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RESEARCH NOTES

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Observations on the Sporozoite Transmission of *Plasmodium vivax* to Monkeys

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ABSTRACT: *Saimiri boliviensis* monkeys were infected via sporozoites with the Salvador I strain of *Plasmodium vivax* that had been stored frozen for periods ranging from 12 to 5,312 days. Prepatent periods ranged from 16 to 53 days.

Previously, we reported on the development of models for the testing of antimalarial vaccines using splenectomized *Saimiri boliviensis* monkeys with varying numbers of sporozoites of the Salvador I strain of *Plasmodium vivax* (Collins et al., 1988). In total, 20 monkeys were used, and the results indicated that when 100,000 sporozoites were injected intravenously into these animals, the prepatent periods averaged 16.6 days versus prepatent periods of 19.4 days after injections of 10,000 sporozoites. When 5 monkeys were injected with 1,000 sporozoites, only 4 developed detectable parasitemia, and the prepatent periods in the 4 infected animals ranged from 24 to 35 days. It seemed that the threshold for infection was between 1,000 and 10,000 sporozoites, and the prepatent period decreased with an increase in challenge dosage.

Subsequently, additional studies were conducted with many more animals and different numbers of sporozoites (Sullivan et al., 1996). Prepatent periods ranged from 10 to 63 days. By 1996, 193 *S. boliviensis* monkeys in total had been infected, 4 via bites, and the rest by the intravenous injection of 10,000 or more sporozoites of the Salvador I strain of *P. vivax*. The requirement for such large numbers of sporozoites and the resultant long prepatent periods contrasted markedly with the results of studies with *Plasmodium knowlesi* in this same animal model (Collins et al., 2004). As we reported, when *S. boliviensis* monkeys were given intravenous injection of 50 sporozoites of the H strain of *P. knowlesi* that had been stored frozen, the prepatent periods ranged from 11 to 16 days. In addition, sporozoite numbers ranging from 450 to 45,000 that had been frozen for over 11 years induced infections with prepatent periods of 10 to 13 days.

The objective of this current study was to determine whether sporozoites of the Salvador I strain of *P. vivax* that had been stored frozen for varying periods could be used to induce infections in splenectomized *S. boliviensis* monkeys for challenge in vaccine trials.

The *S. boliviensis* were colony-born animals. On arrival at the facility, all animals were quarantined for a 2-mo conditioning period, weighed, and tested for tuberculosis. Parasitologic and serologic examination indicated that the animals were free of infection with malarial parasites before primary inoculation. The animals had previously been splenectomized, infected with *Plasmodium* spp., and cured of their infections before participation in this study. All animals were fed a diet that has been proven to provide adequate nutrition and calories to captive *S. boliviensis* used in malaria-related research. Feed was free of contaminants and freshly prepared. Daily observations of the animals' behavior, appetite, stool, and condition were recorded. An attending veterinarian treated all animals as medical conditions arose.

Anopheles stephensi (originally from India) were laboratory reared and maintained at the CDC/DPD insectaries. Mosquito infection was obtained by allowing the caged anophelines to feed through a membrane on blood obtained from an infected chimpanzee. Mosquitoes were held in an incubator at 25 C until sporozoites were present in the salivary glands. Glands were dissected into sterile 50% fetal bovine serum/50% phosphate-buffered saline from groups of 10 mosquitoes, triturated, and an aliquot was counted in a Neubauer cell-counting chamber. Dilutions were made, and the sporozoites were distributed into sterile vials in volumes of 0.5 ml. They were directly stored in the vapor-phase of a liquid nitrogen freezer. For challenge, a vial was removed from storage, thawed quickly, drawn into a syringe, and injected intravenously into the monkey.

Beginning 14 days after sporozoite injection, thick (Earle and Perez, 1932) and thin blood films were made daily and stained with Giemsa. Parasite counts were recorded per microliter of blood. At the end of the study, all animals were administered 30 mg of chloroquine (base) by oral intubation over a period of 3 days and 2.5 mg of primaquine daily for 7 days.

Fifteen of the 21 animals that were injected with sporozoites that had been stored frozen became infected (Table I). The prepatent periods that ranged from 16 to 53 days (median of 32 days) were, in most instances, at the high end of the reported period that we had seen in previous studies (Collins et al., 1988; Sullivan et al., 1996). In our report on studies with frozen sporozoites of *P. knowlesi*, we concluded that the survival of frozen sporozoites may be less that 10% using the current freezing and storage procedures. With a parasite model system that requires between 1,000 and 10,000 sporozoites to predictably ensure infection, such as the *S. boliviensis*/*P. vivax* combination, the number of sporozoites available was apparently insufficient and thus we obtained only approximately 70% infection. The extended prepatent periods and the 6 failed infections are indications that improvement in the freezing procedure is needed.

Nonetheless, it was demonstrated that sporozoites of *P. vivax* could be stored frozen for greater than 14 yr and be made available for chal-

TABLE I. Endohelminths recollected of 7 fish species from La Mintzita Reservoir, Michoacán, Mexico.

Monkey No.	Previous infections with *Plasmodidum spp.*†	Number of sporozoites	Period Frozen	Prepatient Perior
SI-0260	None	25,000	12 days	32 days
SI-63	None	25,000	12 days	32 days
SI-0277	None	25,000	12 days	41 days
SI-0266	None	25,000	12 days	47 days
SI-0207	None	30,000	159 days	16 days
SI-0080	None	30,000	159 days	19 days
SI-0091	None	30,000	159 days	NI*
SI-0192	None	30,000	159 days	NI
SI-0967	*P. fra., P. fal.*	90,000	296 days	39 days
SI-0966	*P. fra., P. fal.*	90,000	296 days	43 days
SI-0975	*P. fra., P. fal.*	90,000	296 days	45 days
SI-0964	*P. fra., P. fal.*	90,000	296 days	NI
SI-0276	None	130,000	405 days	53 days
SI-0258	None	130,000	405 days	NI
SI-2045	*P. viv., P. kno.*	75,000	5,312 days	23 days
SI-2047	*P. viv., P. sim.*	75,000	5,312 days	32 days
SI-2028	*P. fal., P. viv, P. kno., P. sim.*	75,000	5,312 days	NI
SI-2145	*P. viv.*	75,000	5,312 days	22 days
SI-2175	*P. viv.*	75,000	5,312 days	NI
SI-2192	*P. sim.*	75,000	5,312 days	28 days
SI-2194	*P. viv.*	75,000	5,312 days	27 days

† *P. Fra. = Plasmodium fragile; P. fal.* = P. *Falciparum; P. viv. = P. vivax; P. kno. = P. knowlesi; P. sim. = P. simium.*
* NI = No infection.

lenge of *S. boliviensis* monkeys either for vaccine or other biologic studies.

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LITERATURE CITED

COLLINS, W. E., J. C. SKINNER, M. PAPPAIOANOU, J. R. BRODERSON, V. FILIPSKI, H. M. MCCLURE, E. STROBERT, B. B. SUTTON, P. S. STANFILL, AND A. Y. HUONG. 1988. Sporozoite-induced infections of the Salvador I strain of *Plasmodium vivax* in *Saimiri sciureus boliviensis* monkeys. Journal of Parasitology **74:** 582–585.

———, J. S. SULLIVAN, D. NACE, T. WILLIAMS, A. WILLIAMS, G. G. GALLAND, AND J. W. BARNWELL. 2004. Additional observations on the sporozoite transmission of *Plasmodium knowlesi* to monkeys. Journal of Parasitology **90:** 866–867.

EARLE, W. C., AND M. PEREZ. 1932. Enumeration of parasites in the blood of malarial patients. Journal of Laboratory and Clinical Medicine **17:** 1124–1130.

SULLIVAN, J. S., C. L. MORRIS, H. M. MCCLURE, E. STROBERT, B. B. RICHARDSON, G. G. GALLAND, I. F. GOLDMAN, AND W. E. COLLINS. 1996. *Plasmodium vivax* infections in chimpanzees for sporozoite challenge studies in monkeys. American Journal of Tropical Medicine and Hygiene **55:** 344–349.

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Estimation of the Endohelminth Parasite Species Richness in Freshwater Fishes From La Mintzita Reservoir, Michoacán, Mexico

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ABSTRACT: In total, 9 endohelminth species were found to parasitize 7 fish species (2 cyprinids, 4 goodeids, and 1 poeciilid) from La Mintzita Reservoir, Michoacán, in central Mexico; 5 were larvae, including 3 allogenic species (*Clinostomum complanatum*, *Tylodelphys* sp., *Posthodiplostomum minimum*) and 2 autogenic species (*Serpinema trispinosum*, *Spiroxys* sp.). Four were enteric autogenic adults, i.e., *Margotrema bravoae*, *Bothriocephalus acheilognathi*, *Proteocephalus longicollis*, and *Rhabdochona lichtenfelsi*. The metacercariae of *P. minimum* reached the highest levels of prevalence and mean abundance among host species. Our results confirm the depauperate nature of the helminth communities of freshwater fishes from central Mexico. On the basis of this data set, we estimated the total endohelminth species richness for each component community by using 7 nonparametric estimators whose performance was evaluated with the unscaled measures of bias, precision, and accuracy. We found that Chao1 and Bootstrap are the most precise and least biased methods for the 7 component communities; however, species richness was consistently underestimated. The underestimation was an unavoidable consequence of the patchy distribution of helminth species among different component communities, particularly at the small sample size used in our study.

Central Mexico is a region where surveys of freshwater fish parasites have been conducted extensively over the last decades. Most of these studies are focused on taxonomic records of a particular host species (Osorio-Sarabia et al., 1986), a host group in 1 locality (Peresbarbosa-Rojas et al., 1994), or a host group along its distributional range (Mejía-Madrid et al., 2005). Available information indicates that component communities (sensu Bush et al., 1997) of freshwater fishes in this geographical region are generally species poor and numerically dominated by 1 species, frequently the metacercariae of the digenean *Posthodiplostomum minimum*, a species that matures in piscivorus birds (Espinosa-Huerta et al., 1996; Rojas et al., 1997; Pérez-Ponce de León et al., 2000; Sánchez-Nava et al., 2004). Even though a great amount of information has been produced, the total helminth species richness of only 3 host species has been estimated using empirical data and the same methodological approaches (Martínez-Aquino et al., 2004, 2007). The objectives of the present work are (1) to describe the endohelminth parasites of 7 fish species sampled concurrently in La Mintzita, Michoacán, a natural water reservoir located in central Mexico; (2) on the basis of the number of endohelminth species recorded in these 7 fish species (observed richness), to estimate the total species richness for each component community (observed species richness + number of missing species remaining to be found in each component community) using 7 nonparametric richness estimators; and (3) to compare the usefulness of these estimators in predicting the species richness in each component community by evaluating their performance with the unscaled measures of bias, precision, and accuracy (see Walther and Moore, 2005).

In total, 166 adult fishes representing 7 of the 13 fish species inhabiting La Mintzita Reservoir were collected in September 2003: Cyprinidae: *Notropis calientis* (12), *Yuriria alta* (30); Goodeidae: *Alloophorus robustus* (27), *Skiffia lermae* (30), *Xenotoca variata* (7), and *Zoogoneticus quitzeoensis* (30); Poeciliidae: *Xiphophorus helleri* (30). La Mintzita Reservoir is located in the northern portion of the Michoacán State, 7 km SW from Morelia City (19°38′40″N, 111°16′28.7″W). The total area of the reservoir is 7.65 km^2, with a mean depth of 1.5 m (Medina-Nava et al., 2003).

Fishes were captured using seine nets and examined within 4 hr of capture using standard procedures. Voucher specimens of all helminth taxa were deposited at the Colección Nacional de Helmintos, Instituto de Biología, Universidad Nacional Autónoma de México (UNAM), Mexico City, Mexico. Prevalence and mean abundance of infections were calculated following Bush et al. (1997).

By computing 7 nonparametric species richness estimators, we evaluated the total endohelminth species richness in the 7 component communities of fish species from La Mintzita Reservoir; the program EstimateS 7.5 was used (Colwell, 2005). For comparative purposes, we chose ACE, ICE, Chao1, Chao2, Jackknife1, Jacknife2, and Bootstrap estimators, which were assessed by Walther and Morand (1998) on parasite component communities. Each host was considered a sample and the entire component community was considered the data set. For each component community, the sample order was randomized 100 times without replacement; for each new random combination of samples, all estimators were used to calculate the total species richness. The mean values and standard deviation of the 100 resulting estimates at each level of sampling effort were used to calculate the bias, precision, and accuracy of each estimator. The difference between the mean value of the estimates and the total species richness represents the bias of each estimator at each level of sampling effort. Accuracy is the square of these differences; the standard deviation of the estimates yields the precision of each estimator at each level of sampling effort. The performance measures are presented as the average of each performance measure over all levels of sampling effort (Walther and Moore, 2005).

In total, 9 endohelminth species (observed species richness) were

TABLE I. Endohelminths recollected of 7 fish species from La Mintzita Reservoir, Michoacán, Mexico.

		Cyprinidae		Goodeidae				Poeciliidae
Host species	CNHE	*Notropis calientis* (n = 12)	*Yuriria alta* (n = 30)	*Alloophorus robustus* (n = 27)	*Skiffia lermae* (n = 30)	*Xenotoca variata* (n = 7)	*Zoogoneticus quitzeoensis* (n = 30)	*Xiphophorus helleri* (n = 30)
Length (mm)		2.5–3.7	5–15.6	3.8–10.3	3.9–7	2.9–4	2.9–5.7	3.4–7.3
Parameters		%, Ab ± SD	%, Ab ± SD	%, Ab ± SD	%, Ab ± SD	%, Ab ± SD	%, Ab ± SD	%, Ab ± SD
Digenea								
M. bravoae								
Intestine	5038–39	—	—	7.4, 0.07 ± 0.27	—	—	6.67, 0.1 ± 0.4	—
*C. complanatum**	5044–46	—	—	—	3.33, 0.03 ± 0.18	14.29, 0.14 ± 0.38	10, 0.1 ± 0.31	3.33, 0.03 ± 0.18
Body cavity								
Tylodelphys sp.*	5041–43	—	—	—	—	14.29, 0.14 ± 0.38	—	—
Eyes								
*P. minimum**								
Body cavity, brain, liver	5051–53	91.67, 8.58 ± 8.33	100, 473.8 ± 749.54	—	30, 1.27 ± 3	14.29, 0.14 ± 0 .38	—	—
Cestoda								
B. acheilognathi								
Intestine	5054–57	58.33, 0.83 ± 0.94	10, 0.13 ± 0.43	7.4, 0.19 ± 0.79	—	—	—	16.67, 0.43 ± 1.36
P. longicollis								
Intestine	5040	—	—	—	16.67, 0.2 ± 0.48	—	—	—
Nematoda								
*S. trispinosum**								
Intestine	5061	—	—	—	3.3, 0.03 ± 0.18	—	—	—
Spiroxys sp.*								
Body cavity, intestine, mesentery	5062–63	—	—	3.7, 0.07 ± 0.38	—	—	3.33, 0.3 ± 1.64	—
R. lichtenfelsi								
Intestine	5058–60	—	—	3.7, 0.04 ± 0.19	96.67, 11.33 ± 7.93	—	6.67, 0.8 ± 4.2	—

CNHE = Accession number to Colección Nacional de Helmintos; % = Prevalence; Ab = Mean abundance; SD = Standard deviation. * = larvae.

TABLE II. Total endohelminth species richness for 7 fish species from La Mintzita Reservoir, Michoacán, Mexico, obtained with 7 estimators and performance measures of them.

	Sobs	ACE	ICE	Chao1	Chao2	Jack1	Jack2	Bootstrap
Notropis calientis								
Total richness	2	2	2	2	2	2	2	2
Bias	−1.94	−0.03	−0.15	−0.007	−0.04	−0.05	−0.09	−0.03
Precision	0.03	0.16	0.29	0.029	0.15	0.06	0.2	0.15
Accuracy	3.77	0.01	0.07	0.0003	0.019	0.01	0.2	0.0027
Yuriria alta								
Total richness	2	2	2	2	2	2	2	2.04
Bias	−1.78	−0.0003	−0.13	−0.0003	−0.004	−0.97	−0.10	−0.12
Precision	0.16	0.26	0.42	0.04	0.009	0.97	0.90	0.35
Accuracy	3.15	0.0004	0.06	0.0004	0.0008	0.06	0.14	0.018
Alloophorus robustus								
Total richness	4	4.65	6	4	4.32	5.93	6	4.97
Bias	−2.42	−1.3	−2.3	−0.4	−1.2	−1.9	−2.8	−0.9
Precision	2.60	1.8	2.19	0.60	1.61	1.13	2.12	0.94
Accuracy	5.86	2.07	6.61	0.21	1.65	3.91	8.9	0.91
Skiffia lermae								
Total richness	5	9.05	7.24	6*	5.97*	6.93	8.8	5.73
Bias	−3.82	−1.96	−1.81	−0.59	−0.74	−1.29	−2.17	−0.57
Precision	0.89	1.83	2.05	1.25	1.5	0.92	1.67	0.80
Accuracy	14.6	5.21	3.39	0.42	0.56	1.85	5.95	0.35
Xenotoca variata								
Total richness	3	6	5.57	6	5.57*	5.57	7.71	4.02
Bias	−1.71	−1.19	−1.02	−1.10	−1.02	−1.33	−2.19	−0.56
Precision	0.67	1.054	0.97	1.78	1.59	0.70	0.92	0.74
Accuracy	2.94	2.51	1.85	2.38	1.85	2.53	7.85	0.44
Zoogoneticus quitzeoensis								
Total richness	4	4	4.57	4	4	4.97	4.1	4.66
Bias	−2.67	−0.6	−1.59	−0.15	−0.61	−1.36	−1.68	−0.68
Precision	2.72	1.2	1.9	0.32	1.04	0.94	2.11	0.84
Accuracy	7.13	0.52	3.31	0.04	0.52	2.06	3.69	0.52
Xiphophorus helleri								
Total richness	2	2	3.12	2	2*	2.97	3.9	2.37
Bias	−1.38	−0.31	−0.60	−0.02	−0.12	−0.68	−1.11	−0.31
Precision	0.24	0.77	0.82	0.21	0.35	0.62	1.21	0.61
Accuracy	1.89	0.13	0.50	0.001	0.021	0.51	1.49	0.1

Sobs = Observed richness; * Computed using the classic formula.

collected as parasites of 7 fish species from La Mintzita Reservoir (Table I). The helminth fauna include 4 species of digeneans (1 adult and 3 metacercariae), 2 cestodes (adults), and 3 nematodes (1 adult and 2 larvae). The most widely distributed endohelminth species among hosts were the cestode *Bothriocephalus acheilognathi* and the digeneans *Clinostomum complanatum* and *P. minimum*, which parasitized 4 host species; the metacercariae of *P. minimum* reached the highest levels of prevalence and mean abundance (Table I).

Six of the 9 helminth species are transmitted via trophic dynamics, whereas 3 metacercariae, i.e., *P. minimum*, *C. complanatum*, and *Tylodelphys* sp., penetrated their hosts through the skin. The goodeid, *S. lermae*, harbored the richest component community (5 species), whereas the remaining fish species were parasitized by 2–4 helminth species.

On the basis of the values of the 7 estimators used in this study, the minimum number of missing species remaining to be found in the 7 host species studied from La Mintzita Reservoir varies from 1 to 5 (Table II). However, considering performance measures obtained for Chao1 and Bootstrap (the least biased and most precise estimators; see Table II), the total number of species is known for 5 fish species, i.e., *A. robustus*, *N. calientis*, *Y. alta*, *X. helleri*, and *Z. quitzeoensis*, whereas in *S. lermae* and *X. variata*, 1 more species would be expected for each species.

With the exception of the nematode *Serpinema trispinosum*, all other helminth species have been recorded previously in La Mintzita Reservoir (Mejía-Madrid et al., 2005; Garrido-Olvera et al., 2006; Salgado-Maldonado, 2006). Moreover, the larva of *S. trispinosum* had not previously been recorded in fishes from central Mexico; however, adults of this nematode species have been found to parasitize garter snakes (*Thamnophis* spp.) and mud turtles (*Kinosternon* spp.) from 4 lakes located within the same geographical region (see Pérez-Ponce de León et al., 2001).

The helminth fauna of the 7 fish species in La Mintzita Reservoir included only 2 specialist species (*Margotrema bravoae* and *Rhabdochona lichtenfelsi*), which were considered to be part of the biogeographical core helminth fauna of the Goodeidae in accordance with Mejía-Madrid et al. (2005). Additionally, a greater number of generalist species (*C. complanatum*, *P. minimum*, *Tylodelphys* sp., *B. acheilognathi*, *Proteocephalus longicollis*, *S. trispinosum*, and *Spiroxys* sp.) was recorded. The 3 metacercariae, *B. acheilognathi*, and the nematode *Spiroxys* sp. are the most commonly found parasite species in freshwater

fishes from Mexico; each infects more than 20 freshwater fish species (Pérez-Ponce de León et al., 1996; Salgado-Maldonado, 2006). Likewise, the larvae of *S. trispinosum* are widely distributed in southeast Mexico, where they mainly infect cichlid fishes (Vidal-Martínez et al., 2001). Finally, we considered *P. longicollis* as a generalist species because this cestode has been reported from several salmonid fishes in North America (Hoffman, 1999), even though in central Mexico it infects exclusively goodeid fishes.

The taxonomic composition and the low abundances of the helminths in each host species, as well as the number of uninfected hosts in La Mintzita Reservoir, correspond with the pattern described for 12 fish species (3 atherinopsids, 1 centrarchid, 1 cichlid, 2 cyprinids, and 5 goodeids) studied in other water bodies from central Mexico (Espinosa-Huerta et al., 1996; Rojas et al., 1997, Pérez-Ponce de León et al., 2000; Salgado-Maldonado et al., 2001; Martínez-Aquino et al., 2004; Sánchez-Nava et al., 2004). Our results confirm the depauperate nature of the helminth communities of freshwater fishes from central Mexico (Pérez-Ponce de León et al., 2000). These communities have been described as species poor, open to the invasion by allogenic generalist species (particularly avian-transported larval stages such as *P. minimum* and *Tylodelphys* sp.), and with a reduced number of specialist species that are not typically abundant. A further component of these helminth faunas is represented by helminth species anthropogenically introduced, i.e., *B. acheilognathi* (Martínez-Aquino et al., 2004).

Some historical and ecological factors have been identified as determinants of the depauperate character of these component communities: (1) the young geological age of several fish families from central Mexico; (2) the trophic status of the lake; (3) host feeding and behavioral habits; and (4) availability of parasite species and their colonization capabilities (Espinosa-Huerta et al., 1996; Pérez-Ponce de León et al., 2000; Pineda-López et al., 2005). Additionally, in La Mintzita Reservoir, anthropogenic factors such as pollution, introduction of exotic fish species, and extraction of water for domestic use (Medina-Nava et al., 2003) have modified the ecological conditions and the stability of this ecosystem. These conditions would preclude the colonization of new helminth species or would cause the extinction of others from this water body.

The extrapolations obtained using nonparametric estimators are common for many kinds of ecological studies; however, relatively little attention has been focused on their applicability in parasitological investigations. Previous studies have assessed the performance of nonparametric estimators for simulated data of parasite communities, pointing out that Chao1, Chao2, Bootstrap, and Jacknife1 are the most robust estimators (see Poulin, 1998; Walther and Morand, 1998; Zelmer and Esch, 1999). When real data sets of helminth species are used, Chao2 and Jacknife1 were consistently the most precise and least biased methods (see Walther and Morand, 1998). However, for different data sets, different estimators are expected to perform better (Colwell and Coddington, 1994).

When we applied the 7 nonparametric estimators to our data set, we found that Chao1 and Bootstrap are the most precise and least biased methods (Table II). The performance of Chao 1 was better in helminth communities with a high proportion of rare species (*N. calientis*, *Y. alta*, *A. robustus*, *Z. quiteoensis*, and *X. helleri*); likewise, Bootstrap performed better in the component community with a high proportion of quadrants (individual hosts) containing 1 helminth species, i.e., in *S. lermae*, where *R. lichtenfelsi* had a prevalence of 96.67%, as well as in the component community where helminth species infected a similar proportion of hosts, i.e., in *X. variata*, where the digeneans *C. complanatum*, *Tylodelphys* sp., and *P. minimum* had the same prevalence (Table I).

However, the rates of species accumulation of Chao 1 and Bootstrap estimators increased slowly and did not reach a stable value of richness even though all sampling levels were considered. This condition determined that species richness was consistently underestimated, as it is indicated by the negative bias values obtained for both estimators (Table II). The underestimation was an unavoidable consequence of the patchy distribution of helminth species among different component communities, particularly at the small sample sizes used in our study. Therefore, the sample size should increase to account fully for all of the rare species and then, to predict accurately the number of ''unfound'' species (see Chazdon et al., 1998). In general, the patchy distribution of parasites is due to individual host differences in diet, susceptibility, behavior, and immune response, as well as the viability, dispersion, and behavior of individual parasites (Kennedy, 1975, 1977).

Recent helminthological surveys in fishes from La Minzita Reservoir included some of the host species analyzed in our study, i.e., *A. robustus*, *N. calientis*, *S. lermae*, *X. variata*, and *Z. quitzeoensis* (Mejía-Madrid et al., 2005; Garrido-Olvera et al., 2006; Salgado-Maldonado, 2006). Considering these studies, helminth species richness for each component community increased from 1 (in *N. calientis*) to 5 (in *X. variata*) species; this would confirm that the estimators yielded underestimated values of species richness for all component communities analyzed (Table II). However, only 2 endohelminth species reported in these studies are different from those recorded in our sampling efforts, i.e., the cystacanth of *Polymorphus brevis* in *X. variata* and the larval nematode *Eustrongylides* sp. in *S. lermae* (Salgado-Maldonado, 2006).

With the exception of *M. bravoae*, *R. lichtenfelsi*, and probably *S. trispinosum*, the helminth taxa recorded in our study seem to constitute a species pool for this particular area, parasitizing indistinctly all component communities, i.e., host sharing at this level is independent of host phylogeny, being determined primarily by niche sympatry and by the geneneralist character of these helminth species (see Nelson and Dick, 2002). *Eustrongylides* sp. and *P. brevis* (also generalist species) could not be detected with the reduced sample size that we used in this study because of the low levels of infection present in host populations.

On the basis of previous and present information regarding the estimation of helminth species richness in real and simulated communities, it is evident that there is not any estimator that is especially suitable for this particular parasite group. Therefore, further studies are necessary to establish if one of them can be recommended.

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LITERATURE CITED

BUSH, A. O., K. D. LAFFERTY, J. L. LOTZ, AND A. W. SHOSTAK. 1997. Parasitology meets ecology on its own terms: Margolis et al., revisited. Journal of Parasitology **83:** 575–583.

CHAZDON, R. L., R. K. COLWELL, J. S. DENSLOW, AND M. R. GUARIGUATA. 1998. Statistical methods for estimating species richness of woody regeneration in primary and secondary rain forests of NE Costa Rica. *In* Forest biodiversity research, monitoring and modeling: Conceptual background and Old World case studies, F. Dallmeier, and J. A. Comiskey (eds.). Parthenon Publishing, Paris, France, p. 285–309.

COLWELL, R. K. 2005. EstimateS: Statistical estimation of species richness and shared species from samples. Version 7.5. User's guide and application. http://viceroy.eeb.uconn.edu/EstimateS (03/2007).

———, AND J. A. CODDINGTON. 1994. Estimating terrestrial biodiversity through extrapolation. Philosophical Transactions of the Royal Society, London **345:** 101–118.

ESPINOSA-HUERTA, E., L. GARCÍA-PRIETO, AND G. PÉREZ-PONCE DE LEÓN. 1996. Helminth community structure of *Chirostoma attenuatum* (Osteichthyes: Atherinidae) in two Mexican lakes. Southwestern Naturalist **41:** 288–292.

GARRIDO-OLVERA, L., L. GARCÍA-PRIETO, AND G. PÉREZ-PONCE DE LEÓN. 2006. Checklist of the adult nematode parasites of fishes in freshwater localities from Mexico. Zootaxa **1201:** 1–45.

HOFFMANN, G. L. 1999. Parasites of North American freshwater fishes. Cornell University Press, Ithaca, New York, 539 p.

KENNEDY, C. R. 1975. Ecological animal parasitology. Blackwell Scientific Publications, London, U.K., 163 p.

———. 1977. Distribution and zoogeographical characteristics of the parasites fauna of Char *Salvelinus alpinus* in Arctic Norway, including Spitsbergen and Jean Mayen Island. Astarte **10:** 49–55.

MARTÍNEZ-AQUINO, A., G. SALGADO-MALDONADO, R. AGUILAR-AGUILAR, G. CABAÑAS-CARRANZA, AND C. MENDOZA-PALMERO. 2007. Hel-

minth parasite communities of *Characodon audax* and *C. lateralis* (Pisces: Goodeidae) endemic freshwater fishes from Durango, Mexico. Southwestern Naturalist **52:** 125–130.

———, ———, ———, ———, AND M. P. ORTEGA-OLIVARES. 2004. Helminth parasites of *Chapalichthys encaustus* (Pisces: Goodeidae), an endemic freshwater fish from Lake Chapala, Jalisco, Mexico. Journal of Parasitology **90:** 889–890.

MEDINA-NAVA, M., T. ZUBIETA-ROJAS, AND J. RAMÍREZ-HERREJÓN. 2003. Estructura de la comunidad de peces de La Mintzita, Morelia, Michoacán, Cuenca Lerma-Chapala. Biológicas **5:** 19–29.

MEJÍA-MADRID, H., O. DOMÍNGUEZ-DOMÍNGUEZ, AND G. PÉREZ-PONCE DE LEÓN. 2005. Adult endohelminth parasites of Goodeinae (Cyprinodontiformes: Goodeidae) from Mexico with biogeographical considerations. Comparative Parasitology **72:** 200–211.

NELSON, P. A., AND T. A. DICK. 2002. Factors shaping the parasite communities of trout-perch, *Percopsis omiscomaycus* Walbaum (Osteichthyes: Percopsidae) and the importance of scale. Canadian Journal of Zoology **80:** 1986–1999.

OSORIO-SARABIA, D., G. PÉREZ-PONCE DE LEÓN, AND G. SALGADO-MALDONADO. 1986. Helmintos de peces del Lago de Pátzcuaro, Michoacán I: Helmintos de *Chirostoma estor* el ''pescado blanco''. Taxonomía. Anales del Instituto de Biología de la Universidad Nacional Autónoma de México, Serie Zoología **57:** 61–92.

PERESBARBOSA-ROJAS, E., G. PÉREZ-PONCE DE LEÓN, AND L. GARCÍA-PRIETO. 1994. Helmintos parásitos de tres especies de peces (Goodeidae) del Lago de Pátzcuaro, Michoacán. Anales del Instituto de Biología de la Universidad Nacional Autónoma de México, Serie Zoología **65:** 201–204.

PÉREZ-PONCE DE LEÓN, G., L. GARCÍA-PRIETO, V. LEÓN-RÉGAGNON, AND A. CHOUDHURY. 2000. Helminth communities of native and introduced fishes in Lake Pátzcuaro, Michoacán, México. Journal of Fish Biology **57:** 303–325.

———, ———, D. OSORIO-SARABIA, AND V. LEÓN-RÈGAGNON. 1996. Listados faunísticos de México VI. Helmintos parásitos de peces de aguas continentales de México. Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico, 100 p.

———, A. JIMÉNEZ-RUIZ, B. MENDOZA-GARFIAS, AND L. GARCÍA-PRIETO. 2001. Helminth parasites of garter snakes and mud turtles from several localities of the Mesa Central of Mexico. Comparative Parasitology **68:** 9–20.

PINEDA-LÓPEZ, R., G. SALGADO-MALDONADO, E. SOTO-GALERA, N. HERNÁNDEZ-CAMACHO, A. OROZCO-ZAMORANO, S. CONTRERAS-ROBLEDO, G. CABAÑAS-CARRANZA, AND R. AGUILAR-AGUILAR. 2005. Helminth parasites of viviparous fishes in Mexico. *In* Viviparous fishes: Genetics, ecology, and conservation, H. Grier, and M. C. Uribe (eds.). New Life Publications, Homestead, Florida, p. 437–456.

POULIN, R. 1998. Comparison of three estimators of species richness in parasite component communities. Journal of Parasitology **84:** 485–490.

ROJAS, E., G. PÉREZ-PONCE DE LEÓN, AND L. GARCÍA-PRIETO. 1997. Helminth community structure of some freshwater fishes from Pátzcuaro, Michoacán, Mexico. Tropical Ecology **38:** 121–131.

SALGADO-MALDONADO, G. 2006. Checklist of helminth parasites of freshwater fishes from Mexico. Zootaxa **1324:** 1–357.

———, G. CABAÑAS-CARRANZA, E. SOTO-GALERA, J. M. CASPETA-MANDUJANO, G. MORENO-NAVARRETE, P. SÁNCHEZ-NAVA, R. AGUILAR-AGUILAR. 2001. A checklist of helminth parasites of freshwater fishes from the Lerma-Santiago River Basin, Mexico. Comparative Parasitology **68:** 204–218.

SÁNCHEZ-NAVA, P., G. SALGADO-MALDONADO, E. SOTO-GALERA, AND B. JAIMES-CRUZ. 2004. Helminth parasites of *Girardinichthys multiradiatus* (Pisces: Goodeidae) in the upper Lerma River sub-basin, Mexico. Parasitology Research **93:** 396–402.

VIDAL-MARTÍNEZ, V., L. AGUIRRE-MACEDO, T. SCHOLZ, D. GONZÁLEZ-SOLÍS, AND F. MENDOZA-FRANCO. 2001. Atlas of the helminth parasites of cichlid fish of Mexico. Academia, Praha, Czech Republic, 165 p.

WALTHER, B. A., AND J. L. MOORE. 2005. The concepts of bias, precision and accuracy, and their use in the performance of species richness estimators, with a literature review of estimator performance. Ecography **28:** 815–829.

———, AND S. MORAND. 1998. Comparative performance of species richness estimation methods. Parasitology **116:** 395–405.

ZELMER, D., AND G. W. ESCH. 1999. Robust estimation of parasite component community richness. Journal of Parasitology **85:** 592–594.

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Parasite-Induced Changes in Nitrogen Isotope Signatures of Host Tissues

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ABSTRACT: To estimate isotopic changes caused by trematode parasites within a host, we investigated changes in the carbon and nitrogen isotope ratios of the freshwater snail *Lymnaea stagnalis* infected by trematode larvae. We measured carbon and nitrogen stable isotopes within the foot, gonad, and hepatopancreas of both infected and uninfected snails. There was no significant difference in the $\delta^{13}C$ and $\delta^{15}N$ values of foot and gonad between infected and uninfected snails; thus, trematode parasite infections may not cause changes in snail diets. However, in the hepatopancreas, $\delta^{15}N$ values were significantly higher in infected than in uninfected snails. The ^{15}N enrichment in the hepatopancreas of infected snails is caused by the higher ^{15}N ratio in parasite tissues. Using an isotope-mixing model, we roughly estimated that the parasites in the hepatopancreas represented from 0.8 to 3.4% of the total snail biomass, including the shell.

Parasitic infections introduce additional demands on host resources, either through direct competition for energy (Coop and Holmes, 1996; Sorensen and Minchella, 1998) or by stimulating the host's immune system (Moret and Schmidt-Hempel, 2000). Recently, parasites have been shown to modify the feeding patterns of their intermediate hosts (e.g., Thompson, 1990; Miura et al., 2006). Hosts may attempt to compensate for the increased nutritional demands caused by parasites by increasing their foraging effort (Thompson, 1990).

Stable isotope ratios of carbon and nitrogen are increasingly being used to analyze the food sources of macroinvertebrates in various ecosystems (e.g., Vander Zanden and Vadeboncoeur, 2002; Doi et al., 2004, 2006). Stable isotope techniques can provide continuous measures of trophic positions that integrate the assimilation of energy or mass flow through all the various trophic pathways leading to an organism (McCutchan et al., 2003).

The freshwater snail *Lymnaea stagnalis* is an intermediate host for many species of trematode parasites (Yurlova, 2003). *Lymnaea stagnalis* is the first intermediate host for at least 15 trematode species that parasitize the hepatopancreas and the second intermediate host for 18 trem-

TABLE I. The dry weight of the total body, foot, gonad, and hepatopancreas from infected and uninfected *Lymnaea stagnalis* and the percentage of each tissue in the total dry weight of the snails (mean ± 1 SD, n = 10 infected and 10 uninfected snails). The *t*- and *P*-values are for *t*-tests comparing infected and uninfected snails.

	Dry weight (μg)	*t*	*P*	% of Total weight	*t*	*P*
Total body of snail with shell						
Uninfected	794 ± 116					
Infected	838 ± 118	−0.8	0.41			
Foot						
Uninfected	104 ± 26			10.0 ± 1.9		
Infected	88 ± 27	−1.3	0.20	9.0 ± 2.0	−1.1	0.28
Gonad						
Uninfected	59 ± 17			5.6 ± 1.5		
Infected	33 ± 10	−4.2	<0.001	3.3 ± 0.8	−4.2	<0.001
Hepatopancreas						
Uninfected	41 ± 11			3.9 ± 0.8		
Infected	58 ± 15	2.9	0.01	6.0 ± 1.6	4.0	<0.001

atode species that occur in other snail tissues, such as the mantle and foot (Sudarikov et al., 2002; Yurlova, 2003; Yurlova and Serbina, 2004; Yurlova et al., 2006).

We investigated changes in the isotope ratios of infected and uninfected *L. stagnalis* to determine whether parasites can affect the nature and concentration of isotopes. Changes in food sources of hosts because of infection have been reported for marine gastropods (Miura et al., 2006); the phenomenon was observed using stable isotopes. We hypothesized that (1) an infected snail will change food sources because of changes in feeding behavior and habitat, and (2) the isotope ratios in the tissues of infected snails will differ from those of uninfected snails. The confirmation of these hypotheses will provide key information for understanding host–parasite interactions because changes in host food sources and feeding habits because of parasites could affect survival and reproductive rates within a host population.

We studied the common freshwater snail *L. stagnalis* inhabiting the riverine portion of Lake Chany, Siberia, Russia. *Lymnaea stagnalis* is a dominant gastropod snail in western Siberia (Yurlova and Vodyanitskaya, 2005). Lake Chany is located in the Barabinskaya lowland of West Siberia, Russia (54°30′–55°09′N, 76°48′–78°12′E). The lake is located in a forest–steppe region at an altitude of 106 m above sea level. It is a shallow, inland, saline system (average depth, 2.2 m; maximum depth, 8.5 m; Aladin and Plotnikov, 1993; Doi et al., 2004). The study was conducted in the inflow part of the Kargat River (54°37.76′N, 78°13.07′E) of Lake Chany. The substrata at the site varied from detritus to sediment. Information regarding larval trematodes and their effects on the behavior, growth, fecundity, and population dynamics of their snail hosts in West Siberia has been reported previously (Yurlova, 1987, 2003, 2006; Yurlova et al., 2000, 2006).

Specimens of *L. stagnalis* and their parasites were sampled in August 2004. Snails were collected by hand at a depth of 0.1–0.5 m. We collected 20 *L. stagnalis* (10 infected and 10 uninfected). Under laboratory conditions, the snails were measured using a slide caliper (length of shell from apex to aperture), then dissected, and examined for the presence of trematode parasites using a microscope. We saved the foot, gonad, and hepatopancreas for isotope ratio analyses (n = 20). The tissues were first dried at 60 C and then stored at −20 C. Before analysis, the dry weight of each tissue was measured. We calculated the relative contribution of each tissue to the dry weight of the body. We used *t*-tests to examine the effect of infection on tissue dry weight.

Before isotope measurement, the lipids in all tissues were removed using a chloroform:methanol mixture (2:1 by weight) because of the high lipid content in muscle tissue (Kling, 1992). The isotopic ratios of carbon and nitrogen in the samples were measured with a mass spectrometer (Integra CN, SerCon Co., Cheshire, U.K.). The results are presented using common delta notation, calculated as: $\delta^{13}C$ or $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1{,}000$ (‰), where R is the ^{13}C:^{12}C or ^{15}N:^{14}N ratio for $\delta^{13}C$ or $\delta^{15}N$, respectively. Pee Dee Belemnite and atmospheric nitrogen were used as international standards for $\delta^{13}C$ and $\delta^{15}N$, respectively. The errors during the overall analyses were within ±0.2‰ for $\delta^{13}C$ and $\delta^{15}N$. The effects of infection on the carbon and nitrogen isotope values in the different tissues were determined using *t*-tests.

Four of 10 infected *L. stagnalis* had sporocysts of *Plagiorchis mutationis*; 6 of 10 snails were infected by sporocysts of *Plagiorchis* sp. larvae. We determined the total dry weight of each snail by calculating the dry weight and the percentage of total weight represented by each tissue (foot, gonad, and hepatopancreas) from infected and uninfected *L. stagnalis* (Table I). The shell lengths of uninfected and infected snails were 41.9 ± 0.43 and 42.5 ± 0.42 mm, respectively, and were not significantly different ($P = 0.90$). The dry weight and percentage of total weight of infected hepatopancreases were significantly higher than those of uninfected hepatopancreases ($P < 0.01$; Table I). However, the dry weight and percentage of the total weight of infected gonads were significantly lower than those of uninfected snails' gonads ($P < 0.001$; Table 1). The dry weight and percentage of total weight of foot tissue did not differ between infected and uninfected snails (Table I).

The $\delta^{15}N$ values from infected hepatopancreases were significantly higher than those of uninfected hepatopancreases ($P = 0.03$, Table II). However, there were no significant differences in $\delta^{15}N$ between infected and uninfected foot or gonad tissues, or in $\delta^{13}C$ values for foot, gonad, or hepatopancreas tissues ($P > 0.09$; Table II). Except for the $\delta^{15}N$ of the hepatopancreas, there were no significant effects of infection on the $\delta^{13}C$ and $\delta^{15}N$ values of the different tissues (Fig. 1). There were no significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values of tissues between snails infected with *Plagiorchis* sp. and *P. mutationis* ($P = 0.3$). It is possible that our small sample sizes limited our ability to detect differences between snails infected by different trematode species and between infected and uninfected snails. We tested 2 hypotheses: (1) the food sources of host snails are altered by trematode infection, and (2) the isotope ratios of infected tissues differ from uninfected tissues because of parasites. We did not observe the migration of cercariae through the tissues. Parasites can become significantly ^{15}N-enriched from the host because parasites feed mainly on host tissues (Boag et al., 1998; Doucett et al., 1999; Power and Klen, 2004; O'Grady and Dearing, 2006). Changes in host food sources because of infection have been reported for marine gastropods (Miura et al., 2006). Thus, parasites can modify the feeding patterns of snails that serve as their intermediate hosts (Levri, 1999; Levri and Fisher, 2000). However, the $\delta^{13}C$ and $\delta^{15}N$ values of foot and gonad tissues did not differ significantly between the infected and uninfected snails that we examined. Moreover, we did not find differences in the habitat use or feeding behavior of infected or uninfected snails in August. Also, we collected all snails (infected and uninfected) at the same sites and observed that they were moving in many directions. Thus, trematode infection may not have a strong effect on snail diet and may not affect the $\delta^{13}C$ and $\delta^{15}N$ values in snail tissues, expect within the hepatopancreas. An additional explanation for the observed foot and gonad isotope values is that significant isotope changes were not detected because the snails fed on various food sources

TABLE II. The $\delta^{13}C$ and $\delta^{15}N$ values of the foot, gonad, and hepatopancreas of infected and uninfected *Lymnaea stagnalis* (mean ± 1 SD, n = 10 infected and 10 uninfected snails). The *t*- and *P*-values are for *t*-tests comparing infected and uninfected snails.

	$\delta^{13}C$ (‰)	*t*	*P*	$\delta^{15}N$ (‰)	*t*	*P*
Foot						
Uninfected	−32.3 ± 1.6			3.4 ± 1.5		
Infected	−31.1 ± 0.9	−1.77	0.10	4.3 ± 1.7	−1.24	0.23
Gonad						
Uninfected	−32.2 ± 1.2			3.9 ± 1.5		
Infected	−31.5 ± 1.2	−1.36	0.19	3.9 ± 2.0	0.11	0.91
Hepatopancreas						
Uninfected	−31.6 ± 1.5			3.1 ± 1.5		
Infected	−32.3 ± 1.0	0.87	0.39	4.5 ± 1.1	−2.23	0.03

with various isotope ratios, such as sediment organic matter, benthic microalgae, and macroalgae.

In addressing our second hypothesis, the $\delta^{15}N$ values of hepatopancreases from infected snails were significantly higher than those of uninfected hepatopancreases. Moreover, the dry weight of infected hepatopancreases was significantly greater than that of uninfected hepatopancreases. Many generations of trematode asexual larval stages, i.e., sporocysts and/or rediae, develop and reproduce in the hepatopancreas of an infected snail. In most snail–trematode systems, the growth and reproduction of trematode larval sporocysts and rediae occur in resource-rich host tissues, such as the digestive gland and gonads (Kube et al., 2006). Thus, a possible explanation for the greater weight of infected hepatopancreases is the altered composition of tissues such as the increased storage of calcium granules. Also, the dry weight of infected gonads was significantly lower than that of uninfected gonads. Some trematode species possess larval stages that castrate their snail hosts, either chemically or directly (mechanical castration), when the larvae are located in the gonads of the host and cause complete destruction of the gonad (Wilson and Denison, 1980; Sluiters et al., 1984; De Jong-Brink, 1990; Probst and Kube, 1999). In infected snails, the hepatopancreas contains trematode parasite tissues.

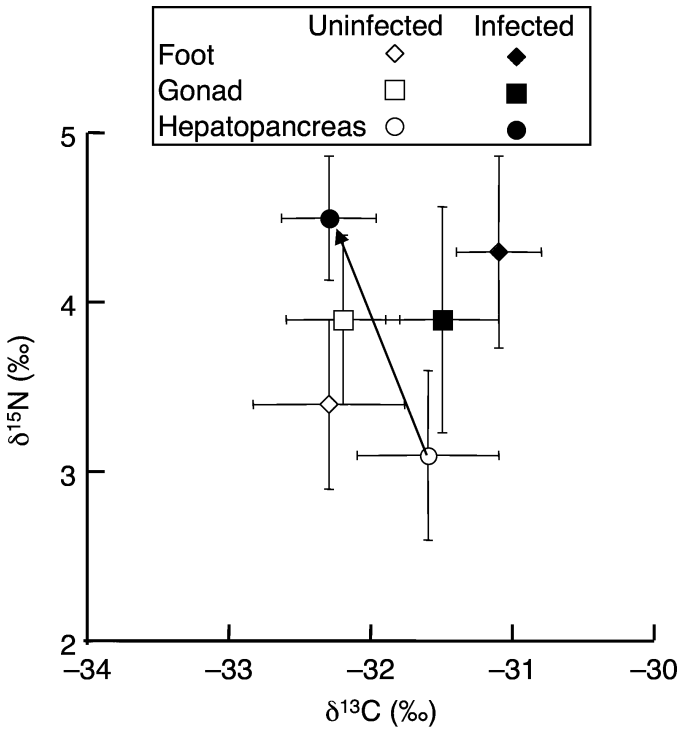

FIGURE 1. $\delta^{13}C$ and $\delta^{15}N$ values of the foot, gonad, and hepatopancreas of infected and uninfected *Lymnaea stagnalis* (mean ± 1 SE). Arrow indicates the significant changes in the $\delta^{13}C$ and $\delta^{15}N$ values of uninfected and infected tissues (*t*-test, $P = 0.03$).

We conclude that the trematode parasites caused the ^{15}N enrichment and increased the weight of infected hepatopancreases, even though Cheng et al. (1983) indicated that the soft tissues of infected and uninfected snails do not differ after trematode parasites are removed. Parasites such as cestodes and nematodes become significantly ^{15}N-enriched from their hosts because the parasites feed mainly on host tissues (Boag et al., 1998; Doucett et al., 1999; Power and Klen, 2004; O'Grady and Dearing 2006). Parasite ^{15}N-enrichment likely causes the $\delta^{15}N$ values of infected and uninfected hepatopancreases to differ, although we did not directly measure the $\delta^{15}N$ values of the trematode parasites.

We calculated the contribution of parasites to the isotope values of infected hepatopancreases using the mass–balance isotope-mixing model of Phillips (2001) with various isotope fractionations of parasites as follows:

$$\delta^{15}N_{\text{infected hepatopancreas}} = a(\delta^{15}N_{\text{uninfected hepatopancreas}} + \Delta^{15}N) + (1 - a)\delta^{15}N_{\text{uninfected hepatopancreas}},$$

where $\Delta^{15}N$ is the isotope fractionation of the cestode and nematode parasites, ranging from +1.0 to +4.0‰ (Boag et al., 1998; Doucett et al., 1999; Power and Klen, 2004; O'Grady and Dearing, 2006). Based on the isotope-mixing model with various isotope fractionations, the contribution of parasites to the isotope values of infected hepatopancreases ranged from 12.5 to 50.0%. Thus, the dry weight of the parasites was calculated using the assumed isotopic contribution from the parasites and the dry weight of the hepatopancreas. In this way, we roughly estimated that the dry weight of parasites in an infected hepatopancreas ranged from 7.3 to 29.0 μg. Parasites in the hepatopancreas thus accounted for between 0.8 and 3.4% of the total snail biomass. The high proportion of parasite biomass within the host probably causes remarkable changes in host reproduction and tissue weight. However, we acknowledge several caveats with this approach that affect its scope. Thus, the species composition of trematode parasites varies among individual snails, and the isotope estimation includes the contributions of many trematode species. In future studies, we plan to estimate and compare parasite biomass in various hosts and parasite species using stable isotopes.

We tested 2 hypotheses using stable isotopes of carbon and nitrogen in snail tissues. We showed that the food sources of snails are not changed by trematode infection, but the nitrogen isotope ratio differs between infected and uninfected hepatopancreases, probably because of the direct effect of parasite biomass. Using an isotope mixing model, we estimated the biomass of parasites in the snails. Our results provide an initial step for estimating the presence and biomass of parasites in host tissues using stable isotope techniques.

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LITERATURE CITED

ALADIN, N. V., AND I. S. PLOTNIKOV. 1993. Large saline lakes of former USSR: A summary review. Hydrobiologia **267:** 1–12.

BOAG, B., R. NEILSON, D. ROBINSON, C. M. SCRIMGEOUR, AND L. L. HANDLEY. 1998. Wild rabbit host and some parasites show trophic-level relationships for a first report. Isotopes in Environmental Health Studies **34:** 81–85.

CHENG, T. C., J. T. SULLIVAN, K. H. HOWLAND, T. F. JONES, AND H. J. MORAN. 1983. Studies on parasitic castration: Soft tissue and shell weights of *Llyanassa obsoleta* (Mollusca) parasitized by larval trematodes. Journal of Invertebrate Pathology **42:** 143–150.

COOP, R. L., AND P. H. HOLMES. 1996. Nutrition and parasite interactions. International Journal for Parasitology **26:** 951–962.

DE JONG-BRINK, M. 1990. How trematode parasites interfere with reproduction of their intermediate hosts, freshwater snails. Journal of Medical and Applied Malacology **2:** 101–133.

DOI, H., E. KIKUCHI, C. MIZOTA, N. SATOH, S. SHIKANO, N. YURLOVA, E. ZUYKOVA, AND E. YADRENKINA. 2004. Carbon, nitrogen, and sulfur isotope changes and hydro-geological processes in a saline lake chain. Hydrobiologia **529:** 225–235.

———, E. ZUYKOVA, E. KIKUCHI, S. SHIKANO, K. KANOU, N. YURLOVA, AND E. YADRENKINA. 2006. Spatial change in carbon and nitrogen stable isotopes of the plankton food web in a saline lake ecosystem. Hydrobiologia **571:** 395–400.

DOUCETT, R. R., D. J. GIBERSON, AND G. POWER. 1999. Parasitic association of *Nanocladius* (Diptera: Chironomidae) and *Pteronarcys biloba* (Plecoptera: Pteronarcyidae): Insights from stable-isotope analysis. Journal of the North American Benthological Society **18:** 514–523.

KLING, G. W., B. FRY, AND J. O'BRIEN. 1992. Stable isotopes and planktonic trophic structure in arctic lakes. Ecology **73:** 561–566.

KUBE, S., J. KUBE, AND A. BICK. 2006. A loss of fecundity in a population mudsnails *Hydrobia ventrosa* caused by larval trematodes does not measurably affect host population equilibrium level. Parasitology **132:** 725–732.

LEVRI, E. P. 1999. Parasite-induced change in host behavior of a freshwater snail: Parasitic manipulation or byproduct of infection. Behavioral Ecology **10:** 234–241.

———, AND L. M. FISHER. 2000. The effect of a trematode parasite (*Microphalus* sp.) on the response of the freshwater snail *Potamopyrgus antipodarum* to light and gravity. Behaviour **137:** 1141–1151.

MCCUTCHAN, J. H. JR., W. M. LEWIS, JR., C. KENDALL, AND C. C. MCGRATH. 2003. Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. Oikos **102:** 378–390.

MIURA, O., A. M. KURIS, M. E. TORCHIN, R. F. HECHINGER, AND S. CHIBA. 2006. Parasites alter host phenotype and may create a new ecological niche for snail hosts. Proceedings of the Royal Society B **273:** 1323–1328.

MORET, Y., AND P. SCHMIDT-HEMPEL. 2000. Survival for immunity: The price of immune system activation for bumblebee workers. Science **290:** 1166–1198.

O'GRADY, S. P., AND M. D. DEARING. 2006. Isotopic insight into host–endosymbiont relationships in Liolaemid lizards. Oecologia **150:** 355–361.

PHILLIPS, D. L. 2001. Mixing models in analyses of diet using multiple stable isotopes: A critique. Oecologia **127:** 166–170.

POWER, M., AND G. M. KLEIN. 2004. Fish host–cestode parasite stable isotope enrichment patterns in marine, estuarine and freshwater fishes from northern Canada. Isotopes in Environment and Health Studies **40:** 257–266.

PROBST, S., AND O. KUBE. 1999. Histopathological effects of larval trematode infections in mudsnails and their impact on host growth: What causes gigantism in *Hydrobia ventrosa* (Gastropoda: Prosobranchia). Journal of Experimental Marine Biology and Ecology **238:** 49–68.

SLUITERS, J. F., E. W. ROUBOS, AND J. JOOSSE. 1984. Increased activity of the female gonadotropic hormone producing dorsal bodies in *Lymnaea stagnalis* infected with *Trichobilharzia ocellata*. Zeitschrift fur Parasitenkunde **70:** 67–72.

SORENSEN, R. E., AND D. J. MINCHELLA. 1998. Parasite influences on host life history: *Echinostoma revolutum* parasitism of *Lymnaea elodes* snails. Oecologia **115:** 188–195.

SUDARIKOV, V. E., A. A. SHIGIN, Y. V. KUROCHKIN, V. V. LOMAKIN, P. P. STENKO, AND N. I. YURLOVA. 2002. Metacercariae of trematodes parasites of freshwater hydrobionts in Central Russia. Nauka, Moscow, Russia, 298 p.

THOMPSON, S. N. 1990. Physiological alterations during parasitism and their effects on host behaviour. *In* Parasitism and host behavior, C. J. Barnard and J. M. Behnke. (eds.) Taylor and Francis, London, U.K., p. 64–94

VANDER ZANDEN, M. J., AND Y. VADEBONCOEUR. 2002. Fishes as integrators of benthic and pelagic food webs in lakes. Ecology **83:** 2152–2161.

WILSON, R., AND J. DENISON. 1980. The parasitic castration and gigantism of *Lymnaea truncatula* infected with the larval stages of *Fasciola hepatica*. Zeitschrift fur Parasitenkunde **61:** 109–119.

YURLOVA, N. I. 1987. Morphology and biology of trematodes *Hypoderaeum cubanicum* comb. nov. and *H. conoideum* (Echinostomatidae). Parazitologiya **21:** 35–42.

———. 2003. The influence of trematodes infections on reproductive potential of *Lymnaea stagnalis* (Gastropoda: Pulmonata) field population. Zoological Journal **82:** 1027–1037.

———, AND E. A. SERBINA. 2004. New data on *Cyathocotyle bushiensis* (Trematoda: Cyathocotylidae). Parazitologia **38:** 191–205.

———, AND S. N. VODYANITSKAYA. 2005. Long-term changes in species composition and abundance of pulmonate snails (Gastropoda) in Lake Chany (south of west Siberia). Sibirskiy Ekologicheskiy Zhurnal **2:** 255–266.

———, ———, AND V. V. GLUPOV. 2000. Analysis of relationships in host–parasite systems (by the example of snails and trematodes). Progress of Modern Biology **120:** 573–580.

———, ———, E. A. SERBINA, V. Y. BISERKOV, B. B. GEORGIEV, AND N. H. CHIPEV. 2006. Temporal variation in prevalence and abundance of metacercariae in the pulmonate snail *Lymnaea stagnalis* in Chany Lake, West Siberia, Russia: Long-term patterns and environmental covariates. Journal of Parasitology **92:** 249–259.

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A Tick From a Prehistoric Arizona Coprolite

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ABSTRACT: Ticks have never been reported in archaeological analyses. Here, we present the discovery of a tick from a coprolite excavated from Antelope Cave in extreme northwest Arizona. Dietary analysis indicates that the coprolite has a human origin. This archaeological occupation is associated with the Ancestral Pueblo culture (Anasazi). This discovery supports previous hypotheses that ticks were a potential source of disease and that ectoparasites were eaten by ancient people.

Nearly 1,000 coprolites from the desert west of the United States have been analyzed for parasite remains (Reinhard, 1990, 1992). Although arthropod parasites are rare, they are occasionally found in coprolites. The discovery of lice in human coprolites led Fry (1977) to conclude that arthropods were consumed to control infestation. In 2005, we commenced the dietary and parasitological analysis of coprolites from Antelope Cave in the northwestern corner of Arizona. At this site, we discovered a tick from a coprolite. This discovery has health, behavioral, and ecological implications for the Puebloan people that once occupied the cave.

Antelope Cave is a large limestone cavern sunk into the gently rolling terrain of the Uinkaret Plateau some 40 km southeast of St. George, Utah. Prehistoric Native Americans occupied it, probably intermittently, for at least 3,000 yr (2028 B.C. to A.D. 1100). The most intense habitation of the cave is attributed to Ancestral Puebloan peoples (Anasazi) who lived there 1,300 to 1,000 yr ago. Antelope Cave lies within the Virgin River Branch of prehistoric western Anasazi territory, and the great majority of artifacts (for example, woven fiber sandals, plaited basketry, Virgin series pottery) in the cave reflects Puebloan (Kayenta) affiliation. There is scant evidence of Fremont cultural influence from the north in Utah. Cultural debris left in the cave by its prehistoric inhabitants forms a 1.52-m-thick layer and contains mostly perishable artifacts, including wooden arrow shafts, basketry, string, netting, sandals, needles and thread, etc., as well as painted pottery and various lithic tools. The Pueblo people used the cave for shelter, and in the surrounding area, they grew corn and beans, gathered wild plant foods, and hunted game, mostly rabbits.

Professional archaeologists have conducted excavations in the cave, off and on, since 1954 (Janetski and Hall, 1983; Janetski and Wilde, 1989). The most extensive excavations were undertaken by the University of California–Los Angeles (UCLA) in 1959–1960 (Johnson and Pendergast, 1960). The coprolite specimen discussed here was recovered by UCLA and came from 1,000-yr-old Pueblo deposits at the rear of the cave. The date is based on cross-dated Anasazi artifacts pending C14 assay.

In 1959, archaeologists from UCLA excavated five 2 × 2 m pits into the midden deposit of Antelope Cave. The excavation units were designated AC59-1 through AC59-5. The coprolite of concern here was recovered from the 60–76-cm level below the surface in pit AC59-2. Along with the coprolite, this level yielded a wide variety of cultural debris, including fragments of Pueblo pointed-toe sandals, sandal ties, a net bag, fiber cordage, feather cordage, and pottery. No features, such as fire hearths, storage pits, latrines, etc., were exposed in this or any other level.

Eight Antelope Cave coprolites have been analyzed to date. Five are consistent with humans and 3 are consistent with canids, probably dogs. Laboratory sample 2 is the focus of this report. Its field context is AC 1516, pit AC 59-2, 60–76 cm below surface. After contextual information was recorded, the coprolite was cleaned of extraneous dirt, photographed, and weighed. Its weight, 2.67 g was then recorded. Observations relative to biological origin were made. The coprolite was then rehydrated in 0.5% trisodium phosphate for 48 hr. It was placed in a 300-ml beaker, and rehydration solution was added until the coprolite was completely immersed. Parafilm was used to cover the beaker to prevent potential modern airborne pollen contamination. Observations were made after 24 hr of rehydration. Rehydration fluid color is sometimes useful in verifying human origin (Reinhard and Bryant, 1992). Human coprolites tend to turn the rehydration solution dark brown or black, although this is not always the case. In addition, the rehydrating coprolite was examined for a mucilage coat, which sometimes forms on dog coprolites after rehydration (Reinhard et al., 1988).

After 48 hr of rehydration, 3 *Lycopodium* sp. spore tablets were added to the coprolite to facilitate quantification (Warnock and Reinhard, 1994; Sianto et al., 2005). For this analysis, *Lycopodium* sp. spore batch 212761 was used. Previous analysis has shown that approximately 12,500 spores are present in each tablet (values presented from different analyses of tablets are 12,432, 12,489, and 12,542). The tablets were dissolved in a few drops of hydrochloric acid and added to the rehydrated coprolite.

The coprolite was then disaggregated. It was transferred to a 600-ml beaker along with the rehydration solution and dissolved *Lycopodium* sp. tablets. A magnetic stir rod was added to the beaker, which was placed on a stir plate. The coprolite in the solution was then stirred for 45 min until it was completely disaggregated.

Microscopic remains were separated from macroscopic remains by pouring the disaggregated coprolite through a 300-μm mesh screen. A stream of distilled water under pressure was used to thoroughly wash the microscopic remains through the screen and into a 600-ml beaker. The macroscopic remains on top of the screen were dried on cotton filter paper. The microscopic remains were sedimented by centrifugation in 50-ml tubes.

The microscopic remains were then analyzed for parasite eggs and microscopic dietary evidence such as plant cells, phytoliths, and starch grains. Nine microscope preparations were made for helminth eggs or protozoan cysts. The dietary residues were categorized and counted. Next, using the following formula, the numbers of each category of dietary residue per gram of coprolite were calculated: concentration = $([i/m] \times n)/w$, where i is items counted, m is marker *Lycopodium* spores counted, n is marker *Lycopodium* spores added, and w is weight of coprolite in grams.

Subsequent to this analysis, about 10 ml of sediment were processed in hydrofluoric acid and acetolysis solution for pollen following the methods of Reinhard et al. (2006). Pollen grains were counted, and the numbers of pollen grains per gram were calculated using the above formula. When the analysis of microscopic remains was completed, the dried macroscopic remains were examined for food residues and arthropods.

One fragment of a tick was found. Images of the tick were made with a Syncroscopy Auto-Montage digital microscope system at the University of Nebraska State Museum Biodiversity Synthesis Laboratory, Lincoln, Nebraska. This system eliminates depth of field limitation problems by automatically capturing the in-focus regions from a range of focal planes and combining them into a single, fully focused, high-resolution image. Adobe Photoshop was used to reconstruct the complete view of the dorsal posterior of the tick so that festoons could be more accurately counted.

The specimen was not cleared or mounted. We are saving the specimen for future study, including molecular analysis. Therefore, we felt that it was best to preserve the specimen with no further chemical treat-

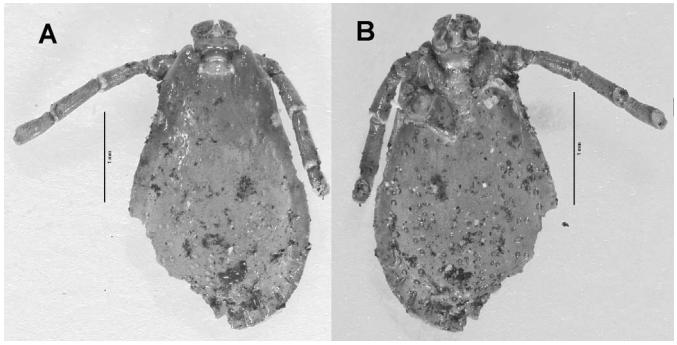

FIGURE 1. Dorsal (**A**) and ventral (**B**) views of the whole specimen. A dorsal scutum is evident. However, the posterior ventral portion of the tick was fractured away and was not discovered in extensive examination of the coprolite residues.

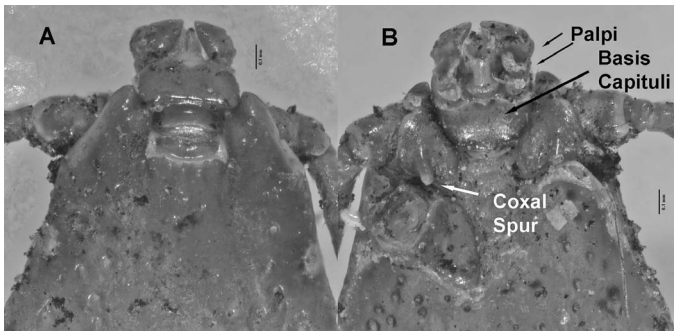

FIGURE 2. In this close-up of the dorsal (**A**) and ventral (**B**) aspects of the tick, one can see the capitulum from both sides. The palpi are short, about as long as the basis capituli. Also, a coxal spur is visible.

ment. A temporary mount was used for microscopy that did not utilize adhesive.

Coprolite Lab 2, FS 1516, is most likely human in origin. Its initial appearance was consistent with human feces, it had a dark rehydration color, and there was no evidence of the mucilage that we often observed in dog coprolites (Reinhard et al., 1988). Moreover, there were no dog hairs in either the macroscopic or the microscopic remains. In contrast, the 3 dog coprolites analyzed so far from the site contained abundant animal hair, lizard bones and scales, and oddities such as fragments of rabbit hide with attached fur. The dogs appear to have hunted small animals, including horned lizards, and supplemented their diet by eating human feces and trash in the cave.

The macroscopic dietary residues from Lab 2, FS 1516, are derived mostly from ground maize, ground sunflower, unknown plant epidermis and fiber, bone, and possibly a female flower from cottonwood. Grass stem/epidermis fragments and maize starch dominate microfossils. For example, 34,300 maize starch grains, 18,724 Poaceae (grass family) cells or phytoliths, and 17,164 vascular bundle fragments were found per gram of coprolite. The pollen has some cottonwood-type grains, but we are uncertain of this identification because fossil cottonwood pollen can resemble many other spores and pollen from other taxa.

A well-preserved tick was found in the microscopic remains (Figs. 1–3). Examination of the tick showed that it was a species of *Dermacentor*. This diagnosis is based on number and size of the remaining festoons, as well as on the fact that the capitulum is visible from dorsal and ventral aspects, a dorsal scutum is present, and the palpi are short (about as long as the basis capituli). Originally, the tick had 11 festoons. Unfortunately, the spiracular plates are not present on this fragmentary specimen, so species-level diagnosis is not possible. However, the basis capituli have short cornua, which is consistent with *Dermacentor andersoni* (Stiles). The white dorsal markings typical of this species are not evident. This is probably a result of passage through a digestive tract.

The incomplete nature if the specimen leaves some doubt as to the identification of species and developmental stage. We believe the tick is an adult or nymph of *D. andersoni* (Stiles). It is 3.4 mm long and 2.0 mm wide. This is in the range for adults and nymphs. The reconstruction of the tick (Fig. 3) shows that it originally had 11 festoons. The ventral surface is fractured posterior to the first left coxa and second right coxa. The outline of a broken-off third coxa is visible, but it is impossible to ascertain if there was a fourth. Three coxa would be consistent with a larva, and 4 would be consistent with a nymph or adult. There are no visible spiracles. If this was an adult, the spiracle plates had to have been present just posterior the fourth coxa. Larvae have no spiracles. We believe that spiracles have been simply fractured away from the specimen since the region of the fourth coxa is missing. To support our identification, there is a distinct spur on the first coxa that is characteristic for larvae and nymphs of *D. variabilis* and *D. andersoni*. Relative to the three-legged larval stage, the general morphology of the scutum is consistent with an adult or nymphal male. The larval scutum is not as elongate as adults and nymphs. We cannot determine whether *D. variabilis* or *D. andersoni* is represented by this specimen. The key anatomical elements, i.e., spiracular plate goblets, are not present. *Dermacentor variabilis* is not present in the Rocky Mountain region and is not endemic to Utah due to climate (Longstreth and Wiseman, 1989). However, Antelope Cave is within the southernmost range of *D. andersoni*. Therefore, in all likelihood, the tick discovered at Antelope Cave is an adult or nymph *D. andersoni*.

From the perspective of the function of a tick as a disease vector, specific identification matters. Tickborne diseases have been suggested as potential health threats for Ancestral Pueblo people (Stodder and Martin, 1992, p. 62). The discovery of *Dermacentor* sp. at Antelope Cave is the first empirical evidence that ticks and humans were in contact. Which diseases were potential threats for the Ancestral Puebloans of Antelope Cave? The fact that we found a *Dermacentor* sp. tick limits the number of disease possibilities (Roberts and Janovy, 2000; Bowman, 2003; PAHO, 2003). Lyme disease is transmitted by *Ixodes* spp. and *Amblyomma* spp. ticks. Ehrlichiosis is transmitted by ticks of 2 genera, i.e., *Ixodes* and *Amblyomma*. Tickborne relapsing fever is transmitted by *Ixodes* spp. and *Ornithodoros* spp. ticks. These diseases probably were not potentialities for Antelope Cave.

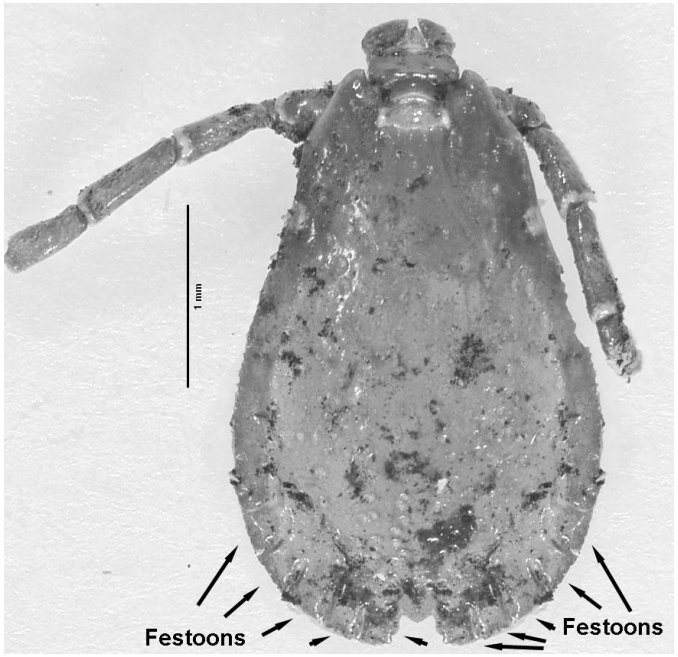

FIGURE 3. Adobe Photoshop reconstruction of the dorsal aspect of the tick. This was made by copying, reversing, and inserting the right lateral edge of the tick over the missing region on the left side. Note that 10 festoons are reconstructed. The posterior-most festoon is lost and could not be inserted in this reconstruction.

Rocky Mountain spotted fever is spread to humans by *Dermacentor* sp. ticks. Tularemia is caused by the bacterium *Francisella tularensis*, which is transmitted by species of *Amblyomma* and *Dermacentor*. Therefore, these 2 infectious diseases could have been transferred by ticks at Antelope Cave. Tick paralysis is a condition that results from neurotoxins secreted by ticks during their feeding process, so tick paralysis or serious skin reactions such as dermatosis, inflammation, swelling, ulceration, and itching are possible.

The question might be raised as to whether this tick was accidentally consumed inadvertently in grain or some other food product. The maize and sunflower found in the coprolite was thoroughly ground. Had the tick been a contaminant of the grain, it would have been ground into powder. It is not likely that ticks would have been in the cave, since *D. andersoni* feeds on small animals in brushy areas. The human coprolites from Antelope Cave contain bones of *Sylvilagus* sp. Rabbits, and the trash deposits contained bones from a variety of small vertebrates. Thus, humans came into contact with the ticks when they hunted small mammals. The fact that this *Dermacentor* sp. was found in an apparent human coprolite and the finding that it was partially crushed indicate that the tick was pinched between the teeth and swallowed. This action reflects a response on the part of one ancient person who chose to remove the tick, bite it, and swallow it, thus suggesting a prehistoric behavior pattern of eliminating arthropod pests by eating them.

We are currently examining more human and dog coprolites from Antelope Cave and anticipate reconstructing the parasite ecology of this site.

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LITERATURE CITED

BOWMAN, D. D. 2003. Georgis' parasitology for veterinarians, 8th ed. Saunders, St. Louis, Missouri, 426 p.

FRY, G. F. 1977. Analysis of prehistoric coprolites from Utah. University of Utah Press, Salt Lake City, Utah, 45 p.

JANETSKI, J., AND M. HALL. 1983. An archaeological and geological assessment of Antelope Cave (NA5507), Mohave County, Northwestern Arizona. Brigham Young University, Department of Anthropology, Technical Series No. 83-73, Provo, Utah, 123 p.

———, AND J. D. WILDE. 1989. A preliminary report of archaeological excavations at Antelope Cave and Rock Canyon Shelter, Northwestern Arizona. Utah Archaeology **2:** 88–106.

JOHNSON, K. L., AND D. M. PENDERGAST. 1983. Archaeological exploration of Antelope Cave, Arizona. *In* An archaeological and geological assessment of Antelope Cave (NA5507), Mohave County, Northwestern Arizona, J. Janetski and M. Hall (eds.). Brigham Young University, Department of Anthropology, Technical Series No. 83-73, Appendix 4, Provo, Utah, p. 71–77.

LONGSTRETH, J. D., AND J. WISEMAN. 1989. The potential impact of climate change on patterns of infectious disease in the United States. *In* The potential effects of global climate change on the United States: Appendix G—Health, J. B. Smith and D. A. Tirpak (eds.). Office of Policy, Planning, and Evaluation, U.S. Environmental Protection Agency, Washington, D.C., p. 3-1–3-41.

PAHO (PAN AMERICAN HEALTH ORGANIZATION). 2003. Zoonoses and communicable diseases common to man and animals, 3rd ed. Parasitoses. Pan American Health Organization, Washington, D.C., 424 p.

REINHARD, K. J. 1990. Archaeoparasitology in North America. American Journal of Physical Anthropology **82:** 145–162.

———. 1992. Patterns of diet, parasitism, and anemia in prehistoric west North America. *In* Diet, demography, and disease: Changing perspectives on anemia, P. Stuart-Macadam, and S. Kent (eds.). Aldine de Gruyter, New York, p. 219–258.

———, AND V. M. BRYANT, JR. 1992. Coprolite analysis: A biological perspective on archaeology. *In* Advances in archaeological method and theory 4, M. B. Schiffer (ed.). University of Arizona Press, Tucson, Arizona, p. 245–288.

———, U. CONFALONIERI, B. HERRMANN, L. F. FERRERA, AND A. ARAÚJO. 1988. Recovery of parasite remains from coprolites and latrines: Aspects of paleoparasitological techniques. Homo **37:** 217–239.

———, T. DAMON, S. K. EDWARDS, AND D. K. MEIER. 2006. Pollen concentration analysis of Ancestral Pueblo dietary variation. Palaeogeography, Palaeoclimatology, and Palaeoecology **237:** 92–109.

ROBERTS, L. S., AND J. JANOVY, JR. 2000. Foundations of parasitology, 6th ed. McGraw-Hill, Boston, Massachusetts, 670 p.

SIANTO, L., K. J. REINHARD, M. L. C. GONÇALVES, AND A. ARAÚJO. 2005. The finding of *Echinostoma* (Trematoda: Digenea) and hookworm eggs in coprolites collected from a Brazilian mummified body dated of 600–1,200 years before present. Journal of Parasitology **91:** 972–975.

STODDER, A. L. W., AND D. L. MARTIN. 1992. Health and disease in the Southwest before and after Spanish contact. *In* Disease and demography in the Americas, J. W. Verano, and D. H. Ubelaker (eds.). Smithsonian Institution Press, Washington, D.C., p. 55–73.

WARNOCK, P., AND K. J. REINHARD. 1994. Methods of extracting pollen and parasite eggs from latrine soils. Journal of Archaeological Science **19:** 261–264.

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Observations on *Cryptosporidium* Life Cycle Stages During Excystation

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ABSTRACT: *Cryptosporidium parvum* (HNJ-1 strain, genotype 2) merozoites were released from oocysts directly during an incubation and excystation procedure without bleach treatment. They were polymorphic, mostly spindle-shaped; others were bean shaped, actively motile, and underwent division. Merozoites survived for short time–period in an in vitro culture system, but could not be established in a subsequent cultivation effort in RPMI medium.

Many vertebrates, including humans, are hosts to the intestinal protozoan parasite, *Cryptosporidium parvum*. Species in this genus have a worldwide distribution and in the last 3 decades have become an increasingly important public health problem. *Cryptosporidium parvum* has been reported as the causative agent of a number of waterborne outbreaks of diarrheal disease and is considered one of the most important contaminants of drinking water (Karanis et al., 2007). The general life cycle and biology of *C. parvum* are comprised of an exogenous stage (oocysts with 4 sporozoites) and an endogenous phase (trophozoites, merozoites, and sexual stages) as extensively described in Fayer et al. (1997). Oocysts of *Cryptosporidium* spp. can be exposed to different media to produce sporozoite excystation. In vitro techniques for sporozoite excystation from oocysts have been reported by several investigators (Upton, 1997). Exposure to an acid pH during in vitro excystation protocols for *Cryptosporidium* spp. mimic host–derived signals, but some of these host–derived triggers seem to be unessential. There is still an insufficient understanding of the hierarchy or synergism of specific

TABLE I. Size and shape of *Cryptosporidium* (HNJ-1 strain) developmental stages described.

	Oocysts of HNJ-1 (one month olds)	
	Sporozoites	Merozoites
Form and properties	Banana shape (''typical sporozoites'')	Pleomorph (a: bean shape, b: short spindle shape, c: long spindle shape)
Average sizes* (length × width) in μm	5.87–6.58 × 0.88–1.20	(a) 2.56–3.85 × 1.27–1.67 (b) 2.71–3.71 × 0.95–1.14 (c) 4.25–5.98 × 1.20–1.98

* For the mean sizes at least 10 single measures were plotted.

triggers because of lack of standardized methods (Smith et al., 2005). Completion of the *C. parvum* life cycle without the need for host cells (Hijjawi et al., 2004) and the discovery of novel extracellular stages in the life cycle of *C. parvum* (Rosales et al., 2005) supported the hypothesis that the organism is not an obligate intracellular parasite. However, recent attempts (Girouard et al., 2006) to propagate *Cryptosporidium* spp. in vitro failed, and the authors concluded that the in vitro propagation of *Cryptosporidium* spp. is not a universal phenomenon.

The aim of the present study is to report observations on *C. parvum* life cycle stages, which could be of interest for the in vitro developmental biology of this protozoon. Oocysts of *C. parvum* (HNJ-1 strain, genotype 2) are potentially infective for humans and other animals such as cattle (Satoh et al., 2005). This isolate was purified from an adult woman in Japan, and has been largely maintained in SCID mice and used in various laboratories in Germany and in Japan. During our observations, oocyst aliquots from the investigated stocks used for DNA extraction were genetically analyzed, identified by nested PCR and PCR-RFLP assays, and confirmed as *C. parvum*, genotype 2 (data not shown). The oocysts were purified by a routine procedure (Kimura et al., 2000; Karanis and Kimura, 2002), cleaned by an immuno–magnetic separation system, and stored at 4 C in Eppendorf tubes in water containing antibiotics (penicillin and streptomycin). Observations during the present study were made using confocal laser scanning microscopy. Oocysts were not pretreated with bleach for decontamination. During excystation, 1×10^6, 1-mo-old oocysts were excysted in a freshly prepared, filter-sterilized medium composed of acidic H_2O containing 0.50% trypsin and 0.75% taurocholate, using a 0.20-μm filter and incubated at 37 C for 40 min. Trials have been performed in Eppendorf tubes in a 0.5 ml suspension. After 20 min incubation, a small drop (2 μl) was resuspended on cover slips and examined microscopically. The suspension was spun in an Eppendorf centrifuge for 5 min at room temperature. A small drop taken from the bottom of the tube containing the parasites was re-suspended on cover slips and examined microscopically. To promote further development in vitro, equal portions of the remaining suspension was added to previously prepared culture flasks containing 40 ml of maintenance RPMI medium according to Hijjawi et al. (2004). Using light microscopy, we observed excysted and unexcysted oocysts (Fig. 1a) with banana-shaped sporozoites inside (Fig. 1b) of the oocysts, as well as a considerable number of banana-shaped sporozoites outside of the oocysts (Fig. 1c, d, e). Merozoites, observed at the same time, were plentiful, active, and pleiomorphic (Fig. 1e–j); they variously measured (Table I) 3.12 μm × 1.47 μm (bean-shaped), 5.07 μm × 1.63 μm (long spindle-shaped), and 3.04 μm × 1.05 μm (short spindle-shaped). In addition, a few spherical stages, approximately 3.5–4 μm in diameter, were likewise seen; these forms were round and smaller than oocysts (not shown). The appearance of these stages was mostly similar to macrogamonts. In vitro propagation in monophasic and biphasic RPMI 1640 maintenance medium according to Hijjawi et al. (2004) was only possible for a limited duration of approximately 48 hr; efforts failed to establish a long–term axenic in vitro culture system using 50-ml culture flasks. Using oocysts pretreated with bleach may kill the very sensitive merozoites, which we report here. In accompanying work (not reported here), we have not observed the same stages when oocysts were pretreated with bleach. Most investigators of *Cryp-*

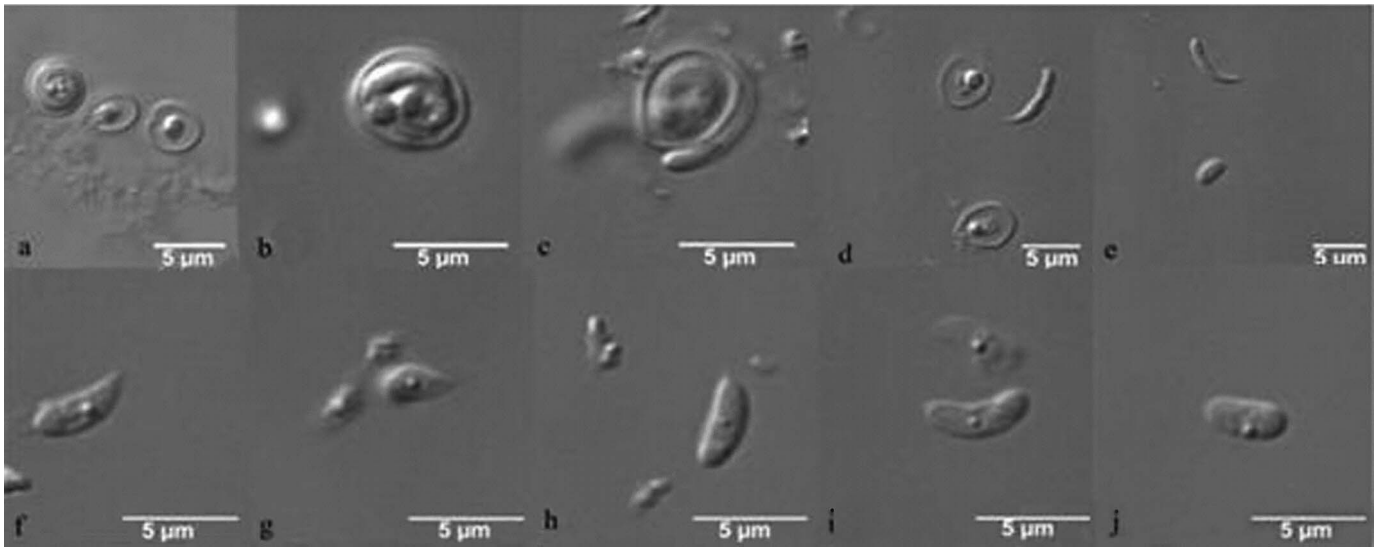

FIGURE 1. *Cryptosporidium* HNJ-1 developmental stages and observations during excystation observed by CLSM. Images of different stages after incubation in excystation solution with all of the mentioned forms: (**a**) 1 unexcysted oocyst, and 2 empty oocysts with a residual body inside; (**b**) oocyst with sporozoites inside; (**c**) oocyst with a sporozoites outside attached the outer part of the oocyst wall; (**d**) oocyst wall with residual body and banana-shaped sporozoite; (**e**) sporozoite and merozoite. Merozoite images: different merozoite images; long spindle shaped (**f**); short spindle shaped (**g**) and bean shaped (**h, i, j**); a prominent nucleus is visible in all stages.

tosporidium spp. biology during in vitro studies treat purified oocysts with sodium hypochlorite in order to prevent microbial contamination. Bleach treatment triggers excystation or may enhance overall excystation rates and provide additional sanitization of the oocyst sample. However, Arrowood (2002) did not find bleach necessary when performing disinfection or survival studies. Moreover, we cannot exclude the possibility that this sort of in vitro emergence and development of *Cryptosporidium* sp. life cycle stages is strain-dependent. In addition, this organism may need several media manipulations to become adapted to axenic culture conditions, e.g., RPMI 1640 medium, mono- or biphasic growth media, or other media. Media manipulation may ultimately enable the development, propagation, and establishment of additional *Cryptosporidium* species in axenic in vitro cultures. Despite the impressive increases in the understanding of this parasite, much more remains to be explored on *Cryptosporidium* spp. evolution, life cycle, and development. We expect that the new findings will contribute to a better understanding of the life cycle of *Cryptosporidium* spp. and of various aspects of its developmental biology.

Some authors have suggested that classification of *Cryptosporidium* species needs to be reconsidered. The uncertain taxonomic position of *Cryptosporidium* species is based on the recent description of new developmental stages in the life cycle, which have characteristics similar to those of gregarines (Carreno et al., 1999; Zhu et al., 2000; Xu et al., 2004; Rosales et al., 2005). Our findings indicate a natural pressure on *Cryptosporidium* spp. for a development outside of its host's cells. Perhaps the endogenous development in the host intestine is not obligate for this parasite to complete its life cycle. Sequence polymorphism for the HNJ-1 strain reflects the extent of subpopulation diversity within the genotype (Satoh et al., 2005). Whether or not the genetic polymorphism of this strain also reflects some biochemical and/or physical properties, and if these findings can be extended to other strains remains to be demonstrated. The possibility of the exogenous in vitro development will offer more possibilities for *Cryptosporidium* spp. research. Data regarding the *Cryptosporidium* spp. genome and on nucleotide biosynthesis will provide significant insight on genetics, physiology, and metabolism for the group (Striepen et al., 2004; Xu et al., 2004). We suggest that future research using in vitro models and the broad array of molecular details should proceed in parallel with detailed work on morphology of the parasite to provide additional insight to the developmental biology of *Cryptosporidium* species. The success of axenic in vitro culture for mass production of the parasite will make the use of experimental animal models unnecessary. It should also be adaptable to drug sensitivity assays and vaccination studies.

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LITERATURE CITED

ARROWOOD, M. J. 2002. In vitro cultivation of *Cryptosporidium* species. Clinical Microbiology Reviews **15:** 390–400.

CARRENO, R. A., D. S. MARTIN, AND J. N. BARTA. 1999. *Cryptosporidium* is more closely related to the gregarines than to coccidian as shown by phylogenetic analysis of apicomplexan parasites inferred using small–subunit ribosomal RNA gene sequences. Parasitology Research **85:** 899–904.

FAYER, R., C. A. SPEER, AND J. P. DUBEY. 1997. The general biology of *Cryptosporidium*. *In Cryptosporidium* and cryptosporidiosis, R. Fayer (ed.). CRC Press, Boca Raton, Florida, p. 1–41.

GIROUARD, D., J. GALLANT, D. E. AKIYOSHI, J. NUNNARI, AND S. TZIPORI. 2006. Failure to propagate *Cryptosporidium* spp. in cell free culture. Journal of Parasitology **92:** 399–400.

HIJJAWI, N. S., B. P. MELONI, M. NG'ANZO, U. M. RYAN, M. E. OLSON, P. T. COX, P. T. MONIS, AND R. C. A. THOMPSON. 2004. Complete development of *Cryptosporidium parvum* in host cell-free culture. International Journal for Parasitology **34:** 769–777.

KARANIS, P., AND A. KIMURA. 2002. Evaluation of three flocculation methods for the purification of *Cryptosporidium parvum* oocysts from water samples. Letters Applied Microbiology **34:** 444–449.

———, C. KOURENTI, AND H. V. SMITH. 2007. Waterborne transmission of protozoan parasites: A review of world-wide outbreaks and lessons we learnt. Journal of Water Health **5:** 1–38.

KIMURA, A., P. KARANIS, E. MALTEZOS, AND H. M. SEITZ. 2000. Bench scale experiments to evaluate the usefulness of sucrose flotation techniques for separation of *Cryptosporidium* oocysts from water. Journal Protozoology Research **10:** 155–165.

ROSALES, M. J., G. P. GORDON, M. S. MORENO, C. M. SANCHEZ, AND C. MASCARO. 2005. Extra cellular like-gregarines stages of *Cryptosporidium parvum*. Acta Tropica **95:** 74–78.

SATOH, M., I. KIMATA, M. ISEKI, AND Y. NAKAI. 2005. Gene analysis of *Cryptosporidium parvum* HNJ-1 strain isolated in Japan. Parasitology Research **97:** 452–457.

SMITH, H. V., A. B. NICHOLS, AND A. M. GRIMASON. 2005. *Cryptosporidium* excystation and invasion: Getting to the guts of the matter. Trends Parasitology **21:** 133–142.

STRIEPEN, B., A. J. PRUIJSSERS, J. HUANG, C. LI, M. J. GUBBELS, N. N. UMEJIEGO, L. HEDSTROM, AND J. C. KISSINGER. 2004. Gene transfer in the evolution of parasite nucleotide biosynthesis. Proceedings of the National Academy of Sciences USA **101:** 3155–3159.

UPTON, S. 1997. In vitro cultivation. *In Cryptosporidium* and cryptosporidiosis, R. Fayer, (ed.). CRC Press, Boca Raton, Florida, p. 181–201.

XU, P., G. WIDMER, Y. WANG, L. S. OZAKI, J. M. ALVES, M. G. SERRANO, D. PUIU, P. MANQUÉ, D. AKIYOSHI, A. J. MACKEY ET AL. 2004. The genome of *Cryptosporidium hominis*. Nature **431:** 1107–1112.

ZHU, G., J. S. KEITHLY, AND H. PHILIPPE. 2000. What is the phylogenetic position of *Cryptosporidium*? International Journal of Systematic and Evolutionary Microbiology **50:** 1673–1681.

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Chinese Liver Flukes in Latrine Sediments From Wong Nim's Property, San Bernardino, California: Archaeoparasitology of the Caltrans District Headquarters

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ABSTRACT: Parasitological analysis of 5 sediment samples from San Bernardino, California latrine deposits spanning the time period from about 1880 to the 1930s are presented. Two sediment samples are from a latrine used by European-Americans. Three sediment samples are from latrines used by Chinese-Americans on the property of Wong Nim, an important member of the Chinese community. Two of the Chinese latrines were positive for human parasites. The human parasites encountered include the human whipworm (*Trichuris trichiura*), the giant intestinal roundworm (*Ascaris lubricoides*, c.f.), and the Chinese liver fluke (*Clonorchis sinensis*). Evidence of the liver fluke is especially important. This parasite cannot complete its life cycle outside of its endemic range in Asia because suitable intermediate hosts are not present in the American continents. Its presence signals that at least some of the Chinese-Americans who used the latrines were immigrants who were infected in Asia and then sustained infections while in the Americas.

Parasitological analysis of archaeological sediments can provide insights into human transhumance (Ferreira et al., 1984; Araújo et al., 1988; Reinhard et al., 1987; Reinhard, 1992; Matsui et al., 2003). The latter authors (Matsui et al., 2003) presented evidence of diplomatic legations in Japan that were parasitized with nonendemic species. Hevly et al. (1979) and Reinhard et al. (1987) reported finding *Trichuris trichiura* in a nonendemic region near modern Flagstaff, Arizona. Ferreira et al. (1984) discovered *Diphyllobothrium pacificum* eggs in coprolites recovered from a Chilean inland site, and this indicates that the prehistoric people who deposited the coprolites used both the coast and inland areas. Araújo et al. (1988) and Ferreira and Araújo (1996) used hookworm evidence to trace prehistoric long-distance migrations. Here, we present evidence of transcontinental introduction of the Chinese liver fluke to California with historic migrations. This study confirms an earlier, unpublished report of Chinese liver flukes from a historic Chinese community in Sacramento, California (Hall, 1982).

Chinese populations moved into San Bernardino in 1867 (Costello and Hallaran, 2004; Costello et al., 2006). By 1880, the countywide Chinese population was about 150. Initially, they lived in various places throughout the town of San Bernardino. They farmed, operated laundries, worked in restaurants and hotels, and were employed as domestic servants or farm laborers. In 1878, the city prohibited laundries within the town limits, and, subsequently, a Chinese quarter was established. By the turn of the twentieth century, as many as 600 Chinese lived in Chinatown. Initially, Chinatown was virtually all male. It was composed of shops, boarding houses, gambling parlors, a temple, labor contractors, and other establishments. By 1893, Chinatown had electricity, and it had piped water by 1900. Human waste disposal was managed by construction of backyard latrines.

Three artifact-filled latrines were discovered during excavations. They were located on property purchased by California-born Wong Nim in 1900. Wong Nim was born in Alameda County, California, and moved to San Bernardino about 1875. He was successful. He first worked as a laundryman but eventually opened a mercantile shop and acted as a labor contractor. He also opened a temple. Wong remained on the corner of Third and B Streets until his death at age 89 in 1941. At that time, he had earned the honorary title of ''Mayor of Chinatown.'' When the State of California purchased Wong Nim's property in 1943, all of the remaining buildings on his property were demolished. At least 1 latrine (number 1035) was filled at this time with debris from the abandoned buildings.

The privies were used by people who built residences and businesses on Wong's original property. However, Wong's house, store, and temple were located a half block away from the latrines and associated houses. It is possible that the latrines were communally used by several Chinese households and businesses.

Processing of the latrine sediment samples was done in 2001, following the methods of Reinhard et al. (1986), Warnock and Reinhard (1992), and Sianto et al. (2005). Sediment was removed from each sample bag. The sediment was freed of large fragments of detritus. From the loose sediments, 30 ml were removed. Next, 3 *Lycopodium* sp. spore tablets were added to each 30-ml sample (about 1,250 *Lycopodium* sp. spores were added to each ml of sediment). For this analysis, *Lycopodium* sp. spore batch 212761 was used. Previous analysis has shown that ~12,500 spores are present in each tablet (values presented from different analyses of tablets are 12,432, 12,489, and 12,542). The tablets were dissolved in a few drops of hydrochloric acid in 300-ml beakers. Next, the 30-ml aliquots of sediment were added to the beakers with 50 ml of distilled water. Subsequently, 20 ml of 10% hydrochloric acid in distilled water was added to dissolve calcium carbonates in the sediment. More water was added until the reaction between the acid and the carbonates in the sediment stopped.

Once the calcium carbonates were dissolved, the samples were treated with the swirl technique. The contents of the beaker were swirled until all particles were in suspension. The beaker was placed on a flat surface for 30 sec. After 30 sec, the fluid was poured through a 300-μm mesh. This was repeated twice. The macrofossils on the mesh were examined for night-soil indicators, especially the presence of *Rubus* sp. seeds. Next, the screened fluid was concentrated by centrifugation in 50-ml centrifuge tubes. The sediments were washed 3 times in distilled water.

Preliminary microscopic examinations were made of the samples to determine if further chemical processing was necessary. It was found that the high content of fine silicates required further processing, so 20 ml of 40% hydrofluoric acid were added to each tube, and the sediments were thoroughly mixed in the acid. The samples were left in the hydrofluoric acid for 24 hr and were stirred occasionally during this period. Next, the sediments were concentrated by centrifugation. The acid was replaced by water, and the sediments were reexamined. The vast majority of silicates were dissolved, and microscopic examination was deemed to be possible. The sediments in the tubes were then washed 3 times in distilled water.

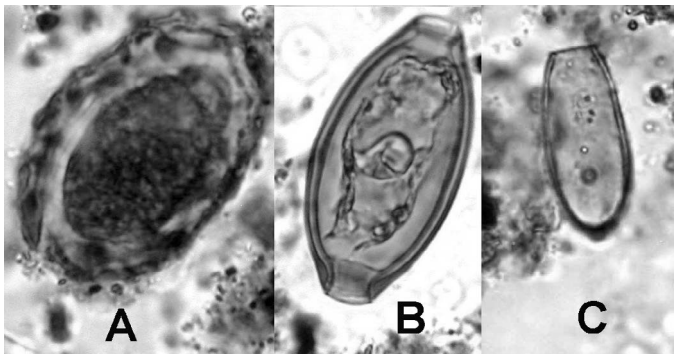

FIGURE 1. Helminth eggs found in the San Bernardino Chinatown latrines. (**A**) *Ascaris lumbricoides* (c.f.); (**B**) *Trichuris trichiura* (c.f.); (**C**) *Clonorchis sinensis*. Note: ''c.f.'' = compares favorably, meaning that the morphology is consistent with human parasites.

Drops of the sediments were transferred to glass microscope slides with Pasteur pipettes. The sediment drops were mixed with glycerin and cover-slipped. For each sample, a total of 25 *Lycopodium* sp. spores was counted along with all parasite eggs found in the process of counting the spores. A count of 25 spores represents 0.02 ml of the sediment sample. After counting, at least 3 more microscope preparations were counted to assess the presence of trace amounts of parasite eggs.

We quantified the parasite eggs for 0.02 ml of processed sediment for each sample in order to standardize the results of each analysis in terms of parasite eggs per ml of sediment. For latrine contexts, we found that 0.02 ml is sufficient to identify parasite egg quantities as low as 50 eggs per ml. We then scanned an additional 0.06 to 0.08 ml of processed sediment to identify trace amounts of parasite eggs.

The concentrations of eggs of each species were calculated using the following formula: concentration = $[(p/m) \times a]/v$, where p is parasite eggs counted, m is marker *Lycopodium* sp. spores counted, a is marker *Lycopodium* sp. spores added, and v is volume of sediment.

Identification of the species of the parasite eggs was done by morphological analysis. In the case of trichurid eggs, the dimensions of the eggs were taken and compared to those of trichurid species from a variety of hosts, including humans, domestic animals, and rodents that commonly infest habitations, outbuildings, and yards. Operculated eggs were compared to the morphology of a variety of cestode and trematode genera. These included species of *Clonorchis*, *Paragonimus*, *Fasciola*, *Diphyllobothrium*, and *Dicrocoelium*.

Based on many years of experimentation (Reinhard et al., 1986; Warnock and Reinhard, 1992; Sianto et al., 2005), we have found this method to be superior to all clinical methods for recovery of parasite eggs from latrine sediments (soil derived from feces). This is because parasite eggs in latrine soils do not respond to flotation in the same way as modern eggs. Parasite eggs are trapped in calcium carbonate deposits and must be freed by chemical means. The calcium carbonate deposits are a special problem in latrines because people added lime to the latrines when they were in use.

We reanalyzed the processed sediments in 2003 to verify the diagnoses based on observations of more eggs. A third analysis was done in 2005 to photograph the eggs.

Samples from the Euro-American latrines were negative for parasite eggs. Two of three samples from Chinese-American latrines were positive for parasite eggs (Fig. 1). Latrine 1056 was the earliest latrine and was used from the 1880s to about 1900. Latrine 1058 was built in 1900 and filled in 1910; number 1035 was the final latrine and was used from 1910 to 1944.

Latrine 1035 yielded 1,065 ascarid eggs and 710 whipworm eggs per ml of sediment, while latrine 1056 contained 3,374 ascarid eggs and 3,552 whipworm eggs per ml of sediment. These fecal-borne roundworm eggs are nearly ubiquitous in historical town sites. These numbers are not high for latrine sediments and are relatively normal for historical sediments. At low or moderate infections, these parasites rarely cause severe disease. No eggs were found in latrine 1058. The whipworm and ascarid eggs were morphologically identical to eggs of *Trichuris trichiura* and *Ascaris lumbricoides*, respectively. We acknowledge that *Ascaris suum* of pigs is morphologically identical to *A. lumbricoides*. We believe that the eggs are from *A. lumbricoides* because pigs did not live at the site and the latrines were used for human waste. There has been a debate concerning the value of egg measurements for diagnosis of *T. trichiura* and *Trichuris suis*. Horne and Tuck (1996) argued that this diagnosis is not possible with archaeological remains. In contrast, Fernandes et al. (2005) presented the majority view that whipworm egg dimensions can be obtained from archaeological sediments for diagnosis.

The most interesting discovery in both of these privies was the delicate eggs of *Clonorchis sinensis*, the Chinese liver fluke. The discovery of these eggs shows that the Chinese immigrants in California brought with them at least 1 species of parasite from Asia. Latrine 1035 contained 710 *C. sinensis* eggs per ml, and latrine 1056 contained 533 eggs per ml.

The 3 latrines were used at different times by the same Chinese community. It is noteworthy that the earliest latrine deposits (1880–1900) and the latest latrine deposit (1910–1941) were positive for parasite eggs, but the 1900–1910 latrine sediment contained no eggs. The absence of evidence of parasitism in the middle period is unexplained.

The Chinese liver fluke, like most trematodes, has a multihost life cycle, which includes fishes and snails. These intermediate hosts have important roles in the life cycle of the parasite. The parasite goes through asexual reproduction in the snails. Thus, the number of parasites produced by a single egg is amplified by the snail stage of the life cycle. The fish is important in conveying the parasites to their definitive host, a fish-eating mammal. The definitive host is the animal that harbors the sexually active stages of the parasite. If the parasite survives the culinary preparation of the fish, it will eventually migrate to the liver of the definitive host and live there for many years, mating and laying eggs. The eggs pass through the bile duct into the digestive tract and are passed with feces.

However, the introduction of this parasite to California was a dead end. The intermediate snail hosts to which it is adapted in Asia are absent in the Americas. The archaeological identification of parasite eggs in latrines that date from the late nineteenth century to the early twentieth century shows that the parasite was possibly introduced by immigrants, who may have lived long lives with their infections during this period. If infections were light, the human hosts probably had no symptoms or mild indigestion with light abdominal pain. If they had heavy infections, then they could have suffered from pronounced abdominal pain, diarrhea, jaundice, hepatomegaly, and/or anorexia. In chronic cases, liver cancer could have resulted from infection. No traditional Chinese remedies for this ailment are known to us. However, many bottles from various medicines were found in the Chinese latrines (Costello et al., 2006). These may be related to the symptoms of parasitic disease.

The absence of *Taenia solium* is important for this community. Two backyard roasting ovens were found near the latrines. The historical evidence and animal bone analysis show that these ovens were used for roasting pigs for weekend parties, ceremonies, and feasts. One of the ovens was in use by 1880. It was probably used by one or more stores to cook meat (Costello et al., 2006). By the 1920s, its use had likely declined but not ceased. When this portion of Wong Nim's property was leveled in the mid-1920s, a new roasting oven was built. This new roasting oven (1036), apparently still important for festival events, was built south of Wong Nim's building, adjacent to the Kuan Yin Temple. This was where people gathered for celebrations and where a pig would be cooked, offered at the altar, and then consumed. The absence of *T. solium* eggs in latrines spatially associated with pig-roasting ovens shows that the preparation of the pigs killed any tapeworm cysts.

Asia is home to many parasites that did not exist in the New World, even after European colonization. These parasites included the Chinese liver fluke (*C. sinensis*), the intestinal fluke (*Fasciolopsis buski*), the oriental lung fluke (*Paragonimus westermani*), and the Asian blood fluke (*Schistosoma japonicum*). These parasites were present in Asia in ancient times, as indicated by archaeoparasitology in Korea, China, and Japan. They infected many people, and, undoubtedly, some immigrants entered the Americas with these parasites. For this reason, the analysis of sediments from the San Bernardino Chinatown latrine is particularly important in demonstrating the cross-continental introduction of parasites into North America. For Chinese liver flukes, this was an ecological dead end.

It may be of further interest to mention that clonorchiasis is, to this day, very much a clinical problem in Asian/Chinese immigrants (Stauffer et al., 2004). We may think this a recent problem, but as this paper points out, it is not.

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LITERATURE CITED

ARAÚJO, A., L. F. FERREIRA, U. CONFALONIERI, AND M. CHAME. 1988. Hookworms and the peopling of America. Cadernos de Saúde Pública **2:** 226–233.

COSTELLO, J. G., AND K. HALLARAN. 2004. The luck of Third Street: Historical archaeology data recovery report for the Caltrans District 8 San Bernardino Headquarters Demolition Project. Preliminary Final Report. California Department of Transportation District 8, San Bernardino, California, 243 p.

———, K. HALLARAN, K. WARREN, M. AKIN, K. J. REINHARD, AND S. GUST. 2008. The luck of Third Street: Archaeology of Chinatown, San Bernardino, California. Historic Archaeology. (In press).

FERNANDES, A., L. F. FERREIRA, M. L. C. GONÇALVES, F. BOUCHET, C. H. KLEIN, T. IGUCHI, L. SIANTO, AND A. ARAUJO. 2005. Intestinal parasite analysis in organic sediments collected from a 16th-century Belgian archeological site. Cadernos de Saúde Pública **21:** 329–332.

FERREIRA, L. F., AND A. ARAÚJO. 1996. On hookworm in the Americas and trans-Pacific contact. Parasitology Today **12:** 454.

———, ———, U. E. CONFALONIERI, AND L. NUNEZ. 1984. The finding of eggs of *Diphyllobothrium* in human coprolites (4,100–1,950 B.C.) from northern Chile. Memórias do Instituto Oswaldo Cruz **79:** 175–180.

HALL, H. J. 1982. Parasitological analysis. *In* Archaeological and historical studies of the IJ56 Block, Sacramento, California: An early Chinese community, M. Praetzellis, and A. Praetzellis (eds.). Cultural Resources Facility, Sacramento, California, p. 113–120.

HEVLY, R. H., R. E. KELLY, G. A. ANDERSON, AND S. J. OLSEN. 1979. Comparative effects of climate change, cultural impact, and volcanism in the paleoecology of Flagstaff, Arizona, A.D. 900–1300. *In* Volcanic activity and human ecology, P. D. Sheets and D. K. Grayson (eds.). Academic Press, New York, New York, p. 487–523.

HORNE, P. D., AND J. A. TUCK. 1996. Archaeoparasitology at a 17th century colonial site in Newfoundland. Journal of Parasitology **82:** 512–515.

MATSUI, A., M. KANEHARA, AND M. KANEHARA. 2003. Palaeoparasitology in Japan—Discovery of toilet features. Memórias do Instituto Oswaldo Cruz **98**(suppl.): 127–136.

REINHARD, K. J. 1992. Parasitology as an interpretive tool in archaeology. American Antiquity **57:** 231–245.

———, U. E. CONFALONIERI, B. HERRMANN, L. F. FERREIRA, AND A. J. G. ARAÚJO. 1986. Recovery of parasite eggs from coprolites and latrines: Aspects of paleoparasitological technique. Homo **37:** 217–239.

———, R. H. HEVLY, AND G. A. ANDERSON. 1987. Helminth remains from prehistoric Indian coprolites on the Colorado Plateau. Journal of Parasitology **73:** 630–639.

SIANTO, L., K. J. REINHARD, M. L. C. GONÇALVES, AND A. ARAÚJO. 2005. The finding of *Echinostoma* (Trematoda: Digenea) and hookworm

eggs in coprolites collected from a Brazilian mummified body dated of 600–1,200 years before present. Journal of Parasitology **91:** 972–975.

STAUFFER, W. M., J. S. SELLMAN, AND P. F. WALKER. 2004. Biliary liver flukes (Opisthorchiasis and Clonorchiasis) in immigrants in the United States: Often subtle and diagnosed years after arrival. Journal of Travel Medicine **11:** 157–159.

WARNOCK, P., AND K. J. REINHARD. 1992. Methods of extracting pollen and parasite eggs from latrine soils. Journal of Archaeological Science **19:** 261–264.

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Seroprevalence of *Neospora caninum* Antibodies in Dogs in India

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ABSTRACT: *Neospora caninum* is one of most important causes of abortion in cattle worldwide, and dogs are an important risk factor for *N. caninum* infection in cattle. Antibodies to *N. caninum* were determined in 184 (126 rural, 58 urban) dogs from the Punjab State, India, using commercial monoclonal antibody-based competitive ELISA and found in 16.8% of the animals. The prevalence of *N. caninum* antibodies was significantly higher in rural dogs (21.4%, 27 of 126) than city dogs (6.9%, 4 of 58). To our knowledge this is the first report of *N. caninum* infection in canines from India.

The domestic dog is a definitive host for *N. caninum*, and the oocysts shed in canine feces are considered essential in the epidemiology of this parasite (McAllister et al., 1998; Dubey et al., 2007). *Neospora caninum* is one of most important causes of abortion in cattle worldwide, and dogs are an important risk factor for *N. caninum* infection in cattle. Therefore, there have been many surveys for *N. caninum* infections in dogs worldwide, and these were recently summarized (Dubey et al., 2007). Here we report the prevalence of *N. caninum* antibodies in dogs from India for the first time.

For the present study, 4 villages from the Punjab State were selected using simple random sampling, without replacement, using the 'Random Village' program of Survey toolbox (Cameron, 1999). Punjab has approximately 500,000 dogs; of these, 378,000 are pets and 119,000 strays (www.husbandrypunjab.org).

Serum samples from all 126 dogs in the 4 villages were collected. Additionally, serum samples were collected from 58 urban dogs brought to the Small Animal Veterinary Clinics of the College of Veterinary Science, Ludhiana, Punjab. In total, 184 serum samples were collected from June to December 2005. Blood (3–5 ml) was collected from a recurrent tarsal vein of dogs, centrifuged, and sera stored at −20 C until assayed. A standardized questionnaire for animals sampled was completed at the time of blood collection.

Sera were tested for presence of *N. caninum*–specific antibodies using a monoclonal antibody–based competitive ELISA (VMRD, Inc., Pullman, Washington) as described by Baszler et al. (1996, 2001) and recently used by us to detect antibodies to this parasite in cattle and buffaloes in Punjab, India (Meenakshi et al., 2007). The ELISA kits were imported from VMRD, and the test was performed according to instructions supplied by the manufacturer. Positive and negative controls were run in duplicate according to manufacturer's instructions. The cut-off value considered positive was 30% inhibition (Meenakshi et al., 2007).

The data were analyzed using SPSS (Statistical Package for Social Sciences, SPSS Inc., Chicago, Illinois) for Windows version 11.0.1. The associations were evaluated between binary outcome variable and a variety of risk factors (age, breed, sex, and spatial distribution) of dogs.

Antibodies to *N. caninum* were found in 31 (16.8%) of 184 dogs. Most dogs had a high SP ratio in the ELISA; percentage inhibition of 31 seropositive dogs was 30–40% (2 dogs), 41–50% (3 dogs), 51–60% (5 dogs), 62–70% (6 dogs), 71–80% (7 dogs), and 81% or more (8 dogs). Seroprevalence was higher in older dogs; 7.3% (3 of 41) of <12-mo-old were seropositive compared with 13.6% (3 of 22) prevalence in 13- to 24-mo-old, and 20.7% (25 of 121) seroprevalence in >24-mo-old dogs, but there was an insufficient number in each age group to establish an age-related relationship. Seroprevalence in males (18.4%, 6 of 48) was higher than in females (12.5%, 25 of 136), but statistically there was no differences between sexes. The seroprevalence was not significantly higher ($\chi^2 = 3.45$; $P = 0.063$) in mongrel dogs (21.1%, 23 of 109) compared with that in defined breeds (10.7%, 8 of 75).

The prevalence of *N. caninum* antibodies in the present study was significantly higher ($\chi^2 = 5.99$; $P < 0.05$) in rural dogs (21.4%, 27 of 126) when compared with that from city dogs (6.9%, 4 of 58); the risk of seroprevalence was 3.5 times higher in rural dogs in comparison to city dogs. These findings are in agreement with reports by others (Sawada et al., 1998; Wouda et al., 1999; for review see Dubey et al., 2007). In India, domestic and stray dogs on farms have easy access to placentas and dead animals, and the ingestion of infected tissues by dogs can lead to shedding of *N. caninum* oocysts (McAllister et al., 1998; Dijkstra et al., 2001). Canids (dogs, coyotes, and possibly red foxes) are the only hosts that can shed *N. caninum* oocysts (McAllister et al., 1998; Gondim et al., 2004; Wapenaar et al., 2006). There are no coyotes in India, and foxes are rarely seen on cattle farms in India. Thus, dogs appear to be the main reservoir of *N. caninum* in India.

LITERATURE CITED

BASZLER, T. V., S. ADAMS, J. VANDER-SCHALIE, B. A. MATHISON, AND M. KOSTOVIC. 2001. Validation of a commercially available monoclonal antibody–based competitive-inhibition enzyme-linked immunosorbent assay for detection of serum antibodies to *Neospora caninum* in cattle. Journal of Clinical Microbiology **39:** 3851–3857.

———, D. P. KNOWLES, J. P. DUBEY, J. M. GAY, B. A. MATHISON, AND T. F. MCELWAIN. 1996. Serological diagnosis of bovine neosporosis by *Neospora caninum* monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. Journal of Clinical Microbiology **34:** 1423–1428.

CAMERON, A. 1999. A practical manual and software package for active surveillance of livestock disease in developing countries. Australian Centre for International Agricultural Research Monograph 54, p. 330.

DIJKSTRA, T., M. EYSKER, G. SCHARES, F. J. CONRATHS, W. WOUDA, AND H. W. BARKEMA. 2001. Dogs shed *Neospora caninum* oocysts after ingestion of naturally infected bovine placenta but not after ingestion of colostrum spiked with *Neospora caninum* tachyzoites. International Journal for Parasitology **31:** 747–752.

DUBEY, J. P., G. SCHARES, AND L. M. ORTEGA-MORA. 2007. Epidemology and control of neosporosis and *Neospora caninum*. Clinical Microbiology Reviews **20:** 323–367.

GONDIM, L. F. P., M. M. MCALLISTER, W. C. PITT, AND D. E. ZEMLICKA. 2004. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. International Journal for Parasitology **34:** 159–161.

MCALLISTER, M. M., J. P. DUBEY, D. S. LINDSAY, W. R. JOLLEY, R. A. WILLS, AND A. M. MCGUIRE. 1998. Dogs are definitive hosts of *Neospora caninum*. International Journal for Parasitology **28:** 1473–1478.

MEENAKSHI, K. S. SANDHU, J. SINGH, S. SHARMA, P. K. SIDHU, C. SREEKUMAR, AND J. P. DUBEY. 2007. Seroprevalence of *Neospora caninum* antibodies in cattle and water buffaloes in India. Journal of Parasitology **93:** 1374–1377.

SAWADA, M., C. H. PARK, H. KONDO, T. MORITA, A. SHIMADA, I. YAMANE, AND T. UMEMURA. 1998. Serological survey of antibody to *Neospora caninum* in Japanese dogs. Journal of Veterinary Medical Science **60:** 853–854.

WAPENAAR, W., M. C. JENKINS, R. M. O'HANDLEY, AND H. W. BARKEMA. 2006. *Neospora caninum*–like oocysts observed in feces of free-ranging red foxes (*Vulpes vulpes*) and coyotes (*Canis latrans*). Journal of Parasitology **92:** 1270–1274.

WOUDA, W., T. DIJKSTRA, A. M. H. KRAMER, C. VAN MAANEN, AND J. M. A. BRINKHOF. 1999. Seroepidemiological evidence for a relationship between *Neospora caninum* infections in dogs and cattle. International Journal for Parasitology **29:** 1677–1682.

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