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Fungi as biocontrol agents of Culicoides biting midges, the putative vectors of bluetongue disease

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Abstract: Biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) are the principal vectors of several notable viral pathogens infecting animal livestock. Sickness and animal deaths caused by the *Culicoides*-transmitted bluetongue virus as well as the recent Schmallenberg virus outbreak have threatened the livestock industry in Europe. Recent studies highlight how, in the near future, the application of 'dry' fungal conidia of *Metarhizium anisopliae* in animal shelters and microenvironment (e.g. dung, manure, leaf litter, livestock surroundings) may be used to control the *Culicoides* vector, thus, reducing the incidence of *Culicoides*-borne diseases.

Keywords: bluetongue virus, biocontrol, *Beauveria bassiana*, *Culicoides*, *Metarhizium anisopliae,* vector control

Introduction

Several microorganisms have been extensively explored for decades in order to develop environmental friendly and cost-effective pest management strategies in agriculture and livestock farming. The well-known bacterial bioinsecticides, *Bacillus thuringiensis* and *Lysinibacillus sphaericus*, are widely used to control many insect species (Jurat-Fuentes and Jackson 2012, Silva-Filha et al. 2014). Several strains of the entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* have been used for the biological control (e.g. ‘Green Muscle’ programme in Africa, Met52 G bioinsecticide) of crop pests (Shah et al. 2007, Ansari et al. 2007, 2008, Skinner et al. 2014), insect species transmitting diseases to livestock (Mochi et al. 2010, Lpez-Snchez et al. 2012, Mishra et al. 2013, García-Munguía et al. 2015, Cruz-Vázquez et al. 2017, Holderman et al. 2017), and with Ceratopogonidae such as biting midge, *Culicoides* spp. (Ansari et al*.* 2010, 2011, de Souza et al. 2014, Nicholas and McCorkell 2014, Narladkar et al. 2015). However, their use against insect vectors of livestock disease is not fully explored.

A few studies have shown the potential of entomopathogenic fungi to control *Culicoides* biting midges, hereafter referred to as ‘midges’, vectors of numerous important livestock diseases including bluetongue, which pose a severe economic risk to the ruminant livestock industry (van Schaik et al. 2008, Velthuis et al. 2010, Zanella et al. 2012, Pinior et al. 2018). The economic impact of the bluetongue serotype 8 (BTV8) epidemics of 2006 and 2007 in the Netherlands alone accounted for 32.4 and 164-175 million, respectively (Velthuis et al. 2010). Whereas, the recent estimates indicate that a total cost of €41.9 million was invested in the bluetongue virus vaccination and surveillance programmes in Austria and Switzerland alone (Pinior et al. 2018).

There are many other fungal entomopathogens, apart from *M. anisopliae* and *B. bassiana*, which have been explored for controlling midges by many authors. For example, de Souza et al. (2014) reviewed thoroughly and gave a detailed account of fungal and oomycete parasites of chironomids, ceratopogonids and simulids. The naturally occurring Oomycete fungal pathogen, *Lagenidium giganteum*, was recorded as biocontrol agent of *Culicoides molestus* larvae, which caused mortality up to 33% in New South Wales, Australia (Wright and Easton 1996). Another dominant marine Oomycetes, *Halophytophthora* species was reported to colonize both living and dead pupae of *C. subimmaculatus* in coastal waters of Hervey Bay region in Queensland, Australia (Stephen and Kurtböke 2011). Yet another deuteromycete fungus, *Culicinomyces clavisporus*, was highlighted as the potential biocontrol agent against European biting midge, *C. nubeculosus* larvae (Unkles et al. 2004).

The impact of *Culicoides*-transmitted viruses such as Akabane in Australia, African horse sickness in Africa, bluetongue (BTV) in North America, Africa and Europe, as well as recently emerged Schmallenberg livestock disease in Europe, highlight the worldwide importance of midges (Elbers et al*.* 2013). The wide distribution of infected vector species of midges contribute to the rapid spread of the virus. At least 83 species of *Culicoides* are found in Europe (Venail et al. 2012), however, only around 30 species have been associated with BTV transmission (EFSA 2017): In Europe, *Culicoides* species that have been implicated as potential vectors of BTV generally belong to the subgenera *Avaritia* and *Culicoides*.

Culicoides (*Avaritia*) *imicola*, *C*. (*Avaritia*) *obsoletus* and *C*. (*Avaritia*) *scoticus* are presently considered confirmed BTV vectors, while *C*. (*Avaritia*) *chiopterus*, *C*. (*Avaritia*) *dewulfi*, *C*. (*Culicoides*) *pulicaris* and *C*. (*Culicoides*) *punctatus* as probable vectors (Purse et al. 2015, Foxi et al. 2016).

Current surveillance measures and control programmes focus on quarantine or movement restrictions of livestock during periods of insect activity as well as animal vaccination (Racloz et al. 2006, OIE 2013, Collins et al. 2016, EFSA 2017). Where disease control by vaccines is not available, midge control by use of fungal biocontrol agents may play an important role in limiting disease outbreaks. Presently, midge control rely predominantly on synthetic pesticides, which pose a risk to humans and the environment (Carpenter et al. 2008a, Webb et al. 2010, Del Rio et al. 2014, Baker et al. 2015, De Keyser et al. 2017). Climate change models predict warmer and wetter weather, which in turn is expected to lead to larger midges densities (Guis et al. 2012, White et al. 2017). Therefore, safe and effective methods of vector control are urgently needed. The application of entomopathogenic fungi may provide potential eco-friendly alternatives for the reduction of midge numbers and consequent reduction in disease transmission.

Case studies

Previous research carried by our group involved several of the commercially viable strains of *Metarhizium*, *Beauveria*, *Isaria* and *Lecanicillium* (Deuteromycotina: Hyphomycetes) to test the ability of these strains in killing an indigenous *C. nubeculosus* (Ansari et al. 2010, 2011). Though *C. nubeculosus* is not a common midge, nor is it considered an important vector species for Schmallenberg or bluetongue viruses, thus it was used as a model insect in our studies, which was sourced from a colonised line. Ansari et al. (2011) demonstrated the biocontrol potential use of fungal application to different substrates (peat, leaf litter, manure) as the representative resting sites for *Culicoides* midges to simulate a more accurate estimation of fungal application in livestock microenvironment. Whereas, Nicholas and McCorkell (2014) obtained 98% reduction in emergence of *C. brevitarsis* adults by incorporating *M. anisopliae* conidia to cattle dung. Superior control was achieved as cattle dung serve substrate for the growth and development of *C. brevitarsis*. Also, Narladkar et al. (2015) reported the use of high dose of fungal spores against unknown species of *Culicoides* larvae (in drainage channel) and adults (resting on cattle shed walls) and claimed LC_{50} values of 3837 mg and 2692 mg (10^8 cfu/g) for *M. anisopliae* and *B. bassiana*, respectively.

Different conidial formulations aimed at improving conidial application and consequently ease of use were tested, i.e. dry conidia dusted uniformly on each substrate (‘dry’ formulation) and conidia suspended in 0.03% aq. Tween 80 (‘wet’ formulation). It was found that conidia attach to the adult midge and infect it by penetrating the cuticle or integument. Once inside the insect, the fungus grows rapidly producing toxins that kill the midges within 24 h (Ansari et al*.* 2011). Following colonisation of the hemocoel, the fungus erupts through the intersegmental sections and produces conidiophores and conidia (Fig. 1). A commercially available strain, *M. anisopliae* F52 (Met52® G bioinsecticide, currently available for control of black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) in horticultural crops, killed 100% of *C. nubeculosus* within 24 h at 10^{11} conidia per m^2. Furthermore, *C. nubeculosus* adults exposed to ‘dry’ or ‘wet’ conidia under semi-field condition showed that dry conidia were more effective than wet conidia, causing 100%

mortality after 5 days compared to 70%, respectively. Met52 granular formulation is approved in several European countries but is not available as dry spores or in a powder form for use in midge control. Irrespective of application method or substrate, all surviving adults collected from *M. anisopliae*-treated substrates in a greenhouse study died from fungal infection. Midges were observed directly transmitting infective conidia between males and females. Similarly, transmission of *M. anisopliae* between adult mosquitoes (*Anopheles gambiae*), has been demonstrated. Further studies in Australia demonstrated the susceptibility of another important species of biting midge (*C. brevitarsis*) to different strains of *M. anisopliae* infection (Nicholas and McCorkell 2014). The authors suggest that *M. anisopliae* has the potential to control *C. brevitarsis* through either surface treatment or topical application to cattle or through incorporation into fresh cattle dung. They found that the two strains of *M. anisopliae* were able to cause 70% mortality in adult *C. brevitarsis* after exposure for 5 days to surfaces treated with approximately 0.6 g/m^2 of dry conidia. These mortalities increased to 96% and 94% after 7 days. Moreover, they showed that when *M. anisopliae* spores were incorporated into fresh cattle dung (between 0.25 and 1 g conidia/kg) the emergence of adult *C. brevitarsis* was reduced by up to 98%.

Importantly, the fungal strains tested pose no obvious risk to humans or the environment (Strasser et al. 2000, Darbro and Thomas 2009). US Environmental Protection Agency conducted risk assessment and found that *Metarhizium brunneum* (=*M. anisopliae* strain F52) was not harmful to earthworms or to such beneficial insects as lady beetles, green lacewings, parasitic wasps, honey bee larvae, and honey bee adults (EPA 2011, Fischhoff et al. 2017). Their production involves relatively low cost and simple technology processes, facilitating the potential for large-scale production. Currently, resting sites are poorly defined for *Culicoides* species and different vector species have different larval habitats and feeding preferences, e.g. adult *C. brevitarsis* prefer grass tussocks (Bishop et al. 1995) whereas *C. impunctatus* prefer downy birch (Carpenter et al. 2008b), therefore, it's impractical for the widespread application of fungal spores. Zimmer et al. (2014) assessed and recorded several substrates which serve as suitable breeding sites and micro-habitats for the larval development of midges, e.g. maize silage residues, cattle dung, ground of flooded meadow, green filamentous algae and underlying substrate, silt from a pond, and ground of hollows. Whereas, Carpenter et al. (2008b) found high levels of lichen, moss and liverwort as commonly resting sites of midge adults near downy birch trees. Breeding sites such as cattle dung could provide a means of exposing midges larvae to *M. anisopliae* in the field via treated dung (Nicholas and McCorkell 2014). However, targeting newly emerged adults prior to their initiation of blood feeding would be preferable to achieve significant reduction in disease transmission rates. Another factor is temperature, which is particularly important as the best time to treat the vector populations would be earlier in the season when midge density is still relatively low and few within the population are infected. The limitation of our studies is that the colonised line is adapted to higher temperatures than wild caught midges, which are cold tolerant, therefore, further studies are required to be conducted with the application testing using wild, field-caught midge populations. Thus, currently control studies has demonstrated for a colonised midges species which has a limited vector capacity in the potential use of entomopathogenic fungi for the reduction of midge-borne disease in livestock (Ansari et al*.* 2010, 2011, Nicholas and McCorkell 2014). The success of midge control programmes using these fungi require large-scale field trials in different microclimate conditions to establish the most effective formulations and application methods for the fungal spores.

Authors' contribution: MA conceptualised and wrote the manuscript. PD and MW provided logistic support and additional information. All authors revised the manuscript and approved the final version.

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Fig. 1. *Culicoides nubeculous* midges at different periods after contact with dry conidia of the entomopathogenic fungus *Metarhizium anisopliae* BNL 102. (A) Healthy adults (B) An adult midge cadaver, 3 days after treatment showing fungal growth through the body wall; (C) An adult cadaver, 6 days after treatment showing fungus sporulation.

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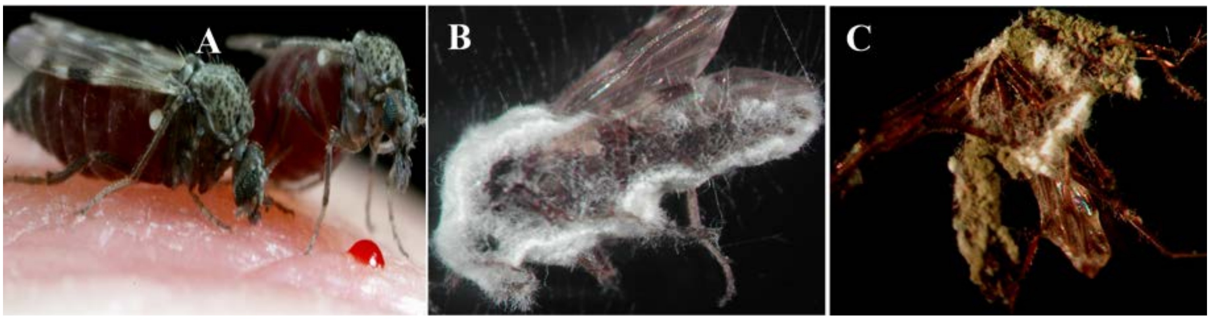

Fig 1.