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RESEARCH ARTICLE

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No amphibian chytrid fungus *Batrachochytrium dendrobatidis* detected in four introduced populations of the midwife toad *Alytes obstetricans* in eastern England

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ABSTRACT – The amphibian chytrid fungus *Batrachochytrium dendrobatidis* threatens amphibian species globally as the causative agent of chytridiomycosis, with the introduction of non-native species being one of the pathways that the pathogen can spread to naive populations. We have monitored and screened the common midwife toad *Alytes obstetricans* in four separate populations in eastern England, to investigate the potential threats to local amphibians. Forty-eight toads across all life stages were swabbed between May 2018 and August 2020, and screened for the presence of *B. dendrobatidis* DNA using qPCR. None of the samples tested were positive, indicating that it is unlikely that any of the *A. obstetricans* swabbed were infected with *B. dendrobatidis*. The populations surveyed represent only a small part of the species range in Britain, consequently a more widespread survey is recommended to increase confidence that British *A. obstetricans* are free of *B. dendrobatidis.*

INTRODUCTION

The amphibian chytrid fungus *Batrachochytrium dendrobatidis*, hereafter *Bd,* was discovered in the late 1990s. It is a non-hyphal zoosporic chytridiomycete fungus that causes amphibian chytridiomycosis, a disease that has been implicated in the decline of amphibian species globally (Berger et al., 1998; Skerratt et al., 2007). *Batrachochytrium dendrobatidis* is present on every continent except Antarctica and is known to affect more than 500 species globally, with the greatest impacts being in Mesoamerica, South America, and Oceania (Scheele et al., 2019). The spread of the fungus is thought to have been facilitated by the pet trade and the introduction of non-native species (Fisher & Garner, 2007).

In Europe, *Bd* is widespread in both geographic and host ranges, with few species displaying the symptoms of chytrid, and the impacts of disease being low for most species (Allain & Duffus, 2019). Several population declines of *Alytes* spp. recorded across continental Europe have indicated that this genus is highly susceptible to *Bd*, with subsequent lethal consequences (Bosch et al., 2001; Walker et al., 2010; Bosch et al., 2013; Doddington et al., 2013). Current data indicate that the *Alytes* spp. are the most susceptible taxonomic group to chytridiomycosis in the Palearctic (Bosch et al., 2001; Walker et al., 2010). Chytridiomycosis was responsible for the decline in the endangered Mallorcan midwife toad *Alytes muletensis*, following the release of infected captive-bred individuals (Walker et al., 2008). More recently, Moroccan midwife toads *Alytes maurus* have been found to be susceptible to chytridiomycosis (Thumsová et al., 2022). The rapid and significant geographic expansion of *Bd* across the entire range of *Alytes dickhilleni* in south-eastern Spain provides a relevant example of how chytridiomycosis severely affects midwife toads (Thumsová et al., 2021).

The salamander chytrid fungus *Batrachochytrium salamandrivorans*, hereafter *Bsal*, is a close relative to *Bd* which was first identified in 2013, after a dramatic decline of European fire salamanders *Salamandra salamandra* in the Netherlands (Martel et al., 2014; Spitzen-van der Sluijs et al., 2013). Since then, research has focused on how this additional pathogen may impact Europe's amphibian species. There is evidence suggesting that infection with *Bsal* in anurans is limited but species such as *A. obstetricans* may act as an intermediary host, infecting urodeles within the same ecological communities (Stegen et al., 2017). It is for this reason that previous screening of *A. obstetricans* in Great Britain targeted both *Bd* and *Bsal* (Allain & Goodman, 2017b; 2018). In the current study, individuals were not tested for *Bsal*, as previous analysis demonstrated that it was not present in *A. obstetricans* (Allain & Goodman, 2018) and in any case populations of *A. obstetricans* were introduced to their current locations before the emergence of *Bsal* in Europe. While *Bd* has previously been found in wild amphibians in Great Britain, *Bsal* is still yet to be detected (Allain & Duffus, 2019).

Alytes obstetricans is one of five recognised species of *Alytes* and is currently the only known species of this group to have established populations in Great Britain over the last century; mainly in southern Britain (Beebee & Griffiths, 2000). There are additional instances of introductions of this species outside of their natural range in Belgium, the Netherlands, and Germany (Speybroek et al., 2016). *Alytes obstetricans* are relatively small stocky toads compared to the native common toad *Bufo bufo*, with adults averaging

5.5 cm in length (Speybroek et al., 2016). Although variation in colour has been observed during surveys, the majority of individuals sampled have been a mottled stone colour, with visible warts and parotoid glands. A series of very small white warts usually 4–5 in number are also generally present along each flank. The native range for this species is western Europe, including the northern half of the Iberian Peninsula (Speybroek et al., 2016).

New populations of *A. obstetricans* are often being recorded in southern Britain, usually following the identification of the males' distinctive call (Allain & Goodman, 2019). This likely indicates an increase in detection rather than an expansion of their range within that same time frame. As a non-native species with an as yet unknown introduction pathway, disease surveillance is essential to ensure that any impacts on native species are minimised.

MATERIALS & METHODS

We surveyed midwife toad populations within urban and/or semi-urban environments at four locations in southern Britain (Fig. 1). Examples of our prime research habitats include, but are not limited to, private gardens and residential alleyways. To locate *A. obstetricans* in these environments during evenings between May and September, manual searches were combined with the use of call playbacks, which had previously been used to assist in the detection of the species (Allain & Goodman, 2017a). Upon capture, all toads were inspected visually for clinical signs of disease such as ulcerations and skin reddening.

To prevent cross-contamination, captured toads were held in individual zip-lock bags whilst each bag contained sufficient moisture and air to minimise stress. In the process of sampling, all appropriate biosecurity measures were taken including the use of nitrile gloves when handling the toads (Mendez et al., 2008), changing of gloves between each site, and the use of Vikron S when disinfecting field equipment (Young et al., 2007). Sterile cotton-tipped swabs (Medical Wire & Equipment, MW-100) were used to take skin surface samples from toads and tadpoles to check for chytrid fungus (Fig. 2). Each of the captured toads were sprayed with a small amount of water as part of the swabbing process in order to free their skin of any accumulated dirt or detritus, which may hinder the qPCR analysis (Kosch & Summers, 2013). The abdomen, thighs, groin, and feet of each individual were swabbed 15 times. After swabbing, data on sex, weight, and snout-to-vent length were recorded. Only in the case of the Cambridge population were tadpoles sampled, they were captured and subsequently released back into ponds using small aquarium nets. The tadpoles were sampled by placing a swab gently in contact with their mouthparts which was then spun for 10 seconds (Retallick et al., 2006). Once the samples had been taken, the swabs were refrigerated at 5 °C until they were sent to the Institute of Zoology at the Zoological Society of London for analysis, following the protocol described by Boyle et al. (2004). Briefly, swabs were tested for the presence of *Bd* DNA in duplicate using qPCR, with a positive result indicated by both duplicates showing amplification.

Figure 1. A map of Great Britain indicating the location of the four populations of common midwife toads *Alytes obstetricans* that were sampled in this study. Locations - 1 = Cambridge, 2 = St. Neots, 3 = Oundle, and 4 = Sutton.

Figure 2. A common midwife toad *Alytes obstetricans* being swabbed for amphibian chytrid fungus *Batrachochytrium dendrobatidis* during this study

RESULTS

A total of forty-eight *A. obstetricans* were swabbed from the four populations between 23 May 2018 and 27 August 2020. Five of these samples were from tadpoles, with the remainder coming from post-metamorphic toads. Of the toads captured, 7 were male, 3 were female, 3 were

Table 1. The qPCR testing for the presence of *Batrachochytrium dendrobatidis* DNA in common midwife toad *Alytes obstetricans* populations from four populations in eastern England, all tests were negative

Date	Location	Life-stage	Positive	Negative
23 May 2018	Cambridge, Cambridgeshire	Post-metamorphic	0	1
21 August 2018	St. Neots, Cambridgeshire	Post-metamorphic	0	1
27 July 2019	Cambridge, Cambridgeshire	Post-metamorphic	0	2
24 August 2019	Oundle, Northamptonshire	Post-metamorphic	0	16
3 September 2019	Sutton, Bedfordshire	Post-metamorphic	0	16
18 September 2019	Cambridge, Cambridgeshire	Post-metamorphic	0	3
18 September 2019	Cambridge, Cambridgeshire	Larval	0	5
27 August 2020	Cambridge, Cambridgeshire	Post-metamorphic	0	4

sub-adults, and 30 were of undetermined sex. None of these showed any clinical signs of chytridiomycosis. The average snout-to-vent lengths of the swabbed toads was 3.60 cm, and the average weight was 7.43 g. All samples tested qPCR negative for the presence of *Bd* DNA (see Table 1).

DISCUSSION

All samples were negative for *Bd* DNA but owing to the small sample size there remains some uncertainty about this result. This is especially true of the St. Neots population where only a single individual was swabbed due to problems associated with surveying; toads are not easily located as they often occur at low population densities and are found in private gardens, access to which depended on the co-operation of local residents.

Between 2018 and 2020 there was repeated testing of the Cambridge population (Table 1) but there was no evidence of chytridiomycosis during this period. The ongoing monitoring and disease screening of amphibian populations, including non-native species, is vital as an early warning system, which will enable swift mitigation to limit the exposure of British amphibian populations to *Bd*. This is especially true given the risk of *Bsal* spillover from captive collections to wild populations of amphibians within Great Britain (Fitzpatrick et al., 2018).

Whilst it is encouraging that there has been no evidence of *Bd* in any population of *A. obstetricans* in eastern Britain to date, the implications of such a discovery must also be considered. If and when *Bd* is detected in a population of *A. obstetricans*, it is unlikely that we would be certain whether they or the native populations of amphibians had been the source of infection. It is unlikely that the sampled populations of *A. obstetricans* mentioned here are vectors of *Bd* due to their high susceptibility to chytridiomycosis, and in any case their populations have persisted in southern Britain for over a century (Beebee & Griffiths, 2000). *Alytes obstetricans* typically inhabit gardens and breeding ponds with the common toad *Bufo bufo*, common frog *Rana temporaria*, and newts such as the smooth newt *Lissotriton vulgaris*. Of the amphibian species encountered on surveys, *A. obstetricans* is the most frequent, likely a consequence of our survey timing. Due to the greater susceptibility of *A. obstetricans* to *Bd*, the screening of *A. obstetricans* is an effective way to check whether or not the other amphibians within the same habitats were likely to be infected.

It is possible that exposure to less virulent strains of *Bd* could be beneficial in avoiding mass die-offs within populations of amphibians, as naive individuals are likely to be more susceptible (Greener et al., 2020). Other species such as the alpine newt *Ichthyosaura alpestris* are a recently established non-native amphibian within Great Britain, with a country-wide distribution (Allain & Lynn, 2021). Alpine newts may act as reservoirs for *Bd* (and other amphibian pathogens) that could potentially expose native amphibians to disease (Greener et al., 2020), highlighting the need to closely monitor amphibian populations where non-native species are known to be present.

This study has provided encouraging results, although it is only a snapshot based on populations in eastern England. Current known populations are considerably more widespread (Beebee & Griffiths, 2000). With adequate funding, all known populations could be screened regularly for *Bd* which would give more certainty of the status of the pathogen in Britain.

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