

FIELD EVIDENCE FOR LEECH-BORNE TRANSMISSION OF AMPHIBIAN *ICHTHYOPHONUS* SP.

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ABSTRACT: Parasites have been implicated in mass mortality events and population declines of amphibians around the world. One pathogen associated with mortality events in North America is an *Ichthyophonus* sp.-like organism that affects red-spotted newts (*Notophthalmus viridescens*) and several frog species, yet little is known about the distribution of this pathogen in wild populations or the mechanism of transmission. In an effort to identify factors influencing the distribution and abundance of this pathogen, we measured *Ichthyophonus* sp. prevalence and a series of factors that could contribute to transmission in 16 newt populations during spring 2004. In contrast to our initial hypotheses of trophic transmission, several lines of evidence suggested a role for the amphibian leech (*Placobdella picta*) in *Ichthyophonus* sp. transmission. We propose the mechanistic hypothesis that a leech acquires *Ichthyophonus* sp. infection when inserting its proboscis into the muscles beneath the skin of infected newts and transmits the infection to other newts in subsequent feeding bouts. We also found effects of host sex, body mass, and breeding condition on *Ichthyophonus* sp. prevalence and the number of attached leeches. The number of leeches attached to newts was strongly related to the proportion of newt habitat containing emergent vegetation, suggesting that anthropogenic eutrophication might lead to more frequent or severe outbreaks of *Ichthyophonus* sp. infection in amphibians.

Amphibian populations throughout the world are declining, many without obvious causes (Stuart et al., 2004). Although a number of factors may contribute to these declines, new or more virulent pathogens have been identified as playing a central role (Kiesecker, Blaustein, and Belden, 2001; Kiesecker, Blaustein, and Miller, 2001; Collins and Storfer, 2003; Daszak et al., 2003). Reports of parasite-induced mortality and morbidity events in amphibian populations have increased in recent decades, most attributed to ranaviruses, trematode metacercariae, and newly recognized fungal pathogens, including a pathogenic chytrid fungus and an *Ichthyophonus* sp.-like organism (Green et al., 2002; Johnson et al., 2004). In particular, the spread of this chytrid fungus has been implicated in declines of amphibian species in Central America, Australia, and California (Berger et al., 1998; Fellers et al., 2001). Disease emergence is often associated with anthropogenic environmental changes (Dobson and Foufopoulos, 2001), and several anthropogenic factors have been implicated as causes of increased disease incidence in amphibians, including introductions of non-native species (Weldon et al., 2004; Jancovich et al., 2005), increased UV radiation (Kiesecker and Blaustein, 1995), pesticide-induced immunosuppression (Kiesecker, 2002; Gilbertson et al., 2003), and anthropogenic eutrophication (Johnson and Chase, 2004).

Several mass mortality and morbidity events have been attributed to infection by an *Ichthyophonus* sp.-like mesomycetozoon fungus first described in bullfrogs (*Rana catesbeiana*) and recorded in 5 additional North American amphibian species, including red-spotted newts (*Notophthalmus viridescens*) (Goodchild, 1953; Herman, 1984; Mikaelian et al., 2000; Green et al., 2002). However, we know little about its distribution, abundance, or impact in normal amphibian populations (Mikaelian et al., 2000). Because no other *Ichthyophonus* sp.-like organism has been described in amphibians, we refer to this organism simply as *Ichthyophonus* sp., although its precise taxonomic status has yet to be determined. Amphibian *Ichthyophonus* sp. is ultrastructurally similar to *Ichthyophonus hoferi*, a trophically transmitted pathogen of marine fish (McVicar, 1982;

Herman, 1984; Spanggaard et al., 1995). However, the few studies of amphibian *Ichthyophonus* sp. have found no evidence of infection in sympatric fish, no evidence of infections in tissues other than the skeletal musculature, no tendency for the organism to germinate after death of the host, and, so far, have not achieved successful transmission by oral or intraperitoneal administration of infected tissue (Herman, 1984; Mikaelian et al., 2000). In red-spotted newts, macroscopic signs of infection range from visible spores to swelling of the axial musculature (Herman, 1984). Severe infections are often associated with ulcerations that lead to secondary infections with bacteria and fungi (Herman, 1984). Amphibian *Ichthyophonus* sp. has been reported from widespread geographic locations in North America and from specimens collected as far back as 1953 (Goodchild, 1953; Herman, 1984; Mikaelian et al., 2000; Green et al., 2002). Because reports have been in response to mass mortality or morbidity events, it has not been possible to evaluate how common this parasite is in amphibian populations or what factors contribute to between-population variation in infection prevalence.

Understanding the transmission process is essential if we expect to examine the consequences of parasitic infections to populations (Hudson et al., 2002). Previous researchers have assumed that amphibian *Ichthyophonus* sp. would be similar to *I. hoferi*, which is transmitted through the ingestion of infected muscle tissue (McVicar, 1982). Consequently, our initial hypotheses were that newts become infected by direct trophic transmission, either by ingestion of infected tissue through necrophagy or by ingestion of spherules dispersed from decaying dead individuals, or via indirect trophic transmission, i.e., ingestion of a carrier host that obtained the infection through predation or scavenging (Mikaelian et al., 2000). Assuming that contacts between individuals occur randomly, direct transmission via infected cadavers is generally predicted to increase with susceptible host density (newts, other amphibians, and possibly fish), although this prediction has not always been corroborated in empirical studies (McCallum et al., 2001). In contrast, indirect trophic transmission may be independent of amphibian density and instead predicts a relationship between disease prevalence and the abundance of some prey species of red-

TABLE I. Characteristics of the wetlands sampled.

Pond/lake	Latitude	Longitude	Wetland type
Beaver 1 (BE)	40°45'52.6"	78°0'43.6"	Landlocked permanent lake
Clearcut Pond (CC)	40°46'27.7"	77°57'0.0"	Ephemeral pond
Catty Ninetails (CN)	40°47'45.5"	77°57'15.5"	Ephemeral pond
Colyer Lake (CO)	40°46'41.8"	77°41'9.2"	Human impoundment
Cranberry Lake (CR)	40°46'2.6"	78°0'15.5"	Landlocked permanent lake
Deep Woods (DW)	40°52'9.0"	78°4'54.6"	Beaver wetland
False Beaver (FB)	40°42'38.3"	77°52'54.3"	Human impoundment
Greenbriar 1 (GB)	40°46'41.3"	78°0'27.4"	Ephemeral pond
Irrigation Pond (IR)	40°42'18.4"	77°56'48.2"	Landlocked permanent pond
Little Acre (LA)	40°48'5.8"	77°56'36.5"	Landlocked permanent pond
Mothersbaugh (MB)	40°39'12.2"	77°54'9.6"	Beaver wetland
Mystery Newt (MN)	40°45'53.0"	78°0'49.2"	Ephemeral pond
Muskrat Pond (MP)	40°53'8.4"	78°4'3.8"	Beaver wetland
Penn Roosevelt (PR)	40°43'36.8"	77°42'8.3"	Human impoundment
Twin Pond (TP)	40°46'49.1"	78°0'13.9"	Ephemeral pond
Turtle Shell (TS)	40°52'26.1"	78°4'35.6"	Beaver wetland

spotted newts. In particular, benthic macroinvertebrates and tadpoles might pick up the infection while foraging. In either case, water quality or hydrological characteristics of ponds might influence parasite survival outside the host.

Preliminary 2002–2003 surveys of several red-spotted newt populations revealed striking between-pond variation in the prevalence of *Ichthyophonus* sp. disease signs. We, therefore, conducted an expanded survey in 2004 of 16 newt populations, measuring a wide range of ecological variables to identify the strongest predictors of infection prevalence. Based on results from these data, we propose the hypothesis that the amphibian leech, *Placobdella picta* (Siddall et al., 2005), is the vector of *Ichthyophonus* sp. infection in red-spotted newts. This hypothesis predicts a positive between-population relationship between disease prevalence and the ratio of leeches to newts, i.e., vector–host ratio (Hudson et al., 1995). In addition, *Ichthyophonus* sp. is predicted to have a similar distribution to other leech-vectored parasites such as *Trypanosoma diemyctili*, a blood-borne parasite known to be vectored by leeches (Mock, 1987), to be found only in peripheral muscles (near the skin surface), to be more frequently observed on body parts regularly bitten by leeches, and to be found growing out from recent leech bites in new infections. In the present article, we present results from field data testing predictions of the trophic transmission hypotheses and of the leech vector hypothesis.

MATERIALS AND METHODS

Study sites

Sixteen lakes and ponds known to support newt populations were chosen in and around Centre County, Pennsylvania, to represent a variety of adult newt habitats, including temporary woodland ponds, permanent landlocked ponds, beaver wetlands, and human impoundments (Table I).

Survey

Sweep surveys were conducted in the late spring (May–June) 2004 within a 24-day period to minimize any seasonal effects on disease prevalence. During each survey, meter-long sweeps of a dip net (30- × 60-cm aperture; 3-mm mesh) were taken at regular 4-step intervals in a gridded pattern in which we sampled perpendicular to the shoreline to a depth of 0.5 m, took another sweep after 4 steps parallel to the

shoreline, worked back to the shoreline taking sweeps every 4 steps, took another 4 steps parallel to the shoreline, and repeated the procedure until we had sampled the entire pond perimeter. Several ponds (BE, CR, IR, CO, and PR) were too large to survey in their entirety, so we undertook a minimum of 40 sweeps along the shore. Numbers of newts, amphibian larvae, and several types of aquatic macroinvertebrates and fish were recorded for each sweep by using a digital voice recorder. Substratum characteristics also were recorded for each sweep, including the approximate depth (<15, 15–30, and 30–50 cm) and the presence of vegetation, leaves, or rocks. The proportion of sweeps containing vegetation, leaves, rocks, and only mud was calculated for each pond as well as the maximum depth of each pond, which was categorized as <1, 1–2, and >2 m. Sex, mass, snout–vent length, and breeding status were recorded for each individual newt. In addition, the number and location of attached leeches and the location and extent (proportion of each body part affected) of *Ichthyophonus* sp. disease signs were recorded. The location and extent of any skin ulcerations also were recorded for each newt. Dissolved oxygen and water temperature were recorded at a depth of 10 cm for each pond at each time point by using a model 95 dissolved oxygen meter (YSI Inc., Yellow Springs, Ohio); and pH was taken with a model 313 pH/temperature meter (Corning Life Sciences, Acton, Massachusetts). Water chemistry analyses were conducted by the Penn State Institutes of the Environments Water Quality Laboratory (University Park, Pennsylvania) (Ca²⁺: standard methods 3500A-Ca AA spectrometric; NO₃-N: standard methods 4500F automated cadmium reduction). After completion of each sweep survey, 10 newts were collected per pond for blood collection and dissection. Newts were transported to the laboratory in 250-ml Nalgene® containers filled with pond water, anesthetized with a drop of 15% benzocaine ointment (Orajel®; Del Laboratories, Inc., Uniondale, New York) on the head, and killed by decapitation within 3 hr of initial capture.

Disease diagnoses

Swelling of the axial musculature (as described by Converse and Green, 2005) and visible spores (0.1–0.5 mm in diameter) under the skin of any body part (generally under the transparent skin of the throat, abdomen, vent, and limbs) were considered diagnostic signs of *Ichthyophonus* sp. infection during sweep surveys (Fig. 1). *Ichthyophonus* sp. spores are spherical or ovoid cells containing endospores that develop in infected tissues (Mendoza et al., 2002). All newts which had been collected for dissection and exhibited these disease signs (n = 22) were examined for the presence of *Ichthyophonus* sp. spores. After fixation in 10% buffered neutral formalin for >24 hr, muscle tissue containing disease signs was embedded in paraffin, sectioned at 10 µm in thickness with a Shandon Finesse® Paraffin microtome (Thermo Electron Corporation, Waltham, Massachusetts), and stained with hematoxylin and eosin. None of the samples was decalcified before sectioning.

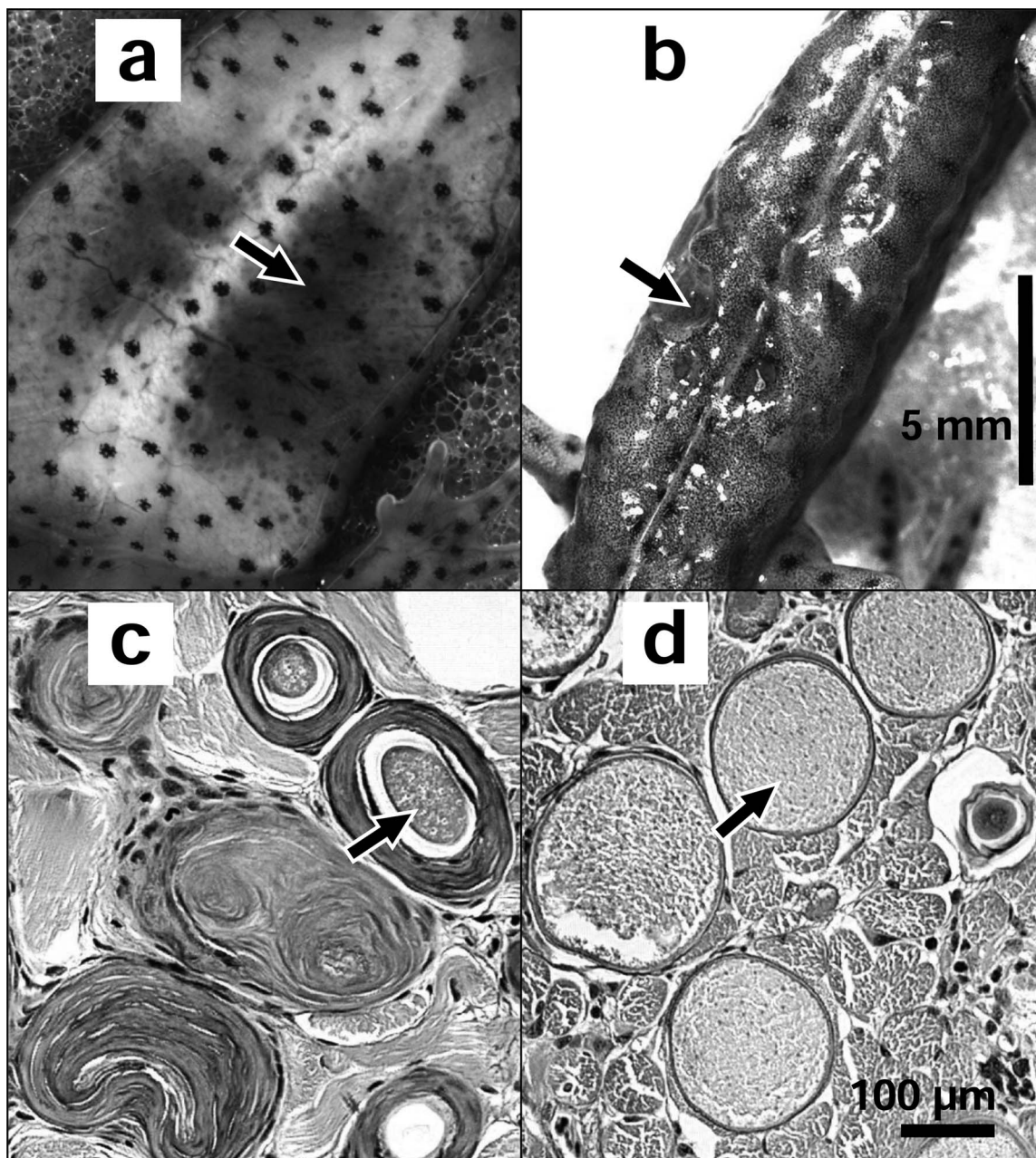


FIGURE 1. Macroscopic signs considered indicative of *Ichthyophonus* sp. infection: (a) visible intramuscular cysts, usually occurring in patches below the translucent skin of the throat, stomach, vent, or limbs (not to be confused with normal black spots on the skin surface), and (b) swelling of the axial musculature (the arrow indicates an ulceration). Histological examination of infected muscles: (c) resting spores (small, dense, with a wrinkled outer layer and often surrounded by a wall of host tissue) and (d) active spores (larger, with a smooth outer layer).

Additional newts collected from 5 of these ponds between March 2003 and June 2005 (Raffel et al., 2006) that showed signs of *Ichthyophonus* sp. infection in gross dissection ($n = 12$) also were examined histologically. Identification of *Ichthyophonus* sp. in photographs of histological sections was confirmed by David E. Green (United States Geological Survey National Wildlife Health Center, Madison, Wisconsin). Active and passive spores were identified according to Mikaelian et al. (2000).

Trypanosome infection was determined from blood smear counts. Blood was collected from newts with a heparinized capillary tube, and a small drop was smeared on a glass microscope slide. Slides were air-dried for 10 min, fixed in methanol for 5 min, and again allowed to dry. Slides were placed in 1% o-dianisidine (3,3'-dimethoxybenzidine; Sigma, St. Louis, Missouri) in methanol for 90 sec, destained in 1%

hydrogen peroxide in 50% ethanol for 90 sec, and rinsed twice in deionized water for 30 sec. Slides were then stained in Giemsa stain for 30 min and rinsed again in deionized water for 10 min. Cells were counted at $\times 400$ magnification starting at the edge of the smear where the initial drop of blood had been placed and working down the slide in a gridded pattern, moving the objective 2 mm between fields and counting all cells in each field until the erythrocyte count reached 5,000. Trypanosomes were quantified as parasites per 5,000 erythrocytes.

Leeches were identified as *Placobdella picta* according to the key of Klemm (1985). Voucher specimens of *Ichthyophonus* sp. (USNPC 98177–98181), *Trypanosoma diemyctyli* (USNPC 98171–98177), and *Placobdella picta* (USNPC 98182; 3 vials) have been submitted to the USNPC, Beltsville, Maryland.

TABLE II. Variables included in between-population analyses of *Ichthyophonus* sp. prevalence and leech abundance (leeches per newt).

Hypothesized predictors of <i>Ichthyophonus</i> sp. prevalence	
Potential intermediate hosts	Host population
Tadpole density	Newt density
Salamander larva density	Seasonality:
Snail density	Date
Fingernail clam density	Water quality
Dragonfly larva density	Temperature (shallow)
Damselfly larva density	Ca ²⁺ concentration
Caddisfly larva density	Nitrate concentration
Leeches per newt*	pH
Fish density	Dissolved oxygen
Total fish density	Pond characteristics
Sunfish density	Water inflow to pond*
Pickrel density	Pond depth
Chub density	
Hypothesized predictors of leech abundance	
Host density	Substrate
Adult newts	Proportion vegetation*
All amphibian larvae	Proportion muddy
Tadpole density	Proportion rocks
Salamander larva density	Proportion leaves
Pond community	Seasonality
Total fish density	Date*
Sunfish density	Water quality
Pickrel density*	Temperature (shallow)
Chub density	Ca ²⁺ concentration
Snail density	pH
Fingernail clam density*	Dissolved oxygen
Total mollusc density*	Pond characteristics
Host behavior	Water inflow to pond
Average newt depth	Pond depth

* $P < 0.1$ in absence of other predictor variables.

Statistical analyses

Newts have high site fidelity to breeding ponds as adults (Gill, 1978), so newts from different ponds were treated as independent breeding populations in the analyses. The best between-population predictors of *Ichthyophonus* sp. prevalence, trypanosome prevalence, and numbers of leeches per newt were determined by backward selection of linear regression models with normal errors and weighted by sample size. Prevalence data were arcsine transformed to correct for non-normality of proportional data, and count data, i.e., leeches per newt and individuals per sweep, were summarized for each pond by calculating the mean natural log of abundance ($\ln[\text{count} + 1]$). Because there were more potential predictor variables than error degrees of freedom (Table II), response variables were first regressed against each predictor individually and only predictor variables with $P < 0.1$ were included in the maximal model (Table II). The minimum adequate model was selected as described by Crawley (2002), excluding variables that did not significantly add to the variance explained by the model (F -statistic; $P < 0.05$). Once the minimal model had been selected, all postulated predictors were tested for significance by using the selected predictor(s) as covariates.

The significance of individual newt characteristics (sex, snout-vent length, mass, body condition, and breeding condition) for predicting *Ichthyophonus* sp. infection, trypanosome infection, and numbers of leeches per newt was determined by backward selection of generalized linear mixed models including pond (population) as a random term. Effects of individual *Ichthyophonus* sp. infection status on the number of currently attached leeches and vice versa also were tested using these models. These models were estimated using restricted marginal quasi-likelihood, with binomial errors for presence/absence of infection and Poisson errors for the number of leeches per newt. Models were selected

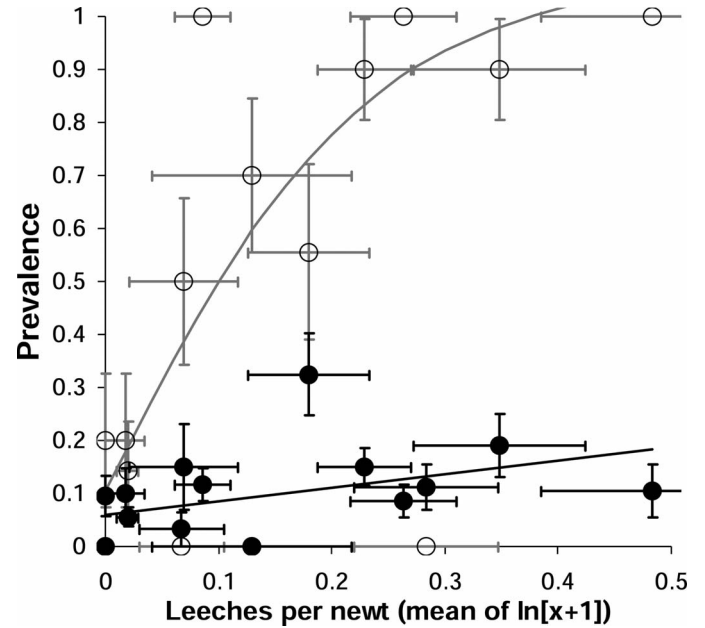


FIGURE 2. Between-population relationships between leech abundance and infection prevalence of *Ichthyophonus* sp. (closed circles and black error bars) and trypanosomes (open circles and gray error bars). The trypanosome data have been fitted with a spline curve ($df = 3$ and omitting the outlying data point for LA [0.28, 0]). Leech counts were natural log transformed. Error bars represent \pm SEs.

as described above for the between-population analyses, except that the significance of predictor variables were determined using submodel deviance tests (chi-square test; $P < 0.05$). *Ichthyophonus* sp.-infected individuals were analyzed separately using generalized linear models to determine whether intensity of infection (proportion of body surface covered by disease signs, arcsine transformed) or the presence of swollen axial musculature (binomial errors) depended on the snout-vent length, sex, mass, breeding condition, or body condition of the affected newt.

One-way chi-square goodness-of-fit tests were used to test the null hypothesis that numbers of leech and *Ichthyophonus* sp. observations on each body part were proportional to the surface area of each body part. The number of *Ichthyophonus* sp. observations on each body part (corrected for surface area) was then regressed against the number of leeches on each part (also corrected for surface area) with normal errors, weighting for the percent surface area of each body part. All analyses were run using S-PLUS® statistical software (Insightful Corp, Seattle, Washington).

RESULTS

Infection by *Ichthyophonus* sp. was recorded from 12 of the 16 newt populations surveyed (875 total newts observed, between 16 and 132 newts from a given pond). The only significant predictor of *Ichthyophonus* sp. prevalence in the between-population analysis was the number of leeches observed feeding on newts ($F = 4.75$; $df = 1, 14$; $P = 0.0468$) (Fig. 2). None of the variables predicted by the trophic transmission hypotheses showed any between-population relationships with *Ichthyophonus* sp. prevalence even before including leeches in the model (all $P > 0.1$). Trypanosomes also showed a highly significant between-population effect of leeches ($F = 17.56$; $df = 1, 14$; $P = 0.0009$) but not of other variables. For the 9 populations in which both *Ichthyophonus* sp. and trypanosomes were detected, trypanosome infection was approximately 6-fold more

TABLE III. Minimal adequate models for within-population variation in *Ichthyophonus* sp. prevalence and leech bite rates.

Response	Predictor	Coefficient	df	X ²	P
<i>Ichthyophonus</i> sp. (presence/absence)	Mass	0.514	1	11.4	0.0007
	Snout-vent length	0.211	1	14.1	0.0001
	Breeding	-0.546	1	38.7	<0.0001
	Sex*	0.199	1	2.3	0.1993
	Breeding:sex	-0.297	1	5.2	0.0220
Leeches (no. attached to newt)	Mass	-0.015	1	34.5	<0.0001
	Sex*	-0.109	1	4.2	0.0400
	Breeding	0.205	1	85.0	<0.0001

* Female = 0, male = 1.

prevalent than *Ichthyophonus* sp. (mean ratio 5.66:1; SE = 1.09) (Fig. 2).

Snout-vent length, mass, and breeding condition were all significant predictors in the within-population analysis of *Ichthyophonus* sp. infection risk (Table III). Snout-vent length and mass were positive predictors of *Ichthyophonus* sp. infection, whereas newts in breeding condition were less likely to be infected (Table III). There was also a significant sex by breeding interaction, with nonbreeding males more likely to be infected than breeding males or females (Table III). With breeding condition removed from the model, male newts had significantly higher *Ichthyophonus* sp. prevalence than female newts ($\chi^2 = 4.5$, df = 1, $P = 0.0342$, coefficient = 0.239). The variance component for the random term pond (SD = 0.589, 95% confidence interval between 0.292 and 1.188) significantly improved the deviance explained by the model ($\chi^2 = 12.2$, df = 1, $P = 0.0005$). Newts were more likely to be infected with *Ichthyophonus* sp. if they had greater snout-vent lengths, had greater mass, or were male (Table III). None of the variables measured for individual newts was significant within-population predictors of trypanosome prevalence ($P > 0.05$).

Both *Ichthyophonus* sp. disease signs and leeches were found to have a nonrandom distribution on the bodies of newts (*Ichthyophonus*: $\chi^2 = 43.6$, df = 7, $P < 0.0001$; leeches: $\chi^2 = 114.2$, df = 7, $P < 0.0001$). Parts of the body on which leeches were observed more often (corrected for surface area of body part) were also more likely to exhibit *Ichthyophonus* disease signs ($F = 20.1$; df = 1, 6; $P = 0.0042$), with both leeches and disease signs more likely to be found on the head and trunk of the body than on the extremities (Fig. 3).

Of 88 newts with visible signs of *Ichthyophonus* sp. infection, 65 (73.9%) had swollen axial muscles and 39 (44.3%) had visible spherules under the skin (16 had both). Of the 65 newts with swollen axial muscles, 14 (21.5%) had skin ulcerations over the affected areas. No other newts with skin ulcerations were observed during this study. The intensity of infection (proportion of body surface covered by disease signs) ranged from approximately 0.35 to less than 0.01, with a mean intensity of 0.090 (SE = 0.008). The mean coverage of the head and trunk was 0.115 (SE = .0128). For newts which were infected, neither the presence of swollen axial muscles nor the intensity of infection (proportion of body surface covered) related significantly with a newt's snout-vent length, mass, body condition, breeding condition, or sex (all $P > 0.05$).

The disease signs of all 22 newts collected for dissection and showing swollen axial muscles or visible spores (16 with swell-

ing, 9 with visible spores, 3 with both) were confirmed by histological examination to be caused by *Ichthyophonus* sp.-like spores. We found no evidence of infection in any dissected newts lacking visible signs of infection, and even those newts with visible infections of the skeletal musculature showed no evidence of *Ichthyophonus* sp. infection in their internal organs or digestive tract. Most of the infected newts with swollen axial musculature had high proportions of active spores (13 of 16 with more than 50% of spores active), but all 6 newts lacking obvious axial swelling (visible spores only) contained predominantly (>50%) passive spores. All 12 additional newts collected during the seasonal survey and having visible signs of infection at the time of dissection also were confirmed to be infected with *Ichthyophonus* sp. by histology. To find no false positives in 34 randomly selected infected newts provides the statistical power to conclude that the true false positive rate was less than 7.4% (based on a binomial test with $\alpha = 0.05$), indicating that the presumptive field diagnoses were accurate indicators of *Ichthyophonus* sp. infection in red-spotted newts. Ten *Ichthyophonus*-infected newts also were found to be infected with trypanosomes, of 22 examined.

Significant predictors were found for leech attachment rates in both the within- and between-population analyses. The proportion of sweeps with emergent vegetation was the only significant between-population predictor of the number of leeches per newt ($F = 17.56355$; df = 1, 14; $P = 0.0009$), with more leeches in ponds with more vegetation (Fig. 4). In the within-population analysis, newts had higher numbers of attached leeches if they were in breeding condition, had lower mass, or were female (Table III). There was no significant effect of *Ichthyophonus* sp. infection on the current number of attached leeches ($\chi^2 = 0.6$, df = 1, $P > 0.4$) or of the number of currently attached leeches on the probability of being infected with *Ichthyophonus* sp. ($\chi^2 < 0.1$, df = 1, $P > 0.8$). The variance due to the random term pond (SD = 1.08, 95% confidence interval between 0.66 and 1.77) significantly improved the deviance explained by the model ($\chi^2 = 76.3$, df = 1, $P < 0.0001$).

DISCUSSION

Several predictions of the leech transmission hypothesis were supported by our results. The number of leeches feeding on newts was the only significant between-population predictor of *Ichthyophonus* sp. prevalence in this study. This pattern is consistent with vector-borne disease models that predict that the basic reproductive number of the parasite (R_0) will be propor-

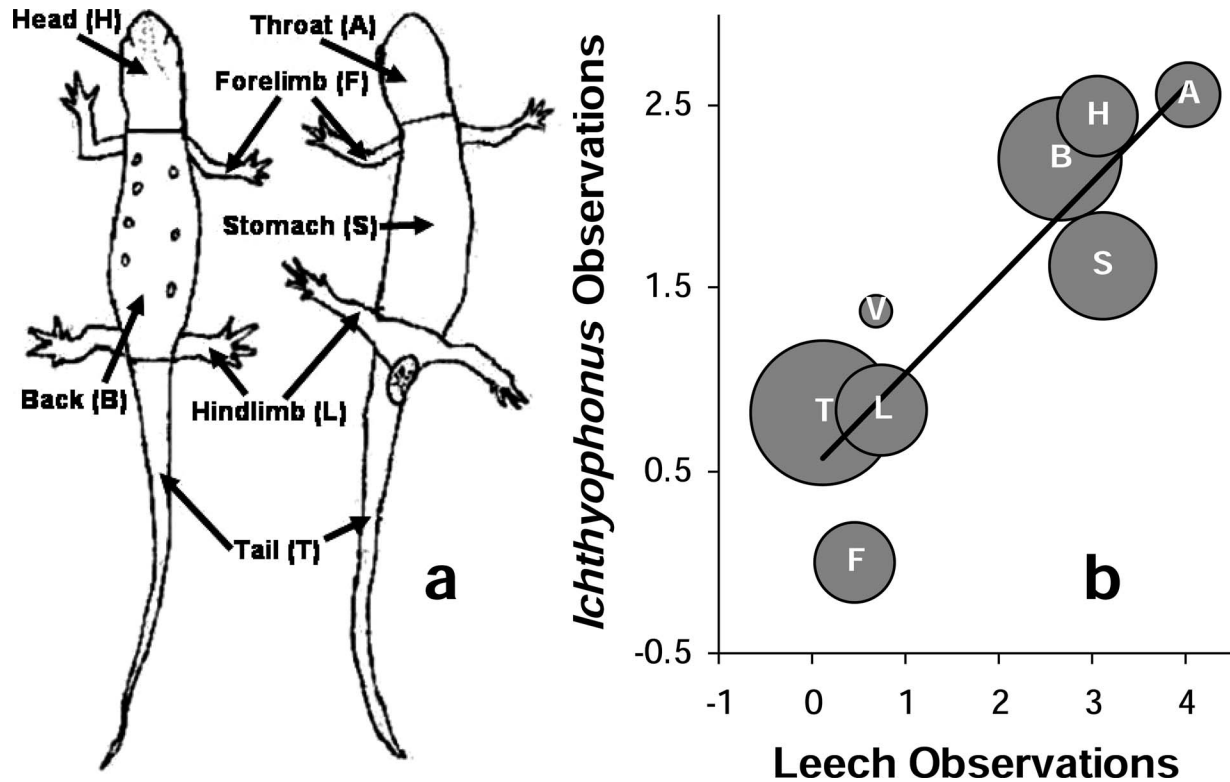


FIGURE 3. (a) The parts of newts' bodies on which leeches and *Ichthyophonus* sp. signs were observed were recorded for all newts. (b) Parts of newts' bodies with higher counts of leeches than expected also were more likely to have signs of *Ichthyophonus* sp. infection. Counts of leeches and *Ichthyophonus* sp. signs have been corrected for the surface area of the body part, which is indicated by the size of the circle. Data point labels in b correspond to letters assigned to parts of the body in a.

tional to the ratio of vectors to hosts (Hudson et al., 1995; Randolph et al., 2002). Trypanosomes, known to be leech-transmitted, exhibited a similar between-pond relationship with leech abundance. An alternative hypothesis, that trypanosome-in-

duced immunosuppression facilitates *Ichthyophonus* sp. infection from some source other than leeches, seems unlikely due to the absence of trypanosome infection in more than half of the *Ichthyophonus* sp.-infected newts examined. In addition, the distribution of *Ichthyophonus* sp. observations on newt bodies was nonrandom, with signs of infection concentrated on those body parts most likely to be bitten by leeches. Finally, during a capture-mark-recapture study of *Ichthyophonus* sp. infection dynamics in newts, Raffel (2006) observed new (less than 1-mo-old) infections growing out from distinctive subcutaneous hematomas. These hematomas frequently form during *Placobdella picta* feeding bouts (Barta and Sawyer, 1990) and are common in ponds and seasons with high leech bite rates (Raffel, 2006), suggesting that leeches are the most likely cause of these haematomas. It should be noted that the leech transmission hypothesis assumes that the prepatent period for *Ichthyophonus* sp. infection is longer than 5- to 48-hr duration of leech feeding (Barta and Sawyer, 1990), because recent *Ichthyophonus* sp. infections have not been observed growing out from sites of actively feeding leeches (T. R. Raffel, pers. obs.), and there was no effect of *Ichthyophonus* sp. infection status on the current numbers of leeches attached to newts. We found no support for either of the 2 trophic transmission hypotheses, with *Ichthyophonus* sp. prevalence showing no relationships with densities of newts, benthic macroinvertebrates, amphibian larvae, or fish.

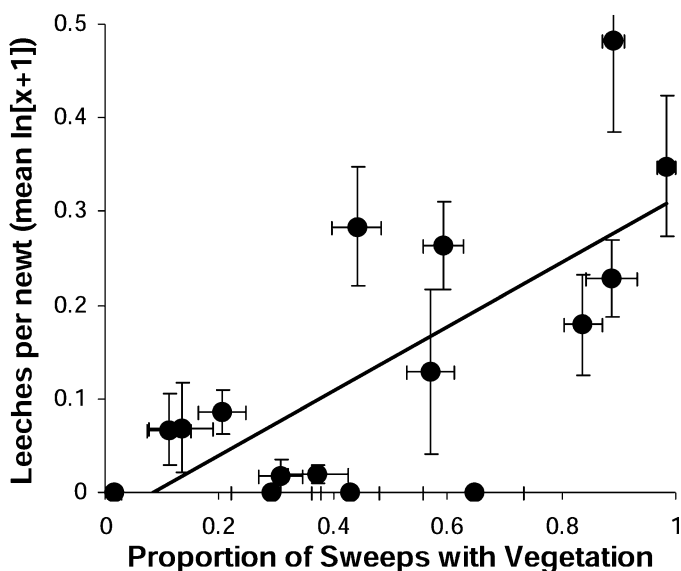


FIGURE 4. More leeches were found per newt in ponds with more emergent vegetation. Leech counts were log transformed. Error bars represent \pm SEs.

The leech transmission hypothesis also helps explain 3 previously unexplained characteristics of this parasite. *Ichthy-*

ophonus sp. can be found in any of the skeletal musculature, including the tail and limbs, but not in the internal organs or digestive tract as predicted by the trophic transmission hypotheses (Mikaelian et al., 2000). Our results corroborated this observation. This distribution is better explained by the leech transmission hypothesis and suggests direct injection of the pathogen into the musculature by leeches. In addition, the absence of *Ichthyophonus* sp. in sympatric fish can be readily explained by the specialization of *P. picta* on amphibian hosts (Sawyer, 1972; Mikaelian et al., 2000). Finally, amphibian *Ichthyophonus* sp. has proven impossible to culture by using methods that have worked for *I. hoferi* (Mikaelian et al., 2000). This difficulty may be due to different conditions in the leech gut from that of the fish gut, where *I. hoferi* spores are induced by acidic pH to form hyphae (Spanggaard et al., 1995).

Most leech-vectored parasites are blood-borne, e.g., *Trypanosoma diemyctyli*, *Haemogregarina balli*, *Lankesterella minima*, and *Babesiosoma stableri*, and they are transmitted to leeches in blood meals from infected hosts (Tse et al., 1986; Mock, 1987; Barta and Dessler, 1989; Siddall and Dessler, 1990). How then might a leech acquire an intramuscular parasite from its host? We think that the proboscis of *P. picta*, used to probe for blood vessels beneath the skin of their amphibian hosts (Barta and Sawyer, 1990), may be crucial for transmission of *Ichthyophonus* sp. from infected amphibians to leeches. This proboscis is approximately 20% of the leech's body length, or 2.6–5 mm for adult leeches (Klemm, 1985; Barta and Sawyer, 1990), easily long enough to reach tissues containing *Ichthyophonus* sp. spores. We propose that a leech acquires *Ichthyophonus* sp. infection by inducing rupture of spores as its proboscis probes into the muscles beneath the skin of an infected newt, either mechanically or by some chemical cue. If this organism is similar to other mesomycetozoeans, this rupture would induce the release of endospores that would develop into a motile infectious stage capable of developing into a new spore containing endospores (Mendoza et al., 2002). Members of the order Ichthyophonida with known transmission mechanisms (*Ichthyophonus hoferi*, *Amoebidium* spp., and *Psorospermium* spp.) have amoeboid infectious stages, unlike members of the related order Dermocystida, whose endospores develop into flagellated zoospores (Mendoza et al., 2002). After rupture, these amoebae or their endospore precursors could infect the leech proboscis, which would then inject infectious amoebae into host tissues during subsequent feeding bouts. This proposed mode of transmission by amoeboid cells attached to the proboscis is not altogether different from that of *Trypanosoma fallisi* in *P. picta*, which migrates to the proboscis following ingestion in a leech blood meal (Martin and Dessler, 1991).

If this hypothesis is correct, transmission of *Ichthyophonus* sp. to leeches should be less efficient than transmission of blood-borne parasites. Any blood meal may contain a small number of trypanosomes, but acquiring *Ichthyophonus* sp. infection would require feeding directly over a patch of infected muscle. The probability of transmission may be somewhat increased by the aggregation of leeches on the head and trunk of newts; however, the average coverage of these body parts was still only 11.5%. Therefore, a leech should be at least 8 times more likely to acquire a blood-borne parasite than *Ichthyophonus* sp. Our data show that trypanosome prevalence is approxi-

mately 6-fold higher than *Ichthyophonus* sp. prevalence in populations with both diseases, consistent with this prediction.

The increased prevalence of *Ichthyophonus* sp. in larger newts (greater snout–vent length) probably reflects an age prevalence relationship in newts. The higher prevalence of infection in nonbreeding newts, however, is more likely reflect an effect of *Ichthyophonus* sp. infection on breeding condition than vice versa. Breeding is generally predicted to increase susceptibility to parasitic infection (Rolff, 2002), an effect unlikely to have led to higher prevalence in breeding newts. Because the breeding season of newts ends in late spring (Petranka, 1998), about the time this survey was conducted, it seems more likely that newts come out of breeding condition sooner when infected. The greater prevalence in male newts and in those with greater mass could be related to differences in susceptibility or exposure to the parasite. These patterns were not apparently due to differences in leech bite rates, which showed the opposite trends. One possibility is that male newts spend more time in the water than females and are less likely to skip a year of breeding, thereby increasing their lifetime exposure to leeches (Gill et al., 1983). This also may be true of individuals with greater mass, which may be more likely to breed in a given year due to better nutritional status. Alternately, male newts may be more susceptible due to suppression of the immune system by testosterone (Zuk and Stoehr, 2002), although the lack of a difference in infection intensity between the sexes contradicts this hypothesis.

Our results suggest that *Ichthyophonus* sp. is a common infection in red-spotted newt populations. Twelve of 16 surveyed populations harbored the infection, and low prevalence has since been observed in 2 of the remaining ponds (data not shown). More than half of the infected newts showed severe swelling of the axial musculature, filled predominantly with active spores and often associated with ulcerations. These ulcerations may lead to secondary infections that act as the immediate cause of death, a phenomenon observed in our laboratory and by other researchers (Herman, 1984; T. R. Raffel, unpubl. obs.). The replacement of muscle tissue by *Ichthyophonus* sp. spores also may increase mortality by lowering foraging ability and escape responses to predators. The near ubiquity and potentially severe consequences of *Ichthyophonus* sp. infection may make this parasite a significant source of mortality for red-spotted newts, but the results of this study are insufficient to draw substantive conclusions about the rate of mortality due to *Ichthyophonus* sp. infection.

The leech transmission hypothesis seems to be the parsimonious explanation of patterns of *Ichthyophonus* sp. infection in red-spotted newts. The current evidence is correlational, however, and more definitive confirmation of this hypothesis will require experimental transmission of *Ichthyophonus* sp. between amphibians. If the leech transmission hypothesis is correct, predicting future outbreaks of *Ichthyophonus* sp. infection may be possible by considering the environmental factors that influence leech abundance. The strongest predictor of the number of leeches attached to newts in our study was the abundance of emergent vegetation. Aquatic vegetation provides the solid footing and structural complexity that glossophoniid leeches need to reach their hosts, since they are incapable of swimming and must crawl inchwormlike along a solid substrate (Sawyer, 1986). *Placobdella picta* also is thought to be host limited, with

high leech attachment rates in years after peaks in amphibian population densities (Gill et al., 1983; Mock, 1983). *Ichthyophonus* sp. outbreaks should therefore be most likely in wetlands with plenty of emergent vegetation and after years of high amphibian reproduction rates. Increased abundance of littoral vegetation is often associated with anthropogenic eutrophication, which also has been linked to increasing rates of trematode-induced limb deformities in amphibians (Rast and Thornton, 1996; Johnson and Chase, 2004). If leeches are indeed the vector of *Ichthyophonus* sp. infection in amphibians, anthropogenic eutrophication might contribute to outbreaks of this parasite.

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