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### ► To cite this version:

Joseph Receveur, Alexandra Bauer, Jennifer Pechal, Sophie Picq, Magdalene Dogbe, et al.. A need for null models in understanding disease transmission: the example of *Mycobacterium ulcerans* (Buruli ulcer disease). *FEMS Microbiology Reviews*, 2022, 46 (1), 10.1093/femsre/fuab045 . hal-03378901

**HAL Id: hal-03378901**

**<https://hal.science/hal-03378901v1>**

Submitted on 25 Nov 2022

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Enclosed is the non-formatted text of the article published ahead on line in September 2021 in the website of *FEMS Microbiology Reviews* – The final published reference is as follows:

Receveur JP, Bauer A, Pechal JL, Picq S, Dodge M, Jordan HR, Rakestraw AW, Fast K, Sandel M, Chevillon C, Guégan J-F, Wallace JR, Benbow MB. **2022**. A need for null model in understanding diseases transmission : the case of *Mycobacterium ulcerans* (Buruli ulcer disease). *FEMS Microbiology Reviews*, 46(1), fuab045

(formatted published text is available at <https://academic.oup.com/femsre/article/46/1/fuab045/6360977>)-

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## **A need for null models in understanding disease transmission: the example of *Mycobacterium ulcerans* (Buruli ulcer disease)**

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**ABSTRACT-** Understanding the interactions of ecosystems, humans and pathogens is important for disease risk estimation. This is particularly true for neglected and newly emerging diseases where modes and efficiencies of transmission leading to epidemics are not well understood. Using a model for other emerging diseases, the neglected tropical skin disease Buruli ulcer (BU), we systematically review the literature on transmission of the etiologic agent, *Mycobacterium ulcerans* (MU), within a One Health/EcoHealth framework and against Hill's nine criteria and Koch's postulates for making strong inference in disease systems. Using this strong inference approach, we advocate a null hypothesis for MU transmission and other understudied disease systems. The null should be tested

against alternative vector or host roles in pathogen transmission to better inform disease management. We propose a re-evaluation of what is necessary to identify and confirm hosts, reservoirs and vectors associated with environmental pathogen replication, dispersal and transmission; critically review alternative environmental sources of MU that may be important for transmission, including invertebrate and vertebrate species, plants and biofilms on aquatic substrates; and conclude with placing BU within the context of other neglected and emerging infectious diseases with intricate ecological relationships that lead to disease in humans, wildlife and domestic animals.

**Keywords:** Buruli ulcer; mycobacteria; neglected disease; emerging disease; environmental pathogens; vector competency

## INTRODUCTION

Understanding and predicting pathogen spread and transmission to humans and wildlife is a key tenet to mitigate and prevent disease epidemics. This need is particularly true, and difficult to achieve, for understudied diseases endemic to many tropical areas of the world. Neglected tropical diseases often do not have high mortality, but have substantial, persistent and widespread morbidity with local, regional and national consequences on public health and economies (Garchitorena *et al.* 2017). A key challenge for predicting spread of newly emerging and neglected diseases is having a confident understanding of how pathogens are transmitted to hosts from the environment, and then spread among hosts and reservoirs. This knowledge of transmission can take decades to decipher, or for some diseases may remain unresolved. These gaps in knowledge are often a product of funding limitations and the need for better multidisciplinary expertise in modeling complex ecological and evolutionary dynamics that are known to affect organismal (and thus pathogen) persistence in nature (Garchitorena *et al.* 2017).

By using a model neglected tropical disease system, Buruli ulcer (BU) disease (Box 1), we evaluate the scientific history and challenges with deciphering the proposed modes of transmission of an environmental mycobacteria to humans and other animals. In the BU system, the pathogen, *Mycobacterium ulcerans* (MU), is reported in association with most aquatic macroinvertebrate taxa; however, for decades research has intriguingly focused only on two insect orders: Hemiptera (Naucoridae and Belostomatidae) and Diptera (Culicidae). Despite this focus, the role of these aquatic insects remains inconclusive as primary vectors of MU. In this review, we posit that transmission may occur upon exposure to contaminated aquatic environments through environmentally related skin puncture or significant sub-dermal trauma through multiple potential mechanisms, as has been suggested previously (Röltgen and Pluschke 2015). To support this argument, we review the knowledge acquired on MU presence in the environment, paying particular attention to its associations with aquatic macroinvertebrates, and outline criteria necessary for testing potential insect vectors hypothesized to transmit MU or other agents of disease. We also identify other biting insect species, neglected in many previous MU literature reviews, known to transmit pathogens that should be investigated within the conditional context of controlled

competency studies. Until such competency studies are undertaken, we propose a null hypothesis of MU transmission defined in [Box 2](#) and summarized as follows: **(i)** transmission occurs via multiple pathways of environmental exposure; **(ii)** observed hosts are simply carriers not involved in transmission to humans and **(iii)** outbreaks of BU are space and time-dependent, mainly driven by landscape and weather interactions (Morris *et al.* 2016a). As argued by other authors, there are likely multiple modes of transmission for MU from the environment to humans and several of those may locally coexist (Garchitorena *et al.* 2015b). In this review, we go beyond hypothesizing multiple modes of MU transmission and advocate placing future research into a null hypothesis, One Health and causal inference context.

Like that for other neglected tropical or newly emerging diseases, deciphering how pathogens emerge, spread and are transmitted to hosts from the environment is important and timely given the resources devoted to and the impacts on local communities. Without strong causal inference (developed from repeatable and multidisciplinary scientific research efforts) based on hypothesis testing, such resources may be squandered when association is confused with causation. In this review, we evaluate how to define unknown transmission pathways for neglected and emerging diseases, and for better elucidating the ecology and evolution of future emerging pathogens predicted to occur with rapid local and global changes. Although based on a model disease system, the epistemological approach we review and advocate, i.e. testing the validity of accumulated scientific arguments to the evidence-based proofs, can be directly and indirectly. In recent years, it has become increasingly evident that an approach connecting human, wildlife and environmental health will be necessary to address some of humanity's most pressing health issues (e.g. pandemics; Morens and Fauci 2020). This integration of multiple disciplines for understanding how, why and when pathogens emerge and how to manage such events has often been referred to as the One Health or EcoHealth approach. The One Health/EcoHealth approach is a way to address complex disease systems that cross traditional disciplinary boundaries, and is a foundational framework for testing multiple, alternative hypotheses in disease ecology (Garchitorena *et al.* 2015b, 2017). The One Health concept is supported by major international agencies concerned with disease prevention and control (Rabinowitz *et al.* 2013). It emphasizes a transdisciplinary approach applied to other disease systems. Thus, we discuss the need to re-evaluate how the scientific community addresses understudied diseases and how researchers position science to better predict and manage future emerging infectious diseases. The synthesis of these concepts and advocacy of conserving the strong inference approach in disease ecology also falls naturally within the One Health and EcoHealth approaches.

## **ONE HEALTH AND ECOHEALTH CONCEPTS IN DISEASE PREVENTION**

In recent years, it has become increasingly evident that an approach connecting human, wildlife and environmental health will be necessary to address some of humanity's most pressing health issues (e.g. pandemics; Morens and Fauci 2020). This integration of multiple disciplines for understanding how, why and when pathogens emerge and how to manage such events has often been referred to as the One Health or EcoHealth approach. The One Health/EcoHealth approach is a way to address

complex disease systems that cross traditional disciplinary boundaries, and is a foundational framework for testing multiple, alternative hypotheses in disease ecology (Garchitorena *et al.* 2015b, 2017). The One Health concept is supported by major international agencies concerned with disease prevention and control (Rabinowitz *et al.* 2013). It emphasizes a transdisciplinary approach towards disease recognizing that pathogen emergence and outbreaks result from a combination of biotic and abiotic relationships mediated by human factors, such as habitat destruction and overpopulation (Conrad, Meek and Dumit 2013; Roger *et al.* 2016). Despite the principles under which it operates, One Health fails, in practice, to truly integrate the three dimensions of medicine, veterinary and ecological sciences (Manlove *et al.* 2016). On the other hand, the WHO-FAO-OIE 'tripartite' has remained a principle of collaboration between specialized agencies, without a specifically funded action program associated with this triangle, and yet it has been shown to be crucial in reducing risk of epidemics (En *et al.* 2008; Morand, Gu'égan and Laurans 2020).

The EcoHealth concept responds in part to these challenges, as it is more integrative and transdisciplinary at its origin, including social science, the humanities and citizen participation. It advocates a system-based approach to the understanding of pathogen transmission and spread (Goto *et al.* 2006; Lerner and Berg 2017). This concept has notably inspired betterdesigned development assistance, but has not resulted in institutional changes, at the multilateral level, that can shape health policies and address the environmental, distal, causes of disease spillovers and emergence.

In the following text, we assimilate the two concepts together and speak about a 'One Health/EcoHealth approach', and we invite the reader to find fruitful discussions of these concepts elsewhere (Manlove *et al.* 2016; Lerner and Berg 2017; Assmuth *et al.* 2020). For many neglected tropical diseases, like BU, a range of factors including land use change, biodiversity loss, environmental or zoonotic reservoir population alteration and human behavior influence how and why pathogens emerge and spread (Gu'égan *et al.* 2020). Gu'égan *et al.* (2020) summarize how a One Health/EcoHealth approach is important for better understanding, and thus managing, infectious diseases (e.g. Nipah virus and malaria) associated with deforestation and changing landscapes. They argue that such information can be better understood for improved management and prevention using a fused One Health/EcoHealth approach that incorporates ecological and evolutionary theory (Webster *et al.* 2016).

As an example, Garchitorena *et al.* (2015b) took a One Health/EcoHealth approach using an ecological and evolutionary-based formalism and a mathematical, hypothetico-deductive approach to assess the relative contribution of two potential transmission routes for MU to the dynamics of BU cases in Cameroon: a biting water bug vector and an environmental association mode. They compared MU spatial and temporal dynamics in surveyed aquatic ecosystems and water bugs (potential vector) to the dynamics of BU human cases and demonstrated BU incidence was better explained by environmental transmission pathways (i.e. 97.14% of transmission) compared to vector-borne transmission by water bugs (i.e. 2.86%). This One Health/EcoHealth work was the first to demonstrate that MU was primarily transmitted from aquatic contaminated environments despite decades of unconfirmed assertion that water bugs were dominant vectors of BU (Garchitorena *et al.*

2014, 2015a). A similar approach could be applied to other neglected diseases (e.g. Chagas disease, leishmaniasis, echinococcosis, schistosomiasis and onchocerciasis) where the vector may be known but ultimate, indirect factors leading to epidemics are less understood (e.g. climate patterns, flooding, spillover hosts and so on); and for those that are presently unknown but predicted to emerge as new pathogens and agents of human and wildlife disease (Gu'egan *et al.* 2020). Here, we review the BU disease system within a broader strong inference and One Health/EcoHealth framework to serve as an epistemological model for other diseases.

## BU DISEASE

BU is the third most important mycobacterial disease in humans, after tuberculosis and leprosy (WHO 2018), and was originally described in 1948 by MacCallum *et al.* (1948; Box 1). Mainly impacting individuals in tropical and subtropical areas, BU endemicity spans over 30 countries (WHO 2018). The etiological agent, MU, is a member of an opportunistic group of environmental pathogens—mycolactone producing mycobacteria (MPMs)—that secrete a polyketide cytotoxic molecule that exhibits analgesic properties and causes tissue damage (George *et al.* 1999; van der Werf *et al.* 2003; Hammoudi *et al.* 2020a). Molecular phylogenomic analyses corroborate a monophyletic clade containing the most important human mycobacterial pathogens, including MU, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Mycobacterium kansasii* and *Mycobacterium marinum*. In 2018, the genus was emended to include only these and other closely related 'slow-growing mycobacteria,' whereas four new genera were erected to comprise the rapid growing and less pathogenic species (Gupta, Lo and Son 2018). MU is a member of the *M. marinum* species complex, which includes multiple environmental pathogens known to cause disease in fishes, amphibians, aquatic invertebrates and tetrapods (Chai 2011; Hashish *et al.* 2018; Davidovich, Morick and Carella 2020). MU is differentiated from other members of the *M. marinum* complex by the presence of the pMUM plasmid, a 170–200 kbp circular DNA molecule containing genes that encode proteins responsible for mycolactone synthesis. Mycolactone is a polyketide cytotoxin best known for its ability to modulate the host immune response, and is associated with enhanced morbidity in human infections (Adusumilli *et al.* 2005; Coutanceau *et al.* 2005). More recently, mycolactone has been implicated in synergistic or antagonistic interactions with quorum sensing bacteria (e.g. *Staphylococcus aureus*), suggesting that animal pathogenesis may have evolved as an artifact of selective forces operating within a polymicrobial network (Dhungel *et al.* 2021). Phylogenetic variation among pMUM lineages is associated with synthesis of at least 10 distinct mycolactone congeners, including three that are primarily associated with disease in mammals (congeners A–D), amphibians (congener E) and fishes (congeners F; Ranger *et al.* 2006; Guenin-Mac'e *et al.* 2019; Hammoudi, Saad and Drancourt 2020b). Traditional taxonomy ascribes the names *Mycobacterium liflandii* and *Mycobacterium pseudoshottsii* to strains producing mycolactone congeners E and F, respectively. More recent reviews consider all MPMs to represent ecovars of MU (Pidot *et al.* 2010; Hammoudi, Saad and Drancourt 2020b).

Since at least the mid-20th century, a group of closely related mycolactone-producing ecovars have caused local human epidemics in Australia and West Africa (Vandelannoote *et al.* 2019).

Phylogeographic analysis of the plasmid and chromosome reveals a concordant pattern that is consistent with iterative introduction and endemic diversification across continents (Buultjens *et al.* 2018). Ecovars known to produce mycolactone congeners A/B and C are associated with human disease outbreaks in Africa and Australia, respectively, and have been the focus of multiple epidemiological investigations. Conversely, ecovars that produce isomer D are associated with sporadic cases, and epidemiology remains virtually unknown. Ecovars that synthesize isomers E and F are not known to infect humans, but are important globally-distributed pathogens of aquatic vertebrates and invertebrates.

Typically associated with aquatic habitats, MU has been detected in a range of invertebrate and vertebrate taxa, as well as in environmental reservoirs (Merritt *et al.* 2010; Röltgen and Pluschke 2015; Combe *et al.* 2017). It is also possible that infected hosts distribute MU into new environments; however, this has not been investigated. The current epidemiological data suggest that MU persists as a saprophyte that infects humans only under particular circumstances (Hubalek 2003). Saprophytic agents have similarities with other host–pathogen systems, such as zoonotic infections, where potential ‘spillover’ events to hosts of human concern have negative impacts (Kuris, Lafferty and Sokolow 2014), and where a strong inference approach is necessary to identifying key aspects of pathogen transmission and spread.

The BU disease system not only provides an example that lends itself to a causal inference approach, but it can showcase how a similar framework could be applied to other neglected or emerging infectious diseases. Recently, there has been an increase in studies into MU ecology (Fig. 1), but the lack of a strong inference framework limits the contributions to the overall body of knowledge regarding transmission mechanisms. While testing individual hypotheses is a cornerstone of the scientific method, it has long been recognized that devising and testing multiple alternative and competing hypotheses (i.e. strong/causal inference) as part of an overall framework is more useful for understanding complex systems associated with disease emergence and spread (Chamberlin 1897; Popper 1959; Platt 1964; Plowright *et al.* 2008). The inclusion of an ecological and evolutionary perspectives on MU/BU studies is dramatically lacking with the majority of work focused on clinical outcomes (Esteban and Garc’ia-Coca 2018). Thus, we focus on the currently understood ecological factors that influence MU persistence and potential dispersal in the environment that leads to human transmission. Because this is a complex system with pronounced local heterogeneity in space and time (Wagner *et al.* 2008; van Ravensway *et al.* 2012; Carolan *et al.* 2014; Garchitorena *et al.* 2014), it is important to consider a synergistic and multidisciplinary approach to this and future emerging diseases.

Other important and related mycobacterial pathogens have obscure transmission that has limited disease management and where a OneHealth/EcoHealth and a strong inference approach would prove useful. For example, *M. leprae* is an obligate intercellular bacterium that causes leprosy or Hansen’s disease. It relies on host cells (e.g. Schwann) for survival, as well as replication. Yet, *M. leprae* transmission pathways are not fully understood despite evidence of disease outbreaks existing since 23 AD (McLeod and Yates 1981). Several studies report risk factors centered around human population density, prolonged contact and deforestation (Bakker *et al.* 2004). Other possible



leprosy transmission routes include wildlife encounters, e.g. nine-banded armadillo (*Dasypus novemcinctus*) in the Americas or red squirrels (*Sciurus vulgaris*) in the British Isles (Avanzi *et al.* 2016); mother to child during pregnancy (Duncan *et al.* 1983); fomite transmission, though this hypothesis has not been proven (Turankar *et al.* 2012) or insect vectors, such as ticks (Ferreira *et al.* 2018) or triatomine bugs (Doannio *et al.* 2011). Experimental evidence also indicates possible transmission for MU by triatomine bugs (Doannio *et al.* 2011). Further complicating known leprosy transmission pathways is the slow growth of *M. leprae* (may take more than 20 days for one division cycle), and the lack of success to culture this bacterium on an artificial substrate. There is elevated concern to identify the hosts, vectors, environment and transmission of *M. leprae* as incidences of drug resistance and multidrug resistance in leprosy have increased reporting (Cambau *et al.* 2018).

### **INSECTS AS POTENTIAL VECTORS OF MU WITH FOCUS ON HEMIPTERA AND CULICIDAE**

Epidemiological studies have identified a variety of risk factors for BU, with substantial coverage given to this topic in other reviews (Merritt *et al.* 2010; Röltgen and Pluschke 2015; Guarnier 2018). In general, activities near slow flowing bodies of water (Barker and Carswell 1973; Brou *et al.* 2008) and environmental disturbance (van Ravensway *et al.* 2012; Garchitorea *et al.* 2014, 2015a; Campbell *et al.* 2015) are commonly reported as risk factors, while wearing long/protective clothing (Raghunathan *et al.* 2005; Debacker *et al.* 2006; Quek *et al.* 2007) and wound care (Pouillot *et al.* 2007; Kenu *et al.* 2014) reduce risk. These associations, as well as the distribution of MU in the environment, wound location and case histories have led to a substantial body of research into aquatic insects as host carriers or potential vectors of transmission (Merritt *et al.* 2010; Röltgen and Pluschke 2015).

Over the last two decades, thousands of macroinvertebrates from various geographic regions have been screened for the presence of MU and other MPMs (see Tables 1 and 2). While some studies focused on specific taxa like mosquitoes (Johnson *et al.* 2007; Singh *et al.* 2019) or water bugs (Mosi *et al.* 2008; Esemu *et al.* 2019), others surveyed entire aquatic communities (Benbow *et al.* 2013; Garchitorea *et al.* 2014; Morris *et al.* 2016a). Despite characteristics of a generalist pathogen, models of MU suggest that infected organisms do play a role in the life cycle of this mycobacterium (Morris *et al.* 2016b), but whether it is associated with transmission to humans remains unresolved. Mosquitoes have been implicated as vectors in Australia, where positivity rates for MU/MPM DNA in adult mosquitoes (Diptera: Culicidae) statistically varied with BU cases (Johnson *et al.* 2007; Singh *et al.* 2019). In Ghana 6.7% of pooled mosquito larvae were MU/MPM DNA positive (Benbow *et al.* 2008; Williamson *et al.* 2008). However, two research groups investigating mosquitoes in Benin found no evidence of MU DNA in either larvae or adults (Djouaka *et al.* 2018; Zogo *et al.* 2015).

In Africa, several studies have named biting water bugs (Hemiptera: Belostomatidae and Naucoridae) the potential culprits in the transmission of MU to humans (Portaels *et al.* 1999), as both tissues (Marsollier *et al.* 2002; Marion *et al.* 2011) and saliva (Marsollier *et al.* 2005; Marion *et al.* 2010; Doannio *et al.* 2011; Konan *et al.* 2015) obtained from environmental specimens tested positive for MU/MPM DNA, and Hemiptera have further been implicated in the dissemination of MU in the



environment (Garchitorena *et al.* 2014; Ebong *et al.* 2017). Despite the occurrence of both Hemiptera families in Australia and their implication in the transmission of BU elsewhere, no studies surveying Australian biting water bugs for the presence of MU DNA have been published to date. In addition, these biting water bugs do not actively search for humans, they do not require a blood meal or protein source to mature their eggs, nor is there any evolutionary history suggesting or supporting a vector borne pathogen transmission or co-evolving host–parasite relationship in semiaquatic Hemiptera (Hungerford 1919; Smith 1997). Therefore, based on the biology and behavior of predaceous aquatic insects, biting humans appears to be a rare event associated with a purely defensive reaction of these bugs (Schaefer and Panizzi 2000; Haddad Jr *et al.* 2010).

The involvement of biting water bugs and mosquitos in BU transmission as either biological or (purely) mechanical vectors has been suggested on several occasions. While laboratory studies have partially supported their potential as vectors (Marsollier *et al.* 2002, 2004a, 2005; Wallace *et al.* 2010, 2017), additional critical evaluation of data derived from environmental samples is warranted (Ebond *et al.* 2017), especially within the context of Hill’s criteria (Box 3 and Table 2).

### **INVERTEBRATES AS ENVIRONMENTAL SENTINELS OF MU**

Epidemiological studies of BU risk factors are informative and provide important statistical associations of factors with disease; however, additional research is needed to differentiate between correlation and causation (Davey Smith and Phillips 2020). This need is particularly true when a specific host or vector species is hypothesized to be a mode of transmission. The utility of epidemiological studies that include potential vector species in the analyses is to provide important data and discussion that could satisfy (in part) one or more of Hill’s criteria (Box 3).

As an example related to a vector hypothesis of MU in Australia, an epidemiological study used written surveys of 49 adult patients (confirmed to have MU via PCR, culture or both) compared to 609 controls (Quek *et al.* 2007). Patients reported self-identified mosquito bites on the forearms and lower legs were risk factor for BU disease and that clothing covering the legs, washing wounds and wearing insect repellent lowered risk; these findings led the authors to conclude the results were consistent with the hypothesis that mosquitoes play a role in MU transmission (Quek *et al.* 2007). As the authors note, no case-control study can establish causation, and so these results were important for developing future research to better test a mosquito vector hypothesis. Additional studies were also correlational (Johnson *et al.* 2007; Johnson and Lavender 2009; Fyfe *et al.* 2010; Lavender *et al.* 2011; Carson *et al.* 2014), except for one that showed mechanical transmission in 2 of 12 mice (17%) under controlled laboratory conditions (Wallace *et al.* 2017). In West Africa, there was no support for correlations of mosquitoes and BU cases (Zogo *et al.* 2015; Djouaka *et al.* 2017), and other work found no significant risk of insect bites near water bodies associated with BU (Raghuathan *et al.* 2005). Importantly, MU has not been shown to persist through metamorphosis from larva-to-pupa or pupa-to-adult stage mosquitoes (Wallace *et al.* 2010), suggesting that MU positive adult mosquitoes were likely inoculated from the environment as they emerged from MU contaminated aquatic habitats. More generally, there is no scientific precedence for any mycobacterial pathogen, or most

bacteria, being transmitted by mosquitoes, but see other discussions (Dieme *et al.* 2015; Laroche, Raoult and Parola 2018). An alternative explanation for mosquito correlations with BU cases is that the mosquitoes themselves (or any other aquatic invertebrate), are simply environmental sentinels of a habitat with a high concentration of MU. Such an interpretation is similar to that suggested for opossums (Carson *et al.* 2014) and other vertebrate species (Tobias *et al.* 2016; Singh *et al.* 2018) considered to be MU environmental sentinels. Overall these findings, and many others from around the world (Table S1, Supporting Information), currently support a null hypothesis of MU transmission.

## ALTERNATIVE MU TRANSMISSION HYPOTHESES AND RESEARCH NEEDS

Vector transmission studies are needed to evaluate against criteria established for strong inference in disease systems (Hill 1965; Plowright *et al.* 2008), and will provide a framework for better understanding BU and other neglected and understudied emerging diseases (Box 3). While we argue that currently there is not enough evidence to support insects as a major transmission mechanism for MU, it remains one hypothesis until additional studies are conducted. To better evaluate alternative hypotheses for transmission mechanisms, or to reject the hypothesis that insects are not directly involved in transmission to humans, there remains several critical research needs:

1. Studies over a range of spatio-temporal scales are needed for a better understanding of MU ecology. Critical work would provide additional information on a series of questions: What habitat or landcover changes occur when MU becomes more abundant? How long after landscape disturbance were BU cases reported? At what time scale? How long is MU in the same interactive space with humans and other animals before it leads to disease? Recent findings on MU have demonstrated this mycobacterium is widely distributed in disturbed and changing tropical aquatic environments (see Tables 1 and 2, and Tables S1 and S2, Supporting Information), suggesting that changes in abundance respond to fluctuation environmental and climate conditions. Additional well-designed experiments in space and time are needed to answer these questions, in order to identify ecological niches of MU near at-risk human communities and how they change over time.
2. It was once thought of microorganisms that *'everything is everywhere, but the environment selects who stays locally'* (Baas- Becking 1934). Such a perspective suggests that microorganisms can be ubiquitous in nature due to their dispersal potential. Thus, to better understand MU ecology it is necessary to know the hosts, reservoirs and habitats in which MU/MPMs resides and replicates. This biogeography of MU may include other microbial assemblages (e.g. polymicrobial biofilms on plants or in soils and suspended solid substrates), macroinvertebrates, vertebrates (e.g. reptiles, amphibians, placental mammals and marsupials) and complex microhabitats (e.g. rhizospheres or hyporheic). For example, the occurrence of BU has been associated with stagnant or lentic bodies of water, such as rivers, swamps, or wetlands (see Table 1 and Tables S1 and S2, Supporting Information). However, no replicative environmental reservoirs have been established. Several studies have

indicated MU may persist in the environment through association with other protective organisms (e.g. amoebae; Eddyani *et al.* 2008; Gryseels *et al.* 2012; Amissah *et al.* 2014), or as inhabitants of polymicrobial biofilms, such as those associated with aquatic plant surfaces (Williamson *et al.* 2012; McIntosh *et al.* 2014). However, the overwhelming majority of publications on specific polymicrobial biofilms associated with NTMs stem from the context of clinical research which are often restricted to culturable biofilm species under highly controlled laboratory conditions (Esteban and Garcia-Coca 2018).

3. To appropriately test alternative hypotheses, such as insect vectors of MU, assessments of vector competence for potential species are needed. Rigorous studies are needed for building strong, causal inference in disease ecology, especially for diseases with unresolved transmission mechanisms. While reservoirs may or may not be the direct source of infection for the (human) hosts, they play a major role in maintaining MU in the environment and likely in the mode(s) of its transmission.

## VECTOR COMPETENCY

The terms 'vectorial capacity' and 'vector competence' are often used interchangeably to describe the ability of arthropods, such as mosquitoes, ticks, black flies, hematophagous hemiptera and other species, to function as a pathogen vector (Beerntsen, James and Christensen 2000; Laroche, Raoult and Parola 2018). By definition, vectorial capacity is quantitative and includes vector competency and environmental, behavioral, cellular and biochemical factors or traits that influence the associations among the vector, pathogen and vertebrate host(s) (Macdonald 1957; Black *et al.* 1996; Beerntsen, James and Christensen 2000). Vector competence is a component of vectorial capacity defined by the complement of intrinsic traits (e.g. genetics) associated with the ability of the arthropod to acquire the pathogen, allow it to replicate and ultimately transmit it to a susceptible host (Beerntsen, James and Christensen 2000; Laroche, Raoult and Parola 2018). Extrinsic factors influencing vector capacity include densities of both vector and host, while intrinsic traits include host genetically mediated preference, longevity, abundance, incubation time and the ability of the vector to become infected after ingesting an infected blood meal (i.e. vector competence), all defining an arthropod species an efficient vector (Hardy *et al.* 1983; Souza- Neto, Powell and Bonizzoni 2019). Vectorial capacity, including competency factors, can be used to calculate rates of transmission (Azar and Weaver 2019). An initial primary assumption in determining vectorial capacity for a candidate species is that there are co-evolutionary relationships among the candidate vector, pathogen, any reservoir(s) and the host(s). The biological steps necessary for a vector to become infective (i.e. able to transmit the pathogen) are illustrated using viral transmission of mosquitoes in Fig. 2 (Azar and Weaver 2019).

There are several physical and physiological barriers that arthropods must overcome to transmit a pathogen. These barriers include how the immune response may limit or inhibit the development of internal microorganisms, and therefore influence success of transmission (Laroche, Raoult and Parola 2018). The most common ways for arthropod vectors to transmit infectious pathogens is via salivation

while biting (as in biological transmission; Mueller *et al.* 2010), horizontal transmission via stercoriation (associated with arthropod feces) and regurgitation (Laroche, Raoult and Parola 2018). Another route is considered mechanical, where pathogen transfer occurs from an infected host by physically contaminated mouthparts or other body parts (Drucker and Then 2015; Sarwar 2015). An important element of disease epidemiology is the interactions of pathogen–vector–host specificity (Graca 1991; Lopes, Daugherty and Almeida 2009). That is, in order to identify which potential vectors are most important for pathogen spread, it is essential to understand if the pathogen has a single or multiple vectors, reservoirs or hosts, and determine vector specificity and efficiencies of potential transmission (Bar-Joseph, Marcus and Lee 1989; Lopes, Daugherty and Almeida 2009). The introduction of pathogens far from their endemic regions (e.g. West Nile, Zika and Dengue viruses) have provided templates for investigating insect-mediated transmission (Colpitts *et al.* 2012). Relating vector capacity in MU transmission is challenging considering that most vector capacity studies have focused on co-evolved viral pathogens with insects (Weaver 2006). Few studies have focused on biological or mechanical bacterial pathogen transmission by insects, although several have been confirmed for other biting arthropods, e.g. ticks (Turell and Knudson 1987; Turell *et al.* 2008).

The experimental variables useful in assessing insect vectorial capacity from a biological transmission perspective are not without problems (Azar and Weaver 2019). Such issues include the compromising ecological relevance with method standardization. An example of this type of compromise within our current body of transmission studies on MU is testing vector competence using an insect predator such as a naucorid, belostomatid or gerrid biting water bugs (Marsollier *et al.* 2002, 2005, 2007) and not an evolved blood feeding insect, such as a mosquito or horsefly. The published studies assuming biological transmission of MU using blood-feeding insects, such as mosquitoes, have not considered important issues such as bacterial coevolution with vectors, bacterial stocks on bloodmeal preparation, bacterial introduction to the vector and passage history of bacteria.

Because of the rarity of biological transmission occurring with insect vectors and bacterial pathogens (Laroche, Raoult and Parola 2018), only two studies have addressed mechanical transmission of MU (Williamson *et al.* 2014b; Wallace *et al.* 2017). Mosquitoes fed on mouse tails covered with MU had a 20% efficacy of producing ulcers via puncture of their proboscis, whereas it was 80% using a sterile needle stick Wallace *et al.* (2017). Further, an abrasion inoculated with MU did not result in disease in a guinea pig model, but the study found that deep punctures were necessary Williamson *et al.* (2014b). We posit that these findings suggest environmental subcutaneous trauma, rather than an insect vector, may explain a greater probability of transmission than an insect-mediated mechanical mode of transmission.

Studies into the vector competence of aquatic insects are severely limited. Of 10 families documented to harbor MU at some point in their life cycle, a systematic search of the literature (Google Scholar, Web of Science and Scopus; using the search terms ‘*Mycobacterium ulcerans*’ and (‘vector competence\*’ or ‘vector capacity’)) did not identify a single study focusing on vector

competence or vectorial capacity and MU. These results show that the ability of insects to become infected and maintain or transmit MU to a host or another environment remains largely unknown.

## OTHER POTENTIAL VECTORS OF MU

To test alternative hypotheses against a null, it is important to recognize other potential arthropod vectors that transmit a variety of pathogens. Numerous arthropod taxa, including Diptera (flies), Hemiptera, Siphonaptera (fleas), Pthiraptera (lice) and Acari (ticks and mites) are known biological or mechanical vectors and reservoirs of several pathogens (Table S1, Supporting Information). Many of the known arthropod vectors of disease pathogens cause superficial trauma to the skin during which the transfer of a pathogen to an uninfected individual occurs.

Bacterial disease agents have generally only been reported to be transmitted by lice, fleas, sand flies, true bugs and others, but not mosquitoes (Laroche, Raoult and Parola 2018). While research documenting bacterial pathogen transmission by insects is limited, there are fascinating similarities with some insect–pathogen relationships and mechanisms by which MU could potentially be transmitted to humans and other animals. For example, mechanical transmission of bacteria pathogens (*Rickettsia prowazeki*, *Bartonella quintana* and *Yersinia pestis*) by lice to humans is known to occur where louse saliva containing the pathogen is scratched by the host into wounds or lesions (Raoult and Roux 1999). Another example of mechanical transmission from host scratching is that of fleas on the skin, where the flea feces is contaminated with pathogens (e.g. *Rickettsia typhi*, *Yersinia pestis* and *Bartonella henselae*) that are forced into wounds or scratches (Perry and Fetherston 1997; Kernif *et al.* 2014; Leulmi *et al.* 2014). Of the two groups of blood-feeding Hemiptera, both Triatomidae and Cimicidae have been connected with transmission of *Bartonella bacilliformis* (causative agent of Carrion Disease) and *B. quintana* (causative agent Trench Fever) through fecal contamination of wounds or lesions (Angelakis, Socolovschi and Raoult 2013). Finally, mosquitoes (e.g. *Anopheles gambiae*) have been suspected to mechanically transmit the obligate intracellular bacterium, *Francisella tularensis* (causes tularemia possibly acquired from their larval habitats (B · · ackman 2015) and the pathogenic bacterium, *Rickettsia felis* (Doyle *et al.* 2011); however, additional studies meeting more of Hill’s criteria are needed to confirm how often bacterial transmission occurs in these examples. Other flies, such as horse flies (Family Tabanidae), were shown to mechanically transmit several species of bacteria including *Bacillus anthracis* (causative agent for Anthrax), *Francisella tularensis* (tularemia) and *Borrelia burgdorferi* (causative agent for Lyme Disease; Foil 1989). Stable flies (Muscidae: *Stomoxys calitrans*) also have been reported to be vectors of certain bacteria, suggesting Diptera may be potential vectors (Mramba, Broce and Zurek 2007). Sand flies (Psychodidae) and filth flies (Muscidae) have been documented with potential to mechanically transmit several species of bacteria during feeding, but more research is needed (Baldacchino *et al.* 2013; Keita *et al.* 2020). Viral and most protist pathogens are generally transmitted biologically, however, the one commonality between the few bacterial disease pathogens transmitted by insects is that it is suspected to be mechanical, and there have been no conclusive biological transmission studies.

There are several insects known to actively seek human hosts for blood meals, and so we hypothesize that Diptera, such as black flies (Simuliidae), sand flies, horse/deer flies (Tabanidae), house/stable/bush flies (Muscidae), Tsetse (Glossinidae), louse flies (Hippoboscidae) and possibly blow flies and flesh flies (Calliphoridae and Sarcophagidae, respectively), could potentially transfer or inoculate MU onto or into the skin of uninfected hosts. The method of inoculation could be either from direct bites or passive feeding on animal or human lesions. Other insects such as hemipterans, both terrestrial (Reduviidae and Cimicidae) and aquatic (Belostomatidae and Naucoridae), can produce significant stabbing bites with probosci that could act as hypodermic puncturing mechanisms to introduce the bacterium subcutaneously. Interestingly, many lepidopteran families (e.g. Anthelidae, Arctiidae, Bombycidae, Europterotidae, Lasiocampidae, Lymantriidae, Megalopygidae, Noctuidae, Notodontidae, Saturniidae and Zygaenidae) possess urticating or stinging spines along with poison glands that can cause severe pain and allergic responses involving significant itching (Diaz 2005). A few beetle (Coleoptera) taxa are known to cause severe skin irritation, blistering and dermatitis, (e.g. Meloidae and Staphylinidae; Selander 1960; Cressey *et al.* 2013). Indeed, it is plausible that certain human immune and behavioral responses to many of these bites and stings may cause sufficient itching and scratching that could introduce MU into host skin, similar to the mechanical transmission examples given above for lice and fleas, but also to how the flagellated protist, *Trypanosoma cruzi* (causative agent of Chagas Disease) is introduced via vigorous scratching of a bite or sting (Teixeira *et al.* 2006). Furthermore, spiders, mites, ticks, scorpions and centipedes have been anecdotally described as mechanical vectors of subcutaneous pathogen inoculation (Meyers *et al.* 1974).

## DECIPHERING HOSTS, RESERVOIRS AND POTENTIAL VECTORS

For ecologists and evolutionists, true host reservoirs of a multihost pathogen are the species allowing, *per se*, the persistence of the pathogen (Ashford 2003). A number of species have been reported to carry MU (Tables 1 and 2). However, not all of these hosts are necessarily reservoirs for MU but might simply be carriers, with some acting as dispersers (Haydon *et al.* 2002). This is probably not the case of all species that have been reported to carry MU (Table 1 and Table S1, Supporting Information). Given the importance of chitin to the development of free-living MU (Sanhueza *et al.* 2016, 2019), most of these carriers should be able to support the development of MU due to the presence of chitinous compounds in their exoskeleton (e.g. insect larvae and snails) or fur (e.g. opossums and grasscutters). They may also feed upon substrates such as plant surfaces covered with biofilm communities that can include MU (McIntosh *et al.* 2014), thereby ingesting the pathogen, as is suspected to be the case for wild grasscutters (i.e. cane rats, Thryonomyidae) or aquatic snails. While the latter has received little attention in BU research to this day, several studies have reported the detection of MU DNA in snails and mussels collected from the environment in various geographic locations: MU DNA was detected in snails of the Planorbidae family (*Planorbis* sp; Marsollier *et al.* 2004a) and *Bulinus* sp. (Kotlowski *et al.* 2004; Marsollier *et al.* 2004a), as well as other Gastropoda, Bivalvia and Basommatophora (Benbow *et al.* 2008; Williamson *et al.* 2008).



Furthermore, Marsollier *et al.* (2004a) were able to experimentally infect *Pomacea canaliculata* (Ampullariidae) and *Planorbis planorbis* (Planorbidae) through aquatic macrophytes with biofilm contaminated with MU. While the snails did not offer conditions supporting the growth of the mycobacterium, they remained infected by viable MU for up to 25 days. Taken together, these data suggest that mollusks and other invertebrates minimally act as transient hosts of MU, and support the hypothesis that they could be part of a larger food chain supporting MU in the environment, as has been previously suggested (Portaels *et al.* 1999; Morris *et al.* 2016b).

Other species may have roles as bridge-hosts, (i.e. not necessarily being replicative hosts but increasing the contacts with other susceptible taxa). Indeed, several species of undomesticated mammals that live in close proximity to humans (e.g. opossums) could act as bridge-hosts (Caron *et al.* 2015). While not sufficient to unilaterally maintain MU in an ecosystem bridge-hosts may facilitate MU dispersal and transmission to humans or into human associated environments. Despite the focus on animals in the search of hosts and reservoirs of MU, a potential role of plants (or, indeed, even specific plant organs) cannot be dismissed, as they can offer specific conditions that might allow MU to grow and persist in the environment. In Benin, samples collected from plants (*Cyperus* sp., *Panicum* sp., *Eichhornia* sp.) were tested for the presence of MU DNA. While none of the plant tissues yielded positive results, MU strains were detected in Naucoridae dwelling in the roots of these plant (Portaels *et al.* 1999). In another investigation, stems and leaves of plants tested positive for MU DNA by qPCR (Zogo *et al.* 2015). Further, MU was detected in aquatic plants collected from emergent zones of both lotic and lentic waterbodies in BU endemic regions of Ghana (McIntosh *et al.* 2014). These observations support the hypothesis that aquatic plants act as a host reservoir of MU, adding a new potential link in the chain of transmission of MU to humans and other vertebrates (Marsollier *et al.* 2004b). In addition, plants can provide shelter for organisms living in their rhizosphere and other soils (e.g. amoebae, which are notably known for allowing many *Mycobacteria* to survive adverse abiotic conditions; Salah, Ghigo and Drancourt 2009). In laboratory studies, several plants have been implicated as a growth factor of and stimulant for biofilm formation by MU (Marsollier *et al.* 2004b; Mougin, Tian and Drancourt 2015). The successful development of a new growth medium utilizing plant extracts (Mougin, Tian and Drancourt 2015) suggests that certain aquatic plants not only contribute to the persistence of MU in the environment but might also play a functional role in the formation of its biofilm.

Mycobacteria are thought to originate from swamps, bogs and other lentic aquatic ecosystems. As an example, peat-rich ecosystems, such as bogs, have been discussed as the natural habitats of *M. tuberculosis* progenitors (Falkinham 2013), a disease lifestyle which is quite close to what we know today regarding extant MU distribution. It is plausible that several mycobacteria were historically aerosolized from soil with increased firemaking and infecting human lungs, or put into contact with individuals from pathogen contaminated freshwater habitats (Honda, Viridi and Chan 2018). Thus, evidence suggests numerous hosts harbor MU (see Tables 1 and 2), which supports invertebrates as secondary or accidental hosts, and that the true natural reservoir for MU is an ecological niche that has not yet been discovered. We suggest that research priority should be directed towards new potential environmental MU reservoir(s).



## ENVIRONMENTAL HABITATS OR RESERVOIRS OF MU REPLICATION: SUPPORT FOR A NULL HYPOTHESIS?

### Molecular and genome characteristics of MU for life in the environment

Understanding the extent and variability of MU in the environment, among different habitats, reservoirs and hosts and how these associations change over time and among geographic regions is critical for testing alternative hypotheses of transmission. For over a decade, there has been considerable interest in identifying and quantifying MU in the environment (Table 1 and Table S1, Supporting Information). These studies identified the DNA of MU among a broad spectrum of taxa and environmental matrices. Identification has been through molecular methods targeting insertion sequence IS2404, followed by targeting pMUM001 genes like ketoreductase (KR) or enoyl reductase (ER) for presumptive presence of mycolactone, the toxin responsible for BU disease (Fyfe *et al.* 2010; Williamson *et al.* 2012; Garchitorena *et al.* 2015a). But despite positive results, these targets are no longer considered specific for MU due to the finding of other MPM from diseased fish and frogs, and other environmental sources. Indeed, a review of data analyzing inter-species relative hybridization ratios between MU and other MPMs led to the suggestion that *Mycobacterium shinshuense*, *M. liflandii*, *M. pseudoshottsii* and mycolactone producing *M. marinum* strains should all be considered a single species (Pidot *et al.* 2010), and suggesting a potential ecological role of mycolactone very different from causing clinical BU disease.

The detection of DNA does not necessarily equate to replicating MU, but is, rather, a marker for MU presence. Indeed, MU has only been cultured from the environment, to our knowledge, in two instances; the first from a homogenized and decontaminated sample of *Gerris* sp., and incubation within the laboratory for 12 months (Portaels *et al.* 2008). The second was from chlorhexidine-decontaminated fecal samples from grasscutters, *T. swinderianus* (aulacode), incubated under microaerophilic conditions for 6 months (Zingue, Panda and Drancourt 2018). This underscores the difficulty in culturing MU from environmental samples, and also that caution should be taken when considering the MU replicative niche.

MU is slow growing under laboratory conditions, and forms biofilms after 7–10 days under lab conditions and in environmental mesocosms (Marsollier *et al.* 2007; Williamson *et al.* 2008). The reduced genome and presence of a large number of pseudogenes suggests that MU diverged from *M. marinum* into a narrower niche range. How and where MU replicates within aquatic environments is still a mystery, though the genome provides clues. For instance, it has been suggested that MU is sensitive to UV due to termination in *CrtL*, a gene responsible for carotenoids production (Stinear *et al.* 2007). However, it is not clear whether MU resides in areas that are protected from UV or has developed molecular mechanisms to counteract UV adverse effects. MU has 771 pseudogenes, including *cydA*, a gene necessary for growth under microaerophilic conditions (Stinear *et al.* 2007). These data, along with the presence of genes for aerobic respiration suggests an oxygen-rich lifestyle. And BU is conspicuously absent from communities near brackish waters in Africa, though this is not the case in Australia (Loftus *et al.* 2018). MU also has a pseudogene, *proV* that is an osmoprotectant

present in *M. marinum*, suggesting that MU does not grow well under conditions of high salinity. All of these findings indicate an aerobic environment where there is protection from UV and high salinity (Doig 2012). Lab experiments on MU growth have shown that mycobacterium colonies benefit from chitin-rich substrates (Sanhueza *et al.* 2019), which suggests a potential association with organisms or substrates with chitin composition.

Whole genome sequencing has provided information necessary to begin to understand the genetic determinants of structural variation among mycolactone isomers, and thus to infer the molecular basis for host-pathogen interactions (Pidot *et al.* 2008; Jenke-Kodama and Dittmann 2009; Quadri 2014; Sarfo *et al.* 2016). Considering that all known MU ecovars exhibit striking genomic similarity, comparative genomic analyses hold high potential to identify the specific variants that impart functional limits to virulence, transmission potential and host range (Qi *et al.* 2009; Röltgen *et al.* 2012; Williamson *et al.* 2014a). Reports of mammalian infection are thus far limited to MU ecovars that synthesize mycolactone isomers A–D. Infection of non-human mammals has been limited to rodents in controlled experiments and wild and captive species from southeastern Australia. Case reports of domestic animals include dogs, cats, horses and alpacas, while reports of wild animals have thus far been limited to marsupials (possums, koalas and so on; Elsner *et al.* 2008; Fyfe *et al.* 2010; Van Zyl *et al.* 2010; O’Brien *et al.* 2011). Considering that infection of outdoor mammals has been limited to Australia (where only the mycolactone C ecovar has been documented), and that isolates from animal cases are genetically nearly indistinguishable from human isolates, it is reasonable to suspect that the mycolactone C ecovar is responsible for all environmentally acquired non-human mammal infections. However, this prediction cannot yet be confirmed, because in most reports the specific mycolactone isomer is not documented. Future research should aim to identify the specific ecovar associated with new case reports, and to identify the molecular determinants of host susceptibility among all MU ecovars, to best predict the role of local environmental reservoirs in human disease risk.

### **Possible non-invertebrate MU dispersal pathways**

The wide distribution of MU in the environment is not limited to BU endemic areas and MU infections are not limited to humans (Hennigan, Myers and Ferris 2013). The possible dispersal of MU to new locations over varying distances is uncertain; however, vertebrates, such as mammals, reptiles, birds, fish and protozoans (e.g. amoeba), have been implicated in the possible dispersal of MU to new locations (Merritt, Benbow and Small 2005; Table S2, Supporting Information).

Fishes are affected by, and display symptoms of mycobacteriosis (i.e. diseases caused by various mycobacteria; Gauthier and Rhodes 2009). These fish mycobacteriosis are caused by mycolactone producers *M. pseudoshottsii*, *M. shottsii* and *M. marinum* (Kaattari and Rhodes 2006; Stragier *et al.* 2008), and are usually manifest in (but not limited to) the liver, kidney and spleen; most external symptoms are not observed until the fish loses its scales, changes pigmentation or develops an ulcer (Gauthier and Rhodes 2009). In the absence of these external indicators of mycobacteriosis, fish can be transported to other locations, sold and eaten by unsuspecting humans or animals who can become infected. This situation was reported using skin ulcer biopsies from 29 patients associated

with fish markets in New York City (Sia *et al.* 2016). A study of aquarium fish (lacking any external signs of mycobacteriosis) from multiple pet shops found 79% (85/107) were positive for non-tuberculous mycobacteria highlighting the potential for anthropogenic dispersal of various mycobacterial species, including MU and other MPMs, through the international pet trade (Kusar *et al.* 2017).

Opossums have been implicated as local environmental reservoirs of MU in the south eastern part of Australia where the genome of MU isolates from the possums differed by only two SNPs when compared to a local human isolate (Fyfe *et al.* 2010). Possums have been reported to travel over 1000 m into forest to feed on pasture (Green and Coleman 1986), with reports of possum movement ranging from 300–390 m for males and 260 m for females (Green 1984; Kerle 1984). This movement could foster the transmission of MU from possums either harboring or infected with MU.

Additionally, the movement of opossums by humans in Australia from one location to another may promote the dispersal of MU into new areas (Stow *et al.* 2006). In Africa, mammalian reservoirs have not been intensively investigated, with one of the only studies failing to detect MU in mammals (Durnez *et al.* 2010), though another study identified MU DNA in a *Mastomys* rat species with ulcer presentation (Dassi *et al.* 2015). Taken together, the large number of ecological matrices positive for MU suggests that insects, other invertebrates and several vertebrate groups are sentinels of a MU rich environment.

### Conclusions & summary for other diseases

In this review, we call for novel epidemiological designs within the framework of One Health/Eco Health to better decipher the transmission and dispersal of MU to better improve our collective understanding of BU disease and to inform management and policy (Roger *et al.* 2016, 2020). Effectively, if the concentration of MU and its seasonal fluctuation in aquatic environments can be associated with temporal risk of BU emergence in local human communities, it may be possible to predict disease risk emergence through systematic environmental screening of the environment for disease control. As in the expression ‘One cannot see the forest for the trees’, concentrating research efforts and funding on searching for a unique vector-borne transmission pathway for MU may continue to prevent a more comprehensive and integrative understanding of disease transmission. This same issue of ignoring indirect, or unknown, ecological or sociocultural factors in disease may be limiting progress in other neglected diseases. A comprehensive and integrative understanding of transmission using a One Health approach is likely a more cost-efficient and effective means of understanding, managing and preventing BU and many other zoonotic and sapronotic diseases. This approach is consistent with what is currently recognized by the United Nations through the Sustainable Development Goals (SDGs), and specifically by SDG#3 on human health and well-being, which clearly recommends proactive actions (e.g. early warning systems) and the development of predictive models of disease emergence and spread that are both less costly and favor community participation and decision-making sharing with citizens (Guégan *et al.* 2018).

With global changes to climate and terrestrial and aquatic ecosystems, it is projected that there will be increases in newly emerging and re-emerging diseases. For those diseases where the transmission

is unknown, it will be important to streamline research efforts into identifying modes of transmission and spread of the infectious agents. We propose that for each disease system there is objective consideration given to developing a null hypothesis of transmission and conceiving rigorous studies that will serve to test against the null hypothesis using a One Health/Eco Health approach and considering Hill's criteria. Using the MU/BU system as a case study, we ask several questions relevant to other neglected or newly emerging disease systems:

1. Is the current evidence enough to call insects (or other animals) important vectors, or are they simply host carriers of an environmental mycobacteria that normally carries out a non-pathogenic existence?
2. Are certain aquatic communities of species indicators of an environment with high abundances of MU, or an environment with the potential to harbor high abundances of MU when conditions change (e.g. deforestation, mining and flooding)?
3. Do certain aquatic communities act as sentinels of potential environmental contamination with MU that when occupied and used by susceptible hosts results in transmission and disease, perhaps months later due to temporal lags in the environment and incubation within hosts?
4. For other diseases, are we certain we understand all of the factors that affect epidemics and pathogen spread? Are there unknown biological or ecological components (e.g. unknown protist, plant or animal reservoirs) that act to facilitate pathogen persistence and transmission to hosts?

Questions from numbers 3 and 4 above provide the conceptual underpinnings for a null hypothesis for most pathogens that have not been shown to have evolved to cause pathogenesis in animal hosts but have evolved other traits that incidentally cause disease while serving an environmental function. Disentangling association and causation has a major role in managing infectious diseases in a changing world and should be a first step when deciphering the environmental roles of hosts, reservoirs and vectors. This process begins with a null hypothesis developed within an ecological and evolutionary context to provide a defensible and convincing demonstration of the scientific method for understanding newly emerging and neglected infectious diseases.

## **FUNDING**

This work was supported by the joint NSF-NIH-NIFA Ecology and Evolution of Infectious Disease program (DEB 1911457) to MEB, JFG, JLP, HRJ and MWS; and by Michigan State University. AgBioResearch, College of Agriculture and Natural Resources, Department of Entomology and Department of Osteopathic Medical Specialties. MEB received an award from an 'Investissement d'Avenir' grant managed by Agence Nationale de la Recherche (LABEX CEMEB: ANR-10-LABX-04-01). CC and JFG were supported by an 'Investissement d'Avenir' grant managed by Agence Nationale de la Recherche (LABEX CEBA: ANR-10-LABX-25-01). CC was supported by Institut de recherche pour le développement (IRD), Centre National pour la Recherche Scientifique (CNRS) and Université de Montpellier. JFG was supported by Institut de recherche pour le développement, Institut national de la recherche pour l'agriculture, l'alimentation et l'environnement (INRAE), Université de Montpellier and Ecole des Hautes Etudes en Santé Publique (EHESP).

## **CONFLICT OF INTEREST**

None

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## BOXES

**BOX 1: Buruli ulcer disease: *Mycobacterium ulcerans* Infection** Buruli ulcer (BU) disease is caused by *Mycobacterium ulcerans* (MU), a pathogen that is related to other agents responsible for tuberculosis and leprosy, in addition to a wide range of other opportunistic skin and respiratory infections caused by *Mycobacterium* species (WHO 2018). The disease was originally described in 1948 by MacCallum *et al.* (1948) and has now been reported in over 33 countries worldwide, mostly restricted to tropical and sub-tropical regions, with greatest numbers of cases being consistently reported from west and central Africa (WHO 2018). While the original description came from Bairnsdale, Australia by Mac- Callum *et al.* (1948), there were early clinical notes from Kampala, Uganda that match the current pathology of BU (Cook 1897). Throughout the middle to late 20th century, there has been considerable research into BU, with much of the early efforts focused in Africa (Clancey, Dodge and Lunn 1962; Lunn *et al.* 1965; Barker *et al.* 1970; Barker 1972; Barker, Clancey and Rao 1972; Revill and Barker 1972).

Infection includes all age groups, but children 7–15 years of age are predominately affected, followed by adults over the age of 50 (Debacker *et al.* 2004). Though there have been recent advances in antibiotic therapy for early lesions, patients sometimes require extensive surgery and hospital stays, creating economic burden for families, with patients suffering social isolation and stigma (Amoakoh and Aikins 2013). Without an effective vaccine and with limited knowledge of transmission, prevention is not yet feasible. Therefore, disease management relies on early case detection, reliable diagnosis and early effective treatment.

The first clinical sign of infection is a firm, painless, nodule or papule that may resemble an insect bite (Feverheiro and Pedrosa 2019). If left untreated, MU produces a cytotoxic macrolide called mycolactone that diffuses through healthy tissue leading to pathology further than the site of bacterial colonization (Sarfo *et al.* 2016). Patient ulcers are characterized by extensive necrosis of the skin and underlying fat. Growth requirements of MU limit infection to subcutaneous tissues in most cases, though osteomyelitis has also been reported (Walsh, Portaels and Meyers 2011).

Mycolactone is cytotoxic to many cell lines and possesses a wide range of molecular targets and mechanisms leading to BU pathogenesis (Sarfo *et al.* 2016). It was recently shown that mycolactone suppresses innate and adaptive immune responses through broad spectrum inhibition of protein

translocation through the endoplasmic reticulum (ER; Hall *et al.* 2014). This mechanism is driven through targeting the Sec61 translocon, blocking the production of cytokines and chemokines, which are subsequently degraded in the proteasome within the cytosol (Ogbechi *et al.* 2015). Mycolactone has also been reported to independently inhibit binding of angiotensin II to its receptor and to elicit toxicity to neurons, prompting analgesia (Marion *et al.* 2014). Finally, mycolactone binds to Wiskott Aldrich Syndromprotein (WASP) and neural-WASP, a family of scaffold proteins, blocking WASP autoinhibition (Guenin-Mac'e *et al.* 2013). This leads to uncontrolled assembly of actin in the cytoplasm and defective cell adhesion and cell migration. More detail of the clinical aspects of BU and mycolactone was recently published in an excellent online, open access book (Pluschke and Röltgen 2019).

**BOX 2: A Null Hypothesis for *Mycobacterium ulcerans* Transmission-** The vast majority of literature on *Mycobacterium ulcerans* (MU) transmission has come from epidemiological and case-control studies, with many fewer mechanistic studies (Tables 1 and 2). As suggested in previous reviews (Merritt *et al.* 2010; Carson *et al.* 2014; Röltgen and Pluschke 2015), MU transmission may occur via multiple pathways of environmental exposure with host direct or indirect interaction(s) with the environment (e.g. plants, soils, water and rhizospheres) or associated biotic reservoirs. The null hypothesis posits that MU transmission to humans, and some other vertebrate species (e.g. opossums and grasscutters), results from routine exposure and contact with certain environmental conditions, or diet consumption, reflective of high MU population abundances in biotic and abiotic reservoirs. For instance, grasscutters (*Thyromys swinderianus*) raised inside and without environmental exposure to MU do not harbor MU in their guts and faeces, whereas wild specimens do because they may feed upon wild plants that contain MU (Hammoudi *et al.* 2020a). This example shows the potentially important roles of trophic habits and foodwebs in MU transmission. In this null hypothesis, aquatic invertebrates and other organisms that harbor MU simply reflect contaminated environments. Depending on seasonality and associated change with human activity patterns, transmission results from contact and exposure with a MU contaminated environment that leads to an infective dose. It is most plausible that aquatic macroinvertebrates, including mosquitoes, are simply reservoirs or host carriers (e.g. amoebae; Gryseels *et al.* 2012), and act as sentinels of BU outbreaks driven more by landscape and weather interactions (Morris *et al.* 2014). Under the null hypothesis, transmission comes in the form of a deep skin puncture or cut that associated with some form of subdermal inoculation through a deep puncture or cut (e.g. vegetation or deep splinters), injury using tools (e.g. machete), insects (e.g. biting water bug) or other forms of skin trauma as discussed in early cases (Meyers *et al.* 1974). Thus, insects and other invertebrates may be one route of environmental transmission, but not the route of transmission. Scenarios for this type of transmission are reported both within and outside Africa with landscape disturbances nearly always cited as risk factors for BU along with frequent, intense and prolonged human exposure to altered habitats (Merritt *et al.* 2010; Carson *et al.* 2014; Röltgen and Pluschke 2015). Indeed, landscape disturbance associated with non-invertebrate routes of transmission were some of the original hypotheses described and tested in the earliest documented outbreaks (Barker *et al.* 1970; Barker

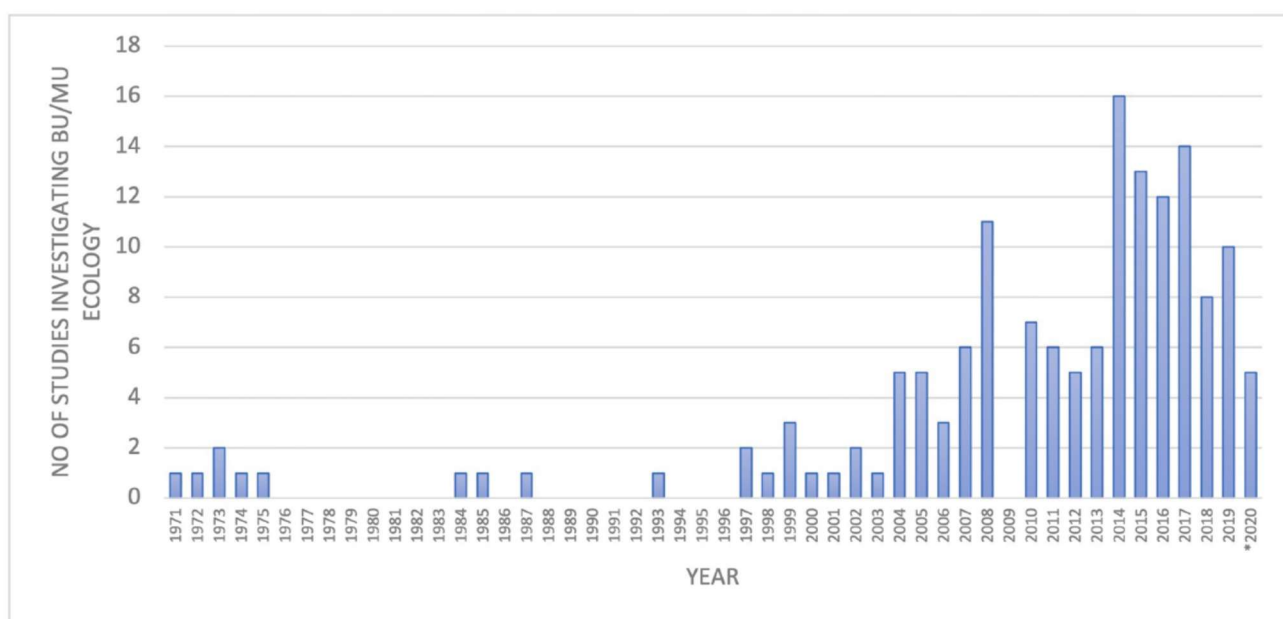
1972; Barker, Clancey and Rao 1972; Revill and Barker 1972; Barker and Carswell 1973; Stanford *et al.* 1976). More recent studies support landscape and weather pattern associations of BU outbreaks (Brou *et al.* 2008; van Ravensway *et al.* 2012; Bratschi *et al.* 2013; Garchitorena *et al.* 2015a, b, 2014), with the mechanisms of transmission likely complex and involving both ecological and pathogenic time delays between environmental cues or conditions and BU case emergences.

**BOX 3: Causal Inference, Hill's Criteria and modified Koch's Postulates-** A systematic review (Merritt *et al.* 2010) of the ecology and transmission of MU summarized both Barnett (1960) and Hill (1965) guidelines as a set of criteria necessary to provide strong evidence for insects as MU hosts (Table 2). Strong causal inference (Platt 1964) is the idea of testing multiple, alternative hypotheses rather than individual hypotheses in isolation and sequentially. This approach involves first defining the plausible hypotheses, and then proceeding to test the hypotheses in the context of the overall system (Greenland, Pearl and Robins 1999; Plowright *et al.* 2008; Röltgen and Pluschke 2019). Strong inference provides several advantages over traditional hypothesis testing, such as reducing the potential for confirmation bias and clarifying assumptions within complex systems and providing evidence for causation (Plowright *et al.* 2008). The difficulty of finding causative agents of disease transmission pathways has led to a variety of strategies for testing candidate agents. Two of the most well-known approaches of establishing strong causal inference in diseases is to fulfill Hill's criteria (Hill 1965) and Koch's postulates (Evans 1978). One of the earliest studies investigating the causative agent of BU disease using causal/strong inference was conducted in Melbourne, Australia (MacCallum *et al.* 1948; Chany *et al.* 2013). Their work examined biopsy specimens from patients in Bairnsdale (hence the alternative name of Bairnsdale ulcer), cultivated the mycobacterium and demonstrated experimentally that the characteristic ulcers could be induced by the bacteria in a mouse model (MacCallum *et al.* 1948; Merritt *et al.* 2010; Ampah *et al.* 2016; Röltgen and Pluschke 2019). These findings were consistent with those of subsequent studies (Fenner 1956; Clancey, Dodge and Lunn 1962) and provided strong support for MU as the causative agent of BU (Chany *et al.* 2013), meeting Koch's postulates: (1) Organism present during disease; (2) Organism is not present in other non-diseased individuals and (3) The isolated organism must be capable of inducing disease in experimental conditions (see Evans 1978 and Plowright *et al.* 2008). In contrast to the bacterial cause of BU disease, the mechanisms underlying transmission remain elusive and with little or inconsistent evidence to meet criteria for a defensible insect vector (Hill 1965; Plowright *et al.* 2008; Merritt *et al.* 2010; Röltgen and Pluschke 2015, 2019; Table 2). Statistical significance alone cannot answer the question of the causal pathways of MU transmission or agents of any other disease.

Without hypothesis-driven investigations designed to address at least Hill's criteria, the definitive mechanisms of MU transmission will likely remain unknown. Thus, we are left with a null hypothesis for MU transmission until if/when it is refuted by rigorous evidence along the lines of Hill's criteria. The most immediate research to be conducted to test a null hypothesis for MU transmission, given the history of insect vector speculation, would be to conduct vector competency studies using multiple, implicated insect taxa—namely, species of Culicidae, Naucoridae and Belostomatidae.

## FIGURES

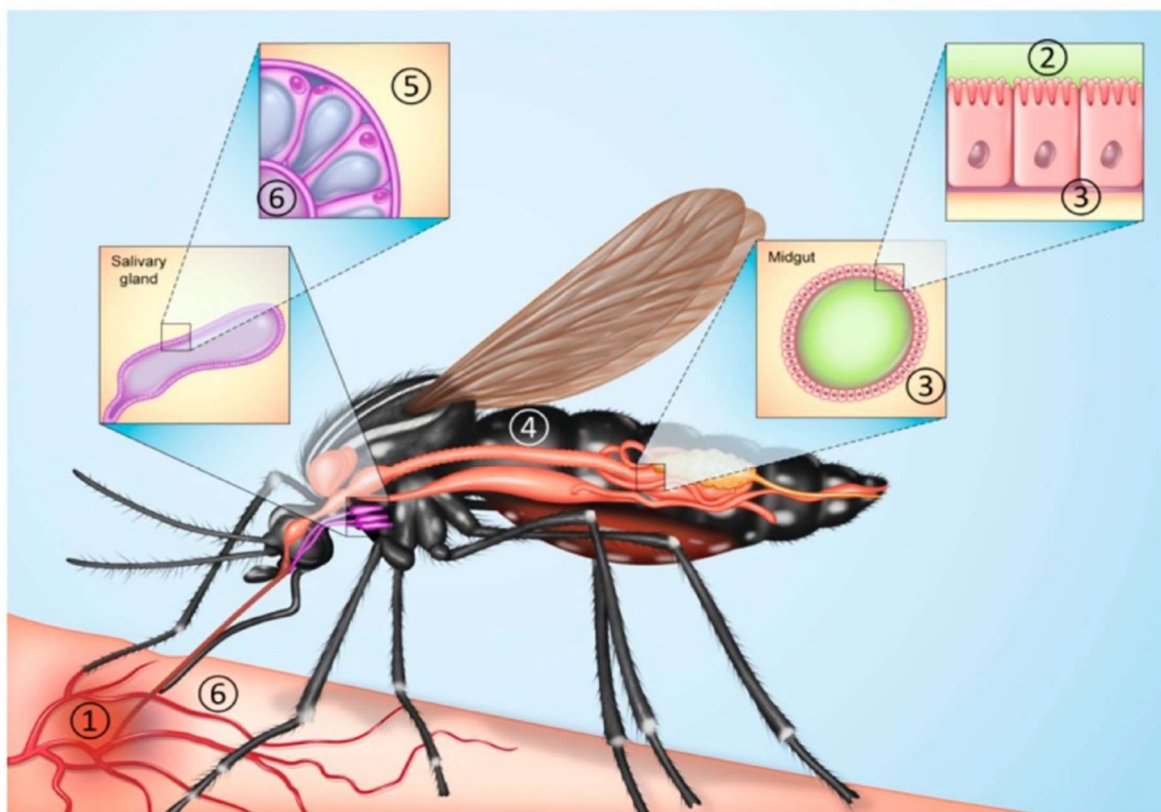
**Figure 1.** Number of studies investigating the ecology of Buruli ulcer (BU) and/or *Mycobacterium ulcerans* (MU) by year of publication. A literature search of the electronic database Scopus (1788–present) was performed on 1 November 2020 to identify articles, letters, notes and short surveys published up to November 2020. No language constraints were applied. Titles, abstracts and keywords of documents were searched using the Advanced Search interface and the following search terms: (TITLE-ABSKEY ('Buruli ulcer') or TITLE-ABS-KEY ('*Mycobacterium ulcerans*')) and (TITLE-ABS-KEY (ecology) or TITLE-ABSKEY (environment\*)). In addition to the database search, the references of identified articles were reviewed for relevance. The resulting literature database was reviewed for duplicates. Studies were selected if they met the following criteria: (1) reported original research content, (2) were not obvious clinical studies related to disease treatment or associated research, or country or regional focused reporting of cases, (3) were not obvious studies of pharmaceutical relevance and (4) focused on the environment and/or ecology or evolution of BU/MU. A total of 281 citation titles were screened for relevance. Where titles and abstracts were considered inconclusive, the full text was retrieved to determine if inclusion criteria were met. After this secondary review, the database comprised 153 citations investigating the ecology or evolution of BU and/or MU



**Figure 2.** Sequence of steps essential for a female mosquito to biologically transmit a virus. (1) female mosquito imbibes the virus from an infective host, (2) once infected inside the midgut, the virus overcomes the midgut infection barrier by replicating inside the midgut epithelial cells (MEC), (3) escaping the MEC, the virus enters the mosquito hemolymph—overcoming the midgut escape barrier developing into an infection, (4) the virus infects and replicates in all peripheral tissues and organs, (5) the penultimate step is to infect the salivary glands overcoming the salivary gland infection barrier and (6) finally shedding into the acinar cells and becoming present in the saliva for



inoculation into a host during the next blood feeding. (Modified from Azar and Weaver 2019).



## TABLES

**Table 1-** Detection of MU/MPM in environmental samples of insect families often implicated in MU transmission to humans

(NB: Table 1 expands on the three next pages)

References	Country(ies)	Culicid	Family Belostomatidae	Naisoridae	Notes/Conclusions by authors
Fyfe et al. (2010)	Australia	<ul style="list-style-type: none"> <li>4.8% of pools MU positive</li> </ul>			
Johnson et al. (2007)	Australia	<ul style="list-style-type: none"> <li>4/12 species MU positive</li> <li>Positivity rate of positive pools ranging from 2.0 to 7.0%</li> </ul>			<ul style="list-style-type: none"> <li>Indication of mosquitoes being true productive reservoirs or vectors involved in transmission</li> <li>MU positivity may simply indicate MU presence in environment</li> </ul>
Lavender et al. (2011)	Australia	<ul style="list-style-type: none"> <li>77/3502 pools (2.2%) MU/MFPM positive</li> </ul>			<ul style="list-style-type: none"> <li>Results suggest mosquitoes are involved in transmission to humans</li> <li>MU detection could also simply reflect MU presence in environment</li> </ul>
Röllgen et al. (2017)	Australia	<ul style="list-style-type: none"> <li>2/3 samples positive</li> </ul>			
Singh et al. (2019)	Australia	<ul style="list-style-type: none"> <li>35/900 mosquitoes analysed</li> <li>3/85 pools MU/MFPM positive</li> </ul>			<ul style="list-style-type: none"> <li>Indication of low burden of MU in environment coinciding with relatively low number of human cases of BU observed during trapping period</li> </ul>
Djouakpa et al. (2017)	Benin	<ul style="list-style-type: none"> <li>2/35 larvae and 30/10 adults tested</li> <li>0% of larvae pools positive</li> <li>0% of adult pools positive</li> </ul>			<ul style="list-style-type: none"> <li>Neither mosquito larvae nor adults are likely to act as reservoirs for MU</li> </ul>
Zogo et al. (2015)	Benin	<ul style="list-style-type: none"> <li>0% of larvae pools positive</li> <li>0% of adult pools positive</li> </ul>			<ul style="list-style-type: none"> <li>Main reservoir: aquatic environment</li> <li>Mosquitoes likely not involved in ecology and dissemination of MU in Benin</li> </ul>

References	Country(ies)	Culicidae	Family Belontiiformidae	Naucoridae	Notes/Conclusions by authors
Kodjowald et al. (2004)	Benin		<ul style="list-style-type: none"> <li>6/43 <i>Appasus</i> samples MU/MFPM positive</li> </ul>	<ul style="list-style-type: none"> <li>1/2 <i>Macrocoris</i> samples MU/MFPM positive</li> <li>3/10 <i>Naucoris</i> samples positive</li> </ul>	<ul style="list-style-type: none"> <li>Detection of DNA confirms suggestions implicating aquatic insects in transmission of BU disease</li> </ul>
Marion et al. (2011)	Benin		<ul style="list-style-type: none"> <li>Flying individuals</li> <li>3/35 <i>Diploxychus</i> sp. MU/MFPM positive</li> </ul>		<ul style="list-style-type: none"> <li>Support hypothesis of water bugs involved in spread and transmission of MU outside of aquatic habitat</li> </ul>
Portaels et al. (2008)	Benin		<ul style="list-style-type: none"> <li>1/1 <i>Appasus</i> sp. MU/MFPM positive</li> </ul>	<ul style="list-style-type: none"> <li>1/1 <i>Naucoris</i> sp. MU/MFPM positive</li> </ul>	
Portaels et al. (1999)	Benin, Ghana		<ul style="list-style-type: none"> <li>2/2 individuals MU/MFPM positive</li> </ul>	<ul style="list-style-type: none"> <li>3/3 individuals MU/MFPM positive</li> </ul>	<ul style="list-style-type: none"> <li>Implication of water bugs as a reservoir of MU</li> </ul>
Esemu et al. (2015)	Cameroon		<ul style="list-style-type: none"> <li>0% of samples positive</li> </ul>	<ul style="list-style-type: none"> <li>0% of samples positive</li> </ul>	<ul style="list-style-type: none"> <li>No evidence of biting water bugs serving as environmental reservoir/potential vector</li> </ul>
Marion et al. (2010)	Cameroon		<ul style="list-style-type: none"> <li>51/298 (17.4%) <i>Appasus</i> saliva samples MU/MFPM positive</li> <li>Tissue pools from BU endemic site positive but large seasonal fluctuation</li> <li>No tissue pools from non-endemic site positive</li> </ul>		<ul style="list-style-type: none"> <li>Biting water bugs implicated in MU transmission</li> <li>Could also be used as environmental indicators of BU risk</li> </ul>
Deanainé et al. (2017)	Côte d'Ivoire		<ul style="list-style-type: none"> <li>1/35 (2.85%) <i>Diploxychus</i> sp. saliva samples MU/MFPM positive</li> </ul>		

References	Countries	Culicidae	Family Belontiidae	Naucoridae	Notes/Conclusions by authors
Koman et al. (2015)	Côte d'Ivoire		<ul style="list-style-type: none"> <li>14 taxa MU/MPM positive (16.8% of <i>Diploonychus</i> sp. Thaeu pools &amp; 87% of <i>Diploonychus</i> sp. saliva samples positive)</li> </ul>	<ul style="list-style-type: none"> <li>2/3 taxa MU/MPM positive</li> <li>Positivity rate of 5.1 and 6.3%</li> </ul>	<ul style="list-style-type: none"> <li>Potentially involved in dissemination of MU/MPM in (natural) environment</li> <li><i>Diploonychus</i> sp. potentially reservoir/vector</li> </ul>
Marsdaller et al. (2012)	Côte d'Ivoire			<ul style="list-style-type: none"> <li>5/80 (6.3%) <i>Naucoris</i> salivary glands MU/MPM positive</li> </ul>	<ul style="list-style-type: none"> <li>Implication of aquatic insects in dissemination and transmission of MU</li> <li>Results do not exclude other modes of transmission or reservoirs</li> </ul>
Mesi et al. (2008)	Ghana		<ul style="list-style-type: none"> <li>0/110 samples positive</li> </ul>		
Williams et al. (2008) and Benbow et al. (2008)	Ghana	<ul style="list-style-type: none"> <li>67% of pools MPM positive</li> </ul>	<ul style="list-style-type: none"> <li>167% of pools MPM positive</li> <li>No significant correlation with MU in environment</li> </ul>	<ul style="list-style-type: none"> <li>12.2% of pools MU positive</li> <li>No significant correlation with MU in environment</li> </ul>	<ul style="list-style-type: none"> <li>No confirming evidence biting water bugs have greater importance to MU transmission than passive contact exposure to environment</li> </ul>
Wallace et al. (2010)	USA	<ul style="list-style-type: none"> <li>Collected larvae MU negative</li> </ul>	<ul style="list-style-type: none"> <li>Collected <i>Belostomatina</i> MU negative</li> </ul>		
Morris et al. (2016)	French Guiana	<ul style="list-style-type: none"> <li>Larvae</li> <li>Avg. bacillus/mg 121.96</li> </ul>	<ul style="list-style-type: none"> <li>Avg. bacillus/mg 30.44</li> </ul>		

**Table 2.** Criteria adapted from Hill (1965) and Plowright *et al.* (2008) for vector involvement in transmission of a disease agent. References listed are not exhaustive but serve to highlight the current state of research. See text for additional references. MU = *M. ulcerans*, BU = Buruli ulcer disease.

Hill's criteria	Description	State of MU/BU research	Challenges	References
Strength	A potential vector should be correlated with disease and have explanatory power	Several studies have implicated insects as risk factors with protective measures (i.e. use of bed netting, insect repellent and covering skin) resulting in decreased risk.	Low incidence rates ( $\leq 4$ cases/1000/year in high-risk areas) of disease makes epidemiological surveys difficult	Garchitorena <i>et al.</i> (2015), Meka <i>et al.</i> (2016), O'Brien <i>et al.</i> (2019)
Consistency	Association between vector and disease should be observed and repeatable under varying conditions	Inconsistent results between different epidemiological surveys investigating arthropod bites as risk factors.	Quantifying common interactions between humans and potential vectors (i.e. mosquito bites) during a relatively large gap between infection and disease presentation.	Jacobsen and Padgett (2010), Raghunathan <i>et al.</i> (2005), Quak <i>et al.</i> (2007)
Specificity	Infective vector should be absent/reduced when disease is absent/reduced	Variable results when investigating insect positivity rates in BU endemic regions (see Table 2).	MU DNA has been observed in a wide range of taxa and environmental matrices. Difficulty in culturing limits investigations into whether observed (i.e. PCR) positivity indicates a true replicative host.	Williamson <i>et al.</i> (2008), Esemu <i>et al.</i> (2019), Singh <i>et al.</i> (2019)
Temporality	Interactions between a potential vector and susceptible individual precede disease	Difficult to estimate for common taxa which interact regularly with humans but may be more apparent for investigations of taxa whose interactions with humans are likely more notable; e.g. the bite of a water bug.	Delays in disease presentation and diagnosis limits ability to identify points of transmission in most cases (e.g. those not travel associated).	Garchitorena <i>et al.</i> (2017)
Biological gradient	Shows a dose response relationship with disease	Experimental studies of transmission by potential vectors to date have not tested for a dose-response relationship though higher concentrations of MU on external surfaces results in higher numbers of infection (needle-puncture)	Resources needed for conducting manipulative studies of potential vector species	Wallace <i>et al.</i> (2017)

Table 2 continued on next page



Table 2 continued

Hill's criteria	Description	State of MU/BU research	Challenges	References
Experimental evidence	Manipulation of vector should be able to induce/reduce disease presentation or infection rates	Mixed results correlating MU-positive insects with case numbers Mechanical transmission by mosquito-induced puncture possible though untested in field conditions, Other potential arthropod vectors have received limited study		Marsollier et al. (2002), Wallace et al. (2017)
Plausibility	Method of transmission must make biological sense			
Coherence	Mechanism consistent with current body of knowledge	Rare compared to other vector borne transmission mechanisms, mechanical transmission of bacteria by insect vectors is thought to occur for a couple of pathogens (e.g. Tularemia in Scandinavia)	Limited investigation of mechanical bacterial transmission by arthropod vectors	Abdallahoum et al. (2021)
Analogy	In the case of limited evidence within a disease system, do similar transmission mechanisms exist in other systems?			