

## SMALL FROGS GET THEIR WORMS FIRST: THE ROLE OF NONODONATE ARTHROPODS IN THE RECRUITMENT OF *HAEMATOLOECHUS COLORADENSIS* AND *HAEMATOLOECHUS COMPLEXUS* IN NEWLY METAMORPHOSED NORTHERN LEOPARD FROGS, *RANA PIPIENS*, AND WOODHOUSE'S TOADS, *BUFO WOODHOUSII*

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**ABSTRACT:** Studies on the life cycles and epizootiology of North American frog lung flukes indicate that most species utilize odonates as second intermediate hosts; adult frogs become infected by ingesting odonate intermediate hosts. Newly metamorphosed frogs are rarely infected with these parasites, predominantly because they are gape-limited predators that cannot feed on large intermediate hosts such as dragonflies. We examined the role of the frog diet and potential intermediate hosts in the recruitment of the frog lung fluke, *Haematoloechus coloradensis*, to metamorphosed northern leopard frogs (*Rana pipiens*), Woodhouse's toads (*Bufo woodhousii*), and bullfrogs (*Rana catesbeiana*) from western Nebraska. Because of the uncertain validity of *H. coloradensis* as a distinct species from *Haematoloechus complexus*, morphological characters of both species were reevaluated and the life cycles of both species were completed in the laboratory. The morphological data on *H. coloradensis* and *H. complexus* indicate that they differ in their oral sucker to pharynx ratio, uterine loop distribution, and placement of vitelline follicles. However, in terms of their life cycles, both species are quite similar in their use of physid snails as first intermediate hosts, a wide range of nonodonate and odonate arthropods as second intermediate hosts, and leopard frogs and toads as definitive hosts. These results indicate that *H. coloradensis* and *H. complexus* are generalists at the second intermediate host level and might be able to infect newly metamorphosed leopard frogs and toads by using small nonodonate arthropods more commonly than other frog lung fluke species. Comparisons of population structure of adult flukes in newly metamorphosed leopard frogs indicate that the generalist nature of *H. coloradensis* metacercariae enables it to colonize young of the year leopard frogs more commonly than other *Haematoloechus* spp. that only use odonates as second intermediate hosts. In this respect, the generalist nature of *H. coloradensis* and *H. complexus* at the second intermediate host level is an avenue for the colonization of young of year frogs.

Amphibian parasites are good model systems to address questions of parasite life cycle diversity and evolution. Recent comparative studies on amphibian parasite life cycles, recruitment, and community structure in anuran hosts by Bolek and Coggins (1998, 2000, 2001, 2003), Hardin and Janovy (1988), Muzzall and coworkers (Muzzall, 1991; Muzzall and Peebles, 1991; Gilliland and Muzzall, 1999; Muzzall et al., 2001), Snyder and Janovy (1994, 1996), and McAlpine (1997), have provided baseline data on the distribution, demography, field host specificity, and life history of amphibian parasites. These studies indicate that the parasite communities of aquatic and semiaquatic anurans are dominated by digenetic trematodes with complex life cycles, whereas the parasite communities of terrestrial anurans are dominated by nematodes which are acquired directly from the soil. Semiterrestrial frogs have fewer adult digenetic trematodes and direct life cycle nematodes than do aquatic or semiaquatic and terrestrial anurans. More important, these studies also indicate that within individual anuran species, newly metamorphosed and juvenile anurans are less commonly infected with parasites than are larger adult frogs.

Numerous hypotheses have been advanced for the lack of parasites in small anurans and include such causal factors as lack of time for exposure to parasites, a small body size, which affects the surface area available for skin-penetrating parasites, and small gape size, which affects the size of potential intermediate hosts that can be ingested by these frogs. The few studies that exist on newly metamorphosed northern leopard frogs (*Rana pipiens*), a semiterrestrial anuran and terrestrial toads (*Bufo* spp.) from Canada and the upper midwestern United States, indicate that young of the year frogs and toads are rarely if ever infected with adult trematodes such as lung flukes

(McAlpine, 1997; Gilliland and Muzzall, 1999; Bolek and Coggins, 2003). Contrary to these studies, our observations from western Nebraska indicate that in an arid environment, newly metamorphosed northern leopard frogs are commonly infected with *Haematoloechus coloradensis* (Cort, 1915), with prevalence reaching over 50%. These observations are intriguing from the perspective of complex life cycle evolution because this trematode is acquired through the frogs' diet and, therefore, should not be expected in newly metamorphosed leopard frogs that are gape-limited predators (Dronen, 1975; Bolek and Coggins, 2003). These observations suggest that differences in host and or parasite life histories may be important in parasite population structure and influence selective pressures on parasite life cycle evolution.

Studies on the life cycles and epizootiology of North American frog lung flukes indicate that, in general, adult frogs become infected by ingesting odonate intermediate hosts (Krull, 1930, 1931, 1932, 1933, 1934; Ingles, 1933; Schell, 1965; Dronen, 1975; Kennedy, 1980). More recently, studies by Snyder and Janovy (1994, 1996) on 4 common frog lung flukes from Nebraska show that host specificity at the first and second intermediate host level can be variable among these parasite congeners, indicating that life cycles of closely related species could differ in evolutionarily significant ways. Because of recent confusion in the literature on the taxonomy of *H. coloradensis* and *Haematoloechus complexus*, as seen in papers by Kennedy (1981) and León-Règagnon and Brooks (2003), and the current synonymy of these 2 species by Kennedy (1981), it is unclear whether Snyder and Janovy (1994) were dealing with *H. complexus* or *H. coloradensis*. The morphology and life cycles of these parasites, therefore, must be re-evaluated.

In this study, our 5 main goals are (1) to determine the population structure of frog lung flukes in newly metamorphosed northern leopard frogs, newly metamorphosed Woodhouse's

toads (*Bufo woodhousii*), and 3 other anuran species from western Nebraska; (2) to determine the second intermediate arthropod hosts that serve as a route of infection for *H. coloradensis* to newly metamorphosed leopard frogs and toads in the field; (3) to re-evaluate the second intermediate host specificity of *H. complexus*; (4) to test whether nonodonate arthropods serve as a viable route of infection for *H. coloradensis* and *H. complexus* to newly metamorphosed frogs and toads; and (5) to re-evaluate the diagnostic characteristics of *H. coloradensis* and *H. complexus*.

## MATERIALS AND METHODS

### *Haematoloechus coloradensis* field studies

During July–August 2001, 142 young of the year northern leopard frogs, were collected from Cedar Creek, Keith County, Nebraska (41.18639°N, 101.36276°W), and examined for *Haematoloechus* spp. Stomach content data were also obtained. All frogs were placed on ice as they were being collected and brought into the laboratory. Frogs were killed, the snout vent length (SVL) was measured, and the frogs were examined for parasites and stomach content data within 1–4 hr of collection. Trematodes were removed from the lungs, allowed to release eggs in water, and fixed in alcohol-formalin-acetic acid (AFA); representative specimens were stained with Semichon's acetocarmine (Pritchard and Kruse, 1982). All lung flukes were identified on the basis of the description for *H. coloradensis* by Cort (1915), *H. complexus* by Krull (1933), *Haematoloechus longiplexus* and *Haematoloechus medioplexus* by Stafford (1902), and *Haematoloechus parviplexus* by Irwin (1929). All stomach contents were identified as specifically as possible, and all intact stomach content remains were measured to the nearest 0.5 mm. Stomach content data were grouped as frequencies of individuals ingested according to order, class, or subclass and aquatic or terrestrial ecological habitats. Invertebrates from stomach contents of frogs were identified to family, genus, or species with keys in Borror et al. (1989), Merritt and Cummins (1996), Westfall and May (1996), Dunkle (2000), Needham et al. (2000), and Thorp and Covich (2001).

Additionally, 62 naturally infected frogs collected from August–September 2001 were maintained in the laboratory for 4–6 wk to allow enough time for all immature specimens of *Haematoloechus* spp. naturally infecting these frogs to mature. Frogs were maintained in groups of 5 individuals in small plastic boxes (33 × 9 × 14 cm) on moist paper towels and fed commercially reared crickets 3 times per week.

Frogs were also sampled from Cedar Creek and Breen's Flyway (a pond adjacent to Cedar Creek, 41.18080°N, 101.57973°W) during 2002–2004 and measured and examined for frog lung flukes. These specimens included 25 northern leopard frogs collected during September 2002; 40 northern leopard frogs and 2 bullfrogs collected during August–September 2003; and 20 northern leopard frogs, 25 metamorphosed Woodhouse's toads, and 10 metamorphosed and adult bullfrogs (*Rana catesbeiana*) collected during June–September 2004.

To determine what second intermediate hosts served as reservoirs of infections for metamorphosed northern leopard frogs at Cedar Creek during 2001, numerous arthropods were sampled during June–August 2001 from this location. All aquatic and semiaquatic arthropods were collected by the use of a dip-net, stored in buckets without snails, and brought into the laboratory. Adult odonates were collected with a butterfly net, then immediately placed on ice in plastic jars, and brought back to the laboratory. All adult odonates and aquatic arthropods were isolated within 1 hr of collection, identified, and examined for the presence of metacercariae. Aquatic and semiaquatic arthropods collected from Cedar Creek were identified to family, genus, or species with keys in Borror et al. (1989), Merritt and Cummins (1996), Westfall and May (1996), Dunkle (2000), Needham et al. (2000), and Thorp and Covich (2001).

The following measures of parasitism were calculated for the various amphibians and invertebrates examined (Margolis et al., 1982): prevalence, the percentage of infected organisms in a sample; mean intensity, the mean number of worms per infected host; mean abundance, the mean number of individuals of a particular parasite species per organism of a particular species examined, including infected and noninfected

individuals; or a combination of these measures. Values are reported as a mean ± 1 SD.

### *Haematoloechus coloradensis* snail first intermediate host infections

Adult *H. coloradensis* flukes were obtained from wild-caught northern leopard frogs from Cedar Creek. Worms were placed in 70-ml plastic containers containing aged tap water and allowed to release their 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°N, 96.46000°W). Snails were maintained on a diet of frozen mustard greens and Tetra Min® fish food. Snails were reared from eggs for a period of 6 wk in the laboratory and then infected with *H. coloradensis* eggs by placing individual snails into 70-ml plastic containers with *H. coloradensis* eggs and Tetra Min fish food for 5 min. All snail feces were then checked for hatched *H. coloradensis* eggs. Exposed snails were maintained for a period of 30 days, and all survivors were isolated in 1.5-ml well plates filled with aged tap water and observed daily for shedding cercariae.

### *Haematoloechus coloradensis* nonodonate arthropod second intermediate host infections

Adult male giant water bugs (Hemiptera: *Belostoma* sp.) covered with eggs were collected from Nickol Pond and brought into the laboratory and placed in individual white 22.7-L buckets. Once hatched, young belostomatid bugs were individually isolated in 1.5-ml well plates filled with aged tap water and fed chironomid larvae daily. Three additional nonodonate arthropod species (Diptera: *Tanytarsus* sp., Ephemeroptera: *Callibaetis* sp., and Crustacea: *Hyalella azteca*) used in the second intermediate host infections came from a variety of natural populations, including the toe drains of Lake McConaughy, Keith County, Nebraska (41.23218°N, 101.66973°W), and Dunwoody Pond, Keith County, Nebraska (41.21527°N, 101.5784°W). Larval eastern pondhawk dragonflies (*Erythemis simplicicollis*) collected from Dunwoody Pond were also exposed to *H. coloradensis* cercariae as positive controls. Nonodonate arthropods and dragonflies were divided into 3 equal groups and designated as time 0 controls, experimental, or time *t* controls. Nonodonate arthropods were isolated in 1.5-ml well plates, whereas dragonflies were isolated in 5-ml well plates filled with aged tap water for 24 hr before exposure. Time 0 controls were dissected at the beginning of the experimental infections, whereas time *t* controls were maintained throughout the duration of the experiment and dissected along with the experimental group. For infections, approximately 20–50 cercariae of *H. coloradensis* from lab-reared and infected *P. gyrina* snails were pipetted into each well that contained an experimental nonodonate and odonate arthropod. After exposure to cercariae, water was changed daily for a period of 4 days, after which time all surviving experimentally exposed arthropods and time *t* control arthropods were dissected in insect saline and inspected for the presence of *H. coloradensis* metacercariae. Cercariae attachment and penetration behavior was observed on a number of nonodonate and odonate arthropods, including dragonfly and damselfly larvae.

### *Haematoloechus coloradensis* frog definitive host infections

Young tadpoles (Gosner stage 26–30) of northern leopard frogs were collected from Cedar Creek and 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 beetle (*Tenebrio molitor*) adults and larvae. Lab-reared northern leopard frogs were each exposed to *H. coloradensis* metacercariae reared in nonodonate arthropods. All arthropods were dissected in insect saline (Hoar and Hickman, 1967). On removal from the nonodonate arthropod hosts, metacercariae were divided into groups of 10–15 in insect saline. Ten to 15 metacercariae were drawn into a pipette and placed into the esophagus of an experimental frog and forced down its throat. The pipette was then examined under a dissecting microscope to confirm that no metacercariae remained. Exposed frogs, along with noninfected time *t* controls, were maintained in groups of 2–4 individuals in 45.5-L tanks on moist sand or gravel. They were fed commercial crickets and tene-

brionid beetles daily for a period of up to 30 days, at which time they were killed, necropsied, and examined for frog lung flukes.

Additionally, 3 lab-reared northern leopard frogs, 3 lab-reared bullfrogs, and 2 field-collected Woodhouse's toads, along with 10 time 0 controls, and 10 time *t* controls collected from Beckius Pond, Keith County, Nebraska (41.20835°N, 101.61777°W), were used for experimental infections with *H. coloradensis*. Amphibians were infected with 5–10 metacercariae of *H. coloradensis* from laboratory-infected dragonflies, *Erythemis simplicicollis*, collected from Dunwoody Pond in Keith County, Nebraska. Frogs and toads were maintained in the laboratory on a diet of commercial crickets for 20–30 days, when they were killed and examined for *H. coloradensis* infections.

#### **Haematoloechus complexus life cycle studies**

Because of high mortality of experimentally infected snails, naturally infected snails were collected from Pawnee Lake, Lancaster County, Nebraska (40.84310°N, 96.85700°W). During 2001–2005, 399 frogs and toads of 6 species were examined from this location; *H. coloradensis* was never in these amphibians (Bolek, 2006). The only frog lung fluke that uses physid snails as first intermediate hosts at this location is *H. complexus*. Fifty *P. gyrina* snails were collected by dip-net and were individually isolated in 1.5-ml well plates filled with aged tap water. Of these, 1 snail shed *Haematoloechus* sp. cercariae. Three nonodonte arthropod species (Diptera: *Tanytarsus* sp., Ephemeroptera: *Callibaetis* sp., and Crustacea: *Hyalella azteca*) along with eastern pondhawk dragonflies used in the second intermediate host infections, came from a variety of natural populations collected from toe drains of Lake McCaughy, Keith County, Nebraska, and Dunwoody Pond, Keith County, Nebraska. These arthropods were divided into 3 equal groups and designated as time 0 controls, experimental, or time *t* controls and were isolated in 1.5-ml well plates filled with aged tap water for 24 hr before exposure. All arthropod infections followed the same procedure as previously described for *H. coloradensis*. To be sure that cercariae shed by the single *P. gyrina* were *H. complexus*, 4 newly metamorphosed Woodhouse's toads along with 10 time 0 and 10 time *t* controls collected from Beckius Pond, Keith County, Nebraska, were used for definitive host infections. The 4 experimental toads were each given 5–10 *H. complexus* metacercariae from laboratory-infected nonodonte arthropods. Toads were maintained in the laboratory on a diet of tenebrionid beetle larvae for 20–30 days, when they were killed and examined for *H. complexus* infections.

#### **Other amphibian field surveys**

During May–September 2000–2004, an additional 57 adult bullfrogs and 2 adult plains leopard frogs (*Rana blairi*) were collected from Nevens Pond, Keith County, Nebraska (41.20710°N, 101.40850°W) and 3 adult plains leopard frogs and 10 adult plains spadefoot toads (*Spea bombifrons*) were collected from Cedar Point Biological Station, Keith County, Nebraska (41.21051°N, 101.52220°W) and examined for frog lung flukes. Voucher specimens of lung flukes have been deposited in the H. W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska (accession numbers HWML 48430, *H. coloradensis*, 48429 *H. complexus*, 48432 *H. longiplexus*, 48431 *H. medioplexus*, and 48433 *H. parviplexus*).

#### **Morphological studies**

Morphological data were collected on 20 *H. coloradensis* worms from northern leopard frogs, plains leopard frogs, and Woodhouse's toads, and 20 *H. complexus* worms from northern leopard frogs, plains leopard frogs, Woodhouse's toads, and green frogs (*Rana clamitans melanota*). Worms used for morphological analysis were collected from a number of locations in Indiana, Nebraska, and Wisconsin. These included 3 *H. coloradensis* collected from plains leopard frogs from Nevens Pond and Cedar Point Biological Station, Keith County, Nebraska, and 7 *H. coloradensis* collected from northern leopard frogs from Cedar Creek, Keith County, Nebraska; 1 *H. complexus* collected from a northern leopard frog from West Lafayette, Tippecanoe County, Indiana (40.44472°N, 86.99024°W); 15 *H. complexus* from plains leopard frogs collected from Pawnee Lake, Nebraska; 1 *H. complexus* from an experimentally infected Woodhouse's toads from Nebraska; and 2 *H. complexus* worms from green frogs collected from Genesee Depot, Waukesha County, Wisconsin (42.98984°N, 88.36634°W). Additionally, to

obtain a better geographical representation *H. coloradensis* and *H. complexus*, voucher specimens collected by Dan Brooks were borrowed from the H. W. Manter Laboratory, University of Nebraska State Museum. These included, *H. coloradensis*: HWML 20134 from a northern leopard frog from Antelope County, Nebraska (42.14764°N, 98.048055°W), 2 slides; HWML 20135 from a northern leopard frog from Saunders County, Nebraska (41.3125°N, 96.69583°W), 2 slides; HWML 20136 from a northern leopard frog from Dawes County, Nebraska (42.773055°N, 103.066385°W), 3 slides; and HWML 20137 from a northern leopard frog from Dawes County, Nebraska (42.699865°N, 103.272775°W), 3 slides, and *H. complexus* HWML 20132 from a plains leopard frog from Lancaster County, Nebraska (40.791665°N, 96.675°W), 1 slide, and HWML 20170 from a plains leopard frog from Jefferson County, Nebraska (40.234865°N, 97.069305°W), 1 slide. On the basis of recent and past *Haematoloechus* sp. description by Brooks (1976) and León-Règagnon et al. (2001, 2002), 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 morphological characteristics between *H. coloradensis* and *H. complexus*. An approximate  $t'_s$ -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**

#### **Haematoloechus coloradensis field studies**

Mean  $\pm$  SD SVL of the 142 northern leopard frogs collected during July–August 2001 was  $3.85 \pm 0.72$  cm; range 2.3–8.3 cm. Seventy-five of 142 (53%) frogs were infected with frog lung flukes, with a mean abundance of  $3.7 \pm 7.3$  (range 0–44). In total, 530 worms were recovered—491 immature and 39 mature—indicating that frogs were recruiting parasites during the collection period. Seasonally, worms became gravid by mid-August. There was a statistically significant difference in the mean intensity of worms recovered from frogs that contained immature worms versus frogs infected with gravid worms. Most frogs contained nongravid worms during 24 July–8 August, with a single frog having 1 gravid worm, whereas most frogs contained gravid worms on August 16, with a single frog having 3 immature worms (Fig. 1;  $t'_s = 3.41$ ,  $P < 0.001$ ). Additionally, of the 62 frogs maintained in the laboratory for a period of 4–6 wk, 30 (48%) were infected with 4 immature and 60 mature (mean intensity [MI] =  $2.13 \pm 1.33$ ) *H. coloradensis*, indicating that only 1 species of frog lung fluke infected this frog population. Stomach contents data were obtained from 139 of 142 (98%) frogs, with a total of 576 individual invertebrates recovered. Sixteen different groups of aerial, terrestrial, and aquatic invertebrates were recovered, with odonates making up 0.7% of the total diet (Fig. 2). The average size of invertebrates ingested by these frogs was 6.8 mm (range 0.5–18 mm), with all large invertebrates ingested being soft-bodied oligochaetes or lepidopteran larvae.

In total, 320 aquatic and semiaquatic arthropods were collected, including larval and adult dragonflies and damselflies, adult coleoptera, larval diptera, larval ephemeroptera, adult hemiptera, and adult amphipoda. Of these, larval and adult dragonflies and damselflies were infected; coleoptera, ephemeroptera, hemiptera, and amphipoda were also infected with *Haematoloechus* sp. metacercariae. All metacercariae were lo-

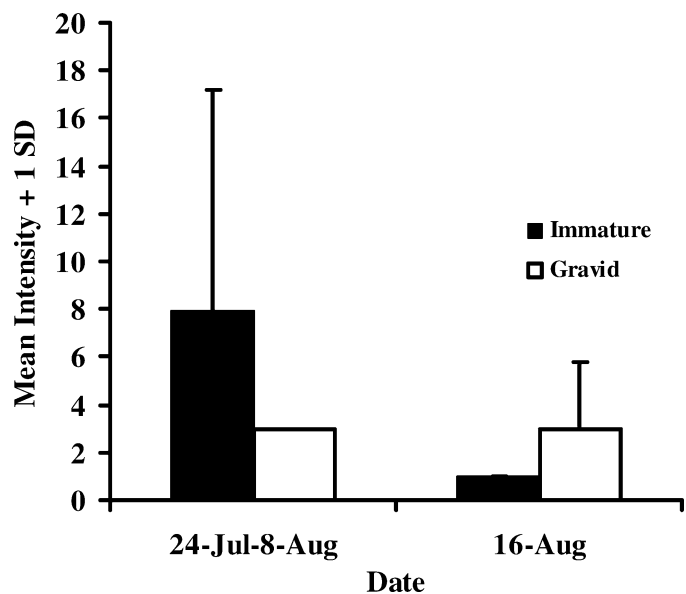


FIGURE 1. Mean intensity of immature and gravid *Haematoloechus coloradensis* recovered from northern leopard frogs infected with predominantly immature worms during late July–early August and predominantly gravid worms during mid-August 2001.

cated in the head, legs, and hemocoel of the arthropods sampled, with prevalence ranging from as high as 94% in larval dragonflies to as low as 0% in dipteran larvae (Table I). None of the metacercariae were encapsulated by the arthropod host. These naturally infected arthropods potentially made up 11.5% of the stomach content data of frogs sampled from this location (Fig. 2).

Mean SVL of the 25 northern leopard frogs collected during September 2002 was  $4.05 \pm 0.59$  cm (range 3.3–6.5 cm); of these, 22 of 25 (88%) frogs were infected with *H. coloradensis*, with a mean abundance of  $5 \pm 4.46$  (range 0–16). Mean SVL of the 40 northern leopard frogs collected during May–September of 2003 was  $4.45 \pm 0.77$  cm (range 3.5–6.5 cm); of these, 27 of 40 (68%) northern leopard frogs were infected with *H. coloradensis*, with a mean abundance of  $2.83 \pm 3.35$  (range 0–16). Two of 40 (5%) northern leopard frogs were infected with *H. medioplexus*, with a mean abundance of  $0.5 \pm 3$  (range 0–19). Additionally, 1 of 2 (50%) bullfrogs (SVL 5.5–6.0 cm) was infected with 19 *H. parviplelexus*. Mean SVL of the 20 northern leopard frogs, 25 Woodhouse's toads, and 10 bullfrogs collected during July–September 2004 was  $4.67 \pm 1$  (2.6–6.2) cm,  $1.6 \pm 0.5$  (1–2.8) cm, and  $7.6 \pm 2.1$  (4.5–10) cm, respectively. Three *Haematoloechus* spp. were recovered from these anurans. Northern leopard frogs shared *H. coloradensis* with Woodhouse's toads, whereas bullfrogs were infected with *H. parviplelexus* and shared *H. longiplelexus* with northern leopard frogs.

#### *Haematoloechus coloradensis* laboratory life cycle studies

Surviving lab-reared and -infected snails began shedding cercariae after a period of 30 days and continued to shed cercariae for up to 2 wk, when observations were stopped. Metacercariae of *H. coloradensis* developed in all 4 species of nonodonate

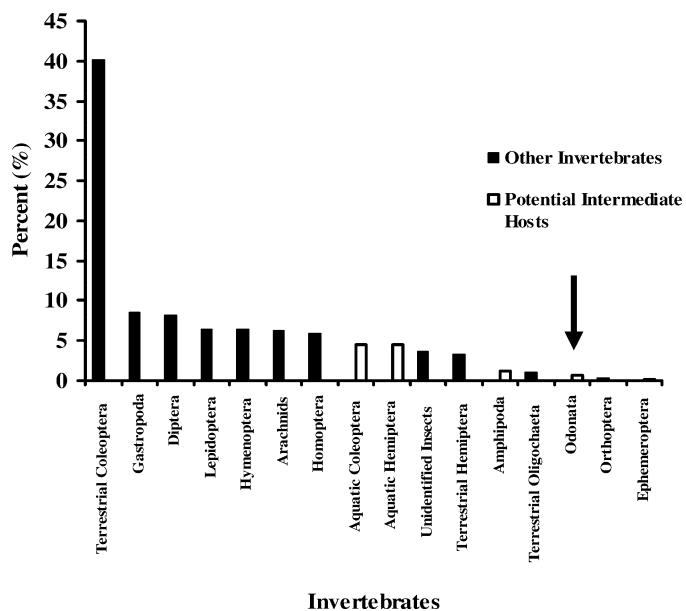


FIGURE 2. Stomach contents frequency data for 142 young of year (YOY) northern leopard frogs indicating the potential intermediate hosts for *Haematoloechus coloradensis* recovered from Cedar Creek, Keith County, Nebraska, during 2001. Note that odonates (arrow) make up only 0.7% of the diet.

arthropod hosts exposed, although not all exposed individuals became infected. Prevalence ranged from a high of 80% for ephemeropterans to a low of 20% for amphipods (Table II). Ten of 10 (100%) dragonflies became infected with *H. coloradensis*. Additionally, 2 chironomid larvae metamorphosed on the day of necropsy; 1 of these insects was infected with a single *H. coloradensis* metacercaria. None of the metacercariae were encapsulated by any of the arthropod hosts. No *Haematoloechus* metacercariae were observed in any of the time 0 or time *t* control groups. Four of 6 (67%) lab-reared northern leopard frogs given 10–15 *H. coloradensis* metacercariae from nonodonate hosts became infected, with a mean intensity of  $1.75 \pm 1$  (range 1–3), whereas none of the 6 time *t* control lab-reared northern leopard frogs were infected.

Additionally, when given metacercariae from experimentally infected dragonfly larvae, 3 of 3 (100%) northern leopard frogs became infected with 1, 2, and 3 *H. coloradensis*, 1 of 2 (50%) Woodhouse's toads were infected with 3 *H. coloradensis*, but none of the 3 (0%) bullfrogs became infected.

Observations on the behavior of *H. coloradensis* cercariae indicated that cercariae stopped swimming on contact with the arthropod host. Cercariae attached to the arthropod with their ventral sucker and began to crawl along the surface of the arthropod body with the aid of their ventral and oral suckers (Fig. 3). Once cercariae encountered an intersegmental membrane, they began to thrust the stylet into the membrane. Some of these were observed to drop their tails, pierce the intersegmental membrane, enter, and develop to the metacercaria stage (Fig. 3).

#### *Haematoloechus complexus* life cycle studies

Metacercariae of *H. complexus* developed in all 3 species of nonodonate arthropod hosts exposed, although not all exposed

TABLE I. Prevalence, mean intensity, mean abundance, total number, and location of *Haematoloechus* spp. metacercariae in 320 arthropods collected from Cedar Creek, Keith County, Nebraska.

Family, genus, or species of arthropods examined	Prevalence (no. infected/no. examined)	Mean intensity $\pm$ 1 SD (range)	Mean abundance $\pm$ 1 SD	No. of metacercariae recovered	Location in host
Insecta					
Odonata: Anisoptera					
Larva <i>Anax junius</i>	94 (15/16)	19.7 $\pm$ 11.3 (1–38)	18.5 $\pm$ 12	296	Head, thorax, leg
Adult Anisoptera					
Libellulidae*	7 (6/81)	4.1 $\pm$ 4.7 (1–12)	0.3 $\pm$ 1.6	25	Head, thorax, leg
Odonata: Zygoptera					
Larva Zygoptera					
Coenagrionidae†	67 (10/15)	3.9 $\pm$ 3.1 (1–11)	2.6 $\pm$ 3.1	39	Head, thorax, leg
Insecta					
Adult Zygoptera					
<i>Hetaerina americana</i>	48 (13/27)	2.4 $\pm$ 2 (1–6)	1.1 $\pm$ 1.8	31	Head, thorax, leg
Coleoptera: Hydrophilidae	11 (3/27)	2.3 $\pm$ 1.5 (1–4)	0.3 $\pm$ 0.9	11	Thorax
Hemiptera: <i>Belostoma</i> sp.	9 (3/33)	1 $\pm$ 0 (1)	0.09 $\pm$ 0.3	3	Head and thorax
Ephemeroptera‡	10 (4/42)	3.5 $\pm$ 1.7 (1–5)	0.3 $\pm$ 1.1	14	Head, thorax, and gills
Diptera: Stratiomyidae larva	0 (0/9)	—	0 $\pm$ 0	0	Not found
Crustacea					
Amphipoda:	4 (3/70)	1.6 $\pm$ 0.6 (1–2)	0.07 $\pm$ 0.4	5	Cephalothorax, leg
<i>Hyalella azteca</i>					

\* *Erythemis simplicicollis* (0/3), *Libellula luctuosa* (0/1), *Plathemis lydia* (0/3), *Sympetrum occidentale* (6/71), *Sympetrum semicinctum* (0/1), *Sympetrum rubicundulum* (0/1), and *Sympetrum vicinum* (0/1).

† *Amphiagrion abbreviatum* (10/14) and *Ischnura verticalis* (0/1).

‡ *Callibaetis* sp. (0/20) and *Caenis* sp. (4/22).

individuals became infected. Prevalence ranged from a high of 67% for chironomids to a low of 21% for amphipods. Metacercariae were located in the head, hemocoel, prolegs, legs, and anal gills of the arthropod hosts. Metacercariae were not encapsulated in any of the arthropod hosts except for a single *Callibaetis* sp., which encapsulated and destroyed all of the metacercariae (Fig. 4). Additionally, 4 of 10 (40%) dragonflies became infected with *H. complexus* (Table III). Two of 4 (50%)

toads became infected with a total of 5 *H. complexus*, 2 of which were adults. No time 0 or time *t* control arthropods or amphibians were infected.

#### Other amphibian field surveys

Of 57 bullfrogs, 5 plains leopard frogs, and 10 plains spadefoot toads examined from Nevens Pond and Cedar Point Bio-

TABLE II. Prevalence, mean intensity, mean abundance, total number, and location of *Haematoloechus coloradensis* metacercariae recovered 4 days postexposure in experimentally infected nonodonate arthropods.

Species of arthropods exposed	Prevalence (no. infected/no. exposed survivors)	Mean intensity $\pm$ 1 SD (range)	Mean abundance $\pm$ 1 SD	No. of metacercariae recovered	Location in host
Hemiptera:					
Belostomatidae					
<i>Belostoma</i> sp.	25 (5/20)	1.8 $\pm$ 1.3 (1–4)	0.45 $\pm$ 1	9	Head, thorax, leg
Diptera:					
Chironomidae					
<i>Tanytarsus</i> sp.	29 (16/55)	4.2 $\pm$ 4.0 (1–15)	1.2 $\pm$ 2.9	67	Head, thorax, anal gill
Ephemeroptera:					
Baetidae					
<i>Callibaetis</i> sp.	80 (8/10)	5.5 $\pm$ 4.5 (1–14)	4.4 $\pm$ 4.6	44	Head, thorax, leg, gill
Crustacea					
Amphipoda					
<i>Hyalella azteca</i>	20 (2/10)	1.5 $\pm$ 0.7 (1–2)	0.3 $\pm$ 0.7	3	Cephalothorax, leg

logical Station, 74% of bullfrogs and 60% of plains leopard frogs were infected with lung flukes, but none of the plains spadefoot toads were infected. Fifty-eight percent of bullfrogs were infected with *H. longiplexus*, with a mean intensity of  $18.6 \pm 18.4$ , and 12% of bullfrogs were infected with *H. parviplexus*, with a mean intensity of  $19.9 \pm 25.5$ , whereas all infected plains leopard frogs were infected with *H. coloradensis*, with a mean intensity of  $1.3 \pm 0.6$ . Overall prevalence and mean intensity of *Haematoloechus* spp. recovered from the 5 anurans sampled from Keith County, Nebraska, during 2000–2004 are given in Table IV.

### Morphological analysis/diagnostic characteristics

*Haematoloechus coloradensis* (Cort, 1915) Ingles, 1932 (Fig. 5): On the basis of 20 mature specimens, body elongate, 4.18–7.28 mm long by 0.82–1.55 mm wide. Oral sucker subterminal and oval 300–500  $\mu\text{m}$  long by 220–420  $\mu\text{m}$  wide. Pharynx 180–340  $\mu\text{m}$  long by 190–330  $\mu\text{m}$  wide. Oral sucker/pharynx width ratio 1.04–1.43. Oral sucker/pharynx length ratio 1.2–1.77. Acetabulum 24–50% body length from anterior end, round to oval 230–340  $\mu\text{m}$  long by 200–380  $\mu\text{m}$  wide. Oral sucker/acetabulum ratio 0.88–1.31. Testes round to oval positioned in tandem in midhindbody, anterior testis 350–600  $\mu\text{m}$  long by 310–750  $\mu\text{m}$  wide and posterior testis 400–650  $\mu\text{m}$  long by 380–720  $\mu\text{m}$  wide. Cirrus sac long, extending to level of acetabulum. Genital pore ventral to pharynx. Ovary oval, rarely round posterior or dorsolateral to acetabulum 130–580

$\mu\text{m}$  long by 70–450  $\mu\text{m}$  wide. Uterus with intercecal loops, never extending past ceca at posterior testis. Vitellaria acinous, follicular forming clusters, distribution differing on each side of body. On ovarian side 7–10 extracecal clusters, rarely 1 intracecal cluster in anterior part of body. On opposite side of body 7–12 extracecal clusters, rarely 1 or 2 intracecal clusters in posterior end of body. Eggs 30–37.5  $\mu\text{m}$  long by 15–21  $\mu\text{m}$  wide.

*Haematoloechus complexus* (Seely, 1906) Krull, 1933 (Fig. 6): On the basis of 20 mature specimens, body elongate, 1.22–6.08 mm long by 0.82–1.19 mm wide. Oral sucker subterminal and oval 110–420  $\mu\text{m}$  long by 110–420  $\mu\text{m}$  wide. Pharynx 60–220  $\mu\text{m}$  long by 70–240  $\mu\text{m}$  wide. Oral sucker/pharynx width ratio 1.47–2.1. Oral sucker/pharynx length ratio 1.67–2.63. Acetabulum 33–48% body length from anterior end, round to oval 80–330  $\mu\text{m}$  long by 80–310  $\mu\text{m}$  wide. Oral sucker/acetabulum ratio 1.1–1.68. Testes round to oval positioned in tandem in midhindbody, anterior testis 250–950  $\mu\text{m}$  long by 150–680  $\mu\text{m}$  wide and posterior testis 160–980  $\mu\text{m}$  long by 150–850  $\mu\text{m}$  wide. Cirrus sac long, extending to level of acetabulum. Genital pore ventral to pharynx. Ovary oval, rarely round posterior or dorsolateral to acetabulum, 300–600  $\mu\text{m}$  long by 200–450  $\mu\text{m}$  wide. Uterus with extracecal loops, always extending past ceca on left and/or right side of body past posterior testis. Vitellaria acinous, follicular forming clusters, distribution differing on each side of body. On ovarian side 3–6 extracecal clusters and 0–4 intracecal clusters located pre-

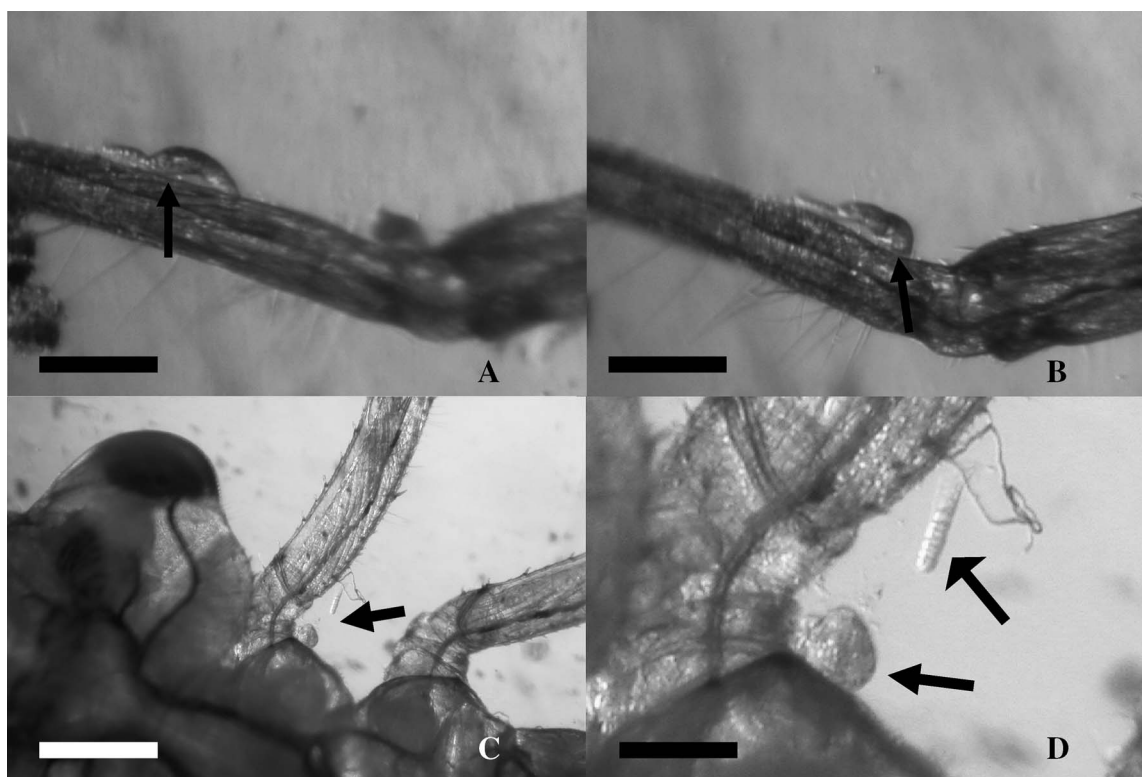


FIGURE 3. *Haematoloechus coloradensis* cercarial attachment, creeping, and penetration behavior on *Ischnura verticalis*. (A) Cercaria attached with its ventral sucker (arrow) to the tibia of *I. verticalis*. (B) Cercaria attached with its oral sucker (arrow) to the tibia of *I. verticalis* after 1 movement up the leg of the damselfly larva. (C) Cercaria beginning to penetrate the intersegmental membrane of the thorax of *I. verticalis*. (D) Enlargement of panel C. Note the penetrating cercaria and lost tail (arrows). Scale bars = 200  $\mu\text{m}$  in panels A and B, 0.5 mm in panel C, and 100  $\mu\text{m}$  panel D.

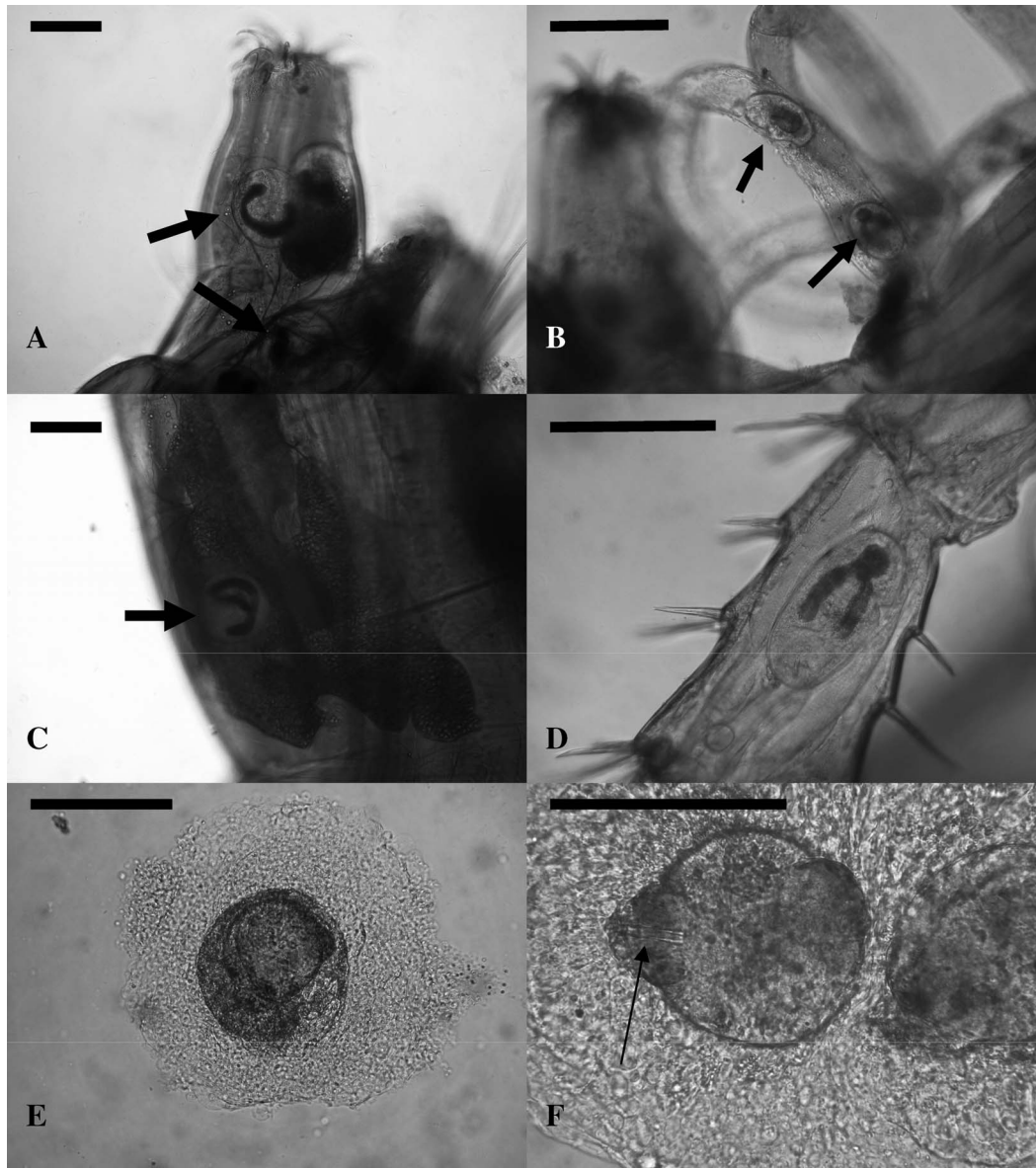


FIGURE 4. Metacercariae of *Haematoloechus complexus* in different body regions of nonodonate arthropods recovered 4–6 days postexposure. (A) Metacercariae in prolegs of *Tanytarsus* sp. (B) Metacercariae in anal gills of *Tanytarsus* sp. (C) Metacercaria in thorax of *Tanytarsus* sp. (D) Metacercaria in leg of *Hyalella azteca*. (E) Encapsulated and dead metacercaria recovered from the thorax of *Callibaetis* sp. (F) Note the stylet. Scale bars = 200  $\mu$ m.

acetabulum and 1 or 2 intracecal postposterior testes. On opposite side of body 4–9 extracecal clusters, 0–4 intracecal clusters preacetabulum, and 0–2 intracecal clusters postposterior testis. Eggs 27.5–35  $\mu$ m long by 15–17.5  $\mu$ m wide.

#### Morphological comparisons among species

Morphological comparisons between *H. complexus* and *H. coloradensis* are presented in Table V. Statistically significant differences were observed in body length, pharynx length and width, OS/PH width and length ratios, acetabulum length and width, OS/AC width ratios, testis length, and vitellaria number among *H. complexus* and *H. coloradensis*. Although these differences were statistically significant, there was overlap among

all of these characteristics except for the oral sucker/pharynx width ratio. However, *H. coloradensis* had a uterus with intercecal loops that never extended past the caeca at the posterior testis level, whereas in *H. complexus*, the uterus extracecal loops always extended past the caeca on the left, right, or both sides of the body past the posterior testis. Finally, the distribution of vitelline follicles differed among these 2 species. *H. coloradensis* always had rows of extracecal vitelline follicles on each side of the body, with rarely 1 group being intracecal in the anterior part of body located preacetabular and rarely 1 or 2 intracecal clusters in the posterior end of the body located posterior of the posterior testis. In contrast, *H. complexus* always had 3–5 groups of intracecal vitelline follicles in the an-

TABLE III. Prevalence, mean intensity, mean abundance, total number, and location of *Haematoloechus complexus* metacercariae recovered 4–6 days post exposure in experimentally infected odonate and nonodonate arthropods.

Species of arthropods exposed	Prevalence (no. infected/no. exposed survivors)	Mean intensity $\pm$ 1 SD (range)	Mean abundance $\pm$ 1 SD	No. of metacercariae recovered	Location in host
Odonata					
Libellulidae					
<i>Erythemis simplicicollis</i>	40 (4/10)	22.3 $\pm$ 20.9 (3–50)	10 $\pm$ 16.2	89	Head, thorax, leg
Diptera:					
Chironomidae					
<i>Tanytarsus</i> sp.	67 (20/30)	6.2 $\pm$ 5.2 (1–18)	4.3 $\pm$ 5.2	124	Head, thorax, anal gill
Ephemeroptera:					
Baetidae					
<i>Callibaetis</i> sp.	40 (2/5)	6.5 $\pm$ 3.5 (4–9)	2.6 $\pm$ 4.0	13	Head, thorax, leg, gill
Crustacea					
Amphipoda					
<i>Hyalella azteca</i>	21 (5/24)	2.4 $\pm$ 2.1 (1–6)	0.5 $\pm$ 1.3	12	Cephalothorax, leg

terior part of the body located preacetabulum, and 1 to 3 intracecal vitelline follicles in the posterior part of the body located posterior of the posterior testis.

## DISCUSSION

### *Haematoloechus coloradensis* recruitment

Field surveys of the 5 amphibian species in Keith County indicate that *H. coloradensis* is the dominant lung fluke in northern leopard frogs and plains leopard frogs, whereas northern leopard frogs are rarely infected with *H. medioplexus* and *H. longioplexus*. Bullfrogs collected from the same locations as northern leopard frogs and plains leopard frogs are commonly infected with both *H. longioplexus* and *H. parvioplexus*. These data support previous surveys on these frogs in Nebraska by Brooks (1976) and Snyder (1996), indicating that *H. longioplexus* and *H. parvioplexus* rarely, or never, infect northern leopard frogs or plains leopard frogs in Nebraska.

The field and laboratory studies we conducted show that in Nebraska, *H. coloradensis* is a generalist at the second intermediate host level and predominantly infects newly metamorphosed leopard frogs through nonodonate aquatic and semi-aquatic arthropods. Until recently, the limits of second intermediate host specificity among species of frog lung flukes were considered to be relatively strict, with most species infecting odonate second intermediate hosts. The life cycle of *H. coloradensis* was initially described from New Mexico by Dronen (1975), who demonstrated that this species also infected dragonflies and damselflies as second intermediate hosts and leopard frogs as definitive hosts. No attempt was made to infect nonodonate arthropods.

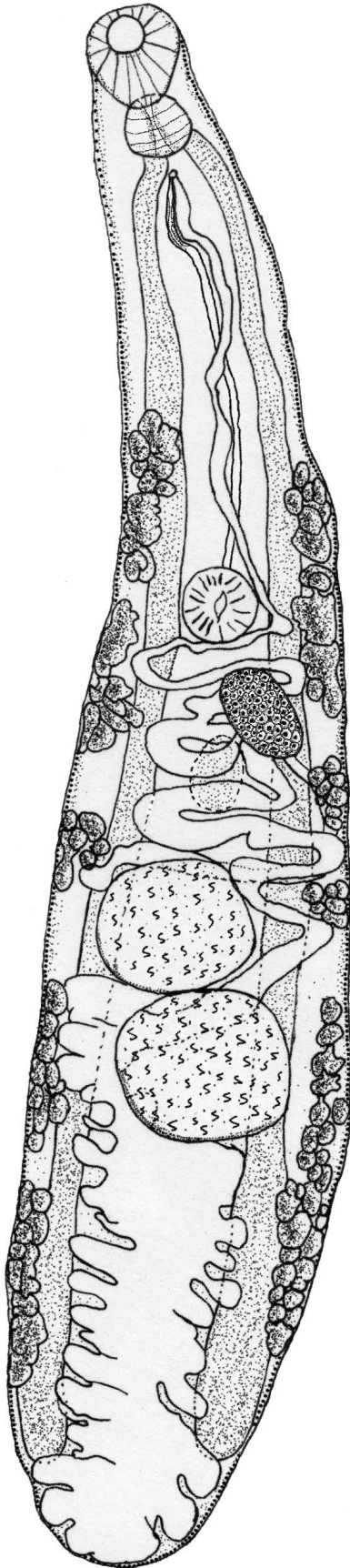
Reports of nonodonate second intermediate hosts for frog lung flukes are rare and restricted to dipteran and plecopteran larvae (van Thiel, 1930; Combes, 1968). However, all other studies on the life histories of frog lung flukes were conducted on odonates as second intermediate hosts, and it is unclear whether nonodonate arthropod infections were attempted in these studies (Krull, 1930, 1933, 1934; Ingles, 1933; Grabda, 1960; Schell, 1965; Dronen, 1975, 1977, 1978; Bourgat and

Kulo, 1979; Kennedy, 1980). Studies by Snyder and Janovy (1994, 1996) on second intermediate host specificity of 4 Nebraska *Haematoloechus* spp. were the first to show that cercariae behavior patterns dictate host specificity at the second intermediate host level among the frog lung flukes. Their studies showed that *Haematoloechus* spp. from Nebraska varied from being generalists (*H. complexus*), infecting dragonflies and

TABLE IV. Population structure of *Haematoloechus coloradensis*, *Haematoloechus longioplexus*, *Haematoloechus medioplexus*, and *Haematoloechus parvioplexus* in 5 species of anurans collected in Keith County, Nebraska during July 2000–September 2004.

Anuran species (n)	<i>Haematoloechus</i> sp.	Prevalence (%)	Mean intensity $\pm$ 1 SD
<i>Bufo woodhousii</i> (25)	<i>H. coloradensis</i>	4	1
	<i>H. longioplexus</i>	0	—
	<i>H. medioplexus</i>	0	—
	<i>H. parvioplexus</i>	0	—
<i>Rana blairi</i> (5)	<i>H. coloradensis</i>	60	1.3 $\pm$ 0.6
	<i>H. longioplexus</i>	0	—
	<i>H. medioplexus</i>	0	—
	<i>H. parvioplexus</i>	0	—
<i>Rana catesbeiana</i> (69)	<i>H. coloradensis</i>	0	—
	<i>H. longioplexus</i>	52	18.6 $\pm$ 18.4
	<i>H. medioplexus</i>	0	—
	<i>H. parvioplexus</i>	33	22.3 $\pm$ 20.2
<i>Rana pipiens</i> (289)	<i>H. coloradensis</i>	58	5.5 $\pm$ 6.6
	<i>H. longioplexus</i>	0.3	1
	<i>H. medioplexus</i>	0.7	10 $\pm$ 12.7
	<i>H. parvioplexus</i>	0	—
<i>Spea bombifrons</i> (10)	<i>H. coloradensis</i>	0	—
	<i>H. longioplexus</i>	0	—
	<i>H. medioplexus</i>	0	—
	<i>H. parvioplexus</i>	0	—





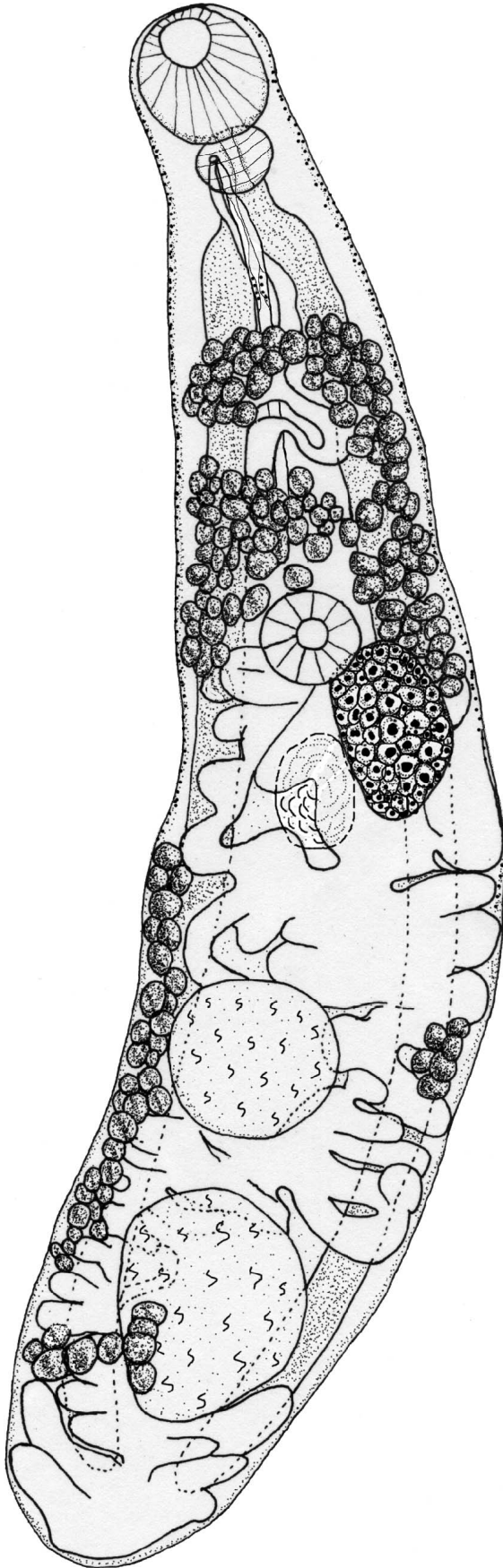
damsselfies as well as nonodonate insects and crustacean species, to specialists (*H. medioplexus* and *Haematoloechus varioplexus*), infecting only dragonflies. The various parasite species, therefore, could have different avenues for host colonization. *Haematoloechus medioplexus* and *H. varioplexus* cercariae are passive host invaders and must be drawn into the unique branchial basket respiratory apparatus of dragonflies, where they encyst and metacercariae are restricted to this region of the host. *Haematoloechus complexus* cercariae are active host invaders; these cercariae stop on contact with the arthropod host and penetrate the hosts at any intersegmental membrane by the use of a stylet and, therefore, can be found in all parts of the body, occurring in a thin hyaline cyst in the abdominal cavity, legs, and head of the second intermediate host. Like *H. complexus*, *H. longiplexus* is also an active host invader but can only penetrate damselfly naiads at the base of the caudal gills; *H. longiplexus* cercariae are also drawn into the branchial basket respiratory apparatus of dragonflies. Interestingly, Snyder (1996) also showed with a rather elegant injection experiment that cercariae of species that only use dragonfly second intermediate hosts can develop in damselfly arthropod hosts, demonstrating that cercariae behavior is the main factor or avenue controlling second intermediate host specificity in these frog lung flukes. More importantly, phylogenetic studies on some North American and European frog lung flukes by Snyder and Tkach (2001) indicate that the use of odonates as second intermediate hosts might be the ancestral condition within this genus. From these and other studies on frog lung fluke life cycles, it is clear that frog lung fluke species can vary in their host specificity at the second intermediate host level.

This study clearly indicates that nonodonate arthropods are a viable avenue for the transmission of *H. coloradensis* to newly metamorphosed leopard frogs and toads in Nebraska. These observations, that *H. coloradensis* can colonize young of year leopard frogs more commonly than other *Haematoloechus* spp. (*H. longiplexus*, *H. medioplexus*, and *H. parvoplexus*) that only use odonates as second intermediate hosts, are important for 2 reasons: (1) the large number of species of second intermediate hosts used that are represented in these frogs' diet; and (2) the range of sizes of second intermediate hosts used.

These observations reveal that this second intermediate host infection pattern is the main reason why newly metamorphosed leopard frogs at Cedar Creek are commonly infected with *H. coloradensis* compared to other studies in which newly metamorphosed frogs are never or rarely infected with *Haematoloechus* spp. (Krull, 1930, 1931; Dronen, 1977; Gilliland and Muzzall, 1999; Muzzall et al., 2001; Bolek and Coggins, 2003). Because small frogs prey primarily on small invertebrates because of gape size limitations, the use of small, nonodonate arthropods as second intermediate hosts allow *H. coloradensis* to colonize newly metamorphosed frogs at Cedar Creek. In fact, of the 289 newly metamorphosed leopard frogs sampled at Cedar Creek during 2001–2004, 168 (58%) were infected with *H. coloradensis*, whereas only 1 (0.3%) and 2 (0.7%) were in-

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FIGURE 5. Line drawing of *Haematoloechus coloradensis* from the northern leopard frog (*Rana pipiens*) collected from Cedar Creek, Keith County, Nebraska. Scale bar = 1.5 mm.



fectured with *H. longiplexus* and *H. medioplexus*, respectively, and none (0%) were infected with *H. parviplexus*, all of which use dragonflies and damselflies as second intermediate hosts. Therefore, this study demonstrates that use of a diversity of small arthropod species as second intermediate hosts by *H. coloradensis* provides an avenue for colonization of small frogs, which prey primarily on small invertebrates presumably because of their limitations in gape size. Interestingly, we never find *H. complexus* in newly metamorphosed Woodhouse's toads, or plains leopard frogs in eastern Nebraska where *H. complexus* is common. However, in eastern Nebraska, both of these anurans breed in small temporary ponds, which usually dry up by the end of the summer when frogs and toads are emerging. Therefore, it is not clear whether second intermediate hosts infected with *H. complexus* are available for young of year anurans to feed on at our study site (Bolek, 2006).

Among the 5 anurans sampled from Keith County, Nebraska, both leopard frog species and, to a lesser degree, Woodhouse's toads became infected with *H. coloradensis* in nature, as well as in the laboratory, whereas bullfrogs were resistant to this species in nature and in the laboratory, confirming previous studies on definitive host specificity of *H. coloradensis* by Dronen (1975).

#### ***Haematoloechus coloradensis* and *H. complexus* taxonomy and distribution**

The morphological data on *H. coloradensis* and *H. complexus* suggest that although morphologically similar, these 2 species are distinct, differing in their oral sucker to pharynx width and length ratios, uterine loop distribution, and placement of vitelline follicles. However, in terms of their life cycles, both species are quite similar. Both utilize physid snails as first intermediate hosts. The cercariae of both of these species are active host invaders and can infect a wide range of nonodonate and odonate arthropods (generalists at the second intermediate host level), and both might be able to infect newly metamorphosed leopard frogs more commonly than other lung flukes that use only odonates as second intermediate hosts. As with *H. complexus*, on contact with a potential arthropod host, the cercariae of *H. coloradensis* attach to the arthropod and are able to penetrate the host at any intersegmental membrane. Finally, both of these species infect northern leopard frogs, plains leopard frogs, and Woodhouse's toads but cannot infect bullfrogs (Krull, 1933; Dronen, 1975; this study).

The similarities in the life history of *H. coloradensis* and *H. complexus* suggest that they could be closely related species. In previous phylogenetic studies on other European and North American species of frog lung flukes (which did not include *H. coloradensis*), host specificity at the first and second intermediate host level has been shown by Snyder and Tkach (2001) to be conserved among related species of *Haematoloechus*. However, recent molecular phylogenetic studies by León-Règagnon and Brooks (2003) on African, European, and North American species of frog lung flukes indicate that *H. complexus*

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FIGURE 6. Line drawing of *Haematoloechus complexus* from the plains leopard frog (*Rana blairi*) collected from Pawnee Lake, Lancaster County, Nebraska. Scale bar = 1.5 mm.

TABLE V. Morphological characteristics of adult *Haematoloechus coloradensis* and *Haematoloechus complexus*. Means and ranges (in parentheses) are in micrometers ( $\mu\text{m}$ ) unless otherwise noted.

Character	<i>H. complexus</i> n = 20 mean (range)	<i>H. coloradensis</i> n = 20 mean (range)	t-Test	P /=/
Shape	Elongate	Elongate	—	—
Body length	4.910 mm (1.220–6.080)	5.644 mm (4.180–7.280)	-2.68	0.046
Body width at acetabulum	0.824 mm (0.260–1.100)	0.851 mm (0.550–1.350)	0.39	0.70
Greatest body width	0.982 mm (0.820–1.190)	1.067 mm (0.820–1.550)	1.33	0.20
Oral sucker length	332 (110–420)	364 (300–500)	1.65	0.11
Oral sucker width	335 (110–420)	304 (220–420)	-1.57	0.12
Pharynx length	162 (60–200)	255 (180–340)	6.5	0.0001
Pharynx width	189 (70–240)	241 (190–330)	4.45	0.0001
OS/PH width ratio*	1.77 (1.47–2.10)	1.26 (1.04–1.43)	-10.28	0.0001
OS/PH length ratio*	2.04 (1.67–2.63)	1.46 (1.20–1.77)	7.99	0.0001
Acetabulum location from anterior end	39% (33–48)	35% (24–50)	0.56	0.57
Acetabulum length	239 (80–330)	275 (230–340)	2.46	0.02
Acetabulum width	241 (80–310)	274 (200–380)	2.14	0.04
OS/AC width ratio*	1.40 (1.10–1.68)	1.11 (0.88–1.31)	-6.52	0.0001
Anterior testis length	625 (250–950)	509 (310–750)	-0.344†	0.001
Anterior testis width	480 (150–680)	507 (400–650)	0.88	0.38
Posterior testis length	685 (160–980)	507 (400–650)	-4.08†	0.0003
Posterior testis width	540 (150–850)	520 (380–720)	-0.52	0.61
Ovary length	420 (300–600)	429 (130–580)	-0.30	0.76
Ovary width	314 (200–450)	310 (70–450)	0.15	0.88
Uterus	With loops extending posterior and lateral to cecal tips	With intercecal loops		
No. of vitellaria	18.60 (16–21)	17.15 (15–21)	-2.67	0.011
Egg length	32.00 (27.50–35.00)	31.50 (28.00–35.00)	-0.63	0.53
Egg width	17.00 (15.00–17.50)	16.60 (15.00–21.00)	-0.91	0.37

\* OS/PH, oral sucker/pharynx; OS/AC, oral sucker/acetabulum.

† Approximate  $t'$ -test.

and *H. coloradensis* form unrelated distinct lineages. We have examined the voucher specimens identified as *H. coloradensis* by León-Règagnon and Brooks (2003) (CNHE4661, 6 slides) and determined that it is not *H. coloradensis*. It differs from *H. complexus* by its oral sucker to pharynx width ratio and from *H. coloradensis* by its distribution of vitelline follicles.

In North America, *H. coloradensis* appears to be a western species, having been reported from 5 anuran species in the western United States: the Chiricahua leopard frog (*Rana chiricahuensis*) in Arizona; the northern leopard frog from Colorado, Idaho, Montana, and New Mexico; Woodhouse's toad, the northern leopard frog, and the plains leopard frog in Nebraska; and Woodhouse's toad, southwestern toad (*Bufo microscaphus*), and northern leopard frog in Utah (Cort, 1915; Frandsen and Grundman, 1960; Parry and Grundman, 1965; Dronen, 1975; Brooks, 1976). In North America, *H. complexus* is an eastern species, being reported from 8 species of anurans: the northern leopard frog in Iowa and North Carolina; the green frog in Maryland and Wisconsin; the northern leopard frog, wood frog (*Rana sylvatica*), and spring peeper (*Pseudacris crucifer*) in Ohio; the northern leopard frog, green frog, and southern leopard frog (*Rana sphenoccephalus utricularia*) in Indiana and Kentucky; and the northern leopard frog, plains leopard frog, Woodhouse's toad, and Cope's gray treefrog (*Hyla chrysoscelis*) in Nebraska (Seely, 1906; Cort, 1915; Krull, 1933, 1934; Odlaug, 1954; Ulmer, 1970; Brooks, 1976; Catalano and White, 1977; Whitehouse, 2002; M. Bolek, pers. obs.). In North America,

Nebraska appears to be the eastern geographic limit for *H. coloradensis* and the western geographic limit for *H. complexus*.

It is unclear why these 2 species exhibit eastern versus western distributions, because their life cycles are so similar. However, except for northern leopard frogs, *H. coloradensis* has been reported from species of frogs and toads that primarily have a distribution west of the Mississippi River (Platz and Mecham, 1979; Brown, and Morris, 1990; Sullivan et al., 1996; Gergus, 1998). Recent biogeographical studies with mitochondrial DNA on the northern leopard frog also indicate eastern and western lineages of these frogs genetically isolated by the Mississippi River (Hoffman and Blouin, 2004). *Haematoloechus complexus*, on the other hand, has been reported from frogs and toads that overlap in their distributions east and west of the Mississippi River and appears to be found in some strictly western species, such as Woodhouse's toads (Conant and Collins, 1998).

The northern leopard frog in Nebraska has a more arid distribution than its eastern lineage; it predominantly uses clear, sand-bottom streams for reproduction and hibernation (Lynch, 1978; Vogt, 1981). During spring and fall, adult northern leopard frogs remain quite close to water but disperse into grassland during the summer. Importantly, newly metamorphosed northern leopard frogs are found closer to water than are the adults and, therefore, have different habitats than do the adults during the summer months (Vogt, 1981). Our field observations support these findings and, over the last 4 yr during the summer,

we have never observed adult northern leopard frogs at Cedar Creek or any of the other small artificial ponds in the general area. In fact, the only adult leopard frogs we have observed during the summer are the occasional individual plains leopard frog at some of the small artificial ponds. Studies by Kruse (1978) on the life histories of the plains leopard frog and northern leopard frog from Nebraska indicate that the northern leopard frog can readily replenish body moisture from soil capillary water and has a much faster rate of rehydration than does the plains leopard frog, indicating that northern leopard frogs might be more adapted to a semiterrestrial existence in western Nebraska than plains leopard frogs. Indeed, the plains leopard frog is not found west of the confluence of the North and South Platte Rivers in Nebraska, except as an isolate along Lake McConaughy in Keith County (Lynch, 1978, 1985).

The only anurans commonly observed at Cedar Creek during the summer are an occasional bullfrog, newly metamorphosed young of year northern leopard frogs, and Woodhouse's toads, which metamorphose during late June and early July and are found along the margin of Cedar Creek throughout the fall when they go into hibernation. These observations suggest that at this location, the only commonly occurring frogs that can support adult *H. coloradensis* worms and release eggs of this species back into an aquatic environment are newly metamorphosed northern leopard frogs and newly metamorphosed Woodhouse's toads. These observations on the natural history of the anuran hosts in western Nebraska, along with the generalist nature of *H. coloradensis* at the second intermediate host level, show this life cycle is well suited for the western Nebraska environment. The results of this study suggest that a particular life cycle variant will be favored by regional environmental conditions and specific definitive host combinations because these factors influence probabilities of transmission at 1 or more stages during a complex life cycle. This comparative approach to life cycle studies within both single and multiple species of hosts allows us to generate hypotheses about mechanisms that drive evolution of parasite life cycles.

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