Larval trematodes in the Upper Midwest and the Pacific Northwest which are known to induce skeletal malformations in anurans

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ABSTRACT

Recent laboratory studies have shown that exposing developing tadpoles of H*yla regilla* and *Bufo boreas* to cercariae of the trematode *Ribeiroia* elicits malformations similar to those observed in field-caught anuran metamorphs. In the summer of 1999, *Ribeiroia* metacercariae were found for the first time in anurans from Minnesota and Wisconsin. Massive infections of *Ribeiroia* metacercariae in *Rana septentrionalis* from the CWB site in Minnesota were positively correlated with development of satellite limbs and mandibular deformities. *Ribeiroia* was infrequently reported elsewhere in Minnesota and Wisconsin in both deformed and normal *Rana pipiens*. In order to determine the species of *Ribeiroia* that occur in Minnesota, California, Oregon, Washington and Montana, surrogate definitive hosts (rock dove, chick, duckling and laboratory rat) were successfully infected with *Ribeiroia* metacercariae obtained from frogs collected in those states. All adult worms obtained from surrogate feedings conform to the description of *R. ondatrae*. Furthermore, analysis of numerous voucher specimens obtained from throughout the Americas indicates that *R. thomasi, R. insignis* and *R. marini* should be synonymized with *R. ondatrae*.

INTRODUCTION

Amphibian population declines and deformities have received considerable attention from both the scientific community and general public (Souder 2000). This attention is largely due to the perceived magnitude of the phenomena and the concurrent lack of understanding about possible causes. Due to their close association with water, amphibians are often considered to be biological indicators of aquatic ecosystem health. Because most species spend a portion of their lives as fully aquatic larvae and remain semi-aquatic as adults, amphibians can be particularly susceptible to many water-borne hazards.

Theories proposed to explain the causes for amphibian deformities range from anthropogenic to natural events. One natural event proposed to explain certain malformations found in anurans (frogs and toads) is the infection of developing tadpoles by larval trematode parasites (Sessions and Ruth 1990). It has been suggested that these larval parasites (called metacercariae) may either chemically or mechanically inhibit proper limb growth in metamorphosing anurans. Recent laboratory studies have shown that when tadpoles of the Pacific chorus frog (*Hyla regilla)* were exposed to cercariae of the trematode *Ribeiroia*, the metamorphs developed many of the same malformations observed in field-caught Pacific chorus frogs (Johnson et al 1999). Johnson and Lunde recently obtained similar malformations by experimental exposures of *Ribeiroia* to the western toad *(Bufo boreas*) (Kaiser 1999). Whereas, anuran malformations are widespread in Minnesota and Wisconsin (Lannoo 1998), there are relatively few surveys of anuran parasites from the Upper Midwest (Ulmer 1970, Williams and Taft 1980, Coggins and Sajdak 1982, Muzzall 1991 and Yoder and Coggins 1996). These surveys rarely differentiate metacerariae present in anurans.

Four species of *Ribeiroia* have been described from North America: *R. ondatrae, R. insignis, R. marini* and *R. thomasi.* Because metacercariae lack the adult morphological structures used to classify trematodes to species, metacercariae must be introduced into surrogate definitive hosts and allowed to develop in order to determine species.

The focus of this study was to determine: 1) whether *Ribeiroia* metacercariae occurred in anurans collected from Minnesota and Wisconsin; 2) whether *Ribeiroia* or any other trematode metacercariae were correlated with malformations in anuran metamorphs, 3) the taxonomic validity of *Ribeiroia* species described from North America and 4) which species of *Ribeiroia* occurred in anurans from the Upper Midwest and Pacific Northwest.

METHODS

Collection of anurans--Throughout the summer of 1999, Pacific chorus frogs (*Hyla regilla*), spotted frogs (*Rana pretiosa*), mink frogs (*Rana septentrionalis*) and northern leopard frogs (*Rana pipiens*) which were either malformed or occurred at sites that historically had high rates of malformations were shipped by overnight express to the University of Wisconsin, La Crosse. Mink and leopard frogs were collected from Minnesota (CWB, NEY, TRD, ZAG, ROI, CBA and DOR) by either David Hoppe, University of Minnesota, Morris, or personnel of the Minnesota Pollution Control Agency. Pacific chorus frogs and spotted frogs were collected from California, Oregon, Montana and Washington by Pieter Johnson and Kevin Lunde. Local leopard frogs and green frogs (*Rana clamitans*) were collected using dip nets from Myrick Park marsh, Goose Island County Park, Trempealeau National Wildlife Refuge, Van Loon Wildlife Area, Bluebird Springs Campground, Walsh Golf Center, and a farm pond near Arcadia, Wisconsin. Several green frogs and wood frogs (*Rana sylvatica*) were collected from Pigeon Lake Field Station, Bayfield County, Wisconsin.

Examination of anurans—Anurans were euthanized with tricaine methanesulfonate (MS-222) and necropsied for parasites using standard procedures. Each anuran was examined for metacercariae using dissecting microscopes. Representative metacercariae were removed from host tissues, placed into tap water and manually excysted. Excysted metacercariae were then examined as wet-mounts at 100 X or 200 X magnification with a compound microscope.

Infection of surrogate hosts— *Ribeiroia* metacercariae and other selected metacercariae were gavaged into esophagi of surrogate species: rock doves (*Columba livia*), mallard ducklings (*Anas platyrhynchos*), chicks (G*allus domesticus*) and laboratory rats (*Rattus norvegicus*).

Examination of surrogate hosts—Seven days post exposure to *Ribeiroia* all surrogate hosts were euthanized by overdose of carbon dioxide. After death was assured, each host was necropsied using standard procedures to locate adult parasites.

Worm identificationn—Adult *Ribeiroia* were either killed unflattened in hot 10% formalin or flattened with slight cover-slip pressure and killed in cold 10% formalin, stained with

Semichon's acetocarmine and mounted in a synthetic resinous medium. Several morphological characteristics were measured (Table 2) and compared to paratype and holotype specimens obtained from H. W. Manter Parasite Collection, University of Nebraska; U. S. Helminthological Collection, Beltsville, MD and private parasite collections. Voucher specimens included *Ribeiroia* from numerous species of naturally infected and experimentally infected avian hosts and three species of mammalian hosts.

RESULTS

Parasite burdens in anurans were generally moderate to heavy (Table 1). While it has been documented that anurans in the Pacific Northwest harbor relatively low diversity of larval trematodes (personal communication, Pieter Johnson, Stanford University), anurans from Minnesota and Wisconsin contained diverse larval trematode communities (Table 1 and Figs. 2, 3, 4, 5). *Riberioia* were discovered at two Minnesota sites (CWB and NEY) notorious for having a high frequency of anurans with malformations (Fig 1).

At CWB, mink frogs harbored the greatest mean intensity of *Ribeiroia* (100 plus metacercariae per frog) recorded to date. Packets of *Ribeiroia* metacercariae (consisting of approximately 20 to 40 individuals) occurred in the inguinal region of the four mink frogs examined (Fig. 6 and 7). The only other metacercariae found at CWB were a few *Alaria* (Fig. 3). The location of *Ribeiroia* within the frogs at CWB was positively correlated with the origin of satellite limbs (Figs. 8 and 9) and abbreviated mandibles (Figs. 10 and 11). Satellite limbs originated superficially (i.e., there was no skeletal connection between satellite limbs and pelvic girdle) and in close proximity to large concentrations of *Ribeiroia*. One frog from CWB had an incompletely formed mandible (dentary). The proximal areas of the mandible that articulated with the maxilla were normal while the distal elements, which would normally form the mandibular symphysis were absent; numerous *Ribeiroia* occurred in the region where the distal elements of the mandible should have formed.

At the NEY site, *Ribeiroia* occurred in considerably lower prevalence and mean intensity than at CWB. *Ribeiroia* was not identified in anurans from the five other Minnesota sites. In addition, we discovered light infections of *Ribeiroia* metacercariae (<1% prevelance) in normal leopard frogs collected from Myrick Park marsh.

The only successful infections of surrogate hosts involved *Ribeiroia* (Table 2). In avian surrogates, adult *Ribeiroia* were found in the proventriculus, while in mammalian hosts, *Ribeiroia* were located in the stomach. At seven days post exposure, all *Ribeiroia* obtained from surrogate hosts were gravid. The hallmark morphological feature identifying *Ribeiroia* is the presence of a pair of esophageal caecae (Figs. 2 and 12). In adult worms, these caecae frequently were obscured by thick vitellaria which extend anteriorly to the pharynx (Figs. 13 and 14). Voucher specimens obtained from frozen definitive hosts lacked spination of the body; *Ribeiroia* obtained from our surrogate hosts exhibited dense body spination (Fig. 14). Examination of voucher specimens of *R. ondatrae, R. insignis, R. thomasi* and *R. marini* indicates that these species cannot be differentiated according to adult morphologies alone (Table 3). Morphological characteristics measured from laboratory reared *Ribeiroia* adults displayed no significant difference from the type species *R. ondatrae* (Table 3). Lacking convincing evidence of differences in miracidia and cercariae between the various described

Ribeiroia species, we recommend synonymizing all other North American species of *Ribeiroia* with *R. ondatrae*.

DISCUSSION

While *Ribeiroia* has not previously been reported from frogs in Minnesota and Wisconsin, Taft, Suchow and Van Horn (1993) found adult *Ribeiroia* from raptors in these two states. Ours is the first report of *Ribeiroia* from Minnesota and Wisconsin frogs. The Minnesota Pollution Control Agency has maintained that because *Ribeiroia* has not been found in Minnesota, this parasite could not be a cause for widespread malformations reported in that state. In our study *Ribeiroia* was found at two of the seven Minnesota sites. Similar spotty distribution of *Ribeiroia* has been found at other Minnesota sites by Rebecca Cole (National Wildlife Health Center, Madison, WI, personal communication). In three years of examining frogs from the Upper Mississippi River Valley, we have found *Ribeirioa* at only one site near La Crosse. Johnson and Lunde spent the summer of 1999 collecting frogs, toads and salamanders from 103 ponds in six Northwestern states, including 42 ponds where deformity rates were found in six species ranging from 5% to 90% (Kaiser 1999). Malformed amphibians at 40 of 42 ponds harbored *Ribeiroia* , while those from normal ponds almost never harbored the parasite. Johnson and Lunde (personal communication) found *Ribeiroia* at several Minnesota and Wisconsin sites but not at others.

In Minnesota, there is now evidence that *Ribeiroia* can occur in normal frogs and that many deformed frogs lack *Ribeiroia* . While *Ribeiroia* may potentially be a cause for malformations in some Upper Midwest anurans, recent studies indicate that pond water and sediment samples from various sites in Minnesota (including NEY and CWB) were capable of inducing developmental abnormalities in the South African clawed frog (*Xenopus laevis*) (Fort et al. 1999a,b).

The lack of significant morphological differences between our laboratory reared *Ribeiroia* and voucher specimens of *Ribeiroia* lead us to conclude that *R. ondatrae* is the only valid species of *Ribeiroia* present in North America. Our being able to rear *Ribeiroia* in three avian and one mammalian species confirms what other authors have found (i.e., *Ribeiroia* has extremely low host specificity) (Beaver 1939, Basch and Sturrock 1969 and Malek 1975). *R. ondatrae* appears to be a widely distributed trematode in the Americas because of its transmission by migratory birds and its ability to utilize whichever planorbid snails (*Helisoma* spp. or *Biomphalaria* spp.) are present in the area.

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Table 1. Trematode prevalence (% infected) and mean intensity (mean number of parasites per infected host) in four anuran species (*Rana pipiens, Rana clamitans, Rana septentrionalis* and *Rana sylvatica*) from Minnesota and Wisconsin.

	Rana pipiens (n=124)		***Rana clamitans*** (n=57)	
	Prev.	M. I.	Prev.	M. I.
Metacercariae				
Ribeiroia	4.0	4.0	0.0	0.0
Alaria	17.0	15.8	0.0	0.0
Fibricola	31.0	78.9	2.0	6.0
Clinostomum	7.0	1.7	0.0	0.0
Ochetosomatids	29.0	36.2	0.0	0.0
Apharyngostrigea	6.0	10.4	4.0	6.0
Auridistomum	0.0	0.0	4.0	3.0
Echinostome (kidney)	27.0	16.7	5.0	58.6
Echinostome (skin)	9.0	8.7	0.0	0.0
Strigeid (toe)	9.0	60.0	0.0	0.0
Meta A *	13.0	42.6	5.0	40.3
Meta B *	10.0	41.8	0.0	0.0
Unknown metacercariae **	45.0	100.0	30.0	48.1
Adults				
Hematoloechus	25.0	7.2	7.0	7.3
Megalodiscus	8.0	6.1	0.0	0.0
Gorgoderid	7.0	2.7	35.0	3.7
Halipegus	4.0	3.3	7.0	4.7
Glypthelmins	6.0	5.8	5.0	34.0

	Rana septentrionalis (n=10)		***Rana sylvatica*** (n=3)	
	Prev.	M. I.	Prev.	M. I.
Metacercariae				
Ribeiroia	40.0	110.0	0.0	0.0
Alaria	50.0	2.4	0.0	0.0
Fibricola	31.0	91.6	0.0	0.0
Ochetosomatids	10.0	1.0	0.0	0.0
Apharyngostrigea	30.0	58.6	0.0	0.0
Echinostome (kidney)	60.0	191.8	100.0	127.3
Echinostome (skin)	30.0	4.3	0.0	0.0
Meta B *	20.0	1.0	0.0	0.0
Adults				
Hematoloechus	10.0	5.0	0.0	0.0
Glypthelmins	10.0	1.0	0.0	0.0

* distinguishable metacercariae of an unknown taxon

Table 2. Comparisons in morphology of adult *Ribeiroia* obtained from surrogate hosts.

	Host			
	Rock Dove n*=49	Chick n=20	Duckling n=33	Rat n=15
Body Length	1818**	1521	1560	2058
Body Width	670	536	556	783
Oral Sucker length	206	176	194	235
Oral sucker width	193	167	184	210
Acetabulum length	293	210	219	296
Acetabulum width	265	234	249	308
Anterior testis length	269	205	202	295
Anterior testis width	492	358	349	530
Posterior testis length	326	229	227	336
Posterior testis width	459	333	320	491
Ovary length	137	118	125	131
Ovary width	151	130	122	155
Cirrus pouch length	181	175	166	248
Cirrus pouch width	153	125	127	160
Egg length	94	107	97	97
Egg width	51	57	54	52

* number of worms measured
** measurements are averages in micrometers

Table 3. Morphological variation among North American species of *Ribeiroia*.

	Ribeiroia species			
	R. ondatrae	*R. insignis*	*R. thomasi*	*R. marini*
Body length	1400-4200	1650-4530	1050-3075	1070-2380
Body width	320-1400	675-1300	400-875	435-1000
Oral sucker length	150-260	250-380	110-350	180-240
Oral sucker width	150-266	220-400	130-300	170-270
Acetabulum length	210-360	270-600	150-340	220-300
Acetabulum width	210-380	320-580	170-360	230-320
Anterior testis length	260-380	180-430	120-390	140-360
Anterior testis width	410-710	450-780	170-430	500-750
Posterior testis length	260-750	200-630	120-430	200-350
Posterior testis width	340-750	390-980	170-420	560-700
Ovary length	75-220	150-220	60-190	80-150
Ovary width	90-220	160-280	60-190	80-190
Egg length	80-90	80-90	80-100	77-90
Egg width	45-50	50-60	50-65	40-57

Plate 1. Fig. 1. Mink frog (*Rana septentrionalis*) from the CWB site in Minnesota with satellite pelvic limbs. (Specimen collected by David Hoppe; photographed by Rick Gillis)

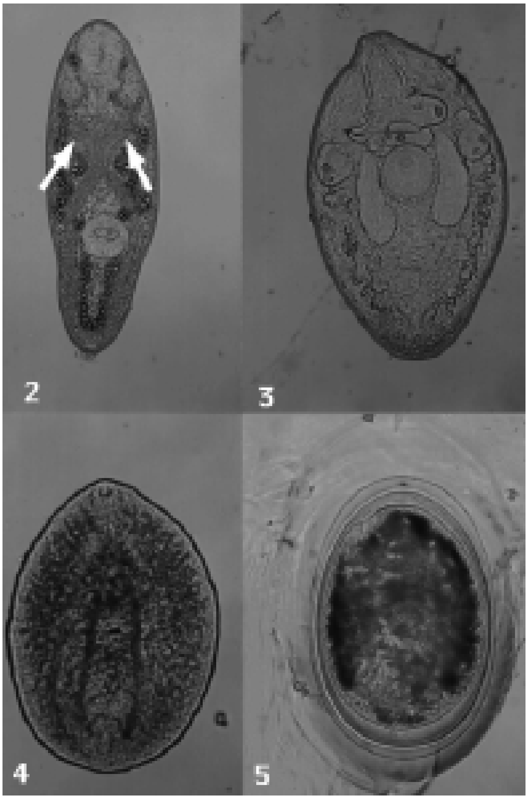

Plate 2. Metacercariae found in Wisconsin and Minnesota anurans. Fig. 2. *Ribeiroia ondatrae*. Fig. 3. *Alaria* sp. Fig. 4. Strigeid metacercariae from toes, Fig. 5. Encysted *Apharyngostrigea pipientis*.

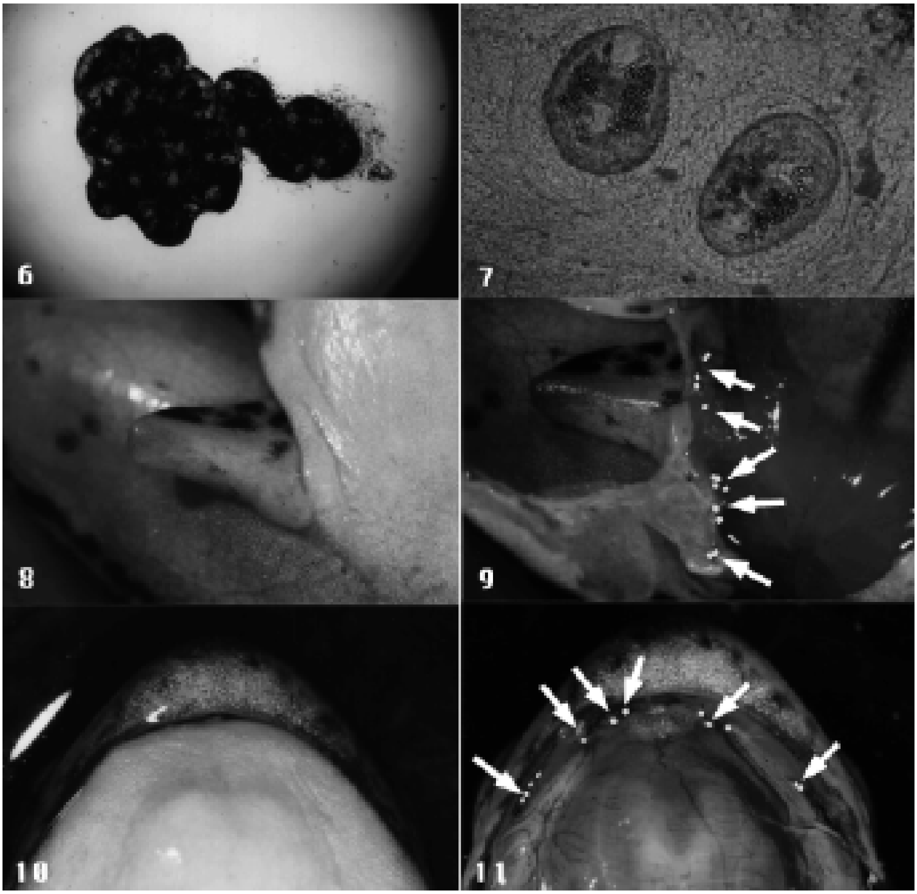

Plate 3. ***Ribeiroia ondatrae*** **from CWB mink frogs. Fig. 6. Packet of metacercariae from the inguinal region. Fig. 7. Two metacercariae attached to underside of skin. Fig. 8. Satellite appendage. Fig. 9. Metacercariae (white spots) asssociated with satellite appendage. Fig. 10. Severe overbite. Fig. 11. Metacercariae associated with overbite. (Note incomplete development of mandible.)**

Plate 4. Fig. 12. Adult *Ribeiroia* "*thomasi*" from great horned owl, northern WI. Fig. 13. *R.* "*thomasi*" from Osprey, Lake Tomahawk, Oneida Co., WI. Fig. 14. Adult *Ribeiroia ondatrae* from surrogate rock dove. (Specimens in Figs. 12 and 13 courtesy of Steve Taft, UW-Stevens Point.)