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Sylvatic Species of *Echinococcus* from Rodent Intermediate Hosts in Asia and South America

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Abstract

During a global survey of the diversity of vertebrates and their parasites including the Gobi and desert/steppe biomes ranging from south central to western Mongolia, we found metacestodes (larvae) of *Echinococcus multilocularis* (Leuckart 1863) in the liver of an individual vole (*Microtus limnophilus* Büchner 1889) collected in grassland habitat at Har Us Lake, southeast of Hovd, Mongolia. Positive identification of *E. multilocularis* from near Hovd was made via comparative cyst morphology, study of hooks from the rostellum derived from protoscolexes, and DNA sequencing of the COX1 mitochondrial gene extracted from tissue of the cysts frozen in the field. This report represents the first record of this species from an arvicolid intermediate host in Mongolia. This report also includes a second record from Bolivia of *E. vogeli* Rausch and Bernstein 1972 (confirmed by measurements of the hooks and cyst morphology) from the common intermediate host, *Cuniculus paca* Linnaeus 1758, collected in the Pilón Lajas Biosphere Reserve, Beni Department.

Key words: Bolivia, cestode, *Cuniculus paca*, cyst, *Echinococcus multilocularis*, *Echinococcus vogeli*, intermediate host, *Microtus limnophilus,* Mongolia

Introduction

Cestodes of the genus *Echinococcus* Rudolphi 1801 (Platyhelminthes: Cyclophyllidea: Taeniidae) occur with a cosmopolitan distribution as adults in the small intestines of wild and domestic carnivores (Rausch 1997). The life-cycle of this cestode (Fig. 1) is typical of species of the family Taeniidae: Adult cestodes live in the small intestine of carnivores that serve as definitive hosts, there producing eggs via sexual reproduction which pass out with the host's feces (Abuladze 1964). A single host (carnivore) may harbor thousands of individual cestodes which may fully cover the intestinal mucosal layer of the small intestine; for example, in the description of *E. felidis* Ortlepp 1937 from an African lion *Panthera leo* (Linnaeus 1758) collected and examined in South Africa, the author stated: "…numerous examples of this parasite were collected

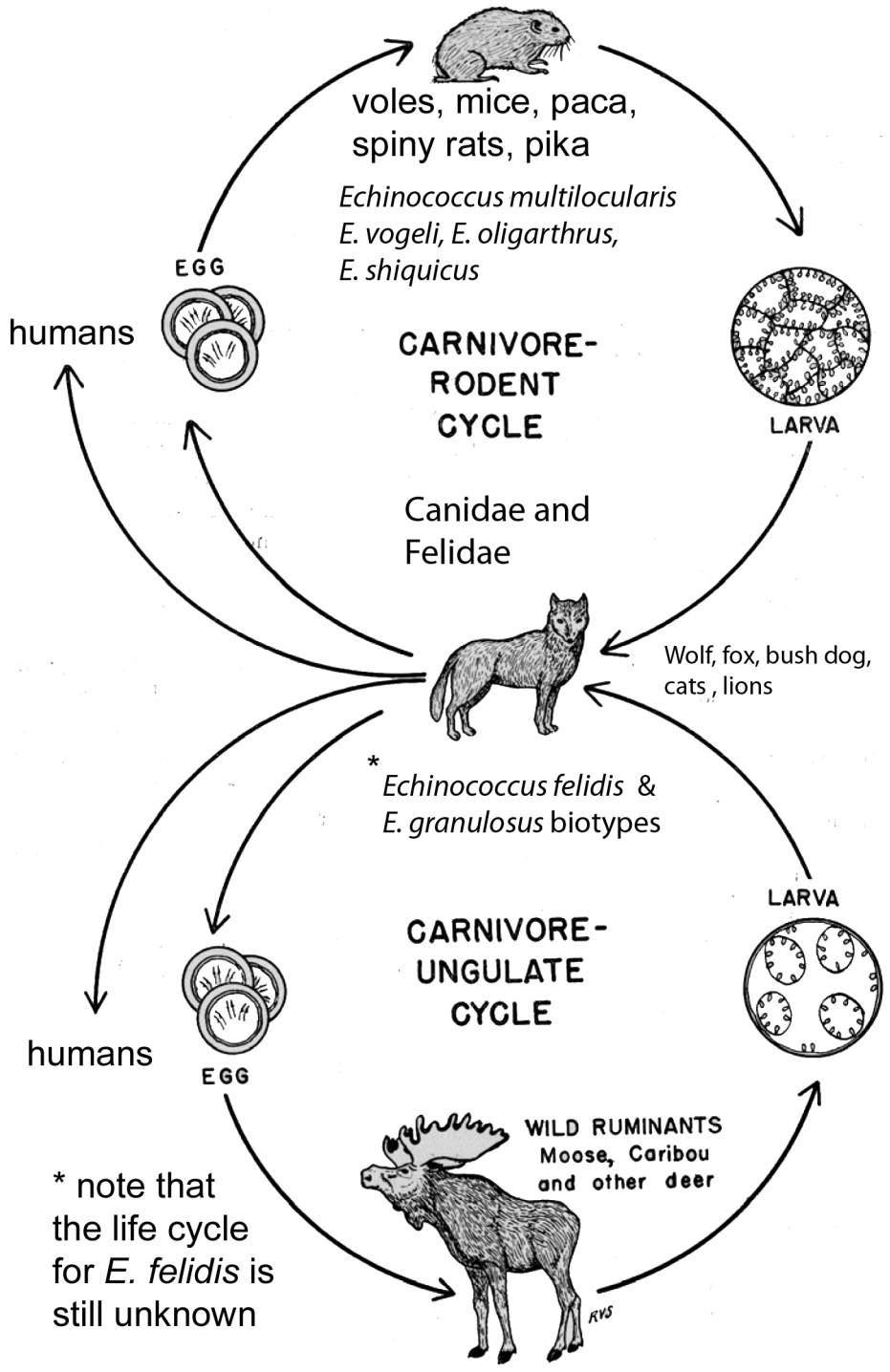

Figure 1. Illustration of the generalized life cycle of cestodes of the genus *Echinococcus*. Modified from Rausch (1952).

from a lion, whose intestine was literally felted over by the parasites. Macroscopically, no lesions were discernible" (Ortlepp 1937). After passing in the feces of the host, the eggs are transmitted, either via coprophagy or by accidental ingestion, to herbivorous mammals that act as intermediate hosts, and develop—usually in the liver—into metacestodes (larvae). After development in the liver, cysts with protosolexes are infective to the definitive host when the infected tissues of the intermediate host are eaten; each protoscolex having the potential to develop into an adult tapeworm in the carnivore (Rausch 1993, 1997).

As part of a biodiversity survey focussing on parasites of sylvatic vertebrates of the Gobi desert region of south and southwestern Mongolia, in July of 2012 we collected six specimens of *Microtus limnophilus* Büchner 1889 in grassland habitat in the Great Lakes region of Mongolia, one of which was infected with larval stages of *E. multilocularis* (Leuckart 1863). Herein we provide the first report of *E. multilocularis* from its natural intermediate host in Mongolia.

In addition, during the course of our concomitant parasite/mammal biodiversity surveys in South America, we recorded and are reporting below, the second record of *E. vogeli* Rausch and Bernstein 1972 from its intermediate host in Bolivia.

MATERIALS AND METHODS

Field Collecting - Mongolia.—From 1999 to 2012, more than four thousand mammals were collected from many sites throughout south-central and southwestern Mongolia to determine the potential distribution of parasites in this region (Fig. 2). After capture using Sherman™ live traps, all mammals were assigned a field number (NK number), and locality, date of capture, and general ecological data were recorded in a field catalog and notebooks. The animal was then killed by chloroform vapor inhalation, brushed and inspected for ectoparasites, and searched for endoparasites (Gardner 1996; Gardner and Jiménez-Ruiz 2009). All mammals collected were examined for endoparasites immediately after death to avoid possible effects of autolytic changes on any helminth parasites present. In Mongolia, the larval stage of *E. multilocularis* was found in the liver of a vole (Fig. 3), Museum of Southwestern Biology (MSB) catalog number MSB264850, collected on 29 June 2012 in a trap set on floating *Phragmites* mats on the southeast shore of Har Us Lake, 50 km SE of Hovd, Mongolia (lat. 47.79733; long. 92.27154; 1,170 m elevation). Among other vertebrates collected and examined for parasites from this locality, we examined 14 voles, including six individuals of *M. limnophilus*, three individuals of *M. oeconomus*, and five as yet unidentified specimens of *Microtus*. All specimens of mammals have been deposited in the collections of the Division of Mammals, Museum of Southwestern Biology, University of New Mexico, Albuquerque, New Mexico. Mammals were collected and processed following accepted protocols published by the American Society of Mammalogists (Gannon and Sikes 2007; Sikes and Gannon 2011).

Field Collecting - Bolivia.—In August 2010, specimens of *E. vogeli* were discovered in an individual paca (*Cuniculus paca* Linnaeus 1758) that was found to contain cysts in the liver. The specimens were collected on the Arroyo Rosario (lat. -14.641803; long. -67.508473; 252 m elevation) located in the Pilón Lajas Biosphere Reserve and Indigenous Territory from the Beni Department, Bolivia. The discovery was part of a wildlife health survey carried out on game hunted for consumption by families of T´simane indigenous hunters. These specimens were fixed in 10% aqueous formalin and processed with standard methods. Hooks were measured from individual protosolexes dissected from the cysts (see Table 3) following Gardner et al. (1988) and Rausch et al. (1978).

Laboratory Investigations.—At necropsy, via macroscopic observation, the liver of the infected vole was found to be almost fully replaced with alveolar cysts of *E. multilocularis* (Fig. 3). The liver, with cysts, was photographed, removed, placed in a clean plastic Petri dish, divided with a new scalpel, and preserved, stored, and transported in each of the following in separate vials or tubes: liquid nitrogen, 10% formalin,

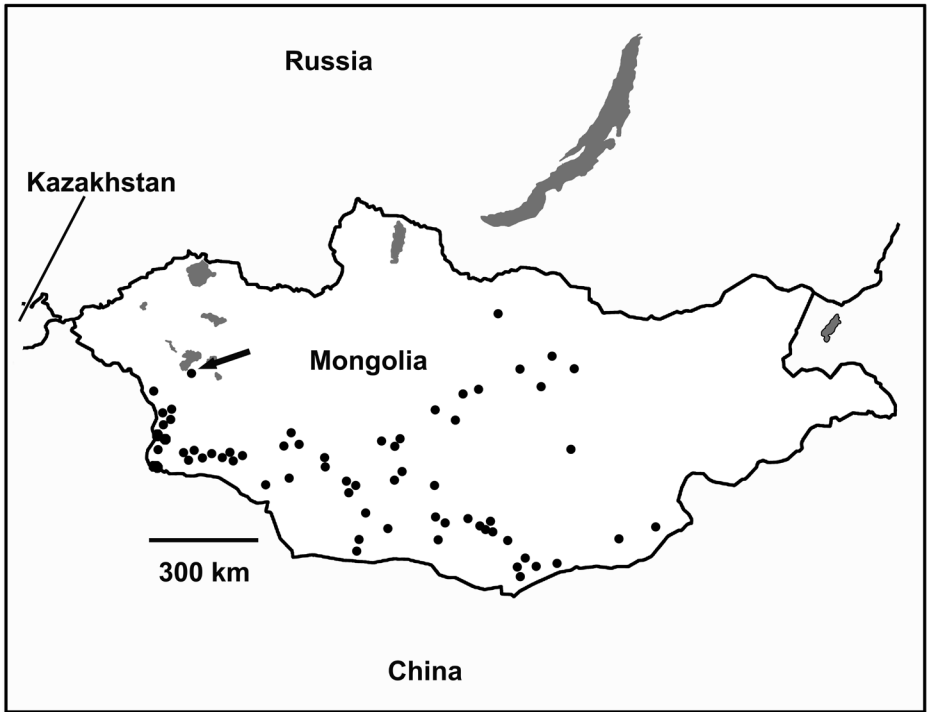

Figure 2. Outline map of Mongolia showing approximate localities where collections were made by our field expeditions, 1999-2012. Arrow indicates area where a single vole infected with *Echinococcus multilocularis* was collected at Har US Lake.

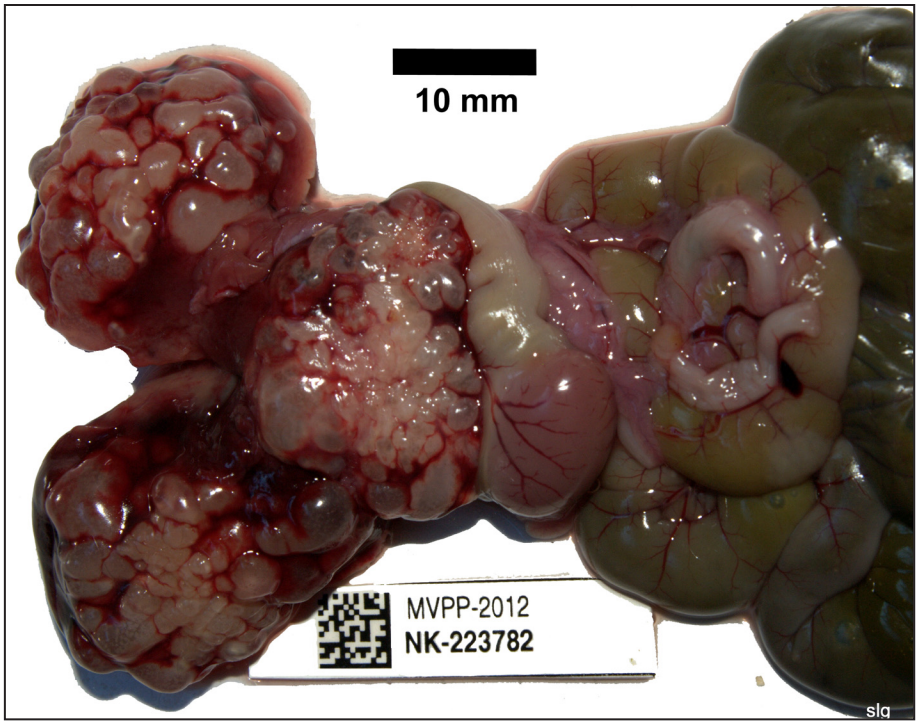

Figure 3. Liver and gastrointestinal tract of *Microtus limnophilus* collected at Har Us Lake, 50 km SE of Hovd, Mongolia (lat. 47.79733; long. 92.27154) showing almost complete involvement of the liver by the characteristic cysts of the metacestode *E. multilocularis*: HWML68052.

and 96% and 70% ethanol. In the laboratory, material preserved in 96% ethanol is maintained in the Parasite Genomic Research Facility at -85° C. For study, cysts were processed by standard methods, sectioned after paraffin embedding, stained with hematoxylin-eosin, and mounted on glass slides (Fig. 4a, b) (Rausch et al. 1978; Humason 1979). Hooks from the protoscolexes studied were measured following Rausch et al. (1978) and Gardner et al. (1988); digital photographs of hooks, stained sections of cyst in liver, and free protosolexes were made with a Zeiss Axiophot™ with 63x objective, and Zeiss™ digital camera. All measurements are given in micrometers.

Methods used to compare DNA sequence of our specimens follow Nakao et al. (2009). DNA was extracted from field-frozen samples and the mitochondrial cytochrome-c oxidase subunit 1 (COX1) was amplified using PCR. The same primer pairs were used for dye-terminator cycle sequencing and an automated reader. PCR successfully amplified the COX1 gene. During the sequencing reaction, reverse primers performed as expected, but forward primers sequence reads failed after running into a repetitive "T" sequence.

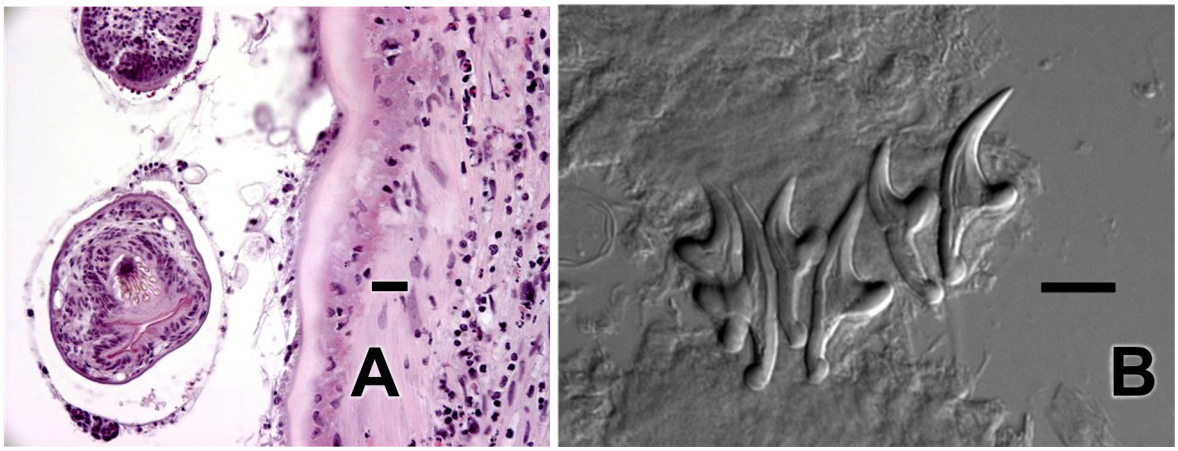

Figure 4. A) Section of cyst from *Echinococcus multilocularis* from *Microtus limnophilus*, showing fully developed protoscolex, germinal layer, cyst wall, and inflammation of liver tissue; HWML49784. Section on slide stained in hematoxylin-eosin. Scale bar = 20 µm. B) Hooks from one protoscolex of *E. multilocularis* removed from cyst of HWML68052, showing characteristic alternating, large and small hooks; HWML49785. Scale bar = 10 µm.

RESULTS

Echinococcus multilocularis *from Voles in Mongolia.*—The cysts in the liver of the infected vole collected at Har Us Lake were initially identified in the field as the larval form of *E. multilocularis*. Confirmation of this identification was made in the Manter Laboratory using DNA sequencing, morphology of the hooks, and histological characteristics of the cysts from the liver of the vole. Measurements of the hooks match well in all dimensions those of published ranges of hook measurements of *E. multilocularis* (Table 1). Measures of central tendency and extent of skewness and kurtosis were made (Table 2) and data concerning normality among morphological characters of hooks from protoscolexes has been included to facilitate future morphological comparisons.

For the specimen we collected in Mongolia, a GenBank "blast search" of the 774 base pair long partial COX1 gene (GenBank accession no. KC893696) showed 98-100% similarity to other *E. multilocularis* sequences and 90-92% similarities to other species (i.e., *E. shiquicus* Xiao et al. 2005; *E. felidis, E. vogeli, E.*

Table 1. Summary of lengths of hooks from protoscolexes of Echinococcus multilocularis *from Mongolia, Montana, and Alaska. Measurements from Vogel (1957) include hooks from protoscolexes from both intermediate and definitive hosts from Germany. Data from Bartel et al. (1992) includes measurements of hooks derived only from study of larval protoscolexes from material originating from both Alaska and Montana. n = number of specimens measured.*

	Large Hooks			Small Hooks			
Geographic Isolate	n	range	mean	n	range	mean	Source of material
Mongolia	112	24-29	27.3	111	21-28	24.5	Present study, cyst from liver of vole, Har Us Lake, Hovd Province, Mongolia
Montana	100	25-31	28.1	100	20-27	24.8	Bartel et al. (1992)
Alaska	100	24-28	25.8	100	17-24	20.7	Bartel et al. (1992)
Germany	65	25-30	27.4	54	22-27	24.4	Vogel (1957)

Table 2. Summary of mensural data of hooks from protoscolexes from metacestodes of Echinococcus multilocularis *from the liver of a vole,* Microtus limnophilus, *collected in Hovd Province, Mongolia. HWML49785. Measurements were made with the microscope following Gardner et al. (1988) and Rausch et al. (1978). n = number of specimens measured, min. = smallest measured structure, max. = largest measured structure, sd = standard deviation, cv = coefficient of variation.*

Character	n	min.	max.	mean	range	sd	cv	skewness g1	kurtosis g2
Large hooks									
Total length	112	24.3	29.4	27.3	5.1	1.1	4.0	-0.2	-0.2
Handle length	108	8.8	12.5	10.6	3.7	0.9	8.2	-0.01	-0.9
Guard width	115	8.0	11.8	9.7	3.8	0.9	9.4	0.1	-0.5
Blade length	102	14.2	19.0	16.7	4.8	1.0	5.7	-0.2	-0.1
Small hooks									
Total length	111	21.2	27.7	24.5	6.5	1.4	5.7	-0.1	-0.2
Handle length	116	8.7	13.4	11.3	4.7	1.0	8.6	-0.02	-0.3
Guard width	118	6.8	11.4	8.5	4.6	0.9	10.2	0.4	0.4
Blade length	107	10.9	15.6	13.2	4.7	1.1	8.0	0.1	-0.5

ortleppi López-Neyra and Soler 1943, and *E. oligarthrus* (Diesing 1863).

Echinococcus vogeli *in* Cuniculus paca*, Second Record for Bolivia*.—The sizes and shapes of the hooks prepared from protoscolexes from the cyst found in the liver of a *Cuniculus paca* hunted for food consumption at the Pilón Lajas Biosphere Reserve and Indigenous Territory enabled a positive identification to be made by one of us (J. L. Mollericona). Because these specimens (*E. vogeli*) had been preserved at time of collection in 10% formalin, no DNA was studied from this species and only measurements of the hooks are presented (Tables 3 and 4). Measurements comparing different collection localities of *E. vogeli* and *E. oligarthrus* from the Neotropics are shown in Table 4.

Table 3. Summary of mensural data of hooks from protoscolexes from metacestodes of Echinococcus vogeli *collected from the Beni Dept., Bolivia (HWML68527). Measurements were made with the microscope following Gardner et al. (1988) and Rausch et al. (1978). n = number of specimens measured, min. = smallest measured structure, max. = largest measured structure, sd = standard deviation, cv = coefficient of variation.*

Character	n	min.	max.	mean	range	sd	cv	skewness g1	kurtosis g2
Large hooks									
Total length	40	40	46.2	44.3	6.2	1.9	4.2	-0.04	-0.9
Handle length	40	13.8	17.5	15.4	3.7	1.3	8.2	-0.6	0.6
Guard width	40	12.5	15	13.3	2.5	1.1	8.0	-1.3	0.7
Blade length	40	25	30	27.1	5	1.3	4.8	0.4	0.1
Small hooks									
Total length	40	26.2	36.2	34.2	10.2	1.8	5.3	8.5	-2.3
Handle length	40	10	16.2	13.7	6.2	1.4	10.4	-0.3	0.1
Guard width	40	8.8	12.5	10.4	3.8	1.0	10	0.2	0.9
Blade length	40	16.2	21.2	19.2	5	1.4	7.5	0.7	-0.9

Table 4. Summary of hook measurements from Echinococcus vogeli *and* E. oligarthrus *from South American rodents.*

	Large Hooks			Small Hooks			
Species	n	range	mean	n	range	mean	Reference
E. vogeli	100	37-44	39.83	100	27-35	32.59	Gardner et al. 1988
E. vogeli	313	39.1-45.6	41.08	283	30.4-36.9	32.98	Rausch et al. 1978
E. oligarthrus	50	30.4-33.9	32	50	24.3-28.7	25.9	Rausch et al. 1978
E. oligarthrus	?	33.83-39.38	35.19	?	27.57-31.66	29.43	Rodríguez et al. 2000
E. vogeli	40	40-46.25	44.34	40	26.25-36.25	34.16	Present study, 2013

DISCUSSION

Species of Echinococcus.—Cestodes of the genus *Echinococcus* are the causative agents of several types of cystic or polycystic disease syndromes in people with metacestodes of the various described species exhibiting different levels and types of proliferation and pathogenesis (Rausch 1997; D'Alessandro and Rausch 2008). Currently, the definition of species in the genus is in flux (see review by Nakao et al. 2013), with four species recognized in the genus *Echinococcus* based mostly on morphological and taxonomic grounds, including: (1) *E. oligarthrus*, the causal agent of unicystic hydatid disease; (2) *E. vogeli*, the causal agent of polycystic hydatid disease; (3) *E. granulosus* (Batsch 1768), the causal agent of cystic hydatid disease; and (4) *E. multilocularis* (Leuckart 1863), the causal agent of alveolar hydatid disease (Rausch 1993, 1997; D'Alessandro and Rausch 2008). However, relatively new information derived from molecular-genetic analyses (especially mitochondrial DNA sequencing) has shown that genetic divergence in populations of some species (i.e., the "*E. granulosus*" biotype or strain complex) may indicate that genetic and phenotypic differentiation is occurring rapidly. Further evidence indicates that the drivers of this differentiation may be anthropogenic in nature (see Rausch 1993, 1997; de la Rue et al. 2011). Additional ongoing work has defined a series of "*E. granulosus* biotypes," with several species being described and others being recognized as potentially distinct, including *E. ortleppi* in South America and others in Africa (Nakao et al. 2013). Previously considered a biotype of *E. granulosus* by Rausch and Nelson (1963), the African species *E. felidis* (Ortlepp 1937) from *P. leo* has recently been shown to be genetically distinct from all other species of *Echinococcus* (see Hüttner et al. 2008, Hüttner et al. 2009, and review by Moro and Schantz 2009).

Distribution of Echinococcus *spp. in the New World.—Echinococcus granulosus* has a cosmopolitan distribution and has been spread world-wide by anthropogenic means occurring wherever sheep/dog associations are common; two forms are recognized, one cycling in sylvatic cervids (as intermediate hosts) and the other in synanthropic hosts (Rausch 1985, 1997). In the Neotropical region, either or both *E. vogeli* and *E. oligarthrus* have been reported as pathogenic parasites of humans from Nicaragua, Costa Rica, Panama, Colombia, Ecuador, Venezuela, Peru, Brazil, Suriname, Uruguay, Argentina, and Chile (D'Allesandro and Rausch 2008; Moro and Schantz 2009). In 1985, cysts of *E. vogeli* were found in the liver of an individual of *Cuniculus paca* collected in the Department of Santa Cruz, Bolivia (Gardner et al. 1988). While *Echinococcus vogeli* has been reported commonly from humans in South America (D'Allesandro and Rausch 2008), most infections have been reported from people from countries with relatively well-developed public health reporting infrastructures. Polycystic hydatid disease is now considered the most pathogenic of all cestodiases (D'Allesandro and Rausch 2008), posing a much greater threat to public health than other forms of taeniiasis or echinococcosis world-wide. In humans, the larvae of *E. vogeli* proliferate as metastatic tumors in the liver, lungs, brain, and other organs (D'Allesandro and Rausch 2008) and if left untreated, hydatid disease can be fatal (Rausch 1993, 1997; Moro and Schantz 2009). In Bolivia, one possible human case of *E. vogeli* was reported (Rivero et al. 2004); all other reports from Bolivia are probably attributable to *E. granulosus* as the etiologic agent.

The absence of reports of human cases of both *E. vogeli* and *E. oligarthrus* from Bolivia (given the fact that *E. vogeli* was collected from a paca in a populated area near San Ramón, northeast of Santa Cruz de la Sierra in 1985) is likely a result from the lack of adequate public health infrastructure in rural parts of Bolivia (D'Alessandro and Rausch 2008). Gardner et al. (1988) speculated that, in view of the distributions of the likely definitive hosts of this species, *E. vogeli* may occur from the Isthmus of Tehuantepec in southern Mexico south to Bolivia, Paraguay, and southern Brazil. Interestingly, in 1995, *E. oligarthrus* was collected from a road-killed *Lynx rufous* Allen 1895 from near San Fernando, Tamaulipas State, Mexico, about 100 km south of Brownsville, Texas (Salinas-Lopez et al. 1996) representing the northernmost extent of the recorded distribution of this cestode in the Nearctic. Additional broad scale biodiversity surveys of mammals and their parasites in the area of the US/Mexico border are necessary to further define the distribution of *E. oligarthrus* in felids and to determine the threat of infection in people in these areas.

Holarctic Distribution of E. multilocularis.—*Echinococcus multilocularis* is now recognized in humans as the second most pathogenic of all cestodes; the more pathogenic species, *E. vogeli*, has a more invasive/proliferative larval stage (see D'Alessandro and Rausch 2008). *Echinococcus multilocularis* has a Holarctic distribution and a range in the Palearctic extending from northern and central Europe east into Asia. In Asia, this species occurs in an area north of the boreal forests south into northern Tibet where it appears to be geographically sympatric with the recently described *E. shiquicus* Xiao et al. 2005 (see Xiao 2005). In the Nearctic, *E. multilocularis* has a disjunct distribution, with a northern population generally co-extensive with Arctic tundra, north of the boreal forest (Rausch 1997). The southern population appears to be spatially and perhaps genetically separate, occurring through central and western Canada (Catalano et al. 2012) and south into the Great Plains of the U.S., being recorded as far south as Nebraska (Storandt et al. 2002; Storandt and Kazacos 2012). Sylvatic definitive hosts for this species are foxes of the genus *Vulpes*; voles or arvicolid (microtine) rodents serve as primary intermediate hosts, although rodents of at least seven families have been shown to act as natural intermediate hosts (Rausch 1985, 1993, 1997). People usually become infected when they interact with family dogs that are passing eggs in feces. The dogs themselves become infected by eating voles that harbor the larvae (Fig. 1).

Echinococcus multilocularis *in Mongolia*.—Recently in Mongolia, Ito et al. (2013) reported the presence of *E. multilocularis* (including several strains) from many wild canids examined from several provinces in the northern part of the country with one record from Gobi Altai province east of Hovd province where we found the infected vole. Previously, hydatid cysts representing larval stages of *E. multilocularis* were described and the DNA sequenced from people who were evidently infected several years earlier (Ito et al. 2010, 2013) and this cestode has been reported to occur commonly in people in Russia to the north of Mongolia (Rausch 1952). Prior to this work, no published records of natural infections of voles have been reported from Mongolia.

This report represents the first definitive record of the larvae of the cestode *E. multilocularis* from a sylvatic vole in Mongolia, which appears to be its natural intermediate host. This record was not unexpected as *M. limnophilus* has been shown to be one of the primary intermediate hosts of *E. multilocularis* in China, where overgrazing appears to promote the transmission of the disease (Vaniscotte et al. 2011). In addition, this cestode also occurs to the north of Mongolia in the Ikurtsk Oblast, Russia (Rausch 1952; Konyaev et al. 2012). As was demonstrated by Rausch (1967) in Alaska, Craig et al., (2000) in China, and Vaniscotte et al. (2011) on the Plateau of Tibet, domestic dogs are the primary sources of amplification and ultimate vectors of transmission of these cestodes to humans. In these areas, human cases result from accidental ingestion of eggs via fecal contamination by dogs infected with adult cestodes. Pathogenesis in people occurs from the larval cestode proliferating in the liver (and, or) other organs (Rausch 1997).

Reported widely in the northern parts of the Nearctic region, *E. multilocularis* appears to be expanding its range south and east, especially in the central grasslands of the continental United States (Rausch and Richards 1971; Storandt and Kazacos 2012); however, few intensive surveys for this species (or for that matter, other species of parasites in mammals) have been conducted in the southern and central Nearctic. The grassland steppe ecosystems of Mongolia are similar in nature to the central Great Plains grasslands of the Nearctic region and these similar ecotypes may show common patterns of geographic expansion/contraction or shifting distributions of *E. multilocularis* as climate warms or otherwise fluctuates through time.

Regarding our discovery of *E. multilocularis* in voles near Har Us Lake, we urge immediate action by health authorities in Hovd Province, western Mongolia, to educate families with dogs as to the risks of infection, especially of children, and to teach safeguards to implement to decrease risk of infection. In addition, we recommend additional focused surveys in the area of western Mongolia to determine prevalence of *E. multilocularis* in both wild and domestic canids in the area.

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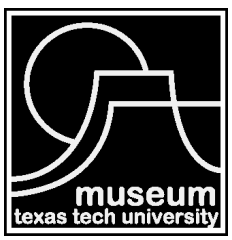

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