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Elevation and vegetation determine *Cryptosporidium* oocyst shedding by yellow-bellied marmots (*Marmota flaviventris*) in the Sierra Nevada Mountains

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A B S T R A C T

Wildlife are increasingly recognized as important biological reservoirs of zoonotic species of *Cryptosporidium* that might contaminate water and cause human exposure to this protozoal parasite. The habitat range of the yellow-bellied marmot (*Marmota flaviventris*) overlaps extensively with the watershed boundaries of municipal water supplies for California communities along the foothills of the Sierra Nevada. We conducted a cross-sectional epidemiological study to estimate the fecal shedding of *Cryptosporidium* oocysts by yellow-bellied marmots and to quantify the environmental loading rate and determine risk factors for *Cryptosporidium* fecal shedding in this montane wildlife species. The observed proportion of *Cryptosporidium* positive fecal samples was 14.7% (33/224, positive number relative to total number samples) and the environmental loading rate was estimated to be 10,693 oocysts animal^{-1} day^{-1}. Fecal shedding was associated with the elevation and vegetation status of their habitat. Based on a portion of the 18s rRNA gene sequence of 2 isolates, the *Cryptosporidium* found in *Marmota flaviventris* were 99.88%–100% match to multiple isolates of *C. parvum* in the GenBank.

1. Introduction

The protozoa of the genus *Cryptosporidium* are obligate, intracellular parasites affecting a large number of vertebrate species including humans. *Cryptosporidium* oocysts are resistant to chlorine disinfection and can cause waterborne enteric disease. Even though humans and livestock are considered major biological reservoirs of *Cryptosporidium* oocysts (Mac Kenzie et al., 1994; Xiao and Ryan, 2004; Atwill et al., 2006a; Feltus et al., 2006; Nichols et al., 2006; Brook et al., 2009; Santin, 2013), wildlife are increasingly recognized as significant sources of water contamination with this parasite (Jiang et al., 2005; Feng et al., 2007; Ruecker et al., 2007, 2012; Chalmers et al., 2010). Wild rodents have been identified as hosts for several *Cryptosporidium* species, including species causing disease in humans (Morgan et al., 1999; Hajdusek et al., 2004; Feng et al., 2007; Kimura et al., 2007; Meireles et al., 2007; Raskova et al., 2013). Specifically in the *Marmota* genus, reported *Cryptosporidium*

species include *C. andersoni* and *C. ubiquitum* (Ryan et al., 2003; Feng et al., 2007), suspected *C. parvum* (Atwill et al., 2003), as well as undetermined species (Ziegler et al., 2007).

The Sierra Nevada is a mountain range extending along the border between California and Nevada with the Sacramento and San Joaquin Valleys to the west and the Basin and Range region to the east (Fig. 1). The Sierra Nevada Range annual snowpack and the surface water generated from snowmelt constitute major sources of municipal and recreational water for California. Above 6,000 feet (~1830 meters), this mountain range encompasses the habitats of several species of mammals including the yellow-bellied marmot (*Marmota flaviventris*) (Sumner and Dixon, 1953). Given the extensive spatial overlap between the range of this rodent and the watershed boundaries for municipal water supplies for California communities along the foothills of the Sierra Nevada, we conducted a cross-sectional epidemiological study to estimate the prevalence of *Cryptosporidium* in yellow-bellied marmots and quantify the Environmental Loading Rate [ELR] (Atwill et al., 2003, 2004) of oocysts from these rodents. Through the construction of a hierarchical Bayesian logistic regression, we tested if habitat characteristics are associated with *Cryptosporidium* oocyst shedding in these rodents.

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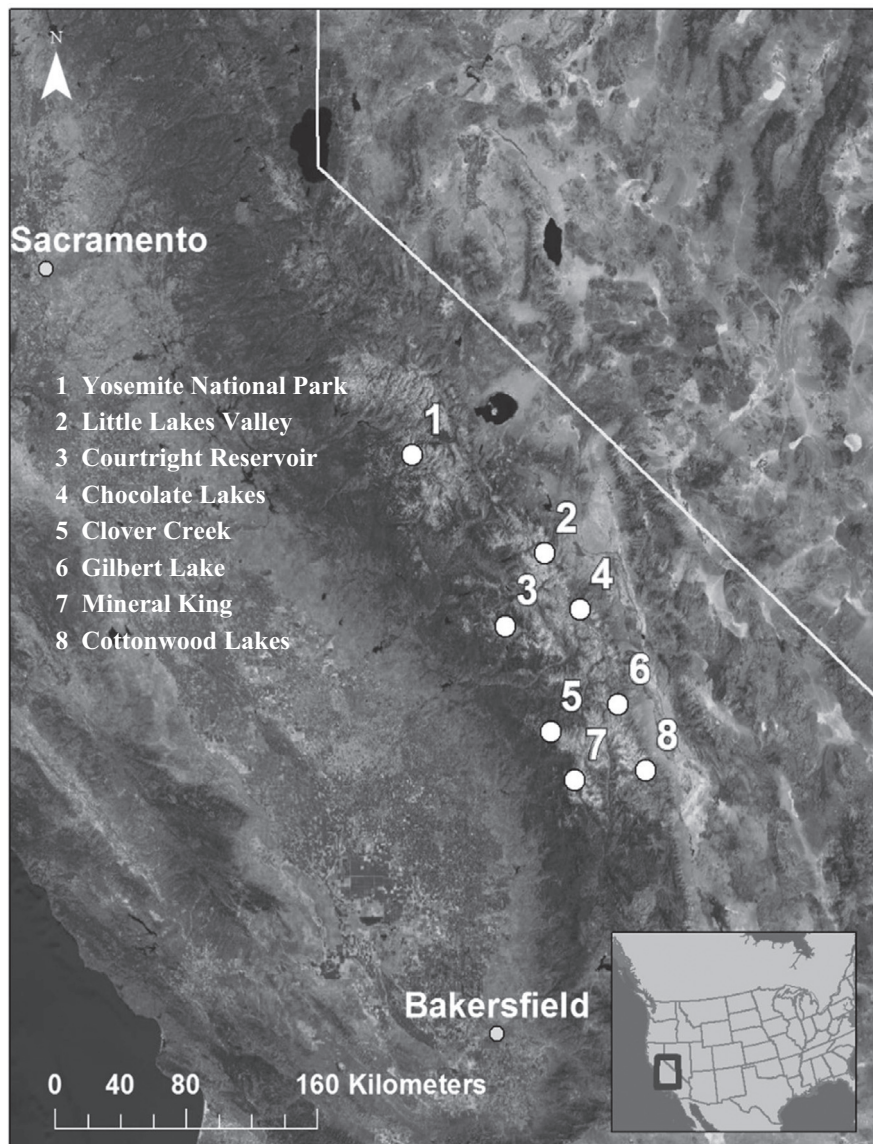*E-mail address:* ratwill@ucdavis.edu (E.R. Atwill).

Fig. 1. Locations in the Sierra Nevada Mountains where scat samples from yellow-bellied marmot were collected. The map contains 14 locations, but some locations clustered together due to the shorter distances between them and the scale of the map. The cluster in Yosemite National Park includes Tenaya Creek, Bridaveil Creek, Pothole Dome, and Lembert Dome. The cluster at Mineral King includes Mineral King, Eagle Lake, White Chief Creek, and Elk Kaweah River.

2. Materials and methods

2.1. Sample collection

Naturally voided fecal samples were collected opportunistically at 14 different locations between June and July 2012 from sites where yellow-bellied marmots defecate. These locations were in the Sequoia National Park, Inyo National Forest, Sierra National Forest, and Yosemite National Park, extending approximately 200 kilometers from north to south, at the eastern and western sides of the Sierra Nevada, California (Table 1, Fig. 1). These locations were selected as suitable habitat for yellow-bellied marmots or were locations where marmots were previously reported. We visited each location once, collecting fresh scat samples found within 4–5 hours. The recognition of feces as from yellow-bellied marmot was based on sighting individuals at every location and by using descriptions and characterizations of marmot scat (Murie and Elbroch, 2005). Only adult sized scat samples were collected in order to avoid collecting feces from other smaller rodent species. To obtain only fresh fecal samples, samples were selected from scat that were moist and with flies present. In

order to minimize the chance of sampling feces from the same animal, only one scat was selected from each site within a location, and no additional samples were collected within 20 m diameter surrounding the scat sampled. Approximately 1.6 g of fecal sample was collected from each sampling site.

Fecal samples were placed into 50 ml polypropylene sterile centrifuge tubes containing 5 ml of antibiotic storage solution (0.1 ml 10% Tween 20, 0.006 g penicillin G, 0.01 g streptomycin sulfate, 1.0 ml amphotericin B solution in 100 ml reagent-grade water). Tubes were kept on ice at 4 °C until transported to the laboratory at University of California at Davis. For each sampling site, coordinates and elevation (ft) above sea level were obtained using a GPS device GPSMAP® 76Cx (Garmin, Olathe, KS, USA) with an accuracy of ≤ 5 m. Location and date of collection were recorded.

2.2. Environmental parameters

Occurrence of vegetation at the sampling sites was recorded as two categories: no vegetation due to predominately rocky substrate; and vegetation, predominantly meadow of grasses and sedges

Table 1
The locations where marmot scat samples were collected in Sierra Nevada Mountains.

Area	Sampling location	Elevation range	Latitude	Longitude
Sierra National Forest	Courtright Reservoir	8131-8365	37° 10' 8.94'' N	118° 33' 57.06'' W
Sequoia National Park	Mineral King	7567-8089	36° 27' 2.25'' N	118° 35' 41.1216'' W
	Eagle Lake	9319-10144	36° 24' 52.6824''N	118° 36' 22.7664'' W
	White Chief Creek	8980-9561	36° 25' 25.1364'' N	118° 34' 30.2412'' W
	Elk Kaweah River	8316-9380	36° 24' 53.1072'' N	118° 35' 11.0976'' W
	Clover Creek	8801-9286	36° 39' 23.5296'' N	118° 43' 24.7224'' W
Inyo	Cottonwood Lakes	11003-11073	36° 29' 30.7428'' N	118° 12' 24.6168'' W
Forest	Gilbert Lake	10478-10538	36° 46' 11.4492'' N	118° 21' 20.6928'' W
	Chocolate Lakes	10112[a]	37° 6' 1.728'' N	118° 58' 21.9036'' W
	Little Lakes Valley	10492-10728	37° 24' 20.2356'' N	118° 45' 31.5612'' W
Yosemite National Park	Tenaya Creek	8421-8696	37° 48' 45.6228'' N	119° 29' 6.7632'' W
	Bridaveil Creek	6985-7164	37° 39' 41.76'' N	119° 37' 7.464'' W
	Pothole Dome	8704-9688	37° 52' 56.5428'' N	119° 22' 31.9224'' W
	Lembert Dome	8610-8941	37° 52' 49.0008'' N	119° 21' 2.7756'' W

[a] Only one sample.

or woody species such as trees. The vegetation category assigned to each sample site was based on direct visual observation and supported by the Normalized Difference Vegetation Index developed by the National Agriculture Imagery Program (California Department of Fish and Wildlife, 2010). Lastly, the mode of human access to each sampling site was recorded as trail, if hiking was necessary to reach the site, or dirt/paved road if the site was accessible by vehicle. We used this variable as an index of human presence in the marmot habitats in order to evaluate its association with *Cryptosporidium* oocysts in the feces. In sampling locations accessible by a dirt/paved road, people are more likely to pass through the location in vehicles, while the presence of a hiking trail suggests more chances of people walking across the location and potentially defecating.

2.3. Detecting Cryptosporidium *oocysts*

The detection of *Cryptosporidium* oocysts from fecal samples was based on published methods (Kuczynska and Shelton, 1999; Pereira et al., 1999) with modifications. Briefly, feces were weighed, resuspended in PBS, and strained into 50 ml centrifuge tubes through 4 layers of cotton gauze. Tubes were centrifuged at 1500 g for 15 min, supernatant was discarded, and pellet was resuspended in 5 ml of deionized water. The fecal solution was mixed with 30 ml of sucrose solution (specific gravity ~1.2) and centrifuged at 1000 g for 25 min. Oocysts were collected from the top of sucrose solution by overlaying 5 ml of deionized water, gently stirring, and pipetting 5 ml from the top. Oocysts were washed by mixing with deionized water and centrifuging at 1500 g for 15 min. The supernatant was discarded by aspiration, leaving a 1:1 ratio of pellet to solution volume. This final solution was weighed and 10 µl was overlaid on commercially prepared slides (Waterborne Inc., New Orleans, LA) and weighed too. Smears were air dried, stained using a direct immunofluorescence antibody kit (Meridian Bioscience, Inc., Cincinnati, OH), and examined using an Olympus BX60 microscope at ×400 magnification. Slides containing one or more oocysts were recorded as positive while those without detectable oocysts were recorded as negative. The crude number of oocysts per gram feces (O), unadjusted by assay percent recovery, was calculated according to the formula: $O = [N \times (W_{susp}/W_{sm})] / F$, where N is the number of oocysts in the smear, W_{susp} and W_{sm} are the suspension and smear weight respectively, and F is the fecal weight. Assuming daily consistent oocyst shedding, the Environmental Loading Rate [ELR] was calculated using the mean number of oocysts per gram of feces found in this study and a crude estimate for total daily fecal production estimated as ~3% of mean body mass or 0.02 kg feces wet weight per day for a typical adult marmot (Atwill et al., 2003).

2.4. Statistical analysis

In order to evaluate the effect of environmental parameters on the likelihood of detecting *Cryptosporidium* oocysts in marmot feces, a hierarchical Bayesian logistic regression model was constructed using JAGS 3.4.0 (http://mcmc-jags.sourceforge.net, [9–15-10], [Plummer, M.]) in R 2.15.1 (http://www.r-project.org, [10-10-11], [Development Core Team]), with R2jags 0.03–08 (http://cran.r-project.org/web/packages/R2jags/index.html, [11–17-2012], [Yu-Su and Masanao]) as the interface. The outcome y_{ij} was the presence of *Cryptosporidium* oocysts (0 = no oocysts / 1 = oocysts present) in the n_i feces collected at the j locations: $y_{ij} \sim Bernouilli\,(p_{ij})$, where the probability of *Cryptosporidium* oocysts being present in the ith yellow-bellied marmot feces sampled at location j, p_{ij} was related to a suite of q fixed predictors at the feces level: elevation and vegetation.

$$logit\,(p_{ij}) = \alpha_{j[i]} + \beta_1 X_{1i} + \ldots + \beta_q X_{qi},$$

where $\alpha_{j[i]}$ are random intercepts shared by feces belonging to location j, defined as:

$$a_j \sim Normal\,(\beta_0 + \gamma_1 \tau_1, \sigma^2)$$

where $\gamma_1 \tau_1$ is the group level predictor mode of human access used to reach each sampling location. We selected a hierarchical model because yellow-bellied marmot fecal samples were grouped by sampling location. This grouping was based on the assumption that the probability of *Cryptosporidium* infection in marmots from the same location may not be independent from each other and therefore may exhibit a level of positive correlation in and above what is accounted for in a fixed effects regression model. Moreover, a Bayesian approach is preferred over the frequentist because the former can avoid problems of identification when the multilevel model is complicated.

Given the continuous nature of the variable elevation, we evaluated graphically the assumption of linearity with respect to the log odds of a fecal sample testing positive for *Cryptosporidium* oocysts by categorizing elevation into 4 strata based on quartiles to get an equal number of observations per stratum elevation: 6,985–8,265 (reference level), 8,265–9,068, 9,068–10,057, and >10,057 ft. Across these four strata, there was a non-linear association between elevation and the log odds of *Cryptosporidium* oocysts shedding, therefore a quadratic term for elevation was considered. Moreover, to determine if effect modification was present between vegetation and elevation, the full dataset was stratified by vegetation status and the relationship reevaluated between elevation and the log odds of *Cryptosporidium* shedding within each strata.

Table 2
Prevalence of *Cryptosporidium* oocysts in yellow-bellied marmot feces in locations with different vegetation status (sampling locations are sorted by ascending elevation).

Location	Total	Overall prev. [%] (95% CI)	Vegetation			No vegetation		
			Total	Pos.	Prev. [%] (95% CI)	Total	Pos.	Prev. [%] (95% CI)
Bridaveil Creek	11	18 (2–52)	6	2	33 (4–78)	5	0	0 (0–52)
Elk Kaweah River	11	18 (2–52)	10	2	20 (3–56)	1	0	0 (0–98)
Courtright Reservoir	39	10 (3–24)	29	3	10 (2–27)	10	1	10 (0–45)
Tenaya Creek	20	30 (12–54)	1	0	0 (0–98)	19	6	32 (13–57)
Lembert Dome	12	25 (5–57)	3	1	33 (1–91)	9	2	22 (3–60)
Pothole Dome	7	43 (10–82)	0	0	-	7	3	43 (10–82)
Mineral King	16	19 (4–46)	16	3	19 (4–46)	0	0	-
Clover Creek	7	43 (10–82)	0	0	-	7	3	43 (10–82)
White Chief Creek	25	12 (3–31)	24	3	12 (3–32)	1	0	0 (0–98)
Eagle Lake	23	0 (0–15)	18	0	0 (0–19)	5	0	0 (0–52)
Chocolate Lakes	1	0 (0–98)	1	0	0 (0–98)	0	0	-
Gilbert Lake	3	0 (0–71)	1	0	0 (0–98)	2	0	0 (0–84)
Little Lakes Valley	6	0 (0–46)	6	0	0 (0–46)	0	0	-
Cottonwood Lakes	43	9 (3–22)	43	4	9 (3–22)	0	0	-
TOTAL	224	15 (10–20)	158	18	11 (7–17)	66	15	23 (13–35)

For model construction, the variable elevation was centered at its mean: 9,068 ft, and the quadratic elevation term was constructed from these centered values. Non-informative priors or hyperpriors were assigned for β_q, β_0 and τ_1 and σ^2: Normal (0, 25) for the first three parameters and LogNormal (0,1) for the last one. Posterior distributions for all parameters were sampled from each of three chains for 60,000 iterations following a 10,000 iteration burn-in, and thinning set to 5, for a total of 30,000 samples. Each chain was assigned random start values from a Normal (0,1) distribution for β_0 and β_q and a Uniform (0,1) for σ. Convergence was assessed by the Gelman–Rubin statistic (Gelman and Rubin, 1992). A backward stepping procedure was used to select terms for the final model, starting with a full model containing the random intercepts, the fixed effects, and two-way interactions: among elevation and vegetation, and quadratic elevation and vegetation. Final model selection was based on the deviance information criterion (DIC) (Spiegelhalter et al., 2002) and credibility of parameters. Given the sample size and the sparse nature of data, fixed regression parameters with 90% Credible Intervals [CrI] that excluded 0 were considered to be credible. We assessed potential confounders by examining the change of the mean parameter estimate when they were included to the selected model. If the inclusion generated a change >10%, we kept the variable in the model.

2.5. PCR and sequencing

Fecal solutions of microscopic positive samples were exposed to 5 cycles of freeze (−80 °C) and thaw (+70 °C), then 0.2 g fecal solutions were used for DNA extraction using the DNA Stool Mini Kit (Qiagen®) according to the manufacturer's manual. A nested PCR was performed using primers and reaction conditions amplifying a fragment of ~830 bp of the 18S rRNA gene according to previously described methods (Xiao et al., 1999). A positive control using *C. parvum* DNA from California dairy calves (GenBank accession no. FJ752165) as template and a negative control without DNA template were included in each PCR. PCR products were verified by electrophoresis in 2% agarose gel stained with ethidium bromide. Verified PCR products were purified using a Qiaquick® spin columns (Qiagen®) and sequenced at the University of California DNA Sequencing Facility, where ABI 3730 Capillary Electrophoresis Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) was used for sequencing. Primers of the secondary PCR of the 18s rRNA gene were used for sequencing. A preliminary analysis of sequences was conducted using Vector NTI Advanced 11 software (Invitrogen Corporation, Carlsbad, CA) followed by a BLAST analysis to compare the sequences to existing *Cryptosporidium* spp. 18S rRNA gene sequences in the GenBank using the National Center for Biotechnology Information [NCBI] online blasting tool (http://blast.ncbi.nlm.nih.gov/).

3. Results

3.1. Fecal shedding of Cryptosporidium oocysts

A total of 224 fecal samples were collected. Thirty three (~15%) fecal samples had detectable levels of *Cryptosporidium* oocysts (95% CI: 10.0–19.4%). Among these 33 positive fecal samples, 22 had one oocyst, 6 had 2 oocysts, 2 had 4 oocysts and one of each had 8, 26 and 35 oocysts per smear. This distribution of oocyst shedding intensity resulted in a mean of 535 oocysts per gram of feces wet weight (95% CI: 204–865), ranging from 35 to 1,646 and a median value of 129 oocysts per gram of feces. The mean ELR was estimated as 10,693 *Cryptosporidium* oocysts per adult marmot per day.

Elevations of the sample collection sites ranged from 6,985 to 11,070 ft, with a median elevation of 9,068 ft. Total sample size of sites with and without vegetation was 158 and 66 respectively, with a median elevation of 8,657 and 9,374 ft, respectively. Bridaveil Creek, Chocolate Lakes, Courtright Reservoir, Lembert Dome, Pothole Dome, and Tenaya Creek were accessed from dirt/paved roads and the remaining locations were reached through hiking trails. The prevalences of *Cryptosporidium* oocysts in fecal samples collected at sites with and without vegetation were 11% and 23%, respectively. A summary of the number of samples collected by location and the positive proportion is presented in Table 2.

3.2. Environmental parameters on fecal shedding of oocysts

Parameter estimates and odds ratios [OR] from the final regression model are presented in Table 3. In the final regression model, the estimates for both two-way interactions were evaluated: the centered elevation and vegetation status, and the square of centered elevation with vegetation status, resulting in credible OR. As a consequence of these credible OR, the variables elevation, quadratic elevation and vegetation status were retained in the selected final model. Simpler models without these interaction terms resulted in larger DICs and the non-credibility of the remaining parameters. The mode of human access to the marmot site was kept in the final model as a confounder because it adjusted elevation's mean parameter estimate by approximately 14%.

The association between elevation and the odds for *Cryptosporidium* oocysts in marmot feces was strongly influenced by the presence or absence of vegetation at the sampling sites. When vegetation was present, the odds of detecting oocysts decreased as

Table 3

Estimated Bayesian hierarchical logistic regression coefficients for environmental factors associated with *Cryptosporidium* oocysts in feces of yellow-bellied marmots in the Sierra Nevada Mountains, California.

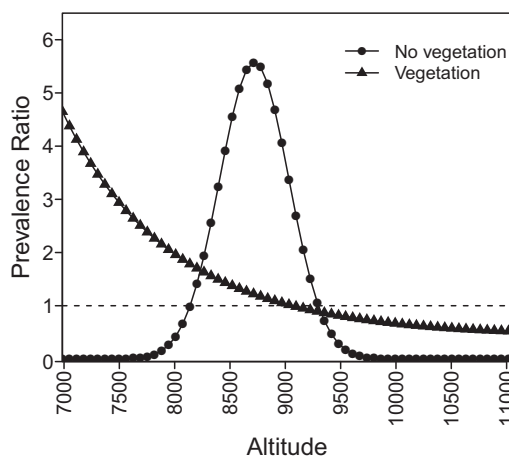| Parameter	Level	Coefficient (90% CrI)	Odds ratio (90% CrI)
Intercept		−2.110 (−2.942, −1.291)	0.121 (0.053, 0.275)
Vegetation	Absence	1.111 (−0.363, 2.454)	3.038 (0.695, 11.638)
	Presence	0	1
Elevation		−0.524 (−1.050, −0.018)	0.592 (0.350, 0.982)
Elevation^2		0.098 (−0.276, 0.443)	1.103 (0.759, 1.557)
Elevation × Vegetation	Absence	−3.013 (−6.555, −0.046)	0.049 (0.001, 0.955)
	Presence	0	1
Elevation^2 × Vegetation	Absence	−5.208 (−9.357, −1.828)	0.005 (0.0001, 0.161)
	Presence	0	1
Access	Road	−0.346 (−1.549, 0.867)	1.413 (0.213, 2.380)
	Trail	0	1
$\sigma^2_{among\text{-}locations}$		0.473 (−0.659, 0.750)	

Fig. 2. Association between *Cryptosporidium* oocyst shedding by yellow-bellied marmots in the Sierra Nevada Mountains and elevation (using 9,068 ft as the reference), stratified by presence or absence of vegetation at sites where scat samples were collected.

site elevation increased (Table 3). For example, at sites where access was via a hiking trail, the odds of detecting oocysts in marmot feces was about 5 times more likely at sites at 7,000 ft elevation compared to feces collected at sites of 9,000 ft elevation. In contrast, for fecal samples collected at rocky location with minimal vegetation, it was rare to find fecal samples with *Cryptosporidium* oocysts from sites at the extreme lower or higher elevations. Instead, positive samples clustered in the middle to upper 8,000 ft range (Fig. 2). The variance among random intercepts was estimated at 0.473; however, the CrI for this estimate covered zero, suggesting that significantly different random intercepts for locations were not present.

3.3. PCR and sequencing

Amplification and sequencing of a fragment of the 18S rRNA gene were successful for two of the microscopic positive samples. The two samples were collected from Bridaveil Creek in Yosemite National Park (isolate 1) and Mineral King in Sequoia National Park (isolate 2). Sequence analysis of the two *Cryptosporidium* isolates demonstrates that they were 99.76% (828/830 bases) similar to each other. Given that the *Cryptosporidium* oocysts were isolated from *Marmota flaviventris* in 2012, the two isolates we named as Mflav12a(1) and Mflav12a(2), respectively. The GenBank accession numbers of the two isolates are KF626380 for Mflav 12a (1) (isolate 1) and KF626381 for Mflav 12a (2) (isolate 2), respectively. Using the NCBI's default algorithm parameters to target maximum 100 sequences in GenBank, the Mflav12a(1) isolate was 100% similar to a *C. parvum* isolate (KM225275) and 99.88% similar to *C. parvum* isolates of the most closely related 10 entries in GenBank (Table 4). Similarly, the Mflav12a(2) isolate was 100% identical to two *C. parvum* isolates (JN247404 and KC476546) and 99.88% similar to *C. parvum* isolates of the other 8 entries in GenBank (Table 4) (based on BLAST analysis completed on December 29, 2014).

4. Discussion

To standardize the sampling procedure, we searched for scat samples at each location during a similar period of time; however, the number of samples by location varied due to the difference in abundance of marmots at each location. We sampled naturally voided fecal deposits and collected only one scat sample per site so as to

Table 4

Comparison of *Cryptosporidium* isolates from yellow-bellied marmots (*Marmota flaviventris*) to *Cryptosporidium* isolates in GenBank.

Location	DNA sequence group (isolate)	GenBank access no.	No. of base pairs	GenBank access no. of closely related isolates	Country of origin	Max. identity [a] (%) by BLAST
Bridaveil Creek (Yosemite National Park)	Mflav12a(1) (isolate 1)	KF626380	830	KM225275 (*C. parvum* Jammu)	USA	100
				AB513857-513863 (*C. parvum* Sakha 102–108)	Egypt	99.88
				AB441687 (*C. parvum* strain Cap (C) 197IR)	Iran	99.88
				EF611871 (*C. parvum* isolate Izatnagar)	India	99.88
				DQ523504 (*C. parvum* clone 3229T)	Spain	99.88
				DQ656355 (*C. parvum* isolate IR-C2)	Iran	99.88
				L16996 (*C. parvum* isolate AUCP-1)	USA	99.88
				AY204238 (*C. parvum* clone HMa)	USA	99.88
				AF161856 (*C. parvum* strain MT)	USA	99.88
				AF108864 (*C. parvum* strain C1)	Australia	99.88
				AF040725 (*C. parvum* strain KSU-1)	USA	99.88
Mineral King (Sequoia National Park)	Mflav12a(2) (isolate 2)	KF626381	830	JN247404 (*C. parvum* BRAsheep53CII)	Brazil	100
				KC476546 (*C. parvum* isolate 98C)	Poland	100
				JX948126 (*C. parvum* isolate A23G)	Thailand	99.88
				KM225275 (*C. parvum* Jammu)	USA	99.88
				AB513857-513863 (*C. parvum* Sakha 102–108)	Egypt	99.88
				AB441687 (*C. parvum* strain Cap (C) 197IR)	Iran	99.88
				EF611871 (*C. parvum* isolate Izatnagar)	India	99.88
				DQ523504 (*C. parvum* clone 3229T)	Spain	99.88
				DQ656355 (*C. parvum* isolate IR-C2)	Iran	99.88
				L16996 (*C. parvum* isolate AUCP-1)	USA	99.88
				AY204238 (*C. parvum* clone HMa)	USA	99.88

[a] Examples of isolates of *Cryptosporidium* that represent entries in the GenBank with the highest percentage match of their 18S rRNA gene sequence with the two new isolates from yellow-bellied marmots.

minimize repeated sampling of the same animal. The home range of yellow-bellied marmots can vary in size and shape (Armitage, 2009) and they can live in colonies or as satellites (Armitage and Downhower, 1974), thus it is not possible to guarantee that each fecal sample belongs to different individuals. Nevertheless, point prevalence estimated in this study (~15%) is similar to the prevalence reported from studies on related mammalian species (Feng et al., 2007; Ziegler et al., 2007; Feng, 2010). The ELR as reported previously for this host species was 208,000 oocysts animal^{-1} day^{-1} (Atwill et al., 2003), which represents a 20-fold higher value compared with the value calculated for this study. However, values reported in the present work are the crude number of oocysts per gram of feces which is unadjusted by percent recovery. In our laboratory, the percent recovery can typically range from 9 to over 30% depending on the vertebrate species being tested (Atwill et al., 2003, 2006a), which results in a 3.3- to 11.1-fold increase in the estimated ERL as reported previously for this host species (Atwill et al., 2003). The estimated ELR from this study is less than the values reported previously for horses (*Equus caballus*), striped skunks (*Mephitis mephitis*), coyotes (*Canis latrans*), and California ground squirrels (*Spermophilus beecheyi*), but higher than the daily loads reported for adult dairy cattle (*Bos taurus*) and beef cattle (*Bos taurus*) (Atwill et al., 2003, 2004).

The role of elevation as a risk factor for *Cryptosporidium* oocysts fecal shedding could be explained by the effects of variables that vary with elevation, such as temperature, desiccation and ultraviolet (UV) radiation. In general, as elevation increases: a) temperature decreases as a result of the air expansion under lower pressure; b) relative humidity decreases as a consequence of lower air temperature and pressure; and c) UV radiation increases due to decreasing density of molecules and particles in the atmosphere. Temperature is a key factor for the survival of intact oocysts on the ground (Fayer, 1994; Jenkins et al., 1999; Olson et al., 1999; Nasser et al., 2007) and in feces (Jenkins et al., 1999; Olson et al., 1999; Kato et al., 2002; Li et al., 2005, 2010). Exposure to ambient temperatures in excess of 30 °C to 40 °C has been shown to inactivate or reduce oocyst infectivity (Anderson, 1985; Fayer, 1994; Li et al., 2005, 2010). Moreover, oocysts have been shown to be susceptible to freeze/thaw cycles (Jenkins et al., 1999) and freezing (Kato et al., 2002). At high elevations, freeze/thaw cycles might occur, but it seems oocysts are more likely affected by heat than cold (Olson et al., 1999; Nasser et al., 2007). Desiccation has been shown to strongly reduce oocyst viability (Anderson, 1986; Robertson et al., 1992; Deng and Cliver, 1999; Nasser et al., 2007). UV radiation inactivates or reduces infectivity of *C. parvum* oocysts (Lorenzo-Lorenzo et al., 1993; Campbell et al., 1995; Craik et al., 2001; Drescher et al., 2001; Linden et al., 2001; Shin et al., 2001; Morita et al., 2002; Rochelle et al., 2004; Connelly et al., 2007; King et al., 2008, 2010; Lee et al., 2008). In this cross-sectional study, we did not include variables of temperature, desiccation, or UV radiation, but these should be considered in future studies of fecal shedding of oocysts by yellow-bellied marmots. In addition, it would be necessary to consider other factors potentially influencing these variables, for example, slope direction.

Buffer strips of vegetation have been shown to retain *Cryptosporidium* oocysts (Davies et al., 2004; Tate et al., 2004; Trask et al., 2004; Atwill et al., 2006b; McLaughlin et al., 2013); therefore, sites with vegetation could potentially have higher oocyst loads to which marmots could become exposed and infected. Vegetation could also influence oocyst inactivation rates by protecting them from higher temperatures, desiccation and UV radiation. We speculate that the negative association between the odds for oocyst shedding and elevation for marmots located in vegetated habitat (Fig. 2) may be the consequence of vegetation reducing oocyst exposure to UV radiation at lower elevation. The effect of vegetation protection is probably related with changes of plant species as elevation increases which lead to higher inactivation rates of oocysts in the environment. With respect to sites with no vegetation, oocysts in fecal material can be removed by the runoff from precipitation, thus reducing oocysts loads (Davies et al., 2004; Tate et al., 2004; Trask et al., 2004); McLaughlin et al., 2013; Davidson et al., 2014). Therefore in these areas, infection of susceptible individuals would likely occur in zones with higher density of marmots and closer contact between animals. According to our results, clusters of positive fecal samples existed at locations harboring a higher density of individuals.

This work describes what we believe to be the first detection of *Cryptosporidium* from yellow-bellied marmots in the unique high elevation locations in Sierra Nevada Mountains. PCR amplification of DNA of *Cryptosporidium* oocysts from fecal samples is frequently hampered due to the many inhibitors present in fecal samples, as experienced by our laboratory and reported by others (Kostrzynska et al., 1999; Xiao et al., 2003; Adamska et al., 2012; Elmore et al., 2013). Although only two isolates were successfully sequenced, our results demonstrate that some of these oocysts recovered are likely to be *C. parvum*. Because *C. parvum* is a species that infects humans and a wide range of vertebrate animals (Fayer, 2010), there is the potential of transmission between mammal species in the Sierra Nevada Mountains, such as marmots, horses, wildlife, and humans. In addition, *Cryptosporidium* shed by yellow-bellied marmots could potentially contaminate water sources used for drinking or recreation in the mountains. However, a valid risk assessment of waterborne outbreaks needs to take into consideration the density of the local yellow-bellied marmot population, the low loads of oocysts shed by these animals, the environmental factors that influence the survival and viability of the oocysts, and hydrological transport of oocysts in the environment.

In summary, yellow-bellied marmots in the Sierra Nevada Mountains are hosts of *Cryptosporidium* spp. with estimated prevalence of 10–20%. Some of the marmots in these populations are likely the hosts of *C. parvum*. The estimated ELR for these animals is lower than values reported for other mammals in California. Fecal shedding of *Cryptosporidium* oocysts by yellow-bellied marmots is associated with elevation and strongly influenced by the presence or absence of vegetation.

Conflict of interest

The authors declared that there is no conflict of interest.

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