MOSQUITOES AS A POTENTIAL VECTOR FOR THE TRANSMISSION OF THE AMPHIBIAN CHYTRID FUNGUS

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Abstract. The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), is an infectious disease responsible for the worldwide decline of amphibian species. To mitigate these declines, it is necessary to identify the various vectors by which the fungus can be transmitted between individuals and populations. The objective of this study was to determine whether adult female mosquitoes can carry and transfer *Bd* fungal cells. Mosquitoes were exposed to netting soaked in a live *Bd* zoospore suspension to determine whether they are able to externally acquire the fungus. Another group was placed into containers with a sterile and *Bd*-inoculated agar plate to determine whether mosquitoes could transfer *Bd* between these surfaces. *Bd* DNA was found to be present on mosquito legs exposed to inoculated netting and agar plates suggesting that *Bd* can be transmitted by the mosquito over short distances. This is the first study to demonstrate that an insect host may be a mechanical vector of *Bd* and suggests that we should begin to consider the role of mosquitoes in the dissemination and control of the fungus.

INTRODUCTION

Emerging infectious diseases have posed a substantial threat to human health and global biodiversity over the past few decades (Daszak, Cunningham, and Hyatt 2000; Patz et al. 1996). Changes in the distribution of these pathogens, as well as their prevalence within host populations, primarily depend upon the extent and rate of transmission between infected and uninfected individuals. Although most infectious agents are specialized for particular modes of transmission, the greatest potential for widespread dissemination is through indirect horizontal transmission via vector organisms, which carry and then transmit the pathogen between hosts (Patz et al. 1996; World Health Organization 2014). With infectious diseases becoming a major cause in the decline and possible loss of entire wildlife populations, recognizing the specific role vectors play in this key threatening process will be necessary for the management of such declines and for species conservation in general (Daszak, Cunningham, and Hyatt 2000).

An example of the dramatic effects infectious diseases can have on wildlife has been the global emergence and rapid spread of chytridiomycosis into naïve amphibian populations, an infectious disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Skerratt et al. 2007; Berger, Speare, and Hyatt 1999). This pathogen affects nearly a third of all amphibians worldwide and is thought to be the leading cause of the dramatic decline or extinction of over 200 species (Skerratt et al. 2007; Bower et al. 2017; Berger et al. 1998). A greater number of amphibian species are likely to be threatened as *Bd* continues to spread

into new geographic areas due to anthropogenic activities (Daszak et al. 1999; Tinsley et al. 2015) and potentially increase in virulence due to climate change (Pounds et al. 2006). To effectively control the spread of *Bd* and understand transmission between individuals, populations and species, modes of dissemination need to be identified.

Bd is generally considered an amphibian specialist, growing in the keratinized epidermis of infected individuals (Voyles, Rosenblum, and Berger 2011). This fungus is typically transmitted through water in the form of motile flagellated zoospores and through contact with infected adults and tadpoles (Longcore, Pessier, and Nichols 1999; Berger, Speare, and Hyatt 1999; Rowley and Alford 2007). Non-amphibian vectors are also thought to play a key role in its spread, and *Bd* has been found to be carried on algae (Johnson and Speare 2003), nematodes (Shapard, Moss, and San Francisco 2012), lizards (Kilburn, Ibáñez, and Green 2011), aquatic birds (Burrowes and De la Riva 2017; Garmyn et al. 2012), and crayfish (Brannelly et al. 2015; McMahon et al. 2013). *Bd* has also been experimentally grown on other keratinised surfaces such as snakeskin (Symonds et al. 2008; Longcore, Pessier, and Nichols 1999), bird feathers (Johnson and Speare 2005; Symonds et al. 2008), and toe scales of aquatic birds (Garmyn et al. 2012). Many of these vectors co-occur with the fungus and their amphibian hosts throughout much of their range, potentially contributing to the spread of *Bd*. The mosquito could be another potential vector since they share these traits such as keratinised surfaces and co-occurrence with amphibians. However, their ability to carry and transfer *Bd* has not been investigated.

Like amphibians, many mosquitoes require seasonal or permanent freshwater habitats in order to breed and oviposit, which means both the host and potential vector occur over similar spatial and temporal scales (Rubbo et al. 2011; Bartlett-Healy, Crans, and Gaugler 2008). Mosquitoes serve as amphibian prey, and adult female mosquitoes require blood-meals to produce eggs, with some species specifically targeting amphibian hosts (Heatwole and Shine 1976; Means 1968). Through such interactions, amphibians can acquire certain diseases, usually occurring via salivary transmission as the mosquito is feeding on the blood of the amphibian host (Crans 1969; Ferguson and Smith 2012), and through trophic transmission when an infected mosquito is ingested by an individual (Ferguson and Smith 2012). However, it is yet unknown whether an amphibian can be infected by a mosquito vector through passive surface-to-surface transmission.

When a mosquito lands on the surface of an infected host to obtain a blood-meal, it is possible that direct physical contact will be made between a region of infected epithelium and the external structures of the insect, providing the means for *Bd* to be collected. Collection of *Bd* zoospores may occur due to the feather-like projections and scales on the mosquito's leg surfaces which may provide a complex surface for dry fungal spores to become attached to the tarsae after landing on an infected surface (Scholte et al. 2003). Moreover, some mosquito species travel many kilometres in a single night (Bailey, Eliason, and Hoffmann 1965) and may acquire blood-meals from multiple hosts during a single blood-feeding cycle (Gillies 2016; Scott et al. 1993; Muturi et al. 2008). Therefore, mosquitoes may be an effective vector to transfer *Bd* zoospores between infected and non-infected individuals and to transfer *Bd* zoospores among infected and uninfected populations in isolated locations. For this study, the main objectives were to determine: i) whether adult female mosquitoes have the potential to carry *Bd* on their external surfaces and ii) whether they can transfer the fungus from one location to another.

MATERIALS AND METHODS

Mosquito rearing
The species used for this study was *Culex quinquefasciatus,* a globally occurring mosquito that feeds on amphibians as well as other hosts (Savage et al. 2007; Irby and Apperson 1988) and serves as a vector for many diseases affecting humans and wildlife (World Health Organization 2014). Egg rafts were produced and provided by the Department of Medical Entomology in Westmead Hospital, Australia. Upon arrival, eggs were placed in small trays with 2 cm of deionized water. Larvae were then reared at 25°C and fed dry food (powdered goldfish

flakes, goldfish pellets, and brewer's yeast) *ad libitum.* Emerging adults were moved to 32 × 22 × 11 cm cages that were kept at room temperature between 20–24°C, on 12/12-hour light-dark cycle, and maintained on a 10% glucose solution. Only females were used in the study, as they are the only gender that requires a blood source and are more likely to make contact with an infected host. Females were distinguished from males based on size and antennae morphology (Fay and Morlan 1959).

Cultivation of Batrachochytrium dendrobatidis
The process of *in vitro* cultivation was carried out by seeding TGhL agar plates as per Longcore, Pessier, and Nichols (1999) (16 g tryptone, 4 g gelatine hydrolysate, 2 g lactose and 10 g bacteriological agar in 1 L distilled water) with 2 ml of a one-week-old actively growing *Bd* broth (strain: Gibbo River-Llesueuri-00-LB-1, derived from pre-existing stock held at the University of Newcastle). Plates were incubated at room temperature conditions between 20–24°C and periodically checked for growth using an inverted light microscope until colonies of zoosporangia and free-swimming zoospores could be detected (approx. 7–10 days). A zoospore suspension was also prepared by flooding inoculated TGhL agar plates with 1.5 ml of liquid media (TGhL). These plates were left to stand for 5 minutes before the supernatant was collected. Strict chytrid hygiene protocols were followed as per Johnson et al. (2003).

Mosquito netting experiment
The ability of adult female mosquitoes to carry *Bd* on external surfaces was examined with an experiment in which a large 9 × 15 × 6 cm section was cut out of the bottom of a small container (11 × 17 × 8 cm) and replaced with a single layer of mosquito netting that was taped to the remaining sections of the container. In the treatment group (n = 10) the netting was then soaked in a *Bd* zoospore solution (1,430,000 ± 420,000 zoospores/ml) for a period of 10 seconds. A control group was also established (n = 8), where netting was soaked in sterile liquid media (TGhL). An adult female mosquito was then introduced into each container using a small opening cut into the lid of the container. The container was placed on its side with the netting in the vertical position, thereby allowing the removal of any excess liquid, while also encouraging the mosquito to land on the *Bd*-soaked netting. To approximate the length of time it takes for a mosquito to obtain a complete blood-meal, individuals were allowed to remain on the netting for a total of 2 minutes (Lahondère and Lazzari 2012), excluding any time periods when the mosquito was on a non-netting surface of the container (Lahondère and Lazzari 2012). Following completion of the experiment, containers were placed at –20°C for five minutes to

euthanize the mosquitoes, whereupon bodies were collected using sterile plastic forceps, placed in separate, sterile containers and stored at –8°C for approximately 2 weeks. For each replicate, the legs of the mosquito were removed using sterile forceps and were placed in an Eppendorf tube for qPCR analysis. The legs of the mosquito were targeted for analysis because they are in regular contact with a blood host during feeding.

Agar plate experiment

The ability of adult female mosquitoes to transfer *Bd* between hosts was examined using TGhL agar plates as a substitute for the exterior surface of an amphibian. Two plates were positioned 10 cm apart on the base of a plastic container (28 × 42 × 16 cm) with a mosquito net lid. To mimic the presence of an infected and non-infected amphibian host, containers in the treatment group (n = 14) were prepared by adding one agar plate with actively growing *Bd* fungus and one that was not inoculated. As mosquitoes were sometimes observed hanging upside down from the netting, and to improve the chance of direct contact being made between mosquitoes and the inoculated agar surface, half of the treatment containers were prepared so that TGhL agar plates were inverted and tied to the overlying netting, while plates in the remaining containers were left upright. To determine whether *Bd* could be transferred in the absence of a mosquito, a control group (n = 7) was established by adding an inoculated and non-inoculated plate type to containers without any mosquitoes (the *Bd* control). A second control group (n = 7) was also established by adding two non-inoculated plates and mosquitoes to determine whether mosquitoes carried a pre-existing load of *Bd* (the mosquito control). Twelve female *C. quinquefasciatus* individuals were introduced to each container (except those in the *Bd* control group) and allowed to move freely between plates over a 96-hour period to maximize the length of time/chance of the mosquito landing on the inoculated surface. To minimize their chance of escape, mosquitoes were temporarily stunned by placing containers at a low temperature (–8°C) for 60 seconds . Each mosquito was then collected using sterile forceps and stored at –8°C for approximately 2 weeks. The legs of each individual were subsequently removed using the previously described methods, with a new pair of forceps used for each container. Inoculated and non-inoculated plates were covered prior to mosquitoes being removed to prevent cross-contamination and any chance of mosquitoes falling into the agar between the interval of being stunned and collected. The non-inoculated plates were then removed from the experimental containers as well and left at room temperature conditions (20–24°C) over a 72-hour period to encourage *Bd* growth. Plates were then swabbed 24 times with a sterile cotton swab using a cross-hatch method for qPCR analysis and kept at freezing temperatures prior to analysis.

Quantification of Bd on plates and mosquitoes

Extraction and quantification of *Bd* from swabs were performed following standard protocols for a qPCR Taqman assay as described by Boyle et al. (2004). DNA from *Bd* that was potentially picked up on the mosquito's leg surfaces was extracted by adding the legs to the extraction solution in place of swabbing material. Mosquito legs were collected and analyzed together for each replicate, showing the amount of *Bd* obtained from a total of 72 legs. A 1/100 dilution of extracted samples from both swabs and mosquitoes was used in the Taqman assay. Each biological sample was analyzed in triplicate to account for natural variation that occurs when pipetting from a sample and in case of false positives or contamination. Samples were then compared against a standard curve using a Rotor-Gene 6000 real-time DNA amplification system (Corbett Life Science, Sydney, Australia). Negative template controls (NTCs) were included in each run to detect contamination, and internal positive controls (IPCs) were used in one replicate of each sample and the NTC to test for inhibition. Following amplification, the mean number of *Bd* genetic equivalents (GE) detected at a standard cycle threshold, across all three replicates was determined, provided no amplification occurred in any NTCs and the sample IPCs amplified within 5 cycles of the NTC and IPC.

To account for false positives, samples were considered positive when amplification occurred in at least two of the three replicates, providing that the PCR reaction was not inhibited. Where two replicates returned a positive value, zero values were included in the mean calculation as it was assumed to be the result of a low quantity of DNA present within the sample. The mean GE value was then multiplied by 100 to account for the dilution step carried out during the extraction process. Samples were considered negative for the presence of *Bd* if no replicates amplified and the sample IPCs amplified within 5 cycles of the NTC IPC. Where inhibition was detected, a further 1/10 dilution of extracted DNA was made and the qPCR process repeated.

RESULTS

In the mosquito netting experiment, *Bd* was detected on mosquito legs in seven out of 10 replicates within the treatment group, with GE loads ranging from 0.59 to 27.04, while no chytrid was found in the control group (Figure 1). In the agar plate experiment, *Bd* DNA was found on the legs of mosquitoes in six out of the 14

treatment replicates, with values ranging between 1.49 and 19.91 GE loads (Figure 2). *Bd* was also detected on swabs from two out of 14 plates from the treatment group, with high GE loads of 20.19 and 26.04 (Figure 2b). All non-inoculated plates from the mosquito and *Bd* control groups tested negative for the presence of *Bd* (Figure 2).

DISCUSSION

The results from this study show that mosquitoes are a potential vector for the transmission of *B. dendrobatidis*. *Bd* was found on the leg surfaces of adult female *C. quinquefasciatus* after exposure to both inoculated agar plates and a zoospore suspension, and mosquitoes were able to transfer *Bd* to sterile agar plates. A high number of zoospores found on plates that were exposed to *Bd* by mosquitoes suggest that mosquitoes are a potential *Bd* vector, and its ability to collect and transfer *Bd* may have implications for its spread. As many mosquito species co-occur globally with amphibians, share similar breeding requirements in aquatic habitats and have positive associations with areas of high precipitation (Rubbo et al. 2011; Bartlett-Healy, Crans, and Gaugler 2008), the potential for *Bd* to be transferred between hosts by mosquitoes could be high. This is further exacerbated by the fact that many mosquito species acquire multiple blood-meals during a single feeding cycle (Gillies 2016; Scott et al. 1993; Muturi et al. 2008), increasing the potential of local transmission between infected and non-infected individuals. *Culex* mosquitoes have been found to disperse over a kilometre on average (Ciota et al. 2012), while other species can travel over 10 kilometres (Bogojević, Merdić, and Bogdanović 2011) (up to 15 kilometres in a single night (Gillies 2016)), and 40 kilometres in one generation (Gillies 2016). This suggests that transmission may also be possible be-

tween distant locations, increasing the risk of isolated populations acquiring the pathogen. In light of this, the next step will be to examine the transmission of *Bd* by mosquitoes to amphibian hosts in the wild.

In order to understand how likely transmission will be between distant host populations, it will be important to know how *Bd* is carried on the leg surfaces of the mosquito. It has been suggested that mosquitoes have the capacity to carry fungal zoospores due to the complex surface of the hair-like projections on their legs (Scholte et al. 2003). Considering this and the results obtained within this study, it is possible that this arrangement of scales on the leg of a mosquito results in a large surface
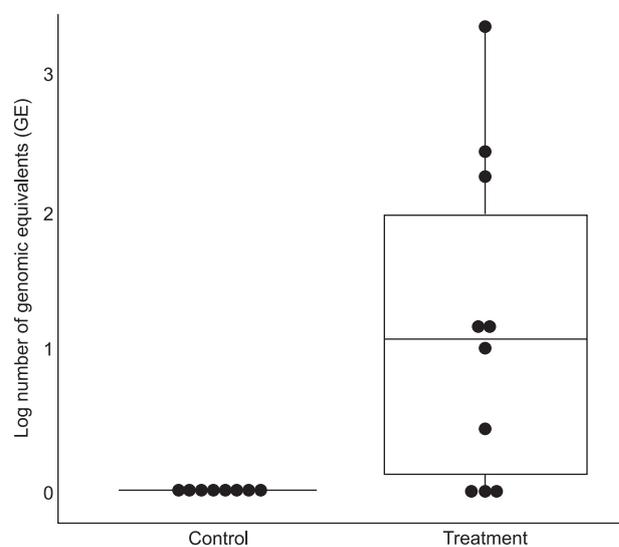

Figure 1. The log mean number of *Batrachochytrium dendrobatidis* (*Bd*) genetic equivalents (GE) detected on the external leg surfaces of *Culex quinquefasciatus* individuals from the mosquito netting experiment. Adult females were individually exposed to netting that had been soaked in sterile TGhL liquid media (Control) or a chytrid suspension (Treatment) for 2 min. Each dot represents the average value obtained from each replicate for the control and treatment group.
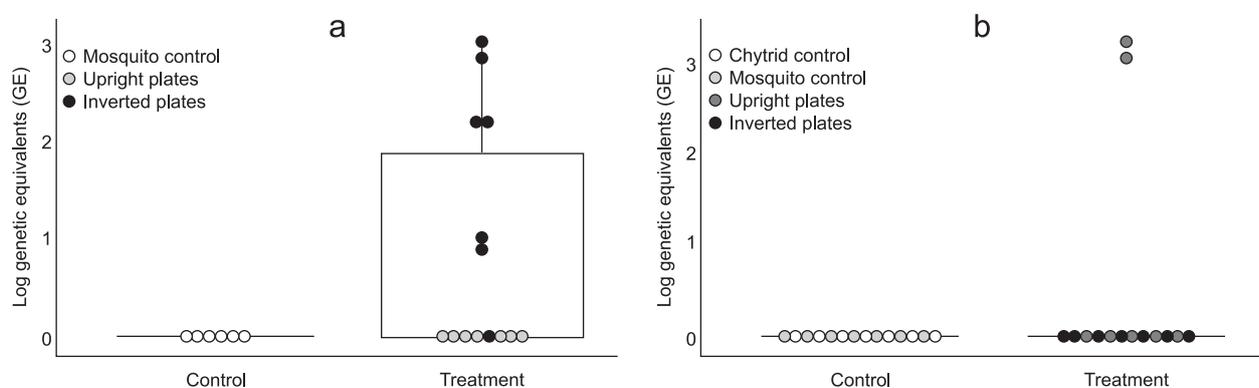

Figure 2. The log mean number of *Batrachochytrium dendrobatidis* (*Bd*) genetic equivalents (GE) detected on (A) external leg surfaces of all *Culex quinquefasciatus* adult females per replicate (n = 12) and (B) non-inoculated TGhL agar plates after swabbing each 24 times. Each dot represents the average value obtained from each replicate exposed to an inoculated agar plate and no mosquitoes (chytrid control), non-inoculated agar plates with mosquitoes (mosquito control), and upright and inverted plates inoculated with chytrid (treatment).

area that is conducive for the transfer of zoospores. However, as leg samples were not examined using light microscopy, it cannot be determined whether Bd was carried by these leg structures, with future research required to determine the method of Bd transfer. As some chytrids are known bio-degraders of chitin and others pathogenic to mosquito larvae (Powell 1993; Shoulkamy and Lucarotti 1998), further research is also required to determine whether *Bd* zoospores are able to breach the cuticle (via enzymatic digestion) and infect cells that underlie the cuticle of mosquitoes. Within this study, *Bd* was more likely to be transferred to inverted plates when compared to those left upright. Although the reason for this discrepancy remains unclear, it does allude to a behavioural preference for the mosquito to land on surfaces in certain positions. As *Bd* tends to infect the ventral surface of frogs (especially the thighs and inguinal region) more than the dorsal surface (Berger, Speare, and Kent 1999), where mosquitoes would be expected to land, these results suggest that host positioning may influence *Bd* transfer. Further research should target the relationship between the location of mosquito feeding and *Bd* infection on amphibian hosts.

If mosquitoes are a significant *Bd* vector, controlling mosquito populations in specific areas may provide refuge for some amphibian species, particularly for remnant and isolated populations. At least one inadvertent example from Australia supports this approach. In Sydney Olympic Park, Australia, mosquito populations have been actively managed since 1998 via annual aerial pesticide applications to freshwater bodies, which has reduced mosquito numbers by up to 80–90% (Webb 2013). In the same area, a large and thriving population of the threatened green and golden bell frog (*Litoria aurea*) persists, despite *Bd* being present in nearby areas and this particular species being highly susceptible to its infection. Controlling mosquito populations may, therefore, be an effective strategy to help prevent the spread and mitigate the effects of *Bd* on threatened amphibians.

This is the first study to demonstrate that an insect host may be a mechanical vector of *Bd* and suggests that we should begin to consider the role of mosquitoes in the dissemination and control of the fungus. This might be particularly important in coming years, as warmer temperatures are known to increase mosquito feeding and biting rates (Lafferty 2009), which means that expected warming from climate change may exacerbate the potential role of mosquitoes as *Bd* vectors. Our study suggests that managing non-amphibian vectors might offer an effective and alternative strategy to mitigate the threat and spread of *Bd* that could be used complementary with those currently in use, providing a multi-fork approach that may be more successful at reducing the presence of *Bd* and its ability to be spread.

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