TITLE: **Development and worldwide use of a non-lethal and minimal population-level impact protocols for the isolation of chytrids from amphibians**

AUTHORS: Matthew C. Fisher[1]*, Pria Ghosh[1], Jennifer M. G. Shelton[1], Kieran Bates[1], Lola Brookes[10], Claudia Wierzbicki[1], Gonçalo M. Rosa[10,39], Rhys A. Farrer[1], David M. Aanensen[1,42], Mario Alvarado-Rybak[2], Arnaud Bataille[3,4], Lee Berger[5], Susanne Böll[6], Jaime Bosch[7], France C. Clare[1], Elodie Courtois[8], Angelica Crottini[9], Andrew A. Cunningham[10], Thomas M. Doherty-Bone[11], Fikirte Gebresenbet[12], David J. Gower[13], Jacob Höglund[14], Timothy Y. James[36], Thomas S. Jenkinson[36], Tiffany A. Kosch[3,5], Carolina Lambertini[37], Anssi Laurila[14], Chun-Fu Lin[15], Adeline Loyau[16,17], An Martel[18], Sara Meurling[14], Claude Miaud[19], Pete Minting[20], Serge Ndriantsoa[21], Frank Pasmans[18], Tsanta Rakotonanahary[21], Falitiana C. E. Rabemananjara[21,40], Luisa P. Ribeiro[37], Dirk S. Schmeller[16,17], Benedikt R. Schmidt[22,41], Lee Skerratt[5], Freya Smith[23], Claudio Soto-Azat[2], Giulia Tessa[24], Luís Felipe Toledo[37], Andrés Valenzuela-Sánchez[2,32], Ruhan Verster[33], Judit Vörös[34], Bruce Waldman[3], Rebecca J. Webb[5], Che Weldon[33], Emma Wombwell[10], Kelly R. Zamudio[38], Joyce E. Longcore[35], Trenton W. J. Garner[10]

[1] Department of Infectious Disease Epidemiology, School of Public Health, Faculty of Medicine (St Mary's campus), Imperial College London, London W2 1PG, UK

[2] Centro de Investigación para la Sustentabilidad, Facultad de Ecología y Recursos Naturales, Universidad Andres Bello, Republica 440, Santiago, Chile

[3] Laboratory of Behavioral and Population Ecology, School of Biological Sciences, Seoul National University, Seoul 08826, South Korea

[4] UMR ASTRE, CIRAD, Montpellier, France

[5] One Health Research Group, College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Queensland, 4811, Australia

[6] Agency for Population Ecology and Nature Conservancy, Gerbrunn, Germany

[7] Museo Nacional de Ciencias Naturales, CSIC c/ Jose Gutierrez Abascal 2, 28006 Madrid, Spain

[8] Laboratoire Ecologie, évolution, interactions des systèmes amazoniens (LEEISA), Université de Guyane, CNRS, IFREMER, 97300 Cayenne, French Guiana.

[9] CIBIO - Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO, Universidade do Porto, 4485-661 Vairão Portugal

[10] Institute of Zoology, Regent's Park, London, NW1 4RY, UK
[11] Conservation Programmes, Royal Zoological Society of Scotland, Edinburgh, UK
[12] Department of Integrative Biology, Oklahoma State University, 113 Life Sciences West, Stillwater, OK 74078, USA
[13] Life Sciences, The Natural History Museum, London SW7 5BD, UK
[14] Department of Ecology and Genetics, EBC, Uppsala University. Norbyv. 18D, SE-75236, Uppsala, Sweden
[15] Zoology Division, Endemic Species Research Institute, 1 Ming-shen East Road, Jiji, Nantou 552, Taiwan
[16] Helmholtz Centre for Environmental Research – UFZ, Department of Conservation Biology, Permoserstrasse 15, 04318 Leipzig, Germany
[17] ECOLAB, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France
[18] Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium
[19] PSL Research University, CEFE UMR 5175, CNRS, Université de Montpellier, Université Paul-Valéry Montpellier, EPHE, Biogéographie et Ecologie des vertébrés, Montpellier, France
[20] Amphibian and Reptile Conservation (ARC) Trust, 655A Christchurch Road, Boscombe, Bournemouth, Dorset, UK, BH1 4AP
[21] Durrell Wildlife Conservation Trust, Madagascar Programme, Madagascar
[22] Department of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
[23] National Wildlife Management Centre, APHA, Woodchester Park, Gloucestershire GL10 3UJ, UK
[24] Non-profit Association Zirichiltaggi - Sardinia Wildlife Conservation, Strada Vicinale Filigheddu 62/C, I-07100 Sassari, Italy
[32] ONG Ranita de Darwin, Nataniel Cox 152, Santiago, Chile
[33] Unit for Environmental Sciences and Management, Private Bag x6001, North-West University, Potchefstroom, 2520, South Africa
[34] Collection of Amphibians and Reptiles, Department of Zoology, Hungarian Natural History Museum, Budapest, Baross u. 13., 1088, Hungary
[35] School of Biology and Ecology, University of Maine, Orono, Maine 04469, USA
[36] Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, Michigan, 48109, USA

[37] Laboratório de História Natural de Anfíbios Brasileiros (LaHNAB), Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, São Paulo, 13083-862, Brazil
[38] Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York, 14853, USA
[39] Centre for Ecology, Evolution and Environmental Changes (CE3C), Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal
[40] IUCN SSC Amphibian Specialist Group-Madagascar, 101 Antananarivo, Madagascar
[41] Info Fauna Karch, Université de Neuchâtel, Bellevaux 51, UniMail Bâtiment 6, 2000 Neuchâtel, Switzerland
[42] Centre for Genomic Pathogen Surveillance, Wellcome Genome Campus, Cambridgeshire, UK

*Corresponding author. Email: matthew.fisher@imperial.ac.uk

ABSTRACT:

Parasitic chytrid fungi have emerged as a significant threat to amphibian species worldwide, necessitating the development of techniques to isolate these pathogens into sterile culture for research purposes. However, early methods of isolating chytrids from their hosts relied on killing amphibians. We modified a pre-existing protocol for isolating chytrids from infected animals to use toe clips and biopsies from toe webbing rather than euthanizing hosts, and distributed the protocol to interested researchers worldwide as part of the BiodivERsA project *RACE* – here called the *RML* protocol. In tandem, we developed a lethal procedure for isolating chytrids from tadpole mouthparts. Reviewing a database of use a decade after their inception, we find that these methods have been widely applied across at least 5 continents, 23 countries and in 62 amphibian species, and have been successfully used to isolate chytrids in remote field locations. Isolation of chytrids by the non-lethal *RML* protocol occured in 18% of attempts with 207 fungal isolates and three species of chytrid being recovered. Isolation of chytrids from tadpoles occured in 43% of attempts with 334 fungal isolates of one species (*Batrachochytrium dendrobatidis*) being recovered. Together, these methods have resulted in a significant reduction and refinement of our use of threatened amphibian species and have improved our ability to work with this important group of emerging fungal pathogens.

100 INTRODUCTION

101 A major consequence of globalisation has been the increase of invasive species owing to
102 trade in live animals and plants. A further outcome of this process is the concomitant rise of
103 novel emerging fungal pathogens (EFPs; (Farrer *et al.* 2017)) as these infections are moved
104 within trade networks and establish in uninfected regions – an example of fungal 'pathogen
105 pollution' (Fisher *et al.* 2012). Whilst EFPs can affect humans, they have also been broadly
106 detrimental to natural populations of plants and animals, leading to worldwide losses of
107 biodiversity. This dynamic has been most apparent across amphibians, where EFPs leading to
108 population extirpation and species extinctions have contributed to amphibians now being the
109 most endangered class of vertebrate (Stuart *et al.* 2004; Mendelson *et al.* 2006). In particular,
110 emergence of parasitic fungi in the genus *Batrachochytrium* (phylum Chytridiomycota, order
111 Rhizophydiales) have played a major role in driving amphibian population and species
112 declines worldwide (Berger *et al.* 1998; Fisher *et al.* 2009).

113 While a single species, *Batrachochytrium dendrobatidis* (*Bd*), was originally thought to have
114 caused the ongoing panzootic (James *et al.* 2009), we now know that amphibian
115 chytridiomycosis is caused by a much broader swathe of phylogenetic diversity than was
116 previously thought (Farrer *et al.* 2011; Schloegel *et al.* 2012). Next-generation sequencing
117 and phylogenomic analyses have shown that *Bd sensu stricto* is composed of deep genetic
118 lineages which are emerging through international trade in amphibians (Fisher *et al.* 2007;
119 Schloegel *et al.* 2009; Schloegel *et al.* 2010). Superimposed upon this background of trade-
120 associated lineages of *Bd* has come the recent discovery of a new species of pathogenic
121 chytrid, also within the Rhizophydiales, *B. salamandrivorans* (*Bsal*; Martel *et al.* 2013). This
122 pathogen has rapidly extirpated European fire salamanders (*Salamandra salamandra*) in the
123 Netherlands and a broad screening of urodeles has shown that *Bsal* occurs naturally in
124 southeast Asia where it appears to asymptomatically infect salamander and newt species
125 (Laking *et al.* 2017).

126 The ability to isolate and culture both *Bd* and *Bsal* has played a key role in catalysing
127 research into their pathogenesis and virulence (Voyles *et al.* 2007; Rosenblum *et al.* 2012;
128 Farrer *et al.* 2017), phenotypic characteristics (Piotrowski *et al.* 2004; Fisher *et al.* 2009;
129 Becker *et al.* 2017) and a wealth of experimental studies on epidemiologically relevant
130 parameters (Garner *et al.* 2009; Ribas *et al.* 2009; Rosenblum *et al.* 2012). Longcore *et al.*
131 (1999) first isolated *Bd* from infected amphibians by modifying techniques used to isolate

132 other chytrids (Barr 1987). Longcore cleaned small (< 0.5mm dia) pieces of *Bd*-infected leg
133 and foot skin by wiping them through agar and then placed skin pieces onto a clean plate of
134 nutrient agar containing penicillin G and streptomycin. This method worked well for isolating
135 from dead animals sent by courier from North and Central America. The method, however,
136 requires euthanizing potentially healthy animals if their infection status was unknown.
137 Further, it is difficult to perform this protocol in remote regions that lack suitable laboratory
138 facilities, and the lethal sampling of amphibians may be contraindicated if the species is
139 endangered, protected or located in protected areas.

140 We confronted this issue in a 2008-2014 project funded by BiodivERsA
141 (http://www.biodiversa.org) – *RACE*: Risk Assessment of Chytridiomycosis to European
142 amphibian biodiversity (Fisher *et al.* 2012). One of the objectives of this project was to adjust
143 the protocol of Longcore *et al*. (1999) to (i) reduce the need to kill adult amphibians, (ii)
144 improve rates of chytrid isolation by allowing the use of more animals, (iii) develop protocols
145 that enabled isolation in a field setting, and, (iv) integrate the data into the GPS-smartphone
146 enabled epidemiological software application *Epicollect* (Aanensen *et al.* 2009; Aanensen *et*
147 *al.* 2014). Further, 'forewarned is forearmed' and we wished to determine whether the
148 protocol was able to isolate other species of chytrid that are part of the amphibian skin
149 microbiota, and that may present a biosecurity risk. This need to more broadly characterise
150 global chytrid biodiversity was met by using resources from *RACE* to train researchers
151 worldwide in chytrid isolation techniques to provide opportunities to characterise novel
152 chytrids as they were discovered.

153 In addition to the non-lethal isolation protocol, a lethal method was developed in parallel to
154 isolate chytrids from the mouthparts of larval amphibians. We describe this method as a
155 refinement to the main isolation protocol.

156 METHODS

157 *Non-lethal field isolation of chytrids*

158 Animals were captured and held in separate plastic bags or suitable containers until ready for
159 processing (Supp. Info. *RML* Protocol 1 and Supp. Info. Swabbing Protocol 2). Using clean
160 gloves and sterilized dissection scissors or scalpel blades, the terminal 1-2mm of the
161 phalanges of the 4th hind toe (counting from the proximal toe) was clipped and laid on the

surface of an mTGhL + antibiotic (200 mg/L penicillin-G and 400 mg/L streptomycin sulphate) agar plate. Alternatively, ~1mm toe-webbing biopsy punches were taken (Sklar instruments, PA, USA) then laid on a plate. This allowed multiple animals to be processed rapidly in the field. Subsequently, each tissue sample was transferred to a second plate with a sterile needle or forceps then cleaned (as far as possible) of surface-contaminating bacteria and fungi by dragging it through the agar-medium. The needle or forceps was then used to place the tissue sample in a sterile 2 ml screw-cap microtube containing liquid mTGhL medium with antibiotics, then stored in a cool, dry place. While 4 ^{o}C appears optimal, we have successfully used shaded regions of streams to cool cultures when refrigeration was not immediately available and have even held tubes and plates for several days at > 10 ^{o}C until suitable storage conditions were available.

Once back in the laboratory, samples in tubes were visually screened for evidence of yeast or bacterial contamination (when the media takes on a 'cloudy' appearance), or mycelial 'balls' around the toe that are evidence of non-chytrid fungal contaminants. Visibly clear samples were decanted into a single well of a sterile 12-well lidded culture plate then incubated at 18°C for up to 4 weeks, topping up with extra medium to counter evaporation as necessary. Depending on the size of the initial tissue sample, toe clips and webbing were divided into several smaller samples before transferring to liquid culture media.

Isolating chytrids from tadpoles

Tadpoles often have higher burdens of infection than adults, especially long-lived tadpoles (Skerratt *et al.* 2008), and have higher densities and encounter rates than adults. In some situations where tadpoles were large and infections heavy, tadpoles were microscopically screened with a dissecting microscope or hand lens for areas of dekeratinization of the mouth parts, especially the jaw sheaths, that indicates infection (Fellers *et al.* 2001; Smith *et al.* 2007). Tadpoles are killed before excising their mouthparts and these preliminary microscopic screens enabled us to use only a small number of animals to isolate chytrids. Additionally, uninfected and naïve tadpoles that were reared in captivity were used as live substrates to bait chytrids from adult amphibians with low levels of *Bd* infection (Bataille *et al.* 2013).

Susceptible tadpoles were reared until gills were resorbed and animals were free-swimming and feeding (developmental Gosner stage 25), because at earlier stages they are still

193 developing the keratinized mouthparts. Each tadpole container was then immersed within a
194 similar but larger container that held at least one chytrid-infected animal. Water exchange
195 between the infected and bait animal containers occurred through small holes (< 0.3 mm)
196 drilled into the bottom of the walls of the smaller internal containers. Animals were held in
197 these conditions for between 2 and 4 weeks at species-appropriate conditions. Tadpoles were
198 periodically examined every fourth day for the presence of the depigmented areas in the jaw
199 sheaths that have been associated with chytrid infection.

200 Isolating chytrids from tadpoles first required killing by immersion in a 5 g/L solution of MS-
201 222 (Torreilles *et al.* 2009) or other approved method. Note that anaesthetics which contain
202 ethanol, such as phenoxyethanol (Gentz 2007), should be avoided as these will kill chytrids
203 while MS222 is not toxic (Webb *et al.* 2005). We then dissected out keratinized jaw sheaths
204 and cleaned the entire sheath, or sections, as above using an agar plate with antibiotics
205 ((Longcore *et al.* 1999); Supp. Info. *RML* Protocol 1). Cleaned sections were then placed
206 singly into sterile 12-well culture plates with 1 mL liquid media + antibiotics, or onto agar
207 plates with 6 – 10 sections per plate, and incubated at 10 – 20 ^{o}C.

208 Because zoospore release may occur immediately, especially from tadpole mouthparts,
209 cultures were examined with an inverted microscope for the presence of active zoospores
210 every day for up to one week following the day that they were initiated. After that, checks
211 every two days were sufficient.

212 *Culture and diagnosis of chytrid isolates*

213 Subsequent culture methods for *Bd* followed those of Longcore *et al.* (1999). When isolation
214 of *Bsal* was anticipated an incubation temperature of 15 ^{o}C was required (Blooi *et al.* 2015)
215 whereas a temperature of 18 – 22 ^{o}C is closer to the measured growth optimum of *Bd*
216 (Longcore *et al.* 1999; Ribas *et al.* 2009). Once growth of zoospores and/or zoosporangia was
217 observed, 100 – 500 µL volume of culture containing zoospores and zoosporangia was
218 transferred by pipette to a new 12-well plate with liquid medium and no antibiotics, and
219 incubated at 15 – 20 °C. All successfully cultured isolates were subcultured into larger
220 volumes, then centrifuged at 1700 g for 10 min before cryopreservation. A portion of the
221 initial pellet was also be used for DNA extraction, while the remaining volume was
222 resuspended in 10% DMSO and 10% FCS in liquid media and transferred into six 2 mL
223 cryotubes for cryopreservation at -80 °C (Boyle *et al.* 2003).

224 We confirmed the identity of *Bd* and *Bsal* by quantitative PCR with an MGB Taqman probe
225 assay in either single-plex or multiplex (Boyle *et al.* 2004; Blooi *et al.* 2013). We identified
226 non-*Batrachochytrium* chytrids was achieved by sequencing appropriate regions of the
227 ribosomal RNA gene with universal fungal primers followed by comparison against OTUs
228 held in UNITE database (Unified system for DNA-based fungal species linked to
229 classification: https://unite.ut.ee) to establish a species-hypothesis for the chytrid isolate in
230 question (Schoch *et al.* 2012). If further genetic data were required, then multilocus analysis
231 or whole-genome sequencing was undertaken using chytrid-specific methods (James *et al.*
232 2009; Farrer *et al.* 2013; Farrer *et al.* 2017; Farrer *et al.* 2017).

233 *Collation of data*

234 To track and report chytrid isolation for the *RACE* project, we used a generic data collection
235 tool that allows the collection and submission of geotagged data forms from field locations,
236 *Epicollect5* (https://five.epicollect.net). This software has the advantage that it can be used on
237 mobile devices with or without internet connection, and allows the immediate sharing of data
238 across the research community. Our database at
239 https://five.epicollect.net/project/bd-global-isolation-protocol included the following data
240 fields: Date; Continent, Country, Site name; Latitude/Longitude; Wild caught or trade?;
241 Amphibian species; Life history stage; Number sampled; Chytrid isolated?; Number isolated;
242 Species of chytrid isolated; Chytrid lineage; Photograph of amphibian; Name of researchers.

243 RESULTS

244 The '*RACE* modified Longcore (*RML*) Protocol' for the non-lethal isolation of chytrids from
245 amphibians is detailed in Supp. Info. 1. Ensure that you have the relevant licences, permits
246 and permissions from ethical committees to follow the *RML* protocol 1, swabbing protocol 2
247 and isolation from larval amphibians.

248 *Non-lethal isolation from adult and juvenile amphibians*

249 Following the formalisation and distribution of the *RACE* protocols, our Epicollect5 project
250 summarised chytrid surveys from 2007 through to 2017 (Table 1). The Epicollect5 database
251 can be spatially visualised at
252 https://five.epicollect.net/project/bd-global-isolation-protocol/data. Figure 1 depicts the

253 isolation of amphibian-associated chytrids using the *RACE* protocols from 5 continents
254 (Africa, Asia, Australia, Europe and South America), 23 countries, 239 sampling episodes,
255 and from latitudes spanning -44.1 S (*Batrachyla antartandica*, Chile) through to 55.6 N (*Bufo*
256 *viridis,* Sweden). Chytrids have been non-lethally isolated from 34 amphibian species, of
257 which 28 were anuran and 5 were caudatan species. The database also contains 5 records of
258 chytrids that were non-lethally sampled from the amphibian trade.

259 In total, 1,152 animals were non-lethally sampled, recovering 207 chytrid isolates and
260 resulting in a recovery rate of 18% (~1 isolate per 5 animals sampled). Of these chytrids, 203
261 (98%) were *Bd*, 2 were *Rhizophydium* sp., 2 were *Kappamyces* sp. and none were *Bsal* (Table
262 1). Of the *Bd* isolated, 42 (88%) were determined to be *Bd*GPL, 5 (10%) were *Bd*CAPE, and
263 1 (2%) was *Bd*CH.

264 *Isolation of chytrids from larval amphibians*

265 In total, 784 tadpoles were sampled recovering 334 chytrid isolates and resulting in a
266 recovery rate of 43% (~1 isolate per 2 – 3 animals sampled). Isolates were recovered from 34
267 species of amphibian, all of which were anurans (Table 2). These chytrid isolates were all *Bd*
268 and, of the lineages recorded, 129 (78%) were *Bd*GPL, 34 (20%) were *Bd*BRAZIL and 3
269 (2%) were hybrids.

270 Baiting chytrid isolates from live adult animals using tadpoles was used successfully in South
271 Korean *Bombina orientalis* as previously described (Bataille *et al.* 2013). Here, six tadpoles
272 were co-housed with adult *B. orientalis*, yielding a single isolate of *Bd* for each attempt
273 equating to a rate of success of ~20%.

274 DISCUSSION

275 The *RML* protocol, based on the original suggestions of Joyce Longcore for the non-lethal
276 isolation of chytrids from amphibians, has been a success with isolates of chytrids recorded
277 from five continents. There are likely many other unrecorded uses of this method because this
278 protocol has been widely dispersed during the 5-year span (2008-2014) of the *RACE* project
279 which trained a cohort of amphibian disease researchers in these techniques.

280 In some circumstances chytrids could not be recovered from toe-clips when sampling
281 populations with persistent infection despite repeated attempts. This was particularly evident

282 when the prevalence and burden of chytrid infections in surveys was low (Swei *et al.* 2011;
283 Bataille *et al.* 2013; Laking *et al.* 2017) or when host species occupied habitats with high
284 bacterial and/or non-target fungal contaminants. In these situations we isolated chytrids from
285 tadpole mouthparts as an associated method to the *RML* protocol. The value of the *RML*
286 protocol in propelling forward research on amphibian chytridiomycosis has been very clear:
287 for instance, of the 59 scientific papers produced by *RACE*, 15 directly used isolates of *Bd*
288 that were generated by this protocol for experimental trials (Supp. Info. 3). Further,
289 subsequently many more studies using these isolates have extended our knowledge of the
290 genetic diversity of *Bd* (James *et al.* 2009; Farrer *et al.* 2011; Farrer *et al.* 2013; Jenkinson *et*
291 *al.* 2016), the development of novel diagnostics (Dillon *et al.* 2017), the genetic repertoire
292 that underpins the virulence of these pathogens (Rosenblum *et al.* 2012; Farrer *et al.* 2017)
293 and the biogeographic distributions of *Bd* diversity worldwide (Farrer *et al.* 2011; Jenkinson
294 *et al.* 2016).

295 Clearly some uncontrolled biases and unanswered questions in these studies need attention.
296 First, the majority of *Bd* isolates belong to the *Bd*GPL lineage. This could be because this
297 lineage is more widespread (and therefore more readily recovered) than other lineages (James
298 *et al.* 2015), or it could be that the intensity of *Bd*GPL infections and/or the rate of zoospore
299 production is higher than for other lineages, which would also equate to a higher rate of
300 isolation. To achieve a true and unbiased understanding of the distribution of these lineages, a
301 lineage-specific diagnostic will need to be developed and deployed. Second, if lineage-
302 specific differences in the probability of successful isolation exist, then mixed infections
303 where these lineages co-occur may not be detected. This can be controlled for by isolating
304 and genotyping many isolates from a single host and population, although this may not fully
305 account for this bias. A related bias is that not all infectious species of chytrid will respond
306 equally to culturing attempts. For instance, despite known attempts to isolate *Bsal* from
307 across its endemic southeast Asian range using the protocol, to date no successful isolations
308 of *Bsal* have been recorded. This is likely due to a combination of the low prevalence and
309 burden of infection in salamanders and newts combined with the low initial growth-rate of
310 *Bsal* (Martel *et al.* 2013; Laking *et al.* 2017). With the *RML* protocol, however, workers have
311 been able to isolate non-*Bd* species of chytrid (*e.g., Kappamyces* spp. and *Rhizophydium* sp.
312 Table 1). This diversity likely represents only a fraction of the diversity of amphibian-
313 associated chytrids that occur, and non-biased estimators of this diversity by, for instance,
314 profiling the nuclear ribosomal RNA cistron (Schoch *et al.* 2012), are sorely needed.

In this age of the global amphibian crisis, research on the effects of chytrid infections is transitioning to attempts to mitigate their impacts (Schmeller *et al.* 2014; Garner *et al.* 2016; Canessa *et al.* 2018). Both of these research streams benefit from the availability of chytrid isolates, but the ethics behind these research programs can be improved. To that end, our data on isolation success suggest that tadpoles are a better target for isolation than metamorphosed animals. This is to some degree unfortunate, because isolation from tadpoles requires killing. However we have outlined one refinement where captive reared tadpoles can be used to 'bait' infections from wild-caught amphibians to isolate chytrids without killing adult amphibians. Here, it is important to recognise that amphibians which have been co-housed in collections should not be returned to the wild due to the danger of cross-transmission of pathogens during husbandry (Walker *et al.* 2008). If it is necessary to isolate chytrids directly from wild tadpoles without using bait animals, we suggest that researchers focus on more fecund species with long larval periods as the focal species in aquatic amphibian communities. Removal of small numbers of tadpoles when clutch sizes are in the hundreds or thousands means that removals will have an insignificant ecological impact; for this reason sacrificing tadpoles is preferable to killing adult animals.

The extent to which toe-clipping effects the fitness of amphibians has been much debated (*e.g.* May (2004) but see Funk *et al.* (2005)). Toe-clipping has been shown to decrease amphibian survival, but this effect, when present, is linearly related to the number of toes removed (McCarthy *et al.* 2004; Ulmar Grafe *et al.* 2011). For the single toe-clip that the *RML* protocol requires, reduction in survival appears to be negligible (Ott *et al.* 1999; Funk *et al.* 2005), and toe clipping is certainly preferred to killing the animal. Attention should be paid to this issue, however, and, where appropriate, survival estimates should be undertaken to determine the health implications of this procedure. Also, antiseptic and analgesic protocols can be considered to ensure that wounds where tissue samples are excised are at low risk of secondary infection (Chevalier *et al.* 2017).

In summary, modification of Longcore's original *Bd*-isolation protocol (Longcore *et al.* 1999) has enabled a broad community of scientists to engage with research on emerging chytrid pathogens of amphibians. This research has had an impact worldwide, and is contributing to the ongoing dialogue that is occurring between scientists, conservationists and policy-makers about how we might mitigate against these infections now and into the future.

ACKNOWLEDGMENTS

Data accessibility: https://five.epicollect.net/project/bd-global-isolation-protocol

Grants: TWJG, MCF, DSS, AL, EC, FCC, JB, AAC, CM, FS, BRS were supported through the Biodiversa project RACE: Risk Assessment of Chytridiomycosis to European Amphibian Biodiversity (NERC standard grant NE/K014455/1 and NE/E006701/1; ANR-08-BDVA-002-03). MCF, JS, CW, PG were supported by the Leverhulme Trust RPG-2014-273, MCF, AC, CW were supported by the Morris Animal Foundation. JV was supported by the Bolyai János Research Grant of the Hunagrian Academy of Sciences (BO/00597/14). FG and DG were supported by the Conservation Leadership Programme Future Conservationist Award. CSA was supported by Fondecyt No. 1181758. MCF and AC were supported by Mohamed bin Zayed Species Conservation Fund Project 152510704. GMR held a doctoral scholarship (SFRH/BD/69194/2010) from Fundação para a Ciência e a Tecnologia (FCT). LFT, CL, LPR KRZ, TYJ, TSJ were supported by São Paulo Research Foundation (FAPESP #2016/25358-3), the National Counsel of Technological and Scientific Development (CNPq #300896/2016-6) and a Catalyzing New International Collaborations grant from the United States NSF (OISE-1159513). CSA was supported by Fondecyt No. 1181758

References

1. Aanensen, D. M., D. M. Huntley, E. J. Feil, F. al-Own and B. G. Spratt (2009). "EpiCollect: Linking Smartphones to Web Applications for Epidemiology, Ecology and Community Data Collection." Plos One **4**(9).
2. Aanensen, D. M., D. M. Huntley, M. Menegazzo, C. I. Powell and B. G. Spratt (2014). "EpiCollect+: linking smartphones to web applications for complex data collection projects." F1000Res **3**: 199.
3. Barr, D. J. S. (1987). Isolation, culture and identification of Chytridiales, Spizellomycetales, and Hypocreales. Zoosporic fungi in teaching and research. M. S. Fuller and A. Jaworski. Athens, Georgia, Southeastern Publishing Corp.**:** p 118-120.
4. Bataille, A., J. J. Fong, M. Cha, G. O. U. Wogan, H. J. Baek, H. Lee, M. S. Min and B. Waldman (2013). "Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians." Molecular Ecology **22**(16): 4196-4209.
5. Becker, C. G., S. E. Greenspan, K. E. Tracy, J. A. Dash, C. Lambertini, T. S. Jenkinson, D. S. Leite, L. F. Toledo, J. E. Longcore, T. Y. James and K. R. Zamudio (2017). "Variation in phenotype and virulence among enzootic and panzootic amphibian chytrid lineages." Fungal Ecology **26**: 45-50.
6. Berger, L., R. Speare, P. Daszak, D. E. Green, A. A. Cunningham, C. L. Goggin, R. Slocombe, M. A. Ragan, A. H. Hyatt, K. R. McDonald, H. B. Hines, K. R. Lips, G. Marantelli and H. Parkes (1998). "Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America." Proceedings of the National Academy of Science, USA **95**: 9031-9036.

387 7. Blooi, M., A. Martel, F. Haesebrouck, F. Vercammen, D. Bonte and F. Pasmans (2015).
388 "Treatment of urodelans based on temperature dependent infection dynamics of
389 *Batrachochytrium salamandrivorans*." Scientific Reports **5**.
390 8. Blooi, M., F. Pasmans, J. E. Longcore, A. Spitzen-van der Sluijs, F. Vercammen and A.
391 Martel (2013). "Duplex Real-Time PCR for Rapid Simultaneous Detection of
392 *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in
393 Amphibian Samples." Journal of Clinical Microbiology **51**(12): 4173-4177.
394 9. Boyle, D. G., D. B. Boyle, V. Olsen, J. A. T. Morgan and A. D. Hyatt (2004). "Rapid
395 quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in
396 amphibian samples using real-time Taqman PCR assay." Diseases of Aquatic
397 Organisms **60**(2): 141-148.
398 10. Boyle, D. G., A. D. Hyatt, P. Daszak, L. Berger, J. E. Longcore, D. Porter, S. G.
399 Hengstberger and V. Olsen (2003). "Cryo-archiving of *Batrachochytrium*
400 *dendrobatidis* and other chytridiomycetes." Dis Aquat Organ **56**(1): 59-64.
401 11. Canessa, S., C. Bozzuto, E. H. C. Grant, S. S. Cruickshank, M. C. Fisher, J. C. Koella, S.
402 Lötters, A. Martel, F. Pasmans, B. C. Scheele, A. Spitzen-van der Sluijs, S. S. and B. R.
403 Schmidt (2018). "Decision making for mitigating emerging wildlife diseases: from
404 theory to practice for an emerging fungal pathogen of salamanders. ." Journal of
405 Applied Ecology.
406 12. Chevalier, H., O. Calvez, A. MArtinez-Silvestre, D. Picard, S. Guerin, F. Isselin-Nondedeu,
407 A. Riberon and A. Trochet (2017). "Marking techniques in the Marbled Newt (
408 *Triturus marmoratus*): PIT-Tag and tracking device implant protocols." Acta
409 Herpetologica **12**(1): 79-88.
410 13. Dillon, M. J., A. E. Bowkett, M. J. Bungard, K. M. Beckman, M. F. O'Brien, K. Bates, M. C.
411 Fisher, J. R. Stevens and C. R. Thornton (2017). "Tracking the amphibian pathogens
412 *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* using a
413 highly specific monoclonal antibody and lateral- flow technology." Microbial
414 Biotechnology **10**(2): 381-394.
415 14. Farrer, R. A. and M. C. Fisher (2017). "Describing Genomic and Epigenomic Traits
416 Underpinning Emerging Fungal Pathogens." Adv Genet **100**: 73-140.
417 15. Farrer, R. A., D. A. Henk, T. W. J. Garner, F. Balloux, D. C. Woodhams and M. C. Fisher
418 (2013). "Chromosomal Copy Number Variation, Selection and Uneven Rates of
419 Recombination Reveal Cryptic Genome Diversity Linked to Pathogenicity." Plos
420 Genetics **9**(8).
421 16. Farrer, R. A., A. Martel, E. Verbrugghe, A. Abouelleil, R. Ducatelle, J. E. Longcore, T. Y.
422 James, F. Pasmans, M. C. Fisher and C. A. Cuomo (2017). "Genomic innovations
423 linked to infection strategies across emerging pathogenic chytrid fungi." Nat
424 Commun **8**: 14742.
425 17. Farrer, R. A., L. A. Weinert, J. Bielby, T. W. J. Garner, F. Balloux, F. Clare, J. Bosch, A. A.
426 Cunningham, C. Weldon, L. H. du Preez, L. Anderson, S. L. K. Pond, R. Shahar-Golan,
427 D. A. Henk and M. C. Fisher (2011). "Multiple emergences of genetically diverse
428 amphibian-infecting chytrids include a globalized hypervirulent recombinant
429 lineage." Proceedings of the National Academy of Sciences of the United States of
430 America **108**(46): 18732-18736.
431 18. Fellers, G. M., D. E. Green and J. E. Longcore (2001). "Oral chytridiomycosis in the
432 mountain yellow-legged frog (Rana muscosa)." Copeia(4): 945-953.

19. Fisher, M. C., J. Bosch, Z. Yin, D. A. Stead, J. Walker, L. Selway, A. J. P. Brown, L. A. Walker, N. A. R. Gow, J. E. Stajich and T. W. J. Garner (2009). "Proteomic and phenotypic profiling of the amphibian pathogen *Batrachochytrium dendrobatidis* shows that genotype is linked to virulence." Molecular Ecology **18**(3): 415-429.
20. Fisher, M. C. and T. W. J. Garner (2007). "The relationship between the introduction of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species." Fungal Biology Reviews **21**: 2-9.
21. Fisher, M. C., T. W. J. Garner and S. F. Walker (2009). "Global Emergence of *Batrachochytrium dendrobatidis* and Amphibian Chytridiomycosis in Space, Time, and Host." Annual Review of Microbiology **63**: 291-310.
22. Fisher, M. C., D. A. Henk, C. J. Briggs, J. S. Brownstein, L. C. Madoff, S. L. McCraw and S. J. Gurr (2012). "Emerging fungal threats to animal, plant and ecosystem health." Nature **484**(7393): 186-194.
23. Fisher, M. C., B. R. Schmidt, K. Henle, D. S. Schmeller, J. Bosch, D. M. Aanensen and T. J. W. Garner (2012). "RACE: Risk Assessment of Chytridiomycosis to European Amphibian Biodiversity. ." Froglog(101): 45-47.
24. Funk, W. C., M. A. Donnelly and K. R. Lips (2005). "Alternative views of amphibian toe-clipping." Nature **433**(7023): 193-193.
25. Garner, T. W., B. R. Schmidt, A. Martel, F. Pasmans, E. Muths, A. A. Cunningham, C. Weldon, M. C. Fisher and J. Bosch (2016). "Mitigating amphibian chytridiomycoses in nature." Philos Trans R Soc Lond B Biol Sci **371**(1709).
26. Garner, T. W., S. Walker, J. Bosch, S. Leech, M. Rowcliffe, A. A. Cunningham and M. C. Fisher (2009). "Life history trade-offs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*." Oikos(118): 783-791.
27. Gentz, E. J. (2007). "Medicine and surgery of amphibians." ILAR J (48): 255-259.
28. James, T. Y., A. Litvintseva, R. Vilgalys, J. A. Morgan, J. W. Taylor, M. C. Fisher, L. Berger, C. Weldon, L. H. du Preez and J. Longcore (2009). "Rapid expansion of an emerging fungal disease into declining and healthy amphibian populations." PLoS Pathogens **5**(5): e1000458.
29. James, T. Y., L. F. Toledo, D. Rodder, D. D. Leite, A. M. Belasen, C. M. Betancourt-Roman, T. S. Jenkinson, C. Soto-Azat, C. Lambertini, A. V. Longo, J. Ruggeri, J. P. Collins, P. A. Burrowes, K. R. Lips, K. R. Zamudio and J. E. Longcore (2015). "Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: lessons from the first 15years of amphibian chytridiomycosis research." Ecology and Evolution **5**(18): 4079-4097.
30. Jenkinson, T. S., C. M. B. Roman, C. Lambertini, A. Valencia-Aguilar, D. Rodriguez, C. H. L. Nunes-de-Almeida, J. Ruggeri, A. M. Belasen, D. D. Leite, K. R. Zamudio, J. E. Longcore, L. F. Toledo and T. Y. James (2016). "Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations." Molecular Ecology **25**(13): 2978-2996.
31. Laking, A. E., H. N. Ngo, F. Pasmans, A. Martel and T. T. Nguyen (2017). "*Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders." Scientific Reports **7**.
32. Longcore, J. E., A. P. Pessier and D. K. Nichols (1999). "*Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians." Mycologia **91**(2): 219-227.
33. Martel, A., A. Spitzen-van der Sluijs, M. Blooi, W. Bert, R. Ducatelle, M. C. Fisher, A. Woeltjes, W. Bosman, K. Chiers, F. Bossuyt and F. Pasmans (2013).

"*Batrachochytrium salamandrivorans* sp nov causes lethal chytridiomycosis in amphibians." Proceedings of the National Academy of Sciences of the United States of America **110**(38): 15325-15329.

34. May, R. M. (2004). "Ecology - Ethics and amphibians." Nature **431**(7007): 403-403.
35. McCarthy, M. A. and K. M. Parris (2004). "Clarifying the effect of toe clipping on frogs with Bayesian statistics." Journal of Applied Ecology **41**(4): 780-786.
36. Mendelson, J. R., 3rd, K. R. Lips, R. W. Gagliardo, G. B. Rabb, J. P. Collins, J. E. Diffendorfer, P. Daszak, D. R. Ibanez, K. C. Zippel, D. P. Lawson, K. M. Wright, S. N. Stuart, C. Gascon, H. R. da Silva, P. A. Burrowes, R. L. Joglar, E. La Marca, S. Lotters, L. H. du Preez, C. Weldon, A. Hyatt, J. V. Rodriguez-Mahecha, S. Hunt, H. Robertson, B. Lock, C. J. Raxworthy, D. R. Frost, R. C. Lacy, R. A. Alford, J. A. Campbell, G. Parra-Olea, F. Bolanos, J. J. Domingo, T. Halliday, J. B. Murphy, M. H. Wake, L. A. Coloma, S. L. Kuzmin, M. S. Price, K. M. Howell, M. Lau, R. Pethiyagoda, M. Boone, M. J. Lannoo, A. R. Blaustein, A. Dobson, R. A. Griffiths, M. L. Crump, D. B. Wake and E. D. Brodie, Jr. (2006). "Biodiversity. Confronting amphibian declines and extinctions." Science **313**(5783): 48.
37. Ott, J. A. and D. E. Scott (1999). "Effects of toe-clipping and PIT-tagging on growth and survival in metamorphic Ambystoma opacum." Journal of Herpetology **33**(2): 344-348.
38. Piotrowski, J. S., S. L. Annis and J. E. Longcore (2004). "Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians." Mycologia **96**(1): 9-15.
39. Ribas, L., M. S. Li, B. J. Doddington, J. Robert, J. A. Seidel, J. S. Kroll, L. B. Zimmerman, N. C. Grassly, T. W. J. Garner and M. C. Fisher (2009). "Expression Profiling the Temperature-Dependent Amphibian Response to Infection by *Batrachochytrium dendrobatidis*." PLoS One **4**(12): -.
40. Rosenblum, E. B., T. J. Poorten, S. Joneson and M. Settles (2012). "Substrate-Specific Gene Expression in *Batrachochytrium dendrobatidis*, the Chytrid Pathogen of Amphibians." Plos One **7**(11).
41. Rosenblum, E. B., T. J. Poorten, M. Settles and G. K. Murdoch (2012). "Only skin deep: shared genetic response to the deadly chytrid fungus in susceptible frog species." Molecular Ecology **21**(13): 3110-3120.
42. Schloegel, L. M., C. M. Ferreira, T. Y. James, M. Hipolito, J. E. Longcore, A. D. Hyatt, M. Yabsley, A. M. C. R. P. F. Martins, R. Mazzoni, A. J. Davies and P. Daszak (2010). "The North American bullfrog as a reservoir for the spread of *Batrachochytrium dendrobatidis* in Brazil." Animal Conservation **13**: 53-61.
43. Schloegel, L. M., A. M. Picco, A. M. Kilpatrick, A. J. Davies, A. D. Hyatt and P. Daszak (2009). "Magnitude of the US trade in amphibians and presence of *Batrachochytrium dendrobatidis* and ranavirus infection in imported North American bullfrogs (Rana catesbeiana)." Biological Conservation **142**(7): 1420-1426.
44. Schloegel, L. M., L. F. Toledo, J. E. Longcore, S. E. Greenspan, C. A. Vieira, M. Lee, S. Zhao, C. Wangen, C. M. Ferreira, M. Hipolito, A. J. Davies, C. A. Cuomo, P. Daszak and T. Y. James (2012). "Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade." Molecular Ecology **21**(21): 5162-5177.
45. Schmeller, D. S., M. Blooi, A. Martel, T. W. J. Garner, M. C. Fisher, F. Azemar, F. C. Clare, C. Leclerc, L. Jager, M. Guevara-Nieto, A. Loyau and F. Pasmans (2014). "Microscopic

Aquatic Predators Strongly Affect Infection Dynamics of a Globally Emerged Pathogen." Current Biology **24**(2): 176-180.

46. Schoch, C. L., K. A. Seifert, S. Huhndorf, V. Robert, J. L. Spouge, C. A. Levesque, W. Chen, E. Bolchacova, K. Voigt, P. W. Crous, A. N. Miller, M. J. Wingfield, M. C. Aime, K. D. An, F. Y. Bai, R. W. Barreto, D. Begerow, M. J. Bergeron, M. Blackwell, T. Boekhout, M. Bogale, N. Boonyuen, A. R. Burgaz, B. Buyck, L. Cai, Q. Cai, G. Cardinali, P. Chaverri, B. J. Coppins, A. Crespo, P. Cubas, C. Cummings, U. Damm, Z. W. de Beer, G. S. de Hoog, R. Del-Prado, D. B, J. Dieguez-Uribeondo, P. K. Divakar, B. Douglas, M. Duenas, T. A. Duong, U. Eberhardt, J. E. Edwards, M. S. Elshahed, K. Fliegerova, M. Furtado, M. A. Garcia, Z. W. Ge, G. W. Griffith, K. Griffiths, J. Z. Groenewald, M. Groenewald, M. Grube, M. Gryzenhout, L. D. Guo, F. Hagen, S. Hambleton, R. C. Hamelin, K. Hansen, P. Harrold, G. Heller, G. Herrera, K. Hirayama, Y. Hirooka, H. M. Ho, K. Hoffmann, V. Hofstetter, F. Hognabba, P. M. Hollingsworth, S. B. Hong, K. Hosaka, J. Houbraken, K. Hughes, S. Huhtinen, K. D. Hyde, T. James, E. M. Johnson, J. E. Johnson, P. R. Johnston, E. B. Jones, L. J. Kelly, P. M. Kirk, D. G. Knapp, U. Koljalg, K. GM, C. P. Kurtzman, S. Landvik, S. D. Leavitt, A. S. Liggenstoffer, K. Liimatainen, L. Lombard, J. J. Luangsa-Ard, H. T. Lumbsch, H. Maganti, S. S. Maharachchikumbura, M. P. Martin, T. W. May, A. R. McTaggart, A. S. Methven, W. Meyer, J. M. Moncalvo, S. Mongkolsamrit, L. G. Nagy, R. H. Nilsson, T. Niskanen, I. Nyilasi, G. Okada, I. Okane, I. Olariaga, J. Otte, T. Papp, D. Park, T. Petkovits, R. Pino-Bodas, W. Quaedvlieg, H. A. Raja, D. Redecker, R. T, C. Ruibal, J. M. Sarmiento-Ramirez, I. Schmitt, A. Schussler, C. Shearer, K. Sotome, F. O. Stefani, S. Stenroos, B. Stielow, H. Stockinger, S. Suetrong, S. O. Suh, G. H. Sung, M. Suzuki, K. Tanaka, L. Tedersoo, M. T. Telleria, E. Tretter, W. A. Untereiner, H. Urbina, C. Vagvolgyi, A. Vialle, T. D. Vu, G. Walther, Q. M. Wang, Y. Wang, B. S. Weir, M. Weiss, M. M. White, J. Xu, R. Yahr, Z. L. Yang, A. Yurkov, J. C. Zamora, N. Zhang, W. Y. Zhuang, D. Schindel and F. B. Consortium (2012). "Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi." Proceedings of the National Academy of Sciences of the United States of America **109**(16): 6241-6246.
47. Skerratt, L. F., L. Berger, H. B. Hines, K. R. McDonald, D. Mendez and R. Speare (2008). "Survey protocol for detecting chytridiomycosis in all Australian frog populations." Diseases of Aquatic Organisms **80**(2): 85-94.
48. Smith, K. G. and C. Weldon (2007). "A Conceptual Framework for Detecting Oral Chytridiomycosis in Tadpoles." Copeia **4**: 1024-1028.
49. Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. Rodrigues, D. L. Fischman and R. W. Waller (2004). "Status and trends of amphibian declines and extinctions worldwide." Science **306**(5702): 1783-1786.
50. Swei, A., J. J. L. Rowley, D. Rodder, M. L. L. Diesmos, A. C. Diesmos, C. J. Briggs, R. Brown, T. T. Cao, T. L. Cheng, R. A. Chong, B. Han, J. M. Hero, H. D. Hoang, M. D. Kusrini, T. T. L. Duong, J. A. McGuire, M. Meegaskumbura, M. S. Min, D. G. Mulcahy, T. Neang, S. Phimmachak, D. Q. Rao, N. M. Reeder, S. D. Schoville, N. Sivongxay, N. Srei, M. Stock, B. L. Stuart, L. S. Torres, T. A. T. Dao, T. S. Tunstall, D. Vieites and V. T. Vredenburg (2011). "Is Chytridiomycosis an Emerging Infectious Disease in Asia?" Plos One **6**(8).
51. Torreilles, S. L., D. E. McClure and S. L. Green (2009). "Evaluation and Refinement of Euthanasia Methods for *Xenopus laevis*." Journal of the American Association for Laboratory Animal Science **48**(5): 512-516.

52. Ulmar Grafe, T., M. M. Stewart, K. P. Lampert and M.-O. Rödel (2011). "Putting Toe Clipping into Perspective: A Viable Method for Marking Anurans." Journal of Herpetology **45**(1): 28-35.
53. Voyles, J., L. Berger, S. Young, R. Speare, R. Webb, J. Warner, D. Rudd, R. Campbell and L. F. Skerratt (2007). "Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis." Diseases of Aquatic Organisms **77**(2): 113-118.
54. Walker, S., J. Bosch, T. Y. James, A. Litvintseva, J. A. O. Valls, S. Pina, G. Garcia, G. Abadie-Rosa, A. A. Cunningham, S. Hole, R. Griffiths and M. C. Fisher (2008). "Invasive pathogens threaten species recovery programs." Current Biology **18**: R853-R854.
55. Webb, R., L. Berger, D. Mendez and R. Speare (2005). "MS-222 (tricaine methane sulfonate) does not kill the amphibian chytrid fungus *Batrachochytrium dendrobatidis*." Diseases of Aquatic Organisms **68**(1): 89-90.

588 **TABLE 1.** Non-lethal isolation of chytrids from adult and juvenile amphibians
589

Continent	Country	*n* Species[1]	*n* Sampled[2]	*n* Chytrid[3]	Chytrid species
Africa	Madagascar	2	145	2	*Kappamyces* sp.
	Cameroon	1	30	1	*B. dendrobatidis*
	Ethiopia	1	5	1	*B. dendrobatidis*
	South Africa	6	179	45	*B. dendrobatidis*
Asia	South Korea	2	28	10	*B. dendrobatidis*
	Taiwan	3	103	13	*B. dendrobatidis/ Kappamyces* sp.
Australia	Australia	1	2	2	*B. dendrobatidis*
Europe	Belgium	1	11	2	*B. dendrobatidis*
	France	2	261	70	*B. dendrobatidis*
	Hungary	1	15	3	*B. dendrobatidis*
	Italy	1	14	4	*B. dendrobatidis*
	Portugal	1	5	1	*Rhizophydium* sp.
	Spain	4	198	37	*B. dendrobatidis*
	Sweden	1	23	5	*B. dendrobatidis*
	Switzerland	1	30	1	*B. dendrobatidis*
	UK	4	50	8	*B. dendrobatidis*
South America	Chile	1	10	1	*B. dendrobatidis*
	French Guiana	2	66	2	*B. dendrobatidis*
Trade	n/a	4	15	5	*B. dendrobatidis*

590
591 [1]Number of amphibian species sampled, [2]total numbers of amphibians sampled, [3]number of
592 chytrids isolated
593
594

595 **TABLE 2.** Isolation of *Batrachochytrium dendrobatidis* from mouthparts of larval
596 amphibians
597

Continent	Country	Host species	Larvae sampled	*Bd* isolates
Africa	Ethiopia	1	36	1
	Uganda	1	20	1
	South Africa	2	88	11
Asia	Taiwan	1	15	1
Australia	Australia	8	54	33
Europe	Belgium	2	2	2
	Netherlands	1	1	1
	France	1	138	38
	Germany	1	10	4
	Spain	3	19	7
	Switzerland	1	42	15
South America	Chile	2	28	4
	Brazil	17	353	217

598
599

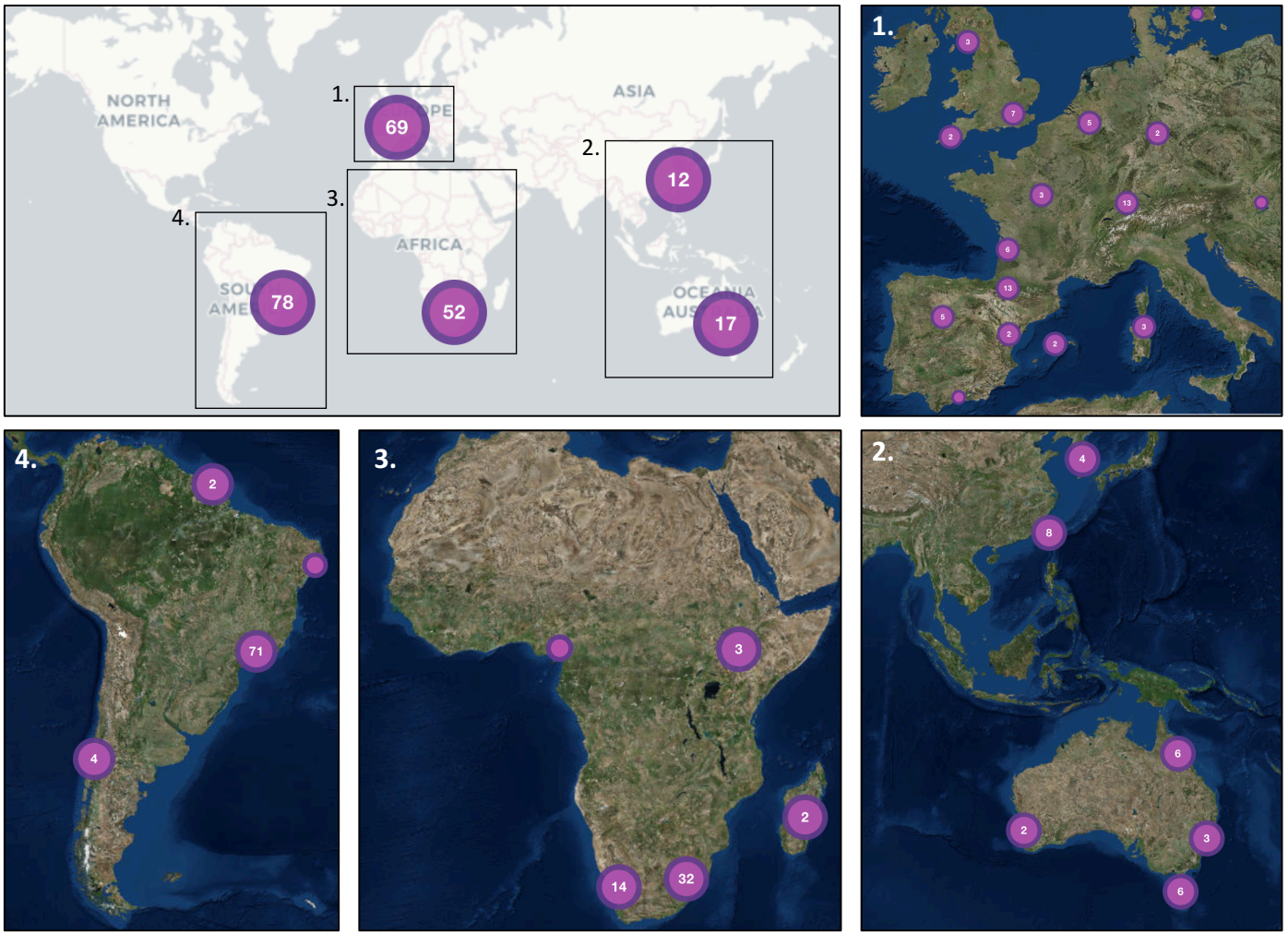

Figure 1. Worldwide distribution of sites where the *RML* Longcore protocol has been used to isolate chytrids. Numbers denote the quantity of amphibian species investigated. A browseable version of this *Epicollect 5* map can be accessed at https://five.epicollect.net/project/bd-global-isolation-protocol