Original Contribution

Ribeiroia Infection Is Not Responsible for Vermont Amphibian Deformities

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Abstract: Reports of limb deformities in amphibians have garnered wide notice from scientists and the public alike. Recent laboratory and field research has supported the hypothesis that infection by the helminth parasite, *Ribeiroia ondatrae*, is associated with deformities, particularly in the western United States. In this study, observational and experimental evidence from eastern United States (Vermont) provides evidence that *Ribeiroia* is absent from a large sample of sites including those with a history of relatively high frequencies of deformity, that the composition of deformities is distinct from that associated with experimental infection by *Ribeiroia*, and that the composition of limb deformities seen in natural populations in Vermont is typical of that reported in the literature. We suggest that while *Ribeiroia* has been shown to be responsible for deformities in some species and locations, other factors may be responsible where the composition of deformities is inconsistent with patterns resulting from known *Ribeiroia* infection.

Keywords: Amphibian, deformities, experiment, observation, *Ribeiroia*, Vermont

INTRODUCTION

During the last decade, reports of amphibians with developmental abnormalities have greatly increased, prompting widespread attention and concern (Souder, 2000). Most reported abnormalities involve the hind limbs. Such deformities have long been described, but systematic evaluations of historical and more recent observations strongly suggest that limb deformities constitute a true emerging disease phenomenon rather than a byproduct of increased attention (Hoppe, 2000; Johnson et al., 2003). A number of

causes for deformities have been nominated including UV radiation, chemical agents, predation, and helminthic infection. The UV radiation hypothesis has largely been discounted as field exposures tend to be low and abnormalities in field-caught individuals rarely show the bilateral symmetry that is the hallmark of experimentally irradiated animals (Meteyer et al., 2000; Ankley et al., 2004). Evidence that exposure to chemicals or predators can lead to high frequencies of deformities in the field is sparse (Ankley et al., 2004), though it is known from experiments that each is capable of causing abnormal limb development (Fort et al., 1999; Johnson et al., 2001b). Both observational (Johnson et al., 2001b; 2002) and experimental (Johnson et al., 1999; Sessions et al., 1999; Stopper et al., 2002)

investigations have provided strong support for a role for infection by the trematode, *Ribeiroia ondatrae* (hereafter *Ribeiroia*), particularly in the western United States (Johnson and Lunde, 2005) and most clearly for the Pacific Treefrog (*Hyla regilla;* Johnson et al., 2002).

It is critically important to better understand the causes of amphibian limb deformities. If current support for the *Ribeiroia* hypothesis can be extended to other species and regions, we can begin to estimate the likelihood that deformities contribute to observed population declines and we can assess the feasibility for control and mitigation of *Ribeiroia*-mediated effects (Ankley et al., 2004). Such intervention may be particularly warranted where human alteration of the environment is implicated in provoking the emergence of disease (Johnson and Chase, 2004; Skelly et al., 2006). If *Ribeiroia* is not involved in deformities in other contexts, strategies for control as well as the health implications for other species, including humans, need to be explored.

The goal of this study is to evaluate the *Ribeiroia* hypothesis for limb deformities in eastern North America (Vermont, USA). During the decade since the first reports surfaced, extremely high frequencies of limb deformities (over 30% of sampled metamorphs in some cases; Levey et al., 2003) have been observed in Vermont amphibian populations. During 2 consecutive years, we sampled 41 wetland sites from across the Champlain Basin of Vermont. In addition to estimating composition of amphibian deformities, we examined amphibians for the presence of metacercarial cysts and collected snails (the prior host of *Ribeiroia*) to evaluate their infection status. Finally, we performed experiments in which we infected Vermont leopard frogs with *Ribeiroia* to compare resulting deformities with those seen in natural populations.

MATERIALS AND METHODS

Composition of Limb Deformities

Between May and August of 2 years (2002, 2003) we collected anuran amphibians representing seven species *(Bufo americanus, Hyla versicolor, Pseudacris crucifer, Rana catesbeiana, R. clamitans, R. pipiens, and R. sylvatica)* from 41 wetlands in the Lake Champlain Basin of Vermont. Wetlands were selected to represent a range of landuse categories, including relatively undeveloped forest (Green Mountain National Forest), rural landscapes, as well as the state's largest urban area centered around Burlington. At each site, we used pipe samplers (steel pipe, 35 cm diam-

eter, 0.91 meters long) and dipnets (46 cm × 23 cm with 1 mm mesh) to capture larval and metamorphic amphibians. During each year, we collected up to 300 specimens from each site (a maximum of 100 during each of three visits). Each individual was later examined in the laboratory to determine species, developmental stage (Gosner, 1960), and presence of abnormalities. For each collected individual between Gosner Stages 40 and 46, limb abnormalities were scored according to Meteyer (2000).

Ribeiroia Infection in Snails

Freshwater snails act as the first intermediate host of *Ribeiroia* (Johnson et al., 2004). We collected snails during both 2005 and 2006. On 2 and 3 June 2005, using dipnets, we collected a total of 444 snails from seven sites. Collection sites in 2005 included six Vermont wetlands (in Alburg, Ferrisburg, Middlebury, North Hero, South Burlington, and West Haven) where high frequencies of amphibian limb deformities occur and a wetland in Fort Edward, NY, where *Ribeiroia* presence had been documented previously [S. Sessions, personal communication]. During 2006, we used dipnets to collect a single snail species, *Planorbella trivolvis*, from four sites including three sites where we collected in 2005 (Alburg, Ferrisburg, Middlebury) as well as a newly identified site (also in Middlebury). Snails were collected on one or more of three dates (7 July: Alburg, Ferrisburg, Middlebury; 23 July: Ferrisburg, Middlebury; 2 August: Ferrisburg, Middlebury). In 2006, we collected a total of 275 *P. trivolvis.*

Snails were immediately brought back to the laboratory and placed in plastic containers held at a constant temperature of 14°C with a 14:10 light dark cycle. Snail infection status was assessed by placing under a light an individual snail in a small container holding 100 ml of water. Containers were checked several times each day for up to 4 days for the presence of shed cercariae. If cercariae were present, they were removed for identification. The identification of *Ribeiroia* was confirmed by the presence of laterally projecting esophageal diverticula (Johnson et al., 2004) as well as their behavior when exposed to live tadpoles (notably their particular tendency to attack sites near the developing hind limb bud; Sessions et al., 1999).

Metacercarial Cysts

We dissected a total of 54 amphibians with hind limb deformities and examined them for the presence of metacercarial cysts. In each amphibian, we examined the

inguinal region for cyst concentrations, which have been reported in *Ribeiroia*-infected animals (Johnson et al., 2003), and where we recovered cysts in animals experimentally infected with *Ribeiroia* (see below).

We used 20 of the 54 dissected larvae in a case-control analysis to compare cyst concentrations on deformed and normal individuals. The 20 selected individuals came from wetlands with the highest deformity frequencies, and were selected at random. Each selected case was compared to a control individual with normal limbs of the same species at the same Gosner developmental stage collected on the same date. The control used was selected at random from among the pool of possible candidates. Beyond examination of the inguinal region, each of the 40 animals was then methodically dissected by removing the skin and examining the tissue beneath for the presence of metacercarial cysts.

Experimental *Ribeiroia* Infection

Leopard frogs were selected for experimental infections because they were the most frequently deformed amphibian species among those we sampled. We collected six leopard frog egg masses from two Vermont locations (Alburg, West Haven) and moved them to the Yale Myers Forest, Connecticut where masses were placed in wading pools holding aged well water and covered with mosquito mesh. Hatchlings were retained in pools until stocked in one of two different venues, laboratory container or cattle-watering tank. After stocking, larvae were reared until they reached the early stages of hind limb bud development (Gosner Stage ranging from 27 to 28) when they were experimentally infected with *Ribeiroia*.

All experimental infections were conducted by exposing individual tadpoles in the laboratory to groups of 40 *Ribeiroia* cercariae for a period of 120 min. This process was repeated three times allowing a period of at least 24 hours between exposure bouts. Following exposure, larvae were reared in one of two types of container: 4-liter plastic laboratory containers, or 1000-liter cattle-watering tanks. Lab reared individuals (*n* = 7) were housed in aged tap water and given an ad libitum ration of a 3:1 mixture of fish flakes and rabbit chow. When individuals reached Gosner Stage 42 (forelimb emergence), they were examined for limb abnormalities. Cattle tanks were set up as artificial ponds using a previously published protocol (Skelly, 2002). Sixty experimentally infected animals were reared in one of three tanks (20 infected individuals per tank). We also stocked three control tanks with tadpoles in which no

Table 1. Frequency of Hind Limb Malformations among 3420 Anuran Amphibians Collected from 41 Vermont Wetlands during 2 Years[a]

Abnormality type	Description	Frequency
Ectromelia	Partially missing limb	63
Ectrodactyly	Missing digit	26
Brachydactyly	Short toe	18
Amelia	Missing limb	17
Hemimelia	Short limb bone	7
Micromelia	Small limb	6
Rotation	1' or 2' limb rotation	4
Other		3
Polydactyly	Extra digit	3
Polymelia	Extra limb	2
Taumelia	Bone bridge	1
Sum		150

[a]Abnormality types are presented in order of decreasing frequency. Terminology modified from Meteyer (2000).

individuals were infected. Once the first larvae neared metamorphosis, all tanks were checked regularly and all individuals at or past Gosner Stage 42 were collected for later examination. All tanks were cleared of remaining individuals on 22 August.

RESULTS

Composition of Limb Deformities

During two years of sampling 41 wetland sites around Vermont, we collected and examined 12,388 anurans. Of these, a total of 3420 were designated metamorphs (Gosner Stages 40–46; Gosner, 1960). Among metamorphs collected, we detected hind limb abnormalities in 117 individuals (overall abnormality frequency: 3.4%) from seven different anuran species (Table 1). Abnormalities were unevenly distributed among species and wetlands. The bulk of the deformities were found in leopard frogs (*R. pipiens*, 52 individuals) and green frogs (*Rana clamitans*, 36 individuals). In the most heavily afflicted population, the limb deformity frequency exceeded 20%. In our sample, 26 of the 117 affected individuals had multiple diagnoses for a total of 150 abnormalities. The most common deformity was a reduction of the hind limb (ectromelia, 63 cases), followed by reduction in the number of digits (ectrodactyly, 26 cases), shortening of digits (brachydactly, 18 cases), and the
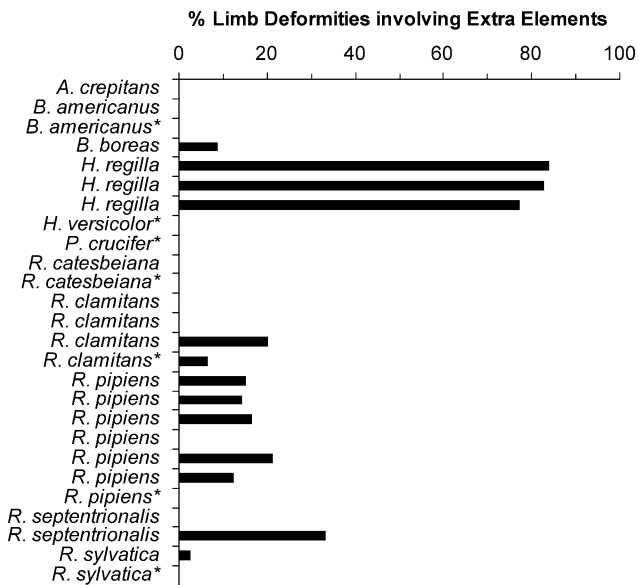
Figure 1. Percent of abnormalities in free living amphibians involving extra hind limbs or limb elements (polymelia, polypody, polyphalangy, polydactyly) recorded from prior published studies. All studies focused on North America and data are presented by species. For records from this study, the species name is followed by an asterisk. Data from a total of 13 studies are included. For each study, we give the reference followed by the species and sample sizes of deformed amphibians examined: (Eaton et al., 2004) *R. sylvatica*, 79; (Helgen et al., 1998) *R. pipiens*, 119; (Johnson et al., 1999) *H. regilla*, 718; (Johnson et al., 2001a) *B. boreas*, 81; (Johnson et al., 2001b) *H. regilla*, 1009; (McCallum and Trauth, 2003) *A. crepitans*, 63; (Ouellet et al., 1997) *B. americanus*, 7, *R. clamitans*, 44, *R. pipiens*, 41; (Meteyer 2000) *R. pipiens*, 70; (Meteyer et al., 2000) *R. pipiens*, 490; (Schoff et al., 2003) *R. clamitans*, 3, *R. pipiens*, 19, *R. septentrionalis*, 3; (Sessions and Ruth, 1990) *H. regilla*, 283; (Sower et al., 2000) *R. catesbeiana*, 2; (Vandenlangenberg et al., 2003) *R. clamitans*, 5, *R. pipiens*, 483, *R. septentrionalis*, 187; (this study) *B. americanus*, 2, *H. versicolor*, 4, *P. crucifer*, 23, *R. catesbeiana*, 4, *R. clamitans*, 46, *R. pipiens*, 39, *R. sylvatica*, 3. Studies in which *Ribeiroia* presence or absence was a condition for sampling, and studies in which data are not separable by species and deformity type, are not included.

entire absence of the hind limb (amelia, 17 cases). Abnormalities involving shortening of limb bones (hemimelia), a reduction in limb size (micromelia), and rotation of the limb (rotation) exhibited lower prevalence (between four and seven cases). Abnormalities involving excess limbs (polymelia, three cases) or digits (polyphalangy, two cases) were rare. A single bone bridge was detected.

We compared the composition of limb deformities observed in this study to prior findings using an index of composition, the fraction of abnormalities involving extra limbs or limb elements (polydactyly, polymelia, polyphalangy, polypody). Figure 1 shows the pattern found in this and prior published studies.

Ribeiroia Infection in Snails

In 2005, we collected a total of 444 snails representing 12 snail species (*Aplexa elongata*, *Bithynia tentaculata*, *Gyraulus circumstriatus*, *Gyraulus parvus*, *Helisoma anceps*, *Physella gyrina*, *Physella heterostropha*, *Physella integra*, *Planorbella trivolvis*, *Planorbula armigera*, *Promenetus exacuous*, *Stagnicola elodes*) from six wetland sites in Vermont and one in New York. *Ribeiroia* cercariae were recovered from *P. trivolvis* collected from Fort Edward, New York. In all, 8 of 31 *P. trivolvis* collected from this site were infected with *Ribeiroia* (infection prevalence of 25.8%). None of the 53 *P. trivolvis* collected from Vermont sites showed evidence of *Ribeiroia* infection, nor did any of the remaining 360 snails of other species.

In 2006, we conducted further snail sampling, concentrating entirely on *P. trivolvis* in Vermont sites. A total of 275 snails were collected on three dates. Consistent with the 2005 results, there was no evidence of *Ribeiroia* infection in any Vermont snails.

Metacercarial Cysts

In none of the 54 deformed metamorphs examined was there any evidence of cyst concentration in the inguinal region.

In the case control analysis, dissection and examination of the entire skin surface revealed that amphibian metamorphs afflicted with hind limb deformities hosted an average of 100 subcutaneous metacercarial cysts, but that cyst load was highly variable ($n = 20$ individuals, SE = 44). Examination of 20 matched control metamorphs (same species, collection date, wetland site, and developmental stage) revealed a comparable pattern of metacercarial infection intensity ($n = 20$, mean = 93, SE = 44). A matched pairs *t*-test revealed that there was no distinction in metacercarial load between cases and controls (df = 19, $P = 0.69$).

Experimental Infections

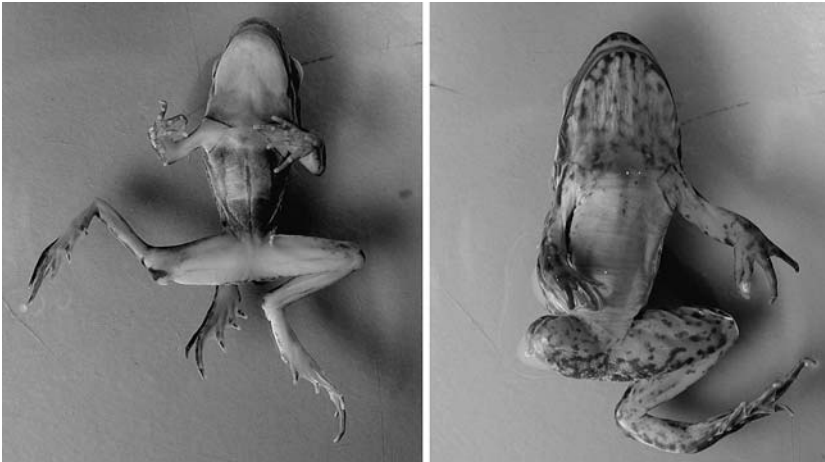We exposed Vermont leopard frogs to *Ribeiroia* (120 cercariae) collected in New York and Connecticut to
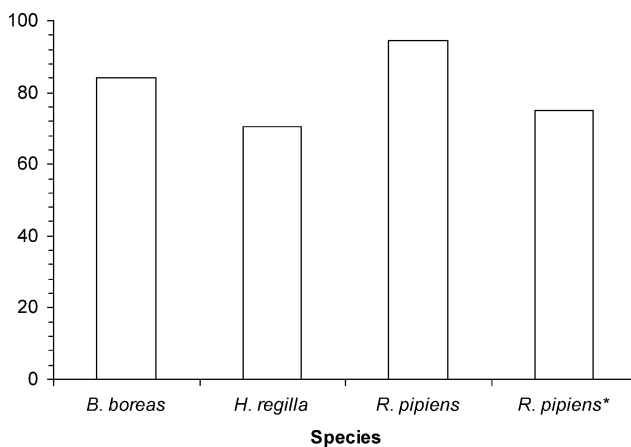
Figure 3. Hind limb abnormalities in three different anuran amphibian species exposed to experimental *Ribeiroia* infection. Bars indicate the prevalence of abnormalities involving extra limbs or limb elements (polymelia, polypody, polyphalangy, polydactyly) from this study (noted by asterisk) and from three prior published experiments (Johnson et al., 1999, 2001a; Schotthoefer et al., 2003).

evaluate the composition of resulting hind limb abnormalities. Larvae were reared in both artificial ponds created in 1000-liter tanks as well as plastic laboratory containers. In the cattle tank experiment, a total of 60 tadpoles were exposed to *Ribeiroia* cercariae, of which 54 survived to metamorphosis (Survival Rate = 90%). Among these, four had hind limb abnormalities (Hind Limb Abnormality Rate = 7.4%). There were no hind limb abnormalities in control tanks. Observed abnormalities included three individuals with polymelia (Fig. 2) and one individual with brachydactly.

Of seven individuals laboratory reared after exposure to 120 cercariae, two died shortly after infection and five survived to metamorphosis. Of the survivors, two had hind limb abnormalities (Hind Limb Abnormality Rate = 40%). Both individuals had duplicate limb elements (polymelia in one case, polyphalangy in the second case). One of these individuals additionally exhibited limb rotation and micromelia.

Across the individuals developing hind limb abnormalities following experimental *Ribeiroia* exposure, all but one showed extra limbs or limb elements. The preponderance of limb and limb element duplication observed here is consistent with prior *Ribeiroia* infection experiments (Fig. 3).

DISCUSSION

A series of observations and experiments fails to support a role for *Ribeiroia* infection in amphibian limb deformities in Vermont, USA. Most critically, there is no evidence for *Ribeiroia* presence in sampled wetlands. A lack of *Ribeiroia* infection in Vermont snails is complemented by a lack of inguinal metacercarial cyst concentrations in metamorphic amphibians. The ready detection of *Ribeiroia* in neighboring New York state suggests both that our sampling methods were robust and that the spatial distribution of *Ribeiroia* is highly heterogeneous. The latter observation is consistent with the results of previous studies of *Ribeiroia* (Johnson et al., 2001b; 2002; Lannoo et al., 2003). Our sampled set of wetlands within Vermont is extensive (41 in number), including several wetlands in which high frequencies of deformities have been detected regularly during the last decade (Levey et al., 2003). We additionally sampled a large number of wetlands with no known records of abnormal amphibian development finding that several of them also yielded substantial frequencies of deformities (Taylor et al., 2005). These observations expand the known

geographic extent of Vermont deformities while simulta-neously extending the recognized range of amphibian species afflicted (Levey et al., 2003).

The deformities we observed in natural populations are strongly patterned despite the wide range of species and wetlands sampled. A few types of limb deformities all characterized by missing or reduced limbs and limb elements (e.g., ectromelia, ectrodactyly) dominated the samples. This pattern is broadly consistent with prior observations of deformities (Ankley et al., 2004; Fig. 1). With the primary exception of some studies conducted in the western United States, most researchers have found that deformities involving extra limbs and limb elements (e.g., polymelia, polyphalangy) are relatively uncommon. The predominance of missing limbs and limb elements is itself inconsistent with a role for *Ribeiroia* infection (Johnson et al., 2001a). While it has been asserted that *Ribeiroia* infection can produce the full range of deformities seen in nature (Stopper et al., 2002; Johnson et al., 2003), infection experiments have predominantly yielded deformities involving extra limbs and limb elements (Fig. 3); it is these deformities that have been closely studied and are now well understood (Sessions and Ruth, 1990; Stopper et al., 2002). The results of our experimental infections are consistent with current understanding of *Ribeiroia* effects yielding primarily malformations involving extra limb elements. This pattern is starkly different from that seen in the field, where of the 150 deformities observed (in the thousands of animals examined), only 5 were extra limbs or limb elements.

Our experimental infections represent the second study of *Ribeiroia* infection effects on leopard frogs. The first (Schotthoefer et al., 2003) uncovered broadly similar patterns including the predominance of deformities involving extra limb elements and limbs (> 94%) and an entire lack of abnormalities such as ectromelia that are the most common in natural populations. While the composition of deformities is similar between the two studies, our mesocosm experiment (prior *Ribeiroia* infection experiments have been conducted in the laboratory) is notable for the low overall frequencies of mortality and deformity observed. This result is unexpected because we exposed vulnerable developmental stages (Gosner Stages 27 and 28) and used a level of exposure (120 cercariae) higher than prior *Ribeiroia* infection experiments (Johnson et al., 1999, 2001a; Schotthoefer et al., 2003). The potential role of context dependence in the consequences of *Ribeiroia* infection deserves further study. Such context dependence

may be widespread in ecological experimentation (Skelly, 2002), arguing for the importance of field-based studies of experimental infection in the future.

Our findings prompt further consideration of the factors responsible for Vermont deformities. Two alternative hypotheses, predation and chemical teratogens, are briefly discussed here.

Predation is a known mediator of hind limb abnormalities including the reduction and absence of limbs common in our samples (Johnson et al., 2003). While it is not straightforward to evaluate whether any given limb reduction or loss is due to a predator, such attacks necessarily involve severe injury to a limb and, consequently, often leave evidence of trauma. We examined each malformed individual closely for any indication of trauma or healing (e.g., exposed bone, irregular pigmentation pattern, scarring). While we did recover animals that had evidently suffered traumatic loss of limb elements, these individuals were not numerous and were not included in the tally of malformed individuals shown in Table 1. We also note that prior radiological studies of metamorphs from Vermont are inconsistent with an important role for limb grazing by predators (Meteyer et al., 2000; Levey et al., 2003; Guderyahn, 2006). As one example, individuals missing an entire limb (amelia) often suffer from deformities of the pelvis that are inconsistent with trauma (Meteyer 2000). It is likely that predators are responsible for some hind limb deformities in our samples. Determining the relative role of predation will require further research.

Most prior consideration of the chemical teratogen hypothesis has focused on evaluating specific compounds, usually in the laboratory. These efforts have largely failed to uncover a candidate capable of causing the types of deformities seen in nature, leaving some to conclude that teratogens are unimportant (Sessions et al., 1999; Stopper et al., 2002). A prior risk analysis of our data suggests a strong association between the incidence of deformities and proximity of a wetland to agriculture (Taylor et al., 2005). This pattern is consistent with that described by Ouellet and colleagues in eastern Canada (Ouellet et al., 1997; Ouellet, 2000). Such an association is a long way from identifying a chemical agent or even establishing that a chemical teratogen, as opposed to some other associated aspect of agricultural landscapes, is responsible. Nevertheless, among hypotheses currently under consideration, these findings imply that the chemical agent hypothesis deserves additional attention. The difficulties encountered in past studies targeting specific teratogens and

mechanisms further suggest that a field-based approach aimed at finding candidate agents associated with high deformity wetlands may provide a productive complement to continued evaluation of agents for which field relevance and toxicity are unknown.

In conclusion, the cause of amphibian limb deformities in some afflicted species and regions remains poorly understood. While reviews note that the *Ribeiroia* hypothesis is the best experimentally supported mechanism among those considered (Blaustein and Johnson, 2003; Ankley et al., 2004), our article and others suggest that there are regions where *Ribeiroia* plays no role and for which the evaluation of other hypotheses must be considered (Meteyer et al., 2000; Johnson et al., 2001a, 2003; Lannoo et al., 2003; Eaton et al., 2004; this study). While it will be difficult to repeat extensive observations and coupled experiments for the many areas where deformities have been reported, we suggest that, where the pattern of deformities is inconsistent with that observed in *Ribeiroia* infection experiments, *Ribeiroia* may be an unlikely cause of deformities. As far back as 2000, Meteyer et al. concluded that it is clear that "multiple limbs are not the distinguishing feature of the recent increase in malformation." While images of multiple-limbed frogs have been widely published, sites where such deformities predominate are less common in the literature than sites where reduction and loss of limbs predominate (Fig. 1). Available evidence suggests that descriptions of deformity events as "an emerging helminthiasis" (Johnson and Sutherland, 2003) are most appropriate where the deformity pattern is consistent with a primary role for *Ribeiroia*.

ACKNOWLEDGMENTS

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