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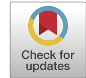
Research Paper

Aqueous Ozone Exposure Inhibits Sporulation in the *Cyclospora cayetanensis* Surrogate *Eimeria acervulina*



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ABSTRACT

Ozone is a potent disinfecting agent used to treat potable water and wastewater, effectively clearing protozoa such as *Giardia* and *Cryptosporidium* spp. It is unclear whether ozone treatment of water or fresh produce can reduce the spread of the emerging parasite *Cyclospora cayetanensis*, which causes cyclosporiasis in humans. Obtaining viable *C. cayetanensis* oocysts to evaluate inactivation methods is challenging because we lack the means to propagate them in vitro, because of delays in case reporting, and because health departments typically add inactivating fixatives to clinical specimens. Research in various surrogate organisms has sought to bolster understanding of the biology of *C. cayetanensis*. Among these surrogates is the poultry parasite *Eimeria acervulina*, a closely related and easily cultured parasite of economic significance. We used this surrogate to evaluate the consequences of ozone treatment, using the sporulation state as an indicator of infectious potential. Treating with ozonated water acidified with citric acid reduced sporulation ability in a dose-dependent manner; treatment with up to 4.93 mg/L initial concentration of ozone resulted in a 93% inactivation of sporulation by 7 days posttreatment. This developmental arrest was accompanied by transcriptional changes in genes involved in regulating the response to reactive oxygen species (ROS) in a time course that is consistent with the production of oxygen free radicals. This study shows that ozone is highly effective in preventing sporulation of *E. acervulina*, a model coccidian used as a surrogate for *Cyclospora*. Furthermore, ozone exposure induced molecular responses to general oxidative stress, documented with several well-characterized antioxidant enzymes.

Cyclospora cayetanensis is a protozoan parasite that causes the human gastrointestinal disease cyclosporiasis, inducing diarrhea, dehydration, and weight loss. Immunocompromised patients and children are particularly vulnerable to severe symptoms associated with cyclosporiasis (Sarfo et al., 2022). While previously associated with travel to endemic regions, increasing numbers of cyclosporiasis outbreaks have been reported in the United States in recent years. The first domestic report of likely cyclosporiasis occurred in Chicago in 1990 and was tentatively linked to a contaminated water source in a physicians' dormitory (Huang et al., 1995). Outbreaks in 1997, 1998, and 1999 were reported in North America, though the infectious vehicle was not conclusively identified (Herwaldt, 2000). A 1997 multistate outbreak was associated with Guatemalan raspberries (Herwaldt et al., 1999). Following this, in 1998 the United States, but not Canada, banned the importation of Guatemalan raspberries, resulting in a multi-cluster outbreak in Ontario, Canada, with no corresponding outbreaks in the United States (CDC, 1998). Additional outbreaks in 1997 were linked to mixed lettuce (mesclun) products (Herwaldt, 2000) and fresh basil (CDC, 1997). In 1999, an outbreak in Missouri was linked epidemiologically to contaminated basil, representing the first instance in which *Cyclospora* was identified on the associated vehicle (Lopez et al., 2001). In 2013 a multi-state outbreak was traced back to bagged salad and cilantro (CDC, 2013), with multistate outbreaks recorded in nearly every subsequent year. *Cyclospora*

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was first identified on domestically grown cilantro in 2018 (Almeria et al., 2019). While this instance was not associated with an outbreak, this highlights an urgent need for research on this emerging pathogen.

Several operational hurdles complicate efforts to study *Cyclospora* biology. The scarcity of viable oocysts for evaluating disinfection measures is a major obstacle since there is currently no *in vitro* method to culture *C. cayetanensis*. A previous attempt to propagate the parasite in willing human volunteers was unsuccessful (Alfano-Sobsey et al., 2004). Furthermore, the submissions through public health departments that serve as the primary source for *C. cayetanensis* samples are typically subject to chemical fixation and preservation, rendering them unsuitable for inactivation assays or transcriptional studies.

Recent efforts to hasten progress in studying *Cyclospora* biology have focused on using the surrogate *Eimeria acervulina*, a close relative of *C. cayetanensis* that causes coccidiosis in poultry (Tucker et al., 2022). Several aspects of *E. acervulina* biology support this approach. Importantly, *E. acervulina* infects regions of the chicken digestive tract that are comparable to infection observed by *C. cayetanensis* in the human digestive tract. Like *C. cayetanensis*, the spread of avian coccidiosis occurs via fecal-oral transmission, with shedding of *E. acervulina* oocysts in the feces of infected birds reaching the infective, sporulated stage in the environment given the right temperature, moisture, and O_2 conditions. The structures that protect *C. cayetanensis* from environmental assaults (chemically resistant oocyst and sporocyst walls) are shared by the *Eimeria* surrogate. Of practical importance, *E. acervulina* is harmless to human researchers and can be propagated in great quantities in poultry. Ingestion of a single oocyst leads to the excretion of 10^3–10^4 oocysts when passed through an avian host (Tucker et al., 2022). *E. acervulina* therefore represents an exceptional experimental platform to study the effects of decontamination procedures for fresh produce, such as the use of ozone.

Ozone is a powerful oxidizing agent that is commonly used for water disinfection and treatment. The ozone molecule is unstable and decomposes rapidly in water. The rapid release of free radicals oxidizes organic and inorganic compounds, making ozone an effective antimicrobial for a wide range of microorganisms, including viruses, bacteria, and protozoan parasites such as *Giardia* (Morrison et al., 2022) and *Cryptosporidium* (Pereira et al., 2008). Ozonated water was shown to reduce the incidence of *Giardia* contamination in drinking water (Kondo Nakada et al., 2020) has proven effective to inactivate *Cryptosporidium parvum* (Morrison et al., 2022), and has shown some promise in reducing sporulation efficiency in *Eimeria* (Morgoglione et al., 2021). Khalifa et al., achieved suppression of *Cyclospora* infectivity following treatment with 1 ppm ozone at various contact times (Khalifa et al., 2001). Several studies have achieved ≥3 log reduction of pathogenic bacteria on berries, peppers, and leafy greens washed with ozonated water (Achen & Yousef, 2001; Bialka & Demirci, 2007; Beltrán et al., 2005). In addition to its disinfecting properties, aqueous and gaseous ozone treatment has demonstrated benefits to produce, including but not limited to the reduction of several types of pesticide residues (Glowacz et al., 2014). Further, strawberries treated with ozone exhibited enhanced textural qualities during storage (Piechowiak et al., 2022). However, as exposure time increases, deleterious consequences begin to arise, such as a reduction in vitamin content or the development of unwanted cosmetic traits (reviewed in Sachadyn-Król & Agriopoulou, 2020).

Here, the effect of aqueous ozone treatment on *E. acervulina* sporulation was evaluated. While sporulation does not always correspond with infectivity, *Cyclospora* oocysts are only infective when sporulated. There are no animal models or *in vitro* culture methods currently developed for *Cyclospora* (Ortega et al., 1998). We therefore relied on the distinct morphological incongruence between unsporulated and sporulated oocysts is readily apparent via light microscopy and used the proportion of sporulated to total oocysts as a metric to evaluate the disinfection capacity of ozone across a range of doses.

Methods

Eimeria purification. HR308 broiler chicks (Longeneckers Hatchery, Elizabethtown, PA) were inoculated with *Eimeria acervulina* oocysts following standard protocols (Tucker et al., 2022). Maintaining *E. acervulina* oocyst viability requires purification from chicken feces immediately upon arrival, as sporulation efficiency rapidly diminishes when stored in source material. Therefore, infected chicken feces were shipped overnight to the Center for Agricultural Synthetic Biology at the University of Tennessee and mixed vigorously, upon receipt, with tap water at a ratio of approximately 1:5 and further mixed into suspension for 30 min using a magnetic stir bar. The resulting mixture was then passed through cheesecloth placed in a funnel and collected in a large beaker. Aliquots were provisioned into 50 mL conical tubes and centrifuged at 935 × *g* for 10 min using a Thermo Scientific TX-1000 rotor (Thermo Scientific X Pro Series, r = 20.9 cm). The pellets were resuspended in saturated sodium chloride (360 g/L), centrifuged again at 935 × *g* for 10 min, and allowed to sit for 10 min. The top fractions were collected, washed with water, and centrifuged at 1460 × *g* for 15 min. This step was repeated twice more to remove residual salt. Finally, the pellets were combined by resuspending in water.

Ozone generation, treatment, and residual measurements. Ozone was produced using an Aqua-8 portable ozone generator (A2Z Ozone Inc.) by passing pure oxygen through the device. The generated ozone was bubbled through a 1-inch diffuser into a graduated cylinder containing 500 mL of sterile water. Ozone depletion was measured by spectrophotometry (Hach DR1900) with AccuVac ampules containing indigo trisulfonate at 10-min intervals for at least 40 min. To quantify the rate of ozone depletion over time, curves were generated by measuring one concentration in tap water and Milli Q deionized water. The addition of 12.1 g citric acid monohydrate to a final concentration of 0.2 M and a pH of 1.925 stabilized the rate of ozone decomposition to first-order kinetics in Milli Q water. The initial ozone concentration of this solution was measured as 4.93 mg/L after a 6-min sparge (bubbling ozone through a porous diffuser into the water for 6 min). Immediately after sparging, ozonated water was added in a 14:1 ratio to suspended *Eimeria* oocysts. Fourteen mL of ozonated water was added to 1 mL of suspended *Eimeria* oocysts (concentration: 2.6×10^8 oocysts/mL) in 10-min intervals during dose (mg/L) measurement, with three biological replicates for each sample. Thus, as the concentration of ozone diminished over time, each oocyst batch received a successively lower dose than the previous, generating a dosage curve. Each replicate group received a noted initial dose, which was allowed to sit for 1 h as the ozone concentration dropped to a negligible amount. After treatment, the oocyst suspensions were centrifuged at 935 × *g* and resuspended in 2.5% potassium dichromate for sporulation. Oocysts were then incubated at 25°C for 7 days until imaging to assess the proportion that matured to the sporulated state.

Gene Targets. Candidate genes were identified using Gene Ontology (GO) searches for categories relevant to ozone response, response to reactive oxygen species (ROS), and regulation of response to ROS. BLASTx searches were used to identify cognate *E. acervulina* genes using mouse or human ortholog queries. An initial set of 23 targets was chosen and further refined to include those genes whose products have obvious roles in mediating response to oxidative stress. BLAST hits that generated unannotated or hypothetical protein results were subject to further reclassification via Conserved Domain Database (CDD; Marchler-Bauer et al., 2011) searches.

RNA isolation and quantitative real-time PCR. RNA was collected from three groups each of treated and control samples at t = 0, 6, and 18 h after ozone exposure. After centrifugation to concentrate oocysts and remove ozonated water, pellets were resuspended in Trizol. Oocyst walls were disrupted using glass beads in an MP Bio bead beater (FastPrep-24 5G, MP Biomedicals) at a velocity of 6 m/s for

60 seconds. RNA was isolated using the Zymo RNA miniprep kit per the manufacturer's instructions. RNA isolates were then subject to purification using the RNA Clean and Concentrate kit from Zymo. Quantitative real-time PCR was performed using the PrimeScript One-step RT kit from Takara on an ABI QuantStudio (3) for 40 cycles with a melting temperature of 60°C. Ct values were normalized to the expression of EF2 as a reference gene and relative quantification was performed using the delta-delta Ct method (Livak & Schmittgen, 2001). Primer sequences are listed in Table 1.

Reactive oxygen species assays. Production of ROS was measured using three separate assays produced by Invitrogen. These included the ROS assay, the Amplex Red Hydrogen Peroxide/Peroxidase assay, and the MitoSOX Red mitochondrial superoxide indicator kits. All assays were performed according to the manufacturer's instructions except for the total ROS kit, for which fluorescence detection on a Cytation 5 (Agilent Technologies) plate reader was substituted for flow cytometry. For this assay, autofluorescence of untreated *Eimeria*, as well as the dichromate substrate, was measured based on work demonstrating that aged *E. maxima* show enhanced autofluorescence in the GFP range during prolonged storage (Beer et al., 2018). For each assay, oocysts were centrifuged in a Fisher Scientific accuSpin Micro 17 at 17,000 × *g* for 5 min and washed to remove residual ozonated water. For the MitoSOX assay specifically, the supernatant was collected and assayed to ensure extracellular superoxide was not detected.

Statistical Analysis. Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS version 29. One-way ANOVA with Tukey's HSD was used to measure differences among groups with $p < 0.05$ considered statistically significant.

Results and discussion

Ozone stabilization and effects on oocyst sporulation. Ozone spontaneously decomposes in water, with its breakdown strongly influenced by various factors including pH, temperature, dissolved organic matter, and the presence of inorganic ions. At low pH, ozone is more stable and reactive. In acidic conditions, the ozone molecule is protonated and exists in the form of ions such as $HOOO^-$ and H_2OO^{2-}, which can react more readily with organic compounds and pathogens, leading to increased disinfection efficiency. Ozone depletion in water was highly dependent on dissolved minerals and/or total organic content (TOC), as well as pH, as previously demonstrated (for reviews, see Kim et al., 1999; Guzel-Seydim et al., 2004). Indeed, in tap water, a precipitous drop was initially seen between $t = 0$ and $t = 10$ min, consistent with the phenomenon of the immediate ozone demand (IOD; Xu et al., 2002) zone (Fig. 1a). In contrast, ozonated deionized water generated using a MilliQ system showed that this steep initial decrease was substantially attenuated but not completely eliminated (Fig. 1b). The addition of citric acid monohydrate to a final concentration of 0.2 M per Hirahara et al. (2019) eliminated the IOD zone and provided a linear decomposition curve (Fig. 1c, d). Consistent with oocysts tolerating acidic conditions in the host alimentary canal, no effect on sporulation capacity was observed in a previous experiment, wherein oocysts were treated with 1 M HCl (data not shown). Therefore, the addition of citric acid was not expected to influence the sporulation rate.

After ozone decomposition was stabilized, *Eimeria acervulina* oocysts were treated with a range of initial ozone doses, from 0 (controls) to 4.93 mg/L, to evaluate ozone's impact on sporulation. Unsporulated *E. acervulina* oocysts are ovoid structures that contain a round cytoplasmic mass surrounded by a protective oocyst wall. Following sporulation, oocysts harbor four distinct, round sporocysts and this morphology is readily distinguishable via light microscopy (Fig. 2a, b). Sporulation efficiency was scored by imaging 96-well plates containing treated oocysts under 40× magnification on an

Table 1
List of primers used for qRT-PCR. Target name is listed according to Genbank annotation status. Reference for primer sequences: this study (all)

Target	Primer name	Primer Sequences (5′-3′)
XM_013396718.1 *Eimeria acervulina* cation-transporting ATPase, putative partial mRNA; EAH_00000740	OZ10_F OZ10_R	GAAGCAAAAAGGCTTCATCG ACCCACAGAACAGCAAATCC
Calcium-dependent protein kinase, related EAH_00014830	OZ12_F OZ12_R	GTCGATTTCGGTCTCTCTGC TCCCTCTCTCAACACGCTTT
Tyrosine kinase-like (TKL) protein, putative EAH_0000640	OZ13_F OZ13_R	AGTTGCGGCTAAGGACAAGA TTCCGCTGTGACTTGAGATG
AGC kinase, putative EAH_00006130	OZ15_F OZ15_R	CGCATGCACTTACAGGAGAA ACCAAATACAAACGCGAAGG
XM_013391092.1 *Eimeria acervulina* superoxide dismutase, putative partial mRNA EAH_00053040	Sod1_F Sod1_R	CTATGAAGGCAGGAGGAGGG TCCGTCGTGTGTCTGTGTAA
XM_013395273.1 *Eimeria acervulina* Superoxide dismutase, related EAH_00009710	Sod2_F Sod2_R	GCAGCTGTTGTGGGGTTTTA AGACAACTGAAGCCCCATCA
Eimeria acervulina peroxiredoxin EAH_00047270	74 75	TCCCTCTTCTTGCAGACGTT GGCAGACGTCTCCGTACTTC
Eimeria acervulina peroxiredoxin 2 EAH_00058070	76 77	TCCATCCTTTACCCAGCAAC CAGGTAAGCCTTGCCAGAAG
peroxisomal catalase, putative EAH_00041420	catF catR	AACGGGGCATCATAGACAAC CACGTGCAAACAGCTTCACT

Olympus Fv1000 confocal microscope. The number of fully sporulated oocysts with distinguishable sporocysts was counted and expressed as a percentage of the total number of oocysts in each field of view.

Liou and colleagues (2002) previously achieved partial inhibition of sporulation in *E. colchici* oocysts challenged with ozone, where lower sporulation ratios and increased survival in birds inoculated with treated oocysts were observed. Inhibition of sporulation was 18.4% in oocysts treated for 15 min and 28.3% in oocysts treated for 60 min (Liou et al., 2002). A more recent study achieved a 23.7% suppression of *Eimeria* spp. following treatment with 2 mg/L ozone for 5 min (Morgoglione et al., 2021). In the present study, dose-dependent sporulation inhibition was observed. At the highest ozone dose of 4.93 mg/L, 93% inhibition of sporulation was achieved by day 7 compared to non-treated controls, and this inhibition activity decreased accordingly with lesser ozone doses (Fig. 2c).

Ozone is a more effective antimicrobial at low pH since its stability is increased. Thus, the effect of buffering aqueous ozone with citric acid may play a role in the relative increase in efficacy achieved in this study. No gross morphological changes to the oocyst wall were readily apparent via confocal microscopy following ozone exposure and incubation. While sporulation at 7 days posttreatment was used as the assay condition, the possibility exists that sporulation was delayed rather than completely blocked. In the context of *E. acervulina* as a proxy for *C. cayetanensis*, which is distributed on fresh produce, a substantial heterochronic shift in time to sporulation remains an effective disinfection method since fresh produce must be consumed before spoilage.

Molecular response to ozone. The mechanism through which ozonation blocks sporulation in *Eimeria* has not been extensively studied. Bacterial aggregation along distinctly misshapen oocyst walls was previously reported for ozone-treated *E. colchici* (Liou et al., 2002). A similar phenomenon of fast attachment of bacteria to the oocyst wall was observed in ozone-treated *Eimeria* spp. oocysts with no external morphological damage (Morgoglione et al., 2021). Bacterial accumulation on treated oocysts was likewise noted in this study, as well as subtle warping of the oocyst wall versus controls, which showed entirely smooth oocysts with no surface anomalies (Fig. 2a, b). To study how ozone influences *E. acervulina* physiology at the molecular level, ROS assays were combined with qRT-PCR targeting transcripts involved in response

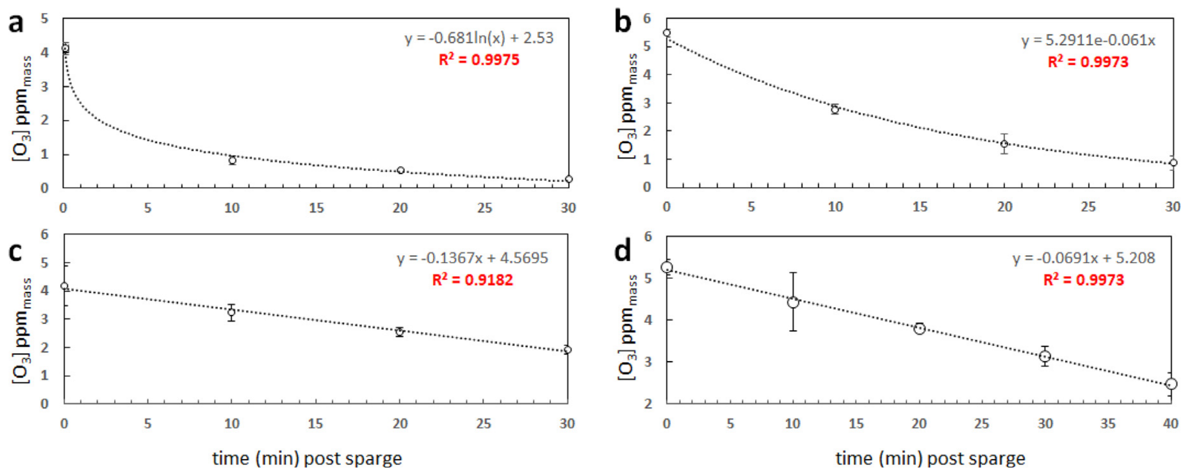

Figure 1. Ozone decomposition in water. a) tap water, pH = 7.0, 3-min sparge; b) MilliQ deionized water, pH = 6.8, 3-min sparge. c) MilliQ deionized water with the addition of citric acid monohydrate to a final concentration of 0.2 M, 3-min sparge, pH = 1.9; d) MilliQ deionized water + citric acid monohydrate to 0.2 M, pH = 1.9, 6-min sparge, data shown to 40 min. This instance was used to generate ozone doses to inactivate *E. acervulina*, including 4.93, 4.44, 3.68, 2.48, 1.26, 0.78, and 0 mg/L. Circles indicate average ± standard error.

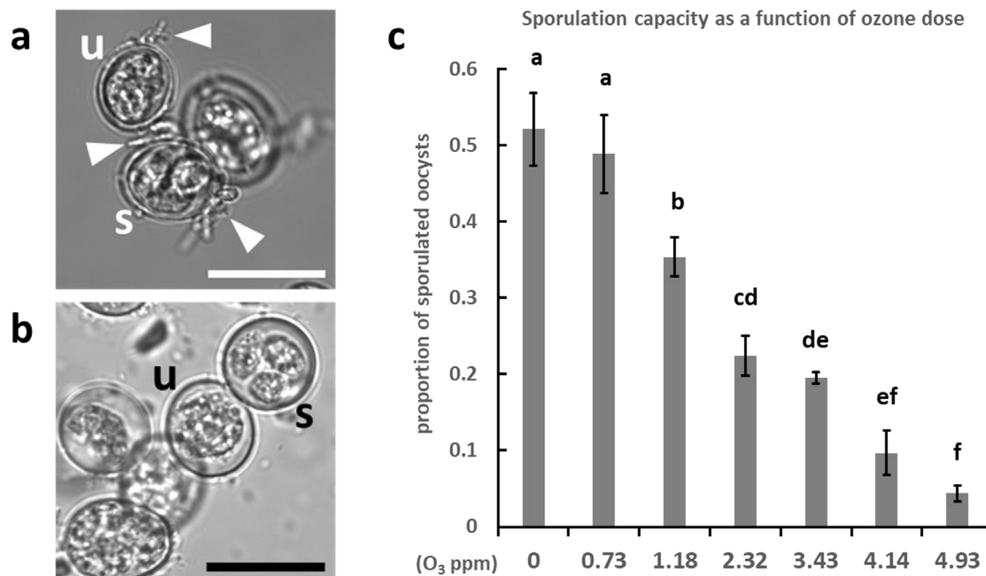

Figure 2. Effect of ozone on sporulation of *Eimeria* oocysts. a) Oocysts treated with approximately 4 mg/L ozone; u: unsporulated; s:sporulated. Arrowheads: bacterial aggregation along oocyst wall, which is subtly misshapen relative to controls shown below. b) Control oocysts received no aqueous ozone. Scale bars: 20 uM. c. proportion of sporulated oocysts relative to ozone dose according to the decomposition kinetics obtained following a 6-min sparge in MilliQ water buffered with citric acid monohydrate to pH = 1.925. Bars represent the mean ± standard error for each initial O_3 dose in parts per million (mg/L; mg/L). Letters above bars indicate $p < 0.05$, repeated one-way ANOVA.

to oxidative stress. Following a challenge with an initial dose of approximately 4 mg/L ozone that was allowed to sit for 1 h, RNA was collected from oocysts at t = 0, 6, and 18 h postexposure for targeted qRT-PCR transcriptional profiling. According to a total ROS assay, the production of oxygen free radicals increased throughout the period of 0–24 h following ozone treatment (Fig. 3b). Conversely, both H_2O_2 and mitochondrial superoxide accumulation decreased during the analysis window, while the abundance of transcripts for genes involved in scavenging free radicals and peroxides increased (Fig. 3a, b).

Peroxiredoxin 1 and 2: EAH_00047270 and EAH_00058070. Peroxiredoxin (Prx) family members are widely represented in all living things and function as H_2O_2 scavengers, catalyzing the conversion of peroxides into water and alcohols. Two Prx family members were identified using BLASTp searches against the *E. acervulina* genome and the expression of each was induced by ozone. Prx I was upregulated in ozone-treated oocysts relative to controls and this elevated expression lasted through the 18-hour time point, with the highest expression evident immediately following exposure at t = 0 h (Fig. 3a, bottom). In mice, $Prx1^{-/-}$ mutants displayed significantly less pulmonary tissue inflammation following ozone exposure than their wild-type congeners, indicating a cellular role for PrxI in mediating the innate immunological response to ozone (Yanagisawa, et al., 2012). Prx 2 expression was also induced by ozone challenge. Transcript abundance was significantly higher than non-treatment controls extending to 18 h, at which point the expression was elevated, but not significantly different from controls (Fig. 3a). In apple fruit treated with ozone, accumulation of 2-Cys Prx protein was observed relative to control fruit (Testempasis et al., 2021), indicating a conserved role for Prx family members' upregulation in response to ozone.

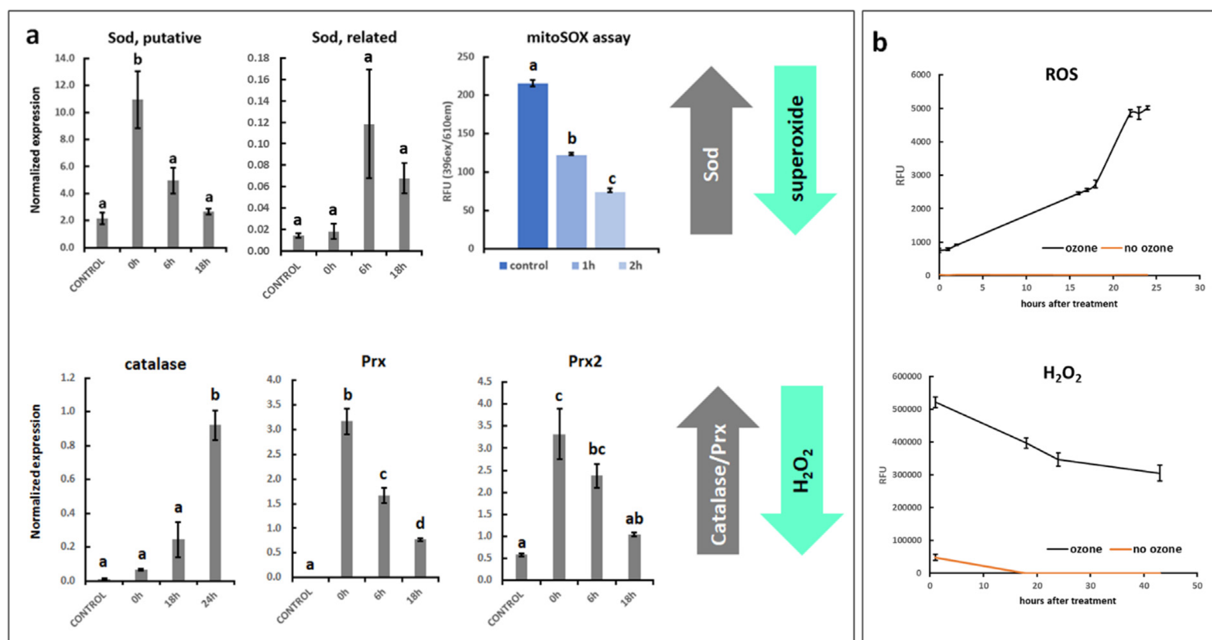

Figure 3. Quantitative PCR results for superoxide dismutase (Sod), catalase, and peroxiredoxin (Prx) transcripts in relation to the intracellular production of oxyradicals and peroxide. a) profiles for several antioxidant enzymes presented with the mitoSOX assay, a readout of mitochondrial superoxide formation. Sod levels are highest at 0–6 h postozone treatment, consistent with reduced superoxide detection in samples exposed to ozone and assayed at 1- and 2 h postexposure. An increase in catalase and Prx expression is concomitant with a reduction in the detection of intracellular peroxide. The mitoSOX readout was limited to 2 h per manufacturer's recommendations. 0 h time points represent the first RNA collection after a 1 h exposure to ozone. b) Temporal profiles of total intracellular reactive oxygen species (top) and hydrogen peroxide (bottom) over the course of 24- and 48 h, respectively. Letters above bars indicate $p < 0.05$, one-way ANOVA. Bars indicate average ± standard error ($n = 3$).

Superoxide dismutase: EAH_00053040 and EAH_00009710. Three major types of superoxide dismutase (SOD), metalloenzymes that catalyze the conversion of superoxides to molecular oxygen and H_2O_2, have been characterized. Every aerobic organism has a set of superoxide dismutase genes. The product of Sod1 is a Cu/Zn variant located in the cytosol, but the presence of H_2O_2 translocates the protein to the nucleus where it acts as a transcription factor (Tsang et al., 2014). Sod2 localizes in the mitochondria and is a Mn or Fe dismutase, while Sod3 was identified as an extracellular form. SOD1 and SOD2 expression was upregulated in response to ozone in rat lung tissue (Rahman et al., 1991). SOD1 tends to be expressed at the highest levels and acts in the largest cellular compartment.

Two Sod annotations were identified in the *E. acervulina* genome: superoxide dismutase, putative (EaSodp) and superoxide dismutase, related (EaSodr). Quantitative RT-PCR analysis showed that EaSodp transcripts were significantly upregulated upon ozone treatment (Fig. 3a, top). According to the Amplex Red Hydrogen peroxide assay, peak H_2O_2 production coincided with maximal EaSodp expression, and both H_2O_2 and EaSodp levels decreased during the postexposure period (Fig. 3a, b). UniProtKB/TrEMBL conserved domains indicate that EaSodp contains Fe or Mn superoxide dismutase domains, suggesting that this annotation may be a mitochondrial SOD. Supporting this notion, mitochondrial superoxide levels, assayed using the MitoSOX kit, were decreased in ozone-treated oocysts, in which the expression profile of EaSodp was shown as elevated relative to control oocysts (Fig. 3a, b).

EaSodr showed substantially lower expression than EaSodp. Furthermore, expression immediately after the ozone challenge for EaSodr was not statistically different from controls (Fig. 3a, top). The relative differences in expression profiles between these identified sod variants support functional divergence.

Peroxisomal catalase, putative: EAH_00041420. Catalase converts H_2O_2 to water and oxygen and showed significant upregulation at 24 h following ozone treatment (Fig. 3a, bottom). EAH_00041420, annotated as a putative peroxisomal catalase (EaCat), was identified in the *E. acervulina* genome, and its expression profile was assayed through 24 h. Catalase induction by ozone, along with SOD, was reported in *E. coli* by Whiteside & Hassan (1987), in which catalase was more responsive than SOD to ozone exposure. Likewise, catalase plays a protective role in *Listeria monocytogenes* in protecting cells from ozone, but mutant strains showed that catalase plays a less significant role than SOD. However, a SOD overexpressing strain lacking catalase was hypersensitive to ozone, highlighting the role of catalase in protecting against oxidative stress (Fisher et al., 2000).

Cation transporting ATPase EAH_00000740. In addition to genes whose products are directly involved in mediating the response to oxidative stress, we identified other target transcripts via GO searches using terms relative to ozone, response to oxygen radicals, and oxidative stress. EAH_00000740, annotated as *Eimeria acervulina* cation-transporting ATPase, harbors a HAD (haloacid dehalogenase) family domain. This superfamily includes ATPases, and Cation transporting ATPase shows close sequence identity with a *Toxoplasma gondii* P-type ATPase. This transcript peaked at 0 h posttreatment, remained high through 6-hours posttreatment, and attenuated by 18 h after ozone exposure (Fig. 4a). P-type ATPases are cation-transporting, membrane-bound proteins with demonstrated roles in maintaining cation homeostasis under oxidative stress, for instance in human erythrocytes treated with ozone (Tükel, 1994). Notably, Na+/K+-ATPase abundance was increased in mice exposed to ozone (Hulo et al., 2011). Unifying this superfamily is the HAD signature motif: DK[TS]GT[LIVM][TS], represented in the *E. acervulina*, *E. mitis*, and *E. necatrix* orthologs as DKTGTLT, which is likewise conserved in the identified *C. cayetanensis* ortholog, LOC34624115.

Tyrosine kinase-like protein: EAH_0000640. Transcripts for EAH_0000640 (EaTKL) showed upregulation beginning at 0 h postozone exposure and this level of expression was maintained through 6 h, finally attenuating at 18 h postexposure to levels statistically similar to controls (Fig. 4b). The protein encoded by this gene contains a

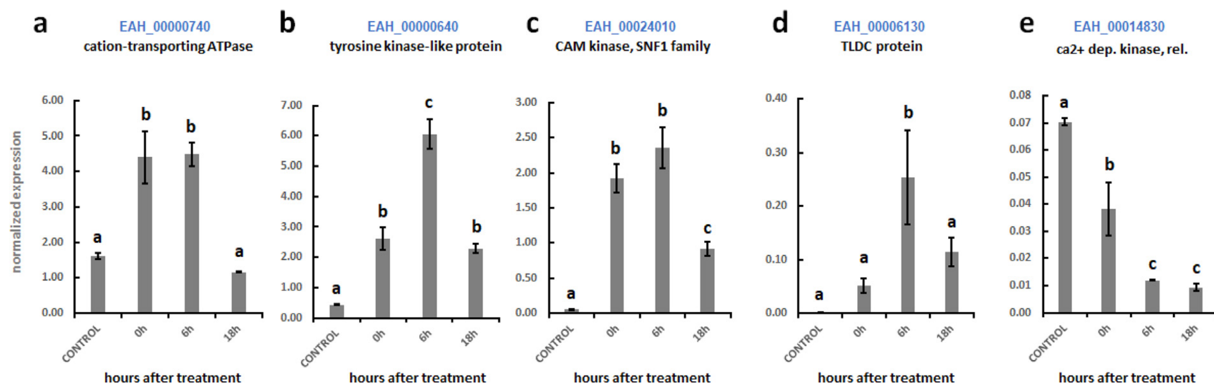

Figure 4. Time courses of the expression of genes identified using GO searches for "response to ozone" and "oxidative stress" are shown with their EAH annotation numbers. 0 h time points represent the first RNA collection after a 1 h exposure to ozone. Letters above bars indicate $p < 0.05$, one-way ANOVA. Bars indicate average ± standard error ($n = 3$).

PKc-like superfamily domain. Proteins containing this domain catalyze the transfer or a phosphoryl group from ATP to serine, threonine, or tyrosine residues of other proteins. BLASTp searches against mammalian genomes identified mitogen-activated protein kinase kinase kinase (MAPKKK) as the closest mammalian, zebrafish, and *Drosophila* ortholog. MAPK7, also known as ERK5, is activated in response to oxidative stress (Abe et al., 1996) and is implicated in antiapoptotic signaling (Pi et al., 2004). Increased transcript abundance for EAH_0000640 in response to ozone stress suggests a cellular attempt at mitigating apoptotic activity via downstream caspases (Pi et al., 2004).

CAM kinase, SNF1 family, putative: EAH_00024010. The expression of CAM kinase, SNF1 family, putative (EaCSFp) showed significant upregulation from 0 to 18 h postozone challenge relative to controls (Fig. 4c). Conserved domain searches identified this gene product as harboring a CAM kinase domain, characteristic of stress-inducible kinases that monitor intracellular energy stores. CAM kinases serve as master regulators of energy homeostasis. SNF1 is a well-characterized family member in yeast that responds to environmental stress. When yeast were treated with a selenium compound to induce oxidative stress and DNA damage, SNF1 was required to maintain the ratio of reduced to oxidized glutathione (Pérez-Sampietro et al., 2013). Thus, EaCSFp upregulation in response to the ozone challenge likely reflects the need for this protein in protecting against oxidative damage to DNA, or activity in other pathways that regulate intracellular energy stores under physiological stress.

Oxidation Resistance 1: EAH_00006130. TLDc [Tre2/Bub2/Cdc16 (TBC), lysin motif (LysM), domain catalytic] domain-containing proteins function to protect against oxidation-related stress in eukaryotes (Finelli & Oliver, 2017). Their mode of action remains unclear, but it was recently shown that TLDc proteins interact with V-ATPase (Eaton et al., 2021), a transmembrane proton pump with demonstrated activity in protecting against oxidative damage (Thorpe et al., 2004). Oxidation resistance proteins (OXR) are key members of this superfamily, and the C-terminal TLDc domain is sufficient to render OXR as fully functional to combat oxidative stress. BLASTp searches using the amino acid sequence for the polypeptide encoded by EAH_00006130 yield OXR1 annotations from zebrafish, human, snowberry fruit fly, and cotton, identifying this protein as an *E. acervulina* OXR1 ortholog (EaOXR1). Oxidation Resistance 1 (OXR1) is a TDLc domain-containing protein whose expression was induced following ozone challenge in *E. acervulina*, with transcript abundance peaking between 6 and 18 h posttreatment (Fig. 4d). The expression profile for the catalase lags the OXR1 expression profile, which peaks at 6 h. EaOXR1 transcripts were induced by ozone in this study. In this study, peak EaOXR1 expression at 6 h may likewise influence catalase levels in *E. acervulina* in response to oxidative insult. Five annotations consisting of three unique *C. cayetanensis* annotations were obtained via BLASTp searches; four of these annotations comprised two sets of duplicate sequences with different names. A multiple alignment of amino acid sequences supports *C. cayetanensis* LOC34621889 as the CcOXR1 ortholog.

Calcium-dependent protein kinase, related: EAH_00014830. The protein product of EAH_00014830 which was downregulated relative to controls from 0 to 18 h posttreatment (Fig. 4e) contains an STKc_CAMK and a Ca^{2+} binding EF-hand superfamily domain. STKc_CAMK members are serine/threonine kinases, and this family includes the well-characterized CamKII. This protein, which is activated by ROS (Anderson, 2015), is a critical sensor of oxidative stress in cardiac tissue (Swaminathan et al., 2012), where sustained CamKII expression is linked to apoptotic pathways. Calcium-dependent protein kinase, related downregulation in treated oocysts, suggests a cellular attempt to mitigate spurious or enhanced apoptosis in coordination with both the upregulation of the presumed antiapoptotic Calcium-dependent protein kinase, related and a suite of antioxidant enzymes under oxidative stress.

In this study, the efficacy of aqueous ozone to disrupt sporulation in a model coccidian was demonstrated. Consistent with previous reports (Liou et al., 2002), bacterial accumulation and mildly malformed oocyst walls were observed, characterized by subtle warping of the near-perfect ovoid structure. A general cellular response to oxidative insult was supported by the upregulation of several enzymes and proteins involved in protecting against oxygen free radicals. The coordinated expression of antioxidant enzymes whose established roles deal specifically with detoxifying oxygen free radicals indicates a cellular attempt to mitigate the deleterious effects of excess reactive oxygen species. Recent genotyping analysis of *C. cayetanensis* resulted in taxonomic distinctions among three species, *C. cayetanensis*, *C. ashfordi*, and *C. henanensis* (Barratt et al., 2023). Despite this new distinction, the findings of this work are likely applicable to these new species, particularly given the deep evolutionary conservation of ozone-responsive genes identified in this study. For instance, superoxide dismutases and peroxiredoxins are found across Archaea and Eukarya.

In contrast to methods that rely on continuous ozone exposure for disinfection, the method presented in this study reaches negligible ozone levels by approximately 1 h but nonetheless achieves superb disinfection capacity. Treatment with 4.93 mg/L ozone resulted in 93.5% relative inhibition of oocyst sporulation. This improvement over prior reports may have been made possible by the addition of 0.2 M citric acid, which stabilized the ozone in water, prolonging parasite exposure to concentrated ozone. Generally, sanitizers are effective at killing microorganisms in wash water rather than directly on produce surfaces. The ozonation method presented in this study could therefore

be used to: 1) disinfect wash water such as that coming off the produce, thereby preventing further contamination, and 2) to sanitize the equipment and facility with no residue. A pH adjustment may be warranted in the latter case to protect the longevity of sensitive machinery. Both of these avenues are future goals, but more data are needed on the effectiveness of ozone in the presence of varying levels of organic matter, shorter treatment times, varying pH, and/or controlled concentration levels.

CRediT authorship contribution statement

Aaron A. Baumann: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Addison K. Myers:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Niloofar Khajeh-Kazerooni:** Methodology, Data curation. **Benjamin Rosenthal:** Writing – review & editing, Resources, Conceptualization. **Mark Jenkins:** Writing – review & editing, Funding acquisition, Conceptualization. **Celia O'Brien:** Resources, Methodology. **Lorraine Fuller:** Resources. **Mark Morgan:** Writing – review & editing, Funding acquisition, Conceptualization. **Scott C. Lenaghan:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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