RESEARCH

The role of *Culex territans* mosquitoes in the transmission of *Batrachochytrium dendrobatidis* to amphibian hosts

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Abstract

Background Mosquitoes are the deadliest organisms in the world, killing an estimated 750,000 people per year due to the pathogens they can transmit. Mosquitoes also pose a major threat to other vertebrate animals. *Culex territans* is a mosquito species found in temperate zones worldwide that feeds almost exclusively on amphibians and can transmit parasites; however, little is known about its ability to transmit other pathogens, including fungi. *Batrachochytrium dendrobatidis (Bd)* is a topical pathogenic fungus that spreads through contact. With amphibian populations around the world experiencing mass die-offs and extinctions due to this pathogen, it is critical to study all potential modes of transmission. Because *Cx. territans* mosquitoes are in contact with their hosts for long periods of time while blood-feeding, we hypothesize that they can transmit and pick up *Bd*.

Methods In this study, we first assessed *Cx. territans* ability to transfer the fungus from an infected surface to a clean one under laboratory conditions. We also conducted a surveillance study of Bd infections in frogs and mosquitoes in the field (Mountain Lake Biological station, VA, USA). In parallel, we determined *Cx. territans* host preference via blood meal analysis of field caught mosquitoes.

Results We found that this mosquito species can carry the fungus to an uninfected surface, implying that they may have the ability to transmit Bd to their amphibian hosts. We also found that Cx. territans feed primarily on green frogs *(Rana clamitans)* and bullfrogs *(Rana catesbeiana)* and that the prevalence of Bd within the frog population at our field site varied between years.

Conclusions This study provides critical insights into understanding the role of amphibian-biting mosquitoes in transmitting pathogens, which can be applied to disease ecology of susceptible amphibian populations worldwide.

Keywords Blood-feeding, Chytrid fungus, Host preference, Mosquito-borne disease, Pathogen transmission

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Background

Mosquitoes are the deadliest animals in the world, spreading many pathogens to their hosts [1, 2]. Although most vectors feed on endothermic hosts such as mammals or birds, some species are known to be ectotherm specialists [3, 4]. A primary example is *Culex territans* Walker 1856, a species of mosquito found in temperate and subtropical zones across the Northern Hemisphere [5–9] that feeds preferentially on amphibians, especially anurans (i.e., frogs and toads) [10–12]. Despite its widespread distribution, little is known about this mosquito. *Culex territans* is a potential vector of pathogens such as giant anuran trypanosomes, hepatozoon parasites [12–14], and viruses [15, 16], but its role in transmitting other pathogens, such as fungi, is unknown. *Batrachochytrium dendrobatidis* (*Bd*) is a topical pathogenic fungus affecting the keratinized tissue in amphibians and is a major contributor to declines in amphibian populations worldwide [17–20]. Chytridiomycosis, the disease caused by *Bd*, affects anuran amphibians more than other amphibian orders, with pathogenic effects varying significantly among species [21, 22]. As an aquatic fungus, *Bd* can be spread environmentally through water or by physical contact with other infected frogs [23]. Fish, crustaceans, and reptiles are known carriers of *Bd* [24–28]; however, the roles that vectors might play in transmitting this deadly fungus remains poorly understood [29, 30]. Toledo et al. [29] found *Bd* DNA present on biting midges (*Corethrella* spp.), showing that arthropod vectors can play a role in the transmission of this fungus. Mosquitoes are known to harbor fungi [31, 32], and *Cx. territans* has a long period of contact time with the skin surface of the frog during blood-feeding (minimum of 30 min to feed to repletion [33]). This amount of time could allow the proboscis and the legs to acquire any fungal spores present on the skin surface of their host. Interestingly, a closely related mosquito species, *Cx. quinquefasciatus*, which feeds on birds and mammals, has been shown to be able to acquire the fungus and transport it to a new surface [30]. Our study tests the ability of *Cx. territans*, the northern frog-biting mosquito, to transfer *Bd* to amphibian hosts, as *Cx. quinquefasciatus* is not known to feed on amphibians.

In the present study, we hypothesized that *Cx. territans* could acquire *Bd* spores and transmit them to a new surface, thus demonstrating the ability to vector this fungal pathogen to anuran hosts through mechanical transmission (i.e., by spreading spores present on the mosquito legs to the skin of their hosts). To test this hypothesis, we first conducted blood meal analyses to determine the primary hosts for this specific population of *Cx. territans*. We then screened field-caught mosquitoes and frogs for the presence of *Bd* at our field site, Mountain Lake Biological Station (MLBS, Pembroke, VA, USA), and carried out a controlled experiment to evaluate the ability of *Cx. territans* to transmit the fungus from one medium surface to another. This work will provide a deeper insight into mosquito-borne disease ecology as well as understanding the potential *Cx. territans* has for vectoring *Bd*, which will enhance our understanding of the spread of this deadly fungus.

Methods

Mosquito sample collection

Mosquitoes were collected at MLBS (37.375654°– 80.522140°, 1160 m above sea level [ASL]) in Giles County, VA, USA. Mosquitoes were collected while at rest around midday from May through September at Sylvatica Pond (37.377079, −80.522245) using a giant bug aspirator (John W. Hock Company, Gainesville, FL, USA) [34]. Following collection, the mosquitoes were brought to the MLBS laboratory, cold-anesthetized, and sorted under a microscope based on species, sex, and reproductive stage [6]. Blood-fed *Cx. territans* females were put in individual 2-ml Eppendorf tubes (Cat #24-283S, Genesee Scientific, Rochester, NY, USA) and were immediately frozen at −80 °C until use for blood meal analysis and pathogen screening for the presence of *Bd* (Fig. 1A), and any bycatch was released.

Blood meal analysis

Blood-fed mosquitoes collected in the field were thawed, and the abdomens (containing the blood meals) were removed using fine forceps. Abdomens were homogenized using a homogenizer and pestle, and DNA was extracted using Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions (without the purification step, eluted in 20 µl AE buffer). We amplified the mitochondrial 16S ribosomal RNA (rRNA) gene from each mosquito blood meal via polymerase chain reaction (PCR) using a 50 µl solution containing 25 µl DreamTaq Green PCR Master Mix (2×) (Cat #FERK1081 Thermo Fisher Scientific, Waltham, MA, USA), 21 µl PCR-grade water (CAS: 7732-1-8-5, Sigma Aldrich, St. Louis, MO, USA), 1 µl of forward and reverse primers (L2513: 3′-GCCTGTTTACCAAAA ACATCAC-5′; H2714: 3′-CTCCATAGGGTCTTCTCG TCTT-5′) [35] and 2 µl DNA. Amplification was carried out using a thermal cycler (MyCycler, Bio-Rad, Hercules, CA, USA) with 35 cycles of 95 °C for 30 s, 57 °C for 15 s, and 72 °C for 30 s. PCR products were run on a 1% agarose gel containing 0.1% GelRed Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) for 30 min at 110 V to confirm successful amplification (Bio-Rad), with the amplified product sent to Genewiz (South Plainfield, NJ, USA) for Sanger sequencing. Sequences were aligned using the Qiagen CLC Genomics Workbench (Qiagen, Hilden,
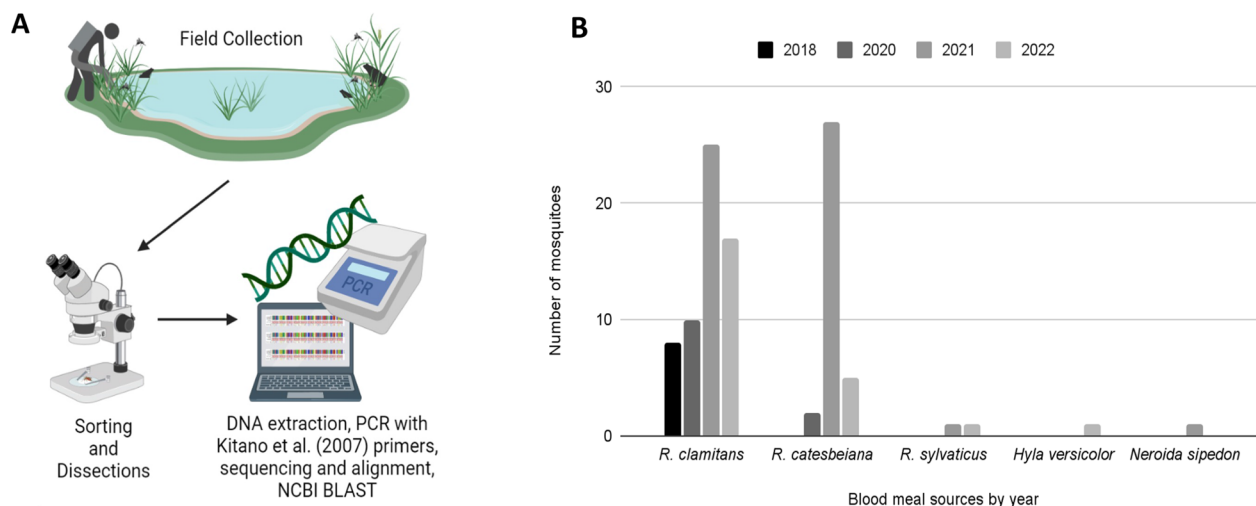
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Fig. 1 **A** Mosquitoes were collected using a giant bug aspirator around a pond occupied by *R. clamitans* and other anurans. Female *Cx. territans* were sorted out, and blood meal analysis was performed. **B** Blood meal sources of *Cx. territans* mosquitoes by year between 2018 and 2022

Germany) [36], and analyzed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). Identification of the source of mosquito blood meals was determined for sequences with a sequence identity of 85% or higher [37] (Fig. 1A).

Anuran collection and swabbing

For taxonomic stability, we follow the nomenclature of AmphibiaWeb (https://amphibiaweb.org). Anurans (frogs) were collected by hand at three permanent artificial ponds (naturalized for over 50 years) at MLBS: Riopel (37.374552, −80.552109), Sylvatica (37.377079, −80.522245), and Horton (37.378483, −80.522078). The largest of these ponds, Riopel, is approximately $80 \times 90 \times 4$ m, whereas the smaller two fluctuate in depth (≤ 1 m) depending on rainfall and measure approximately 30×30 m. None of the ponds contain fish. Frog sampling was conducted under Virginia Department of Game and Inland Fisheries collection permit nos. 064750 and 070446 and Virginia Tech Institutional Animal Care and Use Committee (IACUC) protocols (#19-003 and #22-066). Based on the results obtained for the mosquito blood meal analysis, collections were limited to male and female *Rana* (*Aquarana*) *clamitans* and *Rana* (*Aquarana*) *catesbeiana*. Individuals were placed and kept in unused polyethylene bags (25.4×40.64 cm 4 Mil Industrial Poly Bags, Uline, Pleasant Prairie, WI, USA) with spring water and tied to prevent escape. Larger frogs were kept in individual terrariums ($23.5 \times 15.24 \times 17.78$ cm Kritter Keeper) (Lee's Aquarium & Pet Products, San Marcos, CA, USA), which were cleaned using a 10% bleach solution between uses to prevent contamination. During handling, nitrile gloves were worn and changed between each individual

to prevent potential cross-contamination. Adult frogs were swabbed for *Bd* (described below), measured (snout–vent length), weighed using a spring scale (Pesola, Schindellegi, Switzerland), and marked using the toe clipping method described by Martoff [38] prior to release.

Adult frogs of the two species and sexes were swabbed using rayon-tipped swabs (MW113 series urethral swab, Medical Wire & Equipment Co Ltd, Corsham, UK). The skin of each individual was swabbed five times each on the venter, each medial thigh, and between the digits of each hind leg (Fig. 2A). Swabs were frozen at −80 °C and sent for *Bd* screening at the Genetics Core Facility of the Sam Noble Museum, University of Oklahoma.

Experimental transmission assays

Culex territans mosquitoes were caught as larvae in the ponds at MLBS and reared in the laboratory under conditions as described in Reinhold et al. [39]. Female *Cx. territans* were tested for transmission using a modified experimental setup after Gould et al. [30]. All experiments were conducted in a biosafety cabinet at 25–27 °C using standard sterile practices to maintain the sterility of the plates. *Batrachochytrium dendrobatidis* Global Panzootic Lineage (GPL) strain JEL0423 (Collection of Zoosporic Eufungi at the University of Michigan [CZEUM], Ann Arbor, MI, USA) was plated on sterile 1%T media using CZEUM protocols (10 g Fisher BioReagents tryptone BP1421-500; Fisher Scientific, Waltham, MA, USA) and 10 g bacteriological-grade agar [IBI Scientific CAS #9002-18-0, Dubuque, IA, USA], in 1 L deionized water). Mosquitoes were briefly cold-anesthetized and tethered by the thorax to tungsten rods with ultraviolet (UV)-cured glue (Bondic, Niagara Falls, NY, USA), which
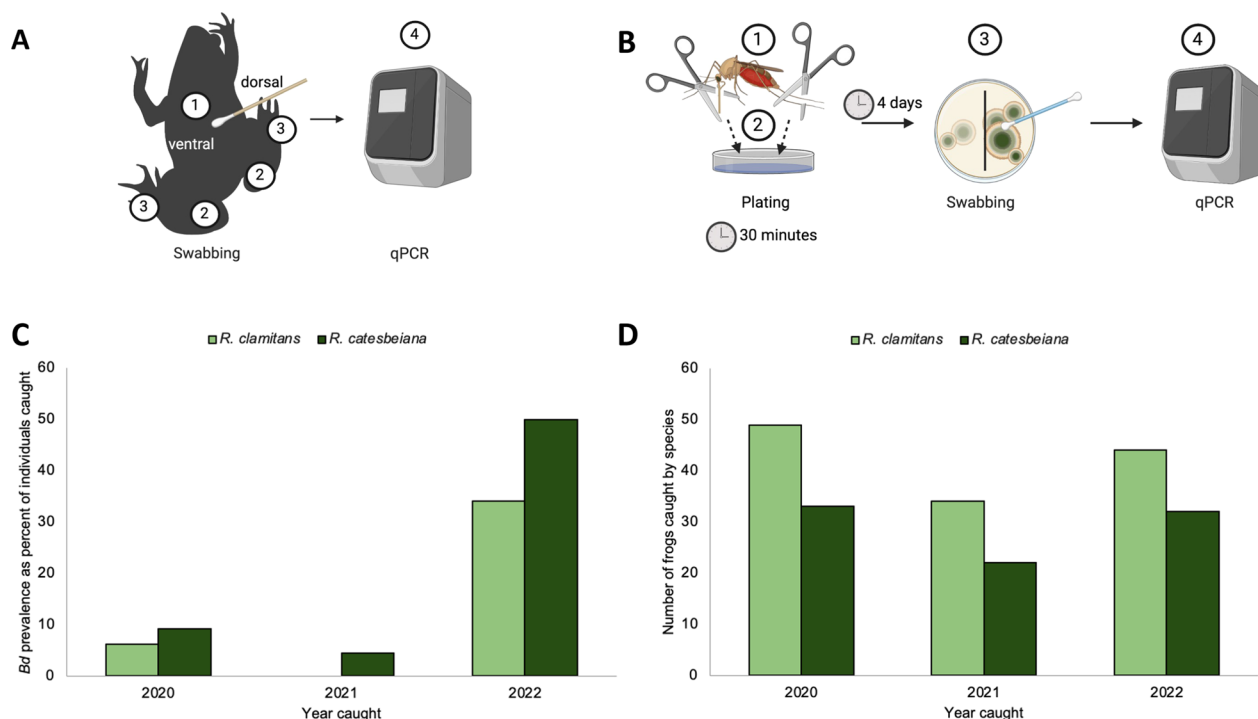
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Fig. 2 **A** Frogs were swabbed five times each on the (1) abdomen, (2) both medial hind thighs, and (3) between the hind digits of both feet. Swabs were (4) sent out for qPCR to confirm *Bd* presence or absence. **B** The (1) proboscis and legs of field-caught mosquitoes were removed and (2) placed on a sterile agar plate, which was divided to separate the two body parts. After 4 days, the plates were (3) swabbed and (4) sent out for qPCR and analysis. **C** Numbers of frogs caught and swabbed per species between 2020 and 2022. **D** Representation of the numbers of frogs that tested positive for *Bd* from 2020 to 2022

held the mosquitoes stationary when exposed to a plate containing *Bd* (cell count: $5.251 \times 10^5 \pm 9.727 \times 10^5$) ($50 \times 11$ mm petri dishes, #EW-14005-24, Advantech, Taipei City, Taipei, Taiwan). These plates were swabbed to confirm the presence of *Bd* after mosquito exposure. An average of ten mosquitoes ($8 < n < 12$) were held on the *Bd* containing plate for 30 min ($N = 123$, 13 replicates, with some mosquitoes lost during transfer). These mosquitoes were then transferred to new individual sterile 1%T agarose plates for 30 min of contact time to simulate the average time needed for this mosquito species to feed to repletion on a frog host [33]. Plates were incubated at room temperature for 96 h before being swabbed. Swabs were sent to the Genetics Core Facility of the Sam Noble Museum, University of Oklahoma, to confirm the transfer of *Bd*. To control for environmental or potential previous *Bd* exposure (during larval or pupal stage), the same experiment was conducted by exposing 50 mosquitoes (in 5 sets of 10) to sterile plates instead of *Bd*-inoculated plates ($N = 50$). These mosquitoes were also transferred to new individual plates after 30 min and allowed to maintain contact for 30 min. Plates were swabbed after 96 h and sent out for processing as described previously (Fig. 3A).

Field mosquito *Bd* sampling

A total of 82 field-caught, blood-fed *Cx. territans* mosquitoes [57 and 25 from 2021 and 2022, respectively] were sampled for *Bd*. Sterile 1%T agar plates (50×11 mm petri dishes, #EW-14005-24, Advantech, Taipei City, Taipei, Taiwan) were made following CZEUM instructions to simulate the landing onto the surface of an amphibian's skin. Each plate was divided into 2 sides for the proboscis and legs respectively. The proboscis was clipped using fine-tipped forceps and put on one side of the plate, and the legs from the same mosquito were pulled and placed on the other side of the plate. Forceps were cleaned and dried before removing any parts of the mosquitoes and in between individuals. Plates were incubated at room temperature for 96 h before being swabbed with MW113 swabs. Swab samples were stored at −80 °C until being sent out for processing as described previously (Fig. 2B).

Screening samples using qPCR

Pathogen screening for *Bd* was conducted at the Genetics Core Facility of the Sam Noble Museum at the University of Oklahoma. Swabs from both agar plates and frogs were first extracted using the PrepMan Ultra Sample Preparation Reagent protocol [40] (Applied Biosystems, Thermo
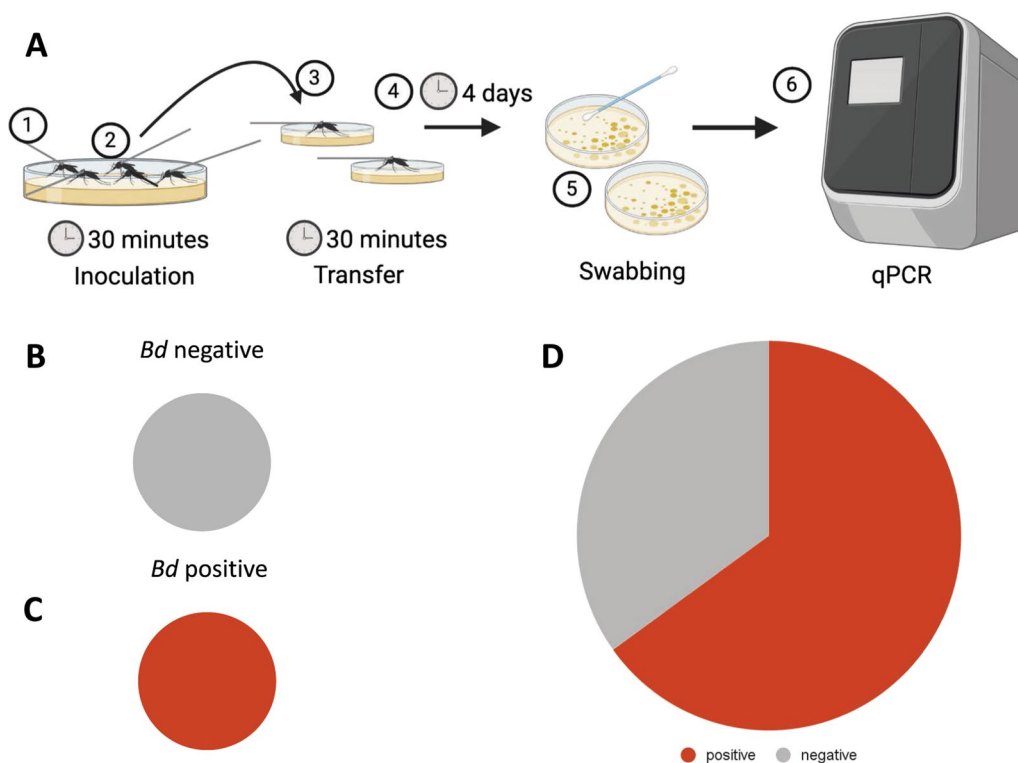
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Fig. 3 **A** Mosquitoes were (1) tethered to a tungsten rod and (2) placed on a *Bd* plate with legs touching the agar. After 30 min, the mosquitoes were (3) transferred to a new, individual sterile plate with limbs in contact with the agar for another 30 min and (4) let sit for 4 days. After 4 days, the plates were (5) swabbed and stored at −80 °C until they were (6) sent out for qPCR and analysis. **B** Representation of negative controls (*n* = 56). **C** Representation of *Bd*-infected plates to confirm the presence of *Bd* (*n* = 6). **D** The number of plates that were positive for *Bd* 4 days after contact with an inoculated mosquito (*n* = 123)

Fisher Scientific). Quantitative PCR (qPCR) techniques were used to determine the presence/absence of *Bd* genetic material and to estimate the number of gene copies per sample, or *Bd* load, using QuantStudio software v3 (Applied Biosystems, Thermo Fisher Scientific). The *Bd* assay primers target the internal transcribed spacer (ITS-1) rRNA gene (forward primer: ITS1-3 Chytr; reverse primer: 5.8S) from Boyle et al. [41]. All samples were run in triplicate, with positive controls of known *Bd* gene copies (gBlock DNA quantities 1e1–1e4) and a negative control (molecular grade sterile water). Samples were considered positive for *Bd* (*Bd*+) if amplification occurred in at least two of the three wells and if the mean *Bd* gene copy number was greater than 1.0, with samples rerun as needed [42, 43].

Results

Blood meal analysis

A total of 538 mosquitoes were collected from Sylvatica Pond at MLBS. Among them, 98 were blood-fed *Cx. territans*. We identified a total of five host species, including *R. clamitans* (61%) (green frog), *R. catesbeiana* (35%) (American bullfrog), *Rana sylvatica* (2%) (wood frog), *H.*

versicolor (1%) (gray treefrog), and *Nerodia sipedon* (1%) (northern water snake). We noted that *Cx. territans* preferentially fed on *R. clamitans* (61%) and *R. catesbeiana* (35%) (Fig. 1B).

Frog collection and swabbing

A total of 127 *R. clamitans* and 87 *R. catesbeiana* were caught and swabbed between 2020 and 2022 (Fig. 2A, C). Among the individuals swabbed, 17.8% were confirmed as *Bd*+. The prevalence of *Bd* infection among the surveyed populations varied between years and species: 9.1% prevalence in *R. catesbeiana* and 6.1% in *R. clamitans* during 2020 dropped to 4.5% and 0% in *R. catesbeiana* and *R. clamitans*, respectively, in 2021. During 2022 we found 38% and 50% prevalence among *R. catesbeiana* and *R. clamitans*, respectively (Fig. 2D). None of the 82 field-caught mosquitoes screened for *Bd* were positive.

Transmission assays

Of the 123 experimental mosquitoes tested, 80 were positive for *Bd* (65%) (cell counts $5.251 \times 10^5 \pm 9.727 \times 10^5$; gene copies $1.94499 \times 10^5 \pm 3.37174 \times 10^5$) (Fig. 3D). None of the five initial control plates or the 50 test controls

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were positive for *Bd*, showing no evidence of prior exposure to the fungus (Fig. 3B). All *Bd*-inoculated plates contained *Bd*, confirming the experimental mosquitoes were exposed to the fungus (Fig. 3C).

Discussion

This study was conducted to assess the prevalence of the pathogenic fungus at our field site, MLBS, and to ascertain whether the amphibian-biting mosquito, *Cx. territans*, is capable of vector transmission of *Bd* to anurans. We determined the primary hosts of *Cx. territans* and tested two focal hypotheses: (1) Pathogen screening efforts would detect *Bd* on *Cx. territans* and their anuran blood meal hosts in the field, and (2) *Cx. territans* is capable of *Bd* transmission from one surface to another in a laboratory setting.

Although *Cx. territans* has been known to feed on a wide variety of hosts [10–12], our results show that the mosquito population at MLBS exhibits a strong preference for *R. clamitans* and *R. catesbeiana* (Fig. 1). Both anuran species are commonly observed around the MLBS ponds. A plethora of other potential hosts have been observed at the MLBS ponds, including *R. sylvatica*, *Rana palustris* (pickerel frog) *H. versicolor*, and *Pseudacris crucifer* (spring peeper). Additional potential hosts present at MLBS include *N. sipedon* (northern water snake), *Crotalus adamanteus* (eastern diamondback rattlesnake), and numerous salamander species (in particular *Notophthalmus viridescens*, and several *Desmognathus* and *Plethodon* spp.) [44]. The strong host preference for *R. clamitans* and *R. catesbeiana* exhibited by *Cx. territans* led us to focus our *Bd* screening on these two species.

Overall, we found that the current prevalence of *Bd* is relatively low among the *R. clamitans* and *R. catesbeiana* populations at MLBS. Previous studies at MLBS found several species of amphibians carrying *Bd*. Rothermel et al. [45] and Wimsatt et al. [46] found *Bd* present in all *Notophthalmus viridescens* (red-spotted newt) individuals tested at MLBS, which is present in all three ponds at the station where *R. clamitans* and *R. catesbeiana* are found. While these studies only tested one species, they demonstrate that *Bd* has been present in the MLBS ponds for more than a decade. Hughey et al. [47] also reported *Bd*-infected individuals of *N. viridescens*, *P. crucifer*, and *R. catesbeiana*, and found lower rates of infection among *R. catesbeiana* than the other two species. None of these studies associated *Bd* infections at MLBS with die-offs or significant population declines. Collectively, the present and previous studies show that *Bd* is established at MLBS. Our results also suggest that infection prevalence and detectability vary from year to year (Fig. 2D). This

kind of variability is not uncommon in areas where *Bd* is considered endemic [47–52] and may be part of natural long-term oscillations described by Talley et al. [49]. Several factors could be playing a role in these oscillations. The presence of carriers in these areas, including (but not limited to) *N. viridescens*, *R. catesbeiana*, and crayfish (e.g., *Cambarus* spp.) may be acting as fungal reservoirs and may partially account for the yearly fluctuations we detected in anuran populations [24, 26, 27, 45–47, 53–55]. Environmental factors could also affect *Bd* prevalence, with colder and drier years potentially causing a shift in *Bd* prevalence [20, 23, 49, 51, 56, 57]. Despite the sudden increase in *Bd* in 2022, no mass die-offs of amphibians were observed at the time, which suggests that *Bd* is not an immediate concern for the amphibians at the site, but future observations and surveillance will provide more insight.

Our experimental transmission test confirmed that *Cx. territans* can transmit *Bd* from one surface to another (Fig. 3D). This suggests that *Cx. territans* can transfer the fungus to a new host after landing and feeding on an infected host. Ecologically, *Cx. territans* is a good candidate to vector a topical fungus, given that it feeds primarily on frogs and is very slow to feed to repletion [10–12, 33]. If the mosquito does not have the opportunity to feed to repletion (i.e., frog jumps into the water), they may seek a new host to complete their meal, allowing them to transmit *Bd* zoospores between hosts. They may also transmit the fungus between gonotrophic cycles, though further testing is needed to determine the maximum time the mosquito can carry the fungus. Because 35% of the mosquitoes did not transmit *Bd* to a new plate, there may be a threshold concentration necessary for transmission. The large range in cell counts on each inoculated *Bd* plate could also have contributed to the variability in individual mosquitoes' ability to transmit zoospores between plates. Another source of variability could be the uneven nature of the way the *Bd* fungus grows. All mosquitoes were placed on visible fungus, but it was difficult to determine which plated area was actively releasing zoospores. This may provide a more ecologically relevant way of testing the ability of a mosquito to transmit *Bd* experimentally than has been previously shown in the literature [30], since the landing area of the mosquito on a frog may not be releasing zoospores. Because more than two-thirds of the mosquitoes transmitted *Bd* to uninfected surfaces, we conclude that *Cx. territans* can carry spores on their appendages and could thus be a competent vector of *Bd* in the field.

We hypothesized that we would find *Bd* on the mosquitoes caught in the field, but all field-caught mosquitoes were negative for *Bd*. These results are not unexpected,

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considering the low prevalence of the fungus in the frog population in 2020 and 2021 and the small mosquito sample size for 2022. However, based on the ability of other arthropod vectors to act as carriers of *Bd* in the wild [29] and the ability of *Cx. territans* to transmit the fungus in a laboratory setting, we would expect to find *Bd*-carrying mosquitoes in a larger sample or among samples from a more highly infected population of frogs. Because this mosquito species lives in close association with frogs throughout the Northern Hemisphere and given that *Bd* is also prevalent in these regions [5–10], transmission in areas of high *Bd* prevalence is likely. The lack of *Bd* found on field mosquitoes may also be due to the location of *Bd* on frogs. *Rana clamitans* and *R. catesbeiana* were often observed resting partially submerged in water, as noted in previous studies [38, 58]. This position results in their ventral surfaces being more likely to be exposed to the water-borne fungus, and consequently, unless the fungus has spread extensively, a mosquito feeding on the dorsum of a frog's body may not make contact with the fungus. Mosquitoes were observed in the field clustering on the frogs' back legs and lower back, possibly to avoid detection and antiparasitic behavior (e.g., grooming) from the host as seen in other mosquito species [59–61]. We also observed mosquitoes on the ventral surfaces of the body and on the toes when those areas were exposed. *Culex territans* is known to feed on more terrestrial amphibians and reptiles [10, 11]. It would therefore be beneficial for future studies to collect additional *Cx. territans* mosquitoes and test for field transmission, especially in areas where *Bd* prevalence is high.

Conclusion

In this study, we determined the host preference of *Cx. territans*, the prevalence of *Bd* at the MLBS field site, the ability of *Cx. territans* to transmit *Bd* in a laboratory setting, and *Bd* prevalence among blood-fed mosquitoes in the field. We found that, at MLBS, *Cx. territans* feeds preferentially on *R. clamitans* and *R. catesbeiana*. We observed varying *Bd* prevalence over the course of 3 years at MLBS within *R. clamitans* and *R. catesbeiana* populations. Most importantly, we found that *Cx. territans* can transmit the fungus to a clean surface. We did not, however, find mosquitoes in the field with *Bd* present on their proboscis or legs. Overall, the results of this study add to the growing understanding of *Bd* epidemiology and disease ecology both on a local and global scale. Future research will help determine whether mosquitoes in the field can carry the fungus and transmit it to a new host as well as the length of time the fungus can stay on the mosquito.

Abbreviations

Bd	*Batrachochytrium dendrobatidis*
Cx	*Culex*
R	*Rana*
MLBS	Mountain Lake Biological Station
H	*Hyla*
N	*Notophthalmus*
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal RNA

Availability of data and materials
The data supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate
Anuran sample collection and handling was conducted under Virginia Department of Game and Inland Fisheries collection permit nos. 064750 and 070446 and under approved IACUC protocols (#19-003 and #22-066) at the Virginia Polytechnic Institute and State University.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References

1. Center for Disease Control and Prevention (CDC). 2018. https://www.cdc.gov/globalhealth/stories/2019/world-deadliest-animal.html. Accessed 26 Jan 2023.
2. Gates B. The deadliest animal in the world mosquito week. Kirkland: The gates notes LLC; 2014.
3. Cebrián-Camisón S, Martínez-de la Puente J, Figuerola J. A literature review of host feeding patterns of invasive *Aedes* mosquitoes in Europe. Insects. 2020;11:848.
4. Reeves LE, Holderman CJ, Blosser EM, Gillett-Kaufman JL, Kawahara AY, Kaufman PE, et al. Identification of *Uranotaenia sapphirina* as a specialist of annelids broadens known mosquito host use patterns. Commun Biol. 2018;1:1–8.
5. Culverwell CL, Uusitalo RJ, Korhonen EM, Vapalahti OP, Huhtamo E, Harbach RE. The mosquitoes of Finland: updated distributions and bionomics. Med Vet Entomol. 2021;35:1–29.
6. Darsie Jr RF, Ward RA. 1981. Identification and geographical distribution of the mosquitoes of North America, north of Mexico. Walter Reed Army Inst of Research Washington.
7. Kitron U, Pener H. Distribution of mosquitoes (Diptera: Culicidae) in northern Israel: a historical perspective II. Culicine mosquitoes. J Med Entomol. 1986;23:182–7.
8. Robert V, Günay F, Le Goff G, Boussès P, Sulesco T, Khalin A, et al. Distribution chart for Euro-Mediterranean mosquitoes (western Palaearctic region). J Euro Mosq Control Assoc. 2019;37:1–28.
9. Zittra C, Waringer J. Species inventory, ecology, and seasonal distribution patterns of Culicidae (Insecta: Diptera) in the National Park Donau-Auen (Lower Austria). Aquat Insects. 2014;36:63–77.
10. Bartlett-Healy K, Crans W, Gaugler R. Temporal and spatial synchrony of *Culex territans* (Diptera: Culicidae) with their amphibian hosts. J Med Entomol. 2008;45:1031–8.
11. Burkett-Cadena ND, Graham SP, Hassan HK, Guyer C, Eubanks MD, Katholi CR, et al. Blood feeding patterns of potential arbovirus vectors of the genus *Culex* targeting ectothermic hosts. Am J Trop Med Hyg. 2008;79:809–15.
12. Crans WJ. The blood feeding habits of *Culex territans* Walker. Mosq News. 1970;30:445–7.
13. Bartlett-Healy K, Crans W, Gaugler R. Vertebrate hosts and phylogenetic relationships of amphibian trypanosomes from a potential invertebrate vector, *Culex territans* Walker (Diptera: Culicidae). J Parasitol. 2009;95:381–7.
14. Ferguson LV, Smith TG. Reciprocal trophic interactions and transmission of blood parasites between mosquitoes and frogs. Insects. 2012;3:410–23.
15. Savage HM, Aggarwal D, Apperson CS, Katholi CR, Gordon E, Hassan HK, et al. Host choice and West Nile virus infection rates in blood-fed mosquitoes, including members of the *Culex pipiens* complex, from Memphis and Shelby County, Tennessee, 2002–2003. Vector-Borne Zoon Dis. 2007;7:365–86.
16. Reinhold JM, Fazal A, Robert J, McLeod D, Paulson S, Auguste J, et al. *Culex territans* Walker as a potential vector of ranaviruses. Virginia: Virginia Polytechnic Institute and State University; 2023.
17. Bienentreu JF, Lesbarrères D. Amphibian disease ecology: are we just scratching the surface? Herpetol. 2020;76:153–66.
18. Forzán MJ, Heatley J, Russell KE, Horney B. Clinical pathology of amphibians: a review. Vet Clin Pathol. 2017;46:11–33.
19. Campbell Grant EH, Miller DA, Muths E. A synthesis of evidence of drivers of amphibian declines. Herpetol. 2020;76:101–7.
20. Voyles J, Johnson LR, Briggs CJ, Cashins SD, Alford RA, Berger L, et al. Temperature alters reproductive life history patterns in *Batrachochytrium dendrobatidis*, a lethal pathogen associated with the global loss of amphibians. Ecol Evol. 2012;2:2241–9.
21. Gahl MK, Longcore JE, Houlahan JE. Varying responses of northeastern North American amphibians to the chytrid pathogen *Batrachochytrium dendrobatidis*. Conserv Biol. 2012;26:135–41.
22. Tobler U, Schmidt BR. Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. PLoS ONE. 2010;5:e10927.
23. Piotrowski JS, Annis SL, Longcore JE. Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycol. 2004;96:9–15.
24. Brannelly LA, McMahon TA, Hinton M, Lenger D, Richards-Zawacki CL. *Batrachochytrium dendrobatidis* in natural and farmed Louisiana

crayfish populations: prevalence and implications. Dis Aquat Org. 2015;112:229–35.
25. Kilburn VL, Ibáñez R, Green DM. Reptiles as potential vectors and hosts of the amphibian pathogen *Batrachochytrium dendrobatidis* in Panama. Dis Aquat Org. 2011;97:127–34.
26. McMahon TA, Brannelly LA, Chatfield MW, Johnson PT, Joseph MB, McKenzie VJ, et al. Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals that cause pathology in the absence of infection. Proc Natl Acad Sci. 2013;110:210–5.
27. Oficialdegui FJ, Sánchez MI, Monsalve-Carcaño C, Boyero L, Bosch J. The invasive red swamp crayfish (*Procambarus clarkii*) increases infection of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*). Biol Invasions. 2019;21:3221–31.
28. Pontes M, Augusto-Alves G, Lambertini C, Toledo L. A lizard acting as carrier of the amphibian-killing chytrid *Batrachochytrium dendrobatidis* in southern Brazil. Acta Herpetol. 2018;13:201–5.
29. Toledo LF, Ruggeri J, Ferraz L, Campos L, Martins M, Neckel-Oliveira S, et al. Midges not only sucks, but may carry lethal pathogens to wild amphibians. Biotropica. 2021;53:722–5.
30. Gould J, Valdez JW, Stockwell MP, Clulow S, Mahony MJ. Mosquitoes as a potential vector for the transmission of the amphibian chytrid fungus. Zool Ecol. 2019;29:36–42.
31. Malassigné S, Valiente Moro C, Luis P. Mosquito mycobiota: an overview of non-entomopathogenic fungal interactions. Pathogens. 2020;9:564.
32. Tawidian P, Rhodes VL, Michel K. Mosquito-fungus interactions and antifungal immunity. Insect Biochem Mol Biol. 2019;111:103182.
33. Reinhold JM, Mcleod D, Lahondére C. Some like it cold: a study of the interactions between *Culex territans* and its amphibian hosts. Virginia: Virginia Polytechnic Institute and State University; 2023.
34. Silver JB. Mosquito ecology: field sampling methods. 3rd ed. Dordrecht: Springer Netherlands; 2007.
35. Kitano T, Umetsu K, Tian W, Osawa M. Two universal primer sets for species identification among vertebrates. Int J Legal Med. 2007;121:423–7.
36. QIAGEN CLC Genomics Workbench 23.0.1 (https://digitalinsights.qiagen.com/).
37. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10.
38. Martoff BS. Territoriality in the green frog. Rana clam Ecol. 1953;34:165–74.
39. Reinhold JM, Chandrasegaran K, Oker H, Crespo JE, Vinauger C, Lahondère C. Species-specificity in thermopreference and CO_2-gated heat-seeking in *Culex* mosquitoes. Insects. 2022;13:92.
40. Cheng TL, Rovito SM, Wake DB, Vredenburg VT. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. Proc Natl Acad Sci. 2011;108:9502–7.
41. Boyle DG, Boyle DB, Olsen V, Morgan JA, Hyatt AD. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Org. 2004;60:141–8.
42. Kerby JL, Schieffer A, Brown JR, Whitfield S. Utilization of fast qPCR techniques to detect the amphibian chytrid fungus: a cheaper and more efficient alternative method. Methods Ecol Evol. 2013;4:162–6.
43. Watters JL, Davis DR, Yuri T, Siler CD. Concurrent infection of *Batrachochytrium dendrobatidis* and ranavirus among native amphibians from northeastern Oklahoma, USA. J Aquat Anim Health. 2018;30:291–301.
44. iNaturalist community. Observations of species list from Mountain Lake Biological Station, USA, observed on/between 6 Jun 2017–31 Aug 2022. https://www.inaturalist.org on 11 May 2023.
45. Rothermel BB, Walls SC, Mitchell JC, Dodd CK Jr, Irwin LK, Green DE, et al. Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. Dis Aquat Org. 2008;82:3–18.
46. Wimsatt J, Feldman SH, Heffron M, Hammond M, Ruehling MP, Grayson KL, et al. Detection of pathogenic *Batrachochytrium dendrobatidis* using water filtration, animal and bait testing. Zoo Biol. 2014;33:577–85.
47. Hughey MC, Becker MH, Walke JB, Startwort MC, Belden LK. *Batrachochytrium dendrobatidis* in Virginia amphibians: within and among site variation in infection. Herpetol Rev. 2014;45:428–38.
48. Kinney VC, Heemeyer JL, Pessier AP, Lannoo MJ. Seasonal pattern of *Batrachochytrium dendrobatidis* infection and mortality in *Lithobates*

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areolatus: Affirmation of Vredenburg's "10,000 Zoospore Rule." PLoS ONE. 2011;6:e16708.

49. Longo AV, Burrowes PA, Joglar RL. Seasonality of *Batrachochytrium dendrobatidis* infection in direct-developing frogs suggests a mechanism for persistence. Dis Aquat Org. 2010;92:253–60.

50. Talley BL, Muletz CR, Vredenburg VT, Fleischer RC, Lips KR. A century of *Batrachochytrium dendrobatidis* in Illinois amphibians (1888–1989). Biol Conserv. 2015;182:254–61.

51. Watters JL, McMillin SL, Marhanka EC, Davis DR, Farkas JK, Kerby JL, et al. Seasonality in *Batrachochytrium dendrobatidis* detection in amphibians in central Oklahoma, USA. Zoo Wildl Med. 2019;50:492–7.

52. Julian JT, Gould VA, Glenney GW, Brooks RP. Seasonal infection rates of *Batrachochytrium dendrobatidis* in populations of northern green frog *Lithobates clamitans* melanota tadpoles. Dis Aquat Org. 2016;121:97–104.

53. Gervasi SS, Urbina J, Hua J, Chestnut T, Relyea A, Blaustein RR. Experimental evidence for American bullfrog (*Lithobates catesbeianus*) susceptibility to chytrid fungus (*Batrachochytrium dendrobatidis*). EcoHealth. 2013;10:166–71.

54. Greenspan SE, Calhoun AJ, Longcore JE, Levy MG. Transmission of *Batrachochytrium* dendrobatidis to wood frogs (*Lithobates sylvaticus*) via a bullfrog (*L. catesbeianus*) vector. J Wildl Dis. 2012;48:575–82.

55. Schloegel LM, Picco AM, Kilpatrick AM, Davies AJ, Hyatt AD, Daszak P. Magnitude of the US trade in amphibians and presence of *Batrachochytrium dendrobatidis* and ranavirus infection in imported North American bullfrogs (*Rana catesbeiana*). Biol Conserv. 2009;142:1420–6.

56. Weather Underground. TWC Product and Technology LLC. 2023. https://www.wunderground.com/dashboard/pws/KVANEWPO68/table/2019-11-1/2019-11-1/monthly. Accessed 22 Jan 2023.

57. Kriger KM, Pereoglou F, Hero JM. Latitudinal variation in the prevalence and intensity of chytrid (*Batrachochytrium dendrobatidis*) infection in eastern Australia. Conserv Biol. 2007;21:1280–90.

58. Emlen ST. Territoriality in the bullfrog *Rana catesbeiana*. Copeia. 1968. https://doi.org/10.2307/1441748.

59. Oduola AO, Awe OO. Behavioural biting preference of *Culex quinquefasciatus* in human host in Lagos metropolis Nigeria. J Vector Borne Dis. 2006;43:16–20.

60. Vinauger C, Lahondère C, Wolff GH, Locke LT, Liaw JE, Parrish JZ, et al. Modulation of host learning in *Aedes aegypti* mosquitoes. Curr Biol. 2018;28:333–44.

61. Wynne NE, Chandrasegaran K, Fryzlewicz L, Vinauger C. Visual threats reduce blood-feeding and trigger escape responses in *Aedes aegypti* mosquitoes. Sci Rep. 2022;12:21354.

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