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Effect of Daily Temperature Fluctuation during the Cool Season on the Infectivity of *Cryptosporidium parvum*▽

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The present work calculated the rate of inactivation of *Cryptosporidium parvum* oocysts attributable to daily oscillations of low ambient temperatures. The relationship between air temperature and the internal temperature of bovine feces on commercial operations was measured, and three representative 24-h thermal regimens in the ~15°C, ~25°C, and ~35°C ranges were chosen and emulated using a thermocycler. *C. parvum* oocysts suspended in deionized water were exposed to the temperature cycles, and their infectivity in mice was tested. Oral inoculation of 10^3 treated oocysts per neonatal BALB/c mouse (~14 times the 50% infective dose) resulted in time- and temperature-dependent reductions in the proportion of infected mice. Oocysts were completely noninfectious after 14 24-h cycles with the 30°C regimen and after 70 24-h cycles with the 20°C regimen. In contrast, oocysts remained infectious after 90 24-h cycles with the 10°C regimens. The estimated numbers of days needed for a 1-$\log_{10}$ reduction in *C. parvum* oocyst infectivity were 4.9, 28.7, and 71.5 days for the 30, 20, and 10°C thermal regimens, respectively. The loss of infectivity of oocysts induced by these thermal regimens was due in part to partial or complete *in vitro* excystation.

It is well recognized that the protozoan parasite *Cryptosporidium parvum* causes waterborne enteric disease and poses a significant threat to public health. Fecal contamination from infected hosts, such as humans and some species of livestock and wildlife (17), can lead to elevated concentrations of *C. parvum* oocysts in drinking, recreational, and irrigation water supplies (6, 8). Once excreted, *C. parvum* oocysts can be eluted from fresh fecal matrices during precipitation events that generate surface flow or runoff conditions (4, 5, 12, 21, 32). During cool moist conditions oocysts can persist for months in the environment (10, 11, 25, 30), but factors such as extremes of temperature, exposure to UV radiation, and desiccation can substantially reduce the number of infective oocysts prior to waterborne transport (2, 7, 9, 11, 19, 24, 25, 29, 30).

To examine thermal stress, most studies have used constant thermal regimens to investigate the effect of temperature on the viability or infectivity of *Cryptosporidium* oocysts (11, 14, 20, 28, 30). To complement this work, we previously investigated the impact of large daily changes in the ambient temperature on *C. parvum* oocyst infectivity, using spring through autumn thermal regimens and temperatures measured inside bovine fecal pats that were exposed to solar radiation at cow-calf and dairy production facilities (23). Under California's summer climatic conditions, internal fecal pat temperatures range from 45°C to 75°C during the day and decrease 10 to 60°C during the night. Exposing oocysts to these large thermal fluctuations results in >3.3-$\log_{10}$ reductions in oocyst infectivity in each 24-h cycle (23). The present study was conducted in order to measure the effect of exposure to oocysts to cool-season daily temperatures (with peaks at temperatures greater than 10°C, 20°C, and 30°C) on the rate of inactivation of *C. parvum* oocysts. Determining the temperature-dependent rate of *C. parvum* oocyst inactivation for these lower temperatures would allow grazing management and source water assessment plans to more properly predict the amount of time needed for exclusion of cattle prior to the onset of winter precipitation in order to inactivate sufficient numbers of oocysts in critical watersheds.

MATERIALS AND METHODS

Acquiring ambient and fecal matrix temperatures. As described previously (23), air temperature and internal temperature data for bovine fecal pats that were exposed to sunlight were collected for 12 months using an Optic StowAway Temp Logger system (Onset Computer Corporation, Bourne, MA) at 11 commercial diary and cow-calf operations throughout California.

Simulating fecal matrix temperatures. From our database of air and fecal matrix temperatures for lower- to middle-elevation regions (100 to 2,500 ft) in California, we selected three typical 24-h profiles of fecal matrix temperatures with maximum midday values of 17.3°C, 27.6°C, and 36.4°C (Fig. 1). Using a custom-made UNIX fitting algorithm, we constructed a time-by-temperature 24-h step function that emulated the three daily thermal profiles. The step functions were then programmed into a 96-well automated thermocycler (GeneAmp 2700 PCR system; Applied Biosystems, Foster City, CA).

Source and purification of *C. parvum* oocysts. Feces were collected from naturally infected calves that were 9 to 21 days old from a commercial dairy in Tulare, CA. Oocysts isolated from the same location and a similar age group were classified as *C. parvum* using a molecular procedure (33). Using an acid-fast protocol with direct fecal smears, samples having more than 25 oocysts per microscopic field (magnification, ×400) were washed using a series of 40-, 100-, 200-, and 270-mesh sieves with Tween water (0.2% [vol/vol] Tween 20 in deionized water). Each resulting suspension was centrifuged at 1,500 × *g* for 20 min in a 250-ml centrifuge tube, the supernatant was discarded, and the pellet was resuspended in Tween water. Oocysts were purified using a discontinuous sucrose gradient (3) and suspended in deionized water. The concentration of purified oocysts was determined by determining the arithmetic mean for six separate counts using a phase-contrast hemacytometer. Stock solutions were prepared by diluting oocysts in deionized water to obtain concentrations of 10^4 and 10^6 oocysts/ml, stored at 4°C, and used within 1 week.

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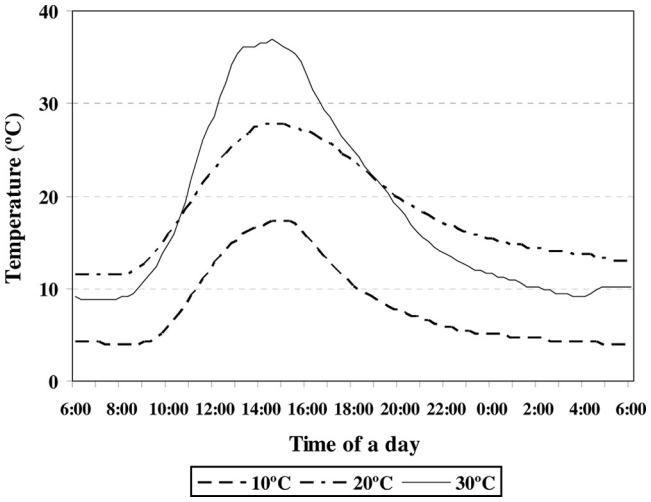

FIG. 1. Representative 24-h 10°C, 20°C, and 30°C thermal profiles for bovine fecal pats located on grazed rangeland throughout California from spring to autumn in 2000.

Treatment of *C. parvum* oocysts with thermal profiles. For each thermal regimen, 96 100-μl MicroAmp reaction tubes (Applied Biosystems, Foster City, CA) were filled with an oocyst stock solution (10^3 oocysts/tube). After a 24-h thermal cycle was completed, a set of 10 tubes was removed from the thermocycler. The remaining tubes were subjected to a replicate 24-h thermal cycle, another set of 10 tubes was removed, and the procedure was repeated until the oocysts were noninfectious. The process of removing tubes and restarting the thermocycler was completed within 1 min so that the break in the continuous temperature was minimized. Oocyst suspensions from replicate temperature-duration tubes were combined, and the percentages of intact, partial, and ghost oocysts were determined using differential interference contrast microscopy at a magnification of ×400 (Olympus BX 60; Olympus America, Inc., New York, NY). One hundred microliters per neonatal mouse was used as an oral inoculum to test the infectivity of the original preparation containing 10^3 oocysts/tube, using the assay described below. The numbers of experiments conducted for each thermal regimen are shown in Table 1. For positive controls, neonatal mice were given 10^3 fresh oocysts; for negative controls, neonatal mice were inoculated with 10^5 heat-inactivated oocysts (exposed to 70°C for 2 h) to monitor possible detection of oocysts in the intestine directly from inocula.

***In vivo* infectivity assay for *C. parvum* oocysts.** A neonatal BALB/c mouse model, modified from the model of Finch et al. (13), was used to test oocyst infectivity (16). Female BALB/c mice with neonatal pups were purchased from Harlan Company (San Diego, CA), housed in cages fitted with air filters, and given food and water *ad libitum*. Intragastric oocyst inocula were delivered in 100 μl of deionized water to 4-day-old neonatal mice, using a 24-gauge ballpoint feeding needle. One hour prior to infection, the neonatal mice were removed from the dam to ensure that their stomachs were empty; following inoculation, the dam was returned to the pups. Litters of mice were randomly assigned to treatment groups, including positive and negative controls as described above.

To determine the *Cryptosporidium* infection status of neonatal mouse pups 7 days postinoculation, the entire intestine was collected and suspended in 5 ml deionized water in a 50-ml tube and homogenized with a tissue homogenizer (Kika Werke Gmbh & Co. KG, Staufen, Germany). The homogenate was centrifuged at 1,500 × *g* for 10 min, the supernatant was discarded, and pellet was resuspended in deionized water and filtered through a 20-μm nylon net filter (Millipore Co., Bedford, MA) that had been fixed on a Swinnex holder (Millipore Co., Bedford, MA). The filtrate was concentrated to 1 ml by centrifugation at 1,500 × *g* for 10 min. Fifty microliters of the final suspension was mixed with 50 μl of fluorescent isothiocyanate-labeled anti-*Cryptosporidium* immunoglobulin M antibodies (Meridian, Cincinnati, OH) and 2 μl of 0.5% Evans blue in phosphate-buffered saline and incubated at room temperature for 45 min in a dark box. Three duplicate wet mount slides were prepared from each sample using 20 μl of reaction mixture per slide. Slides were examined by epifluorescence microscopy (Olympus America, Inc., New York, NY). A mouse was considered infected if one or more oocysts were detected in any of the three slides. Tissue homogenates were shown previously to be twice as sensitive for detecting *C. parvum* infections in neonatal mice as histopathology (16).

Statistical modeling. The infection status of each mouse pup was coded as either infected ($y = 1$) or uninfected ($y = 0$) using the *in vivo* neonatal mouse assay described above. The data resulted in a percentage of infection for litters of mice for each time point (number of days) for each of three 24-h thermal profiles (10, 20, and 30°C). Given the asymmetry (steep decline and then tailing) of the raw data for the percentage of mice infected as a function of time (Fig. 2), we used complementary log-log regression (1) instead of logistic regression to model the effect of daily thermal exposure on the infectivity of *C. parvum* oocysts for neonatal mice. For each 24-h thermal profile (10, 20, or 30°C), this model was formulated using equation 1:

$$P(y = 1 \mid x) = 1 - \exp[-\exp(\alpha + \beta x)] \quad (1)$$

where $P(y = 1 \mid x)$ was the fitted or expected probability that a BALB/c mouse pup was infected with *C. parvum* given that the oocysts had been exposed to x 24-h thermal profiles (e.g., days) and α and β were the maximum likelihood estimates obtained from the complementary log-log regression. Because we used litters of mice for each time point, we adjusted the standard errors for potential clustering of infection status within litters (e.g., lack of independence of infection status for littermates) (15).

To calculate the number of days with each thermal profile (10, 20, or 30°C) needed for a 1-$\log_{10}$ reduction in the number of infective oocysts, the following calculations were used. We established previously the infectious dose curve for *C. parvum* oocysts in neonatal BALB/c mice, defined using equation 2:

TABLE 1. Infectivity of *C. parvum* oocysts in neonatal BALB/c mice after oocysts suspended in water were exposed to 24-h temperature cycles

Temp cycle[a]	Length of exposure (days)[b]	% of infected mice (no. of infected mice/no. of inoculated mice)		
		Treated oocysts	Fresh oocysts	Inactivated oocysts
10°C	1, 3, 5, 7[c]	100 (7/7)[c]	100 (7/7)[c]	0 (0/6)[c]
	15, 30, 45, 60[d]	100 (16/16)[d]	100 (10/10)[d]	0 (0/11)[d]
	75	92.9 (13/14)	100 (11/11)	0 (0/11)
	90	61.5 (8/13)	100 (12/12)	0 (0/11)
20°C	1, 2, 3, 4, 5, 6, 7[c]	100 (7/7)[c]	100 (7/7)[c]	0 (0/7)[c]
	8, 9, 10, 14, 21, 28[c]	100 (7/7)[c]	100 (7/7)[c]	0 (0/7)[c]
	35	53.8 (7/13)	100 (13/13)	0 (0/10)
	42	42.9 (3/7)	100 (7/7)	0 (0/6)
	49	28.6 (4/14)	100 (13/13)	0 (0/10)
	56	33.3 (2/6)	100 (6/6)	0 (0/6)
	63	23.1 (3/13)	100 (13/13)	0 (0/12)
	70	0 (0/14)	100 (12/12)	0 (0/11)
	71, 72	0 (0/6)[c]	100 (6/6)[c]	0 (0/5)[c]
30°C	1, 2, 3, 4, 5[e]	100 (10/10)[e]	100 (6/6)[e]	0 (0/6)[e]
	6	64.7 (11/17)	100 (11/11)	0 (0/11)
	7	26.7 (4/15)	100 (12/12)	0 (0/11)
	8	33.3 (2/6)	100 (6/6)	0 (0/6)
	9	28.6 (2/7)	100 (6/6)	0 (0/6)
	10	14.3 (1/7)	100 (6/6)	0 (0/6)
	11	16.7 (1/6)	100 (6/6)	0 (0/6)
	12	20.0 (1/5)	100 (6/6)	0 (0/6)
	13	16.7 (1/6)	100 (6/6)	0 (0/6)
	14	0 (0/21)	100 (16/16)	0 (0/14)
	15	0 (0/14)	100 (10/10)	0 (0/9)

[a] The 24-h thermal profiles mimicked the temperatures in bovine fecal pats shown in Fig. 1.
[b] One day was equivalent to a single 24-h temperature cycle shown in Fig. 1.
[c] The infectivity data were equivalent for the different lengths of exposure indicated. The number of mouse pups typically was six or seven.
[d] The infectivity data were equivalent for the different lengths of exposure. The number of mouse pups typically ranged from 11 to 16.
[e] The infectivity data were equivalent for the different lengths of exposure. The number of mouse pups ranged from 5 to 17.

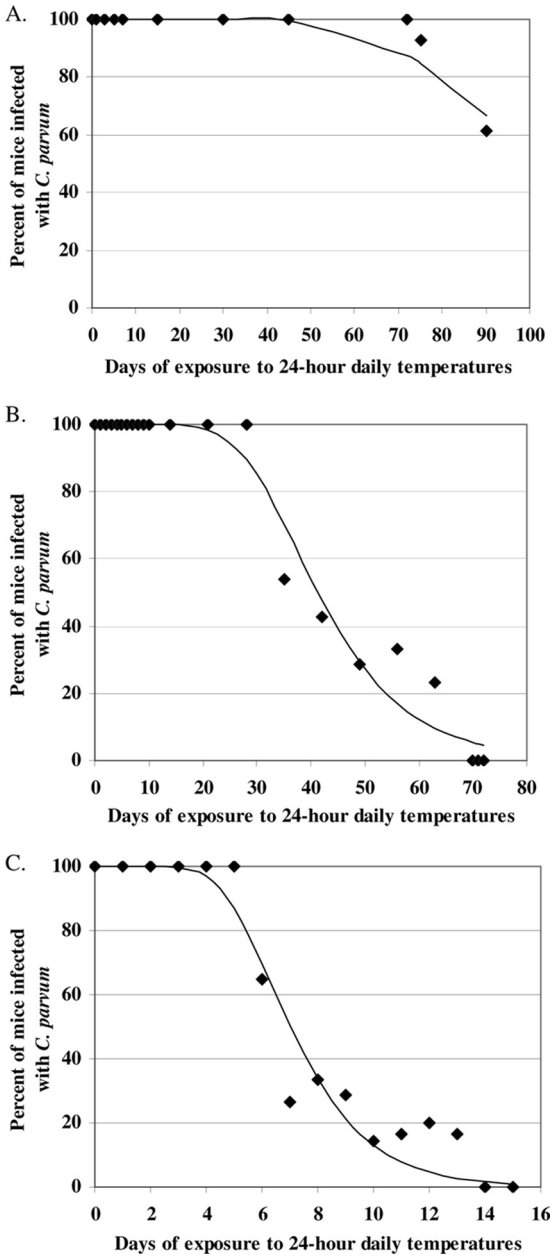

FIG. 2. (A) Effect of daily temperature fluctuations up to ~15°C on the infectivity of *C. parvum* oocysts. (B) Effect of daily temperature fluctuations up to ~25°C on the infectivity of *C. parvum* oocysts. (C) Effect of daily temperature fluctuations up to ~35°C on the infectivity of *C. parvum* oocysts.

$$P(y = 1 | x \text{ oocysts}) = 1/\{1 + \exp[-(\alpha + \beta \times \ln x)]\} \quad (2)$$

where $P(y = 1 | x$ oocysts) is the fitted or expected probability that a BALB/c mouse pup was infected with *C. parvum* if it was given an oral dose of x oocysts, α is -24.244, and β is 5.695. Given that we used an inoculum of 1,000 oocysts per pup, a 1-$\log_{10}$ reduction in the number of infective oocysts was 100 oocysts. Using the dose-response curve described above, a dose of 100 infective oocysts generates a probability of mouse infection of 0.879 ($1/\{1 + \exp[24.244 - 5.695 \times \ln 100]\} = 0.879$). Hence, we calculated the number of days with each thermal profile (10, 20, or 30°C) needed to generate a probability (P) of neonatal mouse infection of 0.879. Using the complementary log-log regression fitted as described above for each thermal profile (10, 20, or 30°C), we then solved for x (days) using equation 3:

$$x = \{\ln[-\ln(1 - P) - \alpha]/\beta\} \quad (3)$$

where P is 0.879 and α and β are the maximum likelihood estimates obtained from the complementary log-log regression (equation 1) that was fitted to the data shown in Table 1.

Nucleotide sequence accession number. The DNA sequence of oocysts has been deposited in the NCBI GenBank under accession no. FJ752165.

RESULTS AND DISCUSSION

Complementary log-log regression models (equation 1) were fitted to the data for each 24-h thermal regimen and are shown in Fig. 2. For the 30°C thermal regimen, the α and β maximum likelihood estimates were 3.37 (95% confidence interval [CI], 2.44 to 4.30) and -0.533 (95% CI, -0.677 to -0.388); for the 20°C thermal regimen, the α and β estimates were 3.30 (95% CI, 2.16 to 4.45) and -0.089 (95% CI, -0.116 to -0.062); and for the 10°C thermal regimen, the α and β estimates were 3.28 (95% CI, 2.18 to 4.37) and -0.035 (95% CI, -0.049 to -0.022). A 1-$\log_{10}$ reduction in *C. parvum* oocyst infectivity would have reduced the ingested dose of infective oocysts from 1,000 to 100 oocysts, resulting in infection of about 88% of the mouse pups based on predictions from equation 2. Therefore, using the values for the α and β coefficients from equation 1 to determine how many days of thermal exposure would result in infection of 88% of the mice (solving equation 3), the estimated numbers of days of each 24-h thermal regimen needed for a 1-$\log_{10}$ reduction in *C. parvum* oocyst infectivity were 5, 29, and 72 days for the 30, 20, and 10°C thermal regimens, respectively.

Using an inoculum containing 10^3 oocysts, which is ~14 times larger than the 50% infective dose (ID_{50}) dose, which is 70.6 oocysts (23), complete loss of infectivity occurred after 70 daily cycles of the 20°C thermal regimen and after 14 daily cycles of the 30°C thermal regimen. These lengths of time for complete loss of infectivity may provide a general guideline for the minimal amount of time needed for oocyst inactivation following exclusion of livestock before the onset of late fall or early winter rains on California rangeland (22). In contrast, despite 90 daily cycles of the 10°C thermal regimen, cryptosporidial infections still occurred in 61.5% of inoculated mice. This period of time exceeds not only the interval between runoff-generating precipitation events for western United States grazed rangelands but also the entire duration of seasonal cooler temperatures (winter) for many grazed locations in California. This work on oocyst inactivation with cooler-season temperatures complements our previous work for warmer seasons, where we demonstrated that exposure to a singe daily cycle of 40°C, 50°C, 60°C, or 70°C thermal regimens (mid-spring through mid-fall fecal pat temperatures) resulted in complete loss of infectivity for an oral dose of 10^5 oocysts in BALB/c mice, or about 1,400 times the ID_{50} (23). Although we did not investigate the effects of winter freezing on oocyst infectivity, data from previous work suggest that *Cryptosporidium* oocysts do not persist over winter under freezing conditions (31) and that 99% of oocysts exposed to soils frozen at -10°C become inactivated within 50 days whether or not freeze-thaw cycles occur (18). Prolonged freezing or repeated freeze-thaw conditions are uncommon in many locations in California utilized for livestock grazing. Hence, the cooler but above-freezing conditions common to much of grazed Califor-

TABLE 2. Percentages of intact, partially excysted, and empty (shell) oocysts induced by exposing *C. parvum* oocysts to 24-h temperature cycles

Temp treatment[a]	Length of exposure (days)[b]	% of[c]:		
		Intact oocysts	Partial oocysts	Empty oocysts
10°C cycle	0[d]	97.0 ± 1.0	2.3 ± 0.6	0.7 ± 0.6
	1	93.0 ± 1.3	4.0 ± 0.6	3.2 ± 0.8
	5	88.2 ± 1.1	5.6 ± 0.3	6.2 ± 0.7
	15	83.9 ± 1.5	7.9 ± 0.9	8.2 ± 0.9
	30	79.3 ± 2.6	9.7 ± 1.1	11.0 ± 1.5
	60	73.5 ± 3.9	11.1 ± 2.4	15.4 ± 2.1
	90	61.6 ± 1.6	20.1 ± 2.1	18.3 ± 2.2
Heat-inactivated oocysts		89.2 ± 0.8	5.7 ± 0.9	5.1 ± 0.5
20°C cycle	0[d]	96.7 ± 0.5	2.3 ± 0.6	1.0 ± 0.9
	1	86.6 ± 2.0	6.5 ± 2.0	6.8 ± 2.0
	5	64.6 ± 2.0	17.7 ± 1.2	17.7 ± 3.1
	10	43.8 ± 6.0	47.2 ± 6.4	9.0 ± 1.3
	28	28.5 ± 5.4	36.4 ± 4.8	35.0 ± 5.8
	56	10.2 ± 2.5	68.08 ± 4.9	21.8 ± 2.5
	70	0	80.7 ± 6.5	19.3 ± 6.5
Heat-inactivated oocysts		90.4 ± 1.8	5.4 ± 1.2	4.2 ± 0.6
30°C cycle	0[d]	95.5 ± 1.4	2.6 ± 0.5	1.9 ± 0.9
	1	56.1 ± 3.6	31.0 ± 3.4	12.9 ± 4.0
	5	42.2 ± 3.7	47.1 ± 3.8	10.7 ± 1.3
	7	15.1 ± 2.4	65.3 ± 6.1	19.6 ± 4.2
	10	4.5 ± 1.0	60.3 ± 1.9	35.1 ± 1.1
	13	0.9 ± 0.9	48.2 ± 4.4	50.9 ± 5.3
	15	0	66.3 ± 5.0	33.7 ± 5.0
Heat-inactivated oocysts		86.0 ± 1.8	6.1 ± 0.8	7.9 ± 1.6

[a] The 24-h thermal profiles mimicked the temperatures in bovine fecal pats shown in Fig. 1.
[b] One day was equivalent to a single 24-h temperature cycle shown in Fig. 1.
[c] The percentages were determined by dividing the number of intact, partially excysted, or empty oocysts by the number of all oocyst forms and then multiplying the result by 100. The data are arithmetic means ± standard deviations.
[d] Fresh oocysts without any thermal treatment were used.

nia rangeland dramatically extend oocyst survival during the season when there are peak runoff conditions, which is typically December through March (22).

The number of days needed for complete loss of infectivity likely depends on the oocyst dose given to mouse pups, so that for larger and smaller doses longer and shorter exposures are required to achieve total loss of infectivity, respectively. This finding underscores the need to describe oocyst survival at specific temperature maxima not only as a function of time but also as a function of the oocyst dose if we are to compare results of different studies and different infectivity or viability methods (vital dyes, cell culture, etc.). Alternatively, comparing data for duration of survival obtained in different studies for similar environmental conditions might be facilitated by calculation of decimal reduction times (DRT) (the time required to reduce survival or infectivity by 1 $\log_{10}$ or 90%) if the inactivation rate follows a first-order decay function and DRT values are independent of dose for assays estimating *Cryptosporidium* infectivity.

The temperature exposure regimens used in this study mimicked the naturally occurring daily fluctuations of temperature experienced by oocysts in bovine fecal material exposed to solar radiation (Fig. 1). Our results were relatively consistent with the results of previous work that used constant temperatures to study thermal inactivation of *C. parvum* oocysts. For example, approximately 30% of oocysts in natural mineral waters remained viable after 12 weeks of incubation at 20°C, as assessed by using inclusion or exclusion of fluorogenic vital dyes (26). Oocysts in reservoir water remained infective for at least 3 months when they were stored between 4°C and 15°C (20), as assessed by a cell culture-TaqMan PCR assay. In sterile distilled water oocysts survived for 10 weeks at 25°C, as assessed using a mouse infectivity assay (27).

Consistent with our previous work (23), the primary mechanism of oocyst inactivation was partial or complete excystation in the environment (Table 2) and presumably rapid inactivation of the released sporozoites. All fresh oocyst inocula contained >95% intact oocysts. The number of days needed for 100% loss of intact oocysts coincided closely with the period of time needed for complete loss of infectivity, despite the fact that 60 to 80% of the original 1,000 oocysts were partially excysted when they were inoculated into the mice, indicating their lack of infectivity (Table 2). Apparently, repeated cycles of daily warming and cooling, like those that occur naturally in fecal deposits exposed to daily doses of solar radiation (Fig. 1), result in partial excystation of substantial numbers of oocysts (Table 2), and either eventual death or some other inactivating mechanism occurs for the sporozoites that are retained within the oocysts.

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