishes and other predators is a widespread problem in amphibian habitats worldwide, but the role of exotic species introduction as an indirect driver of parasite-induced malformations in amphibians has not been examined under field conditions.

Perspective

Over the past eight years, our understanding of the amphibian deformity issue has advanced considerably. Although many agents undoubtedly contribute to the problem, infection by the trematode *R. ondatrae* appears to play a particularly important role. Field studies have identified strong associations between malformation hotspots and the presence of *Ribeiroia*, whereas experimental infection studies have confirmed the teratogenic properties of the parasite, reproducing malformations identical to those from field sites. There are, however, many unanswered research questions, and the focus is shifting away from an all-out search for the 'smoking gun' and toward a more holistic exploration of interactions among environmental agents. Interdisciplinary research teams, representing ecologists, parasitologists, toxicologists and water chemists, are needed to unlock these complex dynamics, identifying the factors that influence *Ribeiroia* hosts and, in turn, the level of infection and the frequency of deformities in amphibians. Toward these goals, there is a profound need for more basic science on the parasite itself. Although *Ribeiroia* has been recorded from >35 birds and five mammals, we still know little about the importance of different definitive host species, and the contribution of fishes relative to amphibians in transmitting the parasite transmission remains an open question. The phylogeny of species within the genus and their connections to related groups, such as *Echinostoma* and *Cathaemasia*, have to be examined carefully. Whether other species of *Ribeiroia* (or other genera) can induce such deformities in amphibians remains unexplored. A more-detailed understanding of the parasite, it hosts and their interactions within a dynamic environment is necessary if we are to determine the long-term implications of

deformities for amphibian populations and overall ecosystem health.

References

1 Kaiser, J. (1997) Deformed frogs leap into spotlight at health workshop. *Science* 278, 2051–2052
2 Souder, W. (2000) *A Plague of Frogs*, Hyperion
3 Blaustein, A.R. and Johnson, P.T.J. (2003) The complexity of deformed amphibians. *Front. Ecol. Environ.* 1, 87–94
4 Johnson, P.T.J. *et al*. Review of the trematode genus *Ribeiroia* (Cathaemasiidae): ecology, life history, and pathogenesis with special emphasis on the amphibian malformation problem. *Adv. Parasitol*. (in press)
5 Nassi, H. *et al.* (1979) Bilan d'un essai de contrôle de *Biomphalaria glabrata* en Guadeloupe á l'aide d'un trématode stérilisant. *Ann. Parasitol.* 54, 185–192
6 Sessions, S.K. and Ruth, S.B. (1990) Explanation for naturally occurring supernumerary limbs in amphibians. *J. Exp. Zool.* 254, 38–47
7 Johnson, P.T.J. *et al.* (1999) The effect of trematode infection on amphibian limb development and survivorship. *Science* 284, 802–804
8 Johnson, P.T.J. *et al.* (2001) *Ribeiroia ondatrae* (*Trematoda: Digenea*) infection induces severe limb malformations in western toads (*Bufo boreas*). *Can. J. Zool.* 79, 370–379
9 Stopper, G.F. *et al.* (2002) How trematodes cause limb deformities in amphibians. *J. Exp. Zool.* 294, 252–263
10 Kiesecker, J.M. (2002) Synergism between trematode infection and pesticide exposure: A link to amphibian deformities in nature? *Proc. Natl. Acad. Sci. U. S. A.* 99, 9900–9904
11 Johnson, P.T.J. *et al.* (2002) Parasite (*Ribeiroia ondatrae*) infection linked to amphibian malformations in the western United States. *Ecol. Monogr.* 72, 151–168
12 Sutherland, D.R. Parasites of North American Anurans. In *Declining Amphibians: A United States' Response to the Global Phenomenon* (Lannoo, M.J., ed.), University of California Press, Berkeley, California (in press)
13 Maden, M. (1996) Retinoic acid in development and regeneration. *J. Bioscience* 21, 299–312
14 Johnson, P.T.J. *et al*. Limb deformities as an emerging parasitic disease in amphibians: evidence from museum specimens and resurvey data. *Conserv. Biol* (in press)
15 Hoppe, D.M. Linking malformations to amphibian declines: history of malformed anurans in Minnesota and interspecific differences in their occurrences. In *Declining Amphibians: A United States' Response to the Global Phenomenon* (Lannoo, M.J., ed.), University of California Press, Berkeley, California (in press)
16 Thiemann, G.W. and Wassersug, R.J. (2000) Patterns and consequences of behavioural responses to predators and parasites in *Rana* tadpoles. *Biol. J. Linn. Soc.* 71, 513–528

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Genes, nitric oxide and malaria in African children

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The unresolved and complex relationship between nitric oxide and falciparum malaria is reflected in recent genetic and immunohistochemical studies in African children. Different genetic associations, perhaps geographically distinctive, are seen between genetic variants of the inducible nitric oxide gene and various disease manifestations in African populations. The picture might not be complete without considering the emerging roles of carbon monoxide, another endogenous gaseous mediator with similar effects to those of nitric oxide. Only when genetic comparisons from across tropical Africa are examined, in conjunction with the newly recognized complexities in the events of systemic inflammation, will this relationship be understood.

Although many African children die of falciparum malaria, they are few in proportion when compared with the absolute number infected. Most survive because of an acquired or innate tolerance to the parasite – the mechanisms of which are not known in detail. Malaria is widely accepted to have been one of the major evolutionary selective pressures on the human genome, and several human genetic variants are thought to be common in the tropics because they provide a survival advantage against *Plasmodium falciparum*. The central role of pro-inflammatory cytokines is now firmly established in the host response against invading pathogens, including malaria, but these mediators also dominate the literature on disease pathogenesis, again including that of malaria. Thus, the downstream mediators generated by these cytokines are much studied in malaria and other infectious diseases. Efforts are being focused on the inducible nitric oxide synthase (iNOS), the enzyme that produces nitric oxide (NO) in response to pro-inflammatory cytokines. As part of this approach, geneticists have been identifying genetic variants, particularly single nucleotide polymorphisms (SNPs), that might modify *NOS2A* (the gene encoding iNOS) expression, and correlating these variants with clinical manifestations of falciparum malaria. These genetic association studies clearly do not provide evidence of causality, so functional data linking these polymorphisms to phenotypes are essential to define their roles in human disease.

Recently, two articles [1,2] have explored variation in the *NOS2A* gene in African children with falciparum malaria. These studies demonstrate the need to define the phenotypes of malaria disease carefully in the study population (e.g. delineating severe malarial anaemia,

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