Influence of *Ribeiroia ondatrae* (Trematoda: Digenea) infection on limb development and survival of northern leopard frogs (*Rana pipiens*): effects of host stage and parasite-exposure level

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Abstract: Recent evidence suggests that infection by larvae of the trematode *Ribeiroia ondatrae* accounts for a significant proportion of limb malformations currently observed in amphibian populations of North America. However, the effects of *R. ondatrae* infection on northern leopard frogs (*Rana pipiens*), one of the species most frequently reported with malformations, have not been adequately explored. Moreover, the risk factors associated with *R. ondatrae*-induced malformations have not been clearly identified. We examined the effects of timing of infection on tadpole survival and limb development. *Rana pipiens* tadpoles were individually exposed to *R. ondatrae* cercariae at the pre-limb-bud (Gosner stages 24 and 25), limb-bud (Gosner stages 27 and 28), or paddle (Gosner stages 31–33) stages of development and monitored through metamorphosis. The effects of infection were stage-specific. Infections acquired at the pre-limb-bud stage resulted in a high mortality rate (47.5–97.5%), whereas tadpoles infected at the limb-bud stage displayed a high malformation rate (16% overall), and the magnitude of effects increased with the level of exposure to cercariae. In contrast, infections acquired at the paddle stage had no effect on limb development or tadpole survival, which suggests that the timing of *R. ondatrae* infection in relation to the stage structure of tadpole populations in the wild is an important determinant of the degree to which populations are affected by *R. ondatrae*.

Résumé : Des indications récentes font croire que des infections par les larves du trématode *Ribeiroia ondatrae* pourraient expliquer une partie importante des malformations des membres que l'on observe actuellement chez les populations d'amphibiens d'Amérique du Nord. Cependant, les effets des infections à *R. ondatrae* sur les grenouilles léopards (*Rana pipiens*), une des espèces le plus fréquemment signalées avec des malformations, n'ont pas été étudiés adéquatement. De plus, les facteurs de risque associés aux malformations causées par *R. ondatra*e ne sont pas clairement identifiés. Nous avons étudié les effets du moment de l'infection sur la survie et le développement des membres chez le têtard. Nous avons exposé des têtards de *R. pipiens* individuellement à des cercaires de *R. ondatrae* au stade de développement précédant l'apparition des bourgeons des membres (stades 24 et 25 de Gosner), au stade des bourgeons des membres (stades 27 et 28 de Gosner) et au stade des membres en palettes (stades 31–33 de Gosner) et les avons suivi jusqu'après la métamorphose. Les effets de l'infection dépendent du stade. Les infections acquises avant l'apparition des bourgeons des membres entraînent une forte mortalité (47,5–97,5 %), alors que les têtards infectés lorsque les bourgeons sont présents ont un taux élevé de malformations (en tout 16 %); l'importance des effets augmente avec le degré d'exposition aux métacercaires. En revanche, les infections acquises lorsque les membres ont la forme de palettes restent sans effet sur le développement des membres et la survie des têtards, ce qui fait croire que le moment de l'infection, en relation avec la structure en stades de la population de têtards en nature, est un facteur déterminant essentiel de l'importance des effets de *R. ondatrae* sur les populations.

[Traduit par la Rédaction]

Introduction

During the past decade, reports of malformed frogs in the wild have increased (e.g., Ouellet et al. 1997; Helgen et al. 1998; Gardiner and Hoppe 1999; Hoppe 2000), leading to concerns that a rise in the frequency of malformations might further threaten declining amphibian populations (Ouellet 2000; Johnson et al. 2002). Although other teratogens, such as UV-B radiation (Ankley et al. 1998, 2000) and pesticides (Ouellet et al. 1997; Fort et al. 1999*a*, 1999*b*; Bridges 2000), including environmental retinoids (Gardiner and Hoppe 1999; Degitz et al. 2000) and endocrine-disrupting chemi-

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cals (Sower et al. 2000), have been proposed as causes of limb malformations, the strongest evidence to date supports the claim that infections by larvae of the trematode *Ribeiroia ondatrae* are responsible for a significant proportion of malformations observed (Johnson et al. 1999; Sessions et al. 1999; Blaustein and Johnson 2003). Both laboratory and field experiments have confirmed that *R. ondatrae* infection in developing anurans induces limb malformations that are consistent with the types observed in wild frog populations (Johnson et al. 1999, 2001*a*, 2001*b*, 2002; Kiesecker 2002; Stopper et al. 2002). Moreover, Johnson et al. (2002) found that of several biotic and abiotic factors, including pesticide contamination, that were measured, the presence and intensity of *R. ondatrae* infections in amphibian populations in the western U.S.A. were the only significant factors that predicted above-baseline frequencies of limb malformations (i.e., >5%).

Ribeiroia ondatrae has a complex life cycle that includes at least three different hosts. Adults live and reproduce in the gastrointestinal tract of birds and mammals. Eggs are deposited in the environment with the feces of these hosts, and larval development proceeds within planorbid snails, where the trematode develops to the free-living cercaria stage. This stage is released in large numbers from the snail, then penetrates and encysts as the next larval stage, known as a metacercaria, in tadpoles or fish (Beaver 1939; Taft et al. 1993). Maturation to adulthood resumes when the infected tadpole or fish is eaten by a suitable avian or mammalian definitive host.

Many parasites that rely on trophic transmission change the morphology or behavior of their intermediate hosts in ways that modify the risk of predation by a definitive host (Holmes and Bethel 1972; Lafferty 1999; Moore 2002). Limb malformations caused by *R. ondatrae* infection, such as supernumerary limbs, skin webs, and bone bridges, are believed to have adaptive significance for the parasite in that they interfere with the locomotory capabilities of affected frogs, thereby increasing the frogs' susceptibility to predation and facilitating completion of the life cycle (Sessions and Ruth 1990; Johnson et al. 2001*b*; Stopper et al. 2002). The occurrence of *R. ondatrae*-induced malformations, therefore, is likely a natural phenomenon; however, increased frequencies of these malformations in amphibian populations may be indicative of recent environmental changes that have resulted in increased stress for the hosts (Blaustein and Johnson 2003). For instance, high intensities of *R. ondatrae* infection and high frequencies of malformations in the western U.S.A have been linked to small man-made impoundments and cattle ponds. Such water bodies receive high nutrient inputs and therefore contain dense populations of snails, which in turn may support prevalences of *R. ondatrae* that are higher than historical ones (Johnson et al. 2002). In addition, Kiesecker (2002) demonstrated that proportions of *R. ondatrae* cercariae encysting in *Rana sylvatica* tadpoles exposed to pesticide in vitro were higher than in control animals, which suggests that contaminants may act synergistically to increase transmission success in the wild. Collectively, these findings highlight the need for a better understanding of environmental factors related to outbreaks of *R. ondatrae*-induced malformations.

One factor that is likely to be an important determinant of *R. ondatrae*-induced limb malformations is the timing of infection (Stopper et al. 2002). Limb-pattern formation is a time-dependent process, and errors that occur at different times during development may cause different types of malformations (Meteyer et al. 2000*b*). Moreover, the ability of cells of the developing limb bud to reproduce amputated limbs declines as tadpole stages progress (Schotté and Harland 1943; Fry 1966), and the likelihood that limb elements will be duplicated in response to tissue damage may be restricted to specific periods of limb development (Bryant et al. 1981; Stopper et al. 2002). Accordingly, the probability that *R. ondatrae* infections will induce malformations likely depends on the stage of limb development at which infections are acquired. Variation in the timing of infection events across geographic regions due to climatic or other local factors might help explain the variation in the distributions of limb-malformation types observed across regions (Loeffler et al. 2001) and among amphibian species that have different breeding phenologies (e.g., Johnson et al. 2001*a*, 2002). However, the developmental period in which infections are likely to cause limb malformations, and the types of malformations induced at different times of development, have not been experimentally defined.

We investigated temporal aspects of *R. ondatrae* – tadpole interactions by exposing northern leopard frog (*Rana pipiens*) tadpoles to *R. ondatrae* cercariae at different stages of limb development. We chose *R. pipiens* as our host species because the greatest percentage of abnormalities has been reported for this species (North American Reporting Center for Amphibian Malformations 2002). In addition, *R. pipiens* displays a wide range of abnormality types across a variety of habitat types, yet few attempts have been made to causally link abnormalities in this species to a particular etiologic agent (but see Stopper et al. 2002). The specific aims of the study were to (*i*) determine when, during metamorphosis, *R. ondatrae* infections are most likely to cause limb malformations, and (*ii*) characterize the types of malformations produced when infections are acquired at different stages of development.

Methods

Study animals

Eggs were obtained from adult *R. pipiens* collected in Minnesota, U.S.A., and artificially fertilized (Nasco, Fort Atkinson, Wis.). In addition, portions of four separate, naturally fertilized egg masses were collected from the wild at a site in southern Wisconsin (Brooklyn Wildlife Area (BWA), Dane County) and kept separate from the other embryos. Embryos hatched in several 20-gal glass aquaria each containing 5–8 cm of water. Following hatching, the water level was raised to fill each aquarium and gentle aeration was initiated. Sponge filters were installed shortly thereafter, and partial water changes were conducted every 2 or 3 days to control ammonia and nitrite concentrations. Tadpoles remained in these holding tanks until the time of exposure to *R. ondatrae*, and were fed boiled romaine lettuce for the first 10 days post hatching, then switched to a diet of Frog Brittle (Nasco) and *Spirulina* pellets ad libitum. The *R. ondatrae* cercariae used in the study were obtained from naturally infected snails (*Planorbella* (syn. *Helisoma*) *trivolvis*) col-

lected from a pond in Urbana, Champaign County, Illinois. These snails were maintained on wafers of ground algae (Hikari, Kyorin Food Inc. Ltd., Himeji, Japan) in individual plastic cups (266 mL) filled with water, which was changed daily. All experimental animals were maintained in carbon-filtered tap water that was diluted from 450 to 200 ppm hardness with deionized water in a 378.5-L reservoir tank and aerated for at least 24 h prior to use. Lighting was maintained on a 12 h light : 12 h dark cycle under fluorescent bulbs, which were changed from Philips 4500K, CRI 62 to Philips 5000k, CRI 85 after the first 100 days of the study to better simulate conditions of natural sunlight in the laboratory and improve vitamin D production and calcification of the anuran skeletal system. All animals were maintained in accordance with the principles and guidelines of the Canadian Council on Animal Care (1993).

Experimental procedures

To evaluate the consequences of acquiring infections at different developmental stages, two experiments were conducted. In the first (experiment 1), tadpoles obtained from Nasco were exposed individually to *R. ondatrae* cercariae at one of three stages of limb development: pre-limb bud (Gosner (1960) stages 24 and 25), limb bud (stages 27 and 28), or paddle (stages 31–33). These stages represent three relatively distinct periods of limb development. The pre-limb-bud stage occurs prior to the onset of limb-bud outgrowth, the limb-bud stage represents the period following limb-bud outgrowth but prior to foot development, and the paddle stage marks the initiation of foot development (Fig. 1).

As large proportions of the tadpoles in each holding tank reached the targeted stages, individuals were randomly selected from tanks, examined for any abnormalities (tadpoles that appeared abnormal in any way were not used in the experiment), staged, measured to the nearest millimetre (total and snout–vent lengths), and isolated in 266-mL plastic cups containing approximately 200 mL of water. Tadpoles (30–73 per treatment; Table 1) were randomly assigned to an exposure treatment, and after a 24-h acclimation period, exposed to the appropriate number of *R. ondatrae* cercariae. For each stage, a control and two exposure treatments were employed. At the pre-limb-bud stage, tadpoles were exposed to 0, 4, or 8 *R. ondatrae* cercariae, whereas tadpoles at the limb-bud and paddle stages were exposed to 0, 8, or 90 cercariae. The exposure levels chosen for analysis were within the range of infection intensities observed in wild amphibian populations (Sessions and Ruth 1990; Johnson et al. 2002).

In all cases except the 90-cercariae treatments, tadpoles were exposed to cercariae in a single dose, with tadpoles remaining in the water with the cercariae for 24 h. Confirmation that the cercariae had infected exposed tadpoles was made 2 days post exposure (PE), when both the control and exposed animals were anesthetized using 1:4000 tricaine methanesulfonate (MS 222) and visually inspected using a dissecting microscope. Metacercariae formed on or just below the surface of the skin, and were therefore easily observed under a microscope. Following this examination, all tadpoles were transferred from the cups to individual 1.8-L plastic containers filled with fresh water, and reared through metamorphosis.

Tadpoles from the 90-cercariae treatments were exposed

Fig. 1. Limb-bud regions of *Rana pipiens* tadpoles at each of the developmental stages (Gosner 1960) used in the study. (A) Pre-limb-bud stage (stages 24 and 25). (B) Limb-bud stage (stages 27 and 28). (C) Paddle stage (stages 31–33). Note that in each photograph the head of the tadpole is to the left and tail is to the right. Scale bars = 1 mm.
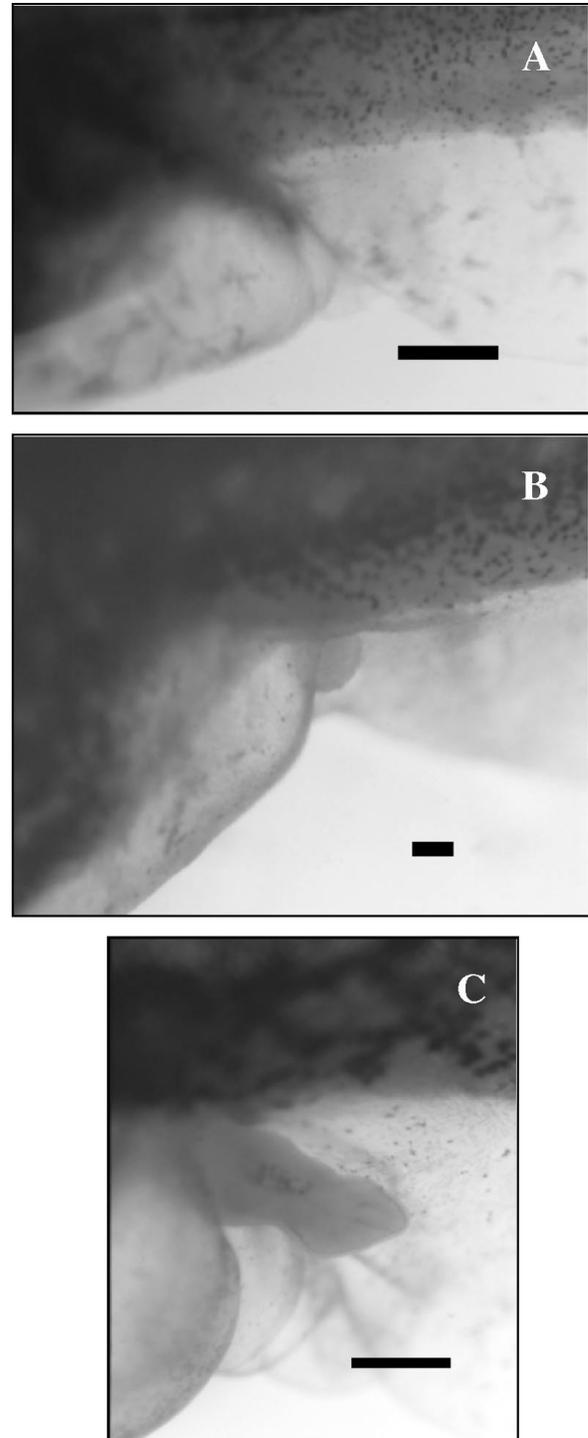
to cercariae in increments of 10 or 20 cercariae each over 12 days. These tadpoles remained in the small, 266-mL plastic cups throughout the 12-day exposure period, during which food and water were refreshed daily prior to the intro-

Table 1. Survivorship and malformation responses observed in *Rana pipiens* tadpoles experimentally exposed at different stages of limb development (Gosner 1960) to various levels of *Ribeiroia ondatrae* cercariae.

Stage	Exposure treatment (no. of cercariae per tadpole)	Exposure period (days)	n	Survivorship (%)	Malformation rate (% of survivors)
Experiment 1					
Pre-limb-bud stage	0	1	60	61.7	0
(stages 24 and 25)	8	1	60	5[a]	0
	0	1	30	86.7	0
	4	1	60	51.7[b]	0
Limb-bud stage	0	1	40	95	0
(stages 27 and 28)	8	1	73	97.3	4.2
	90	12	45	95.6	34.9
Paddle stage	0	1	30	83.3	0
(stages 31–33)	8	1	60	90	0
	90	12	45	82.2	0
Experiment 2					
Pre-limb-bud stage	0	1	40	72.5	0
(stages 24 and 25)	2	1	40	52.5[c]	9.5
	4	1	40	30[b]	16.7
	8	1	40	2.5[a]	0

Note: Tadpoles that survived to stage 42, or to the end of experiment 2, were considered survivors. Tadpoles that did not die, but were euthanized because of inactivity or abnormal behavior were not considered survivors; euthanasia accounts for most of the mortality observed in the limb-bud- and paddle-stage groups in experiment 1.

[a]Comparison with control group: $p < 0.0001$.
[b]Comparison with control group: $p < 0.01$.
[c]Comparison with control group: $p = 0.10$.

duction of cercariae. Tadpoles in these groups were inspected for metacercariae on days 11 and 14 PE. On day 14, tadpoles were transferred to the 1.8-L plastic containers.

After tadpoles were moved to the 1.8-L rearing containers, they were maintained individually on a diet of Frog Brittle and *Spirulina* pellets supplied on alternating days through metamorphosis. Water was changed every other day and containers were cleaned with sponges and rinsed with 70% ethanol approximately every 2 weeks to remove nitrifying bacteria and algae from the surfaces.

Because a high mortality rate was observed in the *R. ondatrae*-exposure treatments of the group at the pre-limb-bud stage in experiment 1, an additional series of treatments was conducted at the pre-limb-bud stage using *R. pipiens* tadpoles from BWA (experiment 2) to further evaluate this effect of *R. ondatrae* infections on tadpole survival. Cercariae were obtained from the infected snails used in experiment 1. Tadpoles (40 per treatment) were individually exposed to 0, 2, 4, or 8 cercariae in a single exposure, and transferred to the 1.8-L containers 2 days PE. Maintenance and examination protocols were as described for experiment 1.

Data collection

In each experiment the number of tadpoles that died in each group was recorded daily. The development of all surviving tadpoles was monitored every 7–10 days for the first 63 days PE by removing the tadpoles from their containers, anesthetizing them in 1:4000 MS-222, and examining them under a dissecting microscope for malformations. Total and snout–vent lengths were recorded at these times. The end of metamorphosis was defined as Gosner stage 42 (time of forelimb emergence; Gosner 1960), at which time final mass

and length were collected. Development continued until stages 44 and 45, when frogs were euthanatized via immersion in 1:1000 MS 222, taped in uniform position onto plastic petri plates, and fixed in 10% neutral buffered formalin as described in Meteyer et al. (2000a).

All tadpoles in experiment 1 completed metamorphosis by 205 days PE. However, not all tadpoles in experiment 2 completed metamorphosis before the study was terminated on day 247 PE. At this time, 90% ($n = 36$) of the controls, 87.5% ($n = 35$) of the 2-cercariae, 97.5% ($n = 39$) of the 4-cercariae, and 100% ($n = 40$) of the 8-cercariae groups had either died or reached stage 42.

Malformed individuals were radiographed using a Faxitron Specimen Radiography System model MX-20 (Elwood, Kans.), followed by clearing and staining using a modification of the technique described by Hanken and Wassersug (1981) to determine which skeletal elements were malformed, duplicated, or deleted. A subset of control and exposed individuals that appeared normal were randomly selected and radiographed and (or) cleared and stained to assess whether malformations were missed during gross examinations. Descriptions of malformations were based on the terminology of Meteyer (2000). Malformations were counted and assigned to categories, and the relative frequency of each category was calculated for each treatment. Following Johnson et al. (1999, 2001b), malformations building on, or forming part of, other malformations were not considered. The severity of malformation was computed as the mean number of discrete malformations per malformed frog for each treatment (Johnson et al. 1999, 2001a).

Statistical analysis

Three-way contingency tables were employed to deter-

Fig. 2. Survivorship curves for the groups of *R. pipiens* tadpoles exposed to different doses of *Ribeiroia ondatrae* cercariae at the pre-limb-bud stage of development in experiment 2. Proportions surviving (mean ± 1 SE) in each group are displayed for the first 14 days post-exposure (PE); mortality rates after this time point were stable. Final cumulative rates of survival are reported in Table 1 for each treatment group.
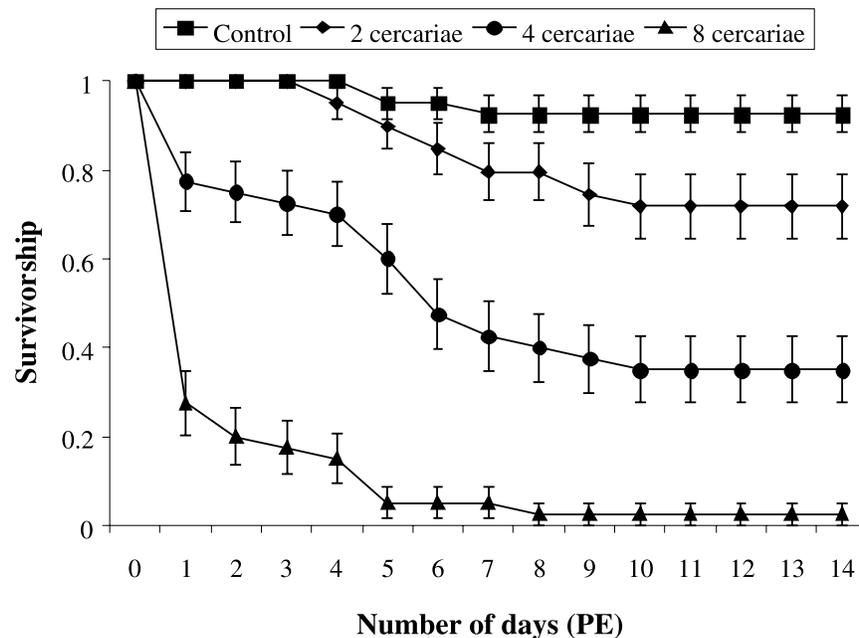
mine the effects of tadpole stage and level of exposure to *R. ondatrae* cercariae on tadpole survival and development. For experiment 1, mortality frequencies were compared across all stages for the 0- and 8-cercariae treatments. The distribution of malformed individuals across treatments was examined for the 0-, 8-, and 90-cercariae treatments for the limb-bud and paddle stages only. Very few individuals exposed to 8 cercariae at the pre-limb-bud stage survived and no individuals were exposed to 90 cercariae at this stage. To adjust for zero cell frequencies in the malformation analysis, ones were added to each cell (Legendre and Legendre 1998). For experiment 2, the effect of level of exposure to cercariae on tadpole survivorship was analyzed using a χ^2 test for trends analysis (Zar 1996), and life-table statistics were also calculated for each treatment (Fox 2001). Because data for the malformation category in this experiment were sparse, data from the exposure treatments were pooled and the frequencies of malformed individuals were compared between control and exposed groups using Fisher's exact test. Analyses were conducted in SAS version 8.02 (SAS Institute, Inc. 1999–2001).

Results

As indicated by visual examination, all the tadpoles exposed to *R. ondatrae* cercariae during the experiments became infected. Mortality of tadpoles was significantly associated with exposure to *R. ondatrae* cercariae as well as with the stage at which they were exposed (df = 7, χ^2 = 166.9, p < 0.00001). Specifically, infections acquired at the pre-limb-bud stage negatively affected tadpole survivorship (df = 1, χ^2 = 43.4, p < 0.0001). In addition, as indicated in both experiments, the proportions of tadpoles that died at

this stage increased with the number of cercariae to which tadpoles were exposed (Table 1). Also, there was a significant positive linear trend in mortality frequencies with increasing exposure level in experiment 2 (χ^2 test for trends, χ^2 = 59.5, p < 0.001), and the rate at which deaths occurred increased with exposure dose. Just 2 days PE, 72.5% of the tadpoles exposed to 8 cercariae had died, whereas only 22.5 and 0% of the tadpoles exposed to 4 and 2 cercariae, respectively, had died, compared with 0% of the controls (Fig. 2). However, *R. ondatrae* infections reduced tadpole survivorship only during this early stage of limb development; the mortality rate was independent of infection status at the limb-bud (df = 1, χ^2 = 0.39, p = 0.534) and paddle (df = 1, χ^2 = 0.83, p = 0.363) stages (Table 1).

In total, 22 of the surviving 273 tadpoles (8.1%) exposed to *R. ondatrae* cercariae in both experiments developed one or more malformations. None of the controls (155) became malformed. Of the malformed individuals, 18 (81.8%) had been exposed to cercariae at the limb-bud stage in experiment 1, and 4 (18.2%) were BWA tadpoles that were exposed at the pre-limb-bud stage in experiment 2. No malformations were observed in tadpoles exposed at the paddle stage (Tables 1 and 2).

The association between exposure at the limb-bud or paddle stage and the development of a malformation was dependent on the level of exposure to cercariae. The frequencies of malformed frogs did not differ significantly between these two stages at the control (Fisher's exact test, p = 1.00) or 8-cercariae exposure level (Fisher's exact test, p = 0.389). However, at the 90-cercariae exposure level, a higher frequency of malformed frogs was observed at the limb-bud stage than at the paddle stage (Fisher's exact test, p < 0.0001). Overall, tadpoles exposed to cercariae at the limb-

Table 2. Composition of malformation types observed in *R. pipiens* tadpoles at each stage of experimental exposure to *R. ondatrae* cercariae.

| Malformation type | Pre-limb-bud stage | Limb-bud stage | | | Paddle stage |
		8 cercariae	90 cercariae	Total	
Hind limb					
Amelia (missing limb)	0	0	0	0	0
Ectromelia (missing limb elements)	0	0	0	0	0
Apody (missing foot)	0	0	0	0	0
Ectrodactyly (missing digits)	0	0	0	0	0
Polymelia (multiple limbs)	0	40 (2)	25 (6)	22.2 (8)	0
Polypody (multiple feet)	0	0	0	0	0
Polydactyly (multiple digits)	0	20 (1)	25 (6)	19.4 (7)	0
Polyphalangy (multiple phalanges)	0	20 (1)	4.2 (1)	5.6 (2)	0
Femoral projection[a]	0	0	8.3 (2)	5.6 (2)	0
Brachydactyly (shortened digits)	75 (3)[b]	0	4.2 (1)	2.8 (1)	0
Skin webbing	0	0	12.5 (3)	8.3 (3)	0
Bone bridge	0	20 (1)	4.2 (1)	2.8 (2)	0
Micromelia (small limbs)	25 (1)	0	0	0	0
Limb hyperextension	0	0	0	0	0
Other[c]	0	0	16.7 (4)	11.1 (4)	0
Ilium					
Reduced	0	0	12.5 (3)	8.3 (3)	0
Misshapen	0	0	16.7 (4)	11.1 (4)	0
Total no. of hind-limb malformations	4	5	24	31	0
Total no. of malformations	4	5	31	36	0
Total no. of malformed frogs	4	3	15	18	0
No. of malformations per malformed frog (mean ± 1 SE)	1.0	1.7 ± 0.33	2.1 ± 0.4	2.0 ± 0.31	0

Note: The values shown are the relative percentages of malformation types within each stage treatment and cercariae exposure level treatments for the limb-bud stage. Numbers in parentheses show the numbers of malformations within each type. The terminology used to describe malformations is according to Meteyer (2000).

[a]Non-articulating microappendage from the dorsal skin of a hind limb (Johnson et al. 2001*b*).

[b]These malformations were bilaterally symmetrical and occurred in Brooklyn Wildlife Area tadpoles only.

[c]Includes fused digit elements (syndactyly) and misshapen tibiale–fibulare.

bud stage were 4.2 times more likely to be malformed than tadpoles exposed at the paddle stage (Mantel–Haenszel estimator of relative risk, 95% confidence limits = 1.16 and 15.12). For the pre-limb-bud stage in experiment 2, the association between exposure to *R. ondatrae* cercariae and the development of a malformation was not significant (Fisher's exact test, $p = 0.118$).

In total, 40 distinct malformations were recorded among the 22 malformed frogs. The majority of these malformations (82.5%) involved the hind limbs. Malformations affecting the ilium were also found (17.5%). Individual malformed frogs had up to five distinct malformations. An average severity of malformation (Johnson et al. 2001*b*) of about 2.0 malformations per malformed frog was maintained across levels of *R. ondatrae* exposure (Table 2). The types of malformations observed differed between the pre-limb-bud and limb-bud exposure groups. Three of the 4 malformed individuals in the pre-limb-bud group developed bilaterally symmetrical malformations. These malformations consisted of shortened or missing phalanges of the fourth digits. The fourth malformed individual in the pre-limb-bud group developed micromelia of the left hind limb (Table 2).

All observed malformations in frogs exposed at the limb-bud stage were asymmetrical, and typically unilateral (16 of

18 malformed frogs, or 88.9%). Polymelia and polydactyly were the most common malformation types at this stage (Table 2), although only one frog developed complete supernumerary limbs. This frog had two extra hind limbs arising ventrally on the right side. Most cases of polymelia were incomplete, involving duplication of the femur and (or) tibiafibula and various elements belonging to the foot. Two of the eight cases of polymelia were associated with partial or complete mirror-image duplications of elements on the anterior–posterior axis, and both patterns were of the anterior mirror-image duplication type (Sessions et al. 1999). Polydactyly was also generally incomplete, involving duplication of a single digit in six cases and two digits in one of the eight cases. Interestingly, 85.7% of the cases of polydactyly were also associated with incomplete or complete duplication of the tibiale or fibulare (Fig. 3). Skin webs and bone bridges accounted for an additional 8.3 and 2.8% of the total observed malformations, respectively. Malformations affecting the ilium, which have not previously been reported in frogs exposed to *R. ondatrae* in the laboratory, consisted of incompletely developed and abnormally shaped ilia (Fig. 3). Typically, malformations of the ilium co-occurred with limb malformations (71.4% of ilium malformations), but also occurred in the absence of limb malformations. No malforma-

Fig. 3. Representative malformed specimens that were cleared and stained. (A) Polydactyly of digit 1 with complete duplication of the tibiale. (B) Polydactyly of digit 4 with incomplete duplication of the fibulare and polyphalangy of digit 5. (C) Misshapen ilium. The arrow indicates a malformation. (D) Reduced ilium and bone bridge of left femur. The arrow indicates a malformation of the ilium.
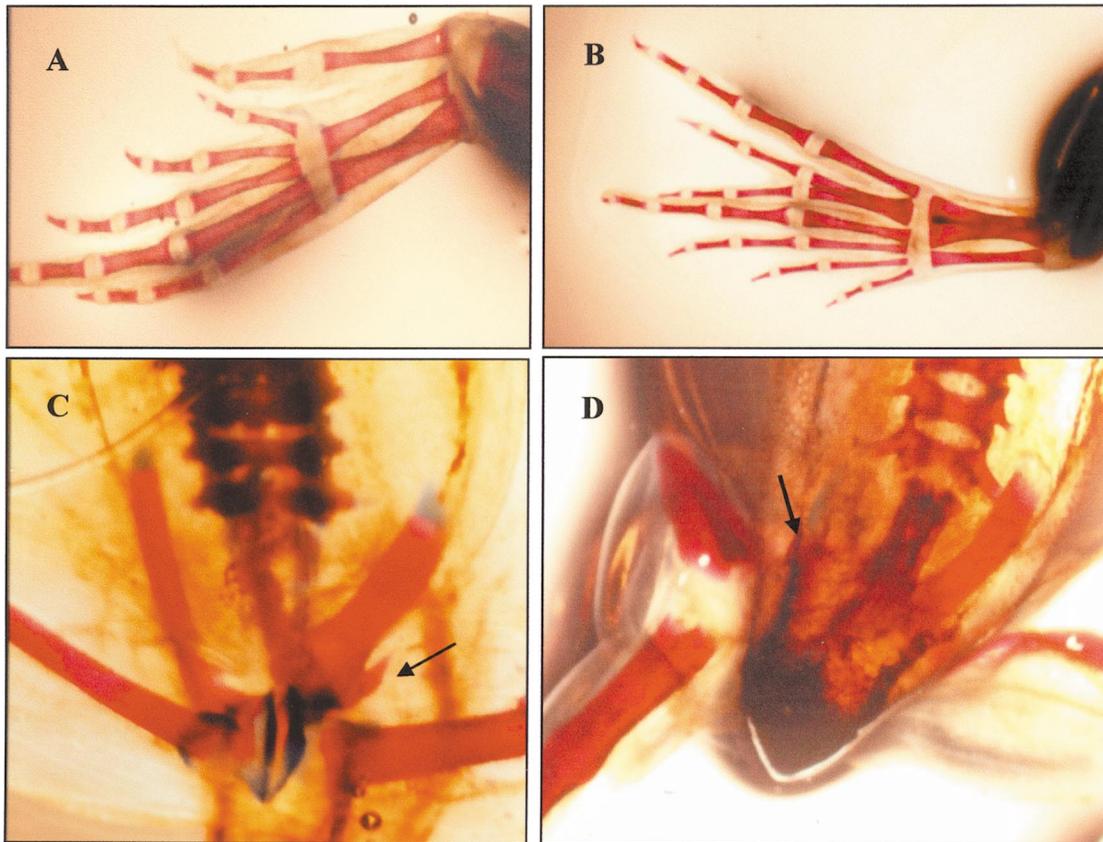
tions that were not detected grossly were revealed following clearing and staining or radiography.

Discussion

Developmental stage is an important mediator of many ecological interactions involving anuran larvae. The effects of predator–prey and competitive interactions, for instance, often depend on the timing of such events with respect to tadpole stage (Werner and Gilliam 1984; Alford and Wilbur 1985; Alford 1989; Fauth 1990). In addition, vulnerability to acidity (Rosenberg and Pierce 1995) and the effects of contaminants (Boone et al. 2001) are influenced by the stage at which individuals are exposed. Moreover, tadpoles display stage-specific preferences for certain microhabitats (Alford and Crump 1982) and thermal environments (Dupre and Petranka 1985; Wollmuth et al. 1987; Wollmuth and Crawshaw 1988). In this study, we demonstrate that the outcomes associated with interactions with a parasite also vary according to the developmental stage at which tadpoles become infected. Specifically, infection by *R. ondatrae* had two distinct detrimental effects on *R. pipiens* tadpoles at different stages of development. Infections acquired early in development, at the pre-limb-bud stage, severely decreased tadpole survivorship. Exposure to as few as 4 cercariae significantly reduced the proportion of tadpoles that survived to metamorphosis, and in both of our experiments, more than 90% of the tadpoles exposed to only 8 cercariae died. Infections ac-

quired by tadpoles at later stages of development, on the other hand, did not cause a decline in survivorship, but did disrupt limb development when infection coincided with the limb-bud stage (stages 27 and 28) of development. Overall, 15.8% of the surviving tadpoles exposed at this stage became malformed compared with 6.2% of all the tadpoles exposed at the pre-limb-bud stage and none of the tadpoles exposed at the paddle stage. Even the low dose of 8 cercariae was sufficient to produce a malformation rate of about 4% in this group, which approaches a rate that has been considered one of concern for amphibian populations (e.g., >5%; Ouellet 2000; Johnson et al. 2002). However, by the time tadpoles had developed to the paddle stage, they had suffered neither infection-induced mortality nor limb malformations, which suggests that the developmental window during which *R. ondatrae* infections potentially harm tadpoles in these ways precedes this stage of development.

The differential responses to *R. ondatrae* infections displayed by tadpoles at different stages were probably related to the developmental status of the hind limbs at the time of infection. For *R. ondatrae*, encystment of cercariae occurs primarily in the limb-bud regions and along the base of the tail (personal observation; Stopper et al. 2002). It was noted that encystment of cercariae caused hemorrhaging and damage to the skin, and in tadpoles exposed at the pre-limb-bud stage (stages 24 and 25), this tissue damage covered a fairly large portion of the tadpole's body because of the relatively large size of metacercariae compared with the small size of

tadpoles at this stage. In many cases, infections destroyed the tissue surrounding the cloaca and caused degeneration of the tail musculature. Such severe damage was probably responsible for the high mortality rate observed at this stage. In contrast, tadpoles at the limb-bud and paddle stages were larger, and tissue damage caused by encystment of cercariae, although present, did not affect a large proportion of the tadpole's body. Alternatively, the limb-bud stage marks a period in limb development when damage of limb-bud tissue can cause intercalation, a process whereby limb-bud cells proliferate and replace lost cells (or apparently lost cells in the case of damage associated with *R. ondatrae* infection). The paddle stage represents a stage of limb development at which damage is unlikely to induce intercalation (Bryant et al. 1981, 1987; Stopper et al. 2002), thus explaining the occurrence of limb malformations at the limb-bud stage but not the paddle stage, as predicted by Stopper et al. (2002).

The responses of *R. pipiens* tadpoles to *R. ondatrae* infections, although similar to those previously reported, differed in several notable ways from those of *Hyla regilla* tadpoles (Johnson et al. 1999) and *Bufo boreas* tadpoles (Johnson et al. 2001*a*). *Rana pipiens* tadpoles at stages 24 and 25 (i.e., pre-limb-bud stage) appeared to be more susceptible to *R. ondatrae* infections than *H. regilla* tadpoles that were exposed to cercariae at comparable stages (stages 23–26; Johnson et al. 1999). Mortality experienced by *R. pipiens* tadpoles ranged from about 52% at an infection density of 2 cercariae to 97.5% at an infection density of 8 cercariae, whereas the mortality sustained by *H. regilla* tadpoles ranged from about 30 to 60% at total infection densities of 16 and 48 cercariae, respectively. *Bufo boreas* tadpoles exposed to the same levels of *R. ondatrae* cercariae as *H. regilla* tadpoles experienced similar mortality frequencies (40–55%), even though they were exposed to cercariae at later stages of development (stages 27–30; Johnson et al. 2001*a*). This is in contrast to the mortality rate of only 2–5% exhibited by *R. pipiens* tadpoles exposed to as many as 90 cercariae at stages 27 and 28 in the present study. Therefore, although *R. pipiens* tadpoles at the pre-limb-bud stage sustained greater losses at lower parasite densities than did *H. regilla* tadpoles, tadpoles at the limb-bud stage suffered much lower mortality rates at higher parasite densities than did *B. boreas* tadpoles.

The ability of *R. ondatrae* to cause limb malformations in *R. pipiens* tadpoles also appeared to differ from that observed in *H. regilla* and *B. boreas* tadpoles, in that infections induced a relatively lower frequency of malformations in *R. pipiens*. Seventy percent of surviving tree frogs exposed to a total of 16 cercariae developed malformations (Johnson et al. 1999), whereas overall, only 4 of the 62 (6.5%) surviving *R. pipiens* tadpoles exposed to between 2 and 8 *R. ondatrae* cercariae at the comparable pre-limb-bud stage became malformed. This difference may be partially related to the lower exposure level used in infecting *R. pipiens* than in infecting *H. regilla*. However, the malformation response of *R. pipiens* tadpoles at stages 27 and 28 also was weaker than that of *B. boreas* tadpoles. Exposure of *B. boreas* tadpoles to 16 cercariae was sufficient to induce malformations at a rate of about 35% (Johnson et al. 2001*a*), which is similar to the rate observed for *R. pipiens* tadpoles exposed to 90 cercariae. It is possible that the different malformation rates observed among the studies result from differences in experimental

conditions associated with *R. ondatrae* exposure, in species-specific responses, or in the geographic strain of *R. ondatrae* used. However, the influence of these types of factors has not been investigated.

Whereas differences in mortality and malformation rates were noted, the types of malformations that developed in *R. pipiens* did not differ substantially from those observed in *H. regilla*, *B. boreas*, or other frog species infected with *R. ondatrae* (Johnson et al. 1999, 2001*a*, 2001*b*, 2002; Kiesecker 2002; Stopper et al. 2002). Polymelia, polydactyly, and skin webs were common in *R. pipiens*, as they were in other species, although the relative frequencies of each type varied among species. Moreover, the types of malformations observed in *R. pipiens* in this study, and by Stopper et al. (2002), have been reported in wild-caught *R. pipiens* tadpoles and metamorphic frogs (Helgen et al. 1998; Meteyer et al. 2000*a*), indicating that *R. ondatrae* infections probably contribute to the malformations observed in wild *R. pipiens* populations.

Notably absent from the malformations observed in *R. pipiens* in this study were missing or partially missing limbs (termed amelia and ectromelia, respectively, by Meteyer (2000) and ectromelia and hemimelia, respectively, by Johnson et al. (2001*b*)). Low percentages of these malformation types were reported in experimentally infected *H. regilla* (Johnson et al. 1999) and *B. boreas* (Johnson et al. 2001*a*), and they have been the most commonly reported malformation types found in free-living *R. pipiens* populations of the eastern U.S.A. and Canada (e.g., Ouellet et al. 1997; Helgen et al. 1998; Converse et al. 2000; Meteyer et al. 2000*a*). Therefore, failure to observe amelia or ectromelia does little to help clarify whether *R. ondatrae* infections adequately explain these types of malformations. The failure to observe such malformations in our study may be related to the small sample of malformed frogs (22) obtained, or to the absence of the appropriate infection conditions. Interestingly, in about 22% of all the tadpoles that survived for more than 2 days following exposure to cercariae at the pre-limb-bud stage (*n* = 159), one of the two limb buds was not detected for some time following infection. These limb buds did eventually appear (the median time for detecting limb buds was 14 days PE (range 9–85 days PE)) and subsequently developed into normal limbs in most cases. One individual missing a detectable limb bud for approximately 85 days PE did develop micromelia (Table 2). These observations suggest that infections acquired at the pre-limb-bud stage are associated with partial or completely missing limbs under certain conditions that do not allow for the repair or delayed emergence of limb buds. However, additional field and laboratory experiments are needed to determine the role of *R. ondatrae* in producing these types of malformations.

Because few malformations developed in tadpoles exposed at stages other than the limb-bud stage, we were unable to determine with any certainty how the timing of infection influences the types of malformations that develop. Despite this, our study clearly indicates that the timing of infection with respect to stage of tadpole development is an important determinant of the outcome of *R. ondatrae* – tadpole interactions. Furthermore, our results may help explain the variation in malformation rates and composition of malformations currently observed in amphibian populations in dif-

ferent geographic regions, as well as among different amphibian species occurring at the same location (e.g., Johnson et al. 2001*b*; Loeffler et al. 2001; Meteyer et al. 2000*a*). Because amphibians vary in their breeding phenologies and life histories, the timing and frequencies of breeding events with respect to periods of high abundance of cercariae may put certain species at greater risk of becoming infected during the critical window of development than others. Moreover, local and interannual variation in climatic factors that regulate amphibian breeding events and cercarial-activity patterns in snails may alter the degree to which the timing of infection events overlaps periods of susceptibility.

Clearly, knowing when during the larval period infections are most likely to cause malformations, as well as when they are no longer expected to induce malformations, and how these traits vary among amphibian species will help elucidate the patterns of increasing frequency of malformations currently observed in amphibian populations in the wild (Blaustein and Johnson 2003). Based on the results of our study, the larval period prior to stages 31–33 represents the period when *R. ondatrae* infections have maximum impact on *R. pipiens* tadpoles. The limb-bud stage (stages 27 and 28) appears to be the most sensitive period of limb development in *R. pipiens*, and malformations induced at this stage are predominantly duplications. The pre-limb-bud stage (stages 24 and 25) also appears to be a vulnerable period of development in that relatively low infection densities can lead to high mortality rates. In fact, mortality may have a more dramatic effect on populations if infections are acquired at these early stages, when tadpoles are at a greater risk of dying from infections than of developing a malformation. As indicated by mortality rates that approached 100% in the pre-limb-bud groups exposed to 8 cercariae, complete cohorts could be eliminated from wild populations if tadpoles are exposed at the right time. Previous investigations have primarily focused on the ability of *R. ondatrae* to cause limb malformations in developing anurans, as malformed individuals are presumed to have a low survival rate (Martof 1956; Merrell 1969; Johnson et al. 2002). Our results suggest that mortality caused directly by *R. ondatrae* infections may also significantly affect tadpole populations, and therefore also deserves more attention in future studies.

In conclusion, the stage-specific outcomes observed in the present study highlight the temporal dynamics of *R. ondatrae* – tadpole interactions, and suggest that the degree to which infections affect tadpole populations depends on the stage structure of populations in relation to the timing of infection events. To fully understand the significance of *R. ondatrae* infections on amphibian populations, future studies should include efforts to identify environmental factors that are related locally to the timing of *R. ondatrae* infection events, as well as the factors that mediate the length of amphibian larval periods, to determine when and for how long tadpoles in the wild are at risk of dying or developing malformations associated with infections. In particular, factors such as the phenology of amphibian breeding events, changes in microhabitat-selection and activity patterns of tadpoles over time, and the effects of environmental conditions on the rate of tadpole development should be documented in relation to the prevalence of infections and cercaria-shedding activity in snail populations.

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