Title: Predation of anurans in southern England by *Batracobdella algira*, a leech previously unknown in the UK.

Abstract

Leech predation of amphibians is known to occur in Europe. Observation of severe leech infestation affecting a common toad (*Bufo bufo*) in southern England in summer 2020, with leeches covering the toad's eyes, throat, and axillae, initiated a collaborative investigation to learn more about the occurrence of such leech predation of anurans in the UK. Soliciting reports from the general public identified leech predation of common toads and common frogs (*Rana temporaria*) in Devon, Greater London, Hampshire, the Isle of Wight, and Somerset in southern England. Through morphological and/or molecular investigation of samples, *Batracobdella algira*, a leech species not previously reported in the UK, was identified in the majority of cases. The known native *Placobdella costata* was also identified, with the observed feeding behaviour on anuran hosts being indistinguishable from that of *B. algira*. Whether the latter is a previously unrecorded or an introduced species has not yet been established. However, sequence data from multiple gene loci were identical to *B. algira* found in Tunisia, suggesting it more likely to be a non-native species to the UK. Further work is required to elucidate the potential origin and distribution of *B. algira* in the UK and whether it has any impact on amphibian populations.

Keywords: amphibians, *Bufo bufo*, Glossiphoniidae, Hirudinea, *Rana temporaria*

INTRODUCTION

Leeches (Hirudinea) are a small, but ecologically important group of highly specialised segmented worms. Around 680 species have been described worldwide, the majority of which are found in, or beside, freshwater bodies (Sket & Trontelj, 2008). Leeches differ from other segmented worms in having anterior and posterior suckers and typically a firm, muscular, segmented body (Govedich et al., 2019).

There are 17 species of freshwater leeches known in the United Kingdom (UK) (Elliott & Dobson, 2015). They range in size at rest from 7 mm for *Piscicola siddalli*, to up to 160 mm for *Trocheta subviridis,* and when fully extended, larger leeches may reach a length of over 200 mm. Morphologically, these species can be identified using a combination of the number and position of the eyes, the size of the caudal sucker, the width and pattern of the annulation, the presence and pattern of the papillae, and the position of the gonopores. Depending on the species, they have been reported to feed on live fish, amphibians, mammals, or birds, as well as invertebrates, but may also scavenge on dead animals (Elliott & Dobson, 2015).

Four British leech species have been reported to be sanguivorous (blood-feeding) on live amphibians: the medicinal Leech (*Hirudo medicinalis*) (family Hirudinidae); and three species of Glossiphoniidae — *Glossiphonia paludosa*, *Hemiclepsis marginata*, and *Placobdella costata* (Elliott & Dobson, 2015). In addition, the horse leech (*Haemopis sanguisuga*) (family Haemopidae) is macrophagous on froglets and toadlets and may also feed on dead or moribund adult amphibians. It is likely that *Erpobdella* spp. and *Dina lineata* (both

Erpobdellidae) may also occasionally feed on dead amphibians (Elliott & Dobson, 2015). Despite increased monitoring of the health of amphibian populations in the UK over recent years, observations of leeches feeding on live amphibians are rarely reported (e.g., Price et al., 2017; Franklinos et al., 2018; Seilern-Moy et al., 2019).

In summer 2020, the sighting of a severe leech infestation affecting a live common toad (*Bufo bufo*) in southern England, with leeches covering both eyes and the throat of the toad (Fig. 1A), raised public concern on social media. Here we report the results of a subsequent collaborative investigation to explore the occurrence of such feeding behaviour on anurans and to identify the leech species involved.

METHODS

Following the initial report of leeches feeding on a common toad in southern England, the "Hampshire and Isle of Wight Amphibian and Reptile Group" (HIWARG) and the "Isle of Wight Reptilarium" launched a regional social media campaign and citizen science survey appealing for reports of leeches feeding on amphibians (www.reptilarium.org/leech-survey). This was combined with a similar national appeal by the "Amphibian and Reptile Groups of the UK" (ARG UK) (www.arguk.org/get-involved/news/a-new-report-of-leech-predation-on-amphibians). Additionally, reports of sick or dead amphibians continued to be solicited from the public by the Garden Wildlife Health project (www.gardenwildlifehealth.org), a national disease scanning surveillance scheme that launched in 2013.

For each sighting, the location and species of amphibian affected and, where possible, the number of amphibians affected and the distribution of the leeches on the body, were recorded. Photographs of affected amphibians accompanied reports when available. In a subset of cases, leech specimens were collected, stored in 70% ethanol, and submitted for species identification.

Morphological identification of 18 leeches from 8 sites (Table 1) was conducted by microscopical examination using a stereomicroscope (Olympus SZ40 Stereomicroscope, magnification x 6.25 – 80). Features including the number and position of the eyes, the width and pattern of the annulation, the presence and pattern of papillae, the size of the caudal sucker, and the position of the gonopores were compared with UK and European taxonomic keys and species descriptions (Sládeček & Košel, 1984; van Haaren et al. 2004; Ben Ahmed et al., 2015; Elliott & Dobson, 2015; Govedich et al., 2019). Additionally, high-resolution pictures of leeches attached to a common frog, taken using macro-photography, were submitted from a single site in Hampshire.

For molecular identification of the *Batracobdella* sp., DNA was extracted from the caudal suckers of 16 leeches from seven sites (Table 1). Each caudal sucker was placed in a microcentrifuge tube and incubated at 37°C for 30 min to evaporate residual ethanol before using the DNeasy Blood & Tissue Kit (Qiagen) as per manufacturer's instructions. A PCR protocol targeting the cytochrome c oxidase subunit I (COI) gene was then conducted (Richardson et al., 2010; Martinsson and Erséus, 2014). Additionally, other multi-loci PCR protocols, as described by Świątek et al. (2023), focusing on the 12S, 28S, and histone H3 genes (Vân Lê et al., 1993; Trontelj and Utevsky, 2005; Martinsson and Erséus, 2014), were carried out on each of three leech samples collected from three separate sites. PCR amplicons were subjected to bidirectional Sanger sequencing and the resulting sequence data were screened against GenBank entries using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Additionally, available COI nucleotide sequences for *Batracobdella* species were retrieved from GenBank and augmented with the sequence data generated in this study. Sequences were aligned in MEGA7 (Kumar et al., 2016) and trimmed in case they exceeded the 'Folmer region' (excluding primer sequences). A web server version of IQ-TREE (Trifinopoulos et al., 2016) was used to estimate the best-fitting models of nucleotide and to subsequently construct a maximum likelihood tree with 1000 replicates. Bootstrap support values were calculated using the ultrafast algorithm with default settings and the SH-aLRT branch test was used to evaluate the tree branch supports. The tree was rooted at *Haementeria ghilianii* and visualized with Interactive Tree of Life v6 (Letunic & Bork, 2007).

Three common frogs from separate sites, from August 2020 (Isle of Wight), September 2020 (Somerset), and January 2023 (Devon), which were observed by members of the public with leeches feeding on them and were subsequently found dead, were submitted for post-mortem examination (PME). A systematic external and internal examination protocol was followed, including parasitological examination of intestinal contents as well as qPCR testing of liver samples for ranavirus and skin swab samples for *Batrachochytrium dendrobatidis* and *B. salamandrivorans* (Franklinos et al., 2018).

RESULTS

From August 2020 to January 2023 inclusive, reports of leeches feeding on common frogs and common toads were received from 41 sites in the counties of Devon, Greater London, Hampshire, the Isle of Wight, and Somerset in southern England (Table 1; Fig. 1). Reports from 37 sites (37/41) involved live anurans, whereas at four sites (4/41) dead common frogs were observed. Estimates of the number of affected amphibians was typically low (range 1—3 per site), however, this information was only available from a minority of sites. The estimated number of leeches per individual varied (1—≥50). Where details were available, for affected common toads, leeches were typically observed to cover the eyes and periocular region (Fig. 1A) as well as attach to the throat, the axillae, and occasionally the ventral body. In common frogs, leeches were reported to be present over the entire body, on occasion also covering the eyes, although most notably on the underside, in the axillae, and inguinal area (Fig. 1B).

Leeches were submitted for species identification from a total of eight sites (eight leeches from three sites in Devon, one from a single site in Hampshire, six from three sites on the Isle of Wight, and three from a single site in Somerset; Fig. 2). All leeches examined microscopically were identified as Glossiphoniidae species. Morphological features, including the number of eyes, the width and patterning of the annulation, the presence of many small papillae, the size of the caudal sucker, and the position of the gonopores (Fig. 3), were indicative of *Batracobdella* sp. (Ben Ahmed et al., 2009) in leeches from seven sites (16/18 individual leeches examined), while they were consistent with those of the native freshwater leech *P. costata* from a single site in Devon (2/18 individual leeches examined). Additionally, high-resolution photographs of leeches from a single site in Hampshire where also morphologically consistent with *P. costata*.

Molecular investigation of the 16 putative *Batracobdella* leeches revealed an identical COI gene sequence (GenBank accession number OR381498; 658 bp), which also was identical to a Tunisian *B. algira* COI sequence (GenBank accession number OR366856, 100% query coverage). Furthermore, the obtained COI sequence shares 99% identity with other Tunisian and Algerian specimens of the same leech species (Fig. 4; GenBank accession numbers: OR366855 and OR367453-OR367454). The obtained COI sequence also exhibits a 92% match (86% query coverage) with a previously published sequence from a leech in Spain, reported as *B. algira* sequence (Trajanovski et al., 2010; GenBank accession number HM246609). Identification as a *B. algira* was further supported through multi-loci PCR sequence analyses of leeches from three sites in England. We successfully amplified fragments of three additional commonly used marker genes, which were identical in all three leeches analysed: nuclear 28S rRNA (GenBank accession number OR381568; 329 bp), histone H3 (GenBank accession number OR453923; 328 bp), and mitochondrial 12S rRNA (GenBank accession number OR381567; 508 bp). The mitochondrial 12S rRNA and histone H3 sequences were found to be identical to the Tunisian *B. algira* specimens (GenBank accession numbers OR388126 and OR453924), and the 28S rRNA sequence was identical to those of Tunisian as well as Algerian *B. algira* specimens (GenBank accession numbers OR371771 and OR371772).

Upon PME, numerous dead leeches (subsequently morphologically identified as *P. costata*) were found loosely attached to the body of a common frog found dead in Devon in January 2023, particularly in the axillae and inguinal area, as well as being free within the plastic bag in which the body was stored. However, no leeches were observed to still be attached to the common frog carcasses submitted from the Isle of Wight and Somerset in August and September 2020 respectively, both of which had been removed from the pond and kept separated from other amphibians prior to death. No macroscopic lesions associated with leech feeding behaviour (e.g., skin abrasions and/or haemorrhages) were observed in these amphibians; however, the compromised state of carcass preservation may have masked subtle changes. All three common frogs that were examined post-mortem were adult males, two in normal and one in thin (August 2020) body condition. No significant abnormalities indicative of disease were observed in two of these common frogs, whereas macroscopic evidence of haemorrhagic enteritis of unknown aetiology was found in the animal submitted from Somerset in September 2020, which might have contributed to ill health and/or death. All three frogs tested negative for ranavirus, *B. dendrobatidis* and *B. salamandrivorans* and their proximate cause of death remains undetermined.

DISCUSSION

A number of different species of native leeches are known to feed on amphibians in the UK. Here, we describe the detection of a leech species novel to the UK, and the first report of *Batracobdella algira* in this country, feeding on anurans in southern England. Like the native *P. costata*, *Batracobdella* spp. belong to the family Glossiphoniidae (order Rhynchobdellida), which includes leeches which feed on the blood of amphibians (Siddall et al., 2005). The species identified in this study appears to be genetically identical to the Tunisian lineage of *B. algira* studied by Ben Ahmed et al. (2009, 2015, 2021), which strongly suggests that the UK *B. algira* may be an introduced, non-native species.

Generally, *B. algira* is described from freshwater habitats in the Mediterranean rim (e.g., Algeria, Tunisia, the Pyrenean Peninsula, Balearic Islands, Corsica, Sardinia, and Sicily) as well as from Bulgaria and Ukraine (Minelli, 1979; Nesemann, 1991; Sori et al., 2011; Ben Ahmed et al., 2015), where it is reported to feed on several species of amphibian (Ben Ahmed et al., 2015; Manenti et al., 2016). However, the true geographic range of *B*. *algira* could be more limited, while the reported broad distribution might be attributed to hidden cryptic diversity of leeches (Raja Ben Ahmed, pers. comm.). Sightings and investigations of *Batracobdella* species remain scarce, and the overall understanding of their distribution and diversity is limited (Nesemann, 1991; Trajanovski et al., 2010).

The apparently restricted southern England distribution of *B. algira* within the UK might suggest this to be the area of arrival in the country, for example through natural routes from continental Europe or anthropogenic movement. The latter route, e.g. through live animal or plant trade, may offer the more likely explanation given the identical sequences of multiple loci of *B. algira* found in the UK and Tunisia despite their geographical distance. However, natural long-range dispersal, possibly mediated by birds, has also been documented in glossiphoniid leeches such as *P. costata* (Kvist et al., 2022), and therefore remains plausible. Additionally, a reporter bias may have occurred due to the regional emphasis of the HIWARG and Isle of Wight Reptilarium social media campaign appealing for sightings of leeches feeding on amphibians, resulting in a perceived localised observation. Potential abiotic environmental features (e.g., temperature, humidity), which influence the regional distribution of leeches, might also restrict the species' distribution (Lunghi et al., 2018).

Based on public reports, the observed distribution on the body and appearance of leeches feeding on common frogs in the two sites from which *P. costata* was morphologically identified were indistinguishable from the reports involving common frogs affected by *B. algira*. In an additional report of leeches attaching to the eyes and throat of a common toad on the Isle of Wight in 2011, another native widespread freshwater leech (*Hemiclepsis marginata*) was morphologically identified (Craig Macadam, unpublished data). The explanation that leeches are generally observed to accumulate mostly on the eyes and periocular areas as well as the throat and axillae of common toads is thought to be due to the avoidance of toxic skin secretions and therefore attaching to areas that are unprotected by skin glands (Kutschera et al., 2010). Our findings highlight the importance of citizen science and the morphological and/or molecular identification of the leech species involved to identify new species records and to differentiate potentially introduced species from known native species that form an important part of the natural fauna diversity (Elliott & Dobson, 2015).

Although interactions between leeches and their hosts are poorly characterised to date, it is known that leeches may use amphibians as an opportunistic food source (Elliott & Dobson, 2015), generally without leading to host death (Getz, 2011; Rocha et al., 2012). However, the impairment of vision caused by leeches covering the eyes of their host will likely affect its ability to forage or evade predators. Other potential health impacts, e.g. by affecting host fitness, pathogen transmission (Siddall & Desser, 1992; Jiménez Sánchez, 1997), or increasing host vulnerability to further infections (Lunghi et al., 2018), have also been previously discussed (Merilä & Sterner, 2002; Stead & Pope, 2010). It has further been proposed that environmental changes (e.g., climate warming or reduced water levels) may enhance negative impacts of leech predation on their hosts (Berven & Boltz, 2001; Ayres & Comesaña Iglesias, 2008).

Although facultative parasitism of the *B. algira* identified in this study seems likely, a phoretic relationship between anurans and this leech species (Khan & Frick, 1997; Maia-Carneiro et al., 2012; Starzecka et al., 2020) remains possible. Molecular investigation of stomach content of leeches could be used to explore the trophic relationship between *B. algira* and anurans in the UK (Marrone et al., 2016). Whilst to date there is no evidence of native leech species affecting amphibians in the UK at a population level, the significance of *B. algira* to amphibian health is yet to be established. In its native habitat, *B. algira* is not known to have negative health or population impacts on amphibians (Ben Ahmed et al., 2014).

Further surveillance across the UK is required to determine the distribution and frequency of occurrence of *B. algira*. Additional genomic sequence analyses across the *Batracobdella* genus might help identify closely related species and determine whether it is a historically unrecorded species in England or if it has originated from elsewhere. Regardless, a risk assessment to elucidate possible incursion pathways of non-native leeches to the UK, combined with a mitigation plan to address anthropogenic routes of introduction, should be developed.

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<u>Data Accessibility</u>

All new DNA sequences generated in the present study are deposited in GenBank under Accession Numbers: OR381498 (COI), OR381567 (12S), OR381568 (28S), and OR453923 (histone H3).

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Figure captions

Figure 1. Common toad (*Bufo bufo*) reported from Devon, England, in August 2020, with leeches covering both eyes and attaching to the throat (A), and common frog (*Rana temporaria*) reported from the Isle of Wight, England, in 2020, with leeches attached to the ventro-lateral body (B). Leech samples from both cases were molecularly identified as *Batracobdella algira*. Image credits: Chryssa M. L. Brown (A); Charis Hayles (B).

Figure 2. Map of Great Britain highlighting the counties from which reports of leeches feeding on common frogs (*Rana temporaria*) and common toads (*Bufo bufo*) were received from the general public (summer 2020 – January 2023 inclusive), including information on the number of sites and leech species identified.

Figure 3. Microscopic images of a leech found feeding on a common frog (*Rana temporaria*) on the Isle of Wight in 2022, with morphological features of a *Batracobdella* sp.: (a) lateral whole body, (b) one pair of eyes, (c) gonopores separated by two annulae (d) large posterior sucker. Olympus SZ40 Stereomicroscope. Magnification x 6.25 – 40.

Figure 4. Maximum Likelihood tree resulting from the analysis of COI nucleotide sequences of *Batracobdella* species. SH-aLRT and UFBoot support values are given below the branches. The sequence obtained from the *Batracobdella* species identified from the UK is identical to sequences from *B. algira* BA2-BA6 from Tunisia and is grouped within the Tunisian-Algerian *B. algira* clade. This clade is considered to represent *B. algira* sensu stricto, which inhabits the type locality.

Table captions

Table 1. Reports received from the general public of leeches feeding on common frogs (*Rana temporaria*) and common toads (*Bufo bufo*) in England, summer 2020 – January 2023 inclusive. Where possible, leech species were identified using morphological and/or molecular methods.