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ARTICLE

Disease Ecology

Colder temperatures augment viability of an indirectly transmitted songbird pathogen on bird feeders

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Abstract

Inanimate surfaces that are contaminated with infectious pathogens are common sources of spread for many communicable diseases. Understanding how ambient temperature alters the ability of pathogens to remain viable on these surfaces is critical for understanding how fomites can contribute to seasonal patterns of disease outbreaks. House finches (*Haemorhous mexicanus*) experience fall and winter outbreaks of mycoplasmal conjunctivitis, caused by the bacterial pathogen *Mycoplasma gallisepticum* (MG). Although bird feeder surfaces serve as an indirect route of MG transmission between sick and healthy individuals, the contributions of feeders to MG transmission in the wild will depend on how ambient temperature affects viability and pathogenicity of MG on feeder surfaces over time. Here, we used two experiments, with identical initial design, to assess such temperature effects. For both experiments, we pipetted equal amounts of MG onto replicate feeder ports held at night-day temperatures representing summer (22–27°C) or winter (4–9°C). We allowed MG to incubate on feeders at either temperature and swabbed remaining inocula from surfaces at 0, 1, 2, 4, or 7 days post-inoculation of the feeder, with each replicate feeder port only swabbed at a single time point. In the first study, we analyzed swabs using a culture-based assay and found that MG incubated at colder versus warmer temperatures maintained higher viability on feeder surfaces over time. In the second study, we replicated the same experimental design but used MG swabs from feeder surfaces to inoculate wild-caught, pathogen-naïve birds and measured resulting disease severity and pathogen loads to determine pathogenicity. We found that MG remained pathogenic on feeder surfaces at cold ambient temperatures for up to one week, much longer than previously documented. Further, MG was significantly more pathogenic when incubated on feeders in colder versus warmer temperatures, with the strongest effects of temperature present after at least four days of incubation on feeder surfaces. Overall, cold ambient temperatures appear to

alter the role of fomites in the MG transmission process, and temperature likely contributes to seasonal disease dynamics in this system and many others.

KEYWORDS
ambient temperature, environmental persistence, fomite transmission, house finches, *Mycoplasma gallisepticum*, pathogenicity, viability

INTRODUCTION

Environmental transmission is a key route of pathogen spread for numerous infectious diseases in both humans and wildlife (Lange et al., 2016; Stephens et al., 2019). Even directly transmitted pathogens (i.e., respiratory viruses) can show notable transmission through indirect pathways when such pathogens survive for sufficiently long on the surfaces of objects, known as fomites (Kraay et al., 2018; Kramer et al., 2006). The relative contributions of fomite transmission to pathogen spread rely on the pathogen's ability to survive long enough on a given surface to encounter and then establish in a susceptible host (Breban, 2013). However, despite the importance of abiotic factors such as temperature to such pathogen survival on fomite surfaces (Riddell et al., 2020), we lack empirical studies of how factors such as temperature influence pathogen survival on fomites for the majority of host–pathogen systems (Lange et al., 2016). Such studies are critical for understanding the role of indirect transmission for pathogen spread and for predicting the effects of seasonal and long-term changes in temperature on host–pathogen dynamics (Altizer et al., 2006; McCallum et al., 2017; Pascual et al., 2002; Wißmann et al., 2021).

Abiotic factors such as temperature are particularly interesting to study for pathogens characterized by seasonal outbreaks because temperature may contribute to seasonality by altering environmental persistence of pathogens. For example, colder temperatures have been associated with increased viability on inanimate surfaces for several pathogens, including SARS-CoV-2 (Riddell et al., 2020), *Escherichia coli* (Wilks et al., 2005; Williams et al., 2005), and *Salmonella typhimurium* (Helke & Wong, 1994). Further, Fine et al. (2011) found that persistence of *Mycobacterium bovis*, the pathogen responsible for bovine tuberculosis, was significantly longer in fall and winter months and suggested that care should be taken during this period to reduce cross contamination between infected and susceptible individuals. Thus, understanding the effects of ambient temperature on pathogen viability outside of the host can give insight into the role of abiotic factors on transmission dynamics and potential management strategies to reduce disease spread.

Fomite transmission is most likely to occur from commonly used surfaces, where infected and susceptible individuals can interact indirectly (McCallum et al., 2001). For wildlife, supplemental feeding stations serve as key fomites for infectious pathogens because they attract large numbers of individuals that then interact with common surfaces to feed (Becker & Hall, 2014; Murray et al., 2016). However, the role of ambient temperature in driving pathogen survival on supplemental feeding surfaces, such as bird feeders, remains unknown. While there is evidence that bird feeding contributes to transmission of diverse pathogens including avian pox, salmonellosis, and trichomoniasis (Lawson et al., 2018; McBurney et al., 2017; Robb et al., 2008; Sorensen et al., 2014; Wilcoxen et al., 2015), the extent to which feeders facilitate environmental transmission will vary strongly with abiotic conditions (Lawson et al., 2018). Therefore, understanding how temperature affects pathogen survival on feeder surfaces can provide insight into the complex relationship between abiotic factors and fomite transmission dynamics.

Here, we examine the relationship between temperature and pathogen viability on fomites using a feeder-transmitted disease system. House finches (*Haemorhous mexicanus*) are common songbirds in the Eastern United States that often experience fall and winter outbreaks of mycoplasmal conjunctivitis, a disease caused by the bacterium *Mycoplasma gallisepticum* ([MG], Dhondt et al., 2005; Ley et al., 1996). Although this pathogen originally emerged in songbirds from poultry, the bacterial strains currently circulating in house finches represent a distinct, monophyletic clade from poultry strains (Hochachka et al., 2013). House finches are highly gregarious and often rely on bird feeders in winter to meet increased energy demands (Bonter et al., 2013; Hawley et al., 2012). House finches often visit tube-style bird feeders (Hartup et al., 1998) which contain small circular ports that birds use to access seed inside. Fomite transmission is thought to occur largely when infected birds feed at ports and deposit MG from infected tissues around the eye as they feed, which susceptible

individuals can then acquire during a subsequent feeding visit. Consistent with this mechanism, prior work shows that infected birds deposit MG onto port feeder surfaces at loads proportional to the amount of pathogen harbored in their conjunctivae (Adelman et al., 2013), and susceptible individuals exposed to a MG-contaminated feeders can become infected (Dhondt et al., 2007).

MG has been shown to survive on and transmit from contaminated feeders for up to 24 h at standard room temperatures (Dhondt et al., 2007), but little is known as to how ambient temperatures affect MG viability on feeder surfaces despite the importance of feeder transmission for this system (Adelman et al., 2015; Moyers et al., 2018; Ruden & Adelman, 2021). To date, the direct effects of temperature on MG survival outside of the host have only been examined in culture for chicken MG strains (Christensen et al., 1994; Nagatomo et al., 2001; Woode & McMartin, 1974) and not for MG strains affecting songbirds. For instance, poultry MG has been shown to have higher viability in colder environments outside of the host (Zain & Bradbury, 1996). This is likely due to the slowing of metabolic activity, which allows limited resources within the external environment to last longer (Chandiramani et al., 1966). Due to similarities in pathogen biology between poultry and songbird strains of MG, we expect to see similar increases in viability at colder temperatures outside of the host, but it is possible that house finch strains differ in their response to environmental factors given the distinct selective pressures operating on poultry versus house finch pathogen strains (e.g., Mugunthan et al., 2023; Reinoso-Pérez et al., 2022).

We tested how ambient temperatures broadly representative of winter versus summer conditions influence the viability of house finch MG strains on feeders using two separate experiments. For both studies, we inoculated replicate feeder ports housed at one of two temperature regimes and sampled ports at 0, 1, 2, 4, and 7 days later to quantify pathogen viability at each temperature. To isolate the potential effects of variable ambient temperature on MG viability while on feeder surfaces only, temperature was held constant once swabs were collected from experimental feeders and then used in two downstream viability assays. In the first experiment, we measured the viability of MG swabbed from feeder ports at a given temperature and time point using culture-based methods. In the second experiment, we measured MG viability with a bioassay by inoculating birds (all housed at identical room temperatures) with swab samples from feeder ports. We predicted that colder ambient temperatures would augment the ability of MG to survive on feeder surfaces, as measured by both cell viability and pathogenicity to wild birds.

METHODS

Experimental design

To test whether ambient temperature influences pathogen viability on feeder surfaces, we conducted two separate experiments using an identical sampling design to incubate MG on feeder surfaces for distinct time periods at distinct ambient temperatures. The experiments differed only in the endpoint assays used to quantify resulting pathogen viability at each temperature–time point combination: (1) we quantified the cell viability of MG in vitro (Experiment 1, October 2022), and (2) we measured in vivo pathogenicity of feeder-incubated MG in wild-caught but pathogen-naive house finches (Experiment 2, January 2023).

For both studies, we placed several tube-style bird feeders in walk-in environmental chambers and set chambers to one of two fluctuating day-night temperature regimes representing summer (22°C [night]–27°C [day]) or winter (4°C [night]–9°C [day]) conditions. We then pipetted equal amounts of MG (or sterile media for control ports) onto replicate bird feeder ports, the most relevant feeder surface for birds to interact with while feeding. We allowed MG to incubate on feeder port surfaces for 0 (swabbing within 30 s after inoculation), 1, 2, 4, or 7 days at their respective temperatures, with each individual feeder port only swabbed once at its assigned time point, such that all feeder ports represent independent samples. At each sample time point, we used a sterile swab to collect any remaining inocula from relevant feeder ports while allowing remaining feeders to continue incubating. The swabs of each port were then used for one of the two viability assays, which differed between the two studies. All temperature–time point combinations were replicated across 2–5 independent feeder ports for Experiment 1 (Table 1) and 4–6 independent feeder

TABLE 1 Number of replicate feeder port inoculations used in Experiment 1.

Incubation time on feeder	Summer (22–27°C)		Winter (4–9°C)	
	MG	Sham control	MG	Sham control
0 days	2	1	2	1
1 day	4	1	4	1
2 days	5	1	5	1
4 days	5	1	5	1
7 days	4	1	4	1

Note: Equal volumes of *Mycoplasma gallisepticum* (MG) or control sterile media were incubated on feeder ports for variable lengths of time at one of the two night-day temperature regimes representing summer and winter. Swabs from each feeder port were used to measure the culture viability of MG in vitro.

TABLE 2 Sample sizes of birds per temperature and incubation treatment ($n = 68$ birds) for Experiment 2.

	Summer (22–27°C)		Winter (4–9°C)	
Incubation time on feeder	MG	Sham control	MG	Sham control
0 days	4	2	4	2
1 day	6		6	
2 days	6	2	6	2
4 days	6		6	
7 days	6	2	6 (5M, 1F)	2 (2F)

Note: All treatments had equal male:female sex ratios, except where indicated (sex ratios in parentheses: M, males; F, females).

ports for Experiment 2 (Table 2). For both experiments, we used fewer replicates for Day 0 versus later time points because we anticipated lower variability from samples that were only exposed to ambient environmental conditions for a brief period (<30 s).

In the first experiment, conducted October 2022, we used the feeder port swabs to conduct a viable cell count in vitro to quantify the number of living MG cells in each sample collected from feeder surfaces. In the second experiment, with identical initial design and conducted in January 2023, we assessed in vivo viability and pathogenicity of the MG collected from feeder surfaces by using a bioassay, whereby feeder port swabs were used to directly inoculate house finches by placing the swabs directly into the conjunctival sacs of wild-caught but MG-naive birds. We then measured the ability of MG to produce infection in the host by quantifying disease severity and pathogen load in each bird post-inoculation. Importantly, all birds were housed at room temperature during the bioassay, such that temperatures only differed during pathogen incubation on bird feeders.

Temperature selection

The experimental temperatures were selected to represent ambient temperatures that bird feeders in many parts of the United States are exposed to in summer versus winter. Because MG is directly deposited onto feeder surfaces (Adelman et al., 2013; Dhondt et al., 2007), MG cells are inherently subject to the same ambient temperatures that the feeders are exposed to. Thus, we ensured that experimental winter temperatures chosen for this study fell within the minimum range of winter temperatures for Southwestern Virginia, USA, where the birds were captured (−10 to 9°C from January to February 2023, National Centers for Environmental Information, 2024a), as well as Durham, North Carolina (−3 to 16°C from January to February 2023, National Centers for Environmental Information, 2024b), where the MG strain used was originally isolated from a bird submitted to the Piedmont Wildlife Center (Ley et al., 2016).

Feeder inoculation and incubation

Tube-style bird feeders (Droll Yankees; Plainfield, Connecticut), each with two feeding ports, were placed in separate environmental chambers set to summer or winter temperature conditions (see *Temperature selection*). All feeders were disinfected, sanitized, and stored until one week prior to the experiment. Immediately before inoculation, feeder ports were wiped down with a dry KimWipe to ensure the inoculation surface was free of debris. We then inoculated replicate feeder ports with MG inoculum (one 5-μL droplet for culture assay in Experiment 1, two separate 5-μL droplets for bioassay in Experiment 2) or sterile Frey's media as a control.

Droplets were then left to incubate on the feeder port surface for 0, 1, 2, 4, or 7 days, depending on the assigned treatment for a given port. For both experiments, we used an MG strain that was first isolated from a male house finch with conjunctivitis near Durham, North Carolina, in 2006, and has since been passaged four total times, but otherwise remained frozen at −80°C (NC2006, 2006.080-5 [4P] 7/26/12). The inoculum used in this experiment contained an initial concentration of 2.49×10^8 color-changing units per mL (CCU/mL) and, prior to use, was thawed, divided into five separate aliquots, and refrozen before feeder inoculation. This allowed us to control for freeze–thaw while also varying the calendar day of inoculation for each feeder time point (0, 1, 2, 4, or 7) such that we could collect all feeder swabs and inoculate birds with those swabs on the same day, regardless of whether the MG had incubated on the feeder for 0 or 7 days.

Experiment 1: Viable cell count assay

At each incubation time point, a sterile flocked swab (Copan FloqSWABS, Copan Diagnostics) was moistened with two drops of sterile eye lubricating drops (CVS Health, propylene glycol 0.6%) to aid in collecting remaining inocula from each feeder port surface. Using a small LED light, we located the MG droplet on each port surface and swabbed the area for 5 s (five turns) to collect any remaining MG inocula or Frey's media. The flocked swab was then swirled in 300-μL triptose phosphate broth and wrung on the side of the tube before freezing at −80°C.

MG swab samples were sterilely titrated in 96-well sterile microtiter plates (Costar 3370) as follows: For each sample, a plate was loaded with 180-μL completed Frey's medium per well (Frey et al., 1968). Frozen MG swab samples were individually thawed from ultralow freezers at time of titration, vortexed, and collected quickly at the bottom of the tube. We inoculated 20 μL/well in column one of the plates, and all eight replicates were then serially titrated 10-fold across the plate using a multichannel micropipette. Plates were sealed with tape, incubated at 37°C, and observed over seven weeks for pH-induced color change indicating growth. Viable count, as CCU/mL, was calculated using a most-probable number (MPN) table (Meynell & Meynell, 1970).

Experiment 2: Bioassay

Bird capture and housing

Hatch-year house finches (identified following Pyle, 2022) were captured using mist nets or feeder traps in Montgomery County, Virginia, and near the campus of Virginia Tech and housed at standard room temperatures (20–22°C). Birds were quarantined for two weeks, during which time they were captured every 3–4 days to visually assess whether clinical signs of mycoplasmal conjunctivitis had developed. Any birds with visible conjunctivitis were isolated along with their cagemates and not used in this experiment. Following the 2-week quarantine, we took blood samples between 14 and 18 days post capture for all birds that did not develop signs of conjunctivitis to screen for MG exposure via seropositivity. To confirm seronegativity for these birds, blood samples were run using an ELISA via a commercial IDEXX kit (IDEXX 99-06729; Westbrook, Maine) following published methods (Hawley et al., 2011). All birds used in this study were MG seronegative at the beginning of the experiment.

Birds were then single-housed at room temperature for the duration of the bioassay. We randomly distributed birds by sex into treatment or sham groups across temperature treatments (summer [22–27°C] or winter [4–9°C]), and incubation time on the feeder (0, 1, 2, 4, or 7 days). All birds were equally divided across treatments by sex, except for one MG treatment group (five males:one female) and one sham treatment group (two females), due to the sex ratios of birds available (Table 2). Birds were given water and food (80% Roudybush Maintenance Diet and 20% sunflower hearts) ad libitum throughout the course of the experiment. Following capture from the wild and up through completion of the experiment, birds were treated with prophylactic medications, Cankerex Plus (Dimetridazole B,P [Vet] 40% m/m powder) and Endocox (2.5% Toltrazuril), to prevent Trichomoniasis and coccidiosis, respectively, which can be fatal to captive finches. Details of medication doses for Cankerex and Endocox can be found in the Supplement of Hawley et al. (2024).

Bioassay inoculation

At each incubation time point, and for each 5-μL droplet (two per port for Experiment 2), a new flocked swab was moistened with two drops of sterile eye lubricating drops to aid in collecting dry inocula from the feeder and to prevent abrasion in the bird's eye during inoculation. After swabbing each feeder port, birds were inoculated by gently pulling open the lower eyelid of each eye using sterile forceps and inserting one of the two flocked swabs (a swab of each droplet was used for each eye) for 5 s (five turns). Although indirect transmission typically occurs between birds and contaminated feeders, by inoculating the bird with the swab directly in the conjunctiva (instead of allowing it to occur through natural interactions with the feeder), we ensured that each bird had equivalent exposure to the inoculum regardless of how much MG was still viable on the feeder surface. Birds were then immediately returned to their cages at room temperatures.

We assessed the viability of the MG inoculum by quantifying both disease severity and pathogen load in birds over the course of infection. We scored disease severity for each bird on Days 2–7 and 10, 14, 21, and 28 days post-inoculation (PID). Following Hawley et al. (2011), each eye of each bird was scored on a scale of 0–3 per eye in 0.5 point increments, where 0 = no swelling, 1 = minor swelling around eye, 2 = moderate swelling, and 3 = severe swelling around the eye ring. Scores for both eyes were then summed for a maximum of six for each bird, per sampling day. All scoring was done by a single individual while blinded to the specific temperature and time treatment of the MG swab used to inoculate each bird. We also quantified pathogen load by taking conjunctival swabs on Days 7, 14, and 21 post-inoculation of the bird. Using sterile forceps, the lower eyelid of each eye was gently pulled back, and a cotton swab (Fisherbrand 22-363-167), moistened with two drops of sterile lubricating eye drops, was inserted into the conjunctiva for 5 s (five turns). The swab was then swirled in 300-μL triptose phosphate broth and rung on the side of the tube prior to being discarded. All samples were then frozen at −20°C.

Conjunctival swab samples were then thawed, and 300 μL was used for genomic DNA extraction using Qiagen DNeasy 96 Blood and Tissue kits (Qiagen; Valencia, California), with a final elution volume of

100 μL. To quantify MG load in conjunctival samples, each 15-μL reaction contained 7.5-μL QuantiNova Probe Master Mix, 2.9-μL DNase-free water, 0.075-μL ROX (1:200), 3-μL extracted DNA sample, and 0.3 μL of 0.20-μM probe and 0.6 μL each of 0.4-μM forward and reverse primers specific to the *mgc2 gene* in *M. gallisepticum* (Grodio et al., 2008). We then used QuantStudio5 to cycle reactions, with 1 cycle at 95°C for 2 min, 40 cycles of 95°C for 5 s, and 60°C for 30 s. Standard curves were generated for each run, based on 10-fold serial dilutions of plasmid that contained a 303-bp *mgc2* insert (Grodio et al., 2008). Serial dilutions consisted of 3.9×10^1–3.9×10^8 copies of plasmid. Reactions were then analyzed using Design & Analysis Software v2.6.0.

Statistical analyses

All statistical analyses were conducted using RStudio Version 4.3.1 (R Core Team, 2023), using the "stats" (R Core Team, 2023) and "tidyverse" (Wickham et al., 2019) packages. Data were visualized using the "ggplot2" (Wickham, 2016) package. For our viable cell count assay, we used an exponential decay model (Brouwer et al., 2017) of the form $y = (a + c \times \text{ind})e^{(b + d \times \text{ind})t}$, where y is the number of CCU/mL remaining at time t in days, a is the parameter for the initial concentration of MG, b is the parameter for the rate of decay, and c and d are offsets that were allowed to change each parameter according to temperature treatment. The variable, ind, represents a binary indicator variable that was set to 0 for the summer condition and 1 for the winter condition. This allowed for different parameters to be fit to each temperature condition. It also allowed for direct statistical comparisons of parameters between groups, since when c or d were statistically different from 0, it could be inferred that the initial conditions or decay rates differed between the temperature treatments, respectively. Starting parameter values for the model were set such that $a = 5$ CCU/mL (log MG load + 1) and b, c, and d were all set to 0. Parameter estimation was performed using the "nlme" package in Rstudio (Pinheiro & Bates, 2000). Nonlinear regression models carry the same assumptions around the model residuals as linear models. Therefore, the assumptions of the nonlinear model were checked graphically, and we found no major deviations from the assumptions. The full set of diagnostics used to assess model assumptions can be found in Appendix S1.

For our bioassay, we used a linear mixed model to examine the additive or interactive effects of temperature (summer or winter), incubation time on feeder (0, 1, 2, 4, or 7 days), and the number of days post-inoculation of the host (PID 7, 14, 21) on disease severity or pathogen load as continuous variables, with bird ID as a random effect since disease metrics of individual birds were evaluated multiple times throughout the experiment. The sex of each bird was initially included as a covariate in each model but had no significant effect on response variables and was thus removed from final models. All final models contained the main variables of temperature, incubation time on feeder, and PID because all had significant effects on response variables. Although we collected data across the entire course of infection, only three post-inoculation time points (Days 7, 14, and 21) were used for analysis because (1) data for both disease severity and pathogen load were available at these points, (2) this period represents peak infection for this MG isolate in this study (Appendix S1: Figure S1) and other studies (Dhondt et al., 2017; Grodio et al., 2012), and (3) given that host responses to this acute infection are inherently nonlinear over time post-inoculation, limiting our analysis to peak timepoints allowed for more robust interpretation of the effects of interest (temperature and incubation time on feeder) in interaction with post-inoculation timepoint. We tested for and confirmed the absence of overdispersion of linear model residuals using the "DHARMa" package (Hartig, 2022). In the case of disease severity, the assumption of a continuous response is technically violated given the bounded nature of the pathology score used in this analysis. However, our use of the linear model in this case is supported by previous literature (Harpe, 2015). Linear mixed models were conducted using the "lme4" package (Bates et al., 2015). We then compared additive and interactive models using likelihood ratio tests in the "stats" package (R Core Team, 2023) to identify the model with the most support. Following each analysis, we used a Type III ANOVA Wald Chi-squared test in the "car" package (Fox & Weisberg, 2019) to determine the overall significance of fixed effects on each response variable (disease severity and pathogen load). We then computed the estimated marginal means for each model using the "emmeans" package (Lenth, 2023) and obtained un-adjusted p-values from post hoc contrasts between interacting predictors. Lastly, we determined the significance of each pairwise contrast using the Bonferroni correction for alpha by dividing $\alpha = 0.05$ by the relevant number of comparisons.

RESULTS

Viable cell count assay

MG incubated on feeder surfaces at colder ambient temperatures had a significantly lower rate of decay (difference = 0.7792, SE = 0.1689, $p < 0.0001$) over time

compared with MG incubated on feeder surfaces at warm ambient temperatures (Figure 1, exponential decay: winter, $b = -0.23 \pm 0.03$ CCU/mL log[MG load + 1], summer, $b = -1.01 \pm 0.17$ CCU/mL log[MG load + 1]; df = 36). As expected, given that equivalent doses of MG were inoculated onto feeder surfaces at each temperature, we did not detect a statistical difference in the number of MG cells swabbed from ports within 30 s of inoculation at time 0 (difference = 0.1177, SE = 0.4980, $p = 0.814$; exponential decay: winter, $a = 4.24 \pm 0.30$ CCU/mL log [MG load + 1]; summer, $a = 4.12 \pm 0.40$ CCU/mL log [MG load + 1]; df = 36). Sham-control ports did not have any viable MG detected at any time point or temperature.

Disease severity

Overall, MG incubated on feeders at colder ambient temperatures caused higher disease severity in birds compared with MG incubated at warmer temperatures, but this relationship is also influenced by the total incubation time of MG on feeders as well the time course of host infection (post-inoculation day, or PID of the host, Figure 2). Specifically, our model indicated a significant three-way interaction between temperature treatment, incubation time of MG on the feeder, and PID (ANOVA; $\chi^2 = 23.04$, df = 8, $p = 0.003$), with post hoc contrasts indicating that across 15 comparisons, peak disease severity in birds (on PID 14) differed by temperature only when MG had incubated on feeder ports for four (post hoc contrasts; $M = 3.5$, SE = 0.99, df = 80.2, $t = 3.55$, $p = 0.0006$, adjusted $\alpha = 0.003$) and seven days (post hoc contrasts; $M = 3.67$, SE = 0.99, df = 80.2, $t = 3.72$, $p = 0.0004$, adjusted $\alpha = 0.003$). Specifically, our model estimated that birds inoculated with MG incubated for 4 days on feeders at colder temperatures caused 15 times higher disease severity on Day 14 post-inoculation than with MG incubated for the same length of time at warm temperatures (estimated marginal means for disease severity at PID 14: winter = 3.75, summer = 0.25). All relevant post hoc contrasts and estimated marginal mean comparisons for disease severity can be found in Appendix S1: Tables S1 and S2. Likelihood ratio tests indicated more support for the interactive model compared

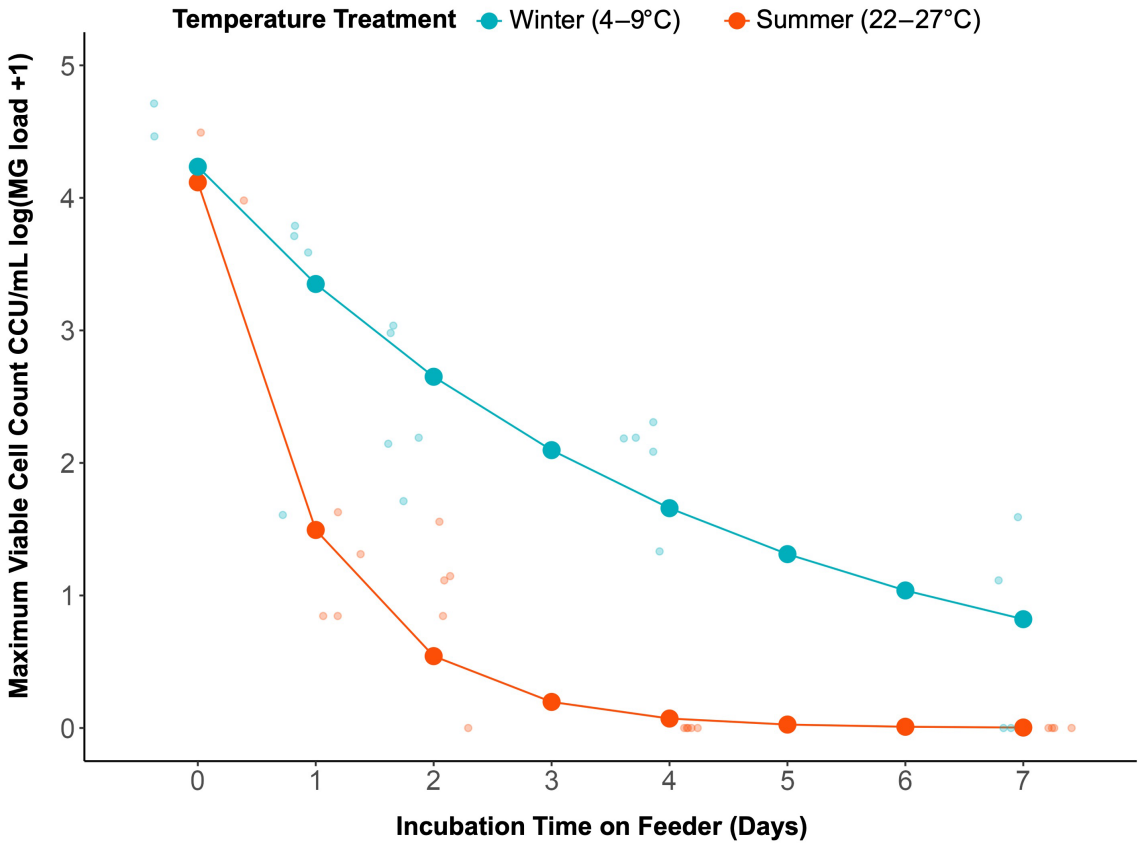

FIGURE 1 *Mycoplasma gallisepticum* (MG) had higher viability on feeder surfaces over time in colder temperatures compared with MG incubated on feeder surfaces held in warmer conditions. Predicted values from the exponential decay model are shown as connected circles and replicate feeder ports are shown as smaller circles. Sham-control ports, which did not have viable MG detected at any time point or temperature, are not shown.

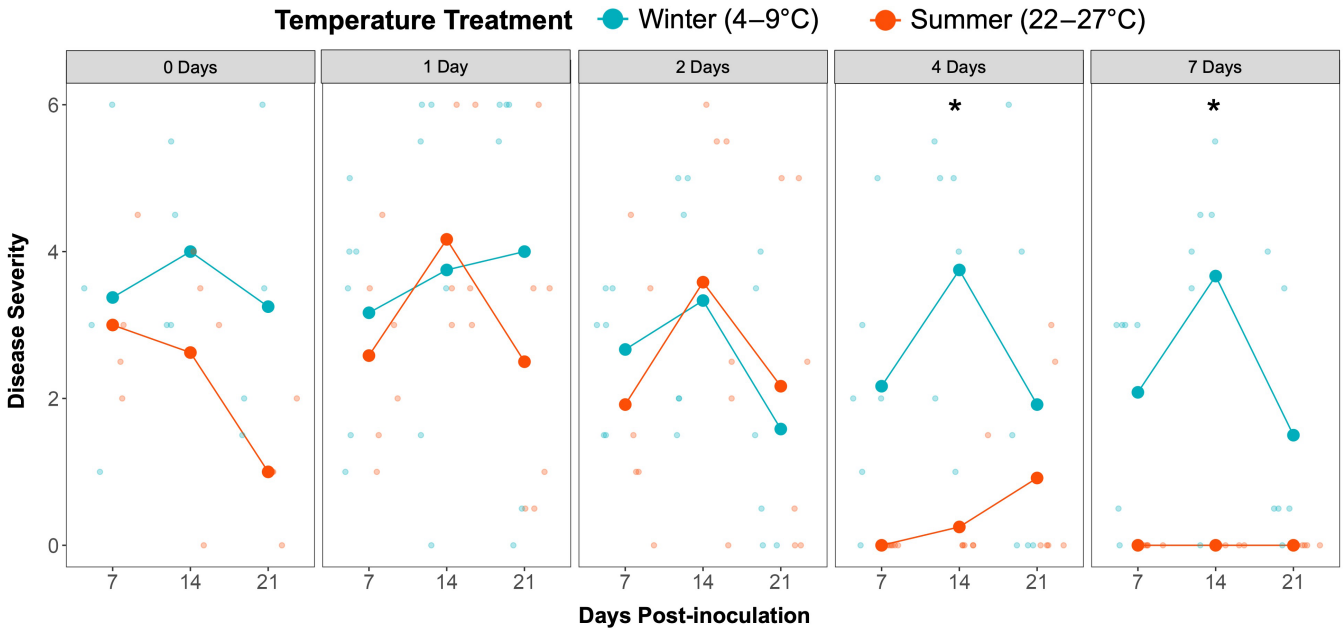

FIGURE 2 After longer incubation times (4 or 7 days) on feeder surfaces, *Mycoplasma gallisepticum* (MG) incubated in colder temperatures produced higher disease severity in pathogen-naive birds over the course of peak infection (Days 7, 14, and 21 post-inoculation [PID]) compared with MG incubated on feeders in warmer temperatures. Facet labels show the length of incubation for MG on feeder surfaces (Days 0, 1 2, 4, or 7), and the feeder incubation temperature for each MG sample is denoted by color. Predicted values are shown as connected circles, and raw data are shown as smaller circles. Significant post hoc pairwise comparisons across temperature treatment, PID, and incubation time are indicated by asterisks. Birds inoculated with swabs from sham-control ports are not shown to better visualize temperature effects, but these birds did not have detectable disease. Note that we converted the predicted value to zero in the one instance when the model predicted value for disease severity was negative (summer, incubation Day 4, PID 7, predicted value $= -8.88 \times 10^{-16}$).

with the additive model (likelihood ratio test: $\chi^2 = 18.7$, df $= 22$, $p = 0.008$), and no additional main effects or interactions between parameters were significant (all $p > 0.05$). Nonzero eye scores were not detected in any birds inoculated with swabs from sham-control ports.

Pathogen load

MG incubated on feeders at colder temperatures produced higher pathogen loads in inoculated birds compared with MG incubated on feeders at warmer temperatures, but the strength of this effect depended on the total length of time that MG incubated on feeder surfaces (Figure 3). Specifically, there was a significant interaction between temperature treatment and incubation time of MG on the feeder surface (ANOVA; $\chi^2 = 11.58$, df $= 4$, $p = 0.020$), with post hoc contrasts indicating that across five comparisons, pathogen load in birds only differed by temperature when MG had incubated on feeder ports for four (estimated marginal means; $M = 2.08$, SE $= 0.64$, df $= 42.2$, $t = 3.27$, $p = 0.002$, adjusted $\alpha = 0.01$) or seven days (estimated marginal means; $M = 2.37$, SE $= 0.64$, df $= 42.2$, $t = 3.73$, $p = 0.0006$, adjusted $\alpha = 0.01$). All relevant post hoc contrasts for pathogen load can be found in Appendix S1: Table S3. There were no significant interactions among other parameters (all $p > 0.05$). Fixed effects for the LMM showed a significant main effect of post-inoculation day on pathogen load ($\chi^2 = 62.44$, df $= 2$, $p < 0.0001$), but no individual effects of temperature ($\chi^2 = 0.39$, df $= 1$, $p = 0.53$) or pathogen incubation time on feeder ($\chi^2 = 3.75$, df $= 4$, $p = 0.44$). Likelihood ratio tests confirmed more support for the model that contained a pairwise interaction between temperature and pathogen incubation time, with PID as an additive variable, compared with the three-way interactive model (likelihood ratio test: $\chi^2 = 30.88$, df $= 14$, $p = 0.006$). Birds inoculated with swabs from sham-control ports either had no MG detected ($n = 30$ total samples) or were below the maximum detected from background contamination in our sensitive quantitative polymerase chain reaction assay (0.83–17.5 total copies of MG in $n = 6$ samples; see Leon & Hawley, 2017).

DISCUSSION

Fomites are a common and potentially underappreciated route of transmission for many infectious diseases

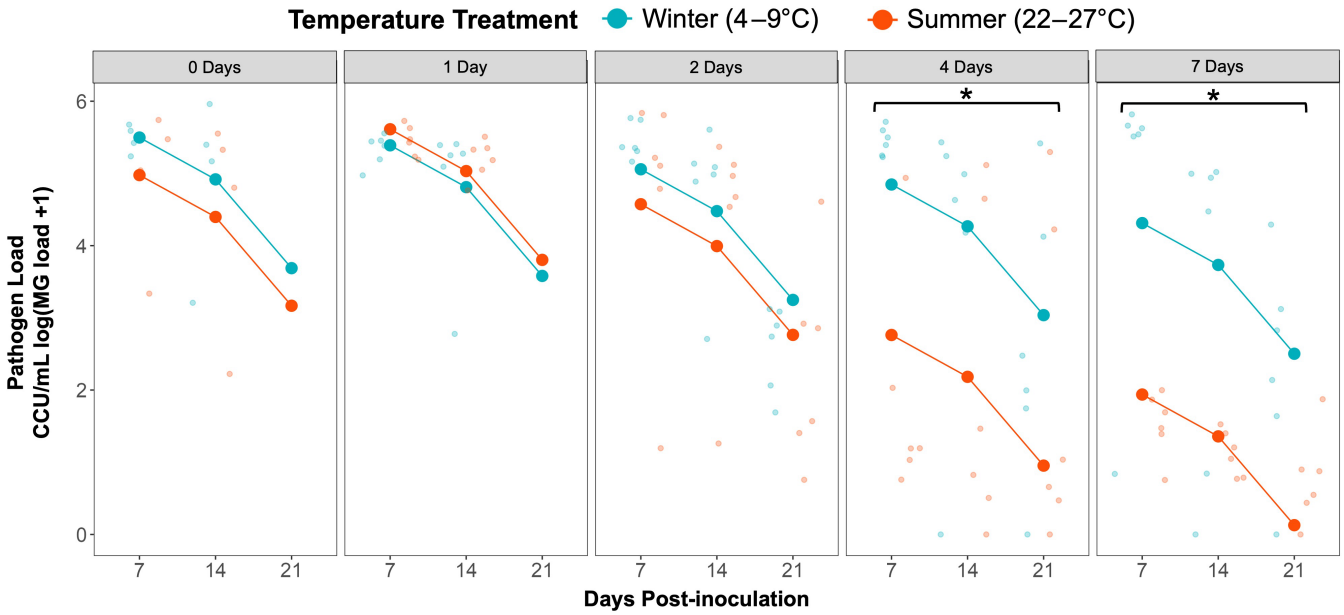

FIGURE 3 After longer incubation times (4 or 7 days) on feeder surfaces, *Mycoplasma gallisepticum* (MG) incubated on feeders at colder temperatures was associated with higher pathogen loads in birds at peak infection (Days 7, 14, and 21), compared with MG incubated on feeders at warmer temperatures. Facet labels show the length of incubation for MG on feeder surfaces (Days 0, 1 2, 4, or 7), and feeder incubation temperature for each MG sample is denoted by color. Significant post hoc pairwise comparisons across temperature treatment and incubation time are indicated by asterisks. Predicted values are shown as connected circles and raw data are shown as smaller circles. Birds inoculated with swabs from sham-control ports are not shown here to better visualize temperature effects on MG treatments.

(Murray et al., 2016). However, the relative importance of fomites to transmission often relies heavily on abiotic conditions, (Breban, 2013; Satterfield et al., 2017) such as temperature, which affect the persistence of pathogens in the environment. Here, we show that ambient temperature had strong effects on the ability of MG to remain viable and pathogenic on bird feeder surfaces, a key fomite in this system. Overall, our results suggest that colder ambient temperatures, at least within the temperature range examined here, augment the role of fomites in the MG transmission process as a whole (McCallum et al., 2017), with temperature effects on fomite survival likely contributing to seasonal disease dynamics in this system (Altizer et al., 2004) and others (Altizer et al., 2006; Wang et al., 2012).

First, using a culture-based assay of viability, we found that MG can remain viable on feeder surfaces for at least seven days in colder ambient temperatures, versus only up to two days in warmer ambient temperatures. For many bacterial pathogens that can survive outside of the host, persistence is often greater at colder versus warmer ambient temperatures (e.g., Brown et al., 2009; Kramer et al., 2006). This may be due to cold temperature conditions facilitating changes in microbial cellular processes that promote greater survival, such as increased cell size (Wiebe et al., 1992) and slowed metabolism (Chattopadhyay, 2006). The mechanisms allowing MG to remain viable longer on feeder surfaces at colder versus warmer temperatures are outside of the scope of this study but represent an area for future research. For pathogenic mycoplasmas in poultry (Catania et al., 2024) and dairy cattle (Justice-Allen et al., 2010), increased viability in the environment may be facilitated by the formation of biofilms, which can offer protection from extreme environments and abiotic conditions (Yin et al., 2019). However, the ability of mycoplasmas to form biofilms can be highly diverse and strain-dependent (Chen et al., 2012). For instance, Bekő et al. (2022) found that 19 of 32 strains of *Mycoplasma anserisalpingitidis* were able to form biofilms and that those strains were more resistant to heat and desiccation relative to strains that did not form biofilms. While some strains of MG have been shown to form biofilms (Chen et al., 2012), whether the strain used in this study has biofilm-forming abilities that may contribute to increased survival on bird feeders remains unknown.

In our bioassay experiment, we found that MG on bird feeders remained pathogenic for significantly longer when incubated in colder versus warmer ambient temperatures, producing high levels of disease severity and pathogen load in birds after incubation on feeders of up to seven days at cold temperatures. Interestingly, temperature effects on pathogenicity were not present until MG had incubated on bird feeder surfaces for at least four

days, suggesting that both temperature and the length of time that MG has been present on a feeder surface are important factors influencing pathogenicity to visiting birds. Notably, ambient temperature did not have detectable effects on pathogenicity of MG that incubated on feeder surfaces for only a short time window (1–2 days), at least within the range of experimental temperatures used here. However, expanding the range of temperatures examined to include extremes such as subfreezing winter temperatures is critical for determining the upper and lower limits of pathogen viability, and for predicting how temperature effects on pathogen persistence scale up to influence transmission dynamics (Satterfield et al., 2017) and seasonality in the system (Altizer et al., 2004). Further, determining how the detected interactive effects of incubation time on feeders and temperature ultimately play out in for free-living birds requires a better understanding of the temporal and spatial heterogeneity in host contacts with feeders (Chen et al., 2013; Scherer et al., 2020; White et al., 2018), as well as an understanding of how much MG is removed from a feeder surface at each visit by a feeding bird. Overall, our results suggest that colder ambient temperatures within the range examined here could allow MG outbreaks to persist at some sites where time intervals of up to one week occur between house finch visits. This spatial heterogeneity may ultimately allow longer population-level pathogen persistence (Swinton et al., 2002), particularly that of virulent strains (Wood & Thomas, 1996), at colder temperatures.

To our knowledge, this is the first study to demonstrate how ambient temperature affects the viability and pathogenicity of MG on bird feeders, which are commonly used resources for backyard birds as well as fomites for pathogen transmission (Hartup et al., 1998). Many studies of microbial survival on fomites use either culture or experimental infection to examine the effects of abiotic factors on viability, such as in cattle (Fine et al., 2011) and rabbit pathogens (Henning et al., 2005). Here, we integrated both culture-based and bioassay approaches, using identical sampling designs, allowing us to make broad comparisons between them. While incubation on fomites at colder temperatures resulted in longer culture-based viability and higher pathogenicity to birds after several days of incubation, there were some interesting qualitative differences in the patterns observed over time for the two assays. In particular, our culture assay detected a steady decline of MG viability on feeder surfaces in cold temperatures, while our bioassay indicated that MG remained almost equivalently pathogenic to live birds after seven days of incubation on feeder surfaces. This discrepancy can result from several possibilities, including a potential dose threshold effect for pathogenic disease, whereby severe disease results whenever birds are exposed to a threshold minimum amount of viable MG (Leon & Hawley, 2017). Further, the minimum dose threshold for infecting live birds may be lower than the minimum color change threshold in the culture assay, resulting in the detected qualitative differences in viability patterns. Lastly, if the strain used in this study has biofilm forming abilities, it may have also had a short-term impact on the discrepancy between MG viable titer and its ability to induce conjunctivitis when sampled from feeders. Regardless of the underlying mechanism, the detected discrepancy suggests that the results of culture-based assays alone must be interpreted with some caution, as such assays may not capture the biologically meaningful effect of variables such as temperature on hosts.

One caveat of our study design is that we only varied ambient temperature in our experiments. Diverse abiotic conditions in addition to temperature may also influence our understanding of when and where bird feeders contribute most to disease transmission. For instance, abiotic factors such as humidity (Qiu et al., 2022; Wright et al., 1968) and increased ultraviolet radiation (Oppezzo et al., 2011) have been shown to negatively affect bacterial cell growth and survival. Because these abiotic factors are often correlated with temperature (e.g., Häder et al., 2015; Mbithi et al., 1991), it is particularly important to examine how covariation in ambient conditions, such as temperature and daylight, influence pathogen viability on feeder surfaces in more natural conditions. Further, our study used a single, well-characterized strain of MG (NC2006) that is known to be virulent in house finches, but future studies should examine temperature effects for other MG strains of variable geographic origin and virulence, including strains directly isolated from wild birds with conjunctivitis. Previous studies show a rapid increase in virulence among songbird strains of MG since emergence in house finches (e.g., Bonneaud et al., 2018; Hawley et al., 2013). Work on other pathogenic microbes hypothesizes that virulence can relate to pathogen persistence in the environment (Walther & Ewald, 2004) or to pathogen thermal tolerance. For example, Ashrafi et al. (2018) found that the fish pathogen, *Flavobacterium columnare*, has increased in virulence since emergence, but these increases were associated with marked decreases in pathogen thermal tolerance. In addition to variation in virulence, MG strains isolated from states with diverse winter conditions (with at least 227 isolates of MG collected from 17 states to date; Ley et al., 2016), may also differ in thermal tolerance as an adaptation to survive outside the host in varying ambient conditions. For instance, Tian et al. (2022) determined that constant warming and temperature

fluctuations in the environment were associated with thermal adaptation in widely distributed soil bacteria. Thus, future studies of MG strains that differ in both virulence and geographic origin are needed to determine whether temperature effects on fomite transmission covary with aspects of strain biology in this system.

Overall, our study indicates that cold ambient temperatures strongly influence the ability of MG to remain viable and pathogenic over time on feeder surfaces. Importantly, under natural conditions, the effects of cold temperatures on MG outside the host shown here may be further compounded by the effects of cold temperatures on other aspects of the transmission process. Winter conditions are particularly challenging for small-bodied endotherms, such as birds, since they must maintain high metabolic demands at a time when natural food sources are scarce. As a result, house finches increase their time spent at supplemental feeding stations (Bonter et al., 2013; Teemer & Hawley, 2024) and increase food intake (Adelman et al., 2013) in colder ambient temperatures. Given that cold temperatures also lengthen pathogen survival on feeder surfaces, as demonstrated here, this could provide longer windows of time for susceptible individuals to encounter pathogens (Murray et al., 2016) and for infected individuals to deposit MG onto feeder surfaces (Adelman et al., 2015) at times of the year when feeders are heavily used to meet thermoregulatory demands. Therefore, future studies should consider how temperature-induced pathogen survival on fomites may interact with other aspects of the transmission process, including feeder use by healthy (Adelman et al., 2013; Altizer et al., 2004) and infected hosts (Hawley et al., 2012; Teemer & Hawley, 2024).

We show that variation in temperature could have key downstream implications for transmission in this system and many others (Sipari et al., 2022). Additionally, we provide insight into one of many links underlying the complex relationship between annual environmental variation and seasonal disease dynamics (Altizer et al., 2006). However, wide scale climate warming has already altered the current patterns of seasonality of several infectious diseases and is expected to continue rapidly (Harvell et al., 2009; Lindgren et al., 2012). Thus, understanding the mechanisms that shape seasonality is not only important for our ability to predict seasonal outbreaks but is also urgent in the face of global climate change (Lafferty, 2009).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Teemer et al., 2024) are available from Figshare: https://doi.org/10.7294/26216252.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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