THE ROLE OF DAMSELFLIES (ODONATA: ZYGOPTERA) AS PARATENIC HOSTS IN THE TRANSMISSION OF *HALIPEGUS ECCENTRICUS* (DIGENEA: HEMIURIDAE) TO ANURANS

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ABSTRACT: *Halipegus eccentricus* is a common hemiurid trematode in the eustachian tubes of North America frogs. However the life cycle of this species has never been completely elucidated. Studies on *H. eccentricus* suggest that it has a 3-host life cycle. Here, we show through fieldwork and host specificity experimental infections that the life cycle of *Halipegus eccentricus* utilizes 4 hosts. Metamorphosed anurans become infected with *H. eccentricus* by feeding on infected damselflies; worms resided in the stomach of anurans, migrate to the eustachian tubes within 32-39 days post-exposure (DPE), and release eggs 50-60 DPE. Cystophorous cercariae develop in *Physa gyrina* snails within 32-35 DPE, infect ostracod (*Cypridopsis* sp.) second intermediate hosts, and develop to metacercariae. Fifteen- to 19-day-old metacercariae from ostracods are infective to both damselfly larvae and metamorphosed anurans. Field surveys of damselflies and tadpoles along with laboratory exposure of damselfly larvae, metamorphosed anurans, and tadpoles with infected ostracods indicated that only metamorphosed anurans and damselflies become infected with *H. eccentricus*, whereas field collected tadpoles and laboratory exposed tadpoles were never infected with *H. eccentricus*. Because little morphological change occurred in the metacercaria stage of *H. eccentricus* between the ostracod second intermediate host and damselfly host, and metamorphosed anurans

became infected with *H. eccentricus* metacercariae recovered from both host groups, we suggest that odonates serve as paratenic hosts in this life cycle. Additionally, our field work and experimental infections provide data on the use of odonates as the route of infection by another North American *Halipegus* sp. that matures in the stomach of frogs. Our data indicate that when the life cycles are known the use of odonates as the route of infection to anurans is common in life cycles of *Halipegus* spp.; and all species exhibit remarkable infection site fidelity in their amphibian hosts.

Species of *Halipegus* infect the intestine, stomach, esophagus, buccal cavity, or eustachian tubes of amphibians and can be progenetic in dragonflies (Rankin, 1944; Macy et al., 1960; Nath and Pande, 1970; Yamaguti, 1971; Prudhoe and Bray, 1982; Moravec and Sey, 1989; Zelmer and Brooks, 2000). Most of these hemiurid trematodes have a typical trematode 3-host life cycle; however, some species add a fourth host in the life cycle (Zelmer and Esch, 1998a). Two types of life cycles have been reported for amphibian *Halipegus* spp. (Goater et al., 1990a). The life cycles of the European *Halipegus ovocaudatus* and the North American *Halipegus occidualis* have been well studied and include frog definitive hosts, snail first intermediate hosts, microcrustacean (copepods and ostracods) second intermediate hosts, and odonate intermediate and/or paratenic hosts. Adult amphibians become infected with these species when they ingest infected dragonflies and/or damselflies (Krull, 1935; Macy et al., 1960; Kechemir, 1978; Goater et al., 1990a; Zelmer and Esch, 1998a). In contrast, other North American species of *Halipegus* have been shown to involve 3 hosts in the life cycle (snails, copepod microcrustaceans, and tadpole stages of amphibians). In the latter case, tadpoles become infected through the accidental ingestion of infected crustaceans and apparently the worms survive tadpole

metamorphosis and migrate to the oral cavity of the metamorphosed frog to mature (Thomas, 1939; Rankin, 1944).

Halipegus eccentricus Thomas, 1939 is a common hemiurid trematode in the eustachian tubes of true frogs in North America (Brooks, 1976; Wetzel and Esch, 1996a, b; Bolek and Coggins, 2001). However, the life cycle of this species has never been completely elucidated (Thomas, 1939). Previous laboratory life cycle studies on *H. eccentricus* by Thomas (1939) suggest that it has a 3-host life cycle. Briefly, Thomas (1939) showed that cystophorous cercariae are shed by *Physa gyrina*, *P. sayii crassa* (a synonym of *P. gyrina* see Dillon and Wethington [2006]), and *Planorbella trivolvis* snail first intermediate hosts, and that cercariae are ingested by species of *Cyclops* and *Mesocyclops* copepod second intermediate hosts in which metacercariae develop. His laboratory studies indicated that cystophorous cercariae attracted the microcrustaceans by thrusting their delivery tube in and out, the cercaria body reached the intestine of the microcrustaceans as the crustaceans fed on cercariocysts, and metacercariae developed in the hemocel of the copepods. Metacercariae in the microcrustacean host were then ingested by tadpoles via respiratory currents. Immature worms resided in the tadpoles' stomach, and it was assumed that worms survived tadpole metamorphosis and migrated to the eustachian tubes of metamorphosed frogs; odonate larvae could not be infected (Thomas, 1939). However, more recent molecular and field data by Wetzel (1995), Wetzel and Esch (1996a, b) and Bolek and Coggins (2001) suggest that *H. eccentricus* uses odonates as the route of infection to frogs, and tadpoles may not be involved in the transmission of *H. eccentricus*.

Until the present, few studies have attempted infecting odonates with the metacercariae of North American species of *Halipegus* and of those none was successful (Krull, 1935; Thomas, 1939; Rankin, 1944; Macy, et al., 1960; Goater et al., 1990a). However, recent advances in our

understanding of the infection mechanism and development of *Halipegus* sp. metacercariae in microcrustacean hosts by Zelmer and Esch (1998a, b, c) indicate that *Halipegus* sp. metacercariae need a considerable amount of development time in the microcrustacean host to be infective to the next host in the life cycle. The study by Zelmer and Esch (1998a) suggested that previous attempts at infecting odonates may have failed because there was not sufficient developmental time allowed within the microcrustacean host to produce a larval stage capable of infecting the next host in the life cycle (Thomas, 1939; Rankin, 1944; Goater et al., 1990a). Taken together, the conflicting field and laboratory studies on the transmission of *H. eccentricus* to anuran hosts and the recent advances in our knowledge of the development of *Halipegus* metacercariae in the microcrustacean second intermediate host suggest that the life cycles of some North American amphibian *Halipegus* spp. should be re-evaluated.

Here, we examined the population structure and route of infection of *H. eccentricus* and a *Halipegus* sp. from the stomach of bullfrogs in a variety of potential snail, damselfly, and larval and metamorphosed amphibian hosts from Nebraska in order to elucidate any differences between the life cycle strategies of these flukes and the original life cycle description of *H. eccentricus* by Thomas (1939). We then completed the life cycle of *H. eccentricus* in the laboratory and compared our results to other life cycle work on *Halipegus* spp. in North America and Europe (Krull, 1935; Rankin, 1944; Macy, et al., 1960; Kechermir, 1978; Goater et al., 1990; Zelmer and Esch, 1998a, b, c). Our study provides laboratory and field data on congeneric flukes and their avenues for, and constraints on, transmission by odonate hosts to frog definitive hosts, data that will allow future testing of hypotheses with respect to the evolution of amphibian hemiurid fluke life cycles.

MATERIALS AND METHODS

Amphibian field surveys

During March-September 2000-2009, 1,135 metamorphosed anurans of 8 species, 637 tadpoles of 7 species, and 50 larval barred tiger salamanders, *Ambystoma tigrinum mavortium*, were collected by hand, dip-net, or seining from Elk Creek (40° 53.145' N, 96° 50.048' W) and Pawnee Lake (40° 51.589' N, 96° 53.468' W), located in Lancaster County, and Beckius Pond (41° 12.523', -101° 37.266'), Breen's Flyway (41° 10.914', -101° 21.654'), Cedar Creek (41° 11.194', -101° 21.820'), and Nevens Pond (41° 12.426', 101° 24.510') in Keith County, Nebraska and examined for *Halipegus* spp. Not all amphibian species or life stages were collected consistently from each site or each yr (Table I). All frogs, toads, and salamander larvae were brought back to the laboratory, killed, the snout vent length (SVL) or SVL and total length (TL) was measured, and examined for worms in the eustachian tubes, and/or the buccal cavity, and digestive system within 72 hr of capture. All tadpoles were taken to the laboratory and the SVL and TL was measured; they were then killed, aged according to Gosner (1960) and McDiarmid and Altig (1999), and examined for worms in the digestive tract and buccal cavity within 72 hr of capture. Additionally, we examined the digestive tracts for the presence of copepod and ostracod microcrustaceans in bullfrog tadpoles, *Rana catesbeiana*, and barred tiger salamander larvae from Nevens Pond, a location were *Halipegus* sp. metacercariae were common in damselflies.

All adult worms removed from amphibian hosts were relaxed in tap water. Gravid individuals were allowed to release eggs; worms were fixed in 95% ethanol or Alcohol-Formalin-Acetic Acid (AFA); representative worms were stained and permanent slides prepared. Worms were stained with acetocarmine, dehydrated in a graded ethanol series, cleared in xylene, and mounted in Canada balsam. Adult worms were identified to genus or species based on

location in the anuran host, as well as adult and cercaria morphology when possible, based on original species descriptions and redescriptions by Krull (1935), Thomas (1939), Rankin (1944), and Zelmer and Esch (1999). Additionally, morphology of released eggs was recorded for representative worms recovered from the stomach and eustachian tubes of naturally and experimentally infected amphibians (see below).

Snail and damselfly surveys at Nevens Pond

During July-August 2006-2008, 200 *Gyraulus parvus* and 200 *Physa gyrina* snails were collected from Nevens Pond by sampling aquatic vegetation with a dip-net. Individual snails were isolated in 1.5-ml well plates filled with aged tap water and observed daily for shedding cystophorous cercariae for a period of a wk. All snails were then measured, crushed, and examined for pre-patent infections. Cercariae were identified based on the cercariae descriptions of amphibian *Halipegus* spp. by Krull (1935) Thomas (1939), and Rankin (1944). Some intra-molluscan stages and cercariae were fixed in AFA and representative stages were stained and permanent slides prepared.

During July-August 2007 and 2008, 19 larvae and 122 adult lyre-tipped spreadwing damselflies, *Lestes unguiculatus*, 140 adult eastern fork tail damselflies, *Ischnura verticalis*, and 55 teneral and adult familiar bluet damselflies, *Enallagma civile*, were collected from Nevens Pond and examined for *Halipegus* sp. metacercariae. Larval damselflies were collected with a dip-net from submerged vegetation and placed in a bucket of water with no apparent snails or microcrustaceans, whereas teneral and adult damselflies were collected with a butterfly net along the edge of Nevens Pond, placed in 3.78-L plastic containers, stored on ice, and taken to the laboratory. All damselflies were identified according to Westfall and May (2006) and May and

Dunkle (2007). Each individual zygopteran larva, teneral, or adult was killed by removing the head. Each odonate was then placed in odonate saline or water, the last segment of the abdomen was cut off on the larvae, or the abdominal sterna were pealed back on teneral and adult damselflies, and the entire gut removed and gently teased apart with forceps, and examined for metacercariae. The rest of the body of individual damselflies was then divided into 3 regions, the head, thorax including the legs, and the abdomen including the anal gills for the larvae, and each body region was teased apart with forceps and examined for metacercariae. Measurements of metacercariae length and width and oral sucker and acetabulum length and width were obtained on relaxed live metacercariae using a calibrated ocular micrometer. A subset of metacercariae was fixed in AFA or 95% ethanol and representative worms were stained and permanent slides prepared.

Frog and toad experimental infections with *Halipegus* sp. metacercariae from damselflies

Three species of anuran were used for experimental infections with *Halipegus* spp. metacercariae recovered from naturally infected damselflies from Nevens Pond. Six bullfrogs were reared in the laboratory from tadpoles collected at Cedar Creek, whereas 9 young of the year northern leopard frogs, *Rana pipiens*, and 21 young of the year Woodhouse's toads, *Bufo woodhousii*, were collected from Cedar Creek and Beckius Pond, respectively. All amphibian species were divided into 3 equal groups, and assigned to time-0 controls, experimental infections, or time-T controls. All time-0 control amphibians were killed and examined for the presence of *Halipegus* spp. before the start of experimental infections. Individual frogs and toads in the experimental group were given 1-7 *Halipegus* spp. metacercariae recovered from naturally infected damselflies from Nevens Pond. For all infections, metacercariae were intubated by a pipette vie the esophagus. The pipette was than examined under a dissecting

microscope to confirm that no metacercariae remained. Based on species, all exposed anurans, along with time-T controls, were maintained in groups in 73.85-L tanks with a gravel substrate, and a 15 cm x 15cm x 5 cm water container at 24 C and 14 hr light:10 hr dark period and fed commercial crickets 3 times a wk. All exposed and time-T anurans were checked twice weekly for the appearance of worms in the eustachian tubes and worm maturity. Additionally, individual frogs and toads were killed and necropsied at 12, 39, 43, 50, 51, 61, 63, and 73 days post-exposure (DPE) and all organs were examined for *Halipegus* spp. Gravid worms were allowed to release eggs for identification and processed as previously described.

Snail first intermediate host specificity study

Thomas (1939) reported naturally and experimental reared and infected *Physa gyrina* and 2 of 11 field collected and experimentally infected *Planorbella trivolvis* snails with *H. eccentricus*-like cercariae. Although we have never encountered *P. trivolvis* snails infected with any hemiurid cercariae over the last 9 yr at any of our collecting sites in Nebraska (Bolek and Janovy, 2008; M. Bolek, pers. obs.), we examined snail host specificity of *H. eccentricus* in laboratory reared *P. trivolvis* and *P. gyrina*. For snail infections, adult *H. eccentricus* flukes were collected from naturally infected bullfrogs from Pawnee Lake and placed in 70-ml plastic containers in aged tap water and allowed to release their eggs, which were processed as previously described. Colonies of *P. trivolvis* and *P. gyrina* snails were established in the laboratory from 25 field collected individual *P. trivolvis* and 25 field collected individual *P. gyrina* from Elk Creek, Lancaster County Nebraska (40° 53.145, -96° 50.048) and Millville Creek (40º 59.611', -96º 33.934'), Lancaster County Nebraska, respectively, according to Bolek and Janovy (2007a, b). Snails were maintained on a diet of frozen lettuce, maple leaves, and Tetra Min® fish food. All snails used in the infections were at least the sixth laboratory

generation since being collected from the wild. For infections, 50 individual snails of each species were exposed to *H. eccentricus* eggs by placing 2 groups of 25 starved snails into 70-ml plastic containers with *H. eccentricus* eggs and Tetra Min® fish food. Snails were allowed to feed on the egg-Tetra Min® fish food mixture for 15 min. After egg ingestion, snail feces were checked for the presence of egg hatching; snails were maintained for a period of 19-35 days in 3.78-L jars with aerated aged tap water at 24 C and 14L:10D period before being crushed and/or examined for developing stages or shedding cercariae of *H. eccentricus*.

Cercaria development, morphology and cercaria body expulsion vie the delivery tube

Adult *H. eccentricus* flukes from a single laboratory infected northern leopard frog and a single laboratory infected bullfrog were placed in 70-ml plastic containers in aged tap water and allowed to release their eggs. Worms were then fixed in AFA, stained, and identified to species based on location in the definitive host and adult worm and egg morphology. Laboratory reared *Physa gyrina* snails were infected with *H. eccentricus* and maintained as previously described. Some surviving snails were crushed and examined for developing stages of *H. eccentricus* at 20, 25, and 28 DPE and developing stages were recorded. At 28 DPE, all remaining snails were isolated in 1.5-ml well plates filled with aged tap water and observed daily for shedding cercariae.

Because it is unclear how *H. eccentricus* cercariae infect microcrustacean second intermediate hosts, we examined the expulsion mechanism of the delivery tube and cercaria body of *H. eccentricus*. Infected snails were allowed to release cercariae, and groups (10) or individual cercariae were placed on slides with aged tap water, and covered by a cover slip. Gentle pressure was applied to the cover slip with forceps and the emerging delivery tube and

cercaria body was recorded as MOV file with a Coolpix 995 Nikon digital camera (Tokyo, Japan) equipped with a Martin Microscope adaptor (MMC00L S/N: 1747). Video MOV files were converted to AVI files with Quick Time Pro 7.0 (Apple Inc.®, Cupertino, California, USA) and then transformed to still images with Windows Movie Maker (Microsoft ®, Redmond, Washington, USA). Length and width measurements of the cercariocysts (cercariae tail membranes), streamer length, and caudal appendage (trigger length) were measured on 15 live undischarged cercariae and the delivery tube length, cercaria body length and width, oral sucker length and width, pharynx length and width, and acetabulum length and width were measured on 15 discharged live cercariae using a calibrated ocular micrometer.

Crustacean second intermediate host specificity studies

Four species of crustaceans (*Cypridopsis* sp., *Phyllognathopus* sp., *Asellus* sp., and *Hyalella azteca*) were chosen for intermediate host specificity studies since these species are commonly recovered from the digestive tract of tadpoles and/or frogs and potentially could transmit metacercariae of *H. eccentricus* to tadpoles and or frogs (Bolek, 1998; Bolek and Janovy, 2007a; M. Bolek**,** pers. obs.). Colonies of ostracods (*Cypridopsis* sp.) and harpacticoid copepods (*Phyllognathopus* sp.) were established in the laboratory in 3.78-L jars with aerated aged tap water, at 24 C and 14L:10D period. Ostracod and copepod cultures were maintained on a diet of frozen lettuce and Tetra Min® fish food before being exposed to cercariae. Additionally, 45 small (3-4 mm) isopods (*Asellus* sp.) and 45 small (3-4 mm) amphipods (*Hyalella azteca*) were collected from the toe drains of Lake McConaughy, Keith County, Nebraska (41° 13.931, -101° 40.184). All isopod and amphipods were divided into 3 equal groups, and assigned to time-0 controls, experimental infections, or time-T controls. All time-0 control isopods and amphipods were killed and examined for the presence of *Halipegus* spp.

before the start of experimental infections. For infections, groups of 10 laboratory reared ostracods and copepods were each placed with approximately 50 fresh (1-2 day after emergence from the snail) *H. eccentricus* cercariae recovered from laboratory reared and infected *P. gyrina* snails in individual 1.5-ml well plates with aged tap water, whereas individual isopods and amphipods were placed with approximately 50 fresh *H. eccentricus* cercariae. Individual well plates were then checked for cercariae and their status, discharged (expelled delivery tube and no cercaria body in the cercariocyst) or not discharged (delivery tube and cercarial body inside the cercariocyst), and the position of the emerged delivery tube (through the caudal appendage or opposite of the caudal appendage) 24 hr after placement with the crustaceans. All exposed ostracods, copepods, isopods and amphipods were maintained in their 1.5-ml well plates at 24 C and 14L:10D period and were examined for the presence of *H. eccentricus* metacercariae, in the hemocel over a period of 2-8 DPE. Additionally, the remaining 35 copepods were examined for the presence of *H. eccentricus* metacercariae 9 DPE, whereas the remaining ostracods were used for frog experimental infections. All exposed ostracods and copepods were individually placed onto slides, and ostracods were gently crushed with a cover slip, and the number of metacercariae was recorded; metacerariae were directly observed in the body cavity of copepods under a cover slip. All exposed and time-T isopods and amphipods were first placed on a microscope slide with a drop of water, gently teased apart with fine forceps, covered with a cover slip and examined on a compound microscope for the presence of *H. eccentricus* metacercariae.

Metacercaria morphology and development in ostracods

Colonies of ostracods (*Cypridopsis* sp.) were established in the laboratory, and maintained, as previously described. For infections groups of 10 laboratory-reared ostracods were placed with approximately 50 fresh (1-2 day after emergence from the snail) *H. eccentricus*

cercariae recovered from laboratory-reared and infected *P. gyrina* snails in individual 1.5-ml well plates with aged tap water and algae. Ostracods were allowed to feed on the algae and cercariae mixture for 24 hr before all groups were removed and maintained in 3.78-L jars with aerated age tap water on a diet of frozen lettuce at 24 °C and 14L:10D period. A sample of 40 exposed ostracods was then removed and examined for developing *H. eccentricus* metacercariae at 2, 12, 14, 19, 20, and 25 DPE, whereas the rest of the exposed ostracods were used for damselfly and tadpole infections. Individual ostracods were examined for *H. eccentricus* metacercariae as previously described. Metacercariae removed from ostracods were observed for the presence or absence of everted bladder villi, the pinching off of the bladder villi, and viability of metacercariae in aged tap water (measured in min). Viability of metacercariae was judged by the ability of metacercariae to continue to move; dead meatacercariae would quickly disintegrate in aged tap water. Measurements were taken on a subset of relaxed live worms recovered from ostracods using a calibrated ocular micrometer.

Damselfly and tadpole infections with *H. eccentricus* metacercariae from ostracods

Thirty ultimate or penultimate larvae of *Ishnura verticalis* damselflies were collected at Millville Creek. Damselflies were divided into 3 equal groups and assigned to 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 damselflies were necropsied for the presence of *Halipegus* metacercariae before the start of experimental infections. For infections, 10-20 laboratory reared and infected ostracods with 19- to 25-day-old metacercariae were pipetted into each 5-ml well plate containing a damselfly larva. Larvae were individually observed ingesting all ostracods using a stereoscopic microscope. Additionally, because damselfly larvae were semi-transparent, we made observations on the escape of *H.*

eccentricus metacercariae from ingested ostracod host in the gut of damselflies. All experimental and time-T control damselflies were maintained in their 5-ml well plates at 24 C and 14L:10D period, then killed and dissected for the presence of *H. eccentricus* metacercariae 2 DPE. Measurements were made using a calibrated ocular micrometer on a subset of relaxed live worms recovered from damselflies. Additionally, all ostracods removed from damselfly intestines were examined for viability and shell valve damage due to damselfly ingestion and digestion.

For tadpole exposures, 15 bullfrog tadpoles Gosner stage (26-40) were collected from Nevens Pond, divided into 3 equal groups, and assigned to time-0 controls, experimental infections, or time-T controls. Time-0 tadpoles were examined for the presence of *Halipegus* sp. infections before the start of the experiment. The 5 experimental tadpoles were all placed with 500 laboratory reared ostracods infected with 19-25 DPE metacercariae in aged tap water in a plastic shoe box (35 cm x 25 cm x 15 cm) and tadpoles were allowed to ingest all ostracods; 5 time-T tadpoles were placed without reared ostracods in aged tap water in a plastic shoe box (35 cm x 25 cm x 15 cm). All tadpoles (experimental and time-T control) were maintained in their respective plastic shoe boxes at 24 C and 14L:10D period before being examined for *H. eccentricus* metacercariae in the stomach, buccal cavity, and remaining organs at 2-7 DPE. Ostracods removed from tadpole intestines were examined for viability and shell valve damage due to tadpole ingestion and digestion.

Frog and toad infections with *H. eccentricus* metacercariae from ostracods

For metamorphosed anuran exposures, 9 newly metamorphosed bullfrogs, 9 newly metamorphosed northern leopard frogs, and 9 newly metamorphosed Woodhouse's toads were

collected from Beckius Pond, divided into 3 equal groups**,** and assigned to time-0 controls, experimental infections, or time-T controls. All time-0 control amphibians were killed and examined for the presence of *Halipegus* spp. before the start of experimental infections. Each experimental frog or toad was restrained and 20 ostracods with 15-20 day old *H. eccentricus* metacercariae (prevalence = 93%; mean abundance = 1.93 $\pm$ 1.3; range = 0-5; all individual metacercariae pinched off their bladder villi when removed from the ostracods and, therefore, were judged to be infective) were pipetted into the mouth of an experimental frog or toad. The pipette was examined to confirm that no ostracods remained. All exposed anurans, along with time-T controls were maintained as previously described and fed crickets 3 times/wk. A single exposed bullfrog and a single exposed northern leopard frog along with time-T controls were killed and examined for the presence of *H. eccentricus* worms in the stomach, buccal cavity, and eustachian tubes 7 DPE. All other exposed and time-T anurans were checked once a wk for the first 4 wk, and then daily for the appearance of worms in the eustachian tubes and worm maturity. All anurans were maintained as previously described, then killed and necropsied at 48-55 DPE.

Voucher specimens of field collected and experimentally infected adult *Halipegus* spp. removed from the stomach and eustachian tubes of metamorphosed anurans and larval salamanders, intra-molluscan stages from *Physa gyrina* snails, and metacercariae removed from damselfly hosts have been deposited in the H. W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska (accession numbers HWML): 49222 *H. eccentricus* from the eustachian tube of a bullfrog from Elk Creek; 49223 *H. eccentricus* from the eustachian tube of a bullfrog from Breen's flyway; 49224 *H. eccentricus* from the eustachian tube of a bullfrog from Nevens Pond; 49225 *H. eccentricus* rediae and cercariae from *Physa gyrina* from Nevens Pond;

49226 *Halipegus* sp. from the stomach of a bullfrog from Nevens pond; 49227 *Halipegus* sp. from the stomach of a barred tiger salamander from Nevens pond; 49228 *Halipegus* sp. metacercariae from the intestine of *Enallagma civile* from Nevens Pond; 49229 *Halipegus* sp. metacercariae from the intestine of *Ischnura verticalis* from Nevens Pond; 49230 *H. eccentricus* from the eustachian tube of a damselfly experimentally infected bullfrog; 49231 *H. eccentricus* from the eustachian tube of a damselfly experimentally infected Woodhouse's toad; 49232 *H. eccentricus* from the eustachian tube of a bullfrog from Pawnee Lake; 49233 *H. eccentricus* from the eustachian tube of a damselfly experimentally infected northern leopard frog; 49234 *H. eccentricus* from the eustachian tube of an ostracod experimentally infected bullfrog; 49235 *H. eccentricus* from the eustachian tube of an ostracod experimentally infected Woodhouse's toad; 49236 *Halipegus* sp. from the stomach of an damselfly experimentally infected Woodhouse's toad; and 49237 *H. eccentricus* rediae and cercariae from an experimentally infected *Physa gyrina*.

RESULTS

Metamorphosed and tadpole amphibian field surveys

Of the 1,135 metamorphosed anurans examined from the 6 locations in Lancaster and Keith Counties Nebraska, only metamorphosed bullfrogs were infected with *Halipegus* species. In Lancaster County, 10 of 111 bullfrogs (9.0%; Mean Abundance [MA]= 0.21 $\pm$ 0.76; Mean Intensity [MI]= 2.4 $\pm$ 1.7; range = 1-4) collected from Pawnee Lake; and 1 of 3 bullfrogs (33%; MA = 0.33 $\pm$ 0.58; I =1) collected from Elk Creek were infected with *H. eccentricus*, whereas in Keith County, 1 of 8 bullfrogs (12.5%; MA = 0.13 $\pm$ 0.35; I = 1) collected from Breen's Flyway; and 5 of 61 bullfrogs (8.2%; MA = 0.22 $\pm$ 0.70; MI = 2.6 $\pm$ 0.89; 2-4) collected from Nevens

Pond were infected with gravid *H. eccentricus*; whereas a single bullfrog (1.6%; MA = 0.01 ± 0.12; I = 1) from Nevens Pond contained 1 non-gravid *Halipegus* sp. worm in the stomach. Gravid worms were located in the eustachian tubes of bullfrogs consistent with the description of *H. eccentricus*. Measurements of 15 eggs from worms removed from the eustachian tubes of bullfrogs were 52 x 21 (45-60 x 20-23) µm long by wide and contained an abopercular spine 62 (53-75) µm long (egg length: spine width ratio 1:1.18-1.25) and were similar to the description of eggs from *H. eccentricus* (Thomas, 1939). Additionally, 1 of 61 (1.6%; MA = 0.01 ± 0.12; I = 1) bullfrogs from Nevens Pond contained a single gravid *Halipegus* species in the upper stomach. Measurements of 15 eggs from this single worm recovered from the bullfrogs upper stomach were 65 x 25 (60-69 x 20-29) µm long by wide and contained abopercular spines that were 140 (112-175) µm long (1:1.86-2.54) and more closely approached the description of eggs from *H. occidualis* which resides under the tongue of frogs (Krull, 1935; Zelmer and Esch, 1998a).

Of the 637 tadpoles and 50 larval salamanders examined for *Halipegus* spp. none of the tadpoles was infected with any species of *Halipegus*; 2 of 50 barred tiger salamander larvae (4%; MA = 0.04 ± 0.19; I = 1) from Nevens Pond each contained 1 non-gravid *Halipegus* worm in the stomach. All bullfrog tadpoles from Nevens Pond contained algae and detritus in their gut content, and occasionally (15/83) 2-3 semi-digested harpacticoid copepods and/or 1-3 live ostracods in their gut contents, whereas all salamander larvae from Nevens Pond contained hundreds of ostracods and copepods with the occasional snail and/or insect in various stages of digestion in their gut content.

Snail and damselfly surveys from Nevens Pond

Of the 200 *Gyraulus parvus* and 200 *Physa gyrina* snails collected from Nevens Pond, 9 of 200 *Physa gyrina* (4.5%) shed cystophorous cercariae. All cystophorous cercariae contained 2 lateral streamers and were identified as the cercariae of *H. eccentricus*. No other patent or prepatent hemiurid infections were detected in any of the snails.

Of the 3 species of damselflies examined for *Halipegus* spp. metacercariae, 4 of 19 larval *L. unguiculatus* (21%; MA = 0.26 $\pm$ 0.56; MI = 1.25 $\pm$ 0.5; 1-2); 1 adult of 55 (53 adult and 2 teneral) *E. civile* (2%; MA = 0.02 $\pm$ 0.1; I =1) and 23 of 140 adult *I. verticalis* (16.4%; MA = 0.5 $\pm$ 1.4; MI =3.1 $\pm$ 2.1; 1-7) were infected with *Halipegus* spp., whereas 0 of 122 (0%; MA = 0) adult *L. unguiculatus* were infected with any *Halipegus* spp. All metacercariae were unencysted and located in the mesenteron of larval and adult damselflies (Fig. 1). Measurements of 5 *Halipegus* spp. metacercariae recovered from adult damselflies indicated that they were 502 (490-520) µm long by 270 (200-300) µm wide, and the oral sucker and acetabulum was 88 (80-90) µm and 174 (160-190) µm in diameter, respectively (oral sucker : acetabulum diameter ratio, 1:1.98-2.11).

Frog and toad experimental infections with *Halipegus* metacercariae from damselflies

A single toad examined 12 DPE contained a single immature *Halipegus* sp. located in the stomach. Of the remaining frogs and toads all species became infected with *Halipegus* spp., however not all individual amphibians became infected (Table II). *Halipegus eccentricus* appeared in the eustachian tubes of frogs and toads between 32-39 DPE, and gravid worms from bullfrogs, leopard frogs, and toads were recovered 50-60 DPE (Fig. 1). Because the eustachian tube openings in Woodhouse's toads were much smaller than the diameter of a single gravid *H. eccentricus*, all gravid worms in toads had a constriction in the mid part of the body, whereas all

worms recovered from the eustachian tubes of bullfrogs and the single infected leopard frog appeared normal. Gravid worms removed from the eustachian tubes of frogs and toads at 50, 60, and 61 DPE released eggs in water; eggs contained fully formed miracidiae (Fig. 1). Measurements of 15 eggs from worms removed from the eustachian tube of a laboratory infected leopard frog 50 DPE were 55 x 22 (53-58 x 18-25) µm long by wide and contained abopercular spines that were 76 (63-105) µm long (1:1.19-1.81).

Additionally 2 of 6 toads exposed to *Halipegus* spp. metacercariae from naturally infected damselflies contained an unidentified species of *Halipegus* in the stomach when examined 51 and 73 DPE. Of these, a single worm contained 30 developing eggs in the uterus at 73 DPE with large spines morphologically similar to those on eggs recovered from the stomach of a naturally infected Nevens Pond bullfrog, but morphologically distinct from those on eggs recovered from worms infecting the eustachian tubes of naturally and experimentally infected frogs and toads (Fig. 1). None of the time-0 or time-T control bullfrogs, leopard frogs, or toads was infected with any *Halipegus* spp.

Snail first intermediate host specificity study

Our snail first intermediate host specificity study indicated that upon ingestion of *H. eccentricus* eggs by snails, most eggs hatched in both *P. trivolvis* and *P. gyrina* snails. Forty-seven *P. tivolvis* and 23 *P. gyrina* snails survived 19 DPE; however, only *P. gyrina* snails became infected with *H. eccentricus*, and all developing stages of *H. eccentricus* were located in the digestive gland of infected *P. gyrina* (15/23). None of the 47 surviving *P. trivovis* (0/47) became infected.

Cercaria development, morphology and cercaria body expulsion vie the delivery tube

Of the 50 laboratory reared *Physa gyrina* snails exposed to eggs of *H. eccentricus* from laboratory infected frogs, 40 snails survived 20 DPE, of which 35 of 40 (87.5%) were heavily infected (Fig. 2). Sporocysts with developing rediae were observed in some of the crushed snails at 20 and 25 DPE located in the digestive gland, whereas rediae with developing cercariae were observed in the digestive gland of crushed snails at 20, 25, and 28 DPE (Fig. 2). Developing cercariae at 20-25 DPE contained the cercaria body and delivery tube outside of the cercariocyst, and the delivery tube appeared annulated; the cercariocyst enclosed the cercaria body and delivery tube 28 DPE. Snails began shedding cystophorous cercariae at 32-35 DPE; cercariae contained 2 lateral streamers and morphologically resembled the description of cercariae of *H. eccentricus* (Fig. 2).

Under slight cover slip pressure, shed cercariae released their cercaria bodies through the delivery tube. The delivery tube emerged first through the caudal appendage or what Thomas (1939) referred to as the excretory appendage, followed by the cercaria body traveling through the delivery tube until its expulsion (Fig. 2). Occasionally, the delivery tube and cercaria body would emerge opposite of the caudal appendage; however, this only occurred in 3 of 100 trials. All cercaria bodies died and disintegrated within 5 min in aged tap water (N = 50). Measurements of 15 live cercariae were as follows: cercariocyst (cyst or bulb) length and width without caudal appendage (trigger or excretory appendage) or the pyriform organ 76 (70-88) x 78 (70-88) µm; streamer length 382 (250-600) µm; caudal appendage length 41 (25-50) µm; delivery tube length after expulsion 374 (325-490) µm and never annulated; cercaria body length and width after expulsion 189 (150-275) x 36 (20-50) µm; oral sucker length and width 28 (23-38) x 27.3 (20-38) µm; acetabulum length and width 26 (18-38) x 27 (18-43) µm; and pharynx length and width 14 (10-18) x 13 (10-18) µm.

Crustacean second intermediate host specificity studies

Twenty-four hr PE, discharged and undischarged cercariae were present in some well plates containing copepods (*Phyllognathopus* sp.) and some ostracods (*Cypridopsis* sp.); undischarged cercariae were present in well plates containing isopods (*Asellus* sp.) and some copepods (*Phyllognathopus* sp.), but no cercariae were present in well plates containing amphipods (*Hyalella azteca*) and some well plates containing ostracods (*Cypridopsis* sp.). These observations suggest that *Hyalella azteca* and some *Cypridopsis* sp. ingested the entire cercariae. Of the 20 discharged cercariae observed in well plates containing copepods and ostracods, all had the delivery tube everted through the caudal appendage. Of the 4 species of crustaceans exposed, only copepods and ostracods became infected. Two of 15 (13.3%; MA = 0.13; I = 1) *Phyllognathopus* sp. became infected with unencysted *H. eccentricus* metacercariae in the hemocel, and 14 of 15 (93.3%; MA = 1.93; MI = 2.1; range 0-5) *Cypridopsis* sp. became infected with unencysted *H. eccentricus* metacercariae, whereas none of the experimental (0/15) and time-T control (0/15) *Asellus* sp. and *Hyalella azteca* was infected with any *Halipegus* sp. metacercariae when examined 2-8 DPE. Examination of the 35 remaining *Phyllognathopus* sp. copepods 9 DPE revealed 1 individual infected with a single dead and degenerating *H. eccentricus* metacercaria.

Metacercaria morphology and development in ostracods

Twenty-two of 40 *Cypridopsis* sp. (55%; MA = 1.8 $\pm$ 2.2; MI = 3.2 $\pm$ 2.1; 1-8) exposed to cystophorous cercariae of *H. eccentricus* became infected; all unencysted metacercariae were located in the hemocel of the ostracod host. Metacercariae recovered from ostracods 2 DPE were 194 (180-200; N = 5) µm in length and did not posses any everted bladder villi.

Unencysted metacercariae recovered from ostracods 12-14 DPE were 360 (200-500; N = 5) µm in length with everted bladder villi (Fig. 3), and all 2-14 day old metacercariae died in aged tap water within 5 min of being removed from ostracods (N = 37). Metacercariae removed from ostracods 19-25 DPE immediately pinched off their everted bladder villi (Fig. 3). Metacercariae recovered from ostracods 19 DPE were fusiform in body shape 481 (440-530; N =7) µm in length, 228 (210-240) µm in width, contained lateral excretory ducts united anterior to the pharynx, and possessed a well developed oral sucker and acetabulum 75 (60-80) µm and 118 (110-120) µm in diameter, respectively, (1:1.5-1.83); all 19 day or older metacercariae were viable in aged tap water for over 1 hr when observations were stopped (N = 15; see Fig. 3).

Damselfly and tadpole infections

Damselfly larvae offered laboratory infected ostracods were immediately attracted to ostracod movement. In all cases, the labium of the larval damselfly was projected out to grasp the ostracod, which was then ingested. During ingestion, all damselfly larvae cracked the ostracod shell with the labium and maxilla, and *H. eccentricus* metacercariae were observed emerging out of infected ostracod and into the foregut of 4 of 10 larval damselflies. Five of 10 larval *I. verticalis* (50%; MA = 0.8 $\pm$ 0.9; MI = 1.6 $\pm$ 0.5; 1-2) examined 2 DPE were infected with metacercaraie of *H. eccentricus*, all located in the mesenteron. All metacercariae were unencysted and were 460 (400-530; N =5) µm in length, 206 (190-230) µm in width, contained lateral excretory ducts united anterior to the pharynx, and possessed a well developed oral sucker and acetabulum 78 (70-80) µm and 119 (115-120) µm in diameter, respectively (1:1.5-1.64; see Fig. 3). All exposed damselflies contained small pieces of ostracod shell exoskeleton in the gut

and all ingested ostracods were dead. None of the time-0 or time-T larval *I. verticalis* was infected with any *Halipegus* spp.

All exposed bullfrog tadpoles were observed ingesting infected ostracods via respiratory currents. However, none of the 5 tadpoles became infected with *H. eccentricus*. Live ostracods were commonly defecated out by tadpoles, and dead ostracods removed from the digestive tract of tadpoles were intact and not cracked (Fig. 3). None of the time-0 or time-T bullfrog tadpoles was infected with any *H. eccentricus*.

Frog and toad infections with *H. eccentricus* metacercariae from ostracods

Of the single experimentally exposed bullfrog and northern leopard frog examined 1 WPE, only the bullfrog contained a single immature *Halipegus* in the stomach. In the remaining exposed frogs and toads, *H. eccentricus* appeared in the eustachian tubes at 32 DPE in 1 bullfrog and 2 Woodhouse's toads, and 36 DPE in the remaining bullfrog and Woodhouse's toad. Overall prevalence, mean intensity $\pm$ 1 SD and range for the 3 exposed bullfrogs and Woodhouse's toads was 100%; 4.3 $\pm$ 4.9; 1-10 and 100%; 6 $\pm$ 5.3; 2-12, respectively, whereas none of the 3 northern leopard frogs became infected. All *H. eccentricus* worms recovered 32-55 DPE occurred in the eustachian tubes of infected anurans, except in a single heavily infected Woodhouse's toad containing 12 worms. In the heavily infected toad, there was physically not enough room for all 12 worms to fit in the eustachian tubes; however, all worms aggregated around the openings of the eustachian tubes. Worms recovered 48-55 DPE from the eustachian tubes of bullfrogs and toads contained 3 to hundreds of eggs in the uterus, and no additional worms were ever found in the stomach. Measurements of 15 eggs from worms removed from the eustachian tube of a laboratory infected Woodhouse's toad at 55 DPE were 56 x 20 (53-63 x

17.5-25) µm long by wide and contained abopercular spines that were 61 (48-73) µm long (1:0.9-1.16). None of the time-0 or time-T control frogs and toads was infected with any *Halipegus* spp.

DISCUSSION

The major contribution of our paper is the elucidation of the mechanism of how anurans become infected with *H. eccentricus* in nature by completing the life cycle in the laboratory, and demonstrating that odonates serve as paratenic hosts in the transmission of *H. eccentricus*. Our field work indicated that adult bullfrogs and salamander larvae were the only amphibians infected with *Halipegus* spp., whereas tadpoles and other metamorphosed anuran species were never infected with *Halipegus* spp. Our field data from Nevens Pond and experimental infections also suggest that bullfrogs become infected with *Halipegus* spp. by feeding on damselfly paratenic hosts and probably other odonates infected with *Halipegus* metacercariae. Clearly, other studies have documented a wide range of odonate species infected with *Halipegus* spp. metacercariae (Krull, 1935; Grieve, 1937; Macy, et al., 1960; Goater et al., 1990a; Wetzel and Esch, 1996a). Although ostracods infected with *H. eccentricus* metacercariae also were infective to metamorphosed anurans in the laboratory, it is unlikely that metamorphosed anurans commonly ingest ostracods in high enough numbers in nature to become infected with *Halipegus* spp. Preliminary stomach content data from Nevens Pond on 20 adult bullfrogs collected on a single night indicate that larval, teneral, and adult odonates can make up 20.9% of bullfrog diets on a given night, with damselflies making up 18.8% of the odonates consumed, suggesting that damselflies are the primary source of *H. eccentricus* infection in Nevens Pond bullfrogs.

Although metamorphosed bullfrogs and barred tiger salamander larvae were the only amphibian species infected with *Halipegus* spp. at our study sites in Nebraska, our laboratory experimental infections indicated that bullfrogs, Woodhouse's toads, and northern leopard frogs were all suitable hosts for *H. eccentricus*, with worms becoming gravid and producing eggs in all 3 anuran species. These observations are similar to those of Brooks (1976) who also reported *H. eccentricus* in 0 of 82 (0%) Woodhouse's toads, 2 of 152 (1.3%) northern leopard frogs, and 14 of 133 bullfrogs (10.5%) from Nebraska, indicating that northern leopard frogs can become infected with *H. eccentricus* in nature although less commonly than bullfrogs. Recent ecological and experimental life cycle studies from our laboratory on other trematodes (frog bladder flukes and lung flukes) that use odontes as a route of infection to anurans suggest that in nature bufonids and leopard frogs feed less commonly on odonates than bullfrogs and other members of the Aquarana Dubois 1992 clade (also known as the *Rana catesbeiana* group) as defined by Hillis and Wilcox (2005). In fact, most reports of gravid *H. eccentricus* in North American anurans are from *R. catesbeiana*, *Rana clamitans,* and *Rana septentrionalis,* all members of the Aquarana clade and *Halipegus* spp. are rarely, if ever, reported from other true frogs, toads, and salamanders (see Thomas, 1939; Macy, et al., 1960; Brooks, 1976; Muzzall, 1991a, b; Russell and Wallace, 1992; Wetzel and Esch, 1996b; McAlpine an Burt, 1998; Bolek and Coggins, 2001; 2003; Bolek and Janovy, 2007a, b; Bolek et al., 2009; Schotthoefer et al., 2009).

When the results of our study are compared to the original life cycle studies of Thomas (1939) and Rankin (1944), questions arise as to the maturity and source of the cercariae of *H. eccentricus* and *H. amherstensis* (in part a junior synonym of *H. eccentricus*; see McAlpine and Burt [1998] and Zelmer and Esch [1999]) used in the descriptions and experimental infections by those investigators. Both Thomas (1939) and Rankin (1944) based their cercaria descriptions of

H. eccentricus on laboratory reared and infected *P. gyrina* snails and cercariae recovered in part (Thomas, 1939), or only, from laboratory infected and crushed snails (Rankin, 1944), whereas their copepod infections originated from cercariae shed from experimentally and naturally infected snails.

In his experimental infections of copepod second intermediate hosts with *H. eccentricus*, Thomas (1939) observed that the cercaria body and delivery tube was always ejected opposite of the caudal appendage, the cercaria body never traveled through the delivery tube, the delivery tube had a jointed or telescopic appearance, and the cercariae occasionally trust the delivery tube in and out of the cercariocyst, apparently attracting copepods. Rankin (1944) observed that the jointed appearing delivery tube of *H. amherstensis* was always out of the developed cercariocyst and the cercaria body emerged in and out of the cercariocyst, attracting copepods to feed on it. Both authors' observations are in contrast to our study, in which we observed the cercaria body and delivery tube packaged inside of mature cercariocysts and during mechanical or microcrustacean induced discharge the cercaria body always emerged through the delivery tube as has been reported for *H. occidualis* by Krull, (1935) and Zelmer and Esch (1998a, b).

Although we cannot comment on Thomas's (1939) and Rankin's (1944) observations on the ability of cystophorous cercariae to attract copepods by movements of the delivery tubes or cercrial bodies because we did not observe these behaviors, we did observe jointed or telescopic appearing delivery tubes outside of the cercarialcysts in immature cercariae that exhibited slow movements. This observation suggests that Rankin's (1944) cercaria description and some of Rankin's (1944) experimental infections were based in part on immature cercariae (see Fig. 4). Finally, we were able to replicate Thomas's (1939) observation on the ejection of the delivery tube and cercaria body opposite of the caudal appendage; however, this only occurred

occasionally when cercariocysts were discharged under cover slip pressure. Previous studies on the ejection of the delivery tube and cercaria body of *H. occidualis* by applying cover slip pressure has been shown to produce abnormal ejections by Zelmer and Esch (1998c) and we never observed this phenomenon when cercariocysts of *H. eccentricus* were discharged by ostracods or harpacticoid copepods.

More intriguing are the discrepancies in our results and the results of Thomas (1939) and Rankin (1944) in the ability or inability of *H. eccentricus* infected copepods or ostracods to infect tadpoles and odonates. In our experimental exposures of tadpoles, metamorphosed anurans, and damselfly larvae with infected ostracods, we observed that ostracods commonly passed through the stomach (manicotti glandular) and remaining digestive tract of tadpoles without being killed and/or digested, whereas ostracods ingested by damselfly larvae were always killed and the shell was cracked open during feeding, releasing *H. eccentricus* metacercariae from the ostracod hemocele into the gut of the odonate host (see Fig. 4). We did not observe the mechanism of how metamorphosed anurans released *H. eccentricus* metacercariae from the hemocel of ostracod hosts. However, studies show that metamorphosed anurans are arthropod predator specialists and during feeding frogs will crack open the exoskeleton of their arthropod prey with their teeth and then digest the fragmented arthropod exoskeleton with their muscular stomach (Duellman and Trueb, 1994). Comparative studies on the digestive capabilities of the stomach of herbivorous tadpoles compared to their carnivorous metamorphosed anuran counterparts also indicate that food passes quickly through the digestive system of tadpoles and hydrochloric acid does not concentrate in the stomach of anuran larvae as it does in metamorphose anurans, suggesting that metamorphosed anurans can digest ostracods more easily than tadpoles can (see Viertel and Richter, 1999).

Even though we have no reason to doubt Thomas's (1939) and Rankin's (1944) experimental infections of tadpoles with *H. eccentricus,* field observations on North American tadpoles from our observations and those of Wetzel and Esch (1996a, b) and studies on the European *H. ovocaudatus* by Kechemir (1978), indicate that anuran larvae are never infected with *Halipegus* spp. in nature. Our observations on the gut content of field collected bullfrog tadpoles from Nevens Pond may be important in that we only observed digested harpactiocoid copepods and/or live ostracods in the digestive tracts of tadpoles. Moreover, laboratory experiments could not support infections of *H. eccentricus* or digestion by tadpoles, whereas Thomas (1939) and Rankin (1944) used cyclopoid copepods in their experimental infections of tadpoles. One explanation for this discrepancy may be that tadpoles do not commonly ingest cyclopoid copepods in nature. Studies on the ecology of ostracods, harpacticoid copepods, and cyclopoid copepods indicate that cyclopoid copepods are found in the open water column, whereas ostracods and harpacticoid copepods are common components of the benthic and epibenthic community and overlap in their habitat with tadpoles (McDiarmid and Altig, 1999; Smith, 2001; Thorp and Covich, 2001).

Finally, as previously pointed out by McAlpine and Burt (1998), our discovery of a gravid *Halipegus* sp. in the upper stomach of a bullfrog from Nevens Pond and our experimental infections of Woodhouse's toads with worms that remained in the stomach of toads 51-73 DPE suggest that there may be a third species of *Halipegus* infecting anurans and caudatans in North America. Previous studies by Macy et al. (1960), Goater et al. (1990b), Wetzel and Esch (1996b), and Zelmer and Esch (1998a) have considered gravid species of *Halipegus* recovered from the upper stomach and esophagus of frogs and salamanders from the western part of the United States as conspecific with *H. occidualis*, which resides under the tongue of frogs in

Canada and United States. The reason for these discrepancies can be traced back to Stafford's (1905) unclear description of the location of *H. occidualis* in the frog host (under the tongue, trachea or eustachian tubes), the morphologically similar eggs and cercariae of the tongue and stomach form of *H. occidualis* reported by Krull (1935) and Macy et al. (1960) and the inability to differentiate most *Halipegus* spp. based on adult morphology (see McAlpine and Burt 1998; Zelmer and Esch, 1999). However, recent genetic studies by Goater et al. (1990b) on *H. occidualis* and *H. eccentricus* and the description of the Central American *Halipegus eschi* by Zelmer and Brooks (2000) have demonstrated that *Halipegus* spp. show remarkable site fidelity under the tongue, eustachian tubes or esophagus of their amphibian host.

More importantly, life cycle studies by 3 independent investigators (Krull, 1935; Goater, 1989; Zelmer and Esch, 1998a) on the form of *H. occidualis* that resides under the tongue of frogs from the eastern United States indicate that when frogs are infected with *H. occidualis* metacercariae from filed collected odonates or laboratory infected ostracods, worms always appeared under the tongue of frogs within 21-22 DPE and become gravid under the tongue within 42-56 DPE. Additional genetic and/or field studies by Goater et al. (1990b), Wetzel and Esch (1996b), Bolek and Coggins (2001) and the present study on the migration of *H. eccentricus* to the eustachian tubes of three amphibian species indicate that *H. eccentricus* always appeared in the eustachian tubes of anurans within 32-39 DPE and gravid worms always reside in the eustachian tubes of their hosts.

In contrast, field and life cycle studies by Macy et al. (1960) on the upper stomach/esophagus form of *H. occidualis* and our *Halipegus* sp. from the upper stomach of anurans and salamanders indicate that when amphibians are infected with *Halipegus* metacercariae from laboratory infected ostrocads or field collected odonates, these worms never

migrate under the tongue or the eustachian tubes of anurans, and worms begin producing eggs in the stomach 71 DPE, suggesting that this is a distinct species. However, because Goater et al. (1990b), Wetzel and Esch (1996b) and Zelmer and Esch (1999) only sampled live green frogs during their studies the presence of worms in the upper stomach/esophagus would have gone unnoticed. Studies by Wetzel and Esch (1996b) on the population biology of *H. occidualis* over time in the same green frog individuals indicate that *H. occidualis* infrapopulations can build over time, and then suddenly drop to zero. Wetzel and Esch (1996b) hypothesized that the large number of worms stimulated a strong inflammatory response under the tongue of green frogs and this caused infrapopulations of *H. occidualis* to be sloughed off and it may be that these worms survive in the stomach of anurans. These data suggest an alternative hypothesis indicating that *H. occidualis* can reside under the tongue and/or the upper stomach/esophagus of anurans. To further confound matters, concurrent infections of gravid worms identified as *Halipegus* sp. from the upper stomach/esophagus, under the tongue and in the eustachian tubes of anurans have been reported from other locations in North America (Muzzall, 1991b; Bouchard, 1951; S. D. Snyder, pers. com.). Clearly, in order to resolve issues in the taxonomy and systematics of North American *Halipegus* spp. residing in the upper stomach/esophagus or under the tongue of North American anurans additional life cycle, morphological and molecular data will be necessary.

Although few complete life cycles are known for amphibian *Halipegus* spp., our data indicate that when known the use of odonates as the route of infection to anurans is common in *Halipegus* spp. These observations suggest a long relationship among *Halipegus* spp. and their odonate paratenic hosts, as is suggested by the ability of the Indian *Halipegus mehransis* to develop progenetically in its odonate host (Krull, 1935; Nath and Pande, 1970; Goater, 1989). Importantly, although most *Halipegus* species are morphologically indistinct, data suggest that

they exhibit incredible site fidelity in their anuran host (Table III). Currently, reports of *Halipegus* spp. exist from Europe, Asia, Papua New Guinea, the Americas, the Middle East, Africa, and Madagascar; once a phylogenetic analysis of the genus is available, it will be interesting to see if site fidelity in the amphibian host of congeners from different continents will be phylogenetically conserved.

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FIGURE 1. Stages of *Halipegus* spp. in the eastern fork tail damselfly *Ischnura verticalis* and bullfrog, *Rana catesbeiana*. (**A**) Phase contrast photomicrograph of an unencysted metacercaria from the gut of a naturally infected *Ischnura verticalis*. Note the large acetabulum compared to the oral sucker and lateral excretory ducts united anterior to the pharynx. Scale bar = 100 µm. (**B**) Two gravid *Halipegus eccentricus* (arrow) in the eustachian tube of an experimentally infected bullfrog 60 days post-exposure. Scale bar = 5 mm. (**C**) Phase contrast photomicrograph of an egg of *Halipegus eccentricus* from worms removed from the eustachian tube of an experimentally infected bullfrog. Note the abopercular spine. Scale bar = 25 µm. (**D**) Same egg after applying pressure to the cover slip. Note the fully formed and unciliated miracidium. Scale bar = 25 µm. (**E**) Phase contrast photomicrograph of an egg of *Halipegus* species recovered from the stomach of a naturally infected bullfrog. Note the abopercular spine difference compared to (C). Scale bar = 30 µm.

FIGURE 2. *Halipegus eccentricus* cercarial development in laboratory infected *Physa gyrina* and emergence of cercaria body through the delivery tube. (**A**) *Physa gyrina* snail with shell removed 28 days post-exposure to *Halipegus eccentricus* eggs. Note the large number of rediae. Scale bar = 5 mm. (**B**) Phase contrast photomicrograph of *Halipegus eccentricus* rediae 25 days

post exposure from a laboratory infected *Physa gyrina*. Note the developing cercaria. Scale bar = 100 µm. (**C**) Phase contrast photomicrograph of a cystophorous cercaria shed from *Physa gyrina* 32 days post-exposure to *H. eccentricus* eggs. Note streamers. Scale bar = 100 µm. (**D**) Same cercaria higher magnification. Note the cyst like tail (t), coiled cercaria body (cb), caudal appendage (ca), and pyriform organ (p). Scale bar = 50 µm. (**E**) Cercaria before delivery tube emergence. (**F**) Delivery tube emerges (arrow). (**G, H**) Cercaria body traveling up the delivery tube (arrow). (**I**) Cercaria body emerging out of delivery tube. Scale bars E-I all = 75 µm. (**J**) Phase contrast photomicrograph of a cercaria after its body emerges. Note degenerating cercarial body in aged tape water. Scale bar = 150 µm.

FIGURE 3. *Halipegus eccentricus* metacercaria development in experimentally infected ostracod and damselfly hosts; and ostracods from the gut of a bullfrog tadpole. (**A**) Cracked *Cypridopsis* sp. 2 days post-exposure to *Halipegus eccentricus* cercariae. Note the 3 metacercariae without everted bladder villi. Scale bar = 200 µm. (**B**) Twelve-day-old metacercaria removed from the body cavity of *Cypridopsis* sp. Note the everted bladder villi (arrow) and absence of lateral excretory ducts united anterior to the pharynx . Scale bar = 50 µm. (**C**) Nineteen-day-old metacercaria removed from the body cavity of *Cypridopsis* sp. Note the pinched off everted bladder villi, and developed lateral excretory ducts united anterior to the pharynx. Scale bar = 50 µm. (**D**) Phase contrast photomicrograph of a 19-day-old metacercaria removed from the body cavity of *Cypridopsis* sp. Note oral sucker acetabulum ratio and lateral excretory ducts united anterior to the pharynx. Scale bar = 75 µm. (**E**) Dead *Cypridopsis* sp. from the intestine of a bullfrog tadpole. Note that the ostracods shells are not cracked and ostracods are not digested. Scale bar = 1 mm. (**F**) Phase contrast photomicrograph of a

Halipegus eccentricus metacercaria removed from the gut of an experimentally infected larval *Ischnura verticalis* 2 days post-exposure. Note the similarity to (D). Scale bar = 75 µm.

FIGURE 4. Original line drawings of cercariae of *Halipegus amherstensis* by Rankin (1944) and photomicrograph of immature *Halipegus eccentricus* cercaria from our study. (**A, B**) Immature and mature cercariae of *H. amherstensis* from Rankin's (1944) original life cycle description. Scale bars = 50 µm. Note the annulated nature of the delivery tube. (**C**) Photomicrograph of 20 DPE developing cercaria of *H. eccentricus*. Note the annulated nature of the delivery tube. Scale bar = 200 µm.

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Figure 1

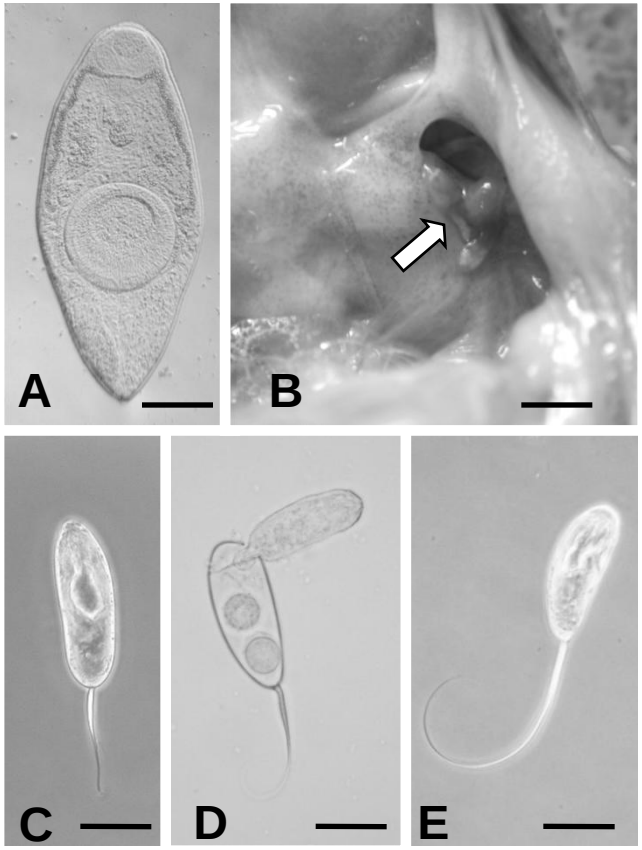

Figure 2

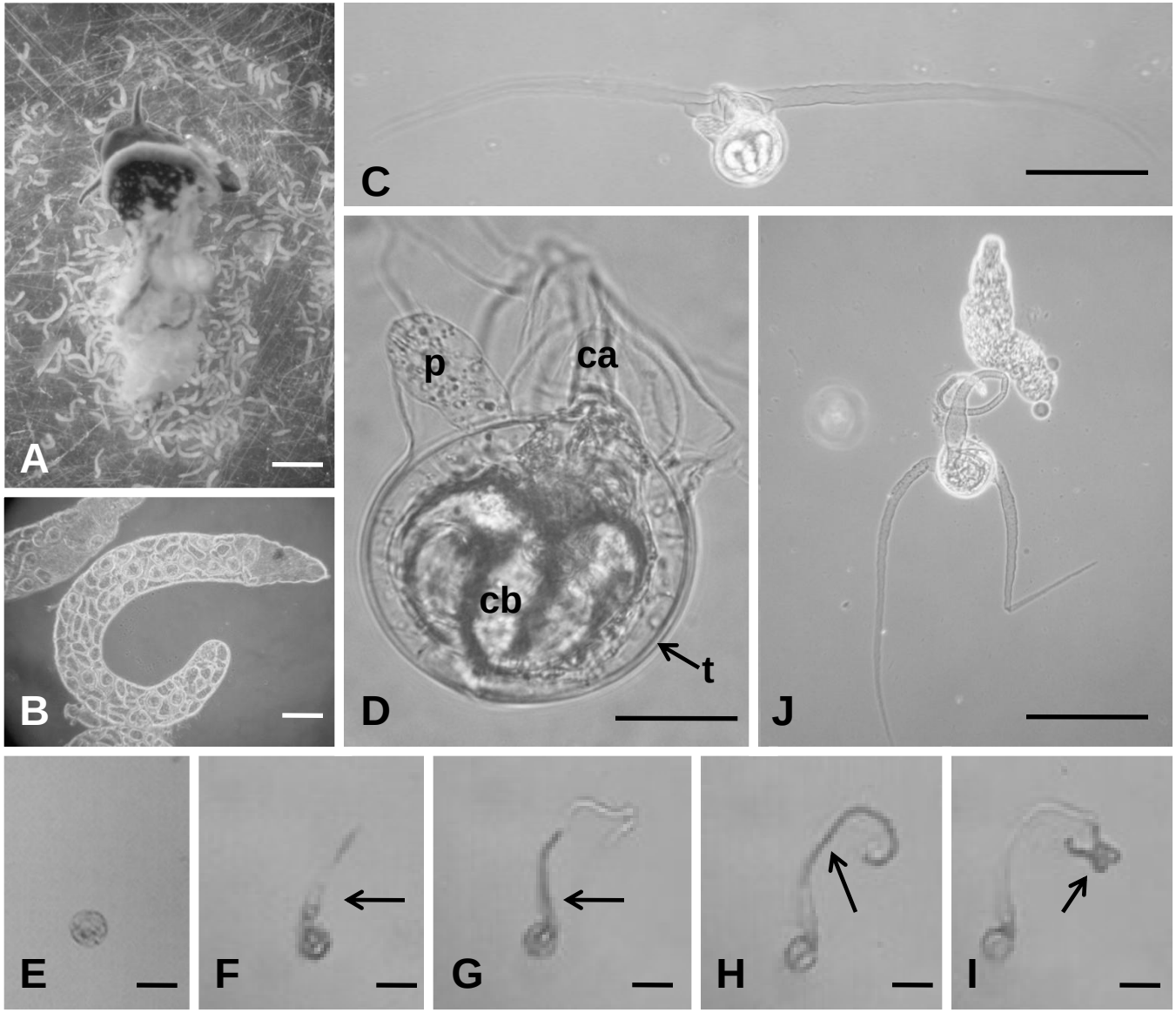

Figure 3

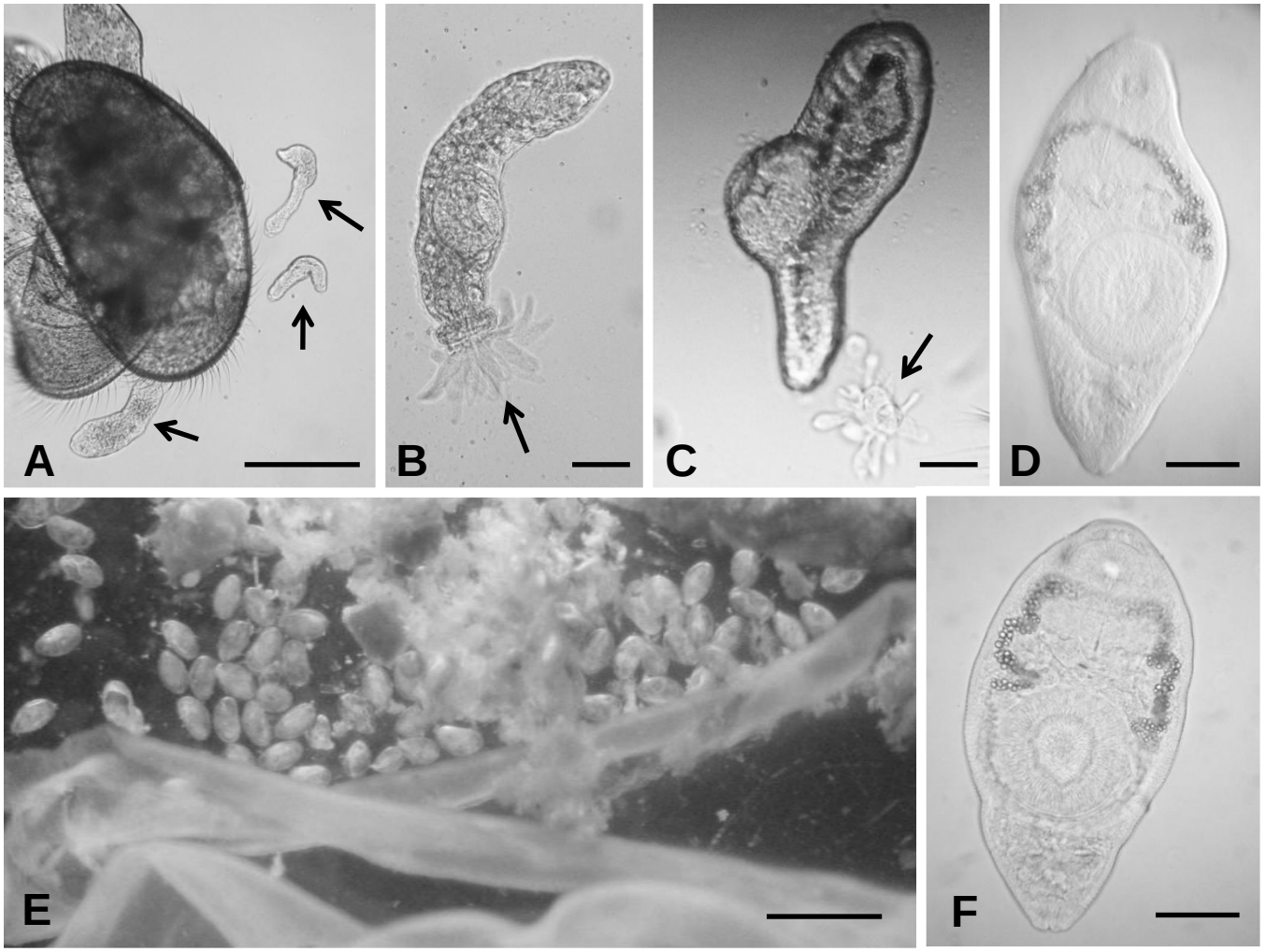

Figure 4

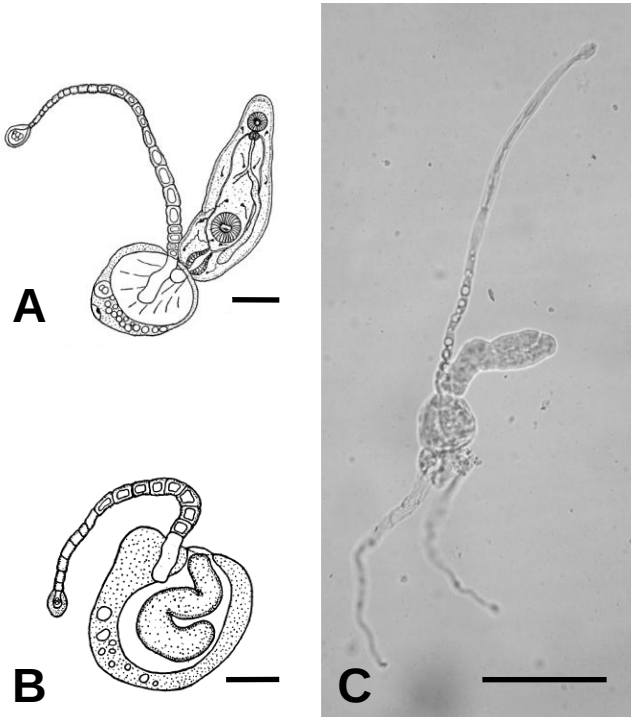

Table I

Table I. Location, species and number of metamorphosed anurans, tadpoles and larval salamanders collected from 6 locations in Keith and Lancaster Counties, Nebraska during May-September 2000-2009 and examined for *Halipegus* species.

Location	Species	Metamorphosed (N)	Tadpoles/Larva (N)
Beckius Pond, Keith Co., NE	*Bufo woodhousii*	100	85
	Pseudacris maculata	25	0
	Rana catesbeiana	0	5
	Rana blairi	3	0
	Spea bombifrans	10	0
Breen's Flyway, Keith Co., NE	*Rana catesbeiana*	8	20

Cedar Creek, Keith Co., NE	*Bufo woodhousii*	25	70
	Rana catesbeiana	2	0
	Rana pipeins	295	122
Nevens Pond, Keith, Co., NE	*Ambystoma tigrinum mavortium*	0	50
	Rana blairi	2	0
	Rana catesbeiana	61	83
	Spea bombifrans	0	60
Elk Creek, Lancaster, Co., NE	*Rana catesbeiana*	3	53

Pawnee Lake, Lancaster Co., NE	*Acris crepitans*	70	0
	Bufo woodhousii	100	0
	Hyla chrysocelis	72	44
	Pseudacris maculata	133	30
	Rana blairi	115	63
	Rana catesbeiana	111	2

Table II

Table II. Location, prevalence, mean intensity, and mean abundance of *Halipegus* spp. in 3 species of laboratory infected frogs and toads with *Halipegus* metacercariae from naturally infected damselflies.

Amphibian species	*Halipegus* spp.	Location in Host	Prevalence (No. infected/No. exposed)	Mean Intensity ± 1 SD	Mean Abundance ± 1 SD (range)
Bufo woodhousii	*H. eccentricus*	Eustachian Tubes	50 (3/6)	1.6 ± 1.1	0.7 ± 1.1 (0-3)
	Halipegus sp.	Stomach	33 (2/6)	1 and 1	0.3 ± 0.5 (0-1)
Rana catesbeiana	*H. eccentricus*	Eustachian Tubes	100 (2/2)	4 and 1	(1-4)
Rana pipiens	*H. eccentricus*	Eustachian Tubes	33 (1/3)	2	0.66 ± 1.15 (0-2)

Table III

Table III. The distribution, anuran site fidelity, and odonate host relationship of amphibian *Halipegus* species.

Species of *Halipegus*	Distribution	Anuran Site Fidelity	Odonate Host Relationship	References
H. africanus	Africa	?	NA	Dollfus, 1950
H. alhaussaini	Middle East	Stomach	NA	Saoud and Roshdy, 1970
H. eccentricus	North America	Eustachian tubes	Paratenic	Thomas, 1939; This study
H. eschi	Central America	Esophagus	NA	Zelmer and Brooks, 2000
H. dubius	South America	Under tongue	NA	Paraense, 1992
H. insularis	Madagascar	Under tongue	NA	Capron et al., 1961
H. japonicus	Asia	Under tongue	NA	Yamaguti, 1936
H. kessleri	India	Under tongue	NA	Grebnitzky, 1872
H. longispina	India	Under tongue	NA	Klein, 1905
H. mehransis	Asia	Stomach	Progenetic	Srivastava 1933; Nath and Pande, 1970
H. occidualis	North America	Under tongue	Paratenic	Krull, 1935; Goater, 1989; Zelmer and Esch, 1998a
H. ovocaudata	Europe	Under tongue	Intermediate	Kechemir, 1978
H. phrynobatrachi	Madagascar	Stomach	NA	Maeder, 1969
H. zweifeli	Papua New Guinea	Intestine	NA	Moravec and Sey, 1989
Halipegus sp.	North America	Upper stomach/esophagus	Paratenic	Macy et al., 1960; This study

?: not given; NA: complete or entire life cycle not known.