PARASITE (*RIBEIROIA ONDATRAE*) INFECTION LINKED TO AMPHIBIAN MALFORMATIONS IN THE WESTERN UNITED STATES

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Abstract. Parasites and pathogens can influence the survivorship, behavior, and very structure of their host species. For example, experimental studies have shown that trematode parasites can cause high frequencies of severe limb malformations in amphibians. In a broad-scale field survey covering parts of California, Oregon, Washington, Idaho, and Montana, we examined relationships between the frequency and types of morphological abnormalities in amphibians and the abundance of trematode parasite infection, pH, concentrations of 61 pesticides, and levels of orthophosphate and total nitrate. We recorded severe malformations at frequencies ranging from 1% to 90% in nine amphibian species from 53 aquatic systems. Infection of larvae by the trematode *Ribeiroia ondatrae* was associated with, and functionally related to, higher frequencies of amphibian limb malformations than found in uninfected populations (≤5%). Parasites were concentrated around the basal tissue of hind limbs in infected anurans, and malformations associated with infection included skin webbings, supernumerary limbs and digits, and missing or malformed hind limbs. In the absence of *Ribeiroia*, amphibian populations exhibited low (0–5%) frequencies of abnormalities involving missing digits or distal portions of a hind limb. Species were affected differentially by the parasite, and *Ambystoma macrodactylum*, *Hyla regilla*, *Rana aurora*, *R. luteiventris*, and *Taricha torosa* typically exhibited the highest frequencies of abnormalities. None of the water-quality variables measured was associated with malformed amphibians, but aquatic snail hosts (*Planorbella* spp.) were significant predictors of the presence and abundance of *Ribeiroia* infection. Morphological comparisons of adult specimens of *Ribeiroia* collected from different sites and raised in experimental definitive hosts suggested that all samples represented the same species—*R. ondatrae*. These field results, coupled with experimental research on the effects of *Ribeiroia* on amphibians, demonstrate that *Ribeiroia* infection is an important and widespread cause of amphibian limb malformations in the western United States. The relevance of trematode infection to declines of amphibian populations and the influence of habitat modification on the pathology and life cycle of *Ribeiroia* are emphasized as areas requiring further research.

Key words: abnormality frequency in amphibians; Ambystoma; *amphibian;* Bufo; Hyla; *limb deformity, amphibians; parasite-induced malformations;* Planorbella *snails vs.* Ribeiroia *infection;* Rana; Ribeiroia ondatrae; Taricha; *trematode.*

INTRODUCTION

In every population a small percentage of individuals with morphological anomalies resulting from genetic mutation, trauma, or developmental disturbances can

be expected. In amphibian populations these "background" abnormalities typically occur at frequencies of 0 to 5% and involve missing digits or portions of a limb (e.g., Martof 1956, Dubois 1979, Meyer-Rochow and Asashima 1988, Read and Tyler 1990, 1994, Tyler 1998). Severe malformations such as supernumerary limbs are less common but often attract greater attention, and frogs and toads with five or more limbs have sparked scientific curiosity for centuries (e.g., Vallisneri 1706, Geoffrey St. Hilaire 1836, Taruffi 1886,

Woitkewitsch 1959, Van Valen 1974). Recently, malformed individuals of >12 species of amphibians have been reported widely in North America (Sessions and Ruth 1990, Ouellet et al. 1997, Meteyer et al. 2000, Johnson et al. 2001*b*). The malformations typically involve the hind limbs, including missing limbs and parts of limbs, extra limbs, and skin webbings, and have been recorded at frequencies of ≥15% (Ouellet et al. 1997, Johnson et al. 2001*b*).

The causes and implications of these malformations remain poorly understood, but several researchers have suggested that such abnormalities may be indicative of an emerging threat to amphibian populations and possibly other wildlife (Ouellet et al. 1997, Gardiner and Hoppe 1999, Burkhart et al. 2000). The declining or threatened status of many North American amphibian populations further emphasizes the importance of determining the factors responsible, as malformed amphibians may suffer reduced survivorship (Johnson et al. 2001*b*). Potential causes under investigation include biotic agents such as parasite infection (Sessions and Ruth 1990, Johnson et al. 1999, 2001*a*, Sessions et al. 1999, Johnson and Lunde, *in press*) and predation (Bohl 1997), and abiotic agents such as UV-B radiation (Ankley et al. 1998, 2000), pesticide contamination (Ouellet et al. 1997, Burkhart et al. 1998, Fort et al. 1999 [but see also Tietge et al. 2000], Bridges 2000), and retinoid exposure (Gardiner and Hoppe 1999, Degitz et al. 2000). While all of these factors can cause malformations under laboratory conditions, efforts to connect them causally on a large scale with malformed amphibians in the wild have remained unsuccessful.

In the western United States, high frequencies of malformations in several amphibian populations have been linked to trematode infection (Sessions and Ruth 1990, Johnson et al. 1999, Sessions et al. 1999). For several species at sites in northern California we have consistently recorded severe limb malformations in >20% of larval and emerging amphibians (Johnson et al. 2001*b*). Although we found no evidence of heavy metals, pesticides, or polychlorinated biphenyls (PCBs) in the surface waters, each pond with malformed amphibians supported the cathamaesiid trematode, *Ribeiroia ondatrae* (hereafter ''*Ribeiroia*''). Larvae (cercariae) of this parasite infect amphibians around the base of the hind limbs where they form subcutaneous cysts (metacercariae). Experimental exposures of larval Pacific treefrogs (*Hyla regilla*) and western toads (*Bufo boreas*) to cercariae of *Ribeiroia* resulted in high frequencies (40–100%) of severe limb malformations similar to those observed in the field, including extra limbs, missing limbs, skin webbings, and bony triangles (Johnson et al. 1999, 2001*a*). Increased exposures to *Ribeiroia* caused higher frequencies of malformation and mortality, whereas larvae exposed to a second trematode species or to no parasites at all exhibited normal limb development and high survivorship (Johnson et al. 1999, 2001*a*).

Ribeiroia has an indirect or multi-host life cycle in which transmission between the second intermediate (amphibian or fish) and final (bird or mammal) hosts depends on predation. After an infected amphibian is consumed by the final host, the trematode sexually matures and releases eggs via the host's feces. The first intermediate hosts in the life cycle, aquatic snails in the genera *Planorbella* and *Biomphalaria*, become infected when the eggs hatch into miracidia and invade a snail's tissues. Inside the snail, miracidia develop into rediae, reproduce asexually, and produce large numbers of mobile cercariae. *Ribeiroia* cercariae preferentially infect and penetrate the tissue surrounding the limbs of amphibian larvae, often causing improper formation of the developing limb bud (Johnson et al. 1999). Indeed, the resulting malformations are suspected to enhance transmission of the trematode by limiting the ability of infected amphibians to escape predation by the final host (Sessions and Ruth 1990, Johnson et al. 2001*a*, Johnson and Lunde *in press*). A growing number of studies have shown that certain parasites with complex life cycles can alter the behavior, appearance, or size of intermediate hosts, thereby increasing the chance that hosts are consumed and the parasite's life cycle completed (Bethel and Holmes 1977, Lafferty and Morris 1996, Poulin 1998, Zimmer 2000).

Although *Ribeiroia* has been implicated in the malformations of a few amphibian populations in California, the parasite's role in malformations at other sites and in other species has not been explored. Amphibians with malformations similar to those induced by *Ribeiroia* have been reported in many parts of North America, but no large-scale field surveys have examined the association between trematode infection and malformations in wild populations. If the frequency of parasite-induced malformations in amphibians has increased, the triggers for this change remain unknown, but human modifications of the environment have been linked to outbreaks of other amphibian pathogens (Kiesecker and Blaustein 1995, Carey 2000). It may be that, as a result of interactions with altered environmental conditions, the density, geographic range, or pathogenesis of *Ribeiroia* has recently changed. Factors such as eutrophication, loss or replacement of natural wetlands with artificial impoundments (e.g., dams, pump chances, and farm ponds), and introductions of nonindigenous species have all been associated with increases in trematode-related diseases (Johnson and Lunde, *in press*).

We investigated the role of *Ribeiroia* infection in population-level malformations in amphibian species in the western United States. Although attention has focused on the midwestern United States and southern Canada, large numbers of malformed amphibians have been reported in the West since the early 1990s. Our study encompassed surveys of more than 12,000 amphibians representing 11 species from 101 sites in the western United States, describing the abnormalities of

>1500 amphibians. At each site we collected descriptive data on the frequency and composition of abnormalities in each species and life-history stage. Sites with frequencies of abnormalities exceeding the established baseline range (0–5%) were compared to those with frequencies <5% to evaluate possible causative factors. Specifically, we examined relationships between the frequency and severity of abnormalities in a species and the abundance of *Ribeiroia* infection, concentrations of pesticides and pesticide metabolites (herbicides and organophosphate insecticides), and the levels of nitrate, orthophosphate, and pH in the corresponding aquatic systems. Finally, we gathered information on the ecology and life history of *Ribeiroia*, including host species (aquatic snails, fishes, and amphibians), geographic distribution, and habitat or species associations.

Methods

Selection of study sites

We obtained information on sites with malformed amphibians in the western United States from three sources: (1) reports submitted to the North American Reporting Center for Amphibian Malformations (Northern Prairie Wildlife Research Center 2000), (2) interviews with herpetologists and field biologists specializing in the region (listed in acknowledgments), and (3) published accounts in the literature. We then identified the subset of reports that documented more than one abnormal amphibian and provided reliable information on the aquatic system from which the affected specimens originated (i.e., the breeding site could be identified and located). Whenever possible, we interviewed the individual(s) who initially reported the site and obtained specific coordinates and identifying features. For each of these ''primary'' sites we also visited one to two haphazardly selected reference sites within a 10-km radius, chosen on the basis of their proximity to a primary site and availability of suitable amphibian habitat. While not selected at random, reference locations provided data on systems with no known reports of malformed amphibians, which could then be compared to sites with malformations to identify potential causative factor(s).

Field surveys

Between June and October 1999 we surveyed aquatic systems in California, Oregon, Washington, Idaho, and Montana (USA). At each site, we recorded data on the temporal status of the pond (permanent or seasonal), wetland origin (natural or constructed impoundment), presence of cattle, presence of fishes, presence of aquatic snails, and surrounding land use. Field data were supplemented with 7.5- and 15-min topographic maps from the U.S. Geological Survey (USGS) and interviews of local landowners. For each pond we also measured the pH, surface area, elevation, levels of nitrate and orthophosphate, and concentrations of 52 pesticides and nine of their metabolites (see *Chemical and pesticide evaluation*, below).

To determine the frequency of abnormalities, we conducted species-specific, time-constrained searches for metamorphosing amphibians along transects at each site. Transects followed the shoreline in a band from 1 m offshore to 2 m inland. Bullfrogs (*Rana catesbeiana*) were captured and examined at night. At the conclusion of each search, we inspected and scored each amphibian for morphological abnormalities. All abnormal specimens and a sample of normal individuals were collected for further examination while the remaining amphibians were released within the transect area. When larval amphibians were observed, net sweeps were performed at 12 evenly distributed points around the shoreline. At each sampling station, a D-frame dipnet (1.4-mm mesh size, 2600-cm^2 opening) was pulled rapidly through the water for 1.5 m in a line perpendicular to the shore (adapted from Heyer et al. 1994). We recorded the identity and relative abundance of amphibian larvae, fishes, and macroinvertebrates (e.g., aquatic snails, predatory insects, and leeches). Amphibian larvae collected in net sweeps also were examined for abnormalities. Following standardized net sweeps, we sampled each wetland exhaustively with dipnets to document amphibian species not recorded during the net sweeps and collect aquatic snails for taxonomic identification and parasite evaluation.

Amphibians were euthanized in MS-222 (tricane methanosulfonate), fixed in 10% phosphate-buffered formalin, and preserved in 70% ethanol. With the aid of a stereo-dissecting microscope, we measured specimens and described their abnormalities using the classification system and terminology presented in Johnson et al. (2001*b*).

Parasite evaluation

Aquatic snails.—Twenty-five to 50 mature snails of each species at a site were dissected and examined for larval trematode infection (i.e., presence of trematode rediae or sporocysts). For taxonomic purposes, a similar number of snails were first relaxed in a solution of water and menthol crystals for 12 h before being fixed in formalin. Upon finding an infected snail, we isolated living cercariae and identified them using a compound microscope. Representative specimens were fixed in Berland's fluid (19 parts glacial acetic acid, one part formalin) for 60 sec and then transferred to 70% ethanol.

Amphibians.—Within 6 h of euthanization, three to five metamorphs and larvae of each amphibian species at a site were dissected and examined for larval trematodes (meta- and mesocercariae). Living metacercariae were counted, carefully removed, and mechanically excysted. Cercariae and metacercariae of *Ribeiroia* were identified by characteristic esophageal diverticula

and lesser features described in Beaver (1939) and Basch and Sturrock (1969).

Surrogate hosts.—To confirm the taxonomic identity of the *Ribeiroia* samples and compare them among sites, we raised metacercariae to adult forms using surrogate definitive hosts. Frogs infected with *Ribeiroia* were shipped to the University of Wisconsin–La Crosse from sites in California, Oregon, Washington, and Montana. Frogs were pithed and their skin removed to expose metacercariae. Between 25 and 50 *Ribeiroia* metacercariae were suspended in tap water and gavaged into the proventriculus of avian surrogate hosts and the stomach of mammalian surrogate hosts. Avian hosts included feral rock doves (*Columba livia*), mallard ducklings (*Anas platyrhynchos*), and chicks (*Gallus domesticus*) obtained from a commercial hatchery. Mammalian hosts were Sprague-Dawley laboratory rats (*Rattus norvegicus*).

At seven days post infection, surrogate hosts were euthanized by an overdose of carbon dioxide followed by cervical dislocation. Specimens of *Ribeiroia* obtained from surrogate hosts were fixed in hot 10% neutral buffered formalin, stained with Semichon's acetocarmine, and mounted in a synthetic resinous medium. For taxonomic verification, we measured the lengths and widths of the body, oral sucker, ventral sucker, ovary, anterior testis, posterior testis, seminal vesicle, and in utero eggs.

Chemical and pesticide evaluation

At each site, we filled two 250-mL dark polyethylene bottles and two 125-mL amber glass bottles with pond water filtered through a 0.7-μm glass-fiber filter. Samples were immediately shipped on ice to the USGS Organic Geochemistry Research Group in Lawrence, Kansas, USA. All samples were coded without information as to whether malformations were observed at the corresponding sites. The following analyses were performed:

GC/MS analysis.—Twenty-four herbicides and herbicide metabolites and 30 organophosphates were analyzed by gas chromatography/mass spectrometry (GC/MS) using the method of Zimmerman and Thurman (1999). The following compounds were extracted by solid-phase extraction, eluted with ethyl acetate, and analyzed by GC/MS using selected-ion monitoring: the herbicides acetochlor, alachlor, ametryn, atrazine, cyanazine, demethylfluometuron, DCA (3,4-dichloro phenylaniline), DCPU (3,4-dichloro phenylurea), DCPMU (3,4-dichlorophenyl methylurea), fluometuron, linuron metolachlor, metribuzin, prometon, prometryn, propachlor, propazine, simazine, terbutryn, TFMA (trifluoromethylaniline), TFMPU (trifluoromethylphenylurea); the herbicide metabolites cyanazine amide, deethylatrazine, deisopropylatrazine; and the organophosphate insecticides azinphos ethyl, carbonphenotion, chlorfenvinphos, chlorpyriphos, chlorpyriphos methyl, coumaphos, diazinon, dichlorvos, dicro-

tophos, ethion, ethoprop, fenchlorphos, fenitrothion, fonofos, leptofos, malathion, methidiathion, methyl parathion, mevinphos, monocrotophos, oxydemeton methyl, parathion, stirophos, sulprofos, thionazin, tokuthion, tribufos, tributyl phosphate, trichlornate, and trifluralin. The chromatographic and mass-spectral conditions are described in detail in Zimmerman and Thurman (1999). The method has a detection limit of 0.05 μg/L using a 125-mL filtered water sample and a precision of $\pm$10% at 0.2 μg/L.

LC/MS analysis.—Diuron and six metabolites of acetochlor, alachlor, and metolachlor were detected through liquid chromatography/mass spectrometry (LC/MS) using the method of Hostetler and Thurman (2000). The metabolites included the ethane sulfonic acid and the oxanilic acid of each of the three parent herbicides. Solid-phase extraction removed the metabolites from each 125-mL filtered water sample and was followed by sequential elution, first with ethyl acetate to remove parent compounds and subsequently with methanol to remove the metabolites. The methanol extracts were evaporated and then re-dissolved in the eluent for the LC/MS, which consisted of 0.3% acetic acid, 24% methanol, 35.7% water, and 40% acetonitrile. Selected-ion monitoring was used to detect the compounds by mass spectrometry. The method has a detection limit of 0.05 μg/L using a 125-mL water sample and a precision of $\pm$10% at 0.2 μg/L.

Nitrate analysis.—Nitrite plus nitrate was measured in filtered, 125-mL water samples by reducing nitrate to nitrite with cadmium metal (Fishman 1993). Imidazole was used to buffer the analytical stream, and samples were treated with sulfanilamide to yield a diazo compound, which coupled with N-1 naphthylethylenediamine dihydrochloride to form an azo dye. Absorbance of the dye was measured colorimetrically, resulting in the sum of the nitrite originally present plus that formed by reduction of nitrate. The method has a detection limit of 0.01 mg/L and a precision of $\pm$10% at the detection limit and $\pm$5% at higher concentrations.

Orthophosphate analysis.—Free orthophosphate, polyphosphates, and a few organic phosphorus compounds were measured after conversion to orthophosphate through acid hydrolysis (Fishman and Friedman 1989). Hydrolysis of the samples with sulfuric acid was followed by reaction of the orthophosphate with ammonium molybdate in acidic solution, which, upon reduction with ascorbic acid, produced an intensely colored blue complex that was measured photometrically. The method has a detection limit of 0.01 mg/L with a precision of $\pm$10% (Fishman and Friedman 1989).

Statistical analysis

Frequency of abnormalities.—For each site and amphibian species, we calculated the frequency of morphological abnormalities as the percentage of abnormal individuals relative to the total number examined. We determined the frequency of abnormalities only for

those species in which ≥25 animals (larvae and metamorphs) were inspected. Observed frequencies of abnormalities were tested against the hypothesized baseline frequency of 5% (goodness-of-fit test, *G* statistic) as a measure of affected sites.

Causal relationships.—Relationships between the frequency of abnormalities and the recorded habitat variables were examined using either nonparametric tests or multiple-regression analysis. We used nonparametric comparisons because we expected substantial variation in the frequency of abnormalities among sites and sought to examine the distribution of rank scores rather than deviations from mean values. Frequency of abnormalities was compared between primary and reference sites, sites with and without pesticide contamination, and sites with and without *Ribeiroia*. Continuous variables hypothesized to cause abnormalities were regressed against the frequency of abnormalities using a least-squares regression model. All variables were first compared for colinearity, transformed logarithmically with the addition of a constant to remove zero values, and their distributions tested for normality. Following each regression, the residual errors were compared against a normal distribution.

We performed a second set of tests to determine which variables were associated with the presence and abundance of *Ribeiroia* infection. Using *G* tests of independence (Sokal and Rohlf 1995), we tested for associations between the presence of *Ribeiroia* and aquatic snail species (detection/non-detection), pesticides (detection/non-detection), cattle (detection/non-detection), temporal status (permanent/temporary), and human modification (natural/constructed impoundment). Abundance of *Ribeiroia* infection was regressed against the levels of nitrate, orthophosphate, individual pesticides, and abundance of snails using a least-squares multiple-regression model.

Abnormality composition.—Abnormalities were described and categorized by species, life-history stage, and site. As each amphibian may suffer from more than one abnormality (e.g., extra right leg and missing left eye), the total number of abnormalities at a site frequently exceeded the total number of abnormal individuals. Patterns of abnormality composition were therefore assembled at the level of individual abnormalities, not individual amphibians (Johnson et al. 2001*b*). The patterns of abnormality composition were compared using a percentage similarity index (PS$_{ij}$ adapted from Eq. 6.5 in Jongman et al. 1995):

$$PS_{ij} = 200 \times \left(\frac{\sum_k \min(y_{ki}, y_{kj})}{\sum_k y_{ki} + \sum_k y_{kj}} \right)$$

where y_{ki} is the number of *k* abnormalities at site or species *i* and y_{kj} is the number of *k* abnormalities at site or species *j*. The index takes the lesser of these two values and divides it by the total number of *k*

abnormalities observed at both sites or in both species. This process is repeated for each abnormality type, and the grand sum multiplied by 200 represents the percentage similarity between sites or species *i* and *j*. We compared patterns of abnormality composition between each species, between sites with and without *Ribeiroia*, and between sites with and without pesticides.

Severity of abnormalities.—The severity of abnormalities, or the mean number of abnormalities per abnormal amphibian, was determined for each species at each site. Severity of abnormalities is distinct from frequency of abnormalities because the latter measures the proportion of abnormal amphibians relative to the total number of individuals inspected, whereas the former examines only the amphibians with abnormalities, scoring the mean number of morphological deviations per individual. Severity of abnormalities was compared between sites with and without either *Ribeiroia* or pesticides and among individual amphibian species. We further examined the relationships between severity of abnormalities and frequency of abnormalities, and abundance of *Ribeiroia* infection using Spearman's rho correlational coefficient.

RESULTS

We surveyed 101 sites across California, Oregon, Washington, Idaho, and Montana (USA) for abnormal amphibians (Fig. 1). We visited 20 sites in seven counties in California, 60 sites in eight counties in Oregon, 11 sites in seven counties in Washington, 6 sites in two counties in Idaho, and 4 sites in three counties in Montana. Thirty-four sites were classified as "primary" and 42 sites were designated as "reference" sites. An additional 25 sites that could not be placed in either category also were surveyed.

We inspected a total of 12 369 amphibians representing 11 species (Table 1) for morphological abnormalities. Among the 64 accounts with sufficient sample sizes (*n* > 25), the frequency of abnormalities varied substantially among sites and species, ranging from 0 to nearly 90%. Primary sites exhibited a higher distribution in the frequency of abnormalities than did reference sites (Wilcoxon two-sample test, $\chi^2 = 7.197$, df = 1, $P = 0.0073$, $n = 49$). Twenty-two sites supported a frequency of abnormalities significantly greater than the expected baseline frequency (5%) in ≥1 species (*G* test, df = 1, $P < 0.001$, $n = 59$). Four of these sites had higher-than-baseline frequencies of malformations in two amphibian species, and two wetlands had frequencies of malformation significantly >5% in three species. When they co-occurred at a site, Pacific treefrogs exhibited a greater frequency of abnormalities than western toads (Wilcoxon signed-rank test, $P = 0.039$, $n = 8$ sites), a marginally higher frequency than bullfrogs (Wilcoxon signed-rank test, $P = 0.062$, $n = 4$ sites), and a lower frequency than California newts (Wilcoxon signed-rank test, $P = 0.028$, $n = 4$ sites).

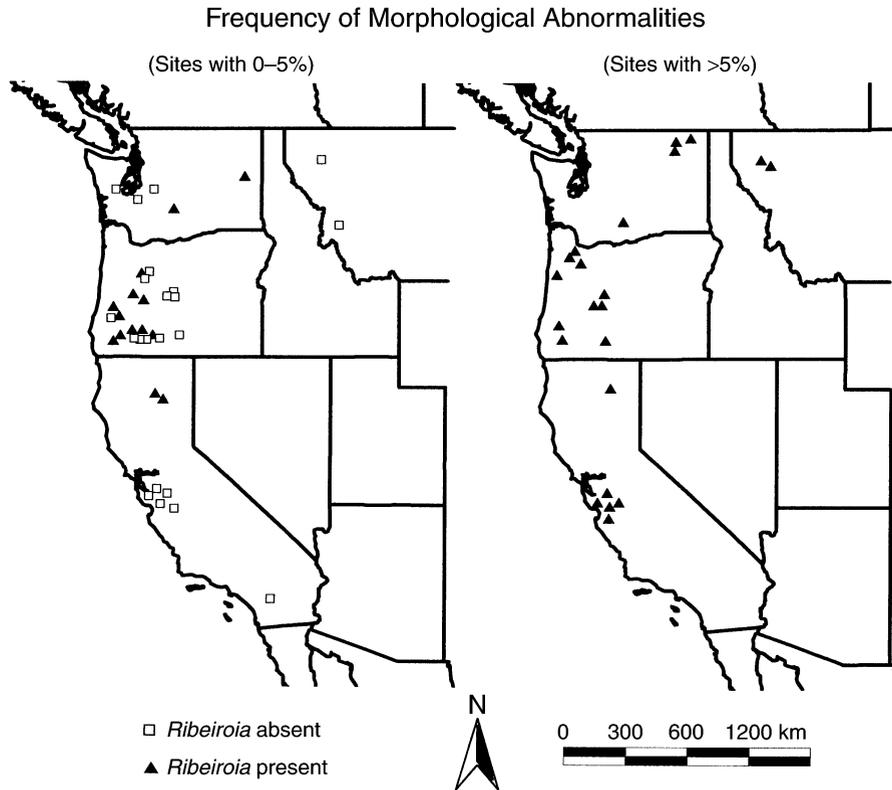## Frequency of Morphological Abnormalities

FIG. 1. Distribution of aquatic sites surveyed for amphibian abnormalities in the western United States. Sites are differentiated by the frequency of abnormalities (significantly greater than 5%; *G* test, df = 2, *P* < 0.05) and by *Ribeiroia* infection (present or absent). Omitted are sites in which <25 amphibians of a species were sampled, including all sites in Idaho.

Treefrog larvae exhibited consistently higher frequencies of malformations than metamorphic treefrogs from the same site (paired *t* test, *P* = 0.048, *n* = 14 sites).

Limb anomalies, including missing limbs and digits (ectromelia and ectrodactyly), skin webbings (cutaneous fusion), and extra limbs and digits (polymelia and polydactyly), were the most frequently encountered abnormalities. Although we also observed amphibians with missing eyes, albinism, jaw malformations, edemas, and skin tumors, these abnormalities accounted for <4% of the total abnormalities. The habitats in which we recorded malformed amphibians encompassed a broad range of aquatic systems, including stock ponds, montane lakes, irrigation canals, impoundments, mitigation ponds, reservoirs, and ephemeral pools.

Determinants of the presence and frequency of malformations

The presence of *Ribeiroia* at a site was associated with above-baseline frequencies (>5%) of abnormalities among the amphibian species we examined (*G* test, χ^2 = 23.302, df = 1, *P* < 0.0001, *n* = 59; Fig. 1). *Ribeiroia* was isolated in amphibians from all of the 22 sites that had a frequency of abnormalities significantly >5% in one or more species (Fig. 2). Corre-

spondingly, sites supporting *Ribeiroia* exhibited higher frequencies of abnormalities than sites without the parasite (Wilcoxon two-sample test, χ^2 = 29.9554, df = 1, *P* < 0.0001, *n* = 59). The frequency of abnormalities at sites with *Ribeiroia* (16.7 ± 2.5% [mean ± 1SE]) was 3 times greater than the maximum baseline level and 6 times greater than the mean frequency at sites without *Ribeiroia* (2.5 ± 2.5%).

The mean number of *Ribeiroia* metacercariae per amphibian (infection abundance [Margolis et al. 1982]) also was a significant predictor of the frequency of abnormalities. All 11 amphibian species surveyed were infected with *Ribeiroia* at one or more sites (Table 1). Pacific treefrogs (*Hyla regilla*) were the most prevalent amphibian species and were present at 83 sites, including 50 of the 54 sites at which malformations were observed in any species. Thus, for analyses comparing abundance of *Ribeiroia* infection to frequency of abnormalities, we used only the data on *H. regilla* to provide a consistent measure of *Ribeiroia* abundance among sites, eliminating differences in abundance of infection among amphibian species. Using a least-squares multiple-regression model, we examined the influence of pH, pond surface area, elevation, orthophosphate levels, and *Ribeiroia* abundance on the frequency of malformations at a site. Path analysis was

TABLE 1. Amphibian species surveyed in our study, together with the range and values of the frequency of abnormalities and the abundance of *Ribeiroia* infection for each species.

Species	No. examined	No. sites	No. Ribeiroia sites	Frequency of abnormalities (%)		Ribeiroia abundance (no. metacercariae/ amphibian)	
				Range	Mean ± 1 SE	Range	Mean ± 1 SE
Long-toed salamander *Ambystoma macrodactylum*†	906	31	17	7.9–24	15.4 ± 4.7	2–108	20.8 ± 10.6
Rough-skinned newt *Taricha granulosa*†	195	12	7	4.2–7.4	5.7 ± 0.9	1–41	12.7 ± 7.5
California newt *Taricha torosa*	289	6	4	5.3–32	16.2 ± 6.2	0.5–1	0.8 ± 0.3
Western toad *Bufo boreas*	1859	15	7	0–12.5	4.5 ± 1.4	1–40	10.2 ± 6.1
Great Basin spadefoot *Scaphiopus intermontanus*†	1	1	1	n.a.	n.a.	25	n.a.
Pacific treefrog *Hyla regilla*	7441	83	46	0–88.5	14.8–3.1	1–236	19.0 ± 4.0
Northern red-legged frog *Rana aurora*†	199	11	5	2.5–25.9	10.8 ± 7.6	1–78	22.7 ± 14.5
Cascades frog *Rana cascadae*†	455	4	1	3.3–5.9	4.6 ± 1.3	1–34	17.5 ± 16.5
American bullfrog *Rana catesbeiana*	777	21	16	1.0–6.5	3.4 ± 0.8	1–73	18.1 ± 4.5
Columbia spotted frog *Rana luteiventris*†	184	8	4	21.1–31.4	26.3 ± 3.0	82–114	95 ± 9.7
Oregon spotted frog *Rana pretiosa*†	63	5	1	n.a.	n.a.	88	n.a.

Notes: For each species, the frequency of abnormalities was derived from all sites in which ≥25 specimens were examined. If 25 specimens of a species were not inspected at any site, ''n.a.'' is listed to indicate ''not applicable.'' Abundance of infection was compiled from all sites supporting amphibians infected with *Ribeiroia*.

† New host species record for *Ribeiroia*.

not used because none of the independent variables exhibited colinearity (Sokal and Rohlf 1995). With the exception of pH, all variables were transformed to a normalized distribution. The model explained 58.9% of the variation, and the residual errors were normally distributed. However, *Ribeiroia* abundance was the only significant predictor variable in the test of effects, and a regression between *Ribeiroia* abundance and frequency of abnormalities revealed a positive relationship with an R^2 value of 0.525 (Fig. 3). Thus, the greater the mean infection abundance of *Ribeiroia* at a site, the higher the frequency of abnormalities in amphibians.

We found no relationship between the pesticide or metabolite compounds and either the presence or frequency of malformed amphibians. Of the 77 sites tested, only 3 sites had measurable levels of any pesticide compound. The first was an orchard pond in Jackson

FIG. 2. Frequency distribution of abnormality frequency accounts in different amphibians species from sites with and without *Ribeiroia* infection. The dashed vertical line represents expected baseline frequency (5%) in ''normal'' amphibian populations.
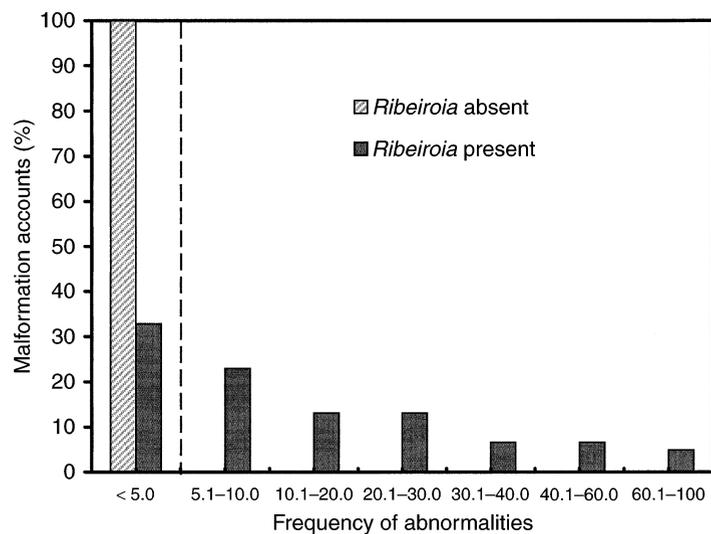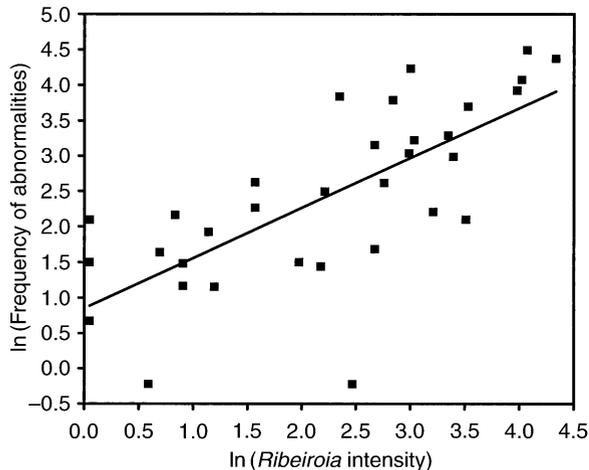
FIG. 3. Relationship between abundance of *Ribeiroia* infection (mean number of metacercariae per amphibian) and frequency of abnormalities (%) in Pacific treefrogs (*Hyla regilla*) at the corresponding site ($y = 0.7049x + 0.8528$, $R^2 = 0.525$, root mean square error $= 0.8462$, $P < 0.0001$, $n = 33$).

County, Oregon, which yielded a simazine concentration of 0.18 µg/L, a deisopropylatrazine concentration of 0.11 µg/L, and a diuron concentration of 0.12 µg/L. One treefrog at this site ($n = 38$) was missing a digit on the hind limb. In Montana, water samples collected from the Bitterroot River in Ravalli County exhibited an alachlor level of 0.53 µg/L. No abnormal amphibians ($n = 25$) were recorded from this site. The third site, a stock pond in Yakima County, Washington, with a diuron concentration of 0.21 µg/L, also supported *Ribeiroia*. However, the frequency of malformations at this site did not significantly exceed the baseline level of 5% (*G* test, $\chi^2 = 1.72$, df $= 1$, $P > 0.05$, $n = 68$), and those malformations that were observed were similar to those recorded from the other 37 sites with treefrogs and *Ribeiroia* but without detectable pesticides.

Abnormality composition

The most common malformations in all species involved the limbs (96%, $n = 2248$ abnormalities). Among anurans, >94% of the abnormalities affected the hind limbs. In urodeles, 41.9% of the abnormalities involved the forelimbs and 56.9% involved the hind limbs. The specific types of limb abnormalities and their relative frequencies differed substantially among species, however.

The greatest number of abnormalities was observed in *H. regilla*. More than 1000 abnormal larvae and metamorphs from 55 sites were described and categorized. Extra limbs and parts of limbs, skin webbings, and missing limbs and digits were the predominant (89.3%) abnormalities observed at the 46 sites supporting *Ribeiroia* ($n = 1749$ abnormalities; Fig. 4; Table 2). Several frogs lacking both hind limbs were col-

lected, while others with as many as five supernumerary limbs or severe skin webbings restricting both hind limbs also were observed. Forelimb abnormalities accounted for <0.5% of the total (Table 2). The pattern of abnormality composition in *H. regilla* was consistent among sites with *Ribeiroia*, and between early life-history stages (Table 2). The abnormalities of treefrog larvae and metamorphs shared an 89% similarity (PS_{ij}); polymelia, cutaneous fusion, and femoral projections accounted for 60–90% of the abnormalities observed in both stages from sites in California, Oregon, Washington, and Montana (Table 2). Furthermore, the combined abnormalities (larval and metamorphic) observed at field sites with *Ribeiroia* agreed closely ($PS_{ij} = 74.3\%$) with those produced in experimental infections using cercariae of *Ribeiroia* and larval treefrogs (Johnson et al. 1999). At the 37 locations supporting *H. regilla* but free of *Ribeiroia*, treefrog abnormalities consisted almost entirely of missing limbs and digits (76.4%, $n = 72$ abnormalities). The pattern of abnormality composition at these sites shared a PS_{ij} of <25% with either the combined abnormalities from all *Ribeiroia* sites or the experimental malformations (Table 3).

We inspected 1859 larval and metamorphic *Bufo boreas* from 15 sites. In contrast to *H. regilla*, the abnormalities of *B. boreas* at sites with *Ribeiroia* were dominated by missing limbs and digits (61.2%, $n = 85$ abnormalities; Table 4; Fig. 4). The composition pattern was similar to the pooled abnormalities of all anurans from sites without *Ribeiroia* (Table 3), but the frequency of these abnormalities at sites with *Ribeiroia* was greater than at sites without the parasite (Wilcoxon two-sample test, $\chi^2 = 4.875$, df $= 1$, $P < 0.027$, $n = 13$). The abnormalities in toads from *Ribeiroia* sites also were moderately similar ($PS_{ij} = 36.0$) to the malformations resulting from laboratory infections of *B. boreas* larvae with *Ribeiroia* cercariae, which consisted almost entirely of skin webbings, extra limbs and digits, and taumelia of the hind limbs (Johnson et al. 2001*a*).

Among the ranid frogs, 69 abnormal *Rana catesbeiana* from 21 sites were observed. Extra and missing fore- and hind limbs accounted for 40.3% of the abnormalities at *Ribeiroia* sites (Table 4; Fig. 4). We documented 42 abnormalities in Columbia spotted frogs (*R. luteiventris*) from two sites in Washington and one in Montana. Each of these sites also supported *Ribeiroia* and high frequencies of severely malformed *H. regilla*. Nearly all of the malformed spotted frogs exhibited cutaneous fusion, often with both hind limbs affected, or polydactyly (Table 4; Fig. 4). The northern red-legged frog (*R. aurora*) had an abnormality composition pattern similar to *R. luteiventris* (Table 3), with 40% of the abnormalities involving cutaneous fusion (Table 4). The Cascades frog (*R. cascadae*), which was observed only at four high-elevation (>1800 m) sites in Oregon, exhibited few abnormalities. Missing and
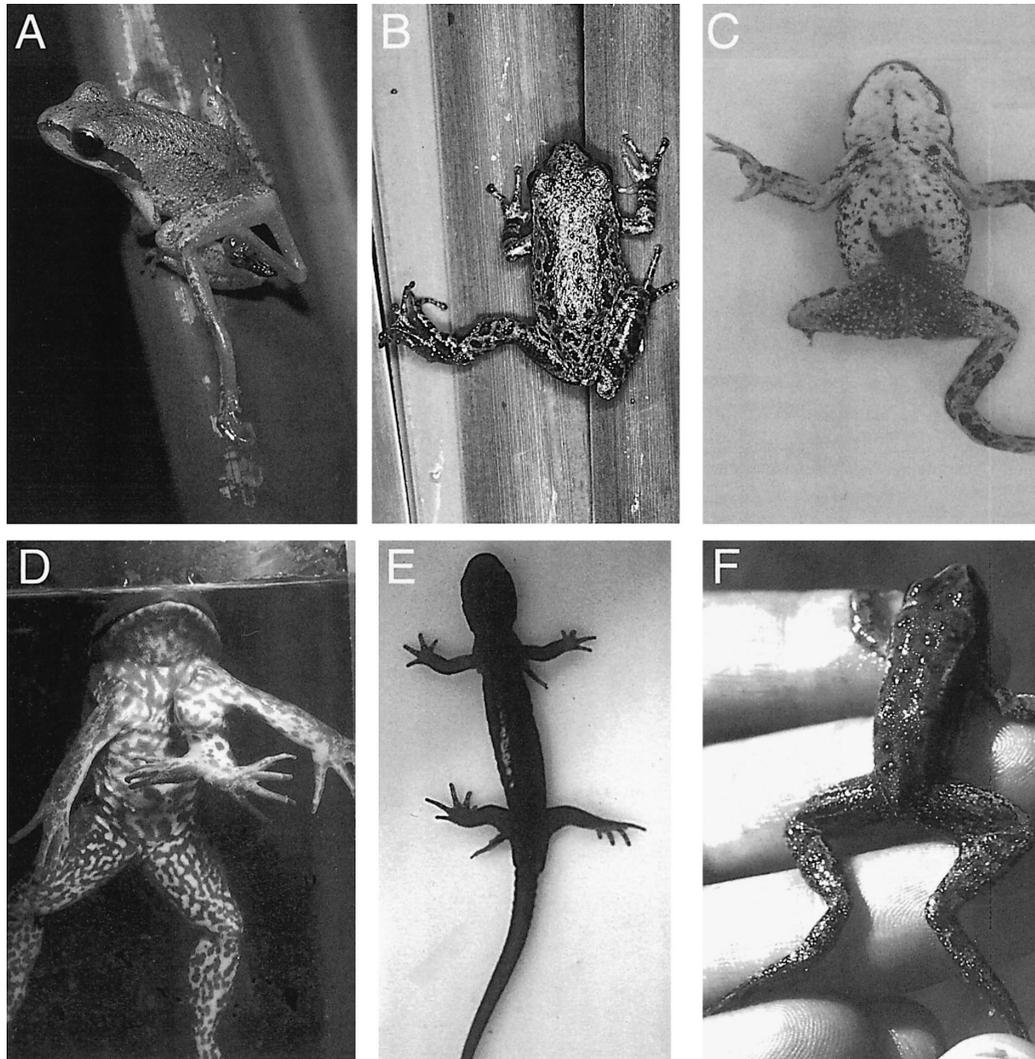
FIG. 4. Representative limb abnormalities observed in amphibians. (A) Pacific treefrog (*Hyla regilla*) with two extra hind limbs (supernumerary pelvis); (B) *H. regilla* with malformed (taumelia) and polydactylous left hind limb; (C) ventral view of western toad (*Bufo boreas*) exhibiting hemimelia in right hind limb; note also abdominal scarring; (D) bullfrog (*Rana catesbeiana*) with two extra forelimbs; (E) long-toed salamander (*Ambystoma macrodactylum*) with poorly developed extra projections posterior to right forelimb and left hind limb; and (F) Columbia spotted frog (*R. luteiventris*) exhibiting cutaneous fusion in right hind limb. Panels A, B, and D are printed with permission of the photographer, Steven Holt.

malformed digits were the most common abnormalities at the single *Ribeiroia* site, and the composition was similar to the combined anuran abnormalities at localities without *Ribeiroia* (Table 3).

Long-toed salamanders (*Ambystoma macrodactylum*), of which 73 abnormal individuals from 17 *Ribeiroia* sites were examined, typically suffered missing and extra limbs or digits in both the fore- and hind limbs (92.7%, $n = 96$ abnormalities; Table 4). Most of the salamanders with extra limbs (Fig. 4) were collected from a pond in western Montana that has supported extra-legged *H. regilla* since at least the 1950s (Hebard and Brunson 1963, Anderson 1977). *Taricha granulosa* and *T. torosa* exhibited patterns of abnor-

mality composition dominated by missing limbs and digits (85.7% and 94.1%, respectively; Table 4). Both species showed patterns most similar to the combined urodele abnormalities from sites without *Ribeiroia* (Table 3).

Severity of abnormalities.—The majority (92.6%, $n = 2248$) of abnormalities in all species were described from sites supporting *Ribeiroia* infection. The greatest number of abnormalities in a single amphibian was seven ($n = 2$), and both cases involved a treefrog with four extra hind limbs, cutaneous fusion, polydactyly, and a hind limb with twisted longbones. The highest rating of abnormality severity for a site with at least 10 malformed individuals occurred in a treefrog pop-

TABLE 2. Composition of morphological abnormalities in larval and metamorphic Pacific treefrogs (*Hyla regilla*) from sites supporting *Ribeiroia* in four states in the western United States.

	Percentage of abnormalities			
	California		Oregon	
Abnormality type	Larvae	Metamorphs	Larvae	Metamorphs
Cephalic and Axial				
Anophthalmy (missing eye)	5.4 (3)	1.6 (4)	0.6 (2)	1.4 (6)
Mandibular dysplasia (abnormal jaw)	0	1.2 (3)	0.3 (1)	0.7 (3)
Open wound	0	0.4 (1)	0	0.9 (4)
Edema	3.6 (2)	0.4 (1)	1.2 (4)	0
Other	0	0.4 (1)	0.6 (2)	0
Forelimb				
Ectrodactyly (missing digit)	0	0	0	0.5 (2)
Hemimelia (partial limb)	0	0.4 (1)	0	0
Ectromelia (missing limb)	0	0	0	0.5 (2)
Other malformed†	0	0	0	0.2 (1)
Hind limb				
Syndactyly (fused digits)	0	0.4 (1)	0.3 (1)	0.2 (1)
Ectrodactyly (missing digit)	5.4 (3)	5.7 (14)	2.3 (8)	2.3 (10)
Polydactyly (extra digit)	1.8 (1)	1.6 (4)	5.0 (17)	2.5 (11)
Apody (missing foot)	1.8 (1)	3.7 (9)	1.7 (6)	2.3 (10)
Polypody (extra foot)	0	2.4 (6)	0.6 (2)	3.0 (13)
Hemimelia (partial limb)	3.6 (2)	4.5 (11)	0.9 (3)	3.0 (13)
Ectromelia (missing limb)	5.4 (3)	6.5 (16)	2.3 (8)	3.9 (17)
Polymelia (extra limb)	42.9 (24)	33.7 (83)	36.7 (126)	47.7 (208)
Femoral projection	16.1 (9)	20.7 (51)	16.0 (55)	11.0 (48)
Cutaneous fusion (skin webbing)	3.6 (2)	8.5 (21)	21.0 (72)	14.0 (61)
Taumelia (bony triangle)	1.8 (1)	3.7 (9)	2.6 (9)	3.2 (14)
Micromelia (small limb)	3.6 (2)	0.8 (2)	2.9 (10)	0.7 (3)
Limb hyperextension	0	0.8 (2)	0.3 (1)	0
Other malformed†	5.4 (3)	2.4 (6)	4.7 (16)	2.1 (9)
Overall				
Right side	51.9 (27)	49.4 (118)	50.9 (170)	49.1 (209)
Left side	48.1 (25)	50.6 (121)	49.1 (164)	50.9 (217)
Total no. abnormalities	56	246	343	436
Total no. abnormal tree frogs	43	178	228	259
Total no. tree frogs inspected	401	1404	1692	1471
No. abnormalities per abnormal frog (mean ± 1 SE)‡	1.30 ± 0.12	1.38 ± 0.05	1.50 ± 0.06	1.68 ± 0.06

Notes: The table presents the percentages of each type of abnormality, with actual numbers of each abnormality observed in parentheses. The total number of abnormalities may or may not equal the number of abnormal animals, as many specimens had more than one abnormality. For complete definitions of the malformations listed, see Johnson et al. (2001*b*).

† Includes anteversion, brachymelia, and brachydactyly.

‡ The numbers of abnormalities per abnormal larva or metamorph, an index of the severity of abnormalities.

ulation in central Oregon and measured 2.82 ± 0.325 abnormalities per abnormal animal (mean ± 1SE). Sites that supported *Ribeiroia* and malformed amphibians had more severe malformations than sites without the parasite (Wilcoxon two-sample test, df = 1, $P <$ 0.0001, n = 56 sites). *Hyla regilla* exhibited the largest mean value of abnormality severity (Table 4), which was significantly larger than those of *A. macrodactylum*, *T. torosa*, *T. granulosa*, *R. catesbeiana*, *B. boreas*, and *R. aurora* (Kruskal-Wallis, χ^2 = 42.7, df = 8, $P <$ 0.0001; Wilcoxon two-sample tests, df = 2, $P <$ 0.005). The mean values of abnormality severity in *R. luteiventris* and *R. cascadae* were not different in distribution from any of the other species (Wilcoxon two-sample tests, df = 2, $P >$ 0.05; Table 4).

The abundance of *Ribeiroia* infection in *H. regilla* was correlated to the mean severity of the abnormalities at the same site (nonparametric correlation = 0.52, Spearman's rho = 0.7121, $P <$ 0.0001). Abnormality severity also was correlated to the frequency of abnormalities in a species (nonparametric correlation = 0.8803, Spearman's rho = 0.8456, $P <$ 0.0001). Taken together, these correlations suggest that, as *Ribeiroia* abundance increases, both the probability that a given amphibian will be abnormal and the numbers of abnormalities per abnormal amphibian likewise increase.

Determinants of Ribeiroia *presence and abundance*

We recorded *Ribeiroia* in amphibians from 59 sites in 21 counties and five states in the western United States. The habitats supporting *Ribeiroia* were diverse, ranging from drying pools to large lakes, from 10 m to >1800 m above sea level, and from isolated, natural systems to agricultural-runoff ponds. Of all the biotic and abiotic habitat factors measured, the presence and abundance of snails in the genus *Planorbella* (Planor-

TABLE 2. Extended.

| | Percentage of abnormalities | | |
| | Washington | | Montana |
Larvae	Metamorphs	Larvae	Metamorphs
0	0	0	0
0	0.9 (3)	0	0
0	0	0	0
0	0.3 (1)	0	0
0	0	0	0
0	0	0	0
0	0	0	0
3.1 (2)	0	0	0
0	0	0	0
0	0	0	0.5 (1)
1.6 (1)	1.5 (5)	2.5 (2)	1.6 (3)
3.1 (2)	3.0 (10)	2.5 (2)	0.5 (1)
1.6 (1)	0.3 (1)	1.2 (1)	0.5 (1)
3.1 (2)	2.1 (7)	0	0
1.6 (1)	1.2 (4)	0	0
9.4 (6)	3.3 (11)	0	0
15.6 (10)	34.3 (114)	40.7 (33)	49.2 (95)
21.9 (14)	14.8 (49)	14.8 (12)	20.2 (39)
28.1 (18)	27.7 (92)	29.6 (24)	23.8 (46)
7.8 (5)	5.4 (18)	3.7 (3)	1.0 (2)
1.6 (1)	1.2 (4)	0	1.0 (2)
0	0.6 (2)	0	0
1.6 (1)	3.3 (11)	4.9 (4)	1.6 (3)
45.3 (29)	43.3 (141)	44.4 (36)	48.2 (92)
54.7 (35)	56.7 (185)	55.6 (45)	51.8 (99)
64	332	81	193
44	161	37	92
134	493	109	243
1.46 ± 0.12	2.06 ± 0.10	2.19 ± 0.19	2.10 ± 0.13

bidae) were the only two variables related to the presence or abundance of *Ribeiroia* infection (Bonferroni corrections, $P < 0.05$). The presence of *Ribeiroia* metacercariae in amphibians at a site was significantly associated with the occurrence of a necessary snail host (G test, $\chi^2 = 43.686$, df = 1, $P < 0.0001$, $n = 83$ sites). Fifty-one of the 52 sites with *Ribeiroia* that were surveyed for snails supported one or more species in the genus *Planorbella*. Correspondingly, at the subset of sites at which we performed net sweep surveys, *Planorbella* snail abundance was the only significant predictor of *Ribeiroia* abundance ($y = -0.0389 + 0.46x$, $R^2 = 0.246$, $F = 6.52$, $P = 0.019$, $n = 22$ sites).

Species of *Planorbella* were associated with wetlands of anthropogenic origin (G test, $\chi^2 = 4.53$, df = 1, $P = 0.033$, $n = 83$ sites), and higher orthophosphate levels (logistic regression, $\chi^2 = 6.0266$, df = 1, $P = 0.014$, $n = 63$ sites). Two of the four species of *Planorbella* were collected at sites beyond their previously known ranges. *Planorbella trivolvis trivolvis*, a common species in the eastern and midwestern United States, was identified at two sites in California and two

sites in Oregon, all of which were artificial habitats and three of which supported *Ribeiroia*. *Planorbella tenuis*, known historically from Texas, Arizona, New Mexico, and in California southward from Santa Clara County (Burch 1989), was found at 12 sites in Oregon, as far northward as Portland (latitude, 45°31′4.22″ N). Eleven of these wetlands were small impoundments and nine of them hosted *Ribeiroia* infection in their amphibian inhabitants.

Ribeiroia *host records and taxonomy*

Metacercariae.—*Ribeiroia* metacercariae were documented in 11 species of amphibians, of which 7 species represent new host records (Table 1). Within infected amphibians, metacercariae were found just beneath the skin, attached to connective tissue or superficially in the musculature. The abundance of infection varied by species and by site, from as few as one metacercaria to more than 200 (Table 1). Within anurans, metacercariae were clustered primarily around the base of the hind limbs and the site of tail resorption, with a secondary cluster around the lower mandible. Within

TABLE 3. Matrix of similarity values (PS$_{ij}$) between the abnormality composition patterns recorded for each amphibian species at sites supporting the trematode *Ribeiroia*.

Species†	HYRE	BUBO	RAAU	RACAS	RACAT	RALU	AMMA	TAGR	TATO	AWR	UWR
HYRE	100	33.1	43.2	7.9	33.1	43.2	30.4	20.9	10.0	21.0	8.0
BUBO		100	25.3	27.3	38.3	20.0	48.8	37.7	47.1	76.4	43.5
RAAU			100	20.0	18.3	47.1	19.4	7.1	8.8	18.6	0
RACAS				100	9.7	4.8	27.1	31.0	33.3	38.0	21.0
RACAT					100	12.7	40.6	14.3	17.7	26.6	17.0
RALU						100	17.9	2.4	4.8	11.4	0
AMMA							100	45.8	53.4	48.5	46.9
TAGR								100	56.7	48.2	81.7
TATO									100	57.5	62.9
AWR										100	51.5
UWR											100

Note: Patterns of abnormality composition were compared using a percentage similarity index (PS$_{ij}$) adapted from Jongman et al. (1995) (see *Methods: Statistical analysis: Abnormality composition*).

† Species key: HYRE = *Hyla regilla*, BUBO = *Bufo boreas*, RAAU = *Rana aurora*, RACAS = *R. cascadae*, RACAT = *R. catesbeiana*, RALU = *R. luteiventris*, AMMA = *Ambystoma macrodactylum*, TAGR = *Taricha granulosa*, and TATO = *T. torosa*. Other abbreviations: AWR = compiled abnormalities of anurans from sites without *Ribeiroia*; UWR = compiled abnormalities of urodeles from sites without *Ribeiroia*.

urodeles, metacercariae were observed at the base of both fore- and hind limbs and among the gills or within the ventral head musculature, with a greater concentration of infection in the head than in the limb region (Wilcoxon signed-rank test, $P = 0.018$). At several sites, metacercariae also were removed from the lateral line canals of fathead minnows (*Pimephales promelas*), although typically at lower abundances of infection than recorded in co-occurring amphibian larvae.

Rediae and cercariae.—*Ribeiroia* rediae and cercariae were found in *Planorbella tenuis*, *P. subcrenata*, and *P. occidentalis*, of which the latter two are new host records. Although frequently infected with other trematodes, none of the other 21 snail species collected hosted *Ribeiroia*, including representatives of 13 genera and five families. Descriptions of the rediae and cercariae of *R. ondatrae* provided by Beaver (1939) and Yamaguti (1975) agreed closely with observations made during our survey.

Adults.—Gravid, adult *Ribeiroia* were obtained from all three experimental avian hosts and the one mammalian host infected with metacercariae. Eighty-five adult worms were examined, measured, and compared morphometrically. The features of all specimens agreed closely with the characteristics and measurements of *R. ondatrae* described in Price (1931), Beaver (1939), and Mettrick (1963) and will be presented elsewhere in greater detail (P. T. J. Johnson, D. R. Sutherland, K. B. Lunde, E. S. Loker, J. M. Kapfer, J. M. Kinsella, and H. Moné, *unpublished manuscript*).

DISCUSSION

Trematode infection and amphibian malformations

Our study supports the hypothesis that trematode infection is a major cause of limb malformations in natural amphibian populations. Among the sites we surveyed, the parasite *Ribeiroia ondatrae* was a powerful predictor of the presence and frequency of malformed amphibians in a population. Within infected anurans, the parasite exhibited a nonrandom distribution, with the majority of metacercariae embedded around the base of the limbs and the tail resorption area. Correspondingly, malformations associated with *Ribeiroia* infection typically involved the limbs, including extra, missing, and malformed fore- and hind limbs, and were recorded in multiple amphibian species across California, Oregon, Washington, and Montana (USA). The mean frequency of abnormalities in amphibians from sites supporting *Ribeiroia* was significantly greater than both the mean frequency in the absence of *Ribeiroia* and the expected baseline frequency. Sites with a frequency of abnormalities significantly above 5% in any species supported the parasite, and the mean abundance of *Ribeiroia* infection was a significant predictor of the frequency of abnormalities within an amphibian population. Simply stated, the greater the amphibian population's infection with *Ribeiroia*, the higher the frequency and severity of limb malformations. This finding agrees with experimental work on the effects of *Ribeiroia* infection on anuran larvae, in which the frequency and severity of malformations depended directly on the level of parasite exposure (Johnson et al. 1999, 2001a). Moreover, the similarity between the malformed amphibians described here and those reported in the Midwest and Canada along with the known occurrence of *Ribeiroia* within these regions (Molnar et al. 1974, Taft et al. 1993, Sutherland 2002), call for study of *Ribeiroia* infection as a cause of amphibian malformations in other parts of North America.

Data on *Hyla regilla* included the largest sample size, the greatest representation from sites with and without *Ribeiroia*, and the most previous field and experimental research on the effects of *Ribeiroia* infection (Johnson et al. 1999, 2001b). At sites supporting *Ribeiroia*, abnormalities in larval and metamorphic *H. regilla* were dominated by extra limbs and digits, skin webbings,

and missing limbs and often occurred at frequencies significantly greater than the baseline range (0–5%). This pattern was highly conserved among 38 sites and across four states, and these same malformations accounted for >80% of the abnormalities produced during experimental infections of larval *H. regilla* with *Ribeiroia* cercariae (Johnson et al. 1999). At sites without *Ribeiroia*, abnormalities were rare and exhibited little similarity to those from sites with the parasite. Taken together, these lines of evidence suggest that *Ribeiroia* infection is both necessary and sufficient to explain higher frequencies of limb malformations in *H. regilla*.

Ribeiroia infection also was implicated in the malformations of *Ambystoma macrodactylum*, *Rana aurora*, *R. luteiventris*, and invasive *R. catesbeiana* in our study. When individuals of these species were infected by *Ribeiroia*, they each exhibited limb malformations similar to those induced in anuran larvae experimentally exposed to *Ribeiroia* (Johnson et al. 1999, 2001*a*). Moreover, malformations in these species occurred at above-baseline frequencies only at sites with *Ribeiroia*. Percentage-similarity (PS_{ij}) comparisons separated the abnormalities of these species when infected with *Ribeiroia* from the compiled anuran and urodele abnormalities at sites without the parasite. Furthermore, at all but one of the sites supporting *Ribeiroia* and high frequencies of abnormalities in these species, we also observed high frequencies of limb malformations in *H. regilla*. Thus, although other agents have not been eliminated as potential causes, infection by *Ribeiroia* remains the most plausible explanation for the malformations we observed in these species.

The role of *Ribeiroia* in causing the morphological abnormalities observed in the remaining four amphibian species is more ambiguous. The abnormalities of *Bufo boreas* from aquatic systems with *Ribeiroia* showed a greater percentage similarity with the compiled abnormalities in anurans from sites without *Ribeiroia* than with the abnormalities of *B. boreas* larvae infected with *Ribeiroia* in the laboratory (Johnson et al. 2001*a*). The frequency of missing limbs and digits in *B. boreas* was greater at sites supporting *Ribeiroia* than at sites without the trematode, however, indicating that *Ribeiroia* increased the occurrence of these abnormalities or that infected toads may be more susceptible to other teratogenic agents such as predators or chemicals. The abnormalities of *Taricha torosa*, *T. granulosa*, and *R. cascadae* exhibited high percentage similarities with the combined abnormalities of urodeles and the combined abnormalities of anurans at sites without *Ribeiroia*. While these abnormalities were generally low in frequency, however, they significantly exceeded 5% only at sites with *Ribeiroia*. Unfortunately, these species were among the least represented, making it difficult to correlate their abnormalities with *Ribeiroia*, alternative environmental factors, or specific life-history patterns.

Additional causes of abnormalities in amphibians

We found no evidence directly or indirectly connecting pesticides or their metabolites to the localities associated with malformed amphibians. Of the 77 sites surveyed, only 3 sites tested positive for any of the 61 herbicide or insecticide compounds examined. The wide variety of compounds tested and low detection limits suggest that agricultural impacts from pesticides at these sites are either very slight or nonexistent. We cannot completely eliminate pesticides as a cause of malformations in some amphibians, however, because of the limited geographic range of our survey, the nonrandom selection of sites, and the finite number of compounds for which we tested. We also found no evidence linking any of the other abiotic habitat variables measured, including acidity, nitrate, orthophosphate, and elevation, to the abnormalities observed in amphibians from sites with or without *Ribeiroia*.

In general, the abnormalities in amphibians collected from aquatic systems without *Ribeiroia* consisted of missing digits, partially missing limbs, and the occasional completely missing limb. These abnormalities usually occurred at low frequencies, and the mean frequency of abnormalities for all sites and species was <3%. Previous studies on background patterns of morphological abnormalities in juvenile amphibians, which collectively examined thousands of specimens representing dozens of species from several continents (Martof 1956, Dubois 1979, Roberts and Verrell 1984, Meyer-Rochow and Asashima 1988, Tyler 1998) suggest a frequency range of 0–5% for missing digits, feet, and parts of limbs for both anurans and urodeles. We therefore suspect that many of the abnormalities from sites without *Ribeiroia* were the result of "normal" levels of trauma, predation, and developmental error.

Host species and ecology of Ribeiroia

Our study also offers new insights into the life cycle and ecology of *R. ondatrae*. Although fishes have traditionally been assumed to be the second intermediate hosts of *Ribeiroia* (Beaver 1939, Simmons 1971, Molnar et al. 1974), we frequently encountered aquatic systems that supported *Ribeiroia* and larval amphibians but were devoid of fish. When amphibians and fish co-occurred, amphibians appeared to exhibit higher infection abundances. Although we found metacercariae in 11 species of amphibians, we observed rediae only in planorbid snails of the genus *Planorbella*. Considering the vagility and abundance of the avian hosts recorded infected by *Ribeiroia*, we suspect that these snails limit the parasite's distribution in aquatic systems.

It has been suggested previously that malformations may increase the likelihood that amphibians infected with *Ribeiroia* are consumed by a suitable definitive host (bird or mammal) (Sessions and Ruth 1990, Johnson et al. 1999), as observed for several other multi-

TABLE 4. Composition of morphological abnormalities in amphibian species in the western United States from the 59 sites supporting the trematode *Ribeiroia*.

Abnormality type	Percentage of abnormalities				
	Bufo boreas	*Hyla regilla*	*Rana aurora*	*Rana catesbeiana*	*Rana luteiventris*
Cephalic and Axial					
Anophthalmy	3.5 (3)	0.9 (15)	0	9.7 (7)	0
Mandibular dysplasia	1.2 (1)	0.6 (10)	10 (1)	2.8 (2)	0
Open wound	1.2 (1)	0.3 (5)	0	0	0
Edema	0	0.5 (8)	0	4.2 (3)	0
Other	1.2 (1)	0.2 (3)	10 (1)	2.8 (2)	2.4 (1)
Forelimb					
Ectrodactyly	1.2 (1)	0.1 (2)	0	0	0
Polydactyly	0	0	0	0	0
Apody	0	0	0	0	0
Hemimelia	0	0.05 (1)	0	0	0
Ectromelia	1.2 (1)	0.2 (4)	0	9.7 (7)	0
Polymelia	0	0	0	6.9 (5)	0
Femoral projection	0	0	0	0	0
Other malformed†	1.2 (1)	0.05 (1)	0	1.4 (1)	0
Hind limb					
Syndactyly	1.2 (1)	0.2 (3)	0	0	0
Ectrodactyly	25.9 (22)	2.7 (47)	0	0	0
Polydactyly	4.7 (4)	2.7 (47)	0	4.2 (3)	9.5 (4)
Apody	8.2 (7)	1.7 (30)	0	0	0
Polypody	0	1.7 (30)	0	0	0
Hemimelia	17.7 (15)	1.9 (33)	10 (1)	0	2.4 (1)
Ectromelia	7.1 (6)	3.3 (57)	0	8.3 (6)	0
Polymelia	3.5 (3)	39.9 (698)	10 (1)	15.3 (11)	0
Femoral projection	1.2 (1)	15.8 (277)	10 (1)	1.4 (1)	2.4 (1)
Cutaneous fusion	5.9 (5)	19.2 (336)	40 (4)	0	78.6 (33)
Taumelia	7.1 (6)	3.6 (62)	0	25.0 (18)	0
Micromelia	2.4 (2)	1.4 (24)	10 (1)	1.4 (1)	0
Limb hyperextension	0	0.3 (5)	0	1.4 (1)	0
Other malformed†	4.7 (4)	2.9 (51)	0	5.6 (4)	4.8 (2)
Overall					
Right side	52.4 (44)	48.0 (821)	66.7 (6)	46.3 (19)	50 (20)
Left side	47.6 (40)	52.0 (891)	33.3 (3)	53.7 (22)	50 (20)
Total no. abnormalities	85	1749	10	72	42
Total no. abnormal animals‡	74	1043	9	58	29
Total no. amphibians inspected	670	5835	114	605	139
No. abnormalities per abnormal animal (mean ± 1se)§	1.15 ± 0.05	1.68 ± 0.03	1.11 ± 0.1	1.24 ± 0.06	1.45 ± 0.11

Notes: The final two columns present the compiled abnormalities of all anurans and urodeles, respectively, from the 42 sites without *Ribeiroia*. For each species, the numbers of each type of abnormality (in parentheses) and its proportion (%) relative to the total number of abnormalities observed are presented.

† Includes anteversion, brachymelia, and brachydactyly.

‡ The total number of abnormalities may or may not equal the number of abnormal animals, as many specimens had more than one abnormality.

§ No. of abnormalities per abnormal larva or metamorph, an index of the severity of abnormalities.

host parasites (e.g., Bethel and Holmes 1977, Lafferty and Morris 1996, Zimmer 2000). Although this hypothesis requires experimental testing, it may help explain the extreme rarity of malformations in adult amphibians and the highly localized distribution of *Ribeiroia* metacercariae around the limbs of amphibians. Interestingly, most malformations and metacercariae in anurans were found around the hind limbs, where they are more likely to be debilitating, whereas in urodeles, which use all four limbs during locomotion, metacercariae and malformations were found in both limb groups at similar frequencies (see also Sessions and Ruth 1990).

Perhaps the most surprising result, however, was the diversity of habitats that supported *Ribeiroia*. *Planorbella* snails and amphibians infected with *Ribeiroia* were documented in a wide array of lentic habitats, including ephemeral ponds, montane lakes, stock ponds, oxbows, drainage canals, and reservoirs. Indeed, we even found malformed anuran larvae infected with *Ribeiroia* in a small, artificial pond less than a year old. The owner and builder of the pond did not stock the system, suggesting a year was sufficient to allow natural colonization by both snail and parasite.

Human influences on trematode infection

Johnson and Lunde (*in press*) reviewed the influences of human activity on trematode-related diseases

Table 4. Extended.

		Percentage of abnormalities		
Ambystoma macrodactylum	*Taricha granulosa*	*Taricha torosa*	All anurans, no *Ribeiroia*	All urodeles, no *Ribeiroia*
0	0	0	6.0 (7)	0
0	0	0	0.9 (1)	0
1.0 (1)	7.1 (1)	0	2.6 (3)	0
0	0	0	0.9 (1)	0
0	0	0	0.9 (1)	0
4.2 (4)	7.1 (1)	20.6 (7)	6.8 (8)	4.4 (1)
3.1 (3)	0	2.9 (1)	0	0
1.0 (1)	7.1 (1)	5.9 (2)	0.9 (1)	13.0 (3)
10.4 (10)	14.3 (2)	8.8 (3)	1.7 (2)	13.0 (3)
6.3 (6)	7.1 (1)	8.8 (3)	1.7 (2)	8.7 (2)
8.3 (8)	0	0	0	0
7.3 (7)	0	0	0	0
0	0	2.9 (1)	0	0
0	0	0	1.7 (2)	0
15.6 (15)	42.9 (6)	20.6 (7)	7.4 (32)	43.5 (10)
8.3 (8)	0	0	1.7 (2)	0
4.2 (4)	7.1 (1)	14.7 (5)	11.1 (3)	8.7 (2)
0	0	0	0	0
5.2 (5)	0	8.8 (3)	15.4 (18)	0
5.2 (5)	0	5.9 (2)	6.8 (8)	8.7 (2)
10.4 (10)	0	0	0.9 (1)	0
3.1 (3)	7.1 (1)	0	0	0
0	0	0	1.7 (2)	0
0	0	0	0	0
1.0 (1)	0	0	4.3 (5)	0
0	0	0	0	0
5.2 (5)	0	0	6.8 (8)	0
53.7 (51)	61.5 (8)	50 (17)	54.7 (58)	52.2 (12)
46.3 (44)	38.5 (5)	50 (17)	45.3 (48)	47.8 (11)
96	14	34	117	23
73	14	27	106	20
818	170	121	3448	281
1.32 ± 0.08	1.0	1.26 ± 0.0	1.10 ± 0.02	1.15 ± 0.08

and evaluated possible hypotheses for an increase in *Ribeiroia*-induced malformations in amphibians. While rejecting the possibility that *Ribeiroia* is a recently introduced parasite or has only recently begun infecting amphibians, they suggested that dramatic and widespread alterations of aquatic ecosystems, particularly the construction of small impoundments or farm ponds, may have created environments conducive to *Ribeiroia* infection. These modified habitats are frequently permanent or semi-permanent and highly productive, facilitating high densities of *Planorbella* snails (Johnson and Lunde, *in press*). As natural wetlands continue to be lost, such aquatic systems may be becoming increasingly important to amphibians and waterfowl (Knutson et al. 2000, Johnson and Lunde, *in press*).

In support of this hypothesis, most malformed amphibians we observed were in artificial impoundments. At the few sites supporting *Ribeiroia* but free of ostensible human impacts, both *Planorbella* snails and malformed amphibians occurred in low numbers.

Ponds sampled in Lassen Volcanic National Park, California, and in Sky Lakes Wilderness, Oregon, each exhibited a malformation frequency of <10% in the resident amphibian species. In contrast, many sites with high frequencies of amphibian malformations were impacted heavily by cattle and supported dense *Planorbella* spp. populations. Several ponds in Washington and Montana, each with a >50-yr grazing history, supported malformations in multiple amphibian species at frequencies of 50–90%. Unfortunately, unimpacted sites were poorly represented in our survey and were usually at high elevations, precluding rigorous comparisons. In addition, we collected *Planorbella trivolvis trivolvis*, a snail native to the eastern United States, at four sites in California and Oregon, and *P. tenuis*, a western species, at 12 sites beyond the northern boundary of its reported range (Burch 1989). All but one of these aquatic systems were human-constructed impoundments that had significantly higher orthophosphate levels than the natural wetlands we sampled. In-

troductions of *Planorbella* snails outside their native range could facilitate an increase in the distribution or local abundance of *Ribeiroia* and amphibian malformations (see Johnson and Lunde, *in press*).

Amphibian decline and conservation

The significance of amphibian malformations to the long-term persistence of affected populations remains conjectural (Wake 1998). High levels of *Ribeiroia* infection and the resulting malformations may increase mortality in wild amphibian populations. In our study and that of Johnson et al. (2001*b*), the frequency of malformations in larval amphibians was significantly higher than that in emergent amphibians from the same system, suggesting that malformed larvae experience greater mortality prior to and during metamorphosis than normal conspecifics. *Ribeiroia* infection may represent a particular threat to amphibian populations or species already in decline. Considering that *Ribeiroia* commonly infects *R. catesbeiana*, a widely introduced ranid in the western United States, as well as a number of commonly stocked game fish (Simmons 1971), the potential for the parasite to be transported is cause for concern.

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