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The Effect of Trematode Infection on Amphibian Limb Development and Survivorship

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The causes of amphibian deformities and their role in widespread amphibian declines remain conjectural. Severe limb abnormalities were induced at high frequencies in Pacific treefrogs (*Hyla regilla*) exposed to cercariae of a trematode parasite (*Ribeiroia* sp.). The abnormalities closely matched those observed at field sites, and an increase in parasite density caused an increase in abnormality frequency and a decline in tadpole survivorship. These findings call for further investigation of parasite infection as a cause of amphibian deformities in other sites and species.

Alarm over increasing reports of deformed amphibians has intensified since the early 1990s (*1, 2*). Over the last decade, abnormalities have been reported in 36 species of amphibians from 42 U.S. states (*3*). Whether abnormalities are contributing to global trends in amphibian population decline or are indicative of environmental threats to human health is still uncertain (*1, 4, 5*). Suggested causes of abnormal amphibians include ultraviolet-B radiation, biocide contamination, retinoids, and parasite infection (*1, 6–9*). However, none of these have been decisively linked to the types of abnormalities most frequently reported in the field: missing, malformed, and extra limbs (*3, 7, 10*).

Between 1996 and 1998, we surveyed 35 ponds in Santa Clara County, California, to determine the prevalence of abnormal amphibians. At 4 of the 13 ponds supporting Pacific treefrogs, severely abnormal frogs were observed. Intensive monitoring programs established at two of these ponds consistently recorded high frequencies (15 to 45%) of metamorphic frogs with polymely (extra limbs) and other hindlimb deformities ($n = 8818$; Table 1). Water tests failed to detect any pesticides, polychlorinated biphenyls (PCBs), or heavy metals, and 200 *H. regilla* eggs collected from the ponds hatched and developed normally in the laboratory (*11*). Community analysis of the 35 ponds revealed that the four ponds with abnormal treefrogs were the only ponds to support both *Hyla regilla* and an aquatic snail, *Planorbella tenuis*, which is a first host of the trematode parasite *Ribeiroia* sp. Upon dissection, we found *Ribeiroia* metacercariae in treefrogs from each of the four ponds. Whereas three other trematode species were also observed in some of the dissections, *Ribeiroia* exhibited a unique distribution within infected frogs: the metacercariae were highly localized in the tissue around the pelvic girdle and hindlimbs, often in close association with abnormal or extra limbs.

We tested the hypothesis that *Ribeiroia* infection is responsible for the limb abnormalities that we observed in *H. regilla*. The experiment operated within an ecologically relevant framework by exposing tadpoles to living parasites at observed field densities, allowing cercariae to freely select a point of penetration, and employing an amphibian host species for which high abnormality rates have been recorded in the field. We collected *H. regilla* egg masses from the Eel River (39°44′N, 123°39′W), an area 300 km north of our monitoring sites with no known records of abnormal frogs (*12*). After hatching, tadpoles were kept individually in 1-liter containers of commercial spring water and randomly assigned to one of six treatments. Those in the experimental treatments were exposed to either 0 (control), 16 (light), 32 (intermediate), or 48 (heavy) *Ribeiroia* cercariae. A fifth group was exposed to 80 cercariae of a second species of trematode (*Alaria mustelae*) also found in frogs from Santa Clara County field sites. The sixth group was exposed to both species: 80 *Alaria* cercariae and 32 *Ribeiroia* cercariae (*13*). Infection levels were selected to encompass the range of parasite densities found in naturally infected abnormal frogs collected from our field sites. Tadpoles were exposed to parasites in four equal doses over a 10-day period, with each dose equal to one-fourth of the total parasite load (*14*).

Exposure of Pacific treefrog tadpoles to *Ribeiroia* cercariae induced severely abnormal limb development in 85% of the frogs surviving to metamorphosis ($n = 71$). The frequency of abnormalities was high in all *Ribeiroia* treatments and showed a positive relationship to parasite density (logistic regression $\chi^2 = 88.16$, df = 3, $P < 0.001$; Fig. 1A). Tadpole survivorship declined with increasing parasite load and fell below 50% in the intermediate and heavy treatments (logistic regression $\chi^2 = 29.86$, df = 3, $P < 0.001$; Fig. 1A). In the control group, 88% of the tadpoles survived, and all survivors were normal. Only 40% of the tadpoles in heavy treatment survived to metamorphosis and

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100% of those developed limb abnormalities (Fig. 1A). *Alaria* cercariae penetrated tadpoles but caused neither limb abnormalities nor an increase in mortality, even at a greater density than that used in the heavy *Ribeiroia* treatment (Fig. 1B). Tadpoles infected with both *Alaria* and *Ribeiroia* had abnormality and mortality rates comparable to *Ribeiroia* alone (G_{adj} = 1.76, df = 1, $P > 0.05$; Fig. 1B). *Ribeiroia* metacercariae were recovered almost exclusively from the pelvic region and hindlimbs of metamorphic frogs while *Alaria* mesocercariae were widely distributed throughout the subcutaneous tissue.

All abnormalities observed in frogs exposed to *Ribeiroia* cercariae involved the hindlimbs (Table 1 and Fig. 2). The specific types of abnormalities recorded in the experiment accounted for 95% of the abnormalities observed in abnormal Pacific treefrogs (n = 1086) caught in the field between 1996 and 1998 (Table 1). The severity of abnormalities in the experiment, as indexed by the mean number of abnormalities per abnormal frog, increased with cercarial dose (*15*) (logistic regression χ^2 = 13.42, df = 2, $P < 0.001$; Table 1). The relative frequency of missing and extra limbs also increased monotonically from light to heavy treatments. Indeed, 30% of the metamorphic frogs in the heavy treatment failed to develop either hindlimb (Fig. 2C).

These results show that parasite infection explains both the frequency and composition of abnormalities observed in *H. regilla* populations in our study system. The trematode *Ribeiroia* was isolated from deformed frogs in the field, was employed at realistic concentrations in experimental exposures, and produced the same range and frequency of amphibian limb abnormalities as observed at field sites. Our results further suggest that trematode infection represents a considerable source of mortality in some amphibian populations. In addition to the high direct mortality accompanying parasite infection, indirect mortality resulting from increased predation of frogs with deformed or missing limbs is likely to be substantial (*4, 8, 9*). Few abnormal adult frogs (<2%) were ever seen at our field sites, even following years in which 25% of the metamorphosing frogs were

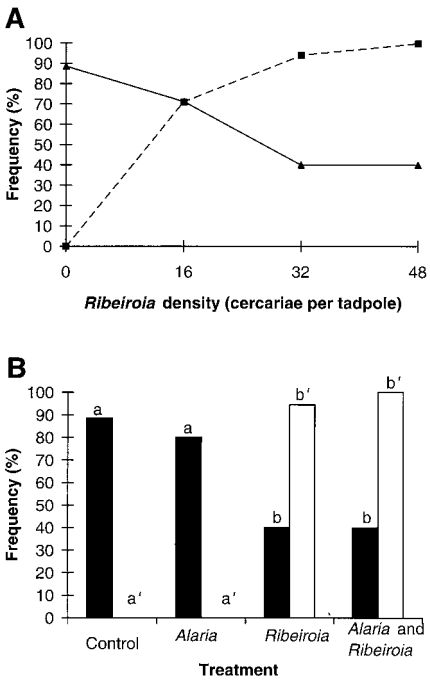

Fig. 1. (**A**) *Hyla regilla* survivorship and abnormality frequency in *Ribeiroia* treatments. Survivorship (▲, solid line) is calculated as number of tadpoles surviving to metamorphosis divided by initial sample size. Abnormality frequency (■, dashed line) is calculated as number of abnormal metamorphosing frogs divided by the total number of metamorphosing frogs within a given treatment. Initial sample sizes in treatments are as follows: control, 35 tadpoles; light, intermediate, and heavy, 45 tadpoles each. Parasite density shows a significant, positive relationship to abnormality frequency and a significant, negative relationship to survivorship (logistic regression, df = 3, $P < 0.001$). (**B**) *Hyla regilla* survivorship and abnormality frequency across parasite treatments. Survivorship (solid bar) and abnormality frequency (open bar) are calculated as above. Within survivorship, differences in the lowercase letters (*a* or *b*) indicate significant differences between treatments (G-test, df = 1, $P < 0.001$). For abnormality frequency, significance groupings are denoted by *a′* or *b′*. Initial sample sizes in the different treatments: control, 35 tadpoles; *Alaria*, 10 tadpoles; *Ribeiroia*, 45 tadpoles; and *Alaria* + *Ribeiroia*, 10 tadpoles.

Table 1. Composition of abnormalities from experimental and field studies of *Hyla regilla*. Numbers represent the proportion of the total abnormalities falling within a particular category (*15*). Treatment categorization by the number of *Ribeiroia* cercariae to which tadpoles were exposed: light (16 cercariae), intermediate (32 cercariae), and heavy (48 cercariae). The numbers in parentheses indicate (number of abnormal metamorphic frogs)/(total number of frogs inspected). The mean number of abnormalities per abnormal frog ± SD, an index of abnormality severity, is displayed for each treatment and for the field data in the final row of the table. Abnormality categories are adapted, in part, from Tyler (*15*).

Abnormality type	Control (%) (0/31)	Light (%) (22/31)	Intermediate (%) (17/18)	Heavy (%) (18/18)	Field sites (%) (1086/8818)
Cephalic and axial					
Anophthalmy (missing eye)	0	0	0	0	0.5
Mandibular hypoplasia (abnormal jaw)	0	0	0	0	0.6
Open wound	0	0	0	0	1.1
Other	0	0	0	0	0.4
Forelimb					
Hemi- and ectromely (missing limb)	0	0	0	0	1.1
Ectrodactyly (missing digit)	0	0	0	0	0.7
Cutaneous fusion	0	0	0	0	0.2
Polydactyly (extra digit)	0	0	0	0	0.1
Polymely (extra limb)	0	0	0	0	0
Other malformed*	0	0	0	0	0.1
Hindlimb					
Hemi- and ectromely (missing limb)	0	11.8	18.6	25.5	6.5
Ectrodactyly (missing digit)	0	0	0	2.1	4.2
Cutaneous fusion	0	17.6	9.3	12.8	5.9
Taumely (bony triangle)	0	5.9	4.7	0	3.4
Polydactyly (extra digit)	0	2.9	6.9	0	4.8
Polymely (extra limb)	0	32.3	44.2	55.3	50.7
Femoral projection†	0	11.7	2.3	2.1	12.2
Other malformed*	0	17.6	13.9	2.1	7.6
No. abnormalities per abnormal frog	0	1.7 ± 0.99	2.52 ± 1.57	2.93 ± 1.73	1.38 ± 0.67

*Other malformed includes brachymely, permanent extension, brachydactyly, syndactyly, and edema. †Femoral projection constitutes a digit-like appendage growing from the dorsal tissue of the femur (Fig. 2H).

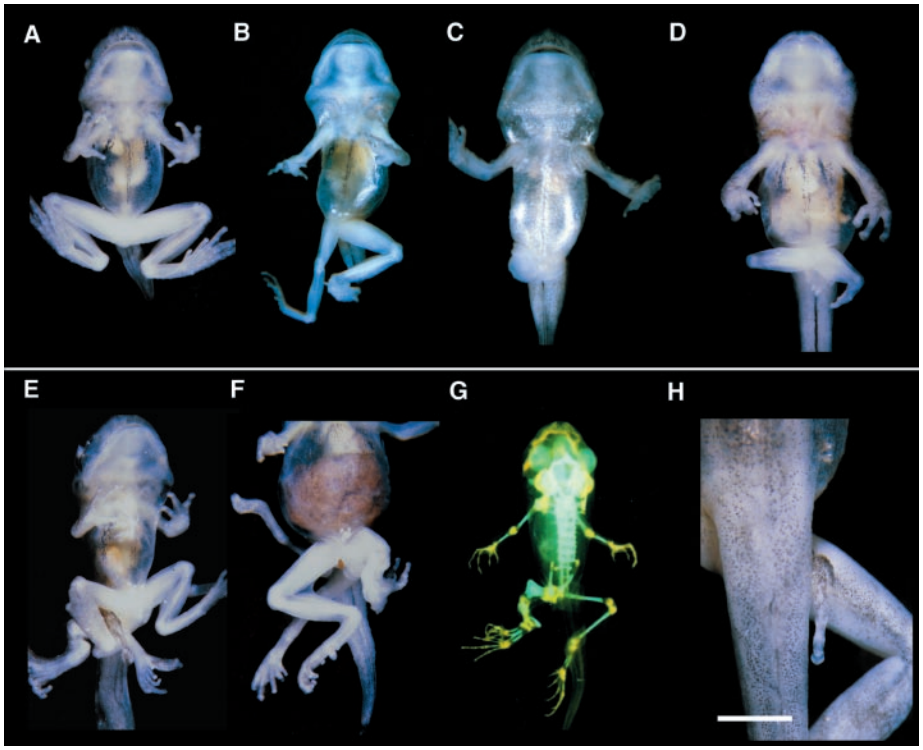

Fig. 2. Composite photograph of the different types of abnormalities produced in tadpoles exposed to *Ribeiroia*. All images show the ventral side of metamorphosing *H. regilla* except (G) and (H), which are dorsal views. (**A**) Control frog with normally developed limbs. (**B**) Metamorphic frog with a permanent extension in right hindlimb. (**C**) Completely missing limbs (ectromely). (**D**) Partially missing limbs (hemimely). (**E**) Four extra hindlimbs (polymely). (**F**) Cutaneous fusion on right limb, with skin webbing connecting femur to tibiofibula. Also note two extra limbs on right side and severely malformed left limb. (**G**) Cleared and double-stained frog with bony triangle in left limb. (**H**) Magnified view of frog with a femoral projection on dorsal side of right femur. Scale bar, 3.90 mm in (A), (B), and (E); 3.60 mm in (C); 3.30 mm in (D); 4.00 mm in (F); 4.40 mm in (G); and 1.70 mm in (H).

abnormal, suggesting that most abnormal frogs die before reaching sexual maturity. Induction of abnormal limbs may therefore function as an evolutionary adpatation enhancing the transmission rate of *Ribeiroia* between its intermediate (amphibian) and final (undetermined predator) hosts (*8*), as previously documented for other parasite taxa and their respective hosts (*16*). The mechanism through which *Ribeiroia* interferes with amphibian limb development remains unknown, but probably involves chemical or physical disturbances—acting independently or in concert—of the developing limb bud (*8, 17, 18*). Elucidation of the mechanism may offer new insights into limb development, especially if the trematode produces a vertebrate growth factor mimic.

The role of trematodes in the occurrence of abnormalities in other amphibian species in North America and the larger issue of amphibian decline is largely unexplored. The types of abnormalities produced in this experiment encompass many of the abnormalities described in reports from across the continent (*1–3*, *6*, *9*). Whether *Ribeiroia* induces limb abnormalities in other amphibian species has yet to be tested, but abnormal bullfrogs (*Rana catesbeiana*) and western toads (*Bufo boreas*) infected with *Ribeiroia* were regularly observed at our study sites. If *Ribeiroia* is involved in the recent increase in abnormal amphibians reported, it could be due to an increase in the density or distribution of one of its host species. Alternatively, other anthropogenic or natural changes in the environment could cause snail population sizes to increase. Accelerated eutrophication due to organic pollution and the removal of molluscivorous predators have both been shown to increase snail abundance and the incidence of parasite infection (*19*). At this point, however, little is known about the distribution, life cycle, or pathogenicity of *Ribeiroia* to other species. These questions, coupled with the extreme mortality and abnormality rates observed in this study, call for an increased research focus on parasite infection and its effects on amphibian host populations.

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13. Snails (*P. tenuis*) infected with *Alaria* or *Ribeiroia* were collected from California field sites, placed in 50-ml vials of commercial spring water, and allowed to shed cercariae for 8 to 10 hours. Within 1 hour after shedding, emerging cercariae were isolated, counted, and added to 100-ml specimen cups containing individual tadpoles. Tadpoles were exposed to cercariae for 90 min. No cercariae were observed in the specimen cups following the exposure period, suggesting that all had penetrated the tadpoles. The control group tadpoles were placed in specimen cups containing only spring water and returned to their containers after 90 min.
14. The experiment began approximately 1.5 weeks after hatching (Gosner Stages 23 through 26). Tadpoles were exposed to trematode cercariae on days 1, 4, 7, and 10 of the experiment. Staging is from K. L. Gosner [*Herpetologica* **16**, 183 (1960)].
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