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EVOLUTIONARY AVENUES FOR, AND CONSTRAINTS ON, THE TRANSMISSION OF FROG LUNG FLUKES (*HAEMATOLOECHUS* SPP.) IN DRAGONFLY SECOND INTERMEDIATE HOSTS

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ABSTRACT: Metacercariae survival patterns and their distribution in second intermediate odonate hosts were examined for 4 species of frog lung flukes. Surveys of aquatic larvae and recently emerged teneral dragonflies and damselflies indicated that prevalence and mean abundance of *Haematoloechus* spp. metacercariae were significantly lower in teneral dragonflies than larval dragonflies, while there was no significant difference in prevalence or mean abundance of *Haematoloechus* spp. metacercariae among larval and teneral damselflies. Experimental infections of dragonflies indicated that metacercariae of *Haematoloechus coloradensis* and *Haematoloechus complexus* were located in the head, thorax, and branchial basket of dragonflies, whereas metacercariae of *Haematoloechus longiplexus* and *Haematoloechus parviplexus* were restricted to the branchial basket of these hosts. Metacercariae of *H. coloradensis*, *H. complexus*, and *H. longiplexus* infected the head, thorax, and abdomen of damselflies, but these insects were resistant to infection with *H. parviplexus*. Subsequent metamorphosis experiments on experimentally infected dragonflies indicated that most metacercariae of *H. longiplexus* were lost from the branchial basket during metamorphosis, but most metacercariae of *H. coloradensis*, *H. complexus*, and *H. parviplexus* survived dragonfly metamorphosis. These observations suggest that the observed ecological host specificity of *H. longiplexus* in semiterrestrial leopard frogs may be due to few metacercariae of *H. longiplexus* reaching these frogs in a terrestrial environment. Because of the uncertain validity of *Haematoloechus varioplexus* as a distinct species from its synonym *H. parviplexus*, their morphological characters were reevaluated. The morphological data on *H. varioplexus* and *H. parviplexus* indicate that they differ in their acetabulum length and width, ovary shape, testes length, and egg length and width. Experimental infections of plains leopard frogs, northern leopard frogs, and bullfrogs with worms from bullfrogs indicate that the synonymy of *H. parviplexus* with *H. varioplexus* is not warranted, and that these flukes are distinct species, i.e., *H. parviplexus* in bullfrogs and *H. varioplexus* in plains leopard frogs and northern leopard frogs.

Field data on the population structure of *Haematoloechus* species among aquatic and semiterrestrial anurans suggests that ecological differences and diet of these hosts affect lung fluke distribution in the definitive hosts (Brooks, 1976; Dronen, 1977; Bolek and Coggins, 2003; Bolek and Janovy, 2007). Studies by Brooks (1976) and Snyder (1996) indicate that in Nebraska, 3 species of *Rana* commonly serve as definitive hosts for 5 species of frog lung flukes. Bullfrogs, *Rana catesbeiana*, are large aquatic species that are commonly infected with 2 species (*Haematoloechus longiplexus* and *Haematoloechus varioplexus*, currently a synonym of *Haematoloechus parviplexus*) but are resistant to infections with *Haematoloechus medioplexus*, *Haematoloechus coloradensis*, and *Haematoloechus complexus*. *Rana blairi*, the plains leopard frog, and *Rana pipiens*, the northern leopard frog, both of which are medium sized semiterrestrial anurans, are infected with *H. coloradensis*, *H. complexus*, and *H. medioplexus*. Field studies indicate that both of these leopard frogs are rarely or never infected with *H. longiplexus* or *H. varioplexus*. Both Brooks (1976) and Snyder (1996) found over 40% of bullfrogs infected with *H. longiplexus*, but only 1–2% of plains leopard frogs and none of the northern leopard frogs was infected in nature. Snyder (1996) in an experimental study infected 7 of 10 (70%) bullfrogs, 2 of 5 (40%) plains leopard frogs, and 3 of 6 (50%) northern leopard frogs with *H. longiplexus*, indicating that they are suitable hosts for this lung fluke. *Haematoloechus varioplexus* has been reported from all 3 of these frog species. However, since Kennedy (1981) synonymized *H. varioplexus* and *H. parviplexus* from leopard frogs and bullfrogs, *H. varioplexus* has not been found in northern leopard frogs and plains leopard frogs in Nebraska when collected from the same locations as bullfrogs that harbor this species (Snyder, 1996).

This ecological host specificity at the definitive host level is particularly interesting, considering that recent studies by Snyder and Janovy (1994, 1996) and Bolek and Janovy (2007) have shown that the cercariae behavior patterns of 5 North American *Haematoloechus* species dictate host specificity at the second intermediate host level. *Haematoloechus coloradensis* and *H. complexus* are generalists within the arthropod host; metacercariae of these species are able to develop in a wide range of aquatic arthropod hosts, including dragonflies (anisopteran) and damselflies (zygopterans), as well as nonodonate arthropods. Metacercariae of *H. medioplexus* and *H. varioplexus* only develop in dragonflies and are considered specialists. Metacercariae of *H. longiplexus* develop in both dragonflies and damselflies, and this species is considered to have intermediate arthropod host specificity.

Studies on the diet of semiterrestrial leopard frogs and aquatic bullfrogs indicate that they commonly feed on odonates; however, leopard frogs predominantly ingest terrestrial stages of odonates, whereas bullfrogs predominantly feed on aquatic stages of odonates (Korschgen and Baskett, 1963; Dronen, 1977). Of the 3 species of *Haematoloechus* that strictly use odonates as second intermediate hosts, only *H. medioplexus* commonly infects semiterrestrial leopard frogs in Nebraska (Brooks, 1976; Snyder, 1996; Bolek and Janovy, 2007). These observations suggest that second intermediate odonate hosts may act as filters or sieves (Euzet and Combes, 1980); only certain lung fluke species are able to pass through all filters and end up in the appropriate terrestrial environment and infect leopard frogs. Therefore, these differences in second intermediate host specificity and second intermediate host life histories may play an important role in parasite movement, distribution, and the observed host specificity in definitive frog hosts.

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To investigate the role of odonate second intermediate hosts in the transmission of frog lung flukes to their anuran hosts, the present study has 4 main goals: (1) determine the distribution of different species of frog lung flukes in 6 amphibian species from a single location; (2) determine the distribution of frog lung fluke metacercariae in different life stages of aquatic and terrestrial odonate second intermediate hosts from a single location; (3) test whether metacercariae of 4 common *Haematoloechus* species can survive metamorphosis in dragonfly and damselfly second intermediate hosts; and (4) reevaluate the definitive host specificity and diagnostic characteristics of *H. parviplexus* and *H. varioplexus* in bullfrogs and leopard frogs. Our study provides laboratory and field data on 4 congeneric flukes and their avenues for, and constraints on, transmission by odonate second intermediate hosts to their frog definitive hosts, data that will allow future testing of hypotheses with respect to the evolution of frog lung fluke life cycles.

MATERIALS AND METHODS

Amphibian field studies

During March 2001–June 2005, some 399 individual amphibians of 6 species were collected from Pawnee Lake, Lancaster County, Nebraska (40.84310, −96.5261), and examined for *Haematoloechus* species. These included 50 Blanchard's cricket frogs, *Acris crepitans blanchardi*, 50 Woodhouse's toads, *Bufo woodhousii*, 36 Cope's gray treefrogs, *Hyla chrysoscelis*, 93 western chorus frogs, *Pseudacris triseriata triseriata*, 70 plains leopard frogs, *R. blairi*, and 100 bullfrogs, *Rana catesbeiana*. Frogs and toads were collected at night by hand, brought back to the laboratory, and killed; the snout vent length (SVL) was then measured, and, on necropsy, all organs were examined for parasites within 1–2 days of collection. Trematodes were removed, allowed to release eggs in tap water, and fixed in alcohol-formalin-acetic acid (AFA). Representative specimens were stained with Seminchon's acetocarmine (Pritchard and Kruse, 1982). All lung flukes were initially identified based on the keys provided by Kennedy (1981) and descriptions of *H. complexus* by Krull (1933) and *H. coloradensis* by Cort (1915). The chi-square test for independence was calculated to compare differences in prevalence among frogs. Voucher specimens have been deposited in the H.W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska (accession numbers HWML 48480, *Haematoloechus coloradensis*; 48481 *Haematoloechus complexus*; 48482 *Haematoloechus longiplexus*; 48483 *Haematoloechus parviplexus*; and 48484 *Haematoloechus varioplexus*).

Odonate field studies

During June–July 2001, some 381 larval, teneral, and adult anisopteran and zygopteran odonates of 10 species (Table I) were collected from Nickol Pond, Cass County, Nebraska (40.81412, −96.46000) and examined for metacercariae of *Haematoloechus* spp. Larval odonates were collected by dip-net, placed in buckets of water with no snails, and brought back to the laboratory. Teneral and adult odonates were collected with a butterfly net along the edges of Nickol Pond, placed in 3.78-L plastic containers, stored on ice, and brought back to the laboratory. All odonates were identified according to Westfall and May (1996) and Needham et al. (2000) before being processed. Each individual zygopteran larva, teneral odonate, and adult odonate was divided into 3 body regions, i.e., the head, the thorax including the legs, and the abdomen including the anal gills for the larvae. All larval, teneral, and adult anisopteran odonates were divided into 4 body regions, i.e., the head, the thorax including the legs, the abdomen, and the branchial basket for larvae or remnants of the branchial basket for the tenerals and adults. All odonates were then teased apart and examined for metacercariae of *Haematoloechus* spp. and their site of infection. All *Haematoloechus* spp. metacercariae were identified to genus based on descriptions provided by Krull (1930, 1931, 1932, 1933, 1934); species identification was not attempted. Owing to low samples sizes of some species and the ability of adult dragonflies and damselflies to colonize ponds from locations other than where they metamorphosed (Conrad et al., 1999), statistical comparisons of infections were only compared among larval and teneral stages of the 2 most commonly collected dragonfly and damselfly species. The chi-square test for independence was calculated to compare differences in prevalence of larval and teneral life stages of odonates, while Student's *t*-test was used to compare differences in mean abundance between larval and teneral life stages of odonates. Approximate *t*-tests were calculated when variances were heteroscedastic (Sokal and Rohlf, 1981).

TABLE I. Prevalence and mean intensity of *Haematoloechus* spp. metacercariae in 10 species of larval, teneral, and adult odonates collected from Nickol Pond, Cass County, Nebraska, during June–July 2001.

Odonate species	Life stage (n)	Prevalence (%)	Mean intensity ±1 SD
Zygoptera			
Enallagma civile			
	Larva (15)	80	1.25 ± 0.6
	Teneral (4)	0	—
	Adult (5)	0	—
I. verticalis			
	Larva (80)	22.5	1.3 ± 0.8
	Teneral (28)	28	1.9 ± 1.5
	Adult (46)	2.3	1
Total Zygoptera			
	Larva (95)	31.6	1.3 ± 0.8
	Teneral (32)	25	1.9 ± 1.5
	Adult (51)	2	1
Anisoptera			
Anax junius			
	Larva (24)	0	—
Celithemis eponina			
	Adult (2)	0	—
E. simplicicollis			
	Larva (75)	18.7	1.5 ± 1.1
	Teneral (74)	5.4	2.2 ± 1.6
	Adult (5)	20	3
Libellula luctuosa			
	Larva (2)	0	—
	Teneral (9)	0	—
	Adult (3)	0	—
Pachydiplax longipennis			
	Larva (9)	33	1.3 ± 0.6
	Adult (2)	0	—
Perithemis tenera			
	Adult (1)	0	—
S. rubicundulum			
	Adult (2)	0	—
Tramea lacerata			
	Larva (1)	0	—
Total Anisoptera			
	Larva (111)	15.3	1.5 ± 1
	Teneral (83)	4.8	2.2 ± 1.6
	Adult (15)	6.6	3

Odonate metamorphosis experiments

Two species of odonates that were commonly infected in nature with frog lung fluke metacercariae were chosen for experimental infections and metamorphosis experiments. These included the eastern forktail damselfly, *Ischnura verticalis*, and the eastern pondhawk dragonfly, *Erythemis simplicicollis*. Both of these species are commonly found in Nebraska and easily maintained in the laboratory; previous reports indicate that they serve as intermediate hosts for frog lung flukes in other

locations throughout North America (Grieve, 1937; McVey, 1985; Snyder and Janovy, 1996; Westfall and May, 1996; Wetzel and Esch, 1996; Corbet, 1999; Needham et al., 2000).

During June–July 2002, some 100 naturally exposed ultimate or penultimate instars of the eastern pondhawk dragonfly, *E. simplicicollis*, and 25 naturally exposed ultimate or penultimate instars of the eastern forktail damselfly, *I. verticalis*, were collected by dip-net at Nickol Pond. Each odonate was placed in an individual 1,000-ml jar with 200 ml of aged tap water along with a single 15-cm wooden applicator stick as a perch; then the jar was covered with a screen lid. All odonates were fed field-collected chironomid larvae, freshwater oligochaetes, or laboratory-reared *Daphnia pulex* crustaceans 3 times a week; water was changed weekly. Cast exuviae were removed and examined for *Haematoloechus* spp. metacercariae. After metamorphosis, all teneral damselflies and dragonflies, along with their respective exuviae, were divided into 3 or 4 body regions, respectively, and examined for *Haematoloechus* spp. metacercariae. Because previous studies indicate that some species of *Haematoloechus* metacercariae take up to 4 days to become infective to the definitive frog host, only dragonflies that were maintained in the laboratory for at least 1 wk before metamorphosis were used in data analysis (Krull, 1930, 1931, 1933). Larval prevalence and mean abundances were calculated by combining any metacercariae left in the exuviae and the metacercariae recovered from the teneral stage. Additionally, 10 *E. simplicicollis* exuviae were collected from Nickol Pond, soaked in water, and examined for *Haematoloechus* spp. metacercariae to confirm that the loss of metacercariae during metamorphosis was not a laboratory artifact. Comparisons among mean abundance of larval and teneral life stages of *I. verticalis* and *E. simplicicollis* were compared with paired *t*-tests, while prevalence was compared with the chi-square test for independence. The Wilcoxon single rank test was calculated when variances were heteroscedastic (Sokal and Rohlf, 1981).

Odonate experimental infections

Gravid *H. coloradensis* flukes were obtained from wild-caught northern leopard frogs, *R. pipiens*, from Cedar Creek, Keith County, Nebraska (41.18639, −101.36276); gravid *H. complexus* flukes were obtained from wild-caught plains leopard frogs, *R. blairi*, in Pawnee Lake, Lancaster County, Nebraska; and gravid *H. longiplexus* and *H. parviplexus* flukes were obtained from wild-caught bullfrogs, *R. catesbeiana*, in Pawnee Lake, Lancaster County and Nevens Pond, Keith County, Nebraska (41.20710, −101.40850), respectively. Individual worms were placed in 70-ml plastic containers containing aged tap water and allowed to release eggs. Worms were then fixed in AFA, stained, and identified to species.

Colonies of *Physa* (*Physella*) *gyrina* snails were established in the laboratory from wild strains collected from Nickol Pond in Cass County, Nebraska (40.81412, −96.46000), whereas *Gyraulus parvus* snails were established in the laboratory from wild strains collected from Dunwoody Pond, Keith County, Nebraska (41.21527, −101.578610). All snail colonies were collected in the spring and were maintained under a 24-hr photoperiod and 25 C. Snails were maintained on a diet of frozen mustard greens, maple leaves, and Tetra Min® fish food and reared from eggs for a period of 6 wk in the laboratory. All *Haematoloechus* spp. eggs were stored for a period of 1–6 wk in 70-ml plastic containers under a 12 hr light : 12 hr dark photoperiod and 25 C, prior to snail infections. Groups of *P. gyrina* snails were exposed to eggs of *H. coloradensis* or *H. complexus* by placing individual snails into 70-ml plastic containers with eggs and Tetra Min® fish food for 5 min, and groups of laboratory-reared *G. parvus* snails were exposed to eggs of *H. longiplexus* or *H. parviplexus* using the same technique.

Owing to high snail mortality of *G. parvus* experimentally infected with *H. parviplexus*, snails naturally infected with *H. parviplexus* were collected from Nevens Pond, Keith County, Nebraska. During 2000–2004, bullfrogs, plains leopard frogs, Woodhouse's toads, and tiger salamanders, *Ambystoma tigrinum mavortium*, were collected from this location; the only amphibian species infected with *H. parviplexus* was the bullfrog (Bolek, 2006; data not shown). Two frog lung fluke species, *H. longiplexus* and *H. parviplexus*, use *G. parvus* snails as first intermediate hosts at Nevens Pond. Therefore, to be sure that snails were infected with *H. parviplexus*, cercariae were identified to species based on morphology and based on the inability to infect damselflies (Krull, 1931; Snyder and Janovy, 1996), and identification was confirmed by feeding developed metacercariae from dragonflies to bullfrogs, plains leopard frogs, and northern leopard frogs and recovering adult worms for identification.

Ultimate and penultimate larvae of *E. simplicicollis* and *I. verticalis*, dragonflies, and damselflies used in the second intermediate host infections came from Nickol Pond, Cass County; Oak Lake, Lancaster County (40.83056, −96.70778); and Dunwoody Pond, Keith County, all in Nebraska. Dragonflies and damselflies were divided into 3 equal groups and assigned to either time-0 controls, experimental infections, or time-*T* controls; the insects were isolated in 5-ml well plates filled with aged tap water for 24 hr before exposure. All time-0 control larval odonates were examined for the presence of frog lung fluke metacercariae before the start of experimental infections. For infections, approximately 20–50 cercariae of each of the 4 species of *Haematoloechus* from lab-reared and field-collected snails for *H. parviplexus* were pipetted into each well.

Twenty-four hours after exposure, all exposed larval odonates and time-*T* controls were transferred to individual 1,000-ml jars with 200 ml of aged tap water along with a single 15-cm applicator stick as a perch, covered with a screen lid, and fed 3 times a week as previously described. Upon metamorphosis, individual tenerals, along with their exuviae, were examined for *Haematoloechus* spp. metacercariae as previously described. Comparisons among infection rates in larval and teneral life stages of *E. simplicicollis* were compared with paired *t*-tests and the chi-square test for independence. The Wilcoxon single rank test was calculated when variances were heteroscedastic (Sokal and Rohlf, 1981).

Haematoloechus varioplexus frog experimental infections

Anuran host specificity experiments were conducted in 2 separate trials. Three anuran species were used. Metacercariae assumed to be *H. parviplexus* recovered from *E. simplicicollis* dragonfly metamorphosis experiments were used in the infections. All metacercariae from *E. simplicicollis* dragonflies were dissected in dishes filled with odonate saline (Fielden, 1960) and randomly mixed. Northern leopard frogs, *R. pipiens*, were reared from tadpoles collected at Cedar Creek, Keith County, Nebraska, and bullfrogs, *R. catesbeiana*, were reared from tadpoles collected from Nevens Pond, Keith County, and Pawnee Lake, Lancaster County, Nebraska; frogs were maintained in the laboratory in 45.5-L tanks filled with aged tap water for a period of 6 wk through metamorphosis. Tadpoles were maintained on a diet of frozen mustard greens and Tetra Min® fish food, whereas metamorphosed frogs were fed commercial lab-reared crickets *Gryllus firmus*, and tenebrionid beetles *Tenebrio molitor* adults and larvae.

In trial I, 4 laboratory-reared northern leopard frogs, *R. pipiens*, and 4 laboratory-reared bullfrogs, *R. catesbeiana*, were each given 10–40 *H. parviplexus* metacercariae. In trial II, 3 laboratory-reared bullfrogs, *R. catesbeiana*, and 3 field-collected young of the year plains leopard frogs, *R. blairi*, along with time-0 and time-*T* controls, were collected from Pawnee Lake, Lancaster County, Nebraska. Frogs in the experimental group were each given 10–20 *H. parviplexus* metacercariae. For all infections, metacercariae were intubated by a pipette via the esophagus. The pipette then was examined under a dissecting microscope to confirm that no metacercariae remained. All exposed frogs, along with time-*T* controls of *R. blairi*, were maintained individually in plastic shoe boxes (35 cm × 25 cm × 15 cm) and fed commercial crickets 3 times a week. Water was changed once or twice a wk. Thirty to 35 days after exposure, all exposed frogs along with the time-*T* controls *R. blairi* were killed and examined for frog lung flukes.

Frog odonate feeding trials

Eighteen newly metamorphosed bullfrogs and 5 adult plains leopard frogs were collected from Pawnee Lake, Lancaster County, Nebraska. Frogs were housed in individual plastic shoe boxes (35 cm × 25 cm × 15 cm) containing 5 cm of water for 24 hr before introduction of a single ultimate *E. simplicicollis* larvae. Shoe boxes were checked every hour on the first day and once daily over a period of a week to see if frogs ingested dragonfly larvae.

Morphological studies

Owing to recent confusion regarding the taxonomy of *H. varioplexus*, worms recovered from bullfrogs and plains leopard frogs were also

compared and identified according to the descriptions of *H. parviplexus* by Irwin (1929) and *H. varioplexus* and *H. similiplexus* by Stafford (1902) and Cort (1915). Morphological data were collected on 20 *H. varioplexus* worms from northern leopard frogs, plains leopard frogs, Woodhouse's toads (*B. woodhousii*), and wood frogs (*Rana sylvatica*) and 20 *H. parviplexus* worms from bullfrogs, and green frogs (*Rana clamitans*). Worms used for morphological analysis were collected from a number of locations in Nebraska, New York, South Dakota, and Wisconsin. These included 1 *H. varioplexus* collected from a northern leopard frogs from Lake Preston, Kingsbury County, South Dakota (44.37581, −97.55479); 6 *H. varioplexus* collected from wood frogs from the University of Wisconsin–Milwaukee Field Station, Ozaukee County, Wisconsin (43.38875, −88.02208); 1 *H. parviplexus* collected from a green frog from Queechy Lake, Colombia County, New York (42.40246, −73.42456); 8 *H. parviplexus* collected from green frogs from Genesee Depot, Waukesha County, Wisconsin (42.98984, −88.36634); 2 *H. parviplexus* collected from a bullfrog from Pawnee Lake, Lancaster County, Nebraska; and 3 *H. parviplexus* from bullfrog experimental infections from Nevens Pond, Keith County, Nebraska. Additionally, to obtain a better geographical representation, *H. varioplexus* and *H. parviplexus* voucher specimens collected by Dan Brooks and Stewart C. Schell were borrowed from the H. W. Manter Laboratory, University of Nebraska State Museum. These included *H. varioplexus* HWML 20151, from plains leopard frogs from Lancaster County, Nebraska (40.791665, −96.675), 6 slides; HWML 20153, from a northern leopard frog from Nance County, Nebraska (41.424305, −97.86542), 1 slide; HWML 20155, from a Woodhouse's toad from Nance County, Nebraska (41.424305, −97.86542), 3 slides; HWML 20157, from a northern leopard frog from Cherry County, Nebraska (42.71139, −100.82528), 1 slide; HWML 20158, from a northern leopard frog from Dawes County, Nebraska (42.699865, −103.272775), 1 slide; HWML 20159, from a northern leopard frog from Grant County, Nebraska (41.99597, −101.63667), 1 slide; HWML 20160, from a plains leopard frog from Webster County, Nebraska (40.12778, −98.509865), 1 slide; *H. parviplexus* HWML 20142, from a bullfrog from Richardson County, Nebraska (40.156945, −95.82236), 5 slides; HWML 20143, from a bullfrog from Rock County, Nebraska (42.49153, −98.872225), 1 slide; and HWML 23879, from a green frog from Wisconsin (no latitude or longitude date given), 1 slide. Based on recent and past *Haematoloechus* species descriptions by Brooks (1976) and León-Règagnon and Brooks (2003), the following characters were recorded: (1) body shape, body length, and body width; (2) oral sucker location, length, and width; (3) pharynx length and width; (4) oral sucker/pharynx ratio OS/PH; (5) acetabulum length from anterior end, and acetabulum length and width; (6) oral sucker/acetabulum ratio OS/AC; (7) testes morphology and location; (8) ovary morphology and location; (9) cirrus sac position and location; (10) uterus and uterine loop morphology; (11) vitellaria number, and location; and (12) egg length and width. Student's 2-tailed *t*-test was used to compare differences in morphology among morphological characteristics of *H. parviplexus* and *H. varioplexus*. An approximate *t*-test was calculated when variances were heteroscedastic (Sokal and Rohlf, 1981). Figures were drawn of representative worms with the aid of a camera lucida.

RESULTS

Amphibian field studies

Of the 6 amphibian species examined from Pawnee Lake, only bullfrogs and plains leopard frogs were infected with frog lung flukes (Table II). Bullfrogs were infected with *H. longiplexus* and *H. parviplexus*, whereas plains leopard frogs were predominantly infected with *H. complexus*, with a single plains leopard frog containing 1 individual *H. complexus* and *H. longiplexus* in each lung. Statistically significant differences were observed in prevalence among all 3 species of frog lung flukes infecting bullfrogs and plains leopard frogs: *H. complexus* $\chi^2 = 52.04$, $P < 0.001$; *H. longiplexus* $\chi^2 = 16.72$, $P < 0.001$; *H. parviplexus* $\chi^2 = 14.97$, $P < 0.001$.

TABLE II. Prevalence and mean intensity of *H. complexus*, *H. longiplexus*, and *H. parviplexus* in 6 species of anurans collected from Pawnee Lake, Lancaster County, Nebraska, during March 2001–June 2005.

Anuran species (n)	*Haematoloechus* species	Prevalence (%)	Mean intensity ±1 SD
A. crepitans (50)			
	H. complexus	0	—
	H. longiplexus	0	—
	H. parviplexus	0	—
B. woodhousii (50)			
	H. complexus	0	—
	H. longiplexus	0	—
	H. parviplexus	0	—
H. chrysoscelis (36)			
	H. complexus	0	—
	H. longiplexus	0	—
	H. parviplexus	0	—
P. triseriata (93)			
	H. complexus	0	—
	H. longiplexus	0	—
	H. parviplexus	0	—
R. blairi (70)			
	H. complexus	42.9	4.2 ± 4.3
	H. longiplexus	1.4	1
	H. parviplexus	0	—
R. catesbeiana (100)			
	H. complexus	0	—
	H. longiplexus	24	5.7 ± 5.7
	H. parviplexus	19	5.1 ± 5.3

Odonate field studies

In total, 387 odonates (209 anisopterans and 178 zygopterans) of 10 species were collected from Nickol Pond; however, not all life stages were collected for each species. Twenty-two of 209 (10.5%) anisopterans and 39 of 178 (22%) zygopterans were infected with *Haematoloechus* spp. metacercariae. Prevalence and mean intensity varied among the different species and life stages (Table I). All metacercariae recovered from zygopteran hosts were located in the head, thorax, and abdomen, while all metacercariae recovered from anisopteran hosts were located in the branchial basket of the larvae or the remnants of the branchial basket of tenerals and adult dragonflies. Of the 2 most commonly collected species, there was no significant difference in prevalence or mean abundance of *Haematoloechus* metacercariae among larvae or tenerals of the eastern forktail damselfly, *I. verticalis* (Fig. 1; $\chi^2 = 0.42$, $P > 0.05$; $t = -1.03$, $P = 0.31$), whereas larval eastern pondhawk dragonflies, *E. simplicicollis*, had a significantly higher prevalence and mean abundance of *Haematoloechus* metacercariae than the tenerals, *E. simplicicollis* (Fig. 1; $\chi^2 = 6.16$, $P < 0.025$; $t = 2.07$, $P = 0.04$).

Odonate metamorphosis experiments

Eleven of 25 (44%) laboratory-metamorphosed eastern forktail damselflies, *I. verticalis*, were infected with *Haematoloechus* metacercariae, with a mean abundance of 1.12 ± 1.69. All metacercariae were located in the head, thorax, and abdomen and all survived metamorphosis. There were no statistically sig-

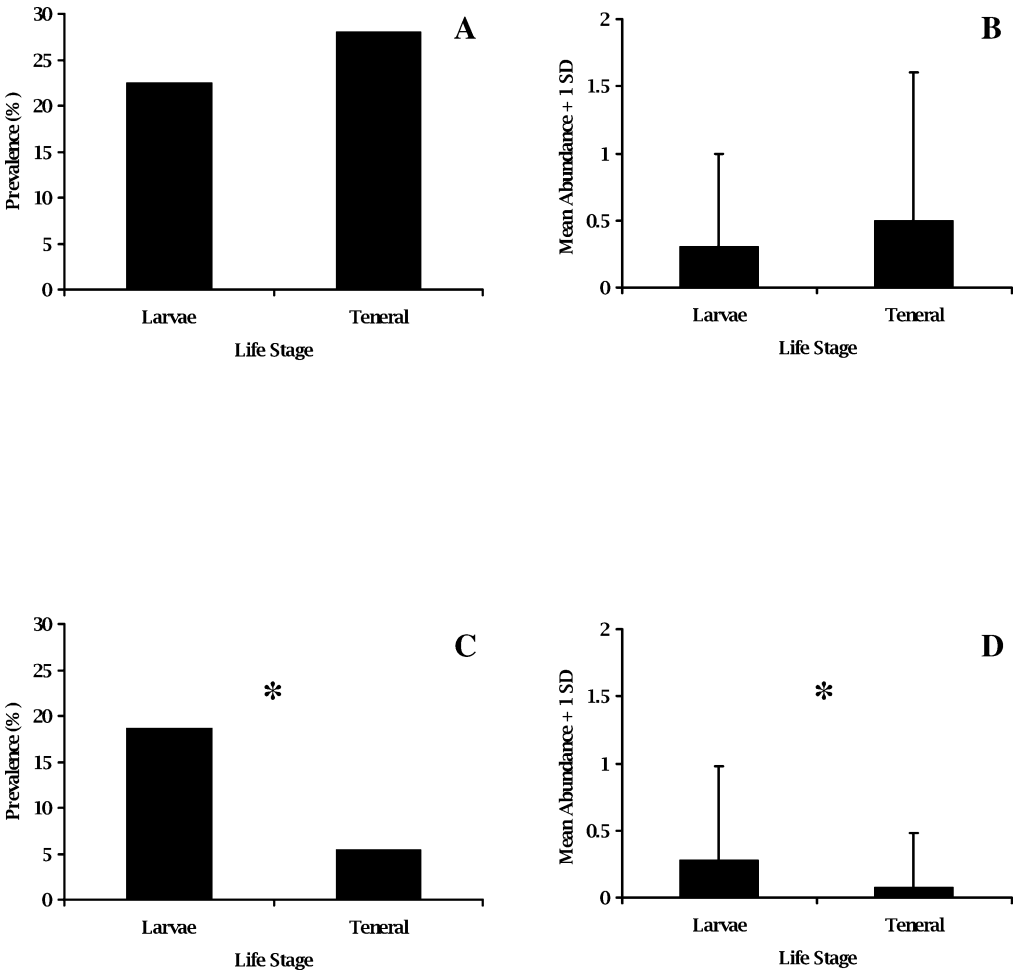

FIGURE 1. Prevalence and mean abundance of *Haematoloechus* metacercariae infecting larva and teneral odonates collected from Nickol Pond, Cass County, Nebraska. (**A**) Prevalence of *Haematoloechus* metacercariae in larval and teneral *I. verticalis*. n = 80 for larvae and 28 for tenerals. (**B**) Mean abundance of *Haematoloechus* metacercariae in larval and teneral *I. verticalis*. n = 80 for larvae and 28 for tenerals. (**C**) Prevalence of *Haematoloechus* metacercariae in larval and teneral *E. simplicicollis*. n = 75 for larvae and 74 for tenerals. (**D**) Mean abundance of *Haematoloechus* metacercariae in larval and teneral *E. simplicicollis*. n = 75 for larvae and 74 for tenerals. Asterisk denotes a statistically significant difference between groups for prevalence and mean abundance ($P < 0.05$).

nificant differences in prevalence or mean abundance between the larval and teneral stages of *I. verticalis* (Fig. 2; $\chi^2 = 0.0$, $P > 0.05$; $t = 0.0$, $P > 0.05$).

Sixty-five of 100 (65%) eastern pondhawk dragonfly larvae, *E. simplicicollis*, were infected with *Haematoloechus* metacercariae. Most metacercariae were located in the branchial basket; however, a few were located in the thorax. All metacercariae located in the thorax survived dragonfly metamorphosis, whereas a significant proportion of metacercariae located in the branchial basket were lost during metamorphosis. There was a statistically significant difference in prevalence and mean abundance of metacercariae located in the branchial basket region of the larvae and tenerals of eastern pondhawk dragonflies, *E. simplicicollis*, whereas there was no statistically significant difference in prevalence and mean abundance of metacercariae located in the thorax of the larvae or tenerals of the eastern pondhawk dragonflies, *E. simplicicollis* (Fig. 2; metacercariae in branchial basket, $\chi^2 = 32.84$, $P < 0.0001$; Wilcoxon signed rank test $Z = -6.34$, $P < 0.0001$; metacercariae in thorax, $\chi^2 = 0.0$, $P > 0.05$; $t = 0.0$, $P > 0.05$).

Of 131 *Haematoloechus* metacercariae located in the branchial basket of larval *E. simplicicollis*, 99 (76%) were lost during metamorphosis. Metacercariae left behind in the remnants of the branchial basket of the exuviae of *E. simplicicollis* were unencysted or lightly encysted with a thin wall (Fig. 3). Of the 32 metacercariae located in the remnants of the branchial basket region of tenerals *E. simplicicollis* that survived metamorphosis, 22 (68%) were encapsulated by the dragonfly (Fig. 3). Additionally, 2 of 10 (20%) *E. simplicicollis* exuviae collected at Nickol Pond contained 1 and 2 *Haematoloechus* spp. metacercariae, indicating that the loss of these metacercariae was not a laboratory artifact.

Odonate experimental infections

All 4 *Haematoloechus* species developed metacercariae in eastern pondhawks, *E. simplicicollis*, although not all exposed

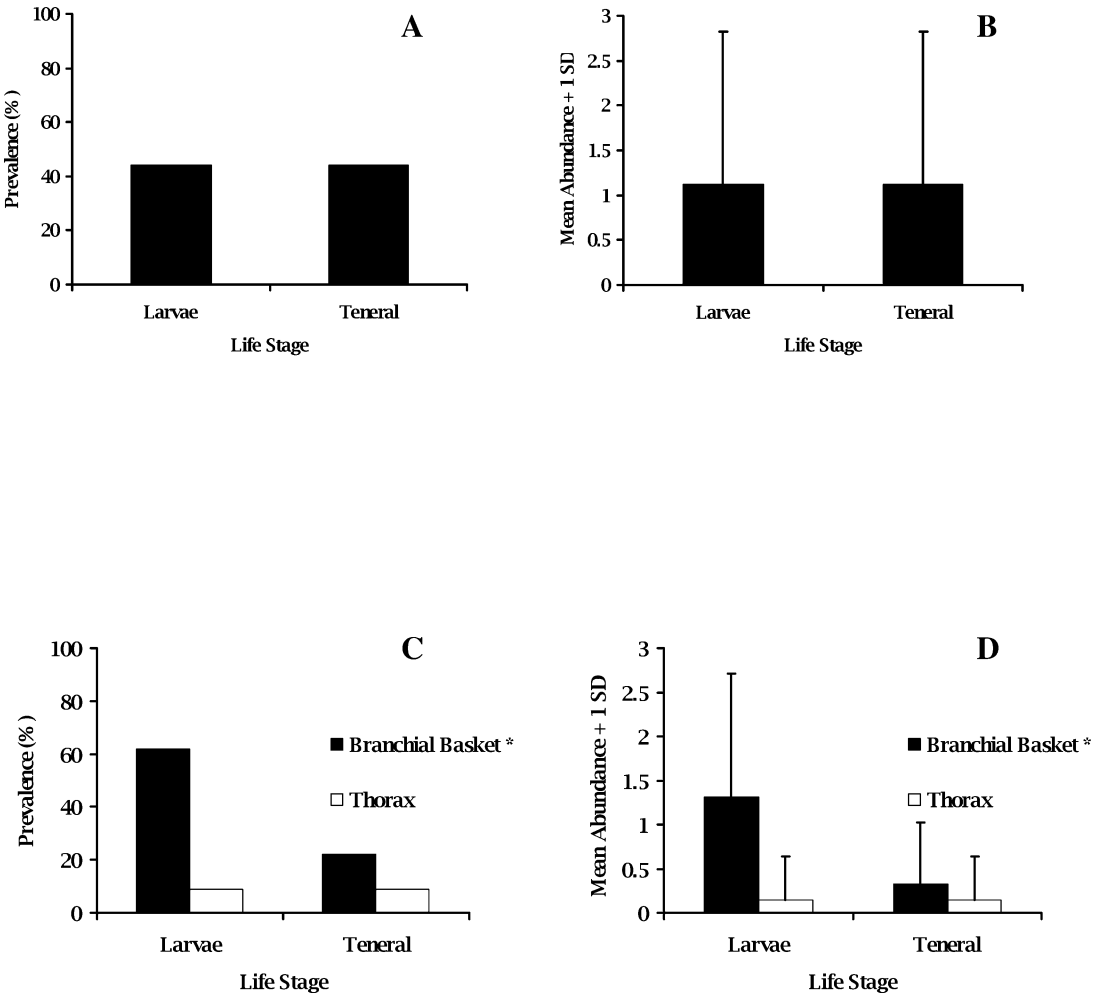

FIGURE 2. Prevalence and mean abundance of *Haematoloechus* metacercariae of laboratory-metamorphosed odonates collected from Nickol Pond, Cass County, Nebraska. (**A**) Prevalence of *Haematoloechus* metacercariae in larval and teneral *I. verticalis*. n = 25 for each life stage. (**B**) Mean abundance of *Haematoloechus* metacercariae in larval and teneral *I. verticalis*. n = 25 for each life stage. (**C**) Prevalence of *Haematoloechus* metacercariae in the branchial basket and thorax of larval and teneral *E. simplicicollis*. n = 100 for each life stage. (**D**) Mean abundance of *Haematoloechus* metacercariae in the branchial basket and thorax of larval and teneral *E. simplicicollis*. n = 100 for each life stage. Asterisk denotes a statistically significant difference between groups for prevalence and mean abundance ($P < 0.05$).

individuals became infected. *Haematoloechus coloradensis*, *H. complexus*, and *H. longiplexus* metacercariae also developed in the damselfly *I. verticalis*, and were located in the head, thorax, and abdomen of this host, whereas *H. parviplexus* was unable to infect this damselfly (Table III). None of the time-0 or time-*T* control dragonflies or damselflies was infected. None of the exposed damselflies survived through metamorphosis, but most exposed dragonflies survived through metamorphosis.

Species specific differences were observed in the location of *Haematoloechus* spp. metacercariae infecting dragonfly hosts among the 4 species of *Haematoloechus* (Table III). *Haematoloechus coloradensis* and *H. complexus* metacercariae were located in the head, thorax, and branchial basket of eastern pondhawk dragonflies, *E. simplicicollis*, whereas *H. longiplexus* and *H. parviplexus* were always restricted to the branchial basket of eastern pondhawk dragonflies, *E. simplicicollis*. During metamorphosis, most metacercariae of *H. coloradensis*, *H. complexus*, and *H. parviplexus* survived metamorphosis in the eastern pondhawk, *E. simplicicollis*, whereas all but 1 metacercaria of *H. longiplexus* were lost during metamorphosis (Figs. 4, 5). There was no significant difference in prevalence or mean abundance of *H. coloradensis* and *H. parviplexus* and prevalence of *H. complexus* among larval and teneral life stages of *E. simplicicollis* (*H. coloradensis*, $\chi^2 = 0.0$, $P > 0.05$; $t = 2.22$, $P > 0.05$; *H. parviplexus*: $\chi^2 = 0.0$, $P > 0.05$; $t = 1.00$, $P > 0.05$; *H. complexus*, $\chi^2 = 0.20$, $P > 0.05$). However, statistically significant differences existed among the larval and teneral life stages of *E. simplicicollis* in prevalence and mean abundance for *H. longiplexus* ($\chi^2 = 5.84$, $P < 0.05$; Wilcoxon signed rank test $Z = -2.201$, $P < 0.05$) and mean abundance for *H. complexus* ($t = 2.3$, $P < 0.05$).

Haematoloechus parviplexus frog experimental infections

In trial I, 4 of 4 (100%) bullfrogs became infected, whereas none of the northern leopard frogs (0%) became infected. In

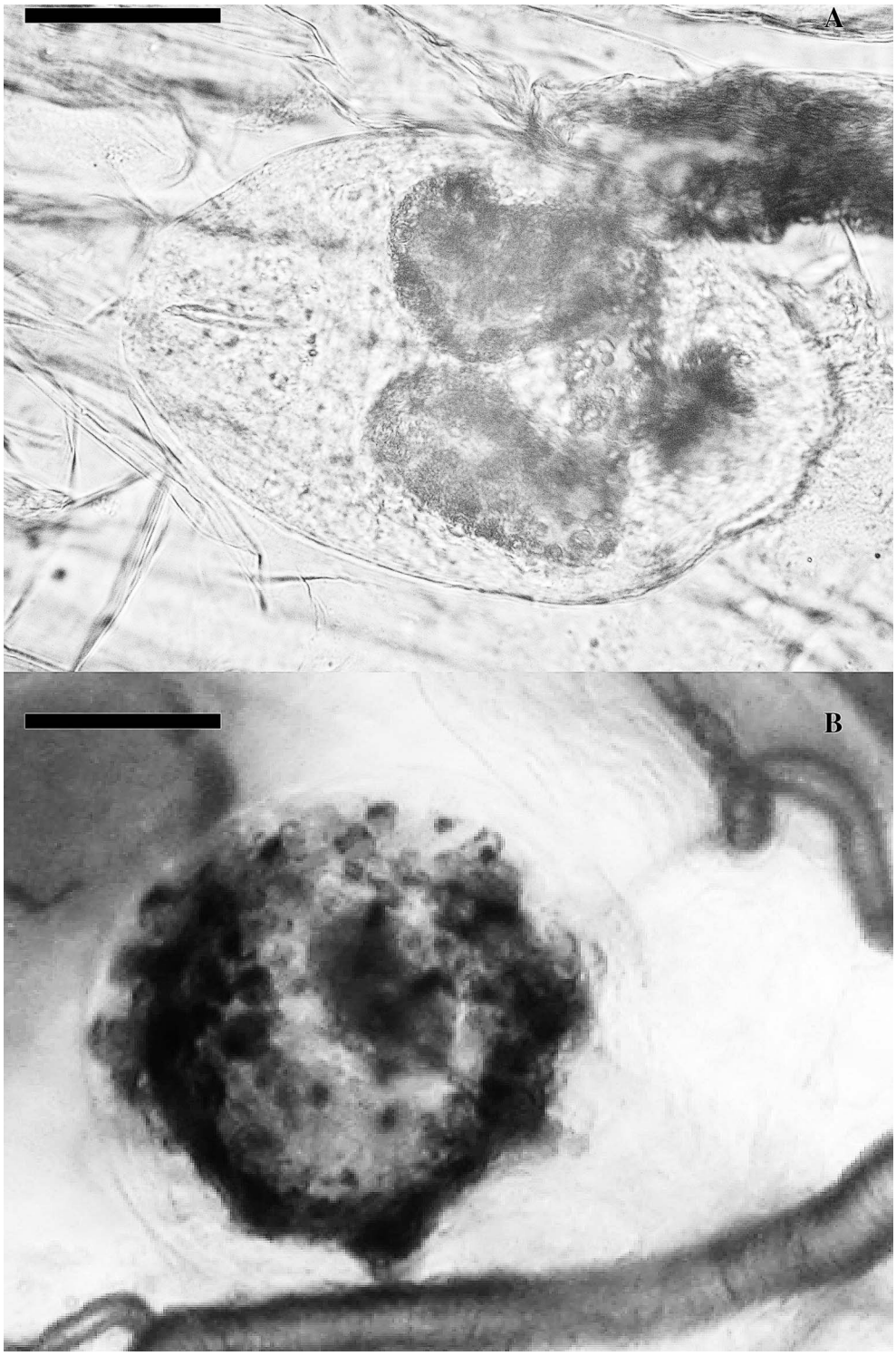

FIGURE 3. *Haematoloechus* metacercariae recovered from (**A**) the remnants of the branchial basket of an exuvia and (**B**) from the remnants of the branchial basket of a teneral *E. simplicicollis*, naturally infected at Nickol Pond, Cass County, Nebraska. Scale bars = 40 μm. Note that the metacercaria from the exuvia is not encysted or encapsulated, while the metacercariae from the teneral dragonfly is encysted and encapsulated.

trial II, 3 of 3 (100%) bullfrogs became infected, whereas none of the plains leopard frogs (0%) became infected (Table IV). None of the time-0 or time-*T* control frogs was infected. All worms recovered from bullfrogs were gravid and most closely resembled the description of *H. parviplexus* (Irwin, 1929).

Frog odonate feeding trials

Of the 18 newly metamorphosed bullfrogs, 8 ingested larval *E. simplicicollis* within 1 hr of placing the larva dragonfly with frogs. Additionally, 5 more newly metamorphosed bullfrogs in-

TABLE III. Number infected and location of *Haematoloechus* spp. metacercariae in experimentally exposed *E. simplicicollis* and *I. verticalis* to cercariae of *H. coloradensis*, *H. complexus*, *H. longiplexus*, and *H. parviplexus*.

	H. coloradensis		*H. complexus*		*H. longiplexus*		*H. parviplexus**	
Experimental odonate	No. infected/no. exposed survivors	Location in host	No. infected/no. exposed survivors	Location in host	No. infected/no. exposed survivors	Location in host	No. infected/no. exposed survivors	Location in host
Odonata: Anisoptera *E. simplicicollis*	10/10	Head, thorax, branchial basket	6/10	Head, thorax, branchial basket	6/9	Branchial basket	4/6	Branchial basket
Odonata: Zygoptera *I. verticalis*	10/10	Head, thorax, abdomen	6/6	Head, thorax, abdomen	3/3	Head, thorax, abdomen	0/4	—

* From naturally infected snails.

gested *E. simplicicollis* larvae within a week. Five bullfrogs did not ingest odonate larvae because dragonflies hid on, or under, the frogs for the duration of the trial. Thirteen of 18 bullfrogs ingested *E. simplicicollis* larvae. Of the 5 adult plains leopard frogs, only a single individual ingested 1 *E. simplicicollis* larva within 6 days of placing the odonate larvae with plains leopard frogs.

DESCRIPTIONS

Haematoloechus parviplexus (Irwin, 1929) Harwood, 1932

(Fig. 6)

Diagnosis (based on 20 mature specimens): Body elongate, 2.32–9.15 mm long by 0.40–1.50 mm wide. Oral sucker subterminal and round to oval 260–510 μm long by 220–480 μm wide. Pharynx 110–260 μm long by 110–250 μm wide. Oral sucker/pharynx width ratio 1.15–1.92. Oral sucker/pharynx length ratio 1.24–1.84. Acetabulum 37–53% body length from anterior end, round to oval 50–150 μm long by 50–130 μm wide. Oral sucker/acetabulum ratio 1.78–4.00. Testes oval positioned in tandem in midhindbody, anterior testis 390–1,290 μm long by 200–790 μm wide and posterior testis 440–1,800 μm long by 150–880 μm wide. Cirrus sac long, extending to level of acetabulum. Genital pore ventral to pharynx. Ovary dorsolateral to acetabulum, deeply lobed 350–1,180 μm long by 120–500 μm wide. Longitudinal extracecal uterine loops reaching intertesticular level. Vitellaria acinous, follicular forming clusters, distribution differing on each side of body. On ovarian side 6–8 extracecal clusters, and 0–3 intracecal clusters located in front of the acetabulum and 0–3 intracecal behind the posterior testis. On opposite side of body 5–10 extracecal clusters, 0–1 intracecal clusters in front or the acetabulum and 0–3 intracecal clusters behind the posterior testis. Eggs 20–25 μm long by 12.5–18 μm wide.

Taxonomic summary

Hosts: Rana catesbeiana and *R. clamitans*.

Haematoloechus varioplexus Stafford, 1902

(Fig. 7)

Synonym: Haematoloechus similiplexus Stafford, 1902; Cort, 1915.

Diagnosis (based on 20 mature specimens): Body elongate, 3.20–7.83 mm long by 0.76–1.63 mm wide. Oral sucker subterminal and oval 260–510 μm long by 220–480 μm wide. Pharynx 160–260 μm long by 150–280 μm wide. Oral sucker/pharynx width ratio 1.37–2.00. Oral sucker/pharynx length ratio 1.62–2.73. Acetabulum 32–52% body length from anterior end, round to oval 180–380 μm long by 180–410 μm wide. Oral sucker/acetabulum ratio 0.85–1.66. Testes round to oval positioned in tandem in midhindbody, anterior testis 350–650 μm long by 260–600 μm wide, and posterior testis 380–760 μm long by 240–550 μm wide. Cirrus sac long, extending to level of acetabulum. Genital pore ventral to pharynx. Ovary oval posterior or dorsolateral to acetabulum, 280–640 μm long by 190–370 μm wide. Longitudinal extracecal uterine loops reaching intertesticular level. Vitellaria acinous, follicular forming clusters, distribution differing on each side of body. On ovarian side 5–8 extracecal clusters, and 1–3 intracecal clusters located in front of the acetabulum and 0–3 intracecal behind the posterior testis. On opposite side of body 4–10 extracecal clusters, 0–2 intracecal clusters in front of the acetabulum and 0–2 intracecal clusters behind the 56 posterior testis. Eggs 30–42.5 μm long by 15–22.5 μm wide.

Taxonomic summary

Hosts: Rana blairi, R. pipiens, R. sylvatica, and *B. woodhousii*.

Remarks

Morphological comparisons among *H. varioplexus* and *H. parviplexus* are presented in Table V. Statistically significant differences were observed between *H. varioplexus* and *H. parviplexus* in oral sucker length and width, OS/PH length and width ratios, acetabulum length and width, OS/AC width ratio, testis length and width, ovary length and width, and egg length and width. Although these differences were statistically significant, there was overlap among these characteristics in all cases except for the acetabulum length and width, OS/AC width ratio, and egg length. Additionally, *H. parviplexus* had a distinctly lobed ovary, while the ovary was never lobed in *H. varioplexus*.

DISCUSSION

Frog lung fluke distribution in frog hosts

Prevalence and mean intensity of frog lung flukes from the 6 species of anurans collected from Pawnee Lake, Lancaster County, Nebraska, indicates that *H. complexus* is restricted to plains leopard frogs, and *H. parviplexus* is restricted to bullfrogs. *Haematoloechus longiplexus* predominantly infects bullfrogs and is rarely found in plains leopard frogs. Previous studies on the life history of *H. complexus* by Krull (1933) indicate that bullfrogs are resistant to this species, explaining the strict host specificity in plains leopard frogs observed at our site, whereas our study indicates that plains leopard frogs and northern leopard frogs are resistant to *H. parviplexus*, explaining the strict host specificity in bullfrogs observed at our study site. The occurrence of *H. longiplexus* in both bullfrogs and plains leopard frogs at our study site supports Snyder's (1996) experimental infections of bullfrogs and leopard frogs in the laboratory with this species and indicates that plains leopard frogs are also susceptible to infections in nature.

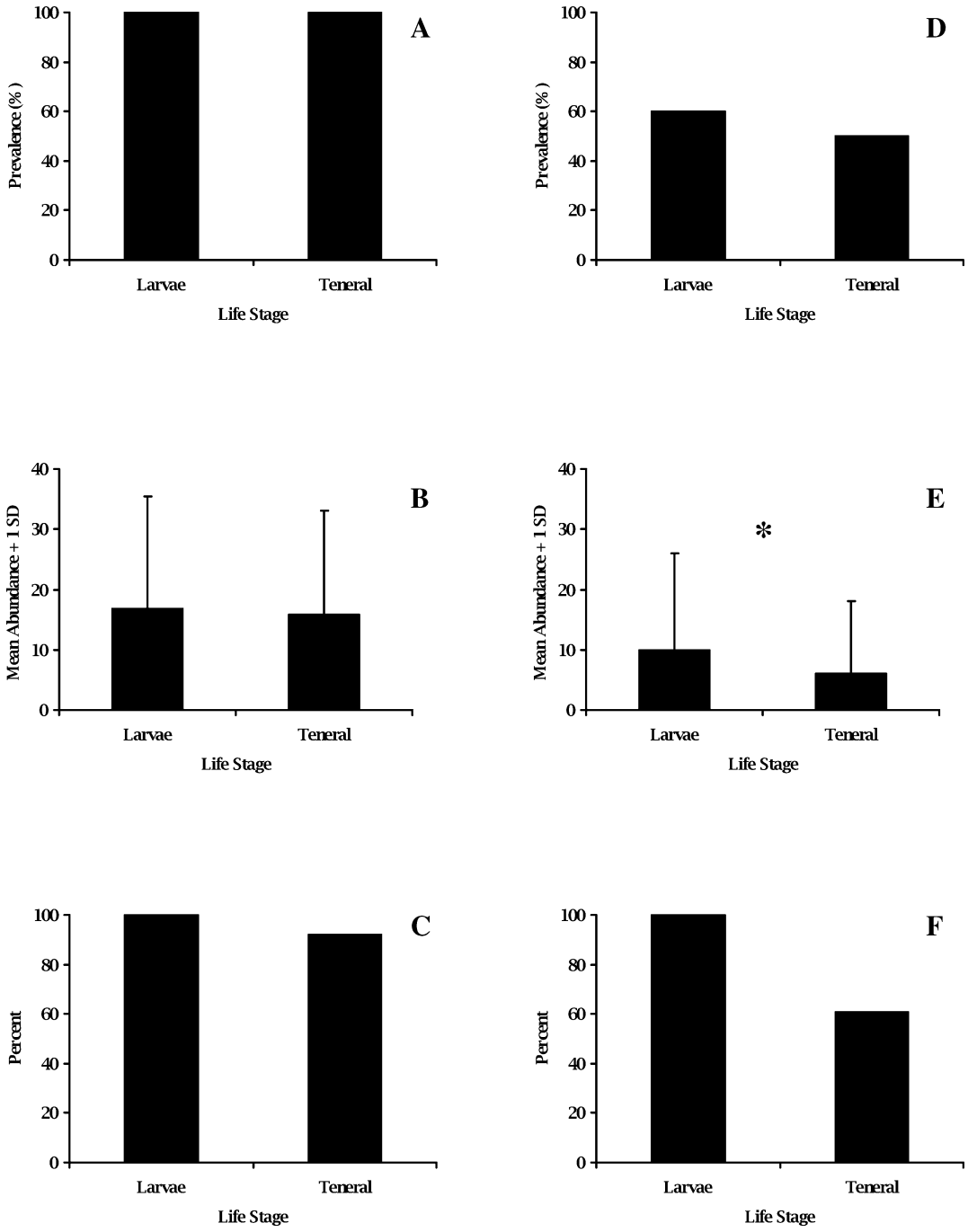

FIGURE 4. Prevalence, mean abundance, and percent of *Haematoloechus* metacercariae recovered from laboratory infected larval and teneral *E. simplicicollis*. *Haematoloechus coloradensis* (n = 10); (**A**) prevalence, (**B**) mean abundance, (**C**) percentage metacercariae. *Haematoloechus complexus* (n = 10); (**D**) prevalence, (**E**) mean abundance, (**F**) percentage metacercariae. Asterisk denotes a statistically significant difference between groups for prevalence and mean abundance ($P < 0.05$).

Haematoloechus parviplexus and *H. varioplexus* taxonomy

Morphological studies on frog lung flukes currently known as *H. varioplexus* and experimental infections of bullfrogs, northern leopard frogs, and plains leopard frogs indicate that the synonymy by Kennedy (1981) of *H. parviplexus* from bullfrogs, green frogs, and toads with *H. varioplexus* from leopard frogs, wood frogs, and toads is not warranted and that these worms represent distinct species. The reason that northern and plains leopard frogs are never infected with *H. varioplexus* when collected from the same location as bullfrogs infected with this species is that frog lung flukes from bullfrogs are actually a distinct species, *H. parviplexus*, which does not establish in Nebraska leopard frogs.

Examination of voucher material or drawings from recent

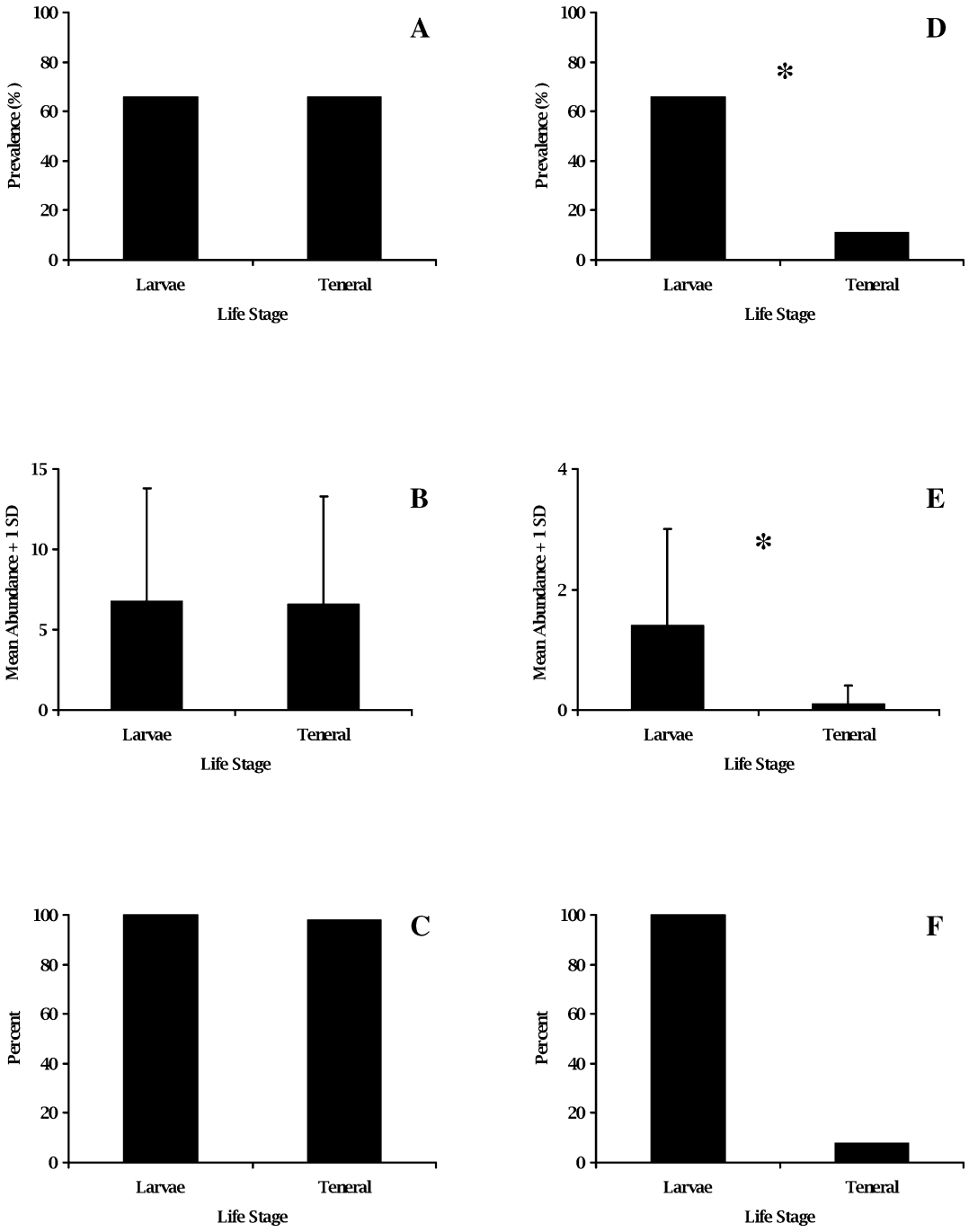

FIGURE 5. Prevalence, mean abundance, and percentage of *Haematoloechus* metacercariae recovered from laboratory infected larval and teneral *E. simplicicollis*. *Haematoloechus parviplexus* (n = 6); (**A**) prevalence, (**B**) mean abundance, (**C**) percentage metacercariae. *Haematoloechus longiplexus* (n = 9); (**D**) prevalence, (**E**) mean abundance, (**F**) percentage metacercariae. Asterisk denotes a statistically significant difference between groups for prevalence and mean abundance ($P < 0.05$).

publications indicates that studies on bullfrogs and/or green frogs by Snyder and Janovy (1996), McAlpine and Burt (1998), Bolek and Coggins (2001), Muzzall et al. (2001), Snyder and Tkach (2001), Yoder et al. (2001), Whitehouse (2002), and in part by León-Règagnon and Brooks (2003) were actually dealing with *H. parviplexus* and not *H. varioplexus*, whereas the study on wood frogs by Muzzall and Peebles (1991) was actually dealing with *H. varioplexus* and not *H. parviplexus*. Although superficially similar, these 2 species differ in their acetabulum length and width, ovary shape, testes length, and egg length and width. *Haematoloechus parviplexus* has a small acetabulum compared with the oral sucker (OS/AC width ratio 2.68; range = 1.78–4.00), the ovary is lobed, and the testes are elliptical in shape, whereas *H. varioplexus* has an acetabulum that is large and comparable in size to its oral sucker (OS/AC width ration 1.25; range = 0.85–1.66), the ovary is never lobed,

TABLE IV. Experimental infections of laboratory-reared *R. catesbeiana* and *R. pipiens* and field-collected *R. blairi* with metacercariae of *H. parviplexus*.

Host	Prevalence (no. infected/no. exposed)	Mean abundance ±1 SD (range)	Number of worms recovered
Trial 1			
R. pipiens	0 (0/4)	0	0
R. catesbeiana	100 (4/4)	19.75 ± 15.5 (4–39)	79
Trial 2			
R. blairi	0 (0/3)	0	0
R. catesbeiana	100 (3/3)	5 ± 4.5 (1–5)	15

and the testes are round to elliptical in shape. Egg length and width also differ among these 2 species, being smaller 24.6 (range = 20–25) × 15.9 (range = 12.5–18) in *H. parviplexus* and larger 36.4 (range = 30–42.5) × 19.1 (range = 15–22.5) in *H. varioplexus*.

The taxonomy of *H. varioplexus* and *H. parviplexus* has been problematic, with confusion in their taxonomy dating back to their original descriptions. Stafford (1902) described *H. varioplexus* from bullfrogs from Toronto and Montreal, Canada, and *H. similiplexus* from northern leopard frogs and American toads, *Bufo americanus*, from numerous locations in Canada, but did not deposit any type specimens in an accredited museum. Cort (1915), in a later study on North American frog lung flukes, emended the description of *H. similiplexus* and considered *H. varioplexus* a species inquirenda. Irwin (1929) described *H. parviplexus* from the green frog, *R. clamitans*, from Minnesota, but did not compare her specimens with the descriptions of *H. varioplexus* and *H. similiplexus* by Stafford (1902). From her description, and from the description and drawing of *H. varioplexus* by Stafford (1902), it is clear that both authors were dealing with the same species of frog lung fluke.

Manter (1938), in a review of amphibian trematodes, synonymized Stafford's (1902) *H. varioplexus* from bullfrogs with Stafford's (1902) *H. similiplexus* from northern leopard frogs and American toads, without giving any justification for this synonymy. Brooks (1976) used Manter's synonymy and reported *H. varioplexus* (originally described as *H. similiplexus*) from northern leopard frogs, plains leopard frogs, and Woodhouse's toads, and *H. parviplexus* from bullfrogs and Woodhouse's toads in Nebraska, and deposited voucher specimens of both species. Finally, Kennedy (1981) synonymized *H. parviplexus* with *H. varioplexus* and 3 other *Haematoloechus* species, based on his own specimens and vouchers deposited by Brooks (1976) and others. Examination of voucher specimens used by Kennedy (1980, 1981) that were available and his drawings in the revision (Kennedy, 1980, 1981) indicate that a number of these were misidentified and apparently Kennedy

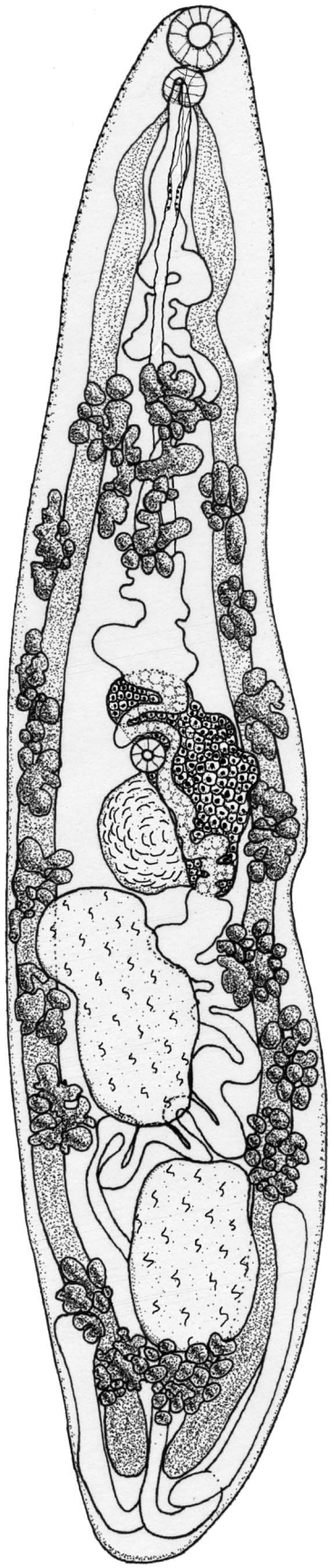

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FIGURE 6. Line drawing of *H. parviplexus* from an experimentally infected bullfrog, *Rana catesbeiana*, with a metacercaria collected from Nevens Pond, Keith County, Nebraska. Scale bar = 1.5 mm.

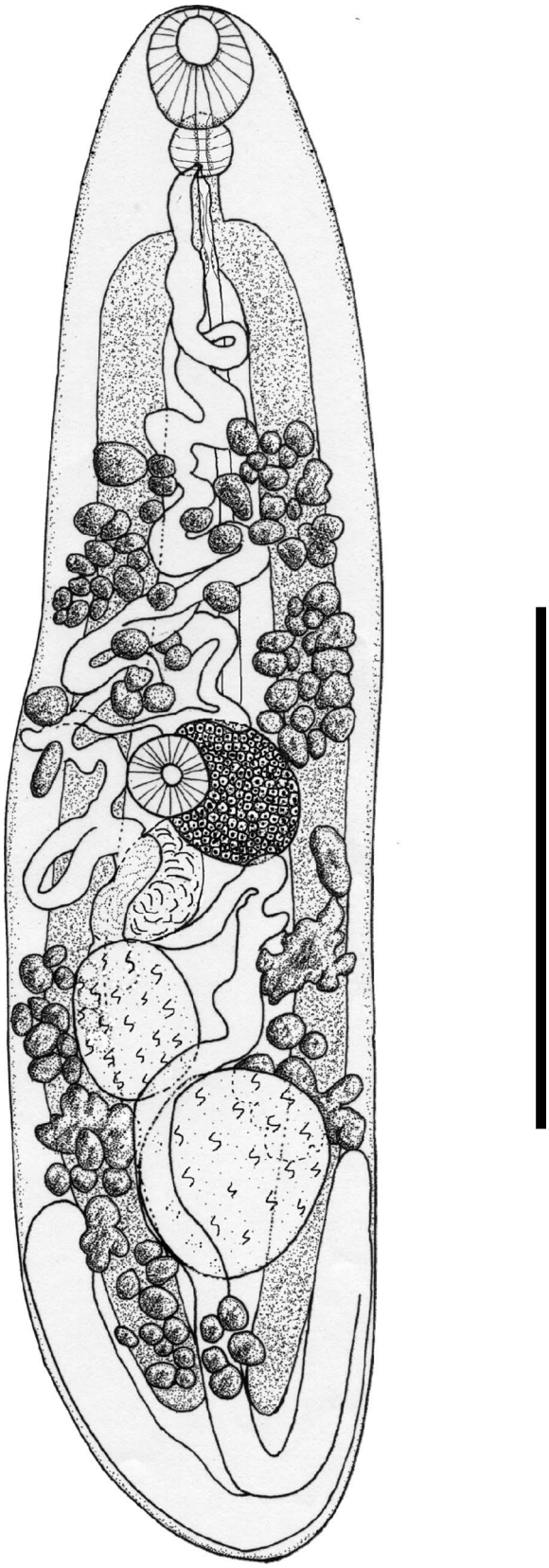

FIGURE 7. Line drawing of *H. varioplexus* from the northern leopard frog, *Rana pipiens*, collected from Nance County, Nebraska. Scale bar = 1.5 mm.

(1981) based his synonymy on these identifications. Most North American workers dealing with *Haematoloechus* species have used Kennedy's (1981) descriptions for *Haematoloechus* species identifications, and, as a result, these reports cannot be trusted. This study on infections of laboratory-reared frogs and previous surveys of anurans in Nebraska by Brooks (1976) and Snyder (1996) clearly indicate that bullfrogs and leopard frogs in Nebraska are infected with 2 distinct species, *H. parviplexus* in bullfrogs and *H. varioplexus* in northern leopard frogs and plains leopard frogs.

A review of the literature, our personal collections of frog lung flukes, and an examination of voucher specimens indicate that *H. parviplexus* has been reported from bullfrogs, *R. catesbeiana*, and green frogs, *R. clamitans*, in Indiana, Kentucky, Louisiana, Michigan, Virginia, and Wisconsin; from green frogs, *R. clamitans*, in Minnesota and New York; from bullfrogs, *R. catesbeiana*, in Toronto, Montreal, New Brunswick, Connecticut, and Idaho; and from bullfrogs, *R. catesbeiana*, and Woodhouse's toad, *B. woodhousii*, in Nebraska (Stafford, 1902; Irwin, 1929; Bennett, 1938; Waitz, 1961; Campbell, 1968; Barbero and Golling, 1974; Brooks, 1976; Snyder, 1996; McAlpine and Burt, 1998; Yoder et al., 2001; M. Bolek, pers. obs.). There is also a report of *H. parviplexus* infecting a single spotted frog, *Rana pretiosa*, and spotted frog–wood frog hybrids, *R. pretiosa* × *R. sylvatica*, from Idaho by Waitz (1961). However, no voucher specimens were deposited, and Waitz (1961) indicated that these worms were immature and not gravid, suggesting that they may not mature in these hosts.

Haematoloechus varioplexus has been reported from northern leopard frogs, *R. pipiens*, from Illinois, Indiana, Michigan, North Dakota, and South Dakota; from wood frogs, *R. sylvatica*, from Michigan and Wisconsin; from the spring peeper, *Pseudacris crucifer*, from Wisconsin; from northern leopard frogs, *R. pipiens*, and American toads, *B. americanus*, from Toronto, Montreal, and Wisconsin; and from northern leopard frogs, *R. pipiens*, plains leopard frogs, *R. blairi*, and Woodhouse's toads, *B. woodhousii*, from Nebraska (Stafford, 1902; Cort, 1915; Fortner, 1923; Brooks, 1976; Muzzall and Peebles, 1991; Yoder and Coggins, 1996; Yoder, 1998; Goldberg et al., 2001; Bolek and Coggins, 2003). The reports of *H. varioplexus* from bullfrogs and green frogs by Campbell (1968) and from green frogs by Bouchard (1951) cannot be verified because voucher specimens were not deposited. However, both investigators used Stafford's (1902) original descriptions for species identification, which suggests that they were dealing with *H. parviplexus*. It is unclear whether bullfrogs and green frogs can become infected with *H. varioplexus*. During the present study, over 200 *P. gyrina*, 100 *G. parvus*, and 1 *Planorbella* (*Helisoma*) *trivolvis* laboratory-reared snails were exposed to *H. varioplexus* eggs recovered from wood frogs, but none became infected; thus, experimental infections of arthropods and frogs could not be attempted.

Metacercariae survival study

Observations on differences in metacercariae survival during dragonfly metamorphosis indicate that certain dragonfly species may act as life cycle 'filters,' or 'sieves.' Thus, only certain lung fluke species are able to pass from an aquatic environment through all filters, then end up in the appropriate terrestrial en-

TABLE V. Morphological characteristics of adult *H. varioplexus* and *H. parviplexus*. Means and ranges (in parentheses) are given in μm unless otherwise noted.

	H. varioplexus (n = 20) mean (range)	*H. parviplexus* (n = 20) mean (range)	*t*-test	*P*
Shape	Elongate	Elongate	—	—
Body length (mm)	4.83 mm (3.20–7.83)	6.61 mm (2.32–9.15)	1.49	0.14
Body width at acetabulum (mm)	1.02 mm (0.65–1.62)	0.96 mm (0.48–1.42)	−0.52	0.60
Greatest body width (mm)	1.09 mm (0.76–1.63)	1.00 mm (0.40–1.50)	−0.98	0.16
Oral sucker length	342 (260–510)	230 (160–310)	−6.02	0.0001
Oral sucker width	303 (220–480)	247 (150–350)	−2.98	0.005
Pharynx length	167 (110–260)	150 (90–250)	−1.35	0.18
Pharynx width	187 (150–280)	174 (110–250)	−1.12	0.27
OS/PH width ratio	1.62 (1.37–2.00)	1.44 (1.15–1.92)	−2.98	0.005
OS/PH length ratio	2.07 (1.62–2.73)	1.56 (1.24–1.84)	−6.66	0.0001
Acetabulum location from anterior end	43% (32–52)	44 (37–53)	0.36	0.72
Acetabulum length	239 (180–380)	93 (50–150)	−11.88*	0.00001
Acetabulum width	245 (180–410)	95 (50–130)	−10.95*	0.00001
OS/AC width ratio	1.25 (0.85–1.66)	2.68 (1.78–4.00)	9.56*	0.00001
Anterior testis length	486 (350–650)	850 (390–1,290)	5.96	0.00001
Anterior testis width	400 (260–600)	492 (200–790)	2.69	0.01
Posterior testis length	566 (380–760)	915 (440–1,800)	4.56*	0.0002
Posterior testis width	427 (240–550)	523 (150–880)	2.25	0.03
Ovary length	405 (280–640)	777 (350–1,180)	6.45*	0.0001
Ovary width	271 (190–370)	350 (120–500)	3.42	0.002
Uterus	Longitudinal extracecal uterine loops	Longitudinal extracecal uterine loops	—	—
Vitellaria number	19.30 (15–23)	19.10 (13–23)	−0.30	0.77
Egg length	36.40 (30.00–42.50)	24.60 (20.00–25.00)	−13.28*	0.00001
Egg width	19.10 (15.00–22.50)	15.90 (12.50–18.00)	−4.18	0.0002

* Approximate *t*-test.

vironment and encounter semiterrestrial leopard frogs. Our study indicates that metacercariae of *H. longiplexus* are more commonly lost during metamorphosis of eastern pondhawk dragonflies than are the other 3 species of *Haematoloechus*. These observations may be important in terms of the observed ecological host specificity of *H. longiplexus*. Both northern leopard frogs and plains leopard frogs are rarely infected with this species in nature, but they can be infected in the laboratory (Brooks, 1976; Snyder, 1996; Bolek and Janovy, 2007; Table II). Diet studies on leopard frogs and bullfrogs indicate that semiterrestrial leopard frogs feed predominantly on adult damselflies and dragonflies, which make up to 15% of the invertebrates reported in their diet (Linzey, 1967; Dronen, 1977), whereas aquatic bullfrogs feed predominantly on larval dragonflies and damselflies, which consist of up to 10–16% of the frequency of their diet, with adult dragonflies being less commonly reported (Korschgen and Baskett, 1963; Fulk and Whitaker, 1969; Stewart and Sandison, 1972). These dietary studies indicate that even though larval *E. simplicicollis* lose most *H. longiplexus* metacercariae during metamorphosis, they can still act as potential intermediate hosts for flukes of aquatic bullfrogs, but they rarely act as hosts for worms infecting semiterrestrial leopard frogs. Clearly, in the laboratory, bullfrogs will ingest larval *E. simplicicollis* when given the chance, indicating that they potentially feed on these insects in nature, whereas semiterrestrial leopard frogs may come in contact less often with aquatic larval odonates in nature.

Previous life history and ecological studies on *H. longiplexus* by Krull (1932) and Wetzel and Esch (1996) in the swamp spreadwing damselfly, *Lestes vigilax*, and eastern pondhawk dragonfly, *E. simplicicollis*, indicate that the metacercariae of this lung fluke were free or only lightly encysted in the head, thorax, and abdomen of damselflies and branchial basket of dragonflies. These observations are in contrast to studies on 4 other species of North American frog lung flukes. Both *H. coloradensis* and *H. complexus* can infect any body region of dragonflies, damselflies, and numerous aquatic arthropods. Life history studies on these 2 species by Krull (1933, 1934), Dronen (1975), Snyder and Janovy (1994, 1996), and Bolek and Janovy (2007) indicate that metacercariae are always encysted in the branchial basket of dragonflies or are found in the body cavity of odonates and other arthropods. Furthermore, the present study indicates that most survive metamorphosis in dragonfly hosts. Life history studies on *H. medioplexus* and *H. parviplexus* by Krull (1930, 1931) and Snyder and Janovy (1994, 1996) also indicate that metacercariae of these species are always found in the branchial basket of dragonflies, where they are covered by a tegument of uniform thickness and encysted in the lamella of the branchial basket. Our study clearly shows that metacercariae of *H. parviplexus* also survive metamorphosis in dragonfly hosts; however, leopard frogs are resistant to infection with this species.

Metamorphosis survival studies on *H. medioplexus*, which infects semiterrestrial leopard frogs, were not conducted as part of this study because too few gravid *H. medioplexus* worms were collected for snail infections (Bolek, 2006). However, Krull (1930, 1931) showed that the metacercariae of *H. medioplexus* survive metamorphosis in 2 dragonfly species, the

white-face meadowhawk, *Sympetrum obrususm*, and the ruby meadowhawk, *Sympetrum rubicundulum*, suggesting that the worms should also survive metamorphosis in *E. simplicicollis*. Our data indicate that the ability of different *Haematoloechus* species to survive odonate metamorphosis is determined by infection site and the ability to encyst, and that such differences in the location of metacercariae within dragonflies or damselflies consequently determine whether the flukes can gain access to terrestrial and semiterrestrial frogs.

European *Haematoloechus* species are known to have metacercariae that do not encyst. One such species is *Skrjabinoeces* (*Haematoloechus*) *similis* (Looss, 1899). Interestingly, it has only been reported to infect 3 species of zygopteran hosts (*Coenagrion hastulatum*, *Coenagrion aramtum*, and *Coenagrion plchellum*). Although no experimental infections were attempted with anisopterans, Grabda (1960) reported that only zygopterans were infected in nature. Her laboratory life cycle studies indicate that cercariae of this species creep along the body of damselflies and penetrate at the base of the head or appendages, and the metacercariae are located in the anterior part of the abdomen where they remain unencysted (Grabda, 1960). These life history observations suggest that the inability to form encysted metacercariae may preclude some *Haematoloechus* species from infecting the branchial basket and/or surviving anisopteran metamorphosis. The similarities in the life history of *H. longiplexus* and *S. similis* also suggest that they may be closely related species. Similarities in host specificity at the first and second intermediate host level have been shown to be conserved among related species of *Haematoloechus* species in previous phylogenetic studies on other European and North American species of frog lung flukes, although those studies did not include *S. similis* (Snyder and Tkach, 2001).

Finally, it is unclear what role adult damselflies play in the recruitment of *H. longiplexus* by semiterrestrial leopard frogs. Our experimental infections clearly indicate that *H. longiplexus* metacercariae are located in the head, thorax, and abdomen of damselfly hosts, and all *Haematoloechus* spp. metacercariae located in these regions of naturally infected damselflies survived metamorphosis. Field data from Nebraska, however, indicate that adult damselflies are rarely infected with *Haematoloechus* spp. metacercariae. Of the 51 adult damselflies collected from Nickol Pond, only 1 was infected. Additionally, of the 64 adult lyre-tipped spreadwing damselflies, *Lestes unguiculatus*, collected from Nevens Pond, none was infected with *Haematoloechus* metacercariae (M. Bolek, person. obs.).

Metacercariae of *H. longiplexus* are commonly found in the head, including the brain, of larval and teneral damselflies, but adult damselflies are rarely infected with *Haematoloechus* spp. in nature, suggesting that infected damselflies may not survive long after infections, or that other factors, such as odonate dispersal to other ponds and feeding sites, reduce the prevalence of *Haematoloechus* spp. metacercariae in adult damselflies. Whatever the reason, it suggests that frogs become infected with *H. longiplexus* close to aquatic habitats where they can feed on infected larvae dragonflies and damselflies or teneral damselflies. Of the 3 *Rana* species in Nebraska, the bullfrog is strictly aquatic (Hudson, 1942); however, among the 2 semiterrestrial leopard frogs in Nebraska, studies by Kruse (1978) indicate that plains leopard frogs are more commonly associated with aquatic habitats than northern leopard frogs. Field data by Brooks (1976), Snyder (1996), Bolek and Janovy (2007), and the present study, indicate that plains leopard frogs are more commonly infected with *H. longiplexus* than northern leopard frogs. Of 271 plains leopard frogs and 514 northern leopard frogs sampled from Nebraska by Brooks (1976), Snyder (1996), Bolek and Janovy (2007), and as part of the current study, 4 plains leopard frogs (1.5%) were infected with *H. longiplexus*, whereas only 1 juvenile northern leopard frog (0.2%) associated with an aquatic habitat was infected with *H. longiplexus*.

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