Emerging Infectious Disease and the Trade in Amphibians

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Abstract

Amphibians are the most threatened class of vertebrate, and rates of species decline and extinction far exceed those seen historically. Habitat loss, climate change, over-exploitation and emerging infectious disease have all been identified as threatening processes. The trade in amphibians has been implicated in over-exploitation through the harvesting of wild animals, and as an important pathway for the global spread of the fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*). However, there are no analyses of how *Bd* may spread through the trade chain. This thesis addresses this issue by (1) determining the prevalence of *Bd* at different stages of the trade chain; (2) examining knowledge, husbandry protocols and biosecurity among retailers and (3) assessing the risk of *Bd* dissemination into the wild in the UK.

Approximately 20,000 amphibians from at least 11 countries enter the UK annually via Heathrow Animal Reception Centre. Overall *Bd* prevalence was 3.6%, but was confined to six of the 43 genera encountered, and only detected in shipments from the USA and Tanzania. Amphibians were sold by 30% of the estimated 3500 livestock retailers in the UK, but made a low contribution to overall income. Disease awareness and knowledge in retailers was found to be lacking, although husbandry standards were deemed to be appropriate. Mortality appeared to be influenced by restocking methods and number of species held, but mass die-offs as a result of disease were generally uncommon. Screening of over 2000 amphibians from 148 retailers for *Bd* revealed a prevalence of 5.8%, but the geographic distribution of infection in the UK was patchy, and was more prominent in aquatic species. A risk assessment conducted according to the framework set out by the World Organisation of Animal Health, identified regions and sections of the trade that pose the greatest threats in terms of introducing *Bd*, and assessed various mitigation measures. The consequences of novel strains of *Bd* and a second, recently discovered *Batrachochytrium* species were found to pose a risk to both native UK and captive amphibians. As trade bans are unlikely to be feasible or effective, alternative measures to mitigate the impact of disease are evaluated.

Keywords: Amphibian trade, pet trade, *Batrachochytrium dendrobatidis*, disease risk assessment, import.

Declaration

The work presented in this thesis is an original contribution by the author, with the following acknowledgments.

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Bd-maps data used in Chapter 5 are freely accessible on-line (www.*Bd*-maps.net), these data are collated by the *Bd*-Maps team based at Imperial College London.

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CHAPTER 1

Introduction: Trade, pet frogs and pathogenic fungus

A Comparison Between the Young Lady and the Bullfrogs. She is holding Female in one hand the Male in the other.

Image from: http://williamgibsonblog.blogspot.co.uk/2009_03_01_archive.html

Broel, A. (1937) *Frog Raising for Pleasure and Profit*. Marlboro House, New Orleans.

Albert Broel was the founder of the American Frog Canning Company.

The trade in wildlife

Wildlife trade has been defined as "the sale and exchange by people of wild animal and plant resources" (Broad et al., 2003). This activity has co-evolved with human societies and has developed from simple transactions between local communities to comprise the complex, and often intertwined network of local, national, and international trade routes that occur today. A major development in the scale of trade coincided with the Industrial Revolution (c.1750-1850), as rapid innovations in transport facilitated global travel (Findlay and O'Rourke, 2007), and continuing advancements in technology promote sustained growth. It is unclear when the detrimental effects of trade in wildlife on natural ecosystems first became apparent, but wildlife trade had certainly become prominent in the literature by the early 1970s. Indeed, in 1974 the International Zoo Yearbook had devoted a section of the publication to "Trade and Transport of Animals" (ZSL 1974 Volume 14). Motivations for the trade in wildlife are almost as diverse as the vast number of species and their derivatives involved; and include, food and medicines, fuel and construction materials, to ornaments, religious icons and pets. Whilst an accurate economic valuation is unattainable, the industry is estimated to be worth several billion dollars per annum (Broad et al., 2003, Engler and Parry-Jones, 2007, Smith et al., 2009a). There is also a notable illegal component to the trade, but given its clandestine nature, it is impossible to quantify the volume or economic value, but rough estimates allude to figures of between \$5-\$20 billion dollars per annum (Rosen and Smith, 2010).

- *Conservation concerns*

The negative effects of trade require consideration in the wider context of conservation, through the understanding that this activity is one of several pressures including, habitat loss, climate change, and pollution, all interacting on the world's biodiversity (Nijman and Shepherd, 2010). Additionally, a significant proportion of natural resources are traded at local and national, rather than international level (Broad et al., 2003), and may constitute a vital livelihood component. The most apparent conservation impact of wildlife trade is the depletion of natural populations through over-exploitation, which has commanded significant research in recent years (e.g. Broad et al., 2003, Adams, 2004, Nijman and Shepherd, 2007, Zhang et al., 2008, Natusch and Lyons, 2012, Herrel and van der Meijden, 2014, Li and Jiang, 2014). The first documented extinction to occur due to over-harvesting and trade, was that of the medicinal Silphion plant (Fig. 1.1) approximately 2000 years ago, recorded by Pliny the Elder (23-79AD) (Kiehn, 2007). More recently the passenger pigeon (*Ectopistes migratorius*), one of the most abundant birds on earth, was hunted to extinction in the USA over the course of a few decades in the 1800s (Johnson et al., 2010). Today, the threat of extinction is very real for a large number of species. These include several species of sturgeon (Order Acipenseriformes) hunted for caviar (Pikitch et al., 2005); hyacinth macaw (*Anodorhynchus hyacinthinus*) illegally collected for the pet trade (Herrera and Hennessey, 2007); tiger (*Panthera tigris*) for use in traditional medicines, amongst others (Morell, 2007); Asian turtle species taken largely for the pet trade (Nijman and Shepherd, 2007, Lyons et al., 2013) and pangolins (e.g. *Manis pentadactyla*) hunted for food and medicinal purposes (Newton et al., 2008). Over-harvesting of some species is also likely to have wider implications such as ecosystem function disruption (Morris, 2010), although specific examples are scarce. In addition to direct biological impacts, the trade in wildlife is associated with indirect factors such as the introduction of non-native species and the dissemination of novel pathogens into naïve areas; which are discussed later, specifically in relation to the amphibian trade.

Fig. 1.1. An ancient coin (Cyrenaic tetradrachm 435 – 375 B.C.) depicting a silphion plant. The plant was the principle trade commodity of Cyrenaica (Libya) for over 200 years, and was prized for its medicinal properties, until it became extinct through over-exploitation. The silphion plant has not been assigned a scientific name, and there is still much debate about its taxonomy.

(Picture and text reference: Kiehn 2007)

- *Wildlife trade regulation*

Global biodiversity is protected at various hierarchical levels, by numerous conventions and legislation. Of pertinence to international wildlife trade is the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). CITES came into force in 1975 with the objective to regulate trade in species which are under threat from over-exploitation, through international co-operation to control of trade volumes using a licensing system (CITES, 2014). Currently, 180 countries have ratified the Convention, and there are over 35000 species (≈5600 animal and 30000 plant) listed under CITES with varying degrees of protection (CITES, 2014).

In addition to CITES protection afforded to particularly vulnerable species, in some cases national or local export quotas exist to prevent over-harvesting. However, the quota setting process may be based on arbitrary figures rather than rigorous data, and implementation and enforcement is rarely adequate (Schoppe, 2009, Natusch and Lyons, 2012, Nijman et al., 2012).

- *UK Legislation*

The UK ratified CITES in 1976 (CITES, 2014). Today, CITES administration falls under the remit of two Management Authorities, the Department for Environment, Food and Rural Affairs (DEFRA), and Animal Health and Veterinary Laboratories Agency (AHVLA), and enforcement duties lie with HM Customs and Revenue, the Border Agency and the police (JNCC, 2014). Legislation for CITES is provided by the European Regulation - EU Council Regulation (EC) no 338/97 and enforced in UK law under 'The Control of Trade in Endangered Species (Enforcement) Regulations 1997 (COTES)'.

The Wildlife and Countryside Act 1981 provides the regulatory framework to implement the Bern Convention (Convention on the Conservation of European Wildlife and Natural Habitats), in the UK. Under this Act, certain native species are protected from trade, and the release of non-native species into the environment is prohibited.

Amphibians

Amphibians represent one of the most diverse vertebrate taxa, comprising three orders; Anura (frogs and toads), Caudata (salamanders and newts) and Gymnophiona (caecilians), and a total of 7312 described species (http://amphibianweb.org; accessed 3/9/14). Amphibians have a widespread global distribution, predominating in the tropics (Fig 1.2) (Halliday, 2008), but are absent in the polar regions. The IUCN global amphibian assessment 2004, revealed approximately a third of all species were threatened (Stuart et al., 2004), and, with extinction rates are as high as 200 times the background rate (McCallum, 2007, Roelants et al., 2007), the situation, together with the general patterns of biodiversity loss, has been described as “the Earth’s sixth mass extinction event” (Wake and Vredenburg, 2008).

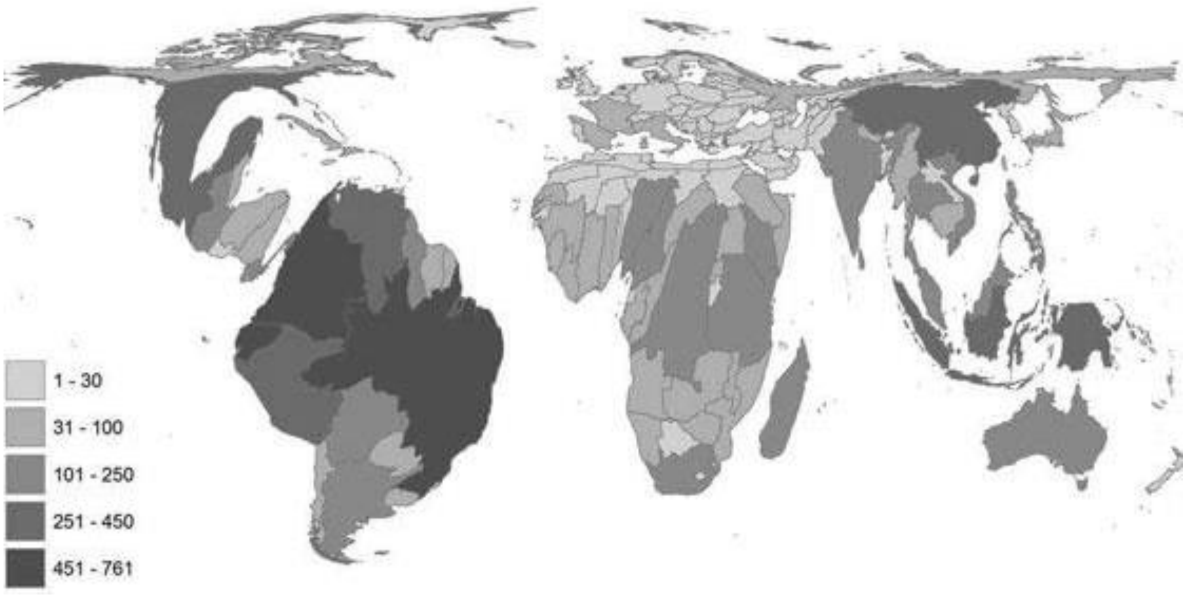


Fig 1.2. Global amphibian diversity illustrated using a diffusion-based cartogram. Geographical boundaries are distorted so the area of the countries represents the diversity of amphibian fauna found there. Figure from: Koo, M.S., Vredenburg, V.T., Gross, J., Spencer, C.L., Tunstall, T., Wake, D.B. "Visualizing AmphibiaWeb Data with Continuous Cartograms" AmphibiaWeb: Information on amphibian biology and conservation. [web application]. 2013. Berkeley, California: AmphibiaWeb. Available: http://amphibiaweb.org/.

- *Threats to amphibians*

Amphibians are facing multiple anthropogenic threats in addition to the 'natural stresses' experienced such as predation, reproduction and competition for resources (Blaustein et al., 2011). Habitat destruction occurs on many levels, from filling in ponds (Beebee, 2014), conversion of land for agriculture, construction for urban development, wetland drainage, to logging and deforestation (Todd et al., 2014), and is deemed to be the greatest overall threat to amphibian diversity (Collins and Storfer, 2003, Beebee and Griffiths, 2005, Gallant et al., 2007). Pollutants such as pesticides, industrial effluents or other environmental contaminants are hypothesised to have a particular impact on amphibians due to their permeable skin, which has a vital function in osmoregulation and gaseous exchange (Hopkins, 2007). Although the sensitivity of amphibians to such pollutants has been disputed (Kerby et al., 2010). Globally, the effects of pollution are likely to be underestimated, as, in both laboratory and field investigations there are heavy biases towards a select number of northern hemisphere species, preventing an accurate assessment of the situation elsewhere such as the tropics (Schiesari et al., 2007). Amphibians are also exploited commercially for a variety of purposes (discussed later). Despite the evolution of life histories that can tolerate high mortality rates, amphibians may suffer population declines due to over-harvesting if the off-take is of high intensity or for prolonged time periods (Mohneke et al., 2009). The introduction of non-native species to amphibian habitats occurs via accidental escapes of pet or laboratory animals, the intentional release for farming or to stock fisheries, or for ornamental purposes. Predation is the most straightforward threat to native species from alien introductions (Collins and Storfer, 2003). However, there a suite of more subtle impacts such as increased competition for resources (food, shelter), and the transmission of infectious disease.

The early 1990's saw an upsurge in amphibian research, largely due to the discovery of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) and its links to dramatic amphibian population declines and extinctions. Of particular interest was the mechanism by which the pathogen was spread, and two main hypotheses were contested. The endemic pathogen hypothesis (EPH), proposed that *Bd* was endemic in the environment, but had undergone an increase in virulence mediated by changing environmental factors. The novel pathogen hypothesis (NPH), explained the observed patterns of mortality by the recent introduction of the new *Bd* pathogen into previously uninfected areas with naïve amphibian populations. Both hypotheses have been comprehensively reviewed by Rachowicz et al. (2005). Fundamental to the NHP, is the movement of *Bd* through anthropogenic mechanisms, such as via amphibian trade, or the translocation of contaminated fomites or water; discussed in detail later.

Anthropogenic activities such as habitat destruction, pollution and biologic resource exploitation have undoubtedly intensified the impacts of disease on ecosystems worldwide (Aguirre and Tabor, 2008). Interactions with other factors such as climate change remain controversial (Rohr and Raffel, 2010), but, there appears to be a general consensus among amphibian researchers that the continuing population declines and extinctions are highly context-dependent and likely to be the result of a complex interactions between multiple factors (Green, 2003, Blaustein et al., 2011).

The uses of amphibians and trade

The importance of amphibians to the functioning of natural ecosystems has been widely recognised (e.g. Brodman et al., 2006 and references therein, Whiles et al., 2006, Beebee, 2014), in addition to which they have been utilised by humans for centuries, appearing in folklore and ancient traditions for a number of cultures around the world. Amphibians have been used in scientific research since the concept of 'science' itself. Aristotle used frogs to demonstrate his hypotheses on the production of sound, in around 4BC (Smith and Stoskopf, 2007), as models in ecological studies (Hopkins, 2007). More recently amphibians have been used in basic anatomy and physiology teaching at schools and university (Tyler et al., 2007), are still important biomedical model organisms (O'Rourke, 2007). The number of uses of the amphibian skin secretions in molecular studies is large (Pukala et al., 2006), and therapeutic properties have long been recognised in traditional medicines. Recent analyses have discovered a number of peptides with antimicrobial properties (Jin et al., 2009) that aid in wound healing (Mashreghi et al., 2013). Secretions from the White's treefrog (*Litoria caerulea*) not only contain some mosquito repellent compounds (Williams et al., 2006), but are effective analgesics (Tyler et al., 2007), and have even been demonstrated to have an inhibitory effect on HIV (Van Compernolle et al., 2005). The giant leaf frog (*Phyllomedusa bicolor*), as well as providing Amazonian tribes with potent skin extracts that result in state of euphoria or "hunting magic" (Daly et al., 1992), produces the peptide Dermaseptin B2. This exhibits antitumor properties by inhibiting proliferation of certain tumour cell types, showing potential for cancer treatment (van Zoggel et al., 2012). Skin secretions have also been used by ‘Western’ recreational drug users, although not always intentionally or successfully (see: Kostakis and Byard (2009) “Sudden death associated with intravenous injection of toad extract”).

Secretions from various frogs of the genus *Notaden* (Fig 1.3) have been found to have powerful adhesive properties, capable of bonding metals, plastics and biological tissues even when the surfaces are damp (Graham et al., 2010, 2013), showing potential future use in human orthopaedic surgery (Millar et al., 2009).

Fig. 1.3. *Notoden bennetti*: the skin secretions of this amphibian have powerful adhesive properties. (http://www.flickr.com/photos/12626159@N05/5050909052)

Perhaps the best known medical application is the use of African clawed frogs (*Xenopus laevis*) as a pregnancy test assay in the mid-1900’s (Gurdon and Hopwood, 2000); a practice that contributed to the spread of *X. laevis* around the globe (Weldon et al., 2004). Whilst more convenient methods have been developed for pregnancy testing, *Xenopus* sp., are still widely traded internationally for other scientific research today.

Perhaps the most familiar use of amphibians is as a food source, typically as frogs’ legs (Jensen and Camp, 2003). The consumption of frogs’ legs occurs all over the world

(Mohneke et al., 2009) and the trade in this commodity is significant at both local and international scales (Warkentin et al., 2009).

Prior to 1987 the international demand for frogs' legs was met almost exclusively by exports of wild caught amphibians from India, but the huge quantities harvested led to population decline and serious concern as early as 1985 (Abdulali, 1985, Niekisch, 1986). As a result India banned the commercial killing of frogs and export of frogs' legs in 1987 (Jensen and Camp, 2003, Anon, 1987), although illegal trade perpetuates in certain regions (Ahmed, 2011). Today, Indonesia and China are now the primary exporters of frogs' legs for the international market (Kusrini and Alford, 2006, Warkentin et al., 2009). Data on the volume of the frogs' leg trade are virtually impossible to attain, but estimates of exports out of Indonesia to the EU fell between 94 and 235 million frogs for 1993 (Veith et al., 2000). The EU and USA continue to import thousands of tonnes of frogs' legs each year (Jensen and Camp, 2003), the majority of which is thought to be wild caught: and a recent study in Indonesia reports that farming of *Lithobates (Rana) catesbeianus*, is decreasing due to "high maintenance costs and vulnerability of the species to disease", suggesting that future exports will continue to consist of wild caught animals (Kusrini and Alford, 2006). However, in response to an ever increasing demand for frog meat, especially from Europe, intensive breeding of non-native species such *as L. catesbeianus* has now been established in a number of countries (Carpenter et al., 2007).

The third main amphibian trade sector is the pet trade, which will be considered in detail.

The pet trade

Although typically considered a hobby of Western societies, pet keeping has historically been practised by an array of cultures globally; Australian aborigines kept an assortment of animals from dingoes, cassowaries to frogs (Zeuner, 1963), Native Americans kept deer, wolves and crows amongst others (Galton, 1883), and certain Malay tribeswomen would even suckle the family monkey (Evans, 1937). A fascinating review of this phenomenon and explanation for its evolution is provided by Serpell (1989). Whilst it is hard to pin down the advent of pet keeping in the UK, the word 'pet' was first seen in the Oxford English Dictionary (OED) in 1539, where it referred to a hand reared lamb or other domestic animal. The definition with which we are more familiar “an animal (typically one which is domestic or tame) kept for pleasure or companionship” did not appear until later in 1710 (Oxford English Dictionary, 2014). Pet keeping was certainly in a form we would recognise at this time, and the early Georgian era (circa 1700s) also saw the coincidental emergence of formally identifiable ‘pet shops’ (Inglis, 2013). Even at this early stage ‘exotic’ animals were not only available to purchase but actively sought after, albeit mainly by the aristocracy and Georgian gentry (Plumb, 2010). The earliest record of a reptilian pet is that of a giant tortoise owned by the Archbishop of Canterbury William Laud in 1633 that resided in the garden of Lambeth Palace until the age of 120, when it was accidentally killed by a gardener (MacCulloch, 2005); an indication that reptiles are not a recent arrival on the pet scene.

In more recent times the pet trade as a whole has grown considerably, especially in Western cultures. Domestic dog and cat populations in the US have risen from approximately 61 and 75 million in 2001, to 76 and 85 million in 2012 respectively (Pet Food Pet Food Institute, 2013), and are currently estimated at 9 million (dogs) and 8 million (cats) in the UK (Pet Food Manufacturer's Association, 2014). Accurate figures for reptiles and amphibians are far harder to obtain, but the Humane Society of the United States claims more than 13 million reptiles were kept as pets in 2009 in the US (The Humane Society of the United States, 2009). In the UK the popularity of keeping exotics, specifically reptiles and amphibians, escalated rapidly from the 1970s (Newman, C. pers comm), with the number of species available increasing from 248 in 1992-3 to 526 in 2004-5 (Tapley et al., 2011). Latest estimates by the Federation of British Herpetologists (FBH) report that 1.2 million UK households were home to over seven million reptiles in 2008 (Federation of British Herpetologists, 2012). Today it is not unusual to see bearded-dragons (*Pogona vitticeps*), corn snakes (*Pantherophis guttatus*) and White’s treefrogs (*L. caerulea*) in unspecialised pet shops and garden centres, with many having the capacity to obtain a far wider variety of species ‘to order’. Specialised reptile shops that routinely stock a far greater range of reptile and amphibian species, along with their often complex housing requirements, can now be found in most UK towns. Despite the requirement for a local authority issued licence in order to operate as a pet shop, the volume and species for sale at these premises is largely unknown as this information is generally not required for such permits.

All native herpetological fauna in the UK is protected by law under the Wildlife and Countryside Act 1981 that makes it an offence to kill, injure, own or sell them. With the small number traded under licence excluded, the UK herpetological trade is therefore comprised exclusively of non-native species. The acquisition of stock for this market therefore results in, along with ethical and welfare concerns (reviewed briefly in Box 1), a number of potential conservation issues for both the UK and exporting countries.

Box 1: *A brief summary of the ethical and welfare concerns regarding the trade in live reptiles and amphibians.*

The herpetological trade has long been at the centre of fiercely contested debates between different independent and governmental groups. On one side opponents argue that the trade is largely comprised of wild caught animals that endure compromised welfare, both pre and post retail, due to inadequate transport facilities and an inability of hobbyists to provide suitable living conditions (RSPCA, 2002, Laidlaw, 2005). Many believe that the removal from their natural environment and treatment of live animals as commodities is simply ethically unjust, and have concerns regarding the potential for transmission of zoonotic diseases. On the other hand, proponents maintain that sustainable utilisation of wildlife has conservation benefits (EUARK 2012), and welfare standards within the trade are necessarily high, as poor survivorship would jeopardise economic feasibility (C. Newman, pers. comm.). It is beyond the scope of this study to investigate the ethical and welfare matters surrounding the trade in detail, but they are inherently linked with conservation aspects so should be considered if amendments to regulations are made.

Harvesting of wild animals can lead to population declines through over-exploitation (as discussed earlier). Of particular relevance to the pet trade, is the potential for specific populations to be at greater risk of depletion where the value of certain species or ‘morphs’ is disproportionally inflated due to perceived rarity therefore increasing demand (Lyons and Natusch, 2013). Newly described species have also been shown to be at risk of severe over-exploitation (Stuart et al., 2004), as a ‘novelty value’ commands high retail prices. This effect has been demonstrated by the rapid depletion of Lao newt (*Laotriton laoensis*), shortly after its discovery (Phimmachak et al., 2012). Although traditional economic models predict that as a species becomes rarer it becomes more difficult to harvest, species extinctions are unlikely: a recent hypothesis - the Anthropogenic Allee Effect (AAE) predicts that consumers will prize rarer species thus putting a disproportionate value on them (Courtchamp et al., 2006). This leads to a shift in equilibrium for hunting/collecting pressure, putting these species as risk. Lyons and Natusch (2013) showed this to be the case, in a study on the harvests and consumer preference of green pythons (*Morelia viridis*), but also revealed that abundance is not the only factor associated with rarity but anomalies in colour type or time since discovery, can also be important attributes. Additional concerns have been raised regarding the possibility of genetic problems of selective over-harvesting (Fenberg and Roy, 2008, Allendorf et al., 2008).

More 'common' species are also susceptible to declines if subjected to persistent harvesting over time (Lyons and Natusch, 2011). Post-import, alien species can damage local ecosystems through: direct predation or competition with native species, or genetic disruption of populations through hybridisation (Sousa et al., 2009, Senn et al., 2010, Lillo et al., 2011, Scott et al., 2012). Krysko et al. (2011) reported 84% of the 137 non-indigenous reptile and amphibian species in Florida were attributed to the pet trade introduction pathway (although, the vast majority of these were reptiles and only three amphibian species had become established). Despite it being an offence in the UK to "release or allow the escape of any animal that is not ordinarily resident in or a regular visitor to Great Britain" (Wildlife and Countryside Act 1981), it is estimated that 50 non-indigenous vertebrate species (15 bird, 17 mammal, 8 amphibian, 3 reptile and 7 fish) are currently established in the UK (Roy et al., 2012). As a significant proportion of these have been introduced through 'ornamental' pathways including the pet trade (Roy et al., 2012), strategies aiming to block these pathways require investigation. The introduction of non-indigenous species has also been implicated in the dissemination of disease into native populations, a process referred to as pathogen pollution (Cunningham et al., 2003). Amphibians have been somewhat overshadowed by reptiles in the literature pertaining to trade studies, but with the emergence of significant diseases such as chytridiomycosis and ranavirus, are starting to fall under intensified scrutiny. The first evidence of the chytridiomycosis in wild UK populations was reported in 2004 in populations of the non-indigenous American bullfrog (*L. catesbeianus*) in Kent (Cunningham et al., 2005), and subsequent studies revealed it to be fairly widespread across the UK (Cunningham and Minting, 2008, Smith, 2013).

- *Amphibians as pets*

Extracting information from the literature, specifically for the amphibian pet trade is challenging for a two reasons. Firstly, most studies on the pet trade investigate both amphibians and reptiles (Schlaepfer et al., 2005, Prestridge et al., 2011, Natusch and Lyons, 2012), but these typically have a strong focus on the latter, or, integrate data for the food and pet trades (Gilbert et al., 2012). Additionally, there is often an emphasis on CITES listed species (Nijman and Shepherd, 2011b), which compounds the problem as proportionally very few of the amphibian species in trade are listed. Nevertheless is it generally accepted that the trade in amphibians for pets is of considerable scale and that in contrast to the food trade, the amphibian pet trade involves a wide diversity of species (Prestridge et al., 2011, Natusch and Lyons, 2012, Tamukai et al., 2014, Chapter 3). The ability to accurately identify the origins of internationally transported amphibians is severely impeded through consignments being re-exported from intermediate countries (Nijman and Shepherd, 2010, 2011b), but the UK and USA certainly import stock from countries all over the globe (Prestridge et al., 2011, Chapter 2). There is little conclusive evidence of population declines caused directly by the collection of wild animals, although this is thought to be an immediate threat for a number of Malagasy species (Andreone et al., 2005). Equally, there is still very little known about the biology and population status of many of the species harvested for the pet trade, as many inhabit remote areas with poor accessibility (Natusch and Lyons, 2012). Globally, Southeast Asia is likely to be the most important source of wildlife (Sodhi et al., 2004, Nijman and Shepherd, 2010), especially Indonesia; but Tanzania and Ghana also appear to be major exporters of African species (Chapter 2). Approximately 20,000 native amphibians were collected in Florida to supply the pet trade in the early 1990s (Enge, 1993), but current data are unavailable. South America is renowned for the Dendrobatidae (poison dart frogs) which are extremely popular in the European herpetological trade (Gorzula, 1996). Consequently all dendrobatidis are listed on CITES Appendix II.

The trade has been implicated in the global dissemination of the fungal pathogen *Bd* (Fisher and Garner, 2007, Kriger and Hero, 2009), and the detection of *Bd* in UK (Cunningham et al., 2005, Peel et al., 2012, Chapter 4), USA (Schloegel et al., 2009) and Japanese amphibian retailers (Tamukai et al., 2014) supports this hypothesis. The pet trade has been, and will remain a contentious issue with some viewing it as an economically important industry (Gonwouo and Roedel, 2008) and others as "for the large part disposable….unnecessary, serving little benefit to society" (Kriger and Hero, 2009).

Frog Pots - *Batrachochytrium dendrobatidis*

Greek: βάτραχος (batracho) = frog, χύτρα (chytra) = earthen pot

- *Taxonomy and Life History*

Chytridiomycosis was first reported by Berger et al. (1998), and the fungal pathogen responsible, *Batrachochytrium dendrobatidis* (*Bd*), was subsequently formally described in 1999 (Longcore et al., 1999). Morphological characterisation ascribed the fungi to a novel genus within the order Chytridiales (Phylum Chytridiomycota, Class Chytridiomycetes). Chytrid fungi are known to parasitise organisms of other taxa including algae and plankton (James et al., 2006), and even the spores of other fungi (Hajek et al., 2013) and marine dinoflagellates (Lepelletiera et al., 2014). Until the recent discovery of *Batrachochytrium salamandrivorans* (Martel et al., 2013), *Bd* was the only known member of the phylum to infect vertebrates (Berger et al., 2005). Recent molecular research has revealed multiple genetically distinct *Bd* isolates (Farrer et al., 2011, Rosenblum et al., 2013, Rodriguez et al., 2014), that vary in virulence (Farrer et al., 2011, 2013). *Bd* infects the keratinised tissues of the amphibian epidermis, forming asexually reproducing zoosporangia that develop in unison with the skin cells (Berger et al., 2005). Prior to parasitising the epidermal cell, *Bd* exists as an aquatic, motile zoospore, which acts as the primary mechanism for dispersal. This highly specialised lifecycle (described in detail by Berger et al., 2005) exhibited by *Bd* suggests a long co-evolutionary history with amphibians. *Bd* has a fairly low an optimal temperature range of 17-23°C, and dies in cultures incubated at 30°C or above (Piotrowski et al., 2004).

- *Pathogenesis*

Bd is keratinophilic, and zoospores exclusively colonise keratinised epithelial cells of post-metamorphic amphibians, and tadpole mouthparts (Blaustein et al., 2005). The exact mechanism by which a zoospore enters an epithelial cell is unknown (Voyles et al., 2011). It has been suggested that nuclear material may enter via a germ tube produced by the zoospore (Longcore et al., 1999), but colonisation strategy may vary between hosts (Van Rooij et al., 2012). The onset of clinical disease and mortality is positively correlated with zoospore burden (Voyles et al., 2009), and is usually associated with abnormalities in epithelial appearance such as hyperkeratosis and excessive sloughing (Berger et al., 2005). Typical behavioural characteristics of severe infection include abnormal posture, anorexia, loss of righting-reflex (Voyles et al., 2009), and in some cases neurological signs (pers. obs.).

Enhanced expression of genes responsible for epithelial repair (Rosenblum et al., 2009), and evidence that *Bd* infection impairs the osmoregulatory function performed by amphibian skin through electrolyte transport disruption (Voyles et al., 2009), suggests epithelial dysfunction contributes to *Bd* pathogenicity. A consequence of this is circulatory cessation and asystolic cardiac arrest through hypokalaemia (Voyles et al., 2009). Fites et al. (2013), found evidence to suggest mature zoosporagia release a soluble mycotoxin capable of activating apoptotic processes in host lymphocytes, thus inhibiting an immune response. Structural changes to the epidermis, including apoptosis, necrosis and disruption to intercellular junctions have also been observed on exposure of amphibian skin to a supernatant of *Bd* in vitro, supporting hypotheses that *Bd* releases toxin or enzymatic substances (Brutyn et al., 2012). As a range of physiological systems are disrupted and fail prior to death, proximate causes of mortality are difficult to establish (Voyles et al., 2009).

- *Detection and diagnosis*

The most commonly utilised method for detection of *Bd* is the real-time polymerase chain reaction (RT-PCR) Taqman assay developed by Boyle et al. (2004) and (Hyatt et al., 2007). This method enables detection of *Bd* using the non-invasive swabbing technique described in Chapter 2. Recent developments to this protocol have included; the addition of bovine serum albumin (BSA) to the reaction, in order to reduce inhibition caused by contaminants (Garland et al., 2010), and the development of duplex methods to simultaneously detect *Bd* and *B. salamandrivorans* (Blooi et al., 2013). Other less widely used methods include analysis of gross pathology, histology, transmission electron-microscopy and antigen/antibody ELISA. These are reviewed in detail in the World Organisation for Animal Health's Aquatic Manual available on-line (Office International des Epizooties (OIE), 2014).

- *Treatment*

Whilst treatment of wild amphibian populations has been attempted (Woodhams et al., 2010), it was unsuccessful, and it is currently not believed to be a viable option (Buck et al., 2011). Other potential options for disease management in the natural environment are being explored, and include the use of zooplankton that consume the fungal zoospores (Buck et al., 2011). In-vitro, there are two primary treatment methods: firstly, the use of elevated temperatures (Chatfield and Richards-Zawacki, 2011), which was used successfully to clear infection from aquatic caecilians (Churgin et al., 2013), and secondly, the use of anti-fungal agents. The identification of the most effective anti-fungal has been the subject of considerable research (Berger et al., 2009, 2010, Martel et al., 2011, Woodhams et al., 2012). Several chemicals have been investigated, but despite concerns regarding incomplete pathogen clearance (Georoff et al., 2013) and its association with depigmentation of tadpoles (Garner et al., 2009a), itraconazole is by far the most common anti-fungal (Berger et al., 2010, Tamukai et al., 2011, Jones et al., 2012, Une et al., 2012, Brannelly, 2014), in current usage. Treatment typically involves bathing the animals in a 0.01% solution of itraconazole, for 10 minutes daily for 10 days (M. Rendle, pers. comm.).

- *Geographic and taxonomic distribution*

Bd-Maps (www.*Bd*-maps.net) is an on-line, fully accessible database, that provides the most comprehensive description of the global distribution of *Bd*. This wide-reaching community project, has collated the results of *Bd* investigations to reveal the pathogen has a worldwide, but heterogeneous distribution (Fig 1.4, Olson et al., 2013). There have been numerous studies that have used ecological and species distribution modelling techniques to predict the distribution of *Bd* (Ron, 2005, Murray et al., 2011), and revealed temperature, (Muths and Hero, 2010), precipitation (Ron, 2005), and high elevations in the tropics (Kriger and Hero, 2007), to be important factors. However, evidence suggesting the underestimation of *Bd* range using these methods (Puschendorf et al., 2013), highlights the need for continued surveillance.

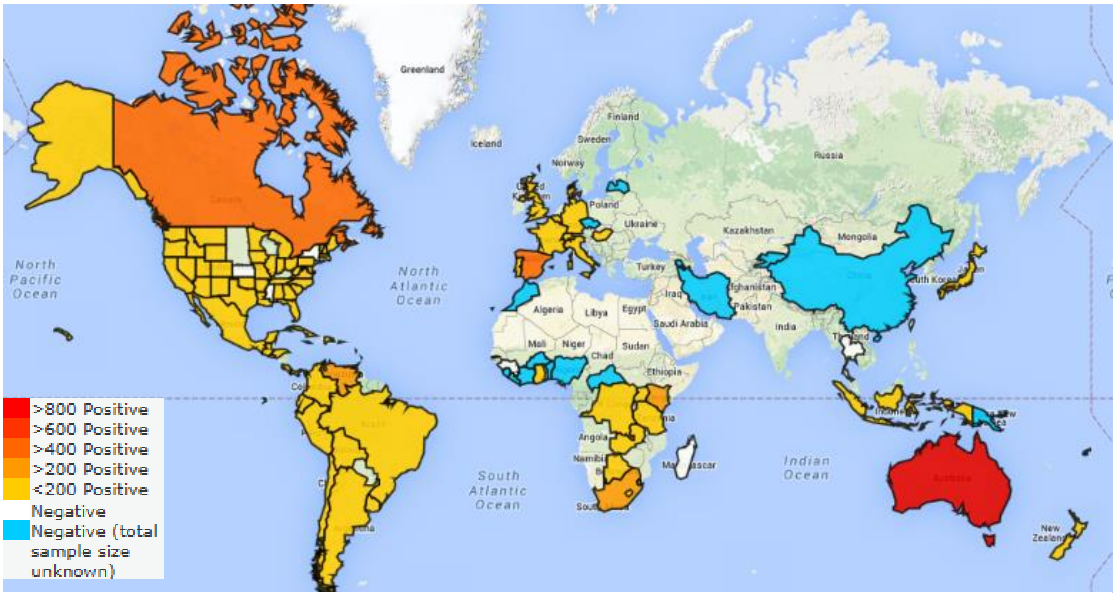


Fig. 1.4. Current global distribution of *Bd* represented by the number of reported positive samples. Screenshot from *Bd*Maps website: www.bdmaps.net accessed 14/01/15.

Bd has been detected in hundreds of species, giving it an unusually broad host-range (Olson et al., 2013), although there are marked inconsistencies in susceptibility to infection and the development of clinical chytridiomycosis between species (Garner et al., 2006, Bancroft et al., 2011, Searle et al., 2011), and between individuals of the same species (Hanselmann et al., 2004). *Bd* can also be present in some species, but not in others at the same site (Zampiglia et al., 2013). The American bullfrog (*L. catesbeianus*), and African clawed frog (*X. laevis*), are among a selection of species that are asymptomatic carriers of *Bd* (Weldon et al., 2004, Garner et al., 2006), that have been implicated in the pathogens global dissemination.

- *The spread of Bd*

Two primary mechanisms exist for spread of *Bd*: (1) movement of infected amphibians into uninfected areas, described as 'vector dissemination' and, (2) transport of *Bd* in water or substrate, or on contaminated fomites, 'mechanical dissemination'.

It is plausible that infected amphibians could introduce *Bd* into previously uninfected areas through natural movements or dispersal, which is relevant at a local scale. Pathogen dissemination on a much wider scale is possible through anthropogenically mediated vector spread, by the movement of infected amphibians via national and international trade for food, scientific purposes and exotic pets (discussed throughout this thesis). Approximately 10000 tonnes of frogs' legs, equating to between 100 and 400 million individuals, are transported around the world each year for human consumption (Gratwicke et al., 2009), and *Bd* has been detected in amphibians destined for the food trade, both on amphibian farming establishments (Mazzoni et al., 2003) and imported consignments and local markets (Schloegel et al., 2009). Whilst the movement of live animals presents a risk of *Bd* dissemination, it is difficult to ascertain the risk of the food trade as a whole, as a proportion of the trade consists of pre-prepared (skinned and frozen) product. *Bd* exclusively colonises epithelial tissue and cannot survive freezing (Gleason et al., 2008), therefore the risk of dissemination through transporting skinned, frozen frogs' legs is considered low (Gratwicke et al., 2009). Weldon et al. (2004) proposed the trade in *Xenopus* toads (historically used as pregnancy assays), as a primary mechanism for the global spread of *Bd*. Amphibians are still widely traded for scientific purposes and *Bd* has been detected in research facilities in the UK (Wombwell, unpublished data). The extent to which dissemination to wild populations occurs is highly dependent on biosecurity measures adopted by the industry, which is largely unknown but likely to be satisfactory.

Movement of live amphibians also occurs for reintroduction programmes for conservation. Indeed, *Bd* was inadvertently disseminated to a native *Alytes muletensis* (Mallorcan midwife toad) population through the release of infected captive bred individuals, with the intention to restock the declining natural population (Walker et al., 2008). Screening for *Bd* prior to release (e.g. Daly et al., 2008), is essential to reduce the likelihood of introducing *Bd* into new areas. While *Bd* screening is likely to be practised in most current reintroduction programmes given the high profile of the pathogen, the risk of introducing as yet unknown pathogens, remains a global concern.

Mechanical transfer can also occur through both anthropogenic and natural means. *Bd* can survive outside a host amphibian in water for up to 7 weeks (Johnson and Speare, 2003), and up to 3 months in sterile substrate (Johnson and Speare, 2005), thus could be unintentionally transferred to uninfected areas on muddy boots of tourists or researchers. Additionally, it has been proposed that water collected and transported by helicopters for extinguishing bush fires could be effective at introducing *Bd* to new areas (Webb et al., 2012), as has transfer on damp recreational equipment such as canoes and angling tackle (Anderson et al., 2014). The potential for pathogen transfer via such anthropogenic means is difficult to quantify, yet efforts to promote disease awareness and encourage appropriate biosecurity measures to people involved in these activities is required to reduce the probability of *Bd* spread. One dissemination route which seems impossible to mitigate is that of mechanical transfer through natural means such has on birds' feathers (Johnson and Speare, 2005) and feet (Garmyn et al., 2012). Identification of processes by which the pathogen is disseminated at both local and global scales is vital in order to investigate mitigation strategies and prevent further spread.

- *The regulation of disease spread through trade*

The movement of animals outside their natural range for trade is commonplace in nowadays, and the risks of inadvertently transporting their pathogens are increasingly being recognised. Indeed, it was the introduction of rinderpest into Europe in 1924 via the importation of cattle that prompted the foundation of the World Organisation for Animal Health (OIE) (MacDiarmid, 2011).

The OIE is an independent organisation that has a vital role in the development and implementation of sanitary standards for the international trade in animals and animal products (Lightner, 2012). These standards are enforced through the World Trade Organisation (WTO), who facilitate international trade through bilateral/multilateral trade agreements and monitor trade policies and procedures. Pathogens considered to pose a significant risk to trade are listed by the OIE as 'notifiable diseases', and countries party to the WTO are obliged to communicate their status regarding these pathogens, and establish appropriate mitigation strategies. *Bd* was listed as notifiable in 2008 (Schloegel et al., 2010), but there has been little evidence of enforced mitigation regulations in any country since.

Summary and Study Aims

There is a significant global trade in amphibians, primarily for food, but also for pets and scientific research, and the trade has been implicated in the spread of *Bd*. The pet trade is considered the primary reason amphibians are imported into the UK, but very little is known about the volume, species composition or disease status of the amphibians in this sector. This lack of information was the main motivation for conducting this research.

Chapter 2 reports the findings of a 30 month study conducted at Heathrow Animal Reception Centre. A consignment sampling protocol was implemented to investigate the *Bd* status of amphibians entering, and transiting through, the UK. Information pertaining to species and country of origin was collated, in order to identify potential patterns in infection status.

The pet trade in the UK is primarily regulated by local councils, and a lack of centralised database for UK pet shop licensing, renders establishing the scale of amphibian retail extremely difficult. In Chapter 3, a Freedom of Information request directed at local councils, together with a telephone and questionnaire survey, established an approximate volume for the trade, as well as determining the number of species available. An assessment of retailers' level of disease awareness and knowledge is made alongside a husbandry evaluation, to indicate the level of risk the industry posed with respect to *Bd* proliferation of dissemination in to wild populations.

The results of a nationwide *Bd* survey, conducted in amphibian retailers are presented in Chapter 4. Here, the overall proportion of infected amphibians is evaluated, and efforts to determine predictors of infection are made.

The results of the three previous chapters, together with a thorough literature review, are utilised to conduct a formal risk analysis for the introduction of *Bd* into the amphibian pet trade in the UK. The methods follow closely the framework set out by the OIE, which, despite being formulated around the trade in agricultural livestock, was found to be satisfactory for this purpose.

In the concluding chapter, a summary of the thesis is contextualised in terms of the international amphibian pet trade, and suggestions and recommendations are made on how the trade could adapt to mitigate disease risk in the future.

CHAPTER 2

Prevalence of *Batrachochytrium dendrobatidis* in amphibians imported into the UK for the pet trade

> Eo anno, infausta lues ovium surrepsit in Anglia, ut ubique repente ovilia, peste grassante, vacuarentur; quæ duravit sequentibus viginti octo annis, ita ut nulla totius regni villa hujus miseriæ clade careret. Causam hujus morbi, prius insuetam incolis, attribuebant multi cuidam diviti de Francorum partibus, qui applicuerat in Northumbriam, adducens secum quamdam ovem Hispaniæ morbidam, quæ totum gregem Angliæ morbi traductione contaminavit; quæ erat de [1] bimalis boviculi quantitate.

This year [in 1275] in England, an unfortunate bane of sheep spread rapidly through the holdings, the plague ravaging the flocks. It lasted twenty-eight years, so that no towns were spared of this misery. The cause of this disease has been unusual inhabitants, which they attributed to a rich Frenchman, who landed in Northumberland, bringing with him a diseased Spanish sheep that polluted all the flocks in England.

Riley, H.T. (Ed) 1863: Historica Anglicana Vol 1: 1272- 1381 by Thomas of Walsingham. Longman, Green, Longman, Roberts, Green. London.

Abstract

There is increasing evidence that the global spread of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been facilitated by the international trade in amphibians. *Bd* was first detected in the UK in 2004, and has since been detected in multiple wild amphibian populations. Amphibians are imported into the UK for the pet trade via Heathrow Animal Reception Centre (HARC), where *Bd* positive animals have been previously detected. Data on the volume, diversity and origin of imported amphibians were collected for 59 consignments arriving at HARC between November 2009 and June 2012, along with a surveillance study to investigate the prevalence of *Bd* in trade animals. Forty three amphibian genera were recorded originating from 12 countries. It was estimated that 5000 – 7000 amphibians are imported through HARC into the UK annually for the pet trade. *Bd* was detected in consignments from the USA and Tanzania, in six genera, resulting in an overall prevalence of 3.6%. This suggests that imported amphibians are a source of *Bd* within the pet trade.

Introduction

Wildlife has been utilised as a commercial resource for thousands of years, and what was primarily once a localised, subsistence activity is now an international, multi-billion dollar industry (Broad et al., 2003). Modern advances in transport, coupled with transnational commerce agreements has resulted in a vast trade network, and dramatically increased the efficiency of within and between country animal movements. Wildlife trade is economically important; perhaps more-so in developing countries where revenue generated from exports makes up a significant part of their GDP (Roe et al., 2002). However, translocation of wild animals (and domestic livestock) often poses significant threats through the facilitation of disease spread (Karesh et al., 2005, Fèvre et al., 2006). Many of these diseases are referred to as emerging infectious diseases (EIDs) due to their recent resurgence or rapid large scale spread (Daszak et al., 2000, 2001). Historically, research has focused on zoonotic EIDs or those that affect domestic livestock and until recently, the impacts on biodiversity have been largely overlooked (Daszak et al., 2000). Wildlife EIDs are now being recognised for their ability to cause population declines, local and global extinctions and subsequent ecosystem disruption (Daszak et al., 1999, Dobson and Foufopoulos, 2001). There have been a plethora of examples of epidemics caused by the movement of infected, non-native animals (Zepeda et al., 2001, Swift et al., 2007, Gummow, 2010, MacDiarmid, 2011 and references therein), often having economically and ecologically disastrous consequences. One such pathogen is the fungal agent *Batrachochytrium dendrobatidis* (*Bd*). Sustained research since the pathogens' discovery in 1998 (Longcore, 1999) continues to provide evidence that *Bd* is a major factor in the recent declines and extinctions of amphibian populations worldwide (Lips et al., 2005, Pounds et al., 2006, Skerratt et al., 2007, Catenazzi et al., 2014). The trade in live amphibians is widely considered a major mechanism for *Bd* dissemination (Fisher and Garner, 2007, Picco and Collins, 2008).

Amphibians are traded on a surprisingly large scale both geographically and economically. Schloegel et al. (2009) estimated that 5.07 million live amphibians are imported in to the US annually via three main ports. Amphibian uses vary widely from scientific research animals, to culinary delicacies, to pets and garden pond embellishments; all involving the translocation of large number of animals (Schlaepfer et al., 2005; Schloegel et al., 2009). The pet trade is a complex system which involves a wide range of both captive bred and wild caught species, originating from multiple countries, involving an estimated six million amphibians per year (OIE, 2006). Amphibians imported into the UK are primarily destined for the pet market and research industries, and approximately 85% of the recorded shipments are processed at Heathrow Animal Reception Centre (HARC) (R, Quest, pers. comm.). Amphibians are generally imported in either 'Reptile' or 'Fish' mixed taxa consignments or rarely as single taxa 'Amphibian' consignments (Peel et al., 2012). HARC is one of four UK border inspection posts (BIPs) licensed to handle amphibians, the others being Gatwick, Manchester and Edinburgh Airports. A recent study estimated that 130000 amphibians are imported into the UK annually (Peel et al., 2012). There has been growing concern regarding the disease risk of non-native amphibian imports to the UK's native amphibian fauna (Cunningham et al., 2005, Garner et al., 2006, Smith, 2013), and *Bd* has already been detected in some incoming shipments destined for the pet trade (Peel et al., 2012). *Batrachochytrium dendrobatidis* was listed as a World Organisation for Animal Health (OIE) notifiable disease in 2008 (OIE, 2008, Schloegel et al., 2010). The OIE advocate the implementation of Disease Risk Analysis (DRA) and mitigation strategies for countries importing animals potentially infected with such pathogens (OIE, 2012), and provide a framework to aid DRA investigations. There are several difficulties to overcome however, when using these frameworks for amphibian import DRA's, the main ones being:

1) There are virtually no data on the volume, species composition, or origins of amphibian consignments entering the UK, as the trade is largely unregulated.
2) Post-import tracking is non-existent, therefore tracing the movements of these animals in country is unachievable.
3) The biosecurity standards of life-long holding facilities (anywhere from zoos to garden pond), are highly variable, thus determining the potential of *Bd* dissemination into wild populations would require extraordinary effort.

Given the problems stated in points 2 and 3, consignment point of entry is a practical place at which to attempt disease mitigation. This in turn has complications relating to regulatory deficiencies including: (1) There is no information regarding the husbandry or packing conditions of the animals prior to arrival. (2) There is very little notice of consignment arrivals.

Additionally, financial (cost of sampling and processing, and potential treatments) and time (man-hours and consignment turnover pressures) constraints, need to be considered in terms of designing feasible mitigation strategies.

Here the findings of a collaborative study conducted at HARC over 30 months from December 2009 to June 2012 are reported. In this study the volume, species composition and country of origin of amphibian imports were recorded, through thorough investigation of 'reptile' consignments arriving at the centre. Skin swabs were taken from a sample of each consignment examined and processed using RT-PCR at the Institute of Zoology (IoZ) to determine the *Bd* infection status.

This study sought to evaluate the risk of *Bd* being imported into the UK via HARC, and examine the feasibility of potential mitigation strategies, by answering the following questions;

1) What is the volume, species composition and origin of amphibian imports into the UK?
2) Can shipment composition be predicted by country of origin?
3) Is infection significantly associated with certain sources, genera or consignments?

Methods

- *Study design and sample size determination*

Staff at HARC were requested to complete an 'Amphibian *Bd* Swab Record Sheet' (see Appendix 1), and collect samples from as many amphibian consignments as possible. Although they may contain amphibians, 'fish' consignments were not sampled as it was not considered logistically feasible, due to the rapid turnover of consignments and their complex packing (sealed, aerated, water filled bags contained within sealed polyboxes).

Amphibian skin swabs were collected by staff at HARC during routine inspection of 'reptile' consignments arriving at the centre. Upon arrival shipment invoices were consulted to determine the number and species present.

Epidemiological sample size calculations based on binomial probability formulae (Cannon and Roe, 1982), were used to determine sample sizes for each consignment. These calculations require values for: i) population size (size of consignment), ii) desired confidence level (99%), and iii) baseline prevalence (20%). The lack of comparative pre-existing data resulted in an estimate of baseline prevalence based on, pet shop prevalence data of 22% (Goka et al., 2009), opportunistic sampling of pet trade imports (3.2%) and laboratory animals (23.5%) (Peel et al., 2012), and laboratory animals 19% (Wombwell, E unpublished MSc thesis). Despite some variation in packing conditions within consignments between species (for example, *Ceratophrys* sp. are always packed individually whereas most other species are carried in conspecific groups), it was assumed that all individuals in each consignment were equally likely to be exposed. Where multiple species were present, a representative number of samples from each species were taken given overall sample size. In addition to swab samples, where possible copies of invoices were made and kept with corresponding shipment samples, to check for inaccuracies on recording sheets.

Fig. 2.1a. Restraint of amphibians for anterior swabbing. (Photo: http://eeblog.lsa.umich.edu/2013/04/on-trail-of-amphibian-chytrid-fungus-in.html)

Fig. 2.1b. Restraint of amphibians for posterior swabbing. (Photo: Trent Garner, ZSL)

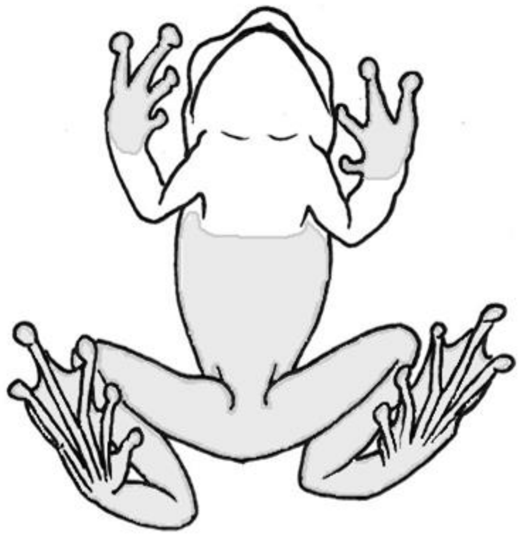


Fig. 2.2. Ventral aspect of an amphibian. Shading illustrates the areas where skin was swabbed for *Bd*.

- *Amphibian skin swabbing*

All samples were collected by trained HARC personnel. Amphibians were randomly selected from containers. A clean pair of powder-free gloves was used for each individual, and amphibians were restrained by gently gripping both hind legs or supported on the dorsum as shown in Fig. 2.1. When necessary the animal was rinsed with aged tap water to remove excess debris. A single use dry swab (MWE: MW100 sterile tubed dry swab) was used to wipe the ventral, lateral surfaces of the amphibian. At least 5 strokes of the feet, drinkpatch, ventral and lateral surfaces were made on each individual, shown in Fig. 2.2.

The sample details (species, number of conspecifics in box, and any notable features) were recorded on the record sheet (Appendix 1), and the amphibian was then placed in a separate container, and another individual selected. This was repeated until the specified number of swab samples had been collected. The swabs were stored in a refrigerator with corresponding swab sheet at HARC until collection or delivery to Institute of Zoology (IoZ).

- *Sample processing*

DNA Extraction

Wearing gloves, the tip of each swab was cut off using a clean scalpel blade in a Petri dish. The tip was then transferred to a labelled 1.5 ml eppendorf containing between 30-40 µg 0.5 mm zirconium/silica microbeads and 60 µl PrepMan® Ultra sample preparation reagent (Applied Biosystems®). The samples were homogenised in a bead beater (Qiagen TissueLyser II) for 45 seconds at 30 r/s, then centrifuged for 1 minute at around 4000 r/min. This step was performed twice. The eppendorfs were then placed in a pre-heated hot-plate for 10 minutes at 100°C. After cooling, the samples were returned to the centrifuge for a further 4 minutes. The supernatant was transferred by pipetting to second set of labelled eppendorfs, before storage at -20°C.

1a	2a	3a	4a	5a	6a	7a	8a	9a	10a	11a	12a
1b	2b	3b	4b	5b	6b	7b	8b	9b	10b	11b	12b
13a	14a	15a	NTC	100a	10a	1a	0.1a	16a	17a	18a	19a
13b	14b	15b	NTC	100b	10b	1b	0.1b	16b	17b	18b	19b
20a	21a	22a	23a	24a	25a	26a	27a	28a	29a	30a	31a
20b	21b	22b	23b	24b	25b	26b	27b	28b	29b	30b	31b
32a	33a	34a	35a	36a	37a	38a	39a	40a	41a	42a	43a
32b	33b	34b	35b	36b	37b	38b	39b	40b	41b	42b	43b

Fig. 2.3. Typical 96 well PCR plate set up. 43 samples, a negative control (NTC), and 4 standards (100, 10, 1 and 0.1 zoospore equivalents), are run in duplicate.

PCR

Samples were diluted to a 1:10 solution using 4 µl of extraction and 36 µl ultrapure DNASE/RNASE-free distilled water, in a labelled eppendorf. This was performed to reduce the inhibitory effect of Prepman ultra on the PCR. A TaqMan® Master Mix was prepared using the following constituents: (per well)

4.74 µl ultrapure DNASE/RNASE-free distilled water
12.5 µl TaqMan® Universal PCR Master Mix
1.25 ITS-1 Forward primer (5’ - CCTTGATATAATACAGTGTGCCATATGTC - 3’)
0.0625 µl TaqMan® MGB probe (Oligo sequence 5’ – 3’ CGA GTC GAA CAA AAT)
1.25 µl 5.8S Reverse primer (5’ - AGCCAAGAGATCCGTTGTCAAA - 3’)
0.2 µl Bovine Serum Albumin (BSA)

The required volume of Master Mix plus 5% was prepared in 1.8 ml cryotubes, then vortexed at a medium speed for a few seconds to ensure the contents were thoroughly mixed. 20 µl of Master Mix was pipetted into each well of a 96 well PCR plate. 5 µl of diluted sample was added to the plate in duplicates. The same volume of the four standards (100, 10, 1 and 0.1 genomic equivalents) and one negative control (distilled water) were included amongst the samples. The placement of samples in a PCR plate is illustrated in Fig. 2.3. A clear PCR plate seal was fitted prior to centrifuging the plate for three minutes at 4000 rpm. The plate was then run on an Applied Biosystems Prism 7300 or OneStep RT-PCR machine. This method follows the protocol of Hyatt et al. (2007) with the exception of the addition of BSA, which was added to reduce inhibition potentially caused by contaminants picked up on the swabs (Garland et al., 2010). The plates were re-run if R2 values were less than 0.9, as were individual samples that returned a single positive result.

- *Data analysis*

1) The volume, species composition and origin of UK amphibian imports.

Total consignment volumes and composition were gleaned from swab record sheets. Import and transit data were compiled separately and used to determine trade routes and volumes. Consignment composition from different countries was extracted, in terms of volume and frequency of occurrence of genera.

2) Prediction of shipment composition by country of origin.

Species data were contracted to genus level in order to reduce the impact of potential species mis-identification. A Sorenson Index was chosen to compare the genera composition between all consignments, as the index is simple to interpret (constrained between 0 - 1), and uses presence/absence rather than abundance data (Kindt and Coe, 2005), as consignment size would otherwise influence the index. The similarity matrix produced was condensed by calculating the mean for indices of the same country, allowing an assessment of compositional similarity between and within countries.

3) Factors associated with Bd infection in imported amphibians.

A Monte Carlo style randomisation analysis was performed to determine the probability of the observed pattern of positive samples amongst the consignments occurring by chance given the overall observed prevalence. A script in ‘R’ (R Core Development Team, 2014) was written to perform 10,000 iterations of a function to compare the variance of the observed distribution (ObsVar) of infection, with the variance of a null distribution generated by randomising the infection data across all consignments. As the variance of a clustered distribution is higher than that of a random distribution, the mean number of times the random variance was greater than the true variance is equal to the probability that that distribution occurred by chance.

There were too few positive samples to produce converged generalised linear mixed models (GLMM’s), so Fisher’s exact tests were performed to test the following null hypotheses:

H_o1: There is no association between country of origin and consignment infection status.

H_o2: There is no association between genus and detection of *Bd* in consignments.

Results

1) The volume, species composition and origin of UK amphibian imports.

Staff at HARC estimated data were collected for at least 80% of consignments received by the centre over the course of the study. Total volume information was available for 54 of the 59 consignments investigated, resulting in a conservative estimate of 14492 amphibians received in ‘reptile’ consignments during the course of the study. Consignments arrived from 12 countries with the USA exporting by far the greatest volume to the UK, followed by Tanzania then Ghana (5600 in 15 consignments; 2880 in 11 consignments; 2106 in 12 consignments respectively), these making up 73% of all arrivals (Fig. 2.4.). Individual consignment volume varied from 19 to 1000 animals (mean = 272, SD = 239). On arrival at HARC consignments where either checked then cleared for entrance into the UK (‘imports’) or checked and returned to a loading area for re-export (‘transits’) to a third country (Fig. 2.5). Forty consignments were imported, equating to 10439 amphibians originating from 10 countries. Approximately 25% of consignments were re-exported, following routine inspection. Given up to 20% of consignments evaded examination, we can estimate that 7000 amphibians are arriving at Heathrow annually in ‘reptile’ consignments, 5050 of which are imported into the UK trade. According to Peel et al. (2012) up to four times as many amphibians are imported in aquatic consignments, resulting in an overall import estimate of 20000.

Overall, 43 genera were recorded. The USA exports the highest diversity of amphibians (Table 2.1), and also the largest number of non-native genera (Fig. 2.7). Only four genera were exclusively seen in ‘transits’ (*Heterixalus*, *Polypedates*, *Mantella* and *Scaphiophryne*). Whilst the most commonly imported genera originate from a variety of exporting countries (Fig. 2.6), transiting shipments are dominated by African genera.

Over 50% of all UK imports consist of only four genera (*Hyla*, *Hyperolius*, *Bombina* and *Cynops*), originating from USA, Tanzania and Ghana, and Hong Kong. The remaining imports comprise smaller volumes of a wide range of genera.

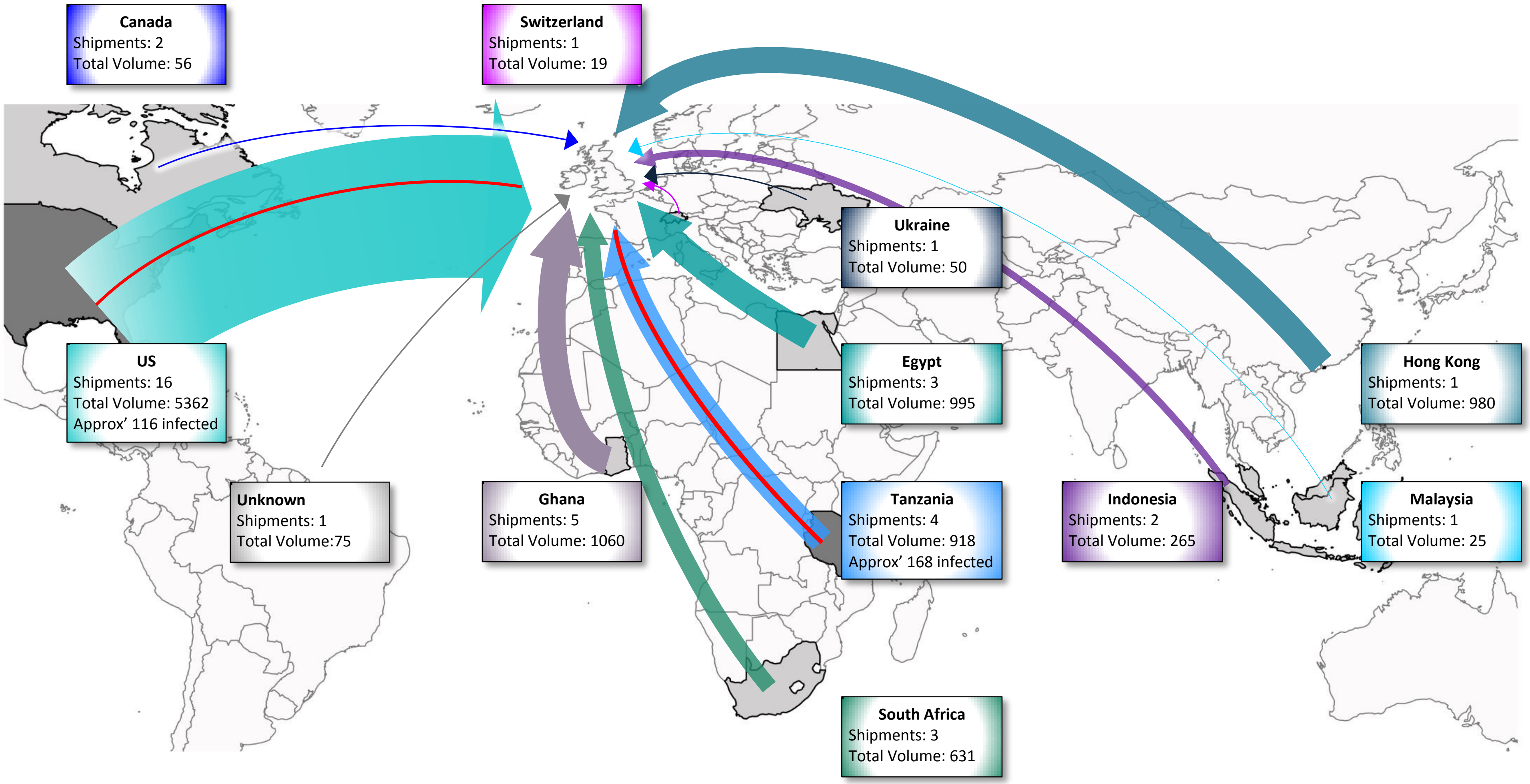
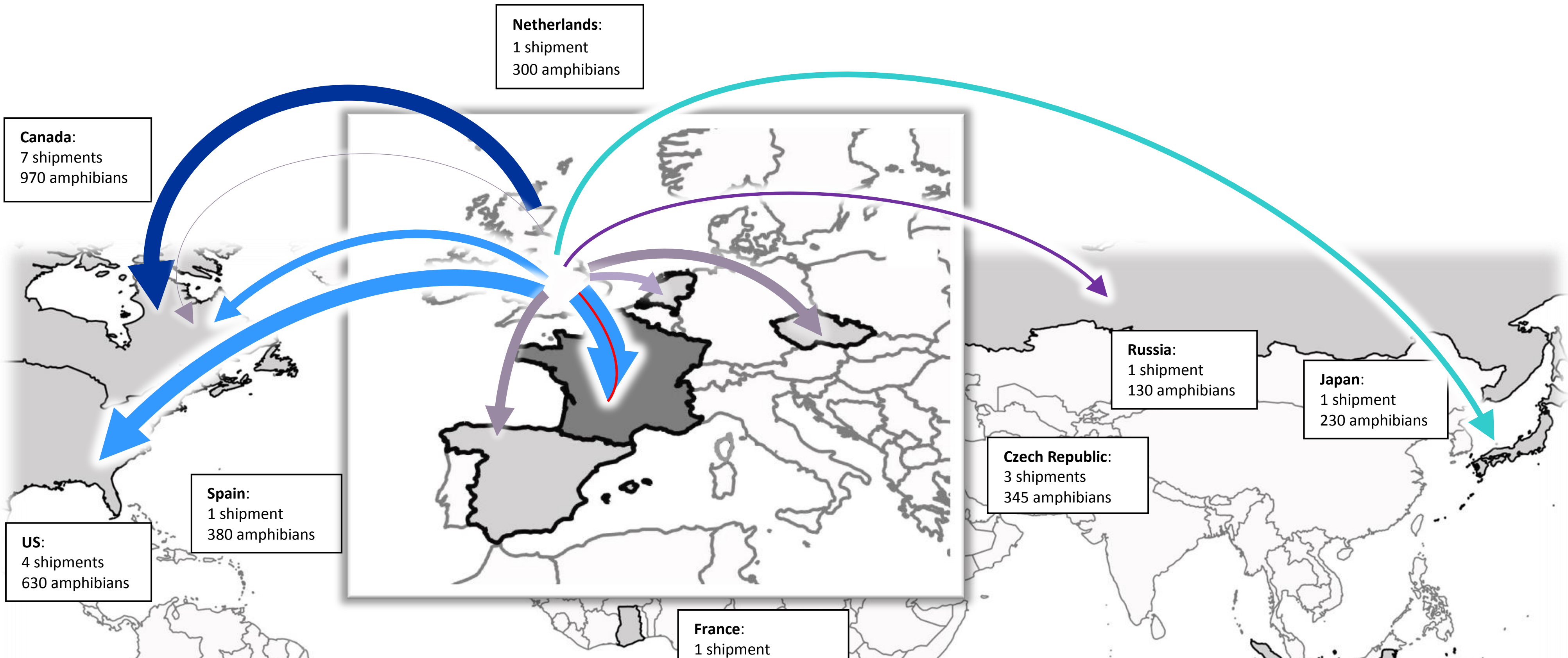


Fig. 2.4. Routes, and volume of sampled amphibian imports into the UK through Heathrow Animal Reception Centre between December 2009 and June 2012. Importing countries with negative shipments shaded light grey, positive shipments shaded dark grey. Arrow thickness represents the total volume of amphibians imported. The proportion of *Bd* infected animals highlighted in red.



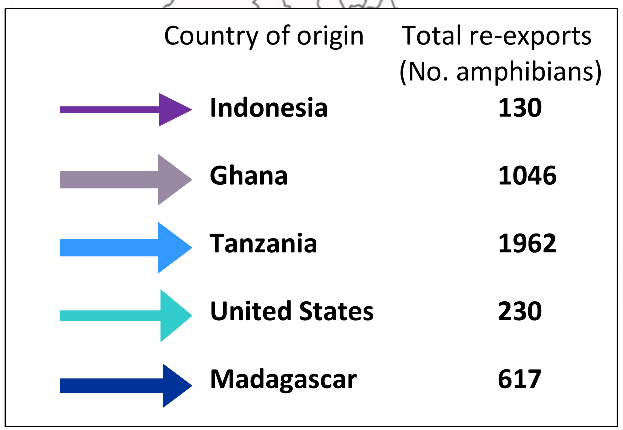


Fig. 2.5. Transit routes of sampled amphibian shipments through Heathrow Animal Reception Centre between December 2009 and June 2012.
Importing and exporting countries with *Bd* negative shipments shaded light grey, with *Bd* positive shipments shaded dark grey. Arrow thickness represents the total volume of amphibians imported. The proportion of *Bd* infected animals highlighted in red.

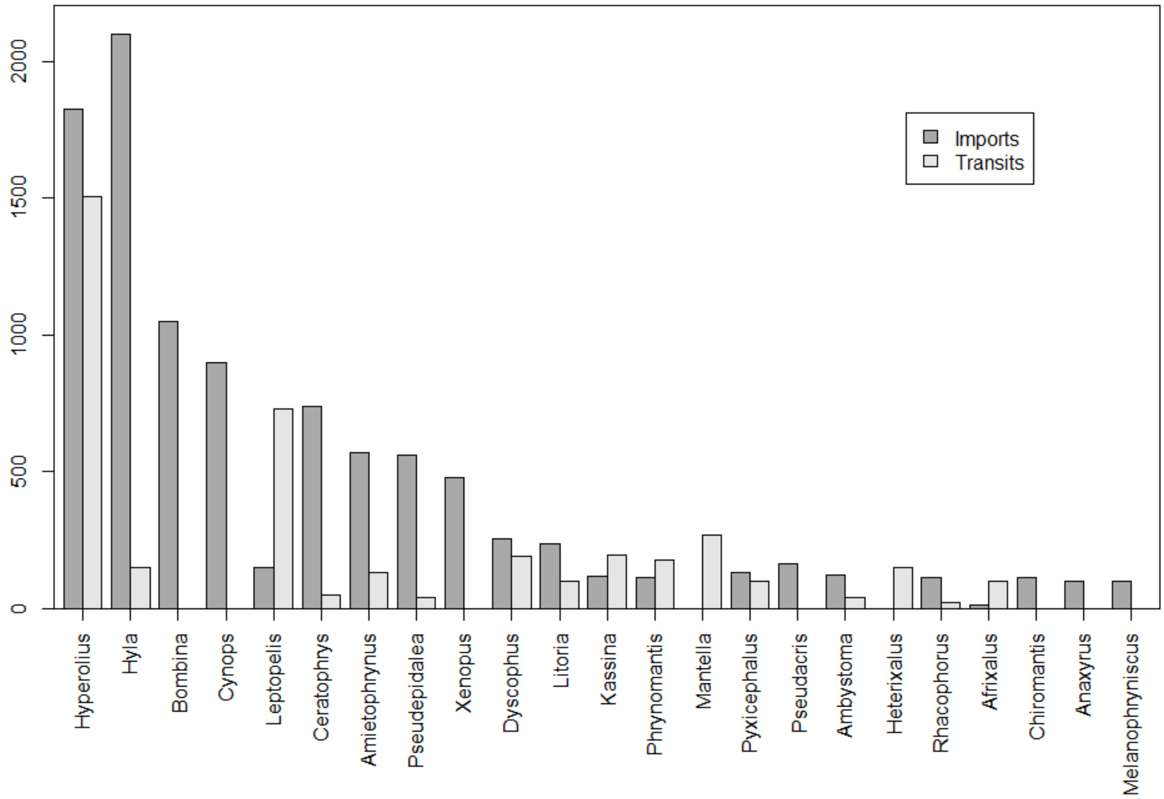


Fig. 2.6. Total volume of amphibians in sampled consignments imported and transiting through HARC, where minimum combined volume exceeded 100.

2) Prediction of shipment composition by country of origin.

Different consignments from the same country of origin were generally similar in composition (Fig. 2.7). Grouping of genera according to their natural geographic distribution showed that, with the exception of the US and Canada, exports consist of native species. The Sorenson index is a measure of ecological distance where values of 0 indicate identical composition between two 'sites', and 1 indicates complete dissimilarity. The mean indices represent the within, and between country, differences in consignment composition. The majority of countries appear to export consignments with genera compliments distinct from those of other countries (values of, or near 1), with a degree of overlap between African countries (Table 2.1). Canada and USA Chicago exported exclusively *Xenopus* sp. thus had an index of 0 (identical consignments). This indicates that country profiles may be useful in estimating the composition of imported amphibians over a number of consignments, but accurately predicting an individual consignment's content is unlikely.

Table 2.1.
Mean Sorenson Indices.
Indices were calculated using the presence or absence of genera for all consignments. Consignments were grouped according to country of origin, and departure airport where possible.

	CA	US C	US M	EG	GH	TZ	ZA	MG	ID
Canada	0.00								
US Chicago	0.00	0.00							
US Miami	1.00	1.00	0.49						
Egypt	1.00	1.00	0.99	0.37					
Ghana	1.00	1.00	0.98	0.81	0.52				
Tanzania	1.00	1.00	0.98	0.87	0.67	0.53			
South Africa	1.00	1.00	0.97	0.84	0.76	0.79	0.36		
Madagascar	1.00	1.00	0.92	1.00	1.00	1.00	1.00	0.22	
Indonesia	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50

3) Factors associated with Bd infection in imported amphibians.

Thirty-six out of the 1010 (3.6%) swabs collected, tested positive for *Bd*. Animals in seven out of 59 (11.8%) consignments were positive for infection, and prevalence within a sample taken from a consignment with infected animals ranged from 5.0 - 85.7%. Extrapolating from the sample prevalence to estimate the number of infected amphibians within a consignment, the overall consignment prevalence was calculated (Table 2.2), which ranged from 1.0 - 85.7%.

The randomisation test showed the observed variance (ObsVar = 0.02), was significantly higher (p = 0.001) than the variance one would expect if infection was randomly distributed amongst consignments ($\bar{x}$(ExpVar) = 0.003). Suggesting infection was clustered within consignments and not evenly distributed. There appears to be further clustering of infection within consignments, at genus level (see Table 2.2), but sample sizes were too small to analyse this statistically.

Table 2.2.
Estimates of numbers of infected individual amphibians, and overall consignment prevalence, entering the UK via HARC between December 2009 and June 2012. Estimated total No. of infected individuals is calculated as, sample prevalence x volume. Estimated overall consignment prevalence is calculated as the sum of the estimated number of infected amphibians in the consignment, divided by the total number of amphibians in the consignment.

Infected Consignment (ID and origin)	Consignment content (genus and volume)	Sample prevalence (%)	Estimated total No. of infected individuals	Estimated overall consignment prevalence (%)
11: TZ	100 *Afrixalus*	0 (0/0)	0	11.1
	550 *Hyperolius*	11.1 (1/9)	61	
	50 *Kassina*	0 (0/4)	0	
	300 *Leptopelis*	16.7 (1/6)	50	
16: TZ	25 *Hemisus*	0 (0/2)	0	22.9
	200 *Hyperolius*	40.0 (4/20)	80	
	100 *Leptopelis*	0 (0/5)	0	
	25 *Pyxicephalus*	0 (0/2)	0	
17: US Miami	70 *Ceratophrys*	0 (0/6)	0	48.8
	100 *Hyla*	83.3 (10/12)	83	
25: TZ	100 *Hyperolius*	85.7 (12/14)	86	85.7
38: US Miami	25 *Calyptocephalella*	0 (0/0)	0	2.8
	90 *Ceratophrys*	0 (0/6)	0	
	450 *Hyla*	0 (0/4)	0	
	25 *Necturus*	85.5 (6/7)	21	
	163 *Pseudacris*	0 (0/2)	0	
50: US Miami	200 *Bombina*	0 (0/4)	0	1.0
	100 *Ceratophrys*	0 (0/4)	0	
	50 *Dyscophus*	0 (0/4)	0	
	200 *Hyla*	0 (0/2)	0	
	25 *Kaloula*	0 (0/0)	0	
	25 *Lepidobatrachus*	0 (0/3)	0	
	12 *Siren*	50.0 (1/2)	6	
53: US Miami	250 *Bombina*	0 (0/10)	0	1.7
	72 *Ceratophrys*	0 (0/5)	0	
	24 *Desmognathus*	25.0 (1/4)	6	

Although *Bd* was detected solely in consignments from the USA and Tanzania (4/17 and 3/11 respectively), there was no significant association between country of origin and consignment infection status (Fisher's exact test: $p = 0.69$). Of the 43 genera encountered during this study, *Bd* was only detected in six: *Desmognathus* (1/4), *Hyla* (10/53), *Hyperolius* (17/207), *Leptopelis* (1/28), *Necturus* (6/7), and *Siren* (1/2). The presence of these genera in a consignment was not however, associated with *Bd* infection in consignment (Fisher's exact test: $p = 0.15$). All positive samples came from genera native to the country of export (see Fig. 2.7), but small overall number of positive samples precluded this from formal statistical analysis.

Figure 7

Discussion

This study revealed a significant number of amphibians, both in terms of volume and diversity, are imported into the UK via HARC. These numbers are undoubtedly underestimates as we acknowledge that data were not collected for all incoming amphibian consignments. Despite accounting for this, our estimate of 5000 - 7000 individuals per year is somewhat lower than the 25000 calculated by Peel et al. (2012) for the year 2006. This inconsistency requires investigation as it may represent an overall decrease in trade, or may indicate other border inspection posts (BIPs) are becoming more favourable for importers. Imports were received from 11 geographically disparate countries and consisted of a large variety of species, highlighting the truly international scope of the trade. With the exception of those from the USA, consignments consisted of amphibians native to the country of export, suggesting wild caught animals. Consignments from the USA (Miami airport), are far more cosmopolitan with respect to genera, indicating a proportion of exports were captive bred animals, or animals re-exported from third-party countries.

Species identification was not always achieved, and it is possible that some species were recorded incorrectly, e.g. *Hyperolius puncticulatus* (listed as ‘Endangered’ by the IUCN) is more likely to be *H. substriatus* (listed as ‘Least Concern’) (IUCN Redlist 2014). Accurate identification is essential in order to monitor the conservation consequences of trade such as unsustainable harvesting, or trade in protected species, which could result in severe population impacts (Rosen and Smith, 2010). As consignment inventories produced in country of origin can be unreliable (pers. obs.), personnel at importing BIP’s should receive adequate training and resources to rectify consignment paperwork.

Whilst there are notable differences in composition between consignments from different countries, there was also some variation within country exports owing to the high diversity of genera exported. This renders the precise prediction of the contents of a consignment prior to its arrival impossible, and necessitates *ad hoc*, rather than pre-planned disease screening.

The detection of *Bd*, in imported and re-exported consignments, supports recent evidence that the global trade in amphibians, coupled with the presence of non-native introduced species, is correlated with the observed global distribution of *Bd* in wild populations (Liu et al., 2012).

The overall prevalence of imports (3.6%) was consistent with results from 2006, where 3.2% (n = 109) of samples were infected (Peel et al., 2012), suggesting ongoing introduction of infected animals into the UK pet trade. Whilst strict biosecurity measures make it unlikely that dissemination of *Bd* into wild populations occurs at point of import (HARC), the level of pathogen containment, post-import, is unknown, and potentially poor. In addition to *Bd*, the continued importation of amphibians risks introducing other pathogens such as ranavirus, which has already been detected in trade amphibians (Schloegel et al., 2009, Kolby et al., 2013), and *Batrachochytrium salamandrivorans* (*Bsal*) a recently discovered, highly virulent chytrid fungus (Martel et al., 2013).

Infection was detected exclusively in USA and Tanzanian consignments, in both imports and transits. Country of origin was, however, not associated with infection probability, but this was likely due to low number of consignments arriving from other countries, resulting in poor statistical power. A collaborative survey from multiple European airports may provide sufficient data to identify countries with statistically higher probabilities of *Bd* infected exports. This would therefore be a beneficial avenue to pursue, as it would allow the implementation of targeted surveillance. *Bd* has recently been detected at higher prevalence (11.7%), in Hong Kong exports (Kolby et al., 2014) in aquatic amphibians (1/5 consignments tested positive). Aquatic consignments were excluded from this study for logistical reasons, but there should be some attempt to investigate these in the near future, as it is likely a large number of amphibians are imported via this route.

The indication that infection is clustered within specific components (specific genera) of specific consignments, is promising for two reasons: Firstly, if high prevalence of infection is contained within components of a consignment, this would reduce the number of samples necessary to detect disease, thus increasing cost effectiveness of disease surveillance. Secondly, if infection is well contained within sub-sections of a consignment, the infected part (rather than the whole) of a consignment could be treated/disposed of, reducing the economic losses associated with detection of infected animals. This requires further investigation through complete consignment swabbing, to fully understand the distribution of disease within a consignment, as very low level infection could have been missed in this study.

Of the 59 consignments investigated, approximately 25% of these were re-exported, making HARC an important ‘hub’ in the amphibian trade network. Identification of such ‘hubs’ is useful in that they provide access to a large number of consignments thus provide a logical place for disease screening, and possible mitigation. The turn-over of consignments at HARC is fast, for three reasons: i) space is at a premium in the busy centre, and there are limited facilities for longer-term housing, ii) wholesalers are keen to take ownership of their stock, and unpack the animals after their journey, and iii) re-exports have to be ready for the connecting flight. Current *Bd* detection methods are unsuited to disease mitigation due to the length of time necessary to receive results (often days), and costs of processing small number of samples. However, even within the time-frame of this study, methods have been developed to sample and detect disease in a time-scale realistic to those at import points. Indeed, it is now possible to detect *Bd* ‘in the field’ with the use of portable PCR machines, at very little relative cost (<£3 per sample), which could be incorporated into fees paid either by importer or exporter. These advances in technology will continue to improve the feasibility of pathogen detection in imports and thus help reduce the risk posed by such trades. Continued surveillance of amphibians imported into the UK is warranted given the increasing reports of *Bd* in trade animals (Schoegel et al., 2005, Kolby et al., 2013). The scope of screening should be broadened to include ranavirus, which has also been detected in trade amphibians and, *Batrachochytrium salamandrivorans* (*Bsal*) the recently discovered, chytrid species (Martel et al., 2013) that could pose a significant threat to great crested newts and other native UK amphibian species.

Findings from this study support the hypothesis that the international trade in amphibians has contributed to the global spread of *Bd*, and reveal a considerable volume of amphibians are imported in to the UK from a range of countries, to supply the pet trade. Whilst research is on-going into the factors influencing the spread of *Bd* and other amphibian pathogens, much of this research has focused on the trade in amphibians for food. This study highlights the presence of disease in the pet trade, and further research is required to evaluate to what extent this could impact on wild amphibian populations.

CHAPTER 3

Conservation implications of amphibians in the pet trade in the UK

"…nearby Covent Garden supplied fruit, vegetable, pots and pans, and gardening tools. Rather alarmingly, it was also London's pet shop. Fish, newts, parrots and even monkeys could all be yours for a price." (Inglis, L. 2013. Georgian London: Into the Streets)

London Pet Shop at Seven Dials Covent Garden (Illustrated London News 1874)

Abstract

Controversy surrounds the amphibian pet trade, with the need for trade bans disputed. Defence for either side is problematic given the paucity of robust data in the UK. To address this issue, this study aimed of to: (1) quantify the volume of trade in the UK, and analyse the species composition; (2) examine husbandry practices, levels of disease awareness, and knowledge among retailers; and (3) evaluate factors influencing mortality. Analysis of 115 questionnaires collected between March 2010 and June 2013, revealed amphibians are routinely stocked in approximately 30% (1000) of pet livestock retailers, but contribute only 5% of gross income. Whilst 127 species were reported in the trade, six accounted for over 50% of the estimated income. Amphibian die-offs were infrequent, and mortality was correlated with number of species and restocking methods. Although specific disease awareness and knowledge was lacking, reported husbandry practices were good. It was concluded that increased communication between the scientific community and stakeholders is vital for reducing disease risk, and pet trade associations will be fundamental in facilitating this.

Introduction

In the UK, amphibian population declines were noted as early as 1973 (Beebee, 1973), however, the scale of global amphibian declines were not fully appreciated until 1989 at the First World Congress of Herpetology. Since that time, and following the Global Amphibian Assessment undertaken by the IUCN in 2004, which placed a third of amphibian species in threatened categories (Stuart et al., 2004), amphibian research and conservation has become significantly more prominent in the literature. Research into the causes of these declines has typically focused on the impacts of single causative factors (Stuart et al., 2004). However, there is growing support for the view that multiple interacting factors, including habitat loss, pollution, introduction of non-native species, over harvesting, and disease, are responsible (Davidson and Knapp, 2007, Rohr and Raffel, 2010, Blaustein et al., 2011, Menendez-Guerrero and Graham, 2013). The trade in amphibians is implicated in the latter three of these factors, and has subsequently been under increased scrutiny in recent years. Amphibians are utilised globally for a wide range of purposes including for food (Mazzoni et al., 2003), as model organisms in scientific and medical research (O'Rourke, 2007), and the pet trade (Schlaepfer et al., 2005, Carpenter et al., 2014).

Frogs' legs do not seem to appeal to the typical British diner, and consequently the UK amphibian trade is predominantly for scientific and pet purposes (Peel et al., 2012). The research market is thought to be of considerable volume, but is composed of few species (e.g. *Xenopus* sp. and axolotls). Conversely, the pet trade involves a wide variety of species, of both captive-bred and wild-caught origins (Tapley et al., 2011), which has led to conservation and welfare concerns. The trade has been implicated in causing declines in wild populations through, (i) unsustainable harvesting (Spellerberg, 1976, Sharifi et al., 2008); a problem amplified if rare or recently described species are targeted (Stuart et al., 2004, Phimmachak et al., 2012); and (ii) exacerbating the spread of pathogens through the transport of amphibians to regions far from their native ranges (Fisher and Garner, 2007). These factors have led to questions regarding the future of the amphibian pet trade, and whether a ban on all or a selection of species should be implemented (Kriger and Hero, 2009). Proponents of the trade refute suggestions a ban is warranted (Reptile and Exotic Pet Trade Association), but both sides face difficulties in providing evidence for their claims. There are very few available data regarding the amphibian trade (but see Peel et al., 2012, Tapley et al., 2011) and consequently, the role amphibians play in the wider pet trade is, to a significant extent, unknown. Before regulatory changes can be considered, the social and economic impacts, such as evaluation of demand and growth trends, need to be determined. In addition, it is imperative to evaluate the risks associated with the trade, and assess potential strategies to curtail future pathogen introduction (Garner et al., 2009b).

In light of these issues, this study sought to provide an analysis of the amphibian pet trade in the UK, using an interdisciplinary approach. Four main questions were identified:

(i) *Does amphibian retail constitute an important proportion of the UK pet trade?*

The proportion of livestock retailers selling amphibians was determined along with the distribution of amphibian retailers between different pet trade sectors. The importance of amphibian sales in this market was estimated by the relative income derived from amphibian sales.

(ii) *What is the volume and species composition in the amphibian trade?*

Analysis of stock lists provided a measure of species diversity in the trade which was compared across shop types. Factors influencing stock purchases were investigated to explain observed patterns.

(iii) *To what extent does mortality impact amphibian trade?*

Evaluation of husbandry protocols and knowledge of amphibian diseases were used to indicate the perceived importance of disease in this trade sector. These factors were used in conjunction with reported mortality events to examine potential predictors of mortality.

(iv) *Is the trade in amphibians likely to increase in the future?*

A forecast for the future of the trade in the UK was estimated using retailer opinion regarding the popularity of amphibians as pets over time, together with the number of pet shop licences issued by local authorities.

Methods

A paper questionnaire was designed in a booklet style with five sections each with a maximum of five questions. The sections comprised discrete categories: 1) ‘Background information’, aimed to provide details of shop ‘type’ e.g. reptile shop or aquatics specialist, as well as information pertaining to size of the business, and income derived from amphibian retail. 2) ‘Amphibian health’, which investigated disease awareness and attitudes, as well as experience of mortality in amphibian stock. 3) ‘Stocklist’ provided actual volume, species available and prices at that time. 4) A ‘Stock acquisition’ section sought to examine the factors influencing stock purchase decisions for both wild caught and captive bred animals, and look at the diversity of supply routes. 5) The ‘Husbandry’ component explored the differences in sanitary protocols between pet shop types, with the aim to identify potential routes for disease spread. The layout and colour printing was chosen for visual appeal, and in order to maintain interest levels, a variety of question formats, e.g. likert-style, tick-box and open-ended, were used throughout the survey (De Vaus, 2002). To retain motivation, questions progressed from simple to more complex in each section, with open-ended questions only used where prediction of responses was not possible (De Vaus, 2002). Initial drafts were piloted at nine pet shops; three pet shops which were visited personally and six that were sent copies by post. On completion, respondents were requested to give feedback on question style, ease of comprehension and length of time to complete. The questionnaire was amended appropriately (see Appendix 2 for the final version), and trialled again at a further two pet shops before the survey began.

A database of the pet shops in the UK was compiled using an internet search of the BT Phonebook, Yellow Pages online (yell.com), and a general google search. Each county was searched individually by postcode prefixes e.g. for Cambridgeshire, CB1, CB2 etc, using search words ‘pet shop’, ‘reptile shop’ and ‘aquatic shop’. From the returns, shop name address details and telephone number were recorded, ordered by postcode and subsequently checked for duplicates. Establishments were assigned a number and were telephoned according to a number list generated using a random number generator (www.random.org). If no contact was made on the first attempt the retailer was checked using the on-line Companies House ‘WebCHeck service’ (www.companieshouse.gov.uk) to confirm their operational status i.e. active, in liquidation or dissolved, if they were registered. For unregistered or active businesses, a further two attempts were made at calling on different days and times before they were discarded from the list. Responding establishments were first asked whether they sold livestock. If they responded ‘yes’, they were then asked if they sold amphibians, and if they did they were asked whether they would be willing to fill in a questionnaire sent by post. Co-operating establishments were sent a questionnaire no longer than three days after the telephone conversation along with a stamped addressed return envelope and compliments slip. Due to the option to remain anonymous it was not possible to identify which establishments had returned their questionnaires so therefore ‘follow-up calls’ to non-responders were not possible. Further questionnaires were collected during visits to pet shops for the collection of amphibian skin swabs (see Chapter 4).

All participants were offered the opportunity to have their amphibians screened for *Bd* and a copy of a summary of the results at the end of the study.

In order to validate the database and investigate temporal changes in trade, freedom of information (FOI) requests were sent via email to all 381 district councils in the UK, requesting the number of pet shop licences issued in 2000, 2005 and 2010 and of these how many allowed the sale of amphibians. Further details of this procedure are described in the following chapter.

In addition to the questionnaire stock list section, stock data collected during shop visits was included in species composition analysis.

- *Data analysis*

All statistical analyses were performed using ‘R’ version 3.1.0 software. Non-parametric tests such as Fisher’s Exact, Kruskal-Wallis and Friedman ANOVA, were performed to investigate differences in responses between ‘reptile’, ‘pet’, and ‘aquatic’ retailers.

Stock and price lists collected during pet shop visits, were collated alongside stock, turnover and price information in the questionnaires, to estimate overall volume and value of the amphibian trade.

Difference in genera composition between shop types was investigated using permutation techniques on grouped similarity indices (Anderson, 2001, Clarke et al., 2008). Genera abundance data (total number of individuals in stock) were transformed in to binary (presence/absence) data, prior to analysis, in order to prevent over emphasis of highly numerous genera. Consequently, the binary ‘Sorenson’ index was used in the calculation (Legendre and Legendre, 1998). Using the ‘com.sim’ function (Jurasinski, 2012) pairwise similarity indices were calculated for all retailer stock lists, data were then grouped by shop type and the similarity means compared. Permutation techniques are used to overcome the lack of in dependence in similarity index data (Jurasinski, 2012), resulting in a matrix identifying significant differences between groups. Constrained (Canonical) correspondence analysis (Oksanen et al., 2013) was performed, to determine the amount of variation in the data explained by retailer type, and visualise the differences graphically.

Seven generalised linear models (GLMs) with binomial errors and the logit link function were used to investigate factors influencing amphibian mortality (Table 3.1). Models, including an intercept only (null) model, were ranked according to AICc values using the ‘MuMIn’ package in ’R’ (Barton, 2014), and selected on the basis of simplicity and rejected if AICc values were greater than six units from the optimum model (Richards, 2008).

Table 3.1.
Models investigating factors affecting amphibian mortality in UK retailers. ‘Type’ is the classification of retailer, either ‘reptile’ ‘pet’ or ‘aquatic’. ‘SpDiv’ is the number of species for sale. ‘RStk’ (restocking) is a binary score indicating whether newly acquired animals were added to existing stock (0) or housed separately from existing stock (1). ‘Exp’ (experience), is the number of years amphibians have been sold by the retailer, and ‘Sup’ (suppliers) is the number of amphibian suppliers used by the retailer. ‘TotVl’ (total volume), is the total number of individual amphibians in stock.

Model	Predictors	Hypothesis
1	Type	Type of retailer (reptile, aquatic or pet) affects the likelihood of observing mortality in amphibian stock.
2	Type + SpDiv	Type of retailer affects observed mortality, and the number of species sold determines the size of this affect.
3	Type + SpDiv + RStk	Type of retailer affects observed mortality, and the number of species and restocking type determines the size of this affect.
4	Exp	Observed mortality is determined by how long amphibians have been sold at the shop.
5	SpDiv + RStk	Observed mortality is affected by the number of species sold, the intercept of which is determined by restocking type.
6	SpDiv + Sup	Number of species sold affects observed mortality, but the intercept is determined by the number of suppliers used.
7	SpDiv+Sup+TotVl	Mortality is affected by the number of species sold, number of suppliers used and the total volume of amphibian stock.
8	Null	Intercept only model.

The ‘captive bred’ or ‘wild caught’ status of amphibians was omitted from analysis, as this information could not be reliably provided by all establishments.

Trends in amphibian trade over time were investigated using GLM’s with quasi-poisson errors for over-dispersed data (Hoef and Boveng, 2007), fitted to data received from local councils on the numbers of pet shop licences; (i) permitting, and (ii) not permitting the sale of amphibians and years 2000, 2005 and 2010. The differences in rate of increase in number of licences issued permitting and not permitting the sale of amphibians (slope of the lines), was compared using analysis of covariance.

Results

(i) Does amphibian retail constitute an important proportion of the UK pet trade?

Freedom of information requests were answered by all local authorities (LA’s) but one, and according to these records, the total number of pet shops selling livestock in 2010 in the UK was 3362.

The internet search resulted in a database of 3231 pet shops, all of which were telephoned over the course of six months from January 2010. Invalid numbers or incorrectly listed businesses accounted for 438 of the calls, resulting in contact with 2789 active pet shop businesses. Approximately 60% (1660) of the pet shops contacted by telephone reported selling livestock (equating to contact with half of all pet shop livestock retailers), of which 495 routinely sold amphibians. Questionnaires were returned by 56 (15%) of the 367 sent to amphibian retailers; a further 60 were collected in person during pet shop visits. One questionnaire was discarded as only the ‘stock list’ section was completed with a selection of reptile species, so the results are reported for a total of 115 establishments (52 reptile, 38 aquatic and 25 pet shops).

Amphibian retailers fell into three distinct categories; ‘reptile’, ‘aquatics’, and a ‘pet’ group that comprised mixed taxa retailers (e.g. small mammals, birds, fish) and the miscellaneous (garden centres, avian specialists) businesses. From this point on, ‘reptile’, ‘aquatic’ and ‘pet shop’ refer only to those that sold amphibians. The number of full-time and part-time employees reported was low across all shop types (Table 3.2), with at least one sole trader in each group. Reptile shops accounted for the highest proportion of amphibian retailers, followed by aquatics then pet stores, although the volume of amphibians in trade was equivalent between reptile and aquatics stores when stock levels were considered (Fig. 3.1). Pet shops constituted proportionally less, both in terms of number of shops and volume of amphibians for sale.

Table 3.2
Number of employees reported by amphibian retailers. n = number of replies.

Employee type	**Aquatic** n = 38	**Pet** n = 25	**Reptile** n = 52
Full time	$\bar{x}$ = 3.8 SD = 2.47	$\bar{x}$ = 1.9 SD = 1.04	$\bar{x}$ = 1.9 SD = 1.33
Part time	$\bar{x}$ = 1.8 SD = 2.07	$\bar{x}$ = 1.7 SD = 1.63	$\bar{x}$ = 1.3 SD = 1.29

Over half of all business types reported income derived from the sale of amphibians to be less than five percent of total income, with a greater than 25% contribution reported by only a single reptile retailer (Fig. 3.2). Approximately a third of reptile shops earned between 6% and 25% of their overall income from amphibian sales, making amphibians most important to this trade group.

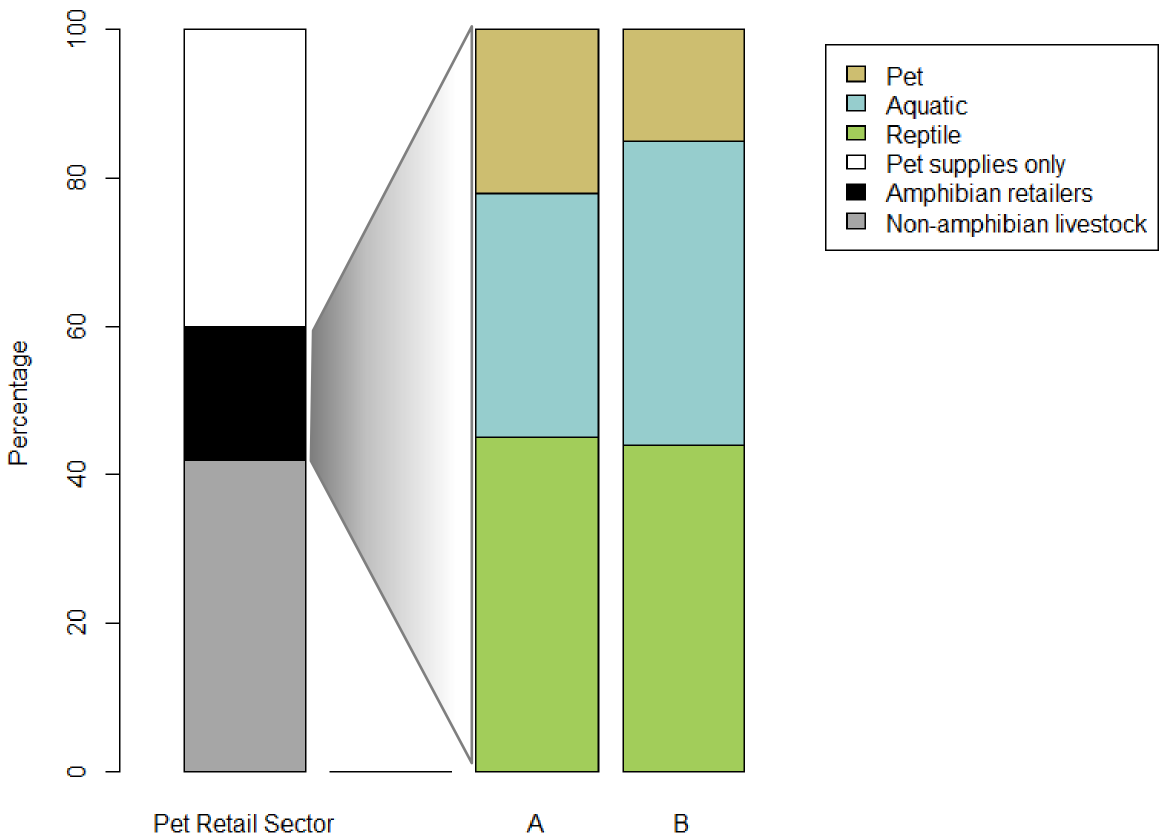


Fig. 3.1. Position of amphibian retail in the UK pet trade. The pet retail sector column illustrates the composition of the pet trade by the percentage of non-livestock (supplies only), livestock excluding amphibians, and livestock including amphibians. Column A represents the proportional distribution of amphibian trade according to number of shops across shop types. Column B represents the proportional distribution of amphibians according to volume

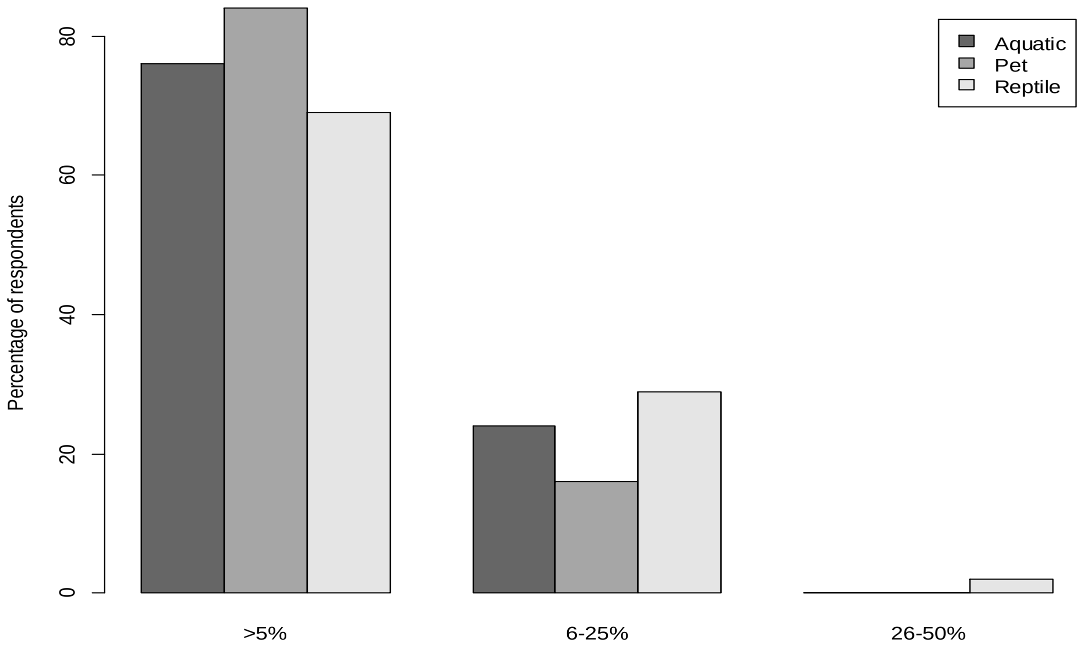


Fig. 3.2. Proportion of shops reporting categories of income derived from the sale of amphibians. Pet shops report the lowest income from amphibian sales, followed by aquatics, than reptile shops.

The reported relative importance of amphibian trade to the different retail groups correlated with estimates of gross income derived from amphibian sales. Based on retail value and annual turnover calculations, the reptile shop sector received double the income gained by aquatics shops, and four times that of pet shops (Table 3.3). The overall contribution of amphibian sales to the pet livestock trade in the UK is likely to be in excess of £3,000,000 a year, given incomplete data prevented inclusion of all recorded species. Aquatics shops had the highest annual amphibian turnover (≈80000), due to a high volume of aquatic Congo frogs (*Hymenochirus boettgeri*) and African clawed frogs (*Xenopus laevis*). The lowest volume of amphibians were sold in pet shops, a third of which were also *H. boettgeri*, suggesting aquatic species are important to this trade group. The annual turnover for the amphibian trade as a whole was estimated to at ≈500000 amphibians per annum. Again, missing data prevented some species being accounted for in turnover calculations, resulting in an underestimate of the true volume.

Table 3.3

Summary of estimates of the annual turnover, and annual gross income derived from amphibians in the retail sector. Price: mean reported retail price. Annual turnover estimate: Mean reported turnover x estimated No. shops selling the species. Estimated annual income: Price x annual turnover. Species accounting for 75% of income specified. Dashed line indicates species accounting for 50% of income.

	Species	Price (£)		Annual turnover		Est' annual income (£)
All shop types						
1	*Litoria caerulea*	20.48		19417		397,683.70
2	*Hyla cinerea*	13.83		20881		288,880.54
3	*Ambystoma mexicanum*	18.76		15323		287,410.63
4	*Bombina orientalis*	11.63		17620		204,852.88
5	*Hymenochirus boettgeri*	3.80		52397		198,876.91
6	*Ceratophrys cranwelli*	33.33		5405		180,143.17
7	*Agalychnis callidryas*	56.66		3157		178,897.41
8	*Polypedates leucomystax*	17.47		7090		123,855.36
9	*Xenopus laevis*	6.84		18097		123,824.08
10	*Trachycephalus resinifictrix*	51.35		1721		88,369.19
11	*Dyscophus guineti*	34.27		2050		70,243.41
12	*Ceratophrys ornata*	40.61		1710		69,451.30
13	*Cynops orientalis*	12.26		5264		64,559.36
		Subtotal		170132		2,277,047.95
			70 species	261559	65 species	675,508.05
			44 species	unavailable	49 species	unavailable
127		TOTAL		**431691**		**2,952,556.00**
Reptile shops						
1	*Litoria caerulea*	19.80		12711		251,647.92
2	*Agalychnis callidryas*	55.00		3667		201,664.10
3	*Hyla cinerea*	12.66		11664		143,442.81
4	*Polypedates leucomystax*	17.47		7239		126,461.45
5	*Ceratophrys cranwelli*	33.26		3641		121,127.06
6	*Bombina orientalis*	10.53		10401		109,505.26
7	*Trachycephalus resinifictrix*	46.87		1676		78,560.16
8	*Dyscophus guineti*	32.49		2212		71,861.22
9	*Ceratophrys ornata*	37.22		1604		59,680.62
10	*Ambystoma mexicanum*	19.58		2611		51,117.94
		Subtotal		57095		1,215,068.53
			47 species	17945	39 species	403,604.83
			61 species	unavailable	64 species	unavailable
113		TOTAL		**75040**		**1,618,673.36**
Aquatics shops						
1	*Ambystoma mexicanum*	17.61		8804		155,062.90
2	*Hymenochirus boettgeri*	3.74		32613		121,877.60
3	*Hyla cinerea*	13.98		7476		104,490.00
4	*Litoria caerulea*	20.99		4726		99,208.22
5	*Xenopus laevis*	5.96		14186		84,510.84
6	*Salamandra salamandra*	59.95		1289		77,277.48
		Subtotal		69,094		642,427.10
			22 species	10940	22 species	156,640.03
			34 species	unavailable	34 species	unavailable
62		TOTAL		**80034**		**799,067.10**
Pet shops						
1	*Bombina orientalis*	13.74		5239		71,994.99
2	*Ambystoma mexicanum*	19.85		2379		47,219.75
3	*Litoria caerulea*	21.85		2066		45,137.08
4	*Hymenochirus boettgeri*	4.00		10963		43,852.80
5	*Ceratophrys cranwelli*	31.67		1308		41,420.00
6	*Hyla cinerea*	18.31		1596		29,228.08
7	*Pyxicephalus adspersus*	46.00		375		17250.00
		Subtotal		23926		296102.70
			15 species	5288	15 species	76,502.16
			28 species	unavailable	28 species	unavailable
50		TOTAL		**29214**		**372,604.90**

(ii) What is the volume and species composition in the amphibian trade?

Analysis of stock composition from the questionnaires (56 completed) and information recorded during shop visits (121 stock lists) resulted in species presence and volume for 62 aquatics, 37 pet and 78 reptile retailers. Despite amphibians accounting for a relatively small percentage of sales income, most stock comprised multiple species, with 127 species overall not including colour morph or hybrid variations, from 65 genera. Species composition varied between establishments, the majority of species being observed infrequently, and at low volumes (requiring statistical analysis to be conducted on genera data rather than species). There were, however, some genera that were generally stocked at higher levels and regularly appeared on stock lists across all shop types (Fig. 3.3 and Appendix 3).

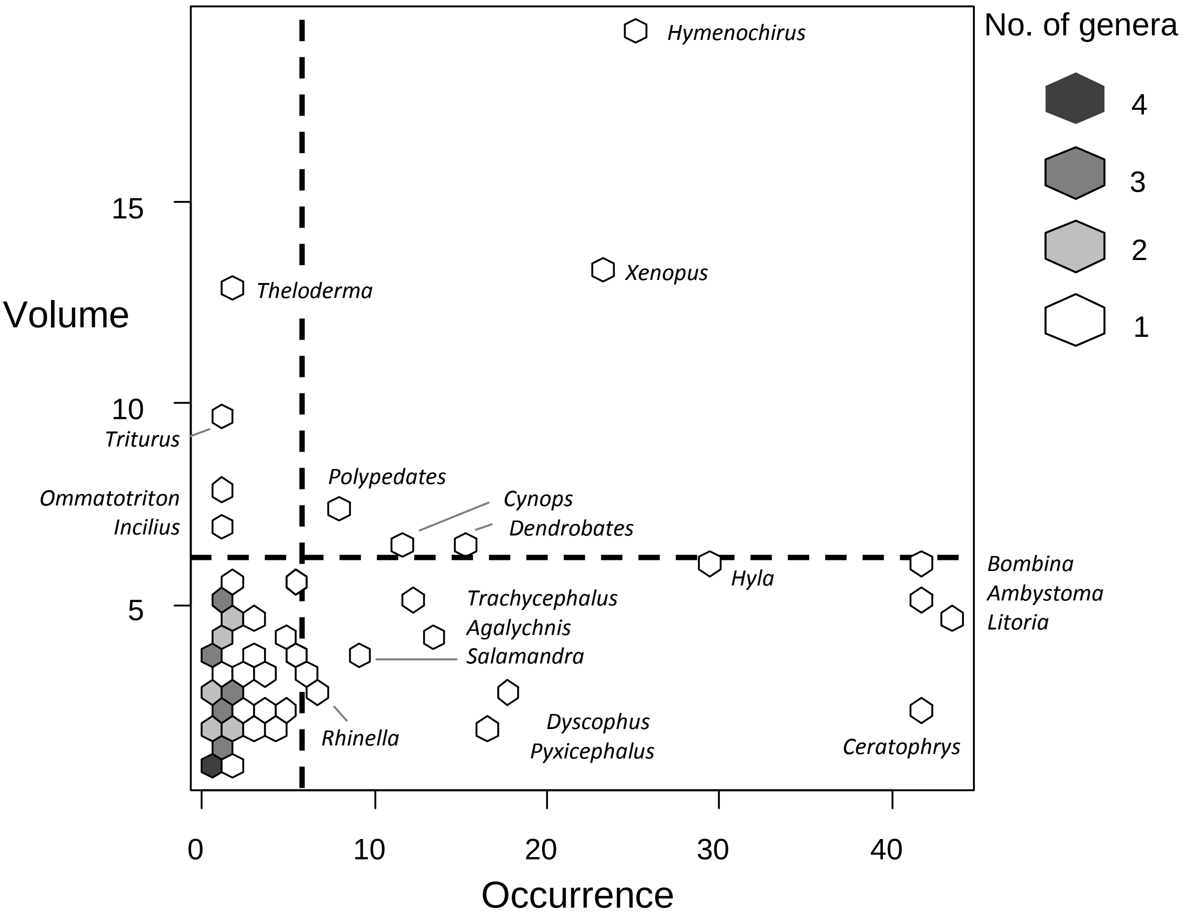


Fig. 3.3. Frequency of occurrence against the mean volume of amphibian genera in stock, where: Occurrence is the percentage of shops in which the genus occurred, and volume is the mean number of individuals of that genus for sale across shops that stock them. The shade of point indicates how many genera fall under that point, e.g. 4 genera were found in 1% of shops with a mean volume of one. The dashed lines show the upper quartile for 'occurrence' (5.65%) and 'volume' (6). Genera in the upper quartile for either occurrence of volume, or both, are specified.

There was significant variation in genera composition between shop types (Table 3.4). Reptile shops were the most distinct in terms of genera composition, with the most commonalities seen between aquatic and pet shops (Fig. 3.4).

Table 3.4
Differences in genera composition between shop types, based on mean Sorensen Indices, inference obtained using 1000 permutations for each level comparison

X	Y	Mean.x	Mean.y	Diff	P	F
Aquatic	Pet	0.2584	0.2144	0.04397	<0.001	13.253
Aquatic	Reptile	0.2584	0.2745	-0.01607	<0.001	5.743
Pet	Reptile	0.2144	0.2745	-0.06004	<0.001	50.019

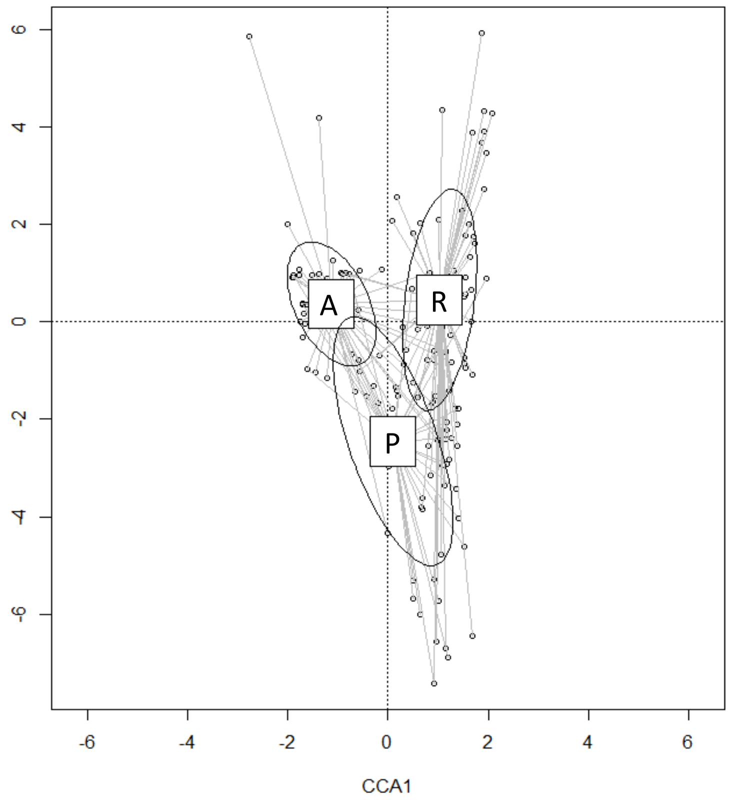


Fig. 3.4. Graphical representation of the results of a canonical correspondence analysis showing variation in genera composition between retailers type. Ellipses indicate the 95% confidence interval for each of the shop types, and the points (individual retailers) are linked to the centre of the corresponding ellipse. A: Aquatics shops. P: Pet shops. R: Reptile shops.

Reptile shops have the most distinct generic composition, with least overlap in variation with other shop types (Fig. 3.4). Aquatics shops and pet shops have the most similar compositions. Although, group variation analysis (Table 3.4), and individual retailer comparisons appear to distinguish between shop type, there is a significant overlap in variation, due to the presence of a ‘core’ group of genera seen across all shop types (Table 3.5).

Table 3.5
The five most commonly observed amphibian genera across all retailer types, and the percentage of shops in which they were stocked (occurrence).

Genus	Occurrence (%)		
	Aquatic	Pet	Reptile
Ambystoma	46.8	37.8	39.7
Bombina	22.6	48.6	53.8
Ceratophrys	25.8	27.0	60.3
Hyla	17.7	21.6	41.0
Litoria	22.6	45.9	59.0

Reptile shops provided a wider range species (Fig. 3.5.), compared to pet and aquatics shops (Kruskal Wallis: $\chi^2 = 26.7$, df = 2, p = < 0.001, post-hoc test = kruskalmc (R)).

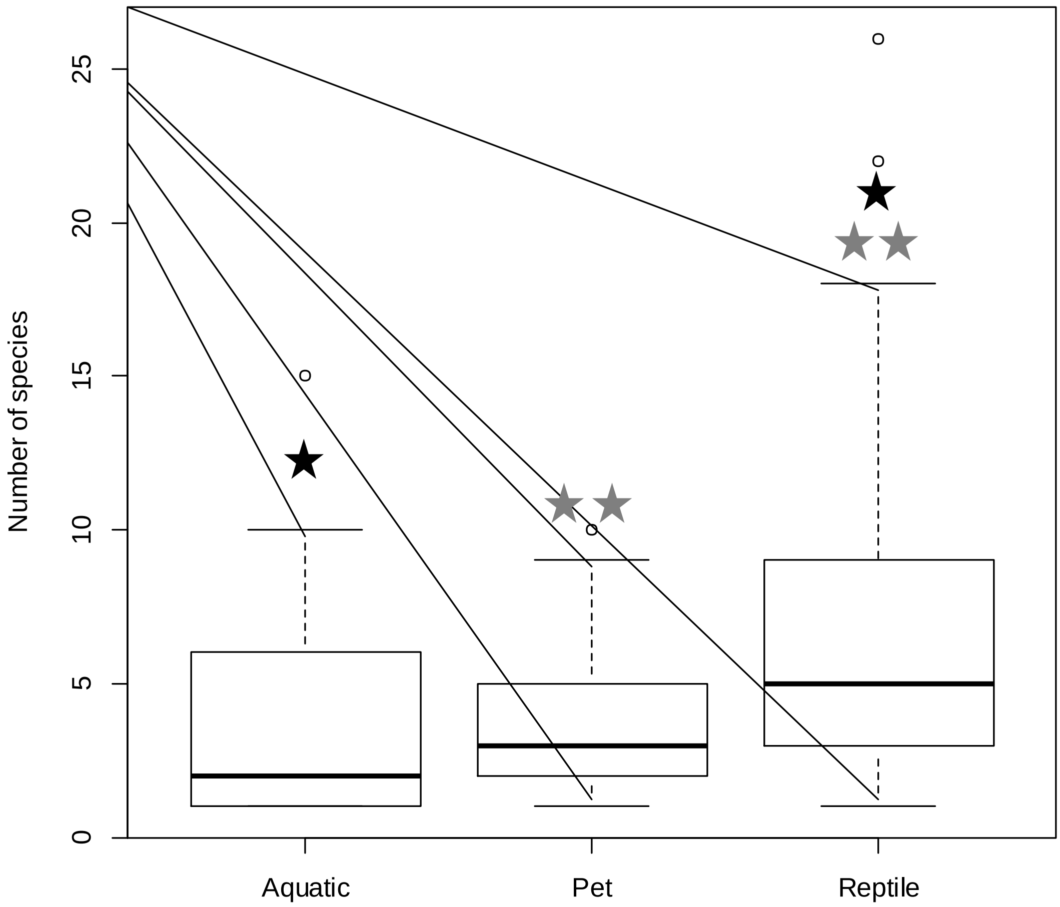


Fig. 3.5. Number of amphibian species available in aquatic, pet and reptile shops: Represented as the median (horizontal line), interquartile range (box), 1.5 times the interquartile range (whiskers), and values greater than 1.5 times the interquartile range plotted individually (outliers). The post-hoc test revealed significant differences between ‘Reptile’ and ‘Pet’(grey stars), and ‘Reptile’ and ‘Aquatic’ (black star) shop species numbers, but not between ‘Aquatic’ and ‘Pet’ shops.

Informal discussions during shop visits revealed the captive-bred or wild caught status of amphibian stock was a contentious issue among retailers, with some refusing to sell wild caught animals. Information from questionnaires corroborated this with 23 retailers explicitly refusing to stock wild caught animals, whilst many others left this section blank. Price was recorded as a consideration in a third of all purchase choices, and was the only factor equivalent in both captive bred and wild caught categories (Fig. 3.6). ‘Quality’; taken to include ‘size and ‘age’ as well as health aspects, and demand; were the main factors influencing the decision to purchase captive bred animals. A comparison of scores (1-5) assigned to the health of newly arrived stock revealed captive bred animals were perceived to be healthier than wild caught counterparts (Mann-Whitney U: $p = < 0.001$). Other factors associated with captive bred animal purchases concerned ease of care, conservation issues and welfare aspects. The main motivation for purchasing wild caught animals was availability of desired species, with genetics, rarity and variety included in the ‘other’ factor group.

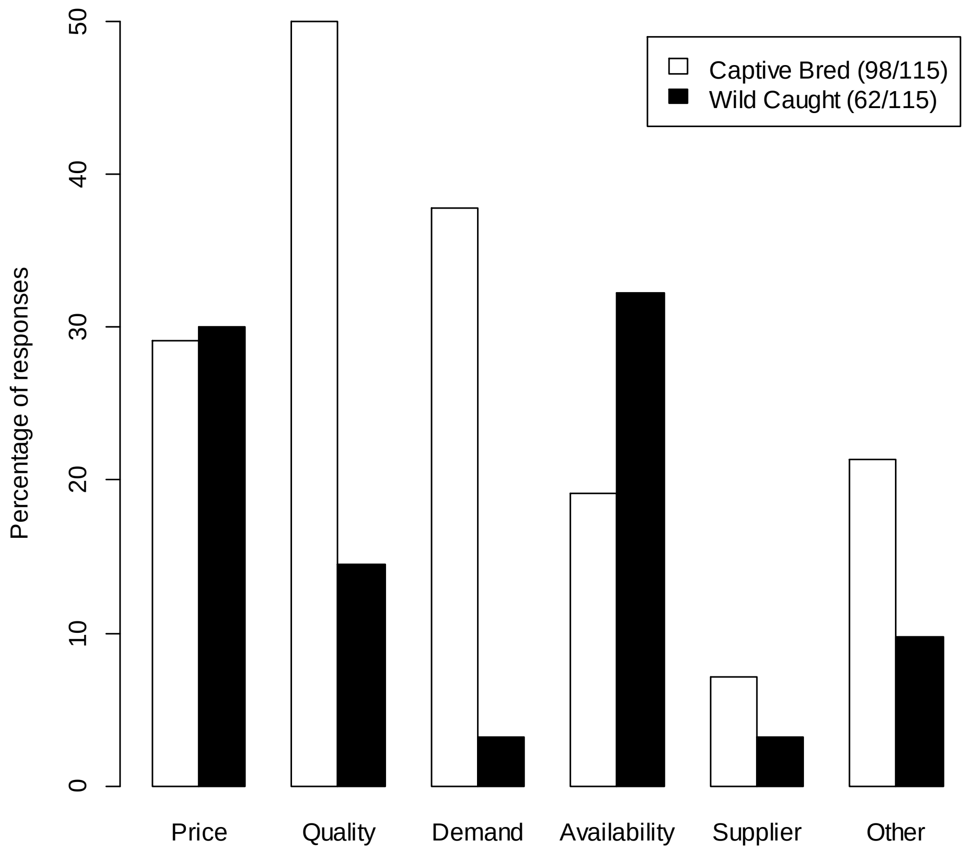


Fig. 3.6. Factors reported by retailers as important in their stock purchase decisions for captive bred (CB) and wild caught (WC) animals, represented as a percentage of total responses for that category (either CB or WC). Total percentage may be >100% as the question allowed more than one factor per category.

(iii) To what extent does mortality impact amphibian trade?

Responses relating to husbandry protocols formed two distinct categories (i) sanitary - relating to routine enclosure maintenance; and (ii) health – relating to the nutritional and physical care of the animal. There was very little variation in sanitary or health care between shop types, with amphibians being fed between daily and twice weekly, sprayed and water bowls changed daily. Three shops (two aquatic and one reptile), reported the routine use of vitamin and mineral supplements. Over 80% of establishments spot clean and inspect their animals daily, with full enclosure cleaning (including substrate change) and disinfection occurring weekly or monthly. Twenty-two responses stated that staff wore gloves when servicing the enclosures, whereas 12 reported no gloves were used, but hand sanitation was frequently mentioned. New amphibian arrivals were generally housed separately to existing stock (81% overall) and to a lesser extent housed with resident animals of the same species (16% overall). Only two pet and one reptile shop reported placing new amphibians with resident animals of different species. Aquatics shops were more likely to add new animals to existing stock than the other shop types. In total eight establishments reported quarantining new arrivals, and time periods for this varied between three days and four weeks, although anecdotal evidence suggests retailers assume amphibians are quarantined at the suppliers prior to receiving them.

The question relating to knowledge of amphibian diseases and awareness of captive animal susceptibility received the lowest response (54/115). Proportionally, aquatics shops gave the fewest answers, whereas reptile shops returned the most replies. The responses for both captive and wild animals were extremely broad resulting in 11 distinct categories (Fig. 3.7). Overall, four specific diseases were named: Chytridiomycosis (variable spellings and abbreviations), ranavirus, red-leg, and metabolic bone disease (abbreviated to MBD). Unspecified 'fungal', 'bacterial' and 'viral' infections, 'don't know' and 'none' were included as separate categories.

Between 10-20% of respondents across all shop types reported having no knowledge of amphibian diseases, and an equivalent number postulated disease did not affect captive animals at all. Unspecified fungal infections were deemed most important to wild amphibians by the aquatics shop community, and between 12-30% of all shops recognised their effect on captive animals. Bacterial infections were the only class to be more frequently regarded as a risk to captive, rather than wild amphibians.

Chytridiomycosis was the most commonly identified specific disease, and was considered to affect wild populations to a greater extent than captive amphibians. Other conditions and pathogens were reported infrequently with no obvious pattern between shop type or amphibian origin (wild or captive).

There was an eclectic array of responses falling into the 'other' category, which did not necessarily relate to disease, but are included here as they could cause morbidity or mortality. The majority related to husbandry failings resulting in physiological problems in captive animals, and environmental disturbances for wild amphibians.

The poor response rate for this question prevented statistical analysis of the data, and interpretation of the figures should acknowledge the low sample size for all shop types.

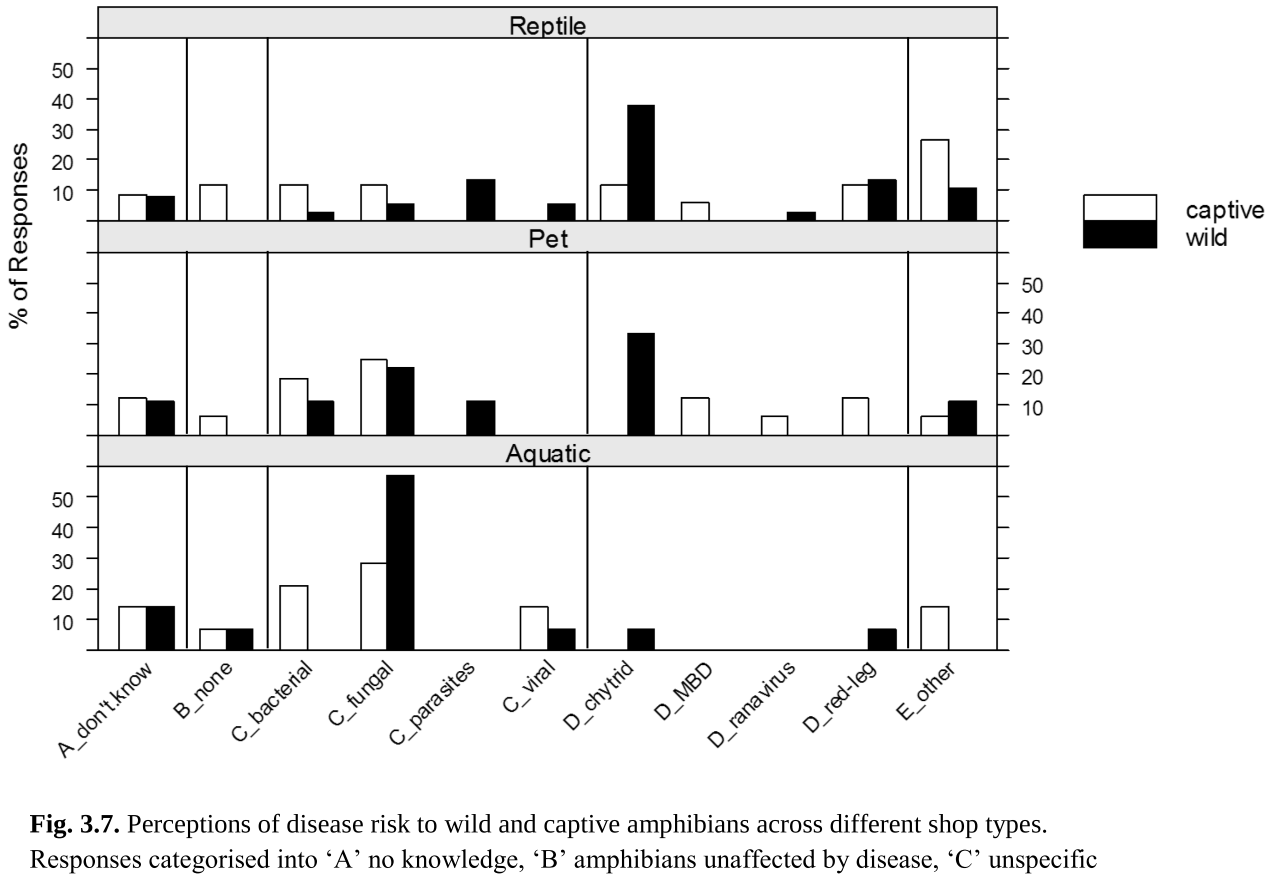


Fig. 3.7. Perceptions of disease risk to wild and captive amphibians across different shop types. Responses categorised into 'A' no knowledge, 'B' amphibians unaffected by disease, 'C' unspecific condition, 'D' specific disease, 'E' other responses mainly relating to husbandry inadequacies, nutritional deficiencies and environmental contaminants. Sample size: Reptile shops 32/52 wild, 29/52 captive; Pet shops 9/25 wild, 13/25 captive; Aquatics shops 13/38 wild 12/38 captive.

Despite the lack of confidence in reporting disease knowledge, 97% of respondents detailed their sources of information regarding disease (Fig. 3.8)

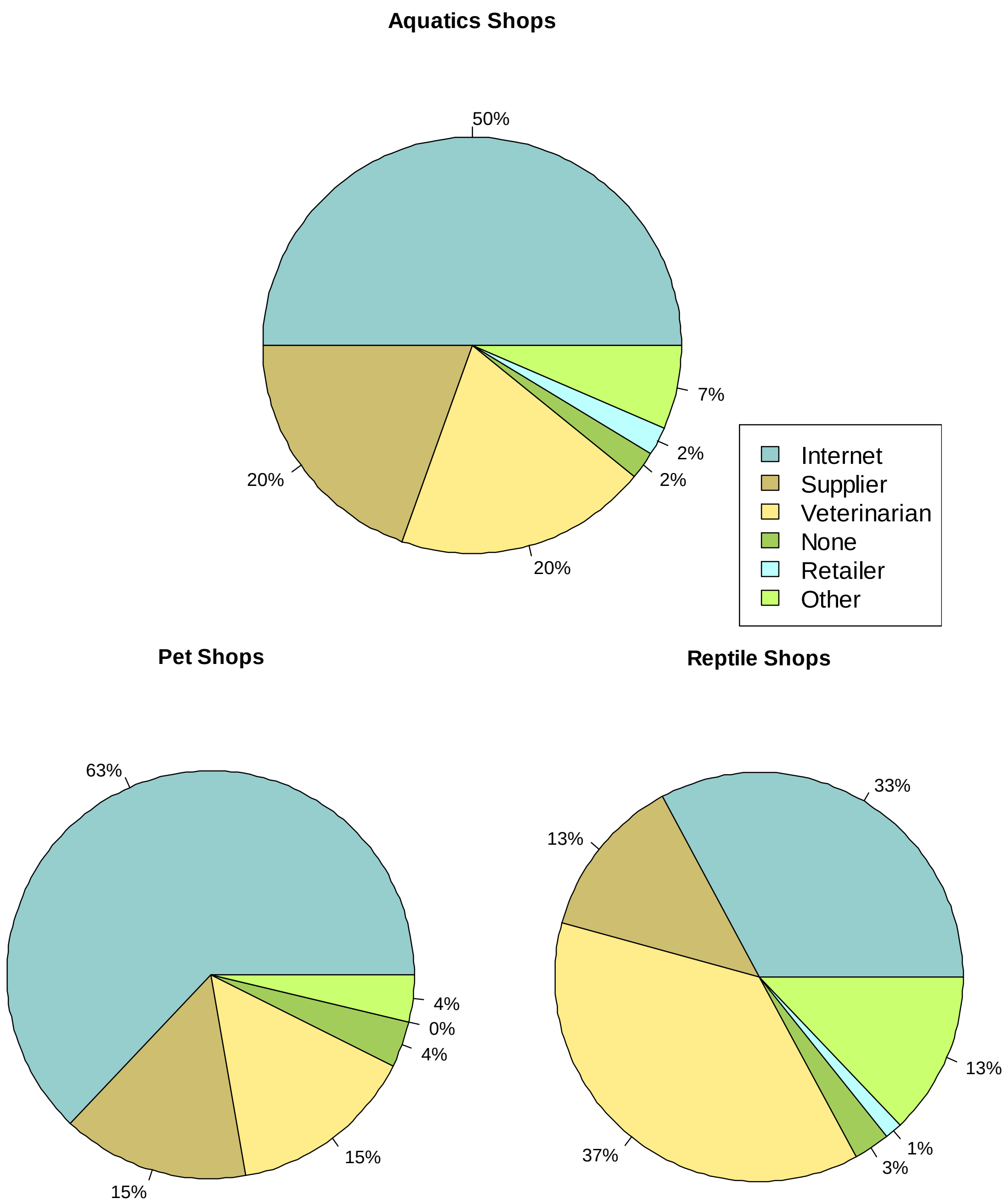


Fig. 3.8. Sources of disease information utilised by amphibian retailers.

There was a significant difference in the sources of disease information between all retailer types (Friedman ANOVA: $\chi^2 = 10.3$, df = 2, $p < 0.01$. Post hoc test: ('friedman ', R package 'agricolae' 2014) aquatic-pet $p < 0.01$, reptile-pet $p < 0.01$, aquatic-reptile $p < 0.05$). Overall the majority of information regarding amphibian health was sought from the internet, with the exception of reptile stores, that more commonly referred to veterinarians. Conversational evidence revealed there was often significant disparity in the confidence and trust afforded to veterinarians between different shops, especially in more general pet stores. Suppliers were considered an important source of disease information for all shop types, and were generally trusted in their competency and considered convenient given the frequent contact between retailer and supplier. Reptile shops used the most diverse information sources including; books, personal experience, hobbyists and customers, and local herpetological society. Less than 5% of shops sought no advice or information regarding disease, suggesting that amphibian health is a topic of interest for retailers. This was reiterated in the mortality section where only one aquatics shop did not respond to the question regarding recent mortality in amphibians. The percentage of shops experiencing amphibian deaths in the last two years ranged from 16% in pet shops, 35% in aquatics, to 52% of reptile retailers. Establishments were asked to describe any amphibian mortality they had experienced over the preceding 2 years, the results of which are summarised in Table 3.6.

In the majority of cases, explanations of mortalities were vague or the aetiology unknown, and on only two occasions specific diseases were reported. Although there appears to be a relatively small number of mortalities, due to the nature of the question this is undoubtedly an underestimate of the true figure, and it is probable that a proportion of the descriptions given are unverified suppositions. However, amphibian mortality clearly occurs across a range of species (>17), and in all types of retail establishment.

Three of the seven models tested to investigate factors influencing mortality fell within $\Delta 6$ (delta) AICc units of the optimum model (Table 3.7). These models contained three of the six factors hypothesised to influence mortality; type of pet shop, restocking method, and species diversity (number of species in stock). Model 3 contained all factors, and was discarded in consideration of the 'nesting rule', which advocates removal of more complex forms of models that attain a lower AICc value (model 5) (Grueber et al., 2011). Model 1 was subsequently rejected on the basis of low model weight combined with AICc value nearing Δ6. Model 5 contained 'species diversity' and 'restocking' variables, and was chosen as best-supported by the data.

Table 3.6

Description of mortality events reported by retailers.

Shop Type	No. Individuals and Species		Description
Aquatics	*4*	*Taricha rivularis*	Arrived in poor condition, bacterial infection suspected.
		Xenopus laevis	Delay in transit. 20% mortality - bacterial infection.
	50	*Cynops orientalis*	Poor packing conditions, from China.
		Xenopus laevis	Poor water quality.
		Litorea caerulea	Red leg.
		Hymenochirus boettgeri	Wild caught individuals occasionally die of anorexia.
	2/6	*Litorea caerulea*	Juveniles died from anorexia.
		Not stated	5 accounts of mortalities cause unknown, dead on arrival (DOA).
Pet	*2*	*C.orientalis, 3 Hyla cinerea*	Unknown cause.
		C.orientalis and H.boettgeri	Fungal infection.
		Not stated	Damaged in transit, bacterial infection.
	1 *1*	*H.cinerea* *Ceratophrys cranwelli*	Old age. Impaction.
Reptile	*3*	*Trachycephalus resinifictrix*	Unknown cause.
		L.caerulea	Wild caught, had internal and external parasites.
	2 *1*	*Dendrobates leucomelas CB* *Dendrobates* sp.	Old age. Anorexia.
		Ceratophrys sp.	Metabolic bone disease.
	2	*Salamandra salamandra*	Sudden death, cause unknown.
		T.resinifictrix, L.caerulea	Fungal infection.
	2	*Agalychnis callidryas*	Unknown cause.
	6	*Bufo debilis*	Unknown cause.
	2 *1*	*Litorea infrafrenata* *Polypedates leucomystax*	Bacterial infection. Unknown cause.
	3/6	*T.resinifictrix*	Bacterial infection.
		Leptopelis vermiculatus	Anorexia.
		Pyxicephalus adspersus	Unknown cause.
		Not stated	6 accounts: 3 DOA's, 2 husbandry, 1 transport problems.

Table 3.7.

A comparison of seven models investigating predictors of mortality in trade amphibians, in order of support for the data (best fitting model at the top). Retained models shaded. The five models below the dashed line were discarded, as they returned AICc values greater than six AICc units difference from the best supported model. As model 5 was a simplification of model 3, but was better supported, model 3 was discarded. Md: Model, Int: Intercept, Sp.Div: Number of species sold, Rstk: Restocking method, Sup: Number of suppliers, TotVl: Total number of amphibians, df: degrees of freedom, logLik: log likelihood.

Md	Int	Type	SpDiv	RStk	Exp	Sup	TotVl	df	logLik	AICc	Δ	wght
5	0.178		0.1335	-1.49				3	-61.752	129.8	0	0.51
3	0.2426	+	0.09295	-1.62				5	-59.840	130.3	0.56	0.384
1	-0.459	+						3	-64.618	135.5	5.73	0.029
2	-0.770	+	0.08391					4	-63.753	135.9	6.17	0.023
6	-1.082		0.09065			0.0813		3	-65.088	136.4	6.67	0.018
Null	-0.405							1	-67.301	136.6	6.89	0.016
7	-1.138		0.05297			0.07762	0.01063	4	-64.485	137.4	7.64	0.011
4	-0.210				-0.0192			2	-67.000	138.1	8.37	0.008

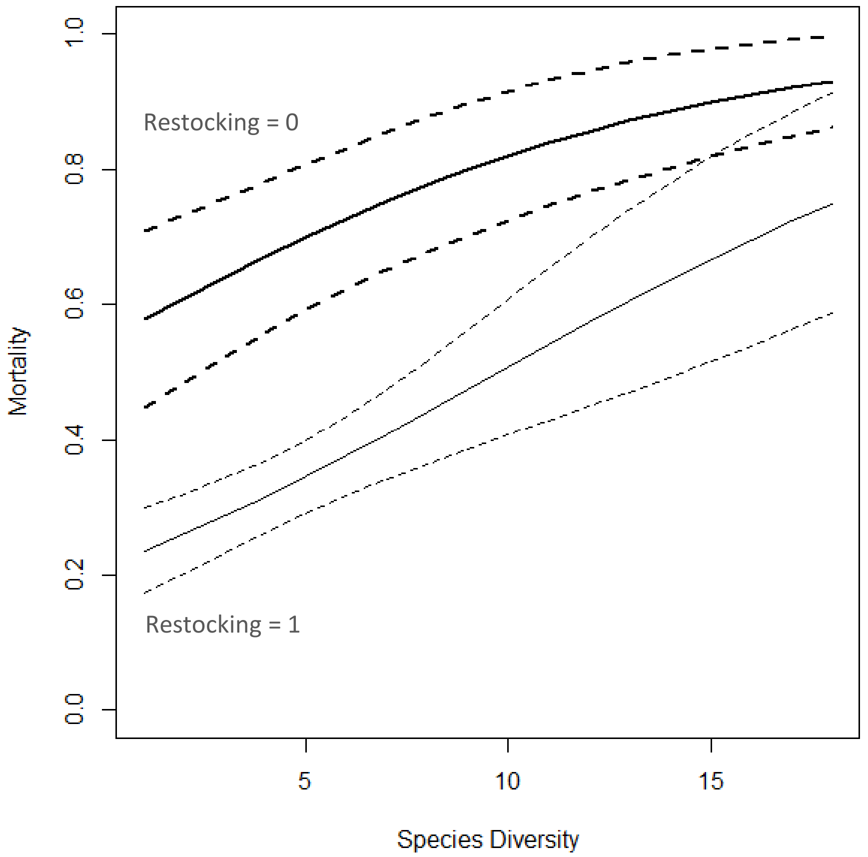


Fig. 3.9. Graph illustrating the effect of species diversity within retailer, and restocking method (0 = newly acquired amphibians are added to current stock, 1 = new stock are housed separately) on mortality. Dotted lines show the SE. Mortality increases with species diversity and is higher in retailers that 'top up' amphibian stock.

The effect of type of retailer on mortality likelihood was unclear from this data, but 'total volume of amphibians', 'number of years selling amphibians', and 'number of suppliers used', were not important for predicting mortality in these models. However, retail establishments that restock by adding newly acquired amphibians to existing stock had an increased likelihood of mortality, compared to those that housed new stock separately; and mortality events are positively correlated with number of species in stock (Fig. 3.9). At high species diversity, the effect of restocking method becomes less discernible.

(iv) Is the trade in amphibian likely to increase in the future?

The mean number of years amphibians had been stocked at individual retailers was 10.7 years, suggesting the amphibian trade was well-established across all shop types. Opinion regarding the popularity of amphibians as pets was consistent between shop types (Fisher's Exact Test: $p = 0.680$). Over 50% of respondents believed there had been no change in the popularity of amphibians as pets during their time in the trade, and a third considered the popularity of amphibians to be increasing. As pet retail is entirely customer demand driven, the popularity of amphibians as pets over time can be inferred by the number of retail outlets. The requirement for all livestock selling pet shops to obtain a licence from their local council allows investigation of these trends. Data were requested on number of pet shops licenced, and number of these permitted to sell amphibians for three years (2000, 2005, 2010), from all UK local authorities.

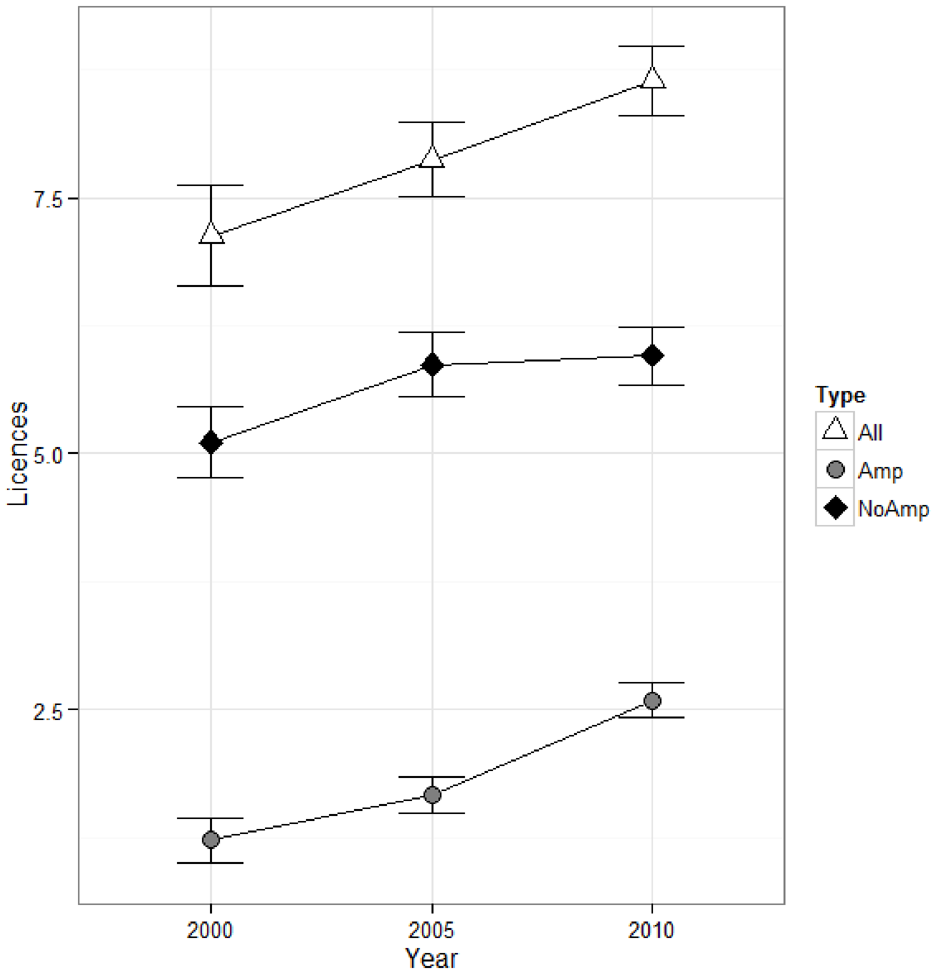


Fig. 3.10. Mean number of pet shop licences by local councils across the UK in the years 2000, 2005 and 2010. The open triangles represent the mean number of licences issued for all livestock retail, the grey circles represent those that permit the sale of amphibians, black diamonds represent those that do not permit the sale of amphibians. Standard errors are indicated by the vertical bars.

An increase in the mean number of pet shop licences issues between 2000 and 2010 (Fig. 3.10) is indicative of growth in the pet trade sector over that time. The rate of increase in licences issued allowing the sale of amphibians, was significantly higher than those not permitting amphibian sales (Aov: deviance -72.9, df = -2, $p < 0.001$); this appears to be especially prominent in between 2005 and 2010. Although an overall increase has been shown, data on too few years were available to make future predictions on number of livestock or amphibian retailers in the UK.

Discussion

Amphibians are sold in approximately 30% of livestock (pet) retailers, from specialist reptile and aquatics shops, to generalist pet shops and garden centres, generating at least £3 million per annum in the UK. Whilst direct comparison is not possible, as income derived from accessories and live food etc. was not included in this study, amphibians appear to comprise a small proportion of the overall pet trade, valued at £5.9 billion in 2010 (European Union Association of Reptile Keepers, 2012). A large proportion of this overall trade value is, however, likely to be attributable to a small number of large chain-stores, such as ‘Pets at Home’ that reported £500 million in 2012 (Pets at Home, 2012). In this study amphibian retail was found to be more prominent in smaller, privately owned businesses, particularly those in the reptile sector, where amphibian derived income was highest.

The number of species recorded in reptile shops was over double that seen in pet or aquatics shops, and stock tended to consist of a few individuals of several species, in addition to the core species seen across all shop types. Three amphibian families (Hylidae, Dendrobatidae and Salamandridae) were over-represented in terms of species numbers seen in the trade, a feature also identified in the trade in Texas, USA (Prestridge et al., 2011). Unsustainable harvesting has been implicated in population declines of a number of amphibian species for example Kaiser’s spotted newt (*Neurergus kaiseri*) (Nijman and Shepherd, 2011a) and the Santa Fe frog (*Leptodactylus laticeps*) (Cortez et al., 2004). Notably, the axolotl (*A. mexicanum*) was the only species regularly stocked in UK pet shops, listed as threatened by the IUCN. However, well established breeding facilities both in the UK and abroad supply the trade exclusively; arguably aiding the species’ survival in light of their demise in the wild. Other threatened species were seen sporadically and in very low numbers, but unfortunately, too few retailers reported the captive-bred or wild-caught status of their stock to enable analysis of the potential impact of UK trade on wild populations.

Amphibian stock in aquatics shops was dominated by Congo frogs (*H. boettgeri*), and African clawed frogs (*X. laevis*). The high volume and turnover of these species are thought to be satisfied through supply from captive breeding farms, mainly in Asia. The volume of *Xenopus* is a particular concern as it is a highly invasive species, known to have established in several countries (Measey et al 2012, and references therein), including the UK (Cunningham et al., 2005). Invasive populations of *X. laevis* have been shown to have detrimental effects on native amphibians (Lillo et al., 2011), and have also been implicated as one of the primary vectors for the global dissemination of *Batrachochytrium dendrobatidis* (Weldon et al., 2004).

Amphibian welfare was not explicitly evaluated in this study, but descriptions of routine husbandry protocols in retail premises and additional comments pertaining to suitable vivarium set-up, indicate that welfare is a fundamental concern for most establishments. There was also a strong preference for captive bred rather than wild caught animals, this was based on ethical, as well as customer demand and quality of livestock. Opponents of the trade have long used ‘high mortality levels’ as an argument to push for a ban on the exotic pet trade. In this study reported mortality was low across all shop types in this sector, and observations made during pet shop visits supported these statements. Of the described mortalities however, a substantial proportion could not be attributed to a specific cause, and no attempt to determine cause was documented. Analyses of coincidental factors revealed high species diversity increased the likelihood of mortality events. This may be an indication that, with more obscure species, specific husbandry requirements are less well understood (Schuppli and Fraser, 2000), resulting in sub-optimum living conditions: rather than an artefact of higher total stock volume, which was not found to be important. The ‘top-up’ method of restocking was also found to increase observed mortality, which intuitively suggests sanitary or disease related issue. The levels of disease awareness and knowledge varied markedly between individual retailers, and are probably best described as ‘misguided’. With very few exceptions, amphibian retailers showed genuine interest in amphibian health, however most sought information primarily from the internet. The reliability of on-line resources to provide accurate information regarding amphibian husbandry and disease has not been assessed, but studies on human medical web-sites have shown quality and accuracy to be highly variable between sources (Craigie et al., 2002, Benigeri and Pluye, 2003). Pathogens such as *B. dendrobatidis* and ranavirus have been detected in pet trade amphibians worldwide (Schloegel et al., 2009, Spitzen-van der Sluijs et al., 2011, Gower et al., 2013, Kolby, 2014, Chapter 4), thus improving awareness of amphibian disease in the retail sector is vital to ensure the welfare of captive animals and prevent dissemination into wild populations. However, communication between amphibian researchers and stakeholders is hindered by current pet shop legislation. In order to sell vertebrates, pet shops are required under the Pet Animals Act 1951 to obtain a licence. Licences are independently issued by local authorities (of which there are nearly 400 in the UK), and the requirements for and information retained by councils varies significantly. Lack of consistency in licensing makes it impossible to determine the number, or identity of amphibian retailers, without first undertaking labour intensive surveys. This issue could be addressed by the formation of a single governing body to which livestock retailers are obliged to belong, a notion supported by trade representatives (C. Newman, pers. comm.). Closer monitoring of pet shops, specifically those that sell exotic animals will become more important in the future if the trade continues to grow. Whilst the number of amphibian retailers appears to have increased between 2000 and 2010, the recent number, complement, and prices of species recorded, was remarkably similar to a study analysing the trade in 2004-2005 (Tapley et al., 2011); indicating a possible increase in volume rather than species diversity.

This study has shown that, despite contributing a small proportion of overall income, amphibian trade is widespread in the UK livestock (pet) trade, and common in smaller, independent businesses. Whilst the majority of sales comprise a small number of ‘core’ species, over 120 species were recorded for sale, the highest diversity seen in reptile shops. It was not possible to assess the impact of the UK trade on wider conservation issues such as over-harvesting, as too few retailers reported the captive-bred or wild-caught status of their stock. This aspect of the trade requires further investigation, as significant acquisition of stock from the wild coupled with future growth of the trade could dramatically increase pressure on wild populations. Additionally, further studies should also consider on-line retail, informal trade and the exchange of amphibians at herpetological shows, as there is evidence that these trade sectors constitute a different, complement of species (Prestridge et al., 2011, pers. obs.). It appears mortality is uncommon in retail outlets, but likelihood increases with species diversity or ‘top up’ restocking methods. This, combined with poor disease awareness and knowledge, highlights the importance of increased communication between stakeholders and amphibian researchers. Trade organisations such as Reptile and Exotic Pet Trade Association (REPTA), and the Ornamental Aquatic Trade association (OATA), may be fundamental in facilitating such communications.

CHAPTER 4

Factors associated with infection by *Batrachochytrium dendrobatidis* in the UK amphibian pet trade

The Calaveras County Frog Jump Competition is believed to have started thanks to the story telling of a man named Mark Twain in the mid 1800's, and still occurs on the third weekend in May today. Competitors 'jockey' their bullfrogs in a bid to out-jump the other frogs. Rules for the modern day competition stipulate amphibians should be returned to where they were caught, to prevent the spread of disease. Image: http://decktheholidays.blogspot.co.uk/2014/04/the-calaveras-county-frog-jump-from.html

Abstract

Bd was first detected in wild UK amphibians in 2004, and recent surveys have revealed a discontinuous but widespread distribution. The hypothesis that *Bd* is spread through international trade has been supported by its detection in amphibian imports, but there have been no widespread surveys conducted post-import. In this study, a UK-wide retailer *Bd* survey, conducted between September 2011 and March 2013, of 148 shops that resulted in over 2000 samples, revealed an overall prevalence of 5.8%. However, infection was uneven taxonomically and geographically. Axolotls and fire-bellied toads were found to be proportionally over-infected, as were come counties e.g. London and Gloucestershire. It is suggested that regional factors, such as stock bred locally, may influence *Bd* infection in certain areas, and potential mitigation practices are discussed.

Introduction

The global declines of amphibians have been a significant cause for concern over the last couple of decades, and the driving factors continue to command a great deal of research. Synonymous to most vertebrate taxa, amphibian biodiversity is primarily threatened by habitat loss (Baillie et al., 2004), in addition to which, climate change, pollution, over-exploitation and emerging infectious disease are also considered important factors (Collins and Storfer, 2003, Stuart et al., 2004, Beebee and Griffiths, 2005, Blaustein et al., 2011). Unlike other taxa however, disease appears to be playing a particularly prominent role in the decline of amphibians, specifically the fungal pathogen *Batrachochytium dendrobatidis* (*Bd*). *Batrachochytium dendrobatidis* is considered a generalist pathogen capable of infecting over 400 species (Aanensen and Fisher, 2014), it has been detected in 56 countries (Olson et al., 2012) and has been implicated in several 'enigmatic declines' observed worldwide (Berger et al., 1998, Lips, 1999, Puschendorf et al., 2006, Lotters et al., 2009).

The trade in live amphibians has been repeatedly implicated in disseminating *Bd*, at local, national and international scales (Mazzoni et al., 2003, Fisher and Garner, 2007, Picco et al., 2008, Schoelgel et al., 2009, Catenazzi et al., 2010, Kolby et al., 2014). Whilst this is intuitively plausible, direct evidence of trade mediated wild amphibian infection has yet to be reported. Globally, the trade in amphibians is of considerable scale (Schaellfer et al., 2005), and varied in purpose, although the food, scientific research and pet trades account for the majority of the trade. In the UK, the food trade is perhaps less important, and amphibians are mainly utilised in scientific research, the predominant species being *Xenopus laevis* and *X. tropicalis* (O'Rourke, 2007), and the pet trade where over 100 species have been recorded (Chapter 3). Amphibians are routinely sold in approximately 30% of pet livestock retailers in the UK, and stock originates from a variety of sources including local breeders and suppliers, to wholesalers acquiring animals via international imports (Chapter 2 & 3).

Several hypotheses have been formulated regarding the global emergence and subsequent spread of *Bd*, and these have been comprehensively reviewed by Daszak et al. (2003), Rachowicz et al. (2005), Skerratt et al. (2007), Young et al. (2007). *Batrachochytrium dendrobatidis* was first detected in wild British amphibians in 2004 (Cunningham et al., 2005), however introduction of the pathogen is likely to pre-date detection (Minting, 2012). Mass mortality events caused by *Bd* seen in mainland Europe (Bosch et al., 2001, Garner et al., 2005, Bovero et al., 2008, Bielby et al., 2009, Rosa et al., 2013) and other parts of the world (Lips et al., 2005, Kriger and Hero, 2007, Woodhams et al., 2008, Kolby et al., 2010), have not been described in the UK, but Minting (2012) suggests less noticeable effects of *Bd* on populations could have gone unnoticed. Two nationwide surveys of the UK have taken place, in 2008 (Cunningham and Minting, 2008) and 2011 (Smith, 2013), both indicating the distribution of *Bd* is "widespread but patchy" (A. Cunningham pers. comm.). Spatially disjunct patterns of infection do not suggest the dispersal of a recently introduced pathogen, which typically present with an 'infection-front' that proceeds in a wave-like pattern (e.g. Lips et al., 2006, 2008); but rather long-term endemicity in an environment where suitable conditions or species are heterogenic, or recurring pathogen introduction from some spatially heterogeneous source e.g. amphibian retail outlets.

The potential for *Bd* to be disseminated from captive to wild amphibian populations was considered by Peel, (2012), who identified four possible transfer pathways:

1) The illegal/accidental release of live infected amphibians.
2) The legal release of live amphibians that may be infected.
3) The release of contaminated water.
4) Release via contaminated equipment, soil or biological samples.

Under the Wildlife and Countryside Act 1981, it is illegal to release any non-native species, unless under specific licence, therefore the 'legal release' pathway is relevant only to conservation re-introductions and mitigation translocations, rather than domestic trade. Contaminated equipment and soil, is again more commonly associated with scientific activities, except perhaps through the disposal of vivarium substrate. The dissemination of *Bd* from domestic trade in amphibians to wild populations could occur however, via illegal release of live animals or contaminated water. For example, non-native amphibian populations have been recorded in the UK for: *Mesotriton alpestris* (Beebee and Griffiths, 2000, Bond and Haycock, 2008), *Lithobates catesbeianus* (Cunningham et al., 2005), and *X. laevis* (Measey, 1998). These introductions are likely the result of escapes or deliberate releases. The detection of *Bd* in these feral populations, together with evidence of disease dissemination through introduction of other non-native aquatic taxa (Peeler et al., 2011), strengthens support for the hypothesis that non-native species introduction pose a significant risk in terms of disease spread. It is unclear to what extent retailers or hobbyists dispose of untreated water from vivaria, directly into the environment, but if it does occur, then *Bd* dissemination is probable (Peel et al., 2012).

Chapter 2 of this thesis revealed the importation of amphibians from at least 11 countries into the UK, all of which, except Madagascar (but see: Kolby, 2014), have confirmed *Bd* infected wild amphibian populations, where surveillance has taken place (*Bd*-Maps: www.*Bd*-maps.net). The infection prevalence in imported amphibians was 3.6%, although, as only 'reptile' consignments were sampled this figure may well underestimate the true prevalence. Combined with findings that amphibian retailers have limited access to reliable disease information, and consequently relatively poor disease knowledge and awareness (Chapter 3), it is vital to assess *Bd* infection situation in the trade. Amphibian retail establishments are the final stage in the trade chain, and the last opportunity to monitor disease, prior to animals becoming part of a private collection. This trade sector therefore represents an 'interface' between captive and wild amphibian populations.

The aims for this study were to test the hypotheses that:

1) Assuming a similar ratio of shop types (and therefore a consistent species composition) between counties, the proportion of *Bd* infected captive amphibians is homogeneous across the UK.

2) Given variation in susceptibility to *Bd* infection in the natural environment can be attributed to any number of extraneous variables, including, specific 'life-histories' and 'environmental conditions' (Searle et al., 2011); and analogous factors such as 'species' or 'vivarium habitat', will influence the likelihood of infection between, i) individual shops, ii) vivaria, and iii) samples.

Methods

A list of all local authority contact details was downloaded from the government website http://local.direct.gov.uk/Data/ (accessed 21/04/11). A request for information regarding the number of pet shops licences issued in 2000, 2005 and 2010, and the number of these permitted to sell amphibians was submitted to each of the local authorities under The Freedom of Information Act 2000 via email or council websites. Councils are obliged by law to reply within 20 working days or receipt of request. Councils that took longer than 1 week to acknowledge receipt of 4 weeks to reply were emailed again. Repeated inaction was reported to the Information Commissioners Office (ICO) which in all but one case prompted reply. This revealed 3275 pet shop licences had been issued in 2010, 982 (30%) of which allowed the sale of amphibians. Information from the telephone survey suggests this is a reasonable estimate of amphibian retailers.

- *Sample size calculation*

Epidemiological sample size calculation relies on prior knowledge, or an estimate of infection prevalence (Fosgate, 2009). As this information was not available for the prevalence of *Bd* in trade amphibians in the UK, a pilot study was undertaken. Amphibian retailers were contacted by telephone and permission was sought to sample their amphibian stock for *Bd*. All the amphibians in stock were swabbed following the protocol described in Chapter 2, and the species, number of individuals in the vivaria, and total number of vivarium in each shop were recorded. A total of forty retailers were included in the pilot study. This initial survey revealed that:

1) When infection was detected, approximately 50% of amphibians in a vivarium would test positive ($\bar{x}$ = 54.11, 95% CI = [39.4, 68.8]).
2) The mean number of amphibians in a single vivarium was 4.7 (95% CI = [3.45, 5.95]).
3) Infection was detected in 8 (20%) of shops.

The distribution of amphibians in the pet trade is clustered over three levels: individuals are grouped within vivarium, vivaria are grouped within shops, and shops are spatially clustered with geographic areas. As the number of amphibians in a vivarium, and number of vivaria in a shop was generally low, all amphibians within a selected shop were swabbed, rather than using a pre-determined ‘individual swab’ sample size. For the purposes of simplifying the study design, the ‘shop’ was then considered as an individual unit, and a target number of shops calculated.

For convenience, an on-line Cannon and Roe table (http://www.scri*Bd*.com/doc/7215883/Sample-Sizes-Cannon-Roe) was used to calculate the number of shops required to estimate the prevalence of *Bd* infected shops, given a population size of 1000 (approximated number of amphibian retailers in the UK), and an assumed prevalence of 20% (percentage of infected shops). To be 95% confident that the true proportion of infected shops would be within +/- 5% of the estimated prevalence, a sample size of 197 was required.

- *Sampling amphibian retailers*

To ensure a representative sample was taken over the UK, county data were used to determine a target number per county based on the relative densities of amphibian retailers between counties (Fig 4.1., details in Appendix 4). County level was chosen as an appropriate scale for displaying results as it is well above the minimum scale of 10 km^2 permitted for the disclosure of commercially sensitive data (Copp et al., 2007), and it was logistically easier to display graphically in a meaningful way.

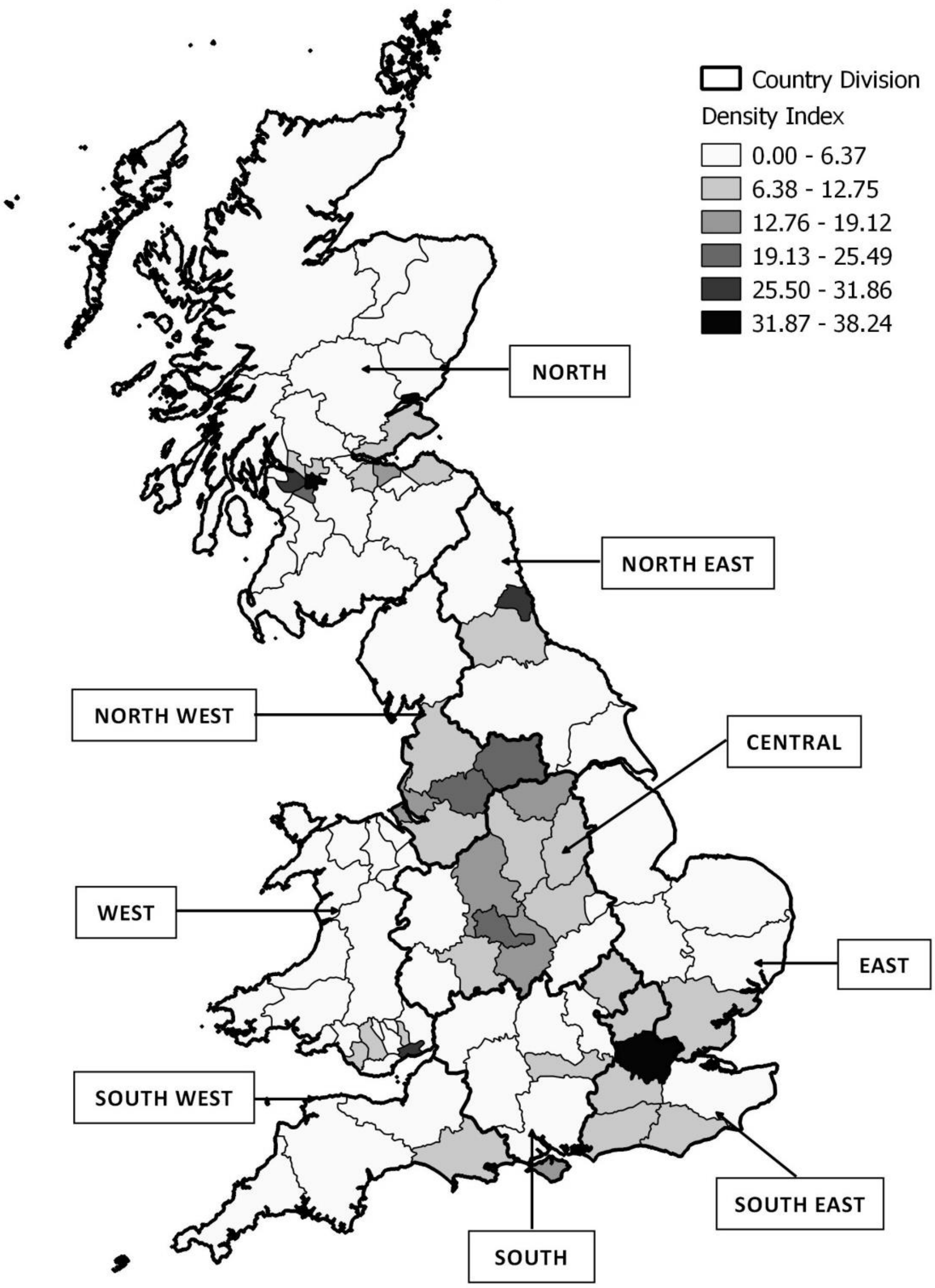
Having identified the target number of pet shops, the pet shop database compiled for Chapter 3 was consulted to get details of potential participants. In addition, a Google search, using ‘reptile shop’, ‘aquatic shop’, or ‘pet shop’, followed by the county name e.g. “reptile shop Cambridgeshire”, was conducted, to ensure no new businesses were missed. Retailers were telephoned, and the presence of amphibians confirmed. The study was explained to amphibian retailers, who were then asked for permission to have their animals screened for *Bd*. A mutually convenient date and approximate time for a visit was then decided with consenting pet shops, who were contacted again, either the day before or morning of, the appointment to confirm. Attempts were made to sample a mix of shop types (reptile, aquatic and pet), from each county.

Sampling was performed by the author with assistance from five students and two colleagues. Training was given prior to shop visits at Institute of Zoology (IoZ), and the students were accompanied on the first few shop visits to ensure the same techniques were adopted by all students.

For logistical reasons students were assigned to specific country divisions e.g. student A + author, to sample ‘North-West’ counties (Fig 4.1). The swabbing procedure was explained and questions from proprietors answered prior to sampling. Proprietors were asked if they could complete a questionnaire, if they had not done so already.

All amphibians in the shop were swabbed, up to a maximum of 30 per vivarium, according to the protocol described in Chapter 2. Where there were several animals in a vivarium, animals were temporarily housed in separate boxes after swabbing so individuals were not sampled more than once, or missed. Gloves were used and changed between individuals, except when retailers requested no gloves to be worn, in which case hands were washed between individuals. The stock information and sample details were recorded before leaving the establishment.

A separate, sealed, plastic bag was used for each shop to store the swabs and data sheet. Samples were transported in a cool-box with ice-packs, to the IoZ, where they were stored refrigerated at 4°C until processing. Samples were analysed at IoZ using the RT-PCR protocol described in Chapter 2.



	North	N.East	N.West	Central	East	West	S.East	S.West	South
No. of shops	100	72	158	209	102	26	139	76	96
Target sample	16	14	32	41	21	5	28	15	20

Fig. 4.1. Map of the UK (excluding Northern Ireland and the Shetland Islands). The nine country divisions demarcate the county groupings used to determine targeted sample size (number of amphibian retailers) for the UK *Bd* retailer survey. The density of amphibian retailers by county is illustrated by shade intensity. Density indices were calculated as: number of amphibian retailers (No. of shops) divided by the area of county (km^2), multiplied by 1000.

- *Data analysis*

1) Is the prevalence of *Bd* uniform across the UK?

Chi-squared tests on counts of positive and negative swab samples in each county were used to investigate association between county and proportion of infected amphibians. However, the low observed overall prevalence resulted in expected counts of positive samples being less than one for several counties. Where expected values are less than one, the conventional chi-squared test is inappropriate as the sampling distribution varies from the chi-square distribution (Campbell, 2007). Fisher's Exact tests were not possible due to the high number of parameters (counties), so p-values were determined by Monte-Carlo simulation (McDonald (2009), specified by 2000 iterations (B = 2000). County prevalence (defined here as, the proportion of positive to negative samples) was illustrated on a UK map, produced using QGIS 'Valmiera' version 2.2 (QGIS Development Team, 2014).

2) What are the predictors of detection of *Bd* in different retail establishments?

Generalised linear models (GLMs) were constructed to determine whether there were any inherent characteristics of shops with detectable infection. 'Shop type' (reptile, aquatic, pet), 'No.Sp' (number of species in stock), 'Tot.amp' (total volume of amphibians in stock), and 'Cty' (County), were identified as potential explanatory factors (Table 4.1). Seven models were compared to a null (intercept only) model using the 'R' package MuMIn (Barton, 2014).

Table 4.1. Predictors of infection at retailer level

Model	Predictors	Hypothesis
1r	Type	Type of retailer (reptile, aquatic or pet) affects the likelihood of samples testing positive for *Bd*.
2r	Cty	The geographic location of the shop influences the likelihood of infection.
3r	Tot.amp	The number amphibians in stock is positively correlated with likelihood of detecting *Bd*.
4r	No.sp	The number of species in stock is positively correlated with likelihood of detecting *Bd*.
5r	Type + Tot.amp	Type of retailer affects the likelihood of detecting infection, the size of effect is determined by number of amphibians in stock.
6r	Type + No.sp	Type of retailer affects the likelihood of detecting infection, the size of effect is determined by number of species in stock.
7r	Type + Cty + No.sp	Type of retailer affects the likelihood of detecting infection, the size of effect is determined by number of species in stock.
8r	Null	Intercept only model.

3) What are the predictors of infection at an individual sample level?

The survey sampling design resulted in a three tier hierarchical data set, where samples were grouped within vivaria and then shop. To account for violation of the 'independence of sample' assumption of standard GLMs caused by the hierarchy effect, generalised linear mixed models (GLMMs) were attempted where 'Shop. No' and 'Viv No' were defined as random effects. However, due to the high number of shops, and zero inflation of the data, model fitting was problematic due to poor convergence. The data were subsequently condensed to vivarium level and models defined using only 'Shop.No' as a random effect, and models accounting for zero-inflation were attempted. Again, models failed to converge, so standard generalised linear modelling was performed, and AICc comparisons, using a delta 6 AICc threshold (Richards, 2008, Grueber et al., 2011), were performed using MuMIn package. As taxonomic information was lost during condensing the data to vivarium level, chi-squared tests were performed across species where sample size was at least 30, to investigate association between taxonomic group and infection with *Bd*. A *post-hoc* test, available from the 'polytomous' package in R (Arppe, 2013), was applied to identify cells where the observed value was significantly greater or less than the expected value.

Table 4.2: Models investigation predictors of infection at vivarium level

Model	Predictors	Hypothesis
1v	Type	Type of retailer (reptile, aquatic or pet) to which the vivarium belongs affects the likelihood of samples testing positive.
2v	Type + Tot.Viv	Type of retailer to which the vivarium belongs affects the likelihood of infection, the size of affect is determined by how many amphibians are in the vivarium.
3v	Tot.Viv + Mix.Sp	The likelihood of infection increases with increasing number of amphibians in the vivarium, this affect is influenced by whether there are single or mixed species.
4v	Tot.Viv + Hab	The likelihood of infection increases with increasing number of amphibians in the vivarium, this affect is influenced by the habitat in the vivarium (predominantly terrestrial (ter) or aquatic (aqu)).
5v	County + Tot.Viv	Geographic location of the vivarium affects the likelihood of infection, the size of affect is determined by the number of amphibians in the vivarium.
6v	County + Mix.Sp	Geographic location of the vivarium affects the likelihood of infection, the size of affect is determined by whether there are multiple species in the vivarium.
7v	County + Tot.Viv + Hab	Geographic location of the vivarium affects the likelihood of infection, the size of affect is determined by the number of amphibians and habitat type in the vivarium.
8v	Null	Intercept only model.

Results

One hundred and forty-eight amphibian retailers participated in the study, resulting in 2207 amphibian swabs (833 from aquatics shops, 282 from pet shops and 1090 from reptile shops), representing 20 families, 63 genera and 113 species.

1) Is the prevalence of *Bd* in trade amphibians uniform across the UK?

The overall UK sample prevalence was 5.8% (128/2207). The distribution of *Bd* in trade amphibians was widespread but patchy across the UK. The proportion of infected amphibians varied significantly between countries ($\chi^2 = 280.49$, B = 2000, $p < 0.001$), with some areas of high prevalence such as London and the parts of the Southwest Midlands. Although statistical analyses were not performed, there was no apparent spatial pattern to counties with different levels of infection (Fig 4.2), and there was no relation to the density of pet shops.

2) Are there any factors associated with detection of *Bd* in different retail establishments?

Batrachochytrium dendrobatidis was detected in 25 (17%, 95% CI = [11,23]) of the shop surveyed. There was no support for any of the factors hypothesised as having an effect on the likelihood of detecting *Bd* at the shop level. Whilst 'number of species' (model 4) and 'total number of amphibians' (model 3) appeared to have a positive relationship with *Bd* detection, the 'null' model is only 3.15 AICc units away from the optimum model (model 4), and was therefore rejected (Table 4.3). Within the limits of the explanatory variables, the likelihood of detecting *Bd* therefore did not differ between establishments.

Table 4.3. A comparison of seven models investigating factors associated with detection of *Bd* in amphibian retailers, in order of support for the data (best fitting model at the top). Models 4 and 3 performed marginally better than the null (intercept only) model. Three models returned AICc values within six AICc units of the best supported model, however, as this group also contained the null model, all models were subsequently rejected. Model numbers and parameters are given in Table 4.1.
Δ: Number of AICc units away from the optimum model. df: degrees of freedom. logLik: log likelihood.

Model	Intercept	Type	No. species	Total amphibians	County	df	logLik	AICc	Δ	Model weight
4	-2.178		0.1083			2	-64.612	133.3	0	0.602
3	-1.850			0.01303		2	-66.160	136.4	3.1	0.128
Null	-1.593					1	-67.217	136.5	3.15	0.124
6	-2.005	+	0.1206			4	-64.192	136.7	3.36	0.112
1	-1.466	+				3	-67.128	140.4	7.12	0.017
5	-1.808	+		0.01302		4	-66.137	140.6	7.25	0.016
7	-24.30	+	0.2977		+	49	-32.799	213.6	80.29	0.000
2	-20.57				+	46	-40.243	215.3	81.99	0.000

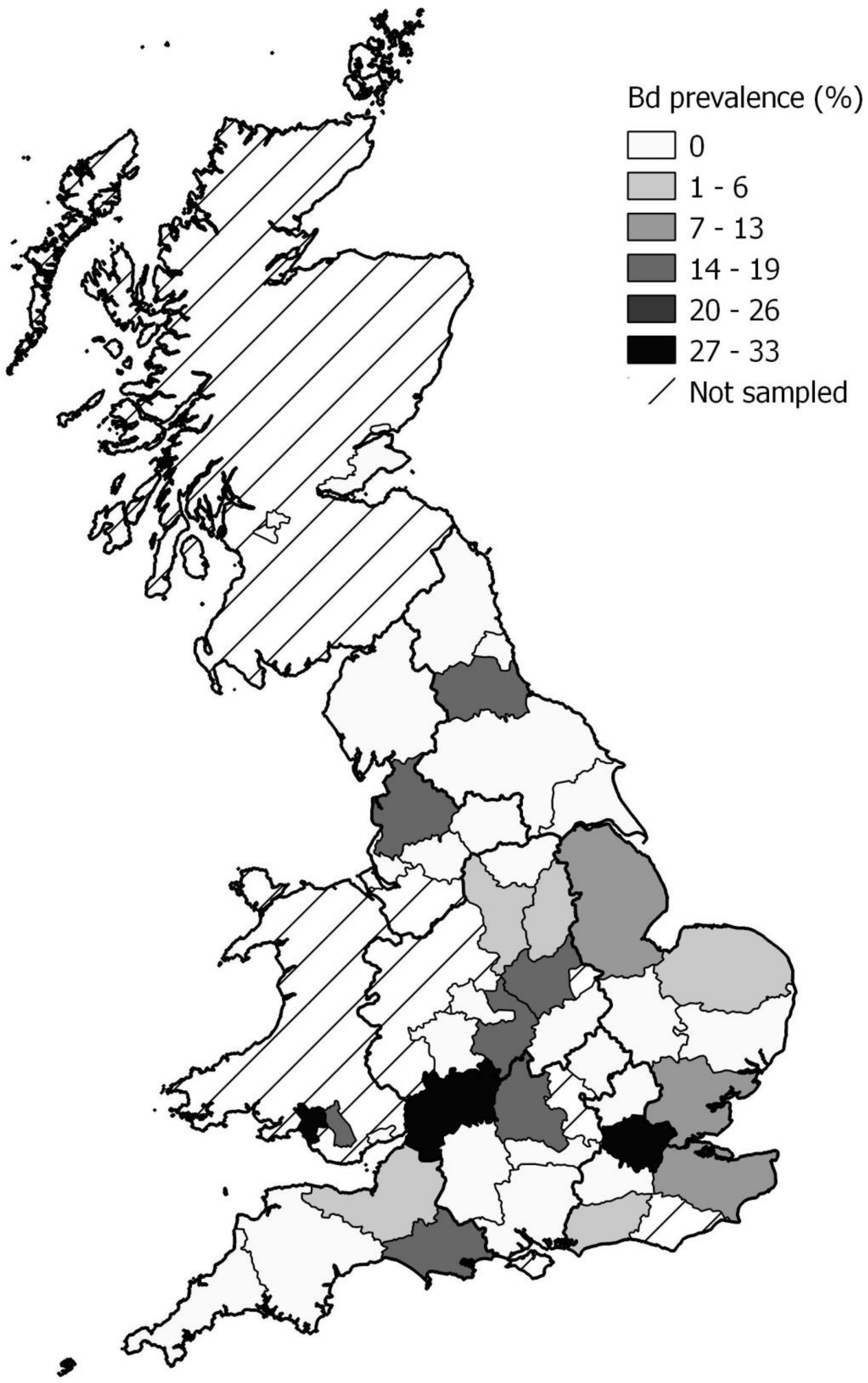


Fig. 4.2. Prevalence of *Batrachochytrium dendrobatidis* in amphibian pet trade animals calculated as the total number of positive samples per county divided by the total number of samples collected from that county.

3) Are there any predictors of infection at the vivarium level?

Overall, the proportion of infected vivaria was 6% (47/794). A single model (model 7) was retained from the set investigating factors associated with infection. This model contained three variables ‘Tot.Viv’ (total number of amphibians in the vivarium), ‘Hab’, the predominant habitat in the vivarium (either aquatic or terrestrial), and county (Table 4). Although models 5 and 4 were discarded as delta AICc>6 (Richards, 2008, Grueber et al., 2011), it is notable they contained a subset of the three variables in the retained model. The model indicates that the likelihood of detecting *Bd* in a vivarium increases with the total number of amphibians in the vivarium, the likelihood is higher in aquatic habitats and varies between counties.

Table 4.4. Comparison of seven models investigating factors associated with likelihood of detectable *Bd* infection in amphibian vivaria. Model 7 (shaded) is the optimum models and is exclusively retained as all other models fall at least 6AICc units away from this model. Model numbers and parameters are given in Table 4.2. Tot.Viv: Total number of amphibians in vivarium. MixSp. Mixed species. df: degrees of freedom logLik: log likelihood. **Δ**: Number of AICc units away from the optimum model.

Model	Intercept	Type	Tot.Viv	Mix Sp.	Habitat	County	df	logLik	AICc	Δ	weight
7	-20.06		0.08336		+	+	49	-114.026	332.6	0	0.931
5	-20.81		0.10760			+	47	-119.506	339.1	6.42	0.038
4	-2.393		0.02762		+		4	-165.682	339.4	6.78	0.031
6	-21.13			1.0580		+	47	-125.013	350.1	17.44	0.000
3	-3.169		0.05098	0.7168			3	-174.313	354.7	22.02	0.000
2	-2.909	+	0.04897				4	-173.875	355.8	23.16	0.000
Null	-2.770						1	-178.447	358.9	26.26	0.000
1	-2.440	+					3	-176.530	359.1	26.45	0.000

Overall *Bd* was detected in 23 (20%) species and the proportion of infected samples varied between <1 – 100% (Fig 4.3), however sample sizes were often very small for individual species, and in all but one case (*N. viridescens*) where prevalence was 50% or more, all the samples were derived from the same establishment.

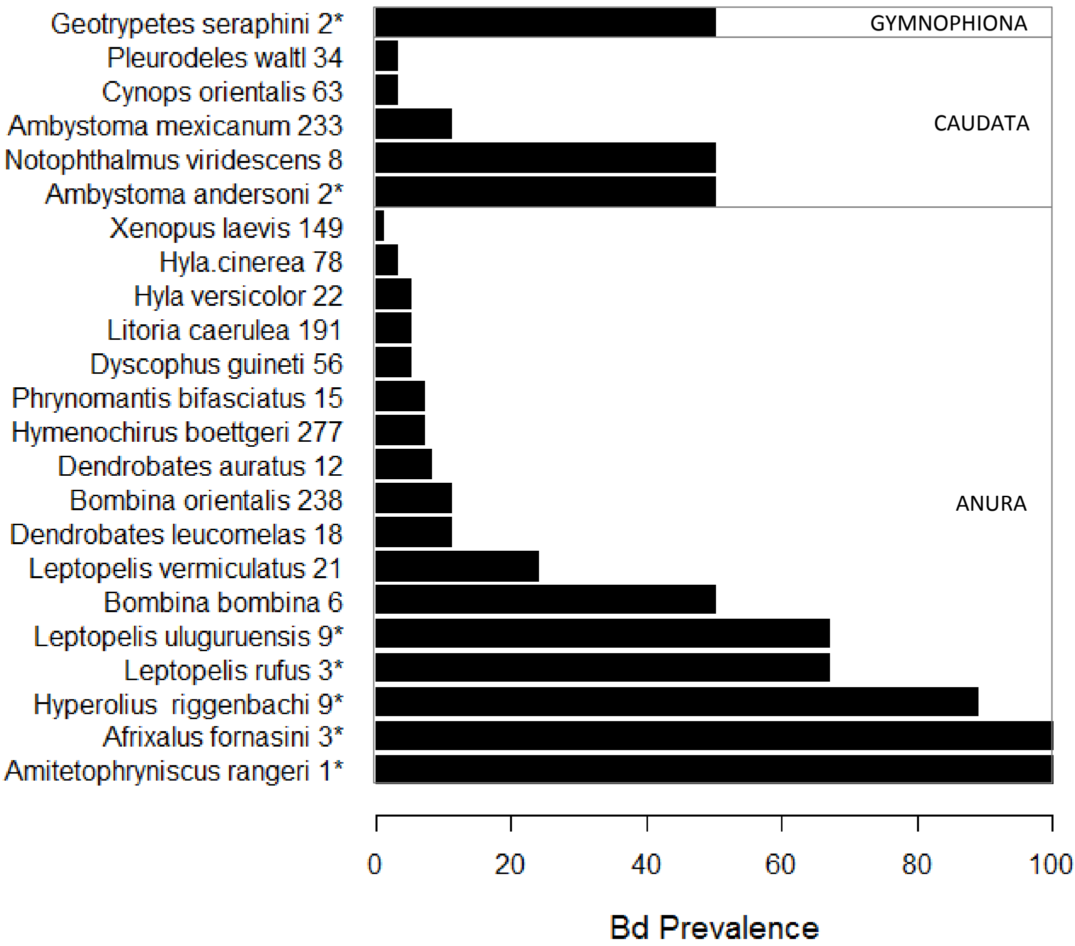


Fig. 4.3. *Bd* prevalence in species sampled in UK retailers. The number of samples analysed is indicated next to the species name. * samples derived from a single shop.

A comparison of *Bd* sample prevalence where species sample size was at least 30, revealed a significant association between species and the proportion of infected samples ($\chi^2 = 61.5$, df = 15, $p < 0.001$). *Post-hoc* analysis revealed that two species (*Ambystoma mexicanum* and *Bombina orientalis*) had a significantly higher than expected proportion of infected samples, and three species (*Ceratophrys* sp., *Trachycephalus resinifictrix* and *X. laevis*) had significantly fewer than expected positive samples (Table 4.5). Samples taken from *X. laevis*, however, frequently returned inconsistent RT-PCR results despite multiple re-runs.

Table 4.5. Results of the chi squared post hoc analysis, performed to identify significant deviations from a homogenous distribution of positive samples, among species where overall sample size was greater than 30. The values indicate the contribution of each variable to the chi-squared statistic, the sign (+ / -) indicates whether the observed value is greater or smaller than the expected value, and significant deviations ($p < 0.05$) are shaded. (*Ceratophrys* species contains *C. cranwelli* and *C. ornata* as these were often difficult to distinguish).

	Agalychnis callidryas	*Ambystoma mexicanum*	*Bombina orientalis*	*Ceratophrys species*	*Cynops orientalis*	*Dendrobates tintorius*	*Dyscophus guineti*	*Hyla cinerea*	*Hymenochirus boettgeri*	*Kaloula pulchra*	*Litoria caerulea*	*Pleurodeles waltl*	*Pyxicephalus adspersus*	*Salamandra salamandra*	*Trachycephalus Resisinifictrix*	*Xenopus laevis*
Bd Pos	-	+	+	-	-	-	+	-	+	-	-	-	-	-	-	-

Discussion

Despite *Bd* being detected in a range of species, and in all three amphibian orders, very few individuals showed clinical signs of infection and, concurrent with questionnaire replies (Chapter 3), there was very little observed mortality. Several reports of asymptomatic infection in captive animals (Goka et al., 2009, Spitzen-van der Sluijs et al., 2011, Churgin et al., 2013), suggest conditions in captivity, e.g. more stable environmental conditions, could favour the amphibian, enabling survivorship despite infection. Undetected infection in the pet trade is concerning not only as it may increase the likelihood of transmission into wild populations, but also as transmission may cause unexpected die-offs in other captive species even after considerable quarantine periods (Forzan et al., 2008).

The ability of *Bd* to infect, and the subsequent response of an amphibian to infection, varies considerably between species (Fisher et al., 2009, Garner et al., 2009c, Gahl et al., 2012, Pasmans et al., 2013), and has been demonstrated both in the field (Smith et al., 2009b), and under experimental conditions (Searle et al., 2011). These differences in responses are further complicated by intra-specific individual variation in response (e.g. Langhammer et al., 2014), although often the mechanisms behind this are unknown (Balaz et al., 2014). Here, two species, *B. orientalis* and *A. mexicanum*, were shown to have a significantly higher than expected proportion of infected individuals. High *Bd* prevalence (85%) has been previously reported in captive *A. mexicanum* in Mexico (Frías-Alvarez et al., 2008), and the lack of observed clinical signs or mortality in UK trade *A. mexicanum* was consistent with those in Mexico. *Bombina orientalis* were also asymptomatically infected, and the identification of *B. orientalis* as an important potential reservoir for *Bd* in the pet trade is supported by studies where *Bd* was detected in wild *Bombina* sp. in Europe (Ohst et al., 2013, Canestrelli et al., 2013, Voeroes et al., 2013, Balaz et al., 2014), and in captive *Bombina* sp. (Spitzen-van der Sluijs et al., 2011, Kolby et al., 2014). The identification of species that show a higher probability of infection is important, as this information could be used to develop targeted surveillance strategies (Balaz et al., 2014), that focus on taxa with a greater potential to act as a reservoir of infection. The lower than expected prevalence observed for *X. laevis* however, contradicts previous published research showing consistent infection (Solis et al., 2010, Kolby et al., 2014); indeed the historical trade of *X. laevis* is implicated as one of the primary instigators of the pathogen's global emergence (Weldon et al., 2004, Soto-Azat et al., 2010). Unfortunately, the origin or captive-bred/wild-caught status data for most of the amphibians sampled were not available, as these data may help to explain this anomaly. Inconsistencies in the output from RT-PCR analysis of samples taken specifically from *X. laevis*, deserve further investigation, as the assay used in this study may be unable to detect all strains of *Bd*.

Detection of *Bd* in less frequently encountered trade species, highlights the diversity of species posing infection risk, but predicting the likelihood of infection in these species, was not possible as small sample sizes precluded statistical analyses. Attempts to identify commonalities at broader scales (vivarium and shop level), irrespective of taxonomy revealed *Bd* was more likely to be detected in vivaria with aquatic set-ups and increased with density of amphibians. As *Bd* is an aquatic fungal pathogen (Longcore et al., 1999), that has a waterborne zoospore life-stage (Berger et al., 2005), the higher proportion of *Bd* positive amphibians occupying aquatic 'habitats' is perhaps not surprising. Similarly, a positive correlation between density and infection detection follows fundamental principles of host-pathogen dynamics and has been demonstrated experimentally in a salmon species (*Oncorhynchus tshawytscha*), infected with disease causing *Aeromonas* sp. pathogen (Ogut and Reno, 2004), and to some extent in amphibian tadpoles infected with *Bd* (Rachowicz and Briggs, 2007). It was, however, not possible to distinguish whether the increased likelihood of infection was actually a result of the aquatic habitat, or certain species (that happened to be aquatic) being predisposed to infection. The evidence that aquatic set-ups, and high density stocking of amphibians increases the likelihood of detecting infection, again helps in targeting surveillance and maximising the impact of such strategies.

In this study the proportion of positive samples was significantly higher in some counties than others, although there was no observable spatial pattern. Informal discussions with retailers revealed that most utilised the same two or three large-scale wholesalers, who supplied amphibians country-wide. Given these wholesalers appear to have a comprehensive UK distribution coverage, if they were the sole source of infection into the trade, a more geographically homogeneous *Bd* distribution would be expected. The additional introduction of *Bd* into retailers stock via local breeders offers a plausible explanation for the localised high proportion of infected animals; and is supported by the commonly infected species *B. orientalis* being commonly acquired via this route (pers. obs.). Identification of possible local sources of infection, particularly facilities regularly supplying amphibians to the trade, is vital, and assistance should be given to eradicate infection at these sites. Retailers should be made aware of the potential for new imports to asymptomatically carry infection, and the risk of novel strains entering the trade; and should be advised to, where possible, keep all species in 'quarantine' conditions.

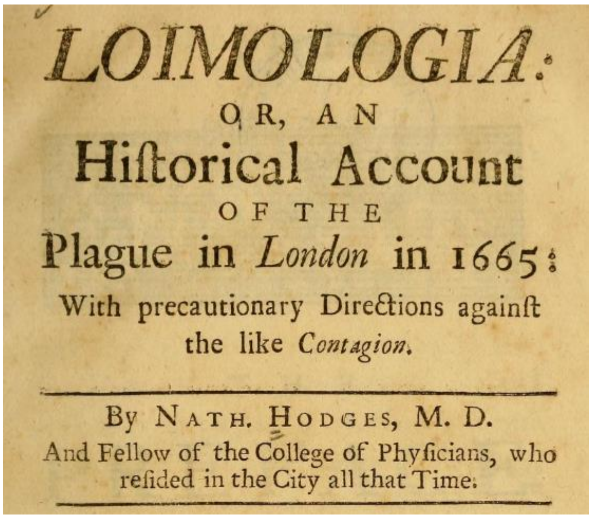
Spatial analyses comparing *Bd* distribution in wild populations and captive amphibians, would further our understanding of the role of trade in disseminating *Bd* into wild populations. Intuitively, areas of high prevalence in captive collections should correlate with sites where *Bd* has been detected in the environment, and further research is required to test this hypothesis.

On a broader scale, describing the geographic distribution of a pathogen is fundamental to understanding its epidemiology, and is a first step in identifying areas at high risk of infection. The spatial distribution of *Bd* has been intensively studied and modelled, using species distribution models (SDMs) and a suite of variables (typically environmental), in order to predict areas of high risk and guide disease management decisions (Ron, 2005, Bielby et al., 2008, Murray et al., 2011, Bielby et al., 2013). Recently, a study in Australia examined the relationship between *Bd* distribution and predicted distributions based on these models, and found SDMs underestimated the distribution of *Bd* especially in areas predicted to be environmentally 'unsuitable' (Puschendorf et al., 2013). This underestimate is explained by shortcomings of opportunistically collected data. However, data relating to trade (e.g. volume of amphibian trade, number of retailers etc.), is excluded as a variable in virtually all of these study types, which is surprising given how much emphasis is placed on trade as a major 'vector' for disease spread (Fisher and Garner, 2007, Picco et al., 2008, Schloegel et al., 2009, Catenazzi et al., 2010, Spitzen-van der Sluijs et al., 2011, Tamukai et al., 2014). As yet, only one study has included trade as a factor in the distribution of *Bd* (Liu et al., 2013), which found *Bd* to be positively associated with coarse scale trade data, as well as climatic and biotic factors. Evidence presented in this thesis indicates that *Bd* is imported into countries through trade and it is present in retail establishments where it is heterogeneously distributed geographically; incorporating trade prevalence data in SDMs, may help explain some of the inconsistencies in current modelling analyses.

CHAPTER 5

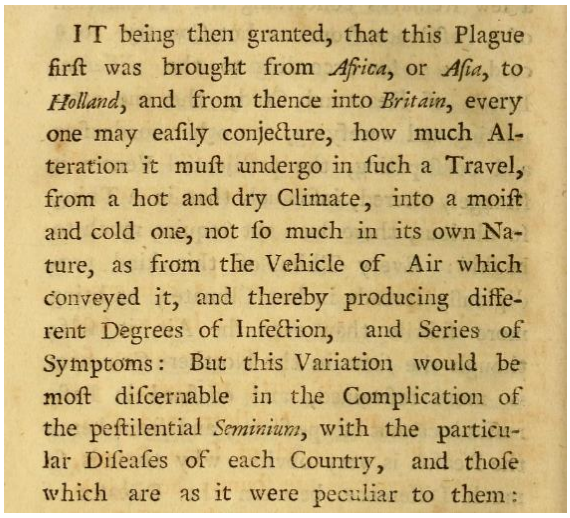
Risk analysis of introducing disease to the UK via the amphibian pet trade

Unintentional anthropogenic movement of pathogens into new areas through trade is not a modern phenomenon. The Second pandemic started with the ‘Black Death’ that originated in Asia and had spread into Europe by the mid-14C via the major trade route known as the ‘Silk Road’. Repeated outbreaks occurred for centuries after, one of which was the Great Plague of London 1665-1666.



LOIMOLOGIA:
OR, AN
Hiſtorical Account
OF THE
Plague in *London* in 1665:
With precautionary Directions againſt the like *Contagion*.

By NATH. HODGES, M. D.
And Fellow of the College of Phyſicians, who reſided in the City all that Time.



IT being then granted, that this Plague firſt was brought from *Africa*, or *Aſia*, to *Holland*, and from thence into *Britain*, every one may eaſily conjecture, how much Alteration it muſt undergo in ſuch a Travel, from a hot and dry Climate, into a moiſt and cold one, not ſo much in its own Nature, as from the Vehicle of Air which conveyed it, and thereby producing different Degrees of Infection, and Series of Symptoms: But this Variation would be moſt diſcernable in the Complication of the peſtilential *Seminium*, with the particular Diſeaſes of each Country, and thoſe which are as it were peculiar to them:

An excerpt from ‘Loimologia’ (Hodges, N. 1721) shows early recognition of international disease spread, and awareness of differential effects of environmental conditions on pathogenicity. Screen-shots taken from: https://archive.org/details/loimologiaorhist00hodg.

Abstract

A number of studies detected *Batrachochytrium dendrobatidis* (*Bd*) in amphibians in the pet trade, supporting the hypothesis that spread of *Bd* is facilitated by international trade. *Bd* is now a World Organisation for Animal Health (OIE) notifiable disease, and guidelines advocate amphibian importing countries undertake a risk assessment. Whilst *Bd* is already present in the UK and no mass mortalities have been observed, the potential for virulent strains to be imported remains a possibility. Additionally, the recent discovery of *B. salamandrivorans* (*Bsal*) in Europe, to which great crested and smooth newts are highly susceptible, is cause for concern. A risk analysis was conducted following OIE guidelines, to assess the impact of *Bd/Bsal* introduction into the UK. Prevalence data obtained from surveys at airports, and in retailers, was supplemented with data from the literature, to inform stages of the assessment. Results indicate the introduction of both pathogens is very likely. Economic impacts may be severe for the trade sector, through loss of stock, and the consequences of *Bsal* introduction to wild populations are likely to be severe, as great crested and smooth newts are highly susceptible. Options for mitigation measures are examined, including point of import screening, in order to reduce the risks posed by *Bd*/*Bsal* introduction.

Introduction

The translocation of animals is now commonplace and increasing in volume annually due to improvements in the efficiency of the transport networks, or 'globalisation' (de La Rocque et al., 2011). Animal movements occur for a variety of reasons including: local and international agricultural livestock trades (Fèvre et al., 2006, Narrod et al., 2011); sporting purposes e.g. transport of competition horses (Leadon and Hodgson, 2014); translocations for conservation or development mitigation (Mathews et al., 2006, Seddon et al., 2007); or zoo stock acquisition and exchange (Lacy, 2013); and for the wildlife or exotic pet trade (Travis et al., 2011, Bush et al., 2014). With all animal movements there is some degree of risk of transmitting pathogens, which may, or may not be zoonotic (Fèvre et al., 2006). The process by which pathogens are moved to - and subsequently infect - new areas or hosts has been termed 'pathogen pollution' (Cunningham et al., 2003), and there have been numerous examples where this has had negative impacts on the health of humans and domestic livestock, as well as native wildlife (Fèvre et al., 2006, Chomel et al., 2007). The exotic pet trade is considered particularly 'risky' in terms of disease dissemination, as there are often not the stringent veterinary checks afforded by the commercial livestock trade (Smith et al., 2009, Narrod et al., 2011). This has been substantiated by a series of reports, including the detection of zoonotic pathogens in pet turtles (Hidalgo-Vila et al., 2008), iguanas (Zehnder et al., 2014), prairie dogs (Phalen, 2004, Petersen et al., 2004), and evidence linking the trade to disease outbreaks in wild animal populations e.g. the introduction of mesomycetozoean parasites to amphibian and fish populations through the aquatics trade (Rowley et al., 2013).

The World Organisation for Animal Health (OIE) is recognised by the World Trade Organisation (WTO) as the "reference body for animal health as it relates to international trade" (Lightner, 2012), and is charged with developing sanitary standards and guidelines for the trade in animals and associated products (Schloegel et al., 2010, Sugiura and Murray, 2011). These guidelines are published as the Terrestrial, and Aquatic, Animal Health Codes (TAHC and AAHC), within which recommendations are made for specific pathogens deemed to pose a risk through trade. The amphibian pathogen *Batrachochytrium dendrobatidis* (*Bd*) was listed as an OIE notifiable disease in 2008 following advice by an *ad hoc* Group on Amphibian Diseases (Schloegel et al.,2010), and guidelines for *Bd* are now included in the AAHC (OIE, 2014). Import authorities are advised to either (1) seek an ‘international aquatic animal health certificate’ from the exporting country verifying the animals have been tested, to confirm absence of disease, or animals are treated where appropriate, or (2) to assess the risk and apply appropriate mitigation measures (OIE, 2014). The feasibility of the certification option is highly questionable, as amphibians are exported from a range of countries, many of which are unable to treat and test amphibians due to lack of financial resources or facilities. Therefore, responsibility falls on importing countries to assess - and if necessary mitigate - the risks (Peel et al., 2012). A previous risk analysis conducted by Peel et al (2012) was a holistic assessment, encompassing imports for the pet trade, scientific research, zoo trade, human consumption, as well as illegal and accidental ‘stowaways’. The authors concluded that the overall risk of introduction of *Bd* to the UK was high. However, limited sample sizes for disease prevalence estimates resulted in a high degree of uncertainty. Such preliminary investigations are useful in directing further research, and the finding that the pet trade was the primary reason for importing amphibians motivated a more detailed analysis of this trade sector.

Risk assessments are employed to inform decisions regarding imports of a variety of biological commodities or their derivatives, from plants (e.g. Andreu and Vilà, 2010), live animals (e.g. Wahlstrom et al., 2002, Whittington and Chong, 2007), foodstuffs such as honey (Mutinelli, 2011) to processed meats (Cobb, 2011); and a number of recognised frameworks exist for such studies. The OIE has published detailed methodologies for both ‘qualitative’ and ‘quantitative’ approaches (OIE, 2010, 2004), that are specifically designed for the import of animals and animal products, and aim to “provide importing countries with an objective and defensible method of assessing the disease risks associated with the importation of animals” (OIE, 2010). Qualitative methods provide a logical examination of a situation, where epidemiological, economical and feasibility factors are considered, resulting in event likelihood estimates expressed non-numerically e.g. high, low or negligible. Quantitative methods are based on probability theory, and employ mathematical models to estimate risk in terms of potential magnitude (Vose, 2001).

Acknowledging the need for further investigation into the risk of pathogen import via the amphibian trade (Peel et al., 2012), this study reports on a detailed qualitative analysis of the risk of importing *Bd* into the UK, specifically focused on the pet trade. This expands on previous work to include: pre-import prevalence data, and additional import and retailer prevalence data, collected over a five year period (2008-2013). Further consideration is given to the potential risk to captive amphibian populations throughout the trade network, and associated economic costs to stakeholders. The report includes an evaluation of the newly discovered amphibian pathogen *Batrachochytrium salamandrivorans* (*Bsal*), in lieu of formal analysis. Evidence gleaned from primary literature and accessible on-line databases support the risk appraisal at each stage of the analysis.

Methods

- *Risk analysis*

The OIE's import risk analysis framework, based upon a model originally designed by Covello and Merkhofer (1993), was chosen for this study as it provides a structured process (Fig. 5.1), yet is more adaptable than other methods, and is commonly used in animal health assessments (OIE, 2010). As a qualitative methodology was adopted, likelihood descriptors are assigned to levels of risk and level of confidence at each stage of the assessment (Table 5.1).

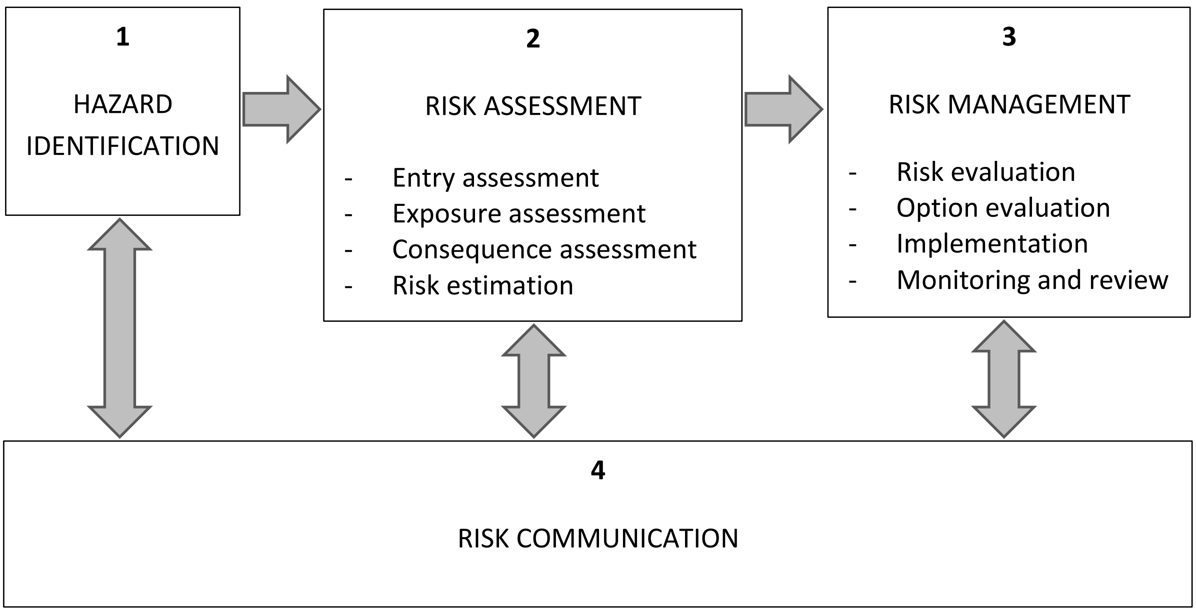


Fig. 5.1. The risk analysis process used by the OIE in the Terrestrial and Aquatic Animal Health Codes. The structure follows that designed by Covello and Merkhofer (1993). Figure re-drawn from OIE (2010).

Component one, ‘Hazard Identification’, typically involves identification of all known pathogenic agents associated with the commodity or product and individual assessment of each to determine which represent a threat, or ‘hazard’ to the importing country. The assessment requires clear identification each pathogen (in this case *Bd* and *Bsal*), reviews the current distribution including presence or absence in trading countries, and summarises relevant epidemiology to arrive at a conclusion on potential risk. As *Bd* and *Bsal* (*Bd/Bsal*) were the focus of this investigation from the outset, pathogens such as ranavirus were not assessed at this stage, and although they undoubtedly deserve attention, it is beyond the scope of this analysis to include them here. The second component, ‘Risk Assessment’ is composed of four steps:

1) ‘Entry Assessment’ describes the likelihood of amphibian consignments being infected with *Bd/Bsal*, and the pathways leading to introduction. A network diagram was drawn, with advice from various stakeholders, to determine important trade foci and pathways, pre- and post-import. Data collected during a 30 month study at Heathrow Animal Reception Centre (Chapter 2), sample data from visits to Ghana and Europe, together with evidence from the literature and on-line resources were utilised for this step.

2) The likelihood of exposure of native UK amphibians or captive collections to *Bd/Bsal*, with each stage of the trade network considered separately. A scenario tree was constructed to identify sites where pathogen dissemination into the environment could occur. Prevalence data obtained from a nationwide survey of retail establishments (Chapter 4), along with evidence from retailer questionnaires (Chapter 3), informed this stage of the analysis.

3) The consequences of *Bd/Bsal* introduction, in terms of biological, environmental and economic factors. Consideration is given to the financial cost to retailers as well as losses suffered through ecosystem disruption or eradication programmes. Published literature and expert opinion forms the basis of this evaluation.

4) The overall conclusions of this component. A summary of the risk assessment, expressed as a series of statements, or decision steps’, determine whether sanitary measures require investigation.

Table 5.1. Terminology used to describe likelihood, level of confidence and significance.

Context	Descriptor	Definition
Likelihood	Very unlikely	The event is not expected to occur.
	Unlikely	The occurrence of the event is reasonably unexpected.
	Likely	The occurrence of the event is reasonably expected.
	Very likely	The event is expected to occur.
Confidence	Low	Less than that required to draw inference.
	Medium	Informed conjecture, but further evidence required for definitive conclusion.
	High	Adequate to draw inference.
Impact	Negligible	Insignificant or inconsequential. No action necessary.
	Moderate	Noticeable or perceptible. Amelioration action necessary.
	Severe	Considerable or critical. Mitigation or preventative action vital.

Informed by the preceding stages, component three, 'Risk Management', explores potential management options required to achieve an acceptable level of risk. Firstly, a 'risk evaluation' step determines whether the risk exceeds the level of risk deemed acceptable by the importing country. This step was extended to include an evaluation of risk of dissemination of *Bd/Bsal* to native amphibians at each stage of the trade network within country. Throughout this process consideration was given to balancing acceptable risk and minimising disruption and economic costs to stakeholders. Identification of possible sanitary measures and evaluation of their effectiveness and feasibility was performed in the 'Option Evaluation' step. Measures were only considered feasible if they could be applied consistently, and were financially viable.

The latter steps to this component 'Implementation' and 'Monitoring and Review', will take place following peer-review of this assessment. Component four, 'Risk Communication' culminates in a written review with input from multiple stakeholders. Whilst communication between stakeholders has occurred throughout the analysis process, further discussions regarding who will undertake the necessary work, and sources of funding, are required prior to distribution of this report.

- *Data collection and interpretation*

Taxonomic nomenclature of the Amphibia according to the IUCN Redlist (accessible from: www.iucnredlist.org) was used in this assessment, but where there is more than one name in common usage all versions will be shown.

Prevalence data were estimated from swab samples taken at different points in the trade network, as described in Chapters 2 and 4, with additional samples collected opportunistically at point of export and European wholesalers and trade shows. Jeffery's confidence intervals (CI_J) were used for all prevalence estimations rather than the standard Wald method, as it performs well with small and large sample sizes, and behaves less erratically than Wald (Brown et al., 2001).

The primary literature was interrogated via an electronic search using the citation indexing service 'Web of Knowledge'. The search term "Batrachochytrium dendrobatidis" was combined sequentially with the terms "trade", "risk analysis", and the names of countries known to export to the UK. The publicly available, on-line *Bd* database *Bd*-Maps (www.*Bd*-maps.net) was utilised to obtain estimated country and species prevalence (sources were cross-checked with results from primary literature searches to ensure accounts were not replicated).

Results

Component 1:

Hazard Identification: Chytridiomycosis

- Aetiological agents

Taxonomy: Family Chytridiales, Class Chytridiomycetes, Genus *Batrachochytrium*, species *dendrobatidis* (Longcore et al., 1999). *Bd*.

Family Chytridiales, Class Chytridiomycetes, Genus *Batrachochytrium*, species *salamandrivorans* (Martel et al., 2013). *Bsal*.

- Infection status in the UK

In wild amphibians, *Bd* was first detected in *Epidalea calamita* (Beebee 2014) and in an invasive population of *Lithobates (Rana) catesbeianus* (Cunningham et al., 2005) in 2004. The *L. catesbeianus* population was subsequently found to be infected with *Bd*, and it is postulated that they introduced *Bd* into the area (Garner et al., 2006). There have been two nationwide surveys since, one in 2008 (Cunningham and Minting, 2008) and one in 2011 (Smith, 2013), both indicated *Bd* to have a widespread yet patchy distribution. To date there have been no reports of chytridiomycosis associated mass mortalities, although it is possible that 'more subtle' effects of population infection have been missed (Minting, 2012). *Bd* has been detected in captive amphibians, in imported amphibian consignments, retailers and in private collections (Chapter 2, Chapter 4), but again, large scale mortalities have not been reported.

There have been no accounts of *Batrachochytrium salamandrivorans* in the UK in either wild or captive amphibians. However, there have been no surveys conducted for *Bsal* to date.

- Epidemiology

Chytridiomycosis is an infectious disease caused by the fungal pathogens *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*).

Batrachochytrium dendrobatidis has a wide-host range, infecting hundreds of amphibian species (Daszak et al., 2003, Olson et al., 2013), including Anurans (frogs and toads), Caudates (newts and salamanders), and Gymnophiona (caecilians). Despite a broad geographic range (Fig. 5.2), encompassing 56 countries (Olsen et al., 2013), there are still areas where the *Bd* has not yet been detected e.g. Madagascar (but see Kolby, 2014), and certain Islands e.g. Fiji (Narayan et al., 2011) and the Seychelles (Labisko, submitted). To date three distinct lineages (Global pandemic - *Bd*GPL, South African - *Bd*CAPE and Swiss - *Bd*CH), have been identified (Farrer et al., 2011), with a possible fourth endemic to Japan (Goka et al., 2009), and fifth in Brazil (Rodriguez et al., 2014). Multiple genotypes have been described within each lineage, with the exception of *Bd*CH (James et al., 2009, Goka et al., 2009, Farrer et al., 2011). *Bd*GPL is the most widespread, and also the most virulent lineage, and is the only lineage associated with mass mortality and amphibian population decline (Farrer et al., 2011).

Bd has a two phase lifecycle, a motile aquatic zoospore stage where transmission occurs, and a sessile reproductive zoosporangium stage that occurs within amphibian epidermal tissue (Berger et al., 2005). *Bd* can infect most amphibian species, however the susceptibility to symptomatic chytridiomycosis is species and population specific (Blaustein et al., 2005, Woodhams et al., 2007, Tobler and Schmidt, 2010), and there are a number of known asymptomatic carriers e.g. *L. catesbeianus* (Daszak et al., 2004) and *Xenopus laevis* (Weldon et al., 2004). In the natural environment, *Bd* tolerates a wide range of thermal and precipitation conditions (Ron et al., 2003); in vitro the optimum temperature range for growth is 17 - 25°C, and long term survival is inhibited at temperatures above 29°C (Piotrowski et al., 2004).

Bd infection resulting in clinical chytridiomycosis generally presents with hyperkeratosis and sloughing skin, along with lethargy and anorexia (Parker et al., 2002). Behavioural symptoms include the animal spending prolonged periods in water, and unusual posture suggesting potential neurological effects (Cunningham et al., 2005). Time to death and precise symptoms are species or population specific (Voyles et al., 2009).

Currently, there are no vaccines against *Bd*. Attempted systemic immunisation of frogs to *Bd* through injections has not proved successful (Stice and Briggs, 2010), and repeated exposure and treatment regimes have had mixed results (Cashins et al., 2013, McMahon et al., 2014). Ex-situ treatment using anti-fungals, specifically itraconazole, have repeatedly proved successful (Brannelly et al., 2012, Brannelly, 2014, Jones et al., 2012, Georoff et al., 2013). However, depigmentation following treatment has been reported in *Alytes muletensis* tadpoles (Garner et al., 2009a) and alternative anti-fungals such as voriconazole have been suggested (Martel et al., 2011). At present no suitable in-situ treatment options have been found.

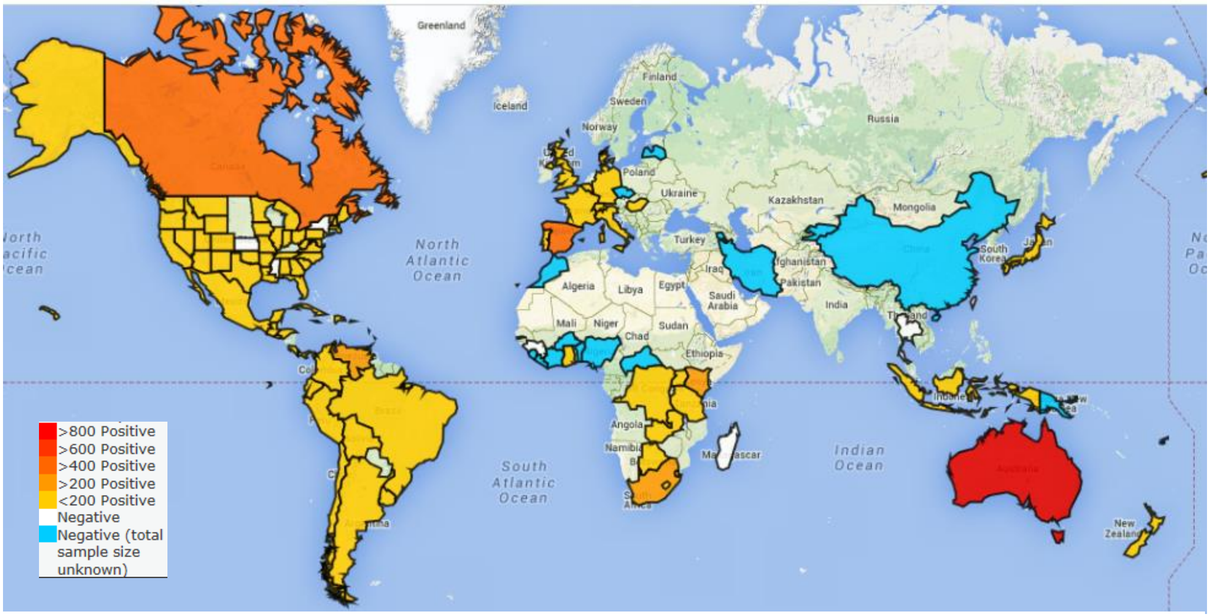


Fig. 5.2. Global distribution of *Batrachochytrium dendrobatidis* where sampling has occurred. The data presented is a count of positive and negative samples, and thus is biased by sampling effort. Map downloaded from www.Bd-maps.net (15 August 2014).

Batrachochytrium salamandrivorans has only recently been discovered, and thus there is comparatively little known about the pathogen's biology or host range. First detected in the Netherlands in 2013 (Martel et al., 2013), research so far indicates it has a narrower host range than *Bd*, with susceptible species confined to the caudates. *Bsal* has a lower thermal preference than *Bd,* of 10-15°C with death at ≥25°C. Experimentally infected fire salamanders (*Salamandra salamandra*) died within 12-18 days, and presented with superficial skin lesions and ulcerations rather than hyperkeratosis associated with *Bd* infection. A wide range of other caudate species were also found to succumb to fatal chytridiomycosis on exposure to *Bsal*, including all UK native species except palmate newts (*Lissotriton* (*Triturus*) *helveticus*). Neurological signs were observed one to two days prior to death (Martel et al., in press). The addition of healthy animals to a vivarium containing infected animals resulted in death, indicating *Bsal* is readily transmitted between infected amphibians.

Whilst OIE sanitary measures have been previously described (OIE, 2014) for *Bd*, an additional risk assessment is warranted specifically for the pet trade, as the measures described by the AAHC are insufficient to cover the broad range of infection and dissemination opportunities that occur in the trade. *Bsal* has yet to be included in the AAHC, and given its potential to cause mortality in both captive and wild amphibians, and an apparent separate niche of amphibians to *Bd* (Martel et al., 2013), an assessment is necessary.

- Conclusion

Whilst *Bd* is already present in the UK, novel strains of *Bd* represent a potential threat to both the UK's native amphibians, and captive amphibians in trade, private collections and zoos. *Bsal* has recently caused rapid declines in salamander populations in The Netherlands and Belgium and it is postulated to be a threat to UK native caudate species. Therefore both *Bd* and *Bsal* are considered to be hazards.

Component 2:

Risk Assessment:

- Entry Assessment

Amphibians have been recorded directly entering UK trade from multiple countries, from four continents through Heathrow Animal Reception Centre (HARC), and indirectly from South America via Europe (Fig. 5.3). Imports from Australia were deemed negligible due to strict legislation, preventing trade in and out of the country. Amphibian stock also derives from captive breeding within the UK, both on a commercial and informal scale. Non-EU commercial amphibian consignments are permitted to enter the UK through four international airports, EU trade can also arrive via seaports e.g. Dover, Kent. Sources of amphibians were assessed separately. Results of the literature review are presented in Appendix 5, and a summary of data collected in this study are shown in Table 5.2.

Africa:

Consignments enter the UK from at least six countries from north, south, east and west Africa. *Bd*-Maps report detection of *Bd* in 13 of 24 countries, and suggest a heterogeneous distribution of the pathogen over the continent (*Bd*-Maps). There was a notable absence of infection in samples from Madagascar (but see Kolby et al., 2014) and Ghana in the literature, which was supported by trade samples from Ghana taken at point of export and import, and from imported samples from Madagascar in the UK. In contrast studies in South Africa, Tanzania and Cameroon, have revealed widespread infection in wild amphibian populations. Positive consignments have been detected at HARC originating from Tanzania (Chapter 2), and a consignment from Cameroon had a prevalence of >75% (Wombwell, unpublished data). South African consignments have not tested positive. However, only a small number of consignments have been sampled, and results from field studies suggest that South African amphibians are likely to be infected. No data were available for in-situ or trade amphibians from Egypt, precluding assessment.

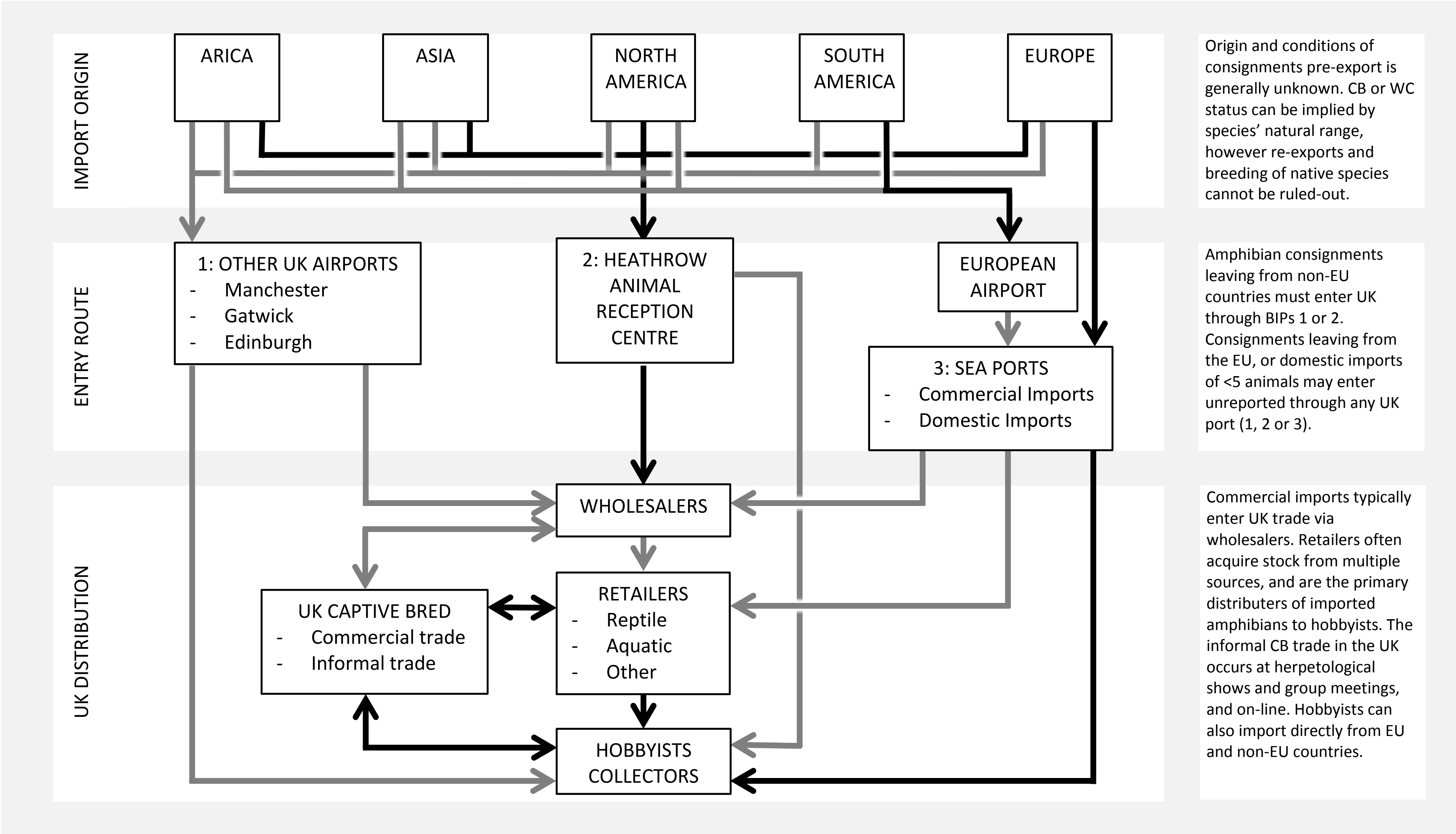


Fig. 5.3. Trade network associated with the UK pet amphibian trade, showing the pathways by which *Bd* (and potentially *Bsal*) could enter, and be transmitted throughout the trade network. Black arrows: data were collected, grey arrows: no data was collected. Australia is not included as amphibian imports and exports are restricted by legislation.

Asia:

Despite evidence that *Bd* had been present in Asia since the early 1900s (Goka et al., 2009, Zhu et al., 2014), formal detection was not reported until 2006 (Une et al., 2008). A single positive consignment was identified in 2008 originating from Indonesia at HARC, samples from other consignments were negative. However, Peel et al., (2012) report up to 75% of imports into HARC comprise of aquatic species that arrive in 'fish consignments', which were not sampled in this study. Anecdotal evidence derived from UK retailers revealed that species such as *Hymenochirus boettgeri, Ambystoma mexicanum, Bombina orientalis* and *X. laevis,* are captive bred on a large scale in China, Indonesia and Hong Kong. The association of these species with aquatic environments and the potential for high densities in breeding facilities, suggest these species pose a high risk of infection. Additionally, reports described in the literature (full details shown in Appendix 5) have identified areas of high prevalence in wild amphibians, which are also exported to a lesser degree.

Recent molecular studies concluded *Bsal* is endemic to Asia, where it has co-evolved with amphibians for approximately 30 million years (Martel et al., in press). An outbreak of chytridiomycosis in native fire salamanders (*S. salamandra*) in Belgium resulting in high mortality, suggests a recent introduction in to Europe (Martel et al., 2013). Detection in a captive amphibian in Europe, together with the extensive trade of amphibians out of Asia (Herrel and van der Meijden, 2014), indicate that consignments from Asia pose a significant threat of introducing *Bsal* into the UK.

North America:

Assessing the risk of consignments from USA being infected with *Bd* is problematic for two main reasons: Firstly, the USA is a principle 'hub' in the international trade network, thus exports may consist of consignments originating 'in-country', or consignments that have entered the country for re-export. Therefore, determining the primary origin of these consignments is difficult. Secondly, there are a number of captive breeding facilities and hobbyists breeding amphibians on a commercial scale. This makes predicting the country of origin of consignment using species geographical ranges unreliable, and precludes the use of native range *Bd* status as an indicator of infection probability. However, the number of potential origins and volume of amphibians exported from the USA, together with a known widespread distribution of

Bd in wild populations (encompassing export of wild caught animals), suggests that infection of consignments from the USA is likely. This is supported by the repeated detection in amphibian consignments at HARC from 2007-2011.

South America:

Some of the first reported mass mortalities of amphibians occurred in the Central American countries of Costa Rica and Panama (Lips, 1998, 1999), where 'waves' of local extirpations appeared to travel southwards. South American imports are not commonly seen at HARC (one sampled in 2008 was *Bd* negative), however amphibians from in this area are imported into the UK via Europe (Anon. pers. comm.).

Europe:

Europe, like USA, functions as a redistribution hub in the trade network, such that the primary origin of amphibian stock is impossible to generalise. Additionally, EU trade agreements allows the unreported trade of amphibians, therefore very little data were available for this assessment. It is plausible that a comparatively small number of amphibians are harvested directly from the wild, by private collectors, who return to the UK via a ferry. Surveys in Europe have identified *Bd* infection in several amphibian populations (Garner et al., 2005, Walker et al., 2007, Bovero et al., 2008, Tessa et al., 2013, Bosch et al., 2013), suggesting that the collection of wild amphibians poses a threat of introduction to the UK. Amphibians acquired from trade fairs or 'expos' in countries such as the Netherlands and Germany, are also informally imported in the same manner. Previous studies have detected *Bd* in amphibians for sale at these events (Spitzen-van der Sluijs et al., 2011), although infection is generally confined to one or two retailers. The South American dendrobatid (dart-frog) species are popular in Europe, a proportion of which are acquired by retailers and hobbyists in the UK. Samples obtained on arrival at a wholesaler from South American, revealed a high prevalence of infection in one of the two species imported. All the animals were routinely treated for *Bd*, and re-tests several weeks later returned negative results (Wombwell, unpublished data). It is unclear how many of the wholesalers routinely treat newly acquired amphibians for *Bd*, or how effective this is in the long term. As *Bsal* has been detected in wild *S. salamandra* populations in the Netherlands (Martel et al., 2013, Spitzen-van der Sluijs et al., 2013), there is a risk of *Bsal* introduction via the import of wild caught amphibians originating from this region.

Table 5.2. Details of the sampling data, collected in this study, used to inform the disease risk assessment.
POI: Point of Import. n = total number of swab samples analysed. WC: wild caught, CB: captive bred, P: overall prevalence, CI_J: Jefferys confidence interval. *PCR results potentially compromised by inappropriate storage conditions prior to analysis.

Point in Trade Chain	**Data collected**	**Details** (including proportion of *Bd*+ samples)	**Results** P (95% CI_J)
Accra Airport, Ghana 2011			
Pre-export at country of origin.	n = 51 Opportunistic sampling of 3 consignments.	WC. 0/9 *Hyperolius* sp., 0/6 *Kassina senegalensis*, 0/31 *Phrynomantis* sp., 0/5 *Pyxicephalus edulis*.	0% (0 – 4.8%)
Accra Airport, Ghana 2012			
Pre-export at country of origin.	n = 25 Opportunistic sampling of 4 consignments.	WC. 3 species.	0%* (0 – 9.5%)
European Supplier			
POI: Europe	n = 168 Samples from one consignment of 300 individuals (2 species).	WC. Direct import from South America, swabbed prior to contact with existing stock. 18/84 *Oophaga pumilio* 0/84 *Dendrobates auratus*	10.7% (6.7 – 16.1%)
European Supplier			
POI: Europe	n = 134, 4 consignments.	Unknown origin. 10 species. 3/7 *Theloderma asperum*	2.2% (0.6 – 5.9%)
European Trade Show			
Pre-import into UK	n = 81, 15 vendors.	Predominantly CB dendrobatids.	6.2% (2.4 – 13.0%)
European Trade Show			
Pre-import into UK	n = 89, 15 vendors.	Predominantly CB dendrobatids.	2.3% (0.5 – 7.0%)
Heathrow Animal Reception Centre 2008			
POI: UK	n = 136. Opportunistic sampling of 21 consignments. 36 species sampled.	*Bd*+: 2/2 *Rhacophorus nigropalmatus* 2/2 *Lithobates pipiens,* 1/6 *Theloderma corticale*, 6/15 *Hyperolius* sp., 1/1 *Afrixalus brachycnemis*, 2/6 *Leptopelis* sp., 1/7 *Phrynomantis* sp.	11.0% (6.6 – 17.1%)
Heathrow Animal Reception Centre 2009-2011 (Chapter 2)			
POI: UK	n = 1010, 59 consignments. See Chapter 2 for details of methods. 43 genera sampled.	*Bd*+:1/4 *Desmognathus* sp., 1/28 *Leptopelis* sp., 6/7 *Necturus* sp., 1/2 Siren sp., 17/207 *Hyperolius* sp., 10/53 *Hyla* sp.	3.6% (2.6 – 4.8%)
UK Retailers (Chapter 4)			
Retailer	n = 2207, 148 retailers. See Chapter 4 for details of methods.	*Bd*+: 23/113 species *Ambystoma mexicanum* & *Bombina orientalis* were proportionally more likely to be *Bd*+.	5.8% (4.9 – 6.8%)
Herpetological Meeting UK			
Hobbyist	n = 36, 8 breeders.	10% of amphibians for sale sampled. 0/30 CB dendrobatids, 1/6 caudates. (*Triturus carnifex Bd*+)	2.8% (0.3 – 12.3%)
Herpetological Meeting UK			
Hobbyist	n = 14, 2 meetings. Opportunistic sampling.	*Bd*+: 1 CB *Bombina varigata*	7.1% (0.8 – 28.8%)
Private Collections UK			
Hobbyist	n = 41. 2 collections. Opportunistic sampling.	CB and WC caudates. 0/29 & 5/12. *Bd*+: 5 species	12.2% (4.8-24.7%)

UK Captive Bred Stock:

UK captive bred amphibians that are incorporated into the trade network are considered a potential pathway for pathogen transmission into the trade. *Bd* was detected in one of two UK breeders that permitted their stock to be screened, neither owner was previously aware of any health problems with the stock and all positive animals were asymptomatic. Furthermore, follow-up investigation into *Bd* positive amphibians detected in retailers (Chapter 4) revealed that, on more than one occasion, infected animals had been sourced from local breeders. Asymptomatic infection in breeding facility amphibians creates a reservoir where *Bd* can proliferate, and subsequently be transmitted into retail stock.

Summary and Conclusions

Bd has been detected in most countries exporting amphibians to the UK, therefore the importation of native wild caught pose a risk of *Bd* introduction. Notable exceptions are Ghana and Madagascar, where *Bd* is yet to be conclusively identified in wild populations and sampling conducted in the UK suggests that import of *Bd* from these countries is very unlikely. In contrast, *Bd* has been detected in consignments from Tanzania and Cameroon, and literature reports indicate high prevalence of infection in wild populations. Consignments from Tanzania and Cameroon are considered very likely to introduce *Bd* into the UK. Estimating the risk of infection in consignments from USA is problematic due to uncertainties regarding the primary origin of amphibians, and their captive bred or wild caught status. However, repeated detection of *Bd* in USA consignments at HARC indicates introduction of *Bd* is likely to occur. Exports posing the greatest risk of infection from China and Singapore comprise aquatic species that are intensively captive bred in this region. Whilst not sampled at point of entry, *Bd* has been identified in stock of this origin in UK retailers, and consignments are considered to be a likely source of infection. Akin to the USA, Europe has a widespread *Bd* distribution and redistributes amphibians from multiple origins. The detection of *Bd* in Europe at trade fairs, suggest this may be an introduction pathway via the domestic trade.

Whilst research into the geographic distribution of *Bsal* is still accruing, preliminary findings suggest with medium confidence, the risk of introducing *Bsal* to UK wild caught caudate amphibians from Northern Europe and Asia is very likely.

- Exposure Assessment

Assimilation of amphibians into the UK trade network constitutes a pathogen exposure risk to two 'susceptible populations': captive amphibian collections (in trade or privately owned), and native wild amphibian populations. The complexity of the trade network is such that the potential for a susceptible population to be exposed to *Bd/Bsal* can occur at a number of points in the network, the three principle points being: wholesalers, retailers and hobbyists/collectors (Fig. 5.4). *Bd/Bsal* are aquatic pathogens that are transmitted directly through contact with infected individuals or indirectly via contaminated fomites, substrate or water; there are no known biological vectors or intermediate hosts, and other forms of transmission e.g. airborne spread, do not occur. Pathogen dissemination to wild or captive populations at point of import is deemed negligible as there is minimal contact made with amphibians during routine inspection of consignments, and appropriate biosecurity measures are in place to prevent cross-consignment contamination, or dissemination into the environment.

Captive amphibian collections

Susceptibility to *Bd* is species and population specific, with some species tolerating long term infection and others succumbing to fatal chytridiomycosis (Bielby et al., 2013). With over 100 species of amphibian in the UK trade (Chapter 3), a generalised 'susceptible population' is undefinable. In the event of a novel hypervirulent strain entering the trade, it is assumed that all species are potentially susceptible to chytridiomycosis. Experimental evidence (Martel et al., 2014) suggests that anuran species are resistant to *Bsal*, whereas caudate species are susceptible.

Probability of exposure of captive amphibians to *Bd/Bsal* is largely dependent on biosecurity measures and husbandry protocols in each establishment. This in turn is influenced by the level of disease awareness and knowledge and of the proprietor and the level of biosecurity exercised within animal management protocols. Re-stocking methods that involve the addition of newly acquired stock to vivaria containing resident animals increase the probability of exposure to infected individuals. Contact rates, and hence dissemination potential, increases with density of animals in each vivarium. Additionally, higher volumes of amphibians and turnover of stock would increase the probability of contact with an infected individual. Aquatic species are more likely to be exposed to infection via this route, as 'top-up' restocking is more common, and the aquatic environment pre-disposes the maintenance of *Bd* over long periods of time. Territorial or species that do not share the same niche would be less likely to transmit infection. Most captive collections are maintained at temperatures suitable to sustain *Bd* growth (pers. obs.). However, the lower thermal preference exhibited by *Bsal*, may preclude infection in some cases, with exception of caudate species kept at cooler conditions, e.g. European species housed outside.

Indirect infection can occur through contact with contaminated fomites, substrate or water (Johnson and Speare, 2003), or through handling of different individuals without washing hands between individuals.

The presence of asymptomatic carriers increases the risk of transmission within collection as the presence of the pathogen would not be known.

Wild amphibian populations

The 'susceptible population' to a novel strain of *Bd* is considered to be all native wild amphibian species, although it is acknowledged that effects are likely to be species specific. *Bsal* is unlikely to have an effect on the anuran species, but two of the three native caudate species (*Triturus cristatus*, and *Lissotriton vulgaris*) are considered susceptible. The widespread distribution of *Bd* in the UK confirms climatic and environmental conditions are suitable for the pathogens survival and establishment. The UK's cool temperate clime suggests suitable conditions may exist for *Bsal*.

The dissemination of pathogens can occur anywhere where there is an 'interface' between wild and captive populations (Fig. 5.4), which represents either direct contact between individuals or through exposure of wild animals to contaminated substrate or effluent. Direct contact can occur through the intentional or accidental release of infected amphibians. This is more likely to occur in a domestic setting at the hobbyist level, with intentional release into garden ponds. The risk of contamination through the release of contaminated effluent increases with the volume released. The volume and frequency of waste produced is likely to be higher at wholesaler and retailer levels, where stock turnover and quantity of animals is higher. Exposure of wild amphibians depends on the release of zoospores and the viability of zoospores released into the environment depends on the methods of disposal. Survival of zoospores is likely to be minimal if water is disposed into the sewage system, as water treatment processes are likely to kill *Bd*.

Conclusion

The risk of exposure of captive amphibians to *Bd* infection (summarised in Table 5.3) through the introduction of infected animals is very likely. Movement of individuals through different establishments throughout the trade chain presents multiple opportunities for pathogen exposure. The risk of exposure of captive amphibians to *Bd/Bsal* increases with turnover, husbandry practices and density of amphibians kept in vivaria, and is ultimately dependent on biosecurity practices. The large volume and high turnover at wholesalers predisposes this stage to be at high risk. However, it is possible that animals kept by hobbyists may be at greater threat from less formal biosecurity measures.

Risk of exposure of wild amphibians (summarised in Table 5.4) is unlikely, and largely depends on the methods of waste disposal from captive collections. Accidental or intentional release of infected amphibians is more likely to happen at the hobbyist level, however, this practice is illegal in the UK, and it is unclear to what extent this occurs. There is unlikely to be any significant geographical biases towards areas of particular risk, due to the widespread distribution of amphibian retailers and hobbyists.

Table 5.3. Captive amphibian population exposure assessment for *Bd/Bsal*.

Exposure pathway	Likelihood	Confidence	Impact	Explanation
Wholesaler	Very likely	Medium	Severe	High volume and turnover of amphibian stock from multiple origins. High density of amphibians in imported consignments, and potential for high contact rates during repacking and redistribution. Wholesalers not formally assessed but economic impacts potentially high through loss of stock and pathogen containment and eradication costs.
Retailer	Likely	High	Moderate	Amphibians acquired from multiple sources, mixed levels of biosecurity. Assessment based on retailer visits. Smaller number of amphibians potentially lost, but pathogen containment and eradication costs applicable.
Hobbyist	Likely	Low	Moderate	Amphibians acquired from multiple sources, mixed levels of biosecurity. Hobbyists have not been formally assessed, but infection may result in veterinarian, as well as decontamination costs.

Table 5.4. Wild amphibian population exposure assessment for *Bd/Bsal*.

Exposure pathway	Likelihood	Confidence	Impact	Explanation
Wholesaler	Unlikely	Low	Moderate	Amphibians housed in enclosed facilities. Economic incentive not to release animals. Potentially contaminated effluent volume high. Small relative number of wholesalers, suggest infection may be initially limited to proximity. Biosecurity standards and practices unknown.
Retailer	Unlikely	Medium	Severe	Amphibians housed in enclosed facilities. Economic incentive not to release animals. High number of retailers suggests widespread dissemination possible, and problematic to trace. Observed biosecurity practices of reasonable standard.
Hobbyist	Likely	Low	Severe	Amphibians generally housed indoors, some outdoors. Escapes or intentional release possible. Number of hobbyists unknown, but likely to be widespread. Biosecurity levels unknown.

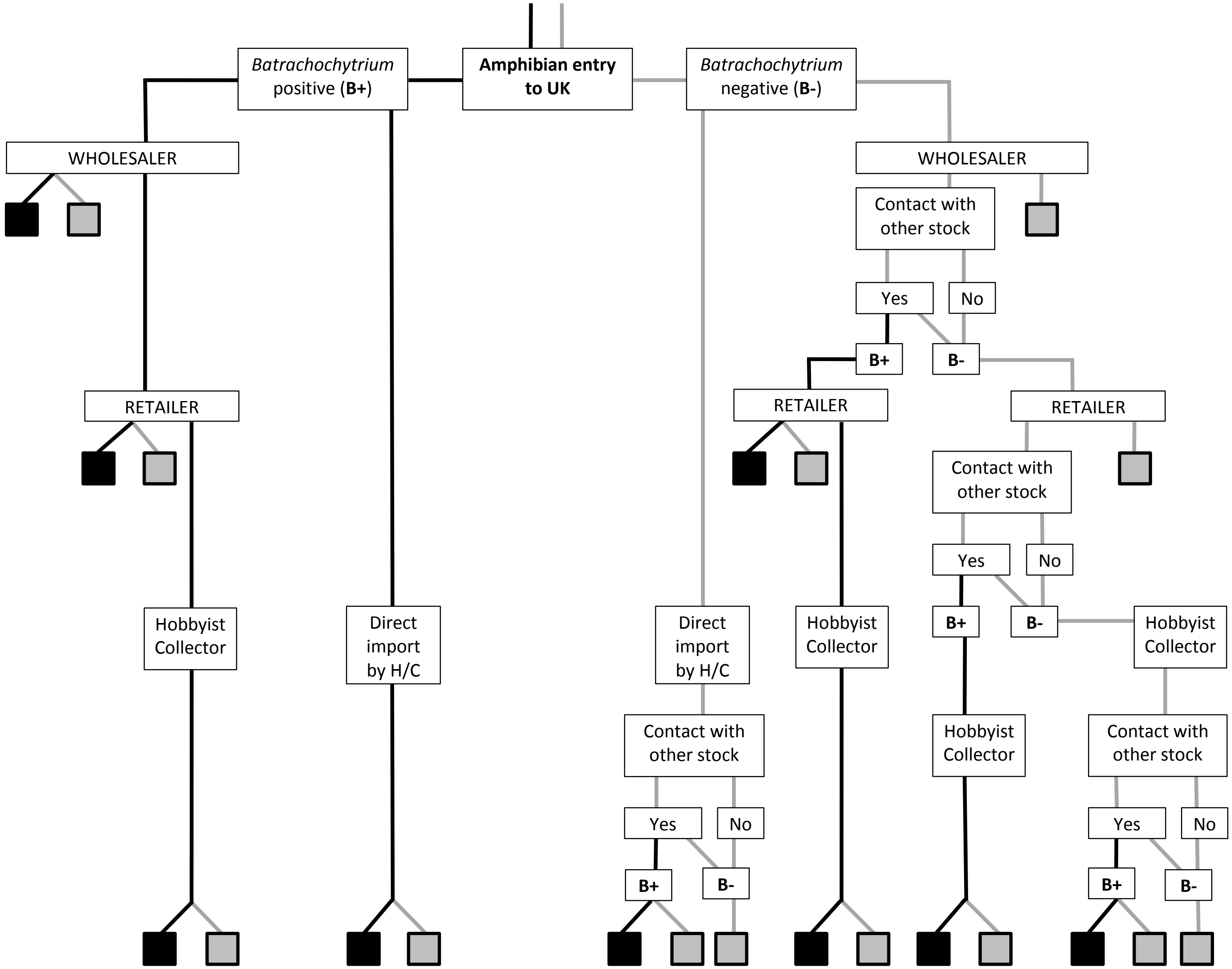


Fig. 5.4. A scenario tree illustrating some of the pathways leading to *Batrachochytrium* dissemination into susceptible amphibian populations, via the pet trade.
'Entry into the UK' represents either commercial or domestic import arriving from any country.
'B+' or 'B-' indicates the infection status of the unit of interest (individual, consignment, or consignment sub-set).
H/C: Hobbyist or collector.
Black lines: infection present.
Grey lines: infection absent.
Black boxes: interface between captive and wild populations where there is a high risk of dissemination from the unit of interest.
Grey boxes: interface between captive and wild populations where there is a low risk of dissemination from the unit of interest.
The interface is defined as 'a point where there is the opportunity for pathogen transmission, either through direct contact of amphibians or the release of contaminated effluent or substrate.
Linked black and grey boxes indicate that 'risk' is determined by biosecurity level at the interface (poor biosecurity = high risk, good biosecurity = low risk).
Contact with other stock may or may not result in infection.
Assumes no prophylactic or reactive treatment, or mortality.

- Consequence Assessment

Batrachochytrium sp. are non-zoonotic, amphibian specific pathogens that do not pose a threat to humans, domestic livestock or other wildlife species, with the possible exception of some crustacean species (McMahon et al., 2013, Terrell et al., 2014).

Captive amphibian populations

Environmental consequences of *Bd/Bsal* dissemination into captive populations are considered small, except, perhaps the loss of rare species in private collections potentially valuable to future ex-situ conservation projects. The most significant impacts are economic implications associated with the loss of stock. In general, amphibian sales comprise a small percentage of overall income for retailers in the UK (Chapter 3), but the contribution is more significant for wholesalers and specialist amphibian breeders and retailers. In addition to the initial loss of stock, there are additional costs involved in decontamination of vivaria, husbandry equipment, and reduction in available housing space for saleable stock. Again, the impacts of these indirect costs will be higher for wholesalers and specialist breeders. Severe disease outbreaks in domestic settings could lead to the extirpation of private collections with substantial financial value.

Wild amphibian populations

The capacity for *Bd* to have dramatic impacts on wild amphibian populations has been illustrated by widespread population declines and extirpations documented on six continents (e.g. Bosch et al., 2001, Lips et al., 2006, Kriger and Hero, 2007, Fisher et al., 2009). Although, *Bd* was not detected during examination of UK native amphibians from 1992-1996 (Cunningham et al., 2005), it now has a countrywide distribution (Smith, 2013); demonstrating the dispersal potential of *Batrachochytrium* sp. pathogens. Introduction and spread of a novel hypervirulent strain of *Bd* could pose a significant risk of high mortality rates in native amphibian species. This is cause for concern given the overall decline in UK amphibian populations over recent decades (Beebee, 2014), especially for species such as the great crested newt (*T. cristatus*), that despite a widespread distribution in the UK, populations in Europe as a whole have experienced a marked decline (Edgar et al., 2005, Beebee, 2014). This has led the species to be protected under the Conservation of Habitats and Species Regulations 2010, making them a European Protected Species. Other notable species that have seen dramatic population declines are the pool frog (*Pelophylax lessonae*) and natterjack toad (*Epidalea calamita*). All three species are listed as 'least concern' by the IUCN but all have decreasing population trends and a high degree of legal protection in the UK. Economic costs arising from virulent *Bd/Bsal* strain introductions would be incurred by the UK government through the implementation of eradication and surveillance programmes, coupled with further assessment and employment of potential mitigation strategies. Local wildlife charities and herpetological groups may also be financially impacted, where required resources would be diverted from other projects to fund amphibian conservation or 'rescue plans'

- Risk Estimation

At each stage of the assessment, risk of *Bd/Bsal* entry and establishment has been identified, and the potential consequences are deemed greater than negligible (Fig. 5.5.), therefore consideration of sanitary measures is warranted.

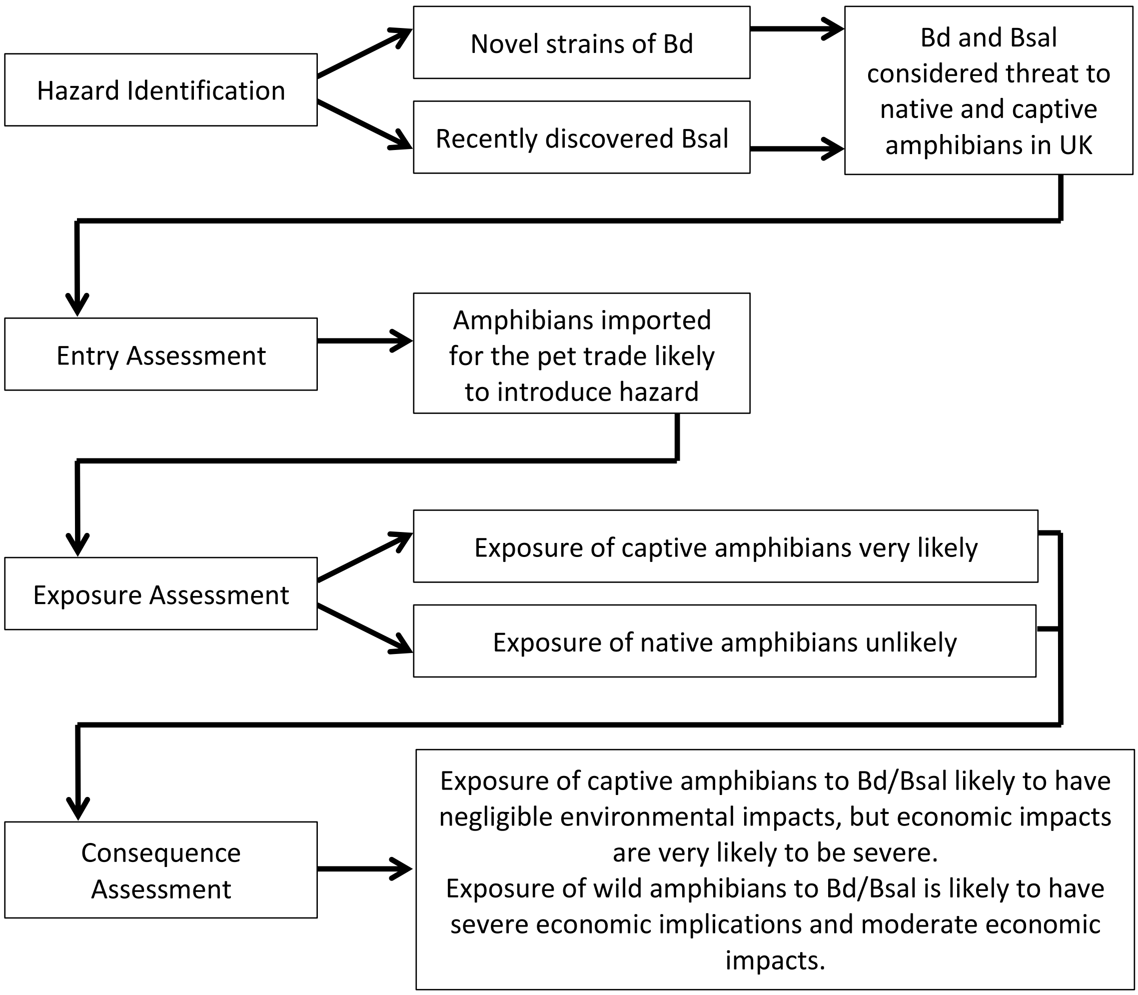


Fig. 5.5. Risk estimation for the introduction of *Bd* into the UK through the amphibian pet trade.

Component 3:

Risk Management:

- Risk evaluation

The 'level of acceptable risk' is not universally defined for all countries (OIE, 2010), and is determined by evaluating the risk of introduction, accounting for likelihood and potential consequences, of a pathogen(s), against the value of the trade to all stakeholders.

The introduction of *Bd/Bsal* into the UK pet trade via imports is considered very likely, having severe economic consequences for stakeholders through the loss of stock, and decontamination costs. As *Bd/Bsal* infection results in significantly different outcomes, from no apparent effect to high mortality, the costs to individual animals cannot be generalised, but overall there are likely to be welfare implications. Environmental costs deriving from pathogen introduction into captive populations are considered low, with the exception of loss of rare species with future potential value in ex-situ conservation projects.

The localised introduction of *Bd/Bsal* into wild amphibian populations is considered likely. However, the spread of the pathogen over wider geographic areas is predicted to result from either (1) natural animal movements or; (2) human mediated translocations of wild amphibians, for example during mitigation programmes for construction projects. In the event of widespread dissemination, the predicted environmental and economic consequences would be severe. Amphibian population declines or extirpations constitute the loss of biodiversity. Valuing 'biodiversity' is problematic, as in addition to direct value e.g. the income from visitors to a national park, there is an indirect or non-use value that describes the "value attributable to the simple knowledge that something exists" (Peeler et al., 2007). Much debate surrounds methods for ascribing numerical values to biodiversity (e.g. Nunes and van den Bergh, 2001, Christie et al., 2006, Martin-Lopez et al., 2008), and it is beyond the scope of this assessment to attain such a figure for the value of wild amphibians in the UK. However, an intrinsic value for wild amphibians is acknowledged in this assessment.

The economic importance of amphibian sales to wholesalers has not been evaluated, but is unlikely to be negligible. At the retailer level, amphibian sales constitute a small proportion of the profits. However, 30% of livestock retailers routinely stock amphibians resulting in an estimated £3million gross annual income derived from amphibian sales in the UK (Chapter 3). Captive or 'pet' amphibians also have an intrinsic value to the individual hobbyist, as well as fulfilling an educational role in amphibian husbandry, care and conservation awareness. Whilst there is a legitimate argument for safe-guarding the amphibian pet trade, the likelihood of introducing *Bd/Bsal* into the UK and the anticipated consequences, bring the risk above the acceptable level, and thus sanitary measures are warranted.

- Option evaluation

As current recommendations put forward by the OIE in the AAHC Chapter 8.1 (OIE, 2014) are generally considered currently unfeasible (Table 5.5) other measures have been considered. All importing countries and routes of amphibian entry into the UK trade are deemed to be 'not declared free from infection with *B. dendrobatidis*', due to the globally widespread distribution of *Bd*. Sanitary measures are unlikely to be feasible pre-export, as a large number of consignments originate from developing countries with severely limited resources and technical capabilities. Sanitary measures applied at point of import will only be possible where appropriate facilities exist, and at formal Border Inspection Posts (BIPs). Regulatory changes are required to either prevent import via other entry points (rail or seaports), or provide inspection and enforcement facilities at these points. The development of rapid molecular diagnostic techniques (Kerby et al., 2013), increases the feasibility of detection at well-established BIPs such as HARC, but funding will be required to implement new protocols. In order to maximise the likelihood of intercepting infected consignments, surveillance should be directed at imports arriving from countries such as Tanzania and USA, where *Bd* has been previously detected. Data are urgently required on the presence of *Bsal* in aquatic consignments, especially those originating from Asia. Detected infected amphibians should not be permitted to enter the UK, however whether consignments are treated or euthanased will largely depend on the economic value of the animals involved. Enforcing mandatory testing or treatment further down the trade chain would be problematic, and education programmes or workshops aimed at stakeholders to improve levels of voluntary testing and biosecurity awareness are likely to more efficient.

Table 5.5. Evaluation of the sanitary measures proposed by the OIE and additional options, with the objective to reduce the risk of importing *Bd/Bsal* into the UK via the amphibian pet trade, and the subsequent dissemination into wild amphibian populations, to achieve a level of acceptable risk.

Sanitary measure	Details	Feasibility
OIE: [Requirement of an international health certificate issued by the competent authority confirming that the animals have been appropriately treated to eradicate infection and have been subsequently tested to confirm the absence of disease…]	Treatment of amphibians prior to export using appropriate anti-fungal agents such as voriconazole, applied by spraying daily for 7 days (Martel et al., 2010). Followed by swabbing of amphibians and identification of *Bd* using TaqMan PCR using protocol by Hyatt et al., (2007). Certification required attesting to the implementation of these measures and absence of *Bd*.	Currently unfeasible. The majority of exporting countries do not have the facilities to house amphibians for the time required to complete the treatment period. Required fungicidal agents are not widely available. Additionally, limited technological, financial and personnel experience precludes molecular diagnostics.
OIE: Direct delivery to and lifelong holding in biosecure facilities for continuous isolation from the local environment.	On entry to the UK, amphibians dispatched directly to biosecure facility or establishment, where they will remain for duration of their lifespan.	Unfeasible. Not conducive to standard trade practices. Amphibians are imported primarily by wholesalers, distributed to retailers and then to hobbyists.
OIE: Treatment of water used in transport and of all effluent and waste materials in a manner that inactivates *B. dendrobatidis*.	Post-import decontamination of waste products prior to disposal. Disinfection protocols recommended in Phillot et al., (2010). *Bd* is inactivated by a range of widely available disinfectants e.g. sodium hypochlorite (bleach), F10, Virkon, Path X. Care should be taken to prevent contamination of local environment through disinfectant use. Heat treatment or complete drying of contaminated substrate may be more appropriate.	Feasible. Disinfectants widely available. Drying does not require specialist equipment. Economically viable.
Additional Options		
Import ban	Implement restrictions on all amphibian imports destined for the pet trade.	Unfeasible. There are currently too few data to justify a ban on imports on the basis of potential disease dissemination. UK is not yet equipped to enforce a ban at all points of entry into the UK.
Disease screening at BIP	Consignment required to have tested negative to *Bd*/*Bsal*, with sufficient samples to detect disease with 99% confidence at prevalence of ≤1%, using molecular (PCR) diagnostics.	Partially feasible. Facilities may not exist at all BIPs. Use of rapid diagnostic techniques limits time required for result. Simultaneous multiple pathogen screening increase the efficiency of diagnostics.
Quarantine at BIP	Consignments quarantined at BIP until absence of the pathogen in confirmed using molecular diagnostics and/or treatment.	Partially feasible. Employment of rapid detection techniques reduces quarantine duration. Variable facilities and staff availability at BIPs.
Treatment of infected consignments	Consignments, or components of consignments that test positive for *Bd*, quarantined and treated with appropriate fungicidals to clear infection.	Currently unfeasible at BIPs. Treatment and quarantine facilities not available. Potentially feasible at wholesalers, if suitable biosecure housing demonstrated and post-treatment disease free status verified.
Euthanasia of infected consignments	Consignments euthanased according to approved guidelines, and disposed of in a biosecure manner e.g. incineration.	Partially feasible. Dependent on the existence of suitable facilities.
Compulsory notification and pathogen screening of all domestic imports (<5 animals imported by individual collectors).	Requirement for individuals entering the UK with amphibians for their personal collection, to inform import authorities, and obligation to have animals screened at point of import.	Partially feasible. Dependent on the existence of suitable facilities.

Primary constraints of the sanitary measures identified are centred around the time, financial and personnel costs involved in detection and mitigation of *Bd*/*Bsal*. The development of rapid molecular diagnostic equipment has greatly improved the potential for disease detection at BIPs, but they are not widely utilised as yet. The installation of these at important import points in the UK are hampered partially through the cost of instrument acquisition and associated reagents, and partially due to lack of technical expertise. Funding for the necessary training and equipment should be made available by the relevant government departments, given the potential for such methods to substantially reduce the risk of importing pathogens such as *Bd/Bsal* into the UK. Distribution of other costs (reagents and laboratory consumables), needs to be discussed with stakeholders and import authorities, but could be incorporated into an 'import fee' paid by importers.

The informal breeding and trade operating in the UK requires immediate attention. Tighter regulation of the trade and disease-free certification of stock, post-import or UK captive bred, should be discussed with wholesalers, retailers, breeders and trade representative groups such as the Reptile and Exotic Pet Trade Association (REPTA).

In order to reduce the risk of dissemination of *Bd/Bsal* into wild UK populations, biosecurity standards should be distributed to stakeholders, with advice on husbandry, sanitation and disease awareness.

- Implementation

Final decisions on the implementation of sanitary measures will require significant input from stakeholders at all levels in the trade, as well as import authorities and government officials. Recommendations based on this risk assessment to be put forward are:

1) Screening of imported amphibians to be undertaken at HARC. Priority to be given to shipments originating from Tanzania, USA and Asia. If implementation is successful after the first year, screening to be carried out at other BIPs.
2) Amphibian consignments, or consignment components, testing positive for *Bd/Bsal* should not leave the BIP unless they are treated on site (requires housing consignment for 7-10 days) and subsequently return negative test results. Alternatively, wholesalers may treat consignments where suitable

biosecure facilities exist, and sold-on when negative test results are obtained. Untreated infected consignments, or consignment components, should be destroyed and disposed of in a biosecure manner.

3) Information on suitable husbandry practices and biosecurity protocols, along with relevant disease information should be distributed to amphibian retailers nationwide. Wholesalers specifically should be advised to conduct regular screening for *Bd/Bsal* where possible.
4) Herpetological groups and trade representatives should be involved in an education programme directed at hobbyists, to iterate the risks associated with the releasing non-native/pet amphibians into the environment, and advocate responsible pet ownership.

Discussion

Conclusions drawn from this assessment indicate that amphibians imported for the pet trade pose a risk in terms of pathogen introduction into the UK. The greatest impact from pathogen introduction will likely be on the trade, and captive populations themselves. Economically, a hypervirulent strain of *Bd/Bsal,* could damage wholesaler and retailer businesses through direct loss of stock, but also through the cost of decontamination; and at the hobbyist level, rare or valuable species may be lost. The presence of known carriers of infection e.g. *X. laevis* and *Rhinella marina* (Cane toad), in trade collections (Pers. obs.), creates the potential for a reservoir of infection, that, in addition to economic losses, has serious welfare implications for susceptible species. Dissemination of *Bd/Bsal* into wild populations is less likely, but still probable, and effects on native amphibians could be severe, potentially affecting protected or declining species. Despite considerable literature coverage of *Bd* distributions in the wild, limited data pertaining to the prevalence in captive and trade amphibians, reduces the degree of confidence in estimates of likelihood of infection for specific trade components. However, the data collected in this study goes a long way to addressing these deficiencies.

Biosecurity planning or disease mitigation strategies at point of import are complicated by multiple entry points (airports, sea ports and rail links), number of exporting countries and diversity of species involved. Currently, HARC have been the only BIP involved in amphibian import research, and a priority is to approach other UK BIPs for their collaboration. Continued broad scale surveillance programmes, in addition to targeted sampling of Tanzanian and USA consignments, will be required, until further specific high risk factors become apparent. Precautionary *Bsal* surveillance requires immediate implementation, targeted at aquatic consignments containing caudate species from Asia, to prevent the establishment of *Bsal* in the UK.

Since the inclusion of *Bd* on the OIE list of notifiable diseases in 2008 (Schloegel et al., 2010), according to the World Animal Health Information Database (WAHID: www.oie.int/wahis_2/public/wahid.php/) a limited number of countries have reported presence of *Bd* in the last few years, although none have implemented any disease control measures. It is not clear to what extent amphibian diseases are recognised as an environmental threat in other countries, and it is likely that for the many, they will be perceived to have little consequence. For countries wishing to address the issue of amphibian pathogen dissemination, a thorough risk analysis must be undertaken prior to in order to abide by WTO obligations.

Import risk assessments are predominantly conducted to assess risks of pathogen introduction posed by single species originating from a single, or small number of countries. Consequently, risk analysis frameworks are designed for that purpose. The OIE import qualitative risk assessment framework (OIE, 2010), used in this analysis was flexible enough to accommodate the additional complexities of multiple species and countries of origin, however, it is unlikely that collection of enough data would be possible to enable a quantitative method. There is still a large amount of information that needs to be gathered, analysed and contextualised before a disease risk assessment can be utilised with high confidence that disease threats from the amphibian trade could be mitigated effectively. Whilst a complex task, it is achievable, with multi-organisational co-operation, interdisciplinary methodologies, and significant stakeholder input.

CHAPTER 6

Discussion

The pet trade has, to some extent, been an enigma in terms of conservation and disease threat, and difficulties in assessing these impacts arise for a number of reasons: 1) Typically, research has focused on the food trade, as it overshadows the pet trade in terms of volume, and is heavily reliant on *Lithobates catesbeianus*, a known carrier of *Bd*, ranavirus (Gratwicke et al., 2010) and notable invasive species (e.g. Abdulali, 1985, Mazzoni et al., 2003, Kusrini and Alford, 2006, Warkentin et al., 2008, Schloegel et al., 2009, 2010, Gratwicke et al., 2010). 2) There is a considerable deficiency of data pertaining to the volume and species composition of the international pet trade. The USA routinely record all amphibian imports and exports on the LEMIS (Law Enforcement Management Information System) (Schlaepfer et al., 2005), and the TRACES (Trade Control and Expert System) electronic application is utilised by the EU, but inadequate reporting may significantly underestimate volumes (Peel et al., 2012). For other countries only CITES listed species are recorded. As very few of the amphibian species in the pet trade are listed under CITES, the use of this database to evaluate overall volumes and trends is limited. 3) Assessing the threats to exploited populations, is not only difficult as the collection volume is unknown, but often there is also a fundamental lack of knowledge regarding the biology or population size of the species involved (Natusch and Lyons., 2012). 4) Within country, amphibian trade regulation appears to be loosely controlled by local councils, for example through the issuing of pet shop licences, with no coordinating central administration. This makes collation of data problematical.

To compound the problem, pet, food and other amphibian trades are, more often than not considered as a single entity “the trade” in publications, despite having discreet supply sources, transport networks, stakeholder groups and end markets. This can result in ambiguous conclusions surrounding the extent of over-exploitation and disease dissemination through different trade sectors.

Peel et al. (2012) estimated 130000 amphibians were imported into the UK annually, some of which were infected with *Bd*. This, together with evidence of *Bd* infecting wild amphibian populations (Cunningham et al., 2005, Minting and Cunningham, 2008, Smith, 2013), highlighted the need for a more thorough investigation. This study aimed to address some of the fundamental questions regarding the amphibian trade in the UK, and research was focused on two key areas, point of import (Heathrow Animal Reception Centre, HARC), and point of sale (amphibian retailers). The main questions asked were:

1) What are the species composition, origin and *Bd* infection status of amphibian consignments arriving at HARC?
2) How important are amphibian sales to the UK pet retail sector, and what is the level of amphibian disease awareness and knowledge amongst retailers?
3) What is the infection status of amphibians in UK retailers, and are there any factors associated with infection?
4) To what extent does *Bd* pose a threat to captive and wild amphibian populations in the UK?

Summary of findings

Approximately 20000 amphibians are imported into the UK for the pet trade via Heathrow Animal Reception Centre annually, from at least 11 countries. Forty-three genera were identified, and, with the exception of consignments from the USA, imports were comprised of genera native to the country of export. Tanzania, USA, Ghana and Hong Kong were the most important exporters in terms of volume. *Bd* was detected in six genera: *Hyperolius* and *Leptopelis* from Tanzania, and *Necturus*, *Siren*, *Desmognathus* and *Hyla* from USA, resulting in overall infection prevalence for imports of 3.6%.

Despite accounting for a relatively small (<5%) percentage of overall income for individual retailers, amphibians were sold in approximately a third of livestock retailers, and contributed at least £3million annually to the pet industry. Amphibian retailers could be broadly categorised into three shop types; ‘reptile’ – predominantly herptile species often with a selection of insect and arachnids, ‘aquatic’ – mostly fish sometimes with corals, and ‘pet’ – generalist stores with small mammals, goldfish, small cage birds, and occasionally kittens and puppies. Six of the 127 amphibian species in the trade, comprised a core sub-set that were frequently observed in the majority of retailers, and accounted for over 50% of the total estimated annual gross income. Specific disease knowledge and awareness was generally limited, but descriptions of husbandry practices suggest reasonable levels of biosecurity, even if this is not the primary motivation. The number of pet shop licences issued that allowed for the sale of amphibians appeared to increase between 2000 and 2010, implying the demand may be increasing in the UK.

The nationwide amphibian retailer *Bd* survey revealed an overall prevalence of 5.8%. Infection was widely distributed geographically, with some counties having a significantly higher proportion of infected animals than others. Two species, *Bombina orientalis* and *Ambystoma mexicanum* were found to be proportionately over infected compared to other species, and infection with *Bd* was associated with species kept in aquatic conditions.

The risk analysis indicated it was very likely that *Bd* would be imported into the UK, and it was concluded with moderate confidence that dissemination into wild populations from the trade was likely, and into captive populations was very likely. The introduction of a virulent strain of *Bd* may have a substantial negative economic impact for the trade sector, especially wholesalers of specialist retailers. The recent discovery of *Batrachochytrium salamndrivorans* (*Bsal*) (Martel et al., 2013) precluded its inclusion in earlier parts of the study, and currently it is unknown in the UK. The introduction of this pathogen via the pet trade is considered very likely, and could lead to significant mortalities in the great crested newt (*Triturus cristatus*), a highly protected species in the UK.

Application of methods used in a wider context and limitations of current study

Border Inspection Posts as sites for disease surveillance

Heathrow Animal Reception Centre (HARC) is the largest of four UK airports permitted to accept amphibian imports, and is equipped to handle the huge variety of domestic and commercial imports. Here, the success of the *Bd* survey relied on the full co-operation and enthusiasm of management and staff at the Centre, and the availability of resources and funding. The identification of *Bd* entering the UK in amphibian consignments suggests that the implementation of mitigation strategies, such as mandatory treatment or euthanasia or infected amphibians, should be a serious consideration in the near future. Additionally, continued sampling would greatly improve our understanding of the disease risks posed by different consignment origins and species contents, which would help target, and therefore increase the efficiency of, surveillance. Through informal discussions with various stakeholders, it became apparent that 'less legitimate' imports may be imported through other airports, intentionally to avoid the rigorous consignment inspections afforded to consignments arriving at HARC. Although these are unsubstantiated claims, attempts to co-ordinate research at other airports should be a priority, not only to conduct disease surveillance, but to verify the legitimacy of imported species. Unfortunately, logistical problems precluded sampling of aquatic consignments at HARC, which are believed to comprise a substantial proportion of amphibian imports. Investigation of these consignments requires urgent attention, as most originate from Asia, the hypothesised origin of *Bsal* (Martel et al., 2014).

Current legislation negates the requirement to declare amphibian imports from the EU. Consequently, at present it is not possible to quantify, determine the species or assess the disease status of amphibians entering from Europe. It is likely that a significant number of the amphibians obtained in Europe enter the UK via seaports in privately owned vehicles. Given the confirmed presence of *Bd* in European trade animals (Spitzen-van der Sluijs et al., 2013), enhanced checks of vehicles entering the UK via seaports, and screening of amphibians detected, would reduce the introduction of *Bd* via this route. Acknowledging limited funds and resources, the periods during and directly following herpetological shows on the continent, should be a priority for increased vigilance.

Acquisition of trade data from retailers

A considerable proportion of the data used in this study was derived directly from amphibian retailers, the vast majority of whom were extremely helpful and co-operative. Questionnaires are a useful tool in gathering trade data, as a large amount of information can be obtained from a wide target population, and a standardised set of questions allow the direct comparison of responses (De Vaus, 2002). Whilst some biases may occur, for example, more replies may be expected from specialist retailers, and mortality rates would likely be under-reported, subsequent shop visits suggested these effects were negligible and a representative sample had been achieved. Again, most retailers were willing to participate in the *Bd* survey. Analysis of infection status at point of sale is vital, as this represents the 'interface' between captive and wild populations. At a broad scale, the results here show that the prevalence of infection in retailers (5.8%) is higher than that at point of import (3.8%), but a limited number of widely traded species are proportionally over-infected. This information has implications for future management options and suggests that: at BIP's, targeted sampling and mitigation would effectively reduce the risk of importing *Bd*, whereas in retailers, a broader treatment scheme may be more effective, focusing on species shown to be proportionally over-infected (e.g. *Bombina* sp. and *A. mexicanum*). Preliminary comparative analysis between imports arriving at HARC and amphibian stock in retailers (Wombwell, unpublished data), indicates substantial differences in overall species composition and the taxonomic distribution of *Bd*, suggesting that data collected at BIP's does not reflect the situation at the retail end of the trade chain, and thus cannot be used to make predictions on disease status post-import. Further research is needed to understand the transmission and possible proliferation of pathogens throughout the trade chain, and will require the inclusion of wholesalers and private breeders, which was not possible in this study. The international trade network presents complexities on a much bigger scale, and has yet to be examined as a whole (Stuart et al 2004). Whilst it would take considerable collaborative efforts, the application of network analysis techniques, which have started to make an appearance in commercial livestock epidemiological studies (Dubé, 2011), are an interesting avenue to pursue that would enable the identification of important trade 'hubs' for disease dissemination or proliferation.

The application of 'Disease Risk Analysis' to the amphibian pet trade.

Formal risk analysis emerged as a distinct discipline in the late 1960s (Kellar, 1993), and evolved alongside veterinary epidemiology to be an important tool in assessing disease risk in international trade (Morley, 1993). The World Organisation for Animal

Health (OIE) provides a framework to assist analysts in conducting assessments in a logical and transparent manner. These frameworks are however, designed for the agricultural livestock industry, and are therefore usually implemented with the focus on one host species and one (or a select few) pathogen from a single country or origin. This study successfully demonstrated the application of the qualitative Covello-Merkhofer framework, advocated by the OIE, to assess the risk posed by multiple species from multiple origins. Whilst, arguably somewhat subjective, the output from analyses like this could be used as a tool to effectively communicate disease risk information to stakeholders and decision makers.

Future prospects for the amphibian pet trade and recommendations

The commercial exploitation of wildlife is, and will remain, a contentious issue, and the amphibian pet trade is no exception. Proponents maintain the trade inspires support for conservation through contact with unusual species, and valuable knowledge is gained regarding the biology and captive husbandry through the experience of keeping these animals. Additionally, there are economic benefits for all levels in the trade chain, which may be substantial especially in developing countries. On the other hand, opponents contend that trade threatens biodiversity through over-exploitation, introduction of non-native species and disease, and is ethically unjust. To a greater or lesser extent, all these factors are likely to be true. However, a constructive evaluation of the trade is only possible through objective research, and transparent analyses, but is often complicated by the paucity of reliable data.

The trade in amphibians has consistently been associated with over-exploitation of wild amphibian populations (Andreone and Luiselli, 2003, Stuart et al., 2004, Andreone et al., 2005, Schlaepfer et al., 2005, Carpenter et al., 2007, Gonwouo and Roedel, 2008, Rowley and Alford, 2009), but it is often unclear, as to which arm of the trade such statements apply. The food trade is the most significant in terms of volume, and supplying this market has resulted in severe population declines especially in Asia (Kusrini and Alford, 2006), although the farming of *L. catesbeianus* may alleviate the pressure on wild populations in the future. The pet trade, whilst associated with a high diversity of species, few make up the bulk of the trade in terms of volume (Prestridge et al., 2011, Herrel and van der Meijden, 2014, Chapter 2,3). It appears that, overall, a similar compliment of species (e.g. *Litoria caerulea*, *B. orientalis*, *Cynops orientalis*, *Hymenochirus* sp.) form the 'core sub-set' that comprise the mainstay of the trade (Japan: Tamukai et al., 2014, Taiwan: Hou et al., 2006, USA : Schlaepfer et al., 2005, Prestwich et al., 2011, Herrell and van der Meijden, 2014 UK: Tapley et al., 2012, Chapter 3). Anecdotal evidence suggests a significant proportion of these species are routinely captive bred in large numbers for the trade; if true, the threat to wild populations may be less than previously thought. However, the species traded in the highest volumes may be less susceptible to over-exploitation than those less commonly seen in trade (Schlaepfer et al., 2005), which are less abundant, have small or restricted distribution or are exclusively wild caught e.g. the Hairy frog (*Trichobatrachus robustus*) (Gonwouo and Roedel, 2008).

Validation of the origins of the most widely traded species should be a priority, to enable assessment of the susceptibility of these species to natural population declines, and infection. Efforts should be made to quantify the extent of harvesting on wild populations and where appropriate, quota systems should be introduced to ensure sustainable off-take (Bulte and Damania, 2005), based on sound biological research.

The international trade in amphibians has been implicated in the global spread of *Batrachochytrium dendrobatidis Bd* (Fisher and Garner, 2007), and detection of the pathogen in a number of studies supports this hypothesis (e.g. Weldon et al., 2004, Picco and Collins, 2008, Schloegel et al., 2009, Catenazzi et al., 2010, Tamukai et al., 2014, Chapter 2,4). Despite the listing of *Bd* as a notifiable disease in 2008 (Schloegel et al., 2010), to-date there have been no formal mitigation strategies implemented. With the recent discovery of *Batrachochytrium salamandrivorans* (*Bsal*), the recurrent detection of other pathogens such as ranavirus, it is imperative that biosecurity measures be adopted to reduce the spread of these and other emerging infectious diseases through the trade.

The most obvious preventative method would be a trade ban, but such a draconian measure may not be feasible or indeed suitable for a number of reasons. 1) Amphibians are utilised for a variety of purposes and it would be problematical to ban trade for some, but not all uses, on the grounds of conservation (over-exploitation and disease spread) or, ethics and welfare. The demand for amphibians as a food commodity has resulted in extensive local and international markets, with considerable economic value

(Warkentin et al., 2008, Kusrini and Alford, 2006). Whilst it is unlikely that there would be any health impacts through loss of protein sources (Kriger and Hero, 2009), in Western countries, economic impacts would be felt by the suppliers in developing countries. As it is unclear to what extent amphibian collectors in range countries rely on the pet trade, it would be irresponsible to enforce a ban without undertaking extensive socio-economic research to determine the level of reliance on this activity. 2) Current levels of amphibian trade regulation fail to even document the industry. It seems implausible that the infrastructure, even in developed countries, will cope with effectively enforcing a ban. This may lead to 3) an increase in the illegal trade, which may have severe conservation impacts (Cooney and Jepson, 2006, Rivalan et al., 2007, Conrad, 2012).

Another option would be to initiate 'disease free' certification for amphibians entering the trade. Ideally, commodities would be declared pathogen free prior to leaving the country of origin, but it is unlikely that the majority of exporting countries will have the resources or facilities to perform the diagnostic procedures required. Therefore the onus would be on importing countries to monitor the health of imports. Regulatory advisors and industry representatives should discuss the feasibility of point-of-import screening, as this may offer an effective method of reducing pathogen introduction via the trade. To supplement such efforts, consumer-based initiatives such as the US Pet Industry Joint Advisory Council's '*Bd*-Free Phibs' campaign (http://www.pijac.org/*Bd*-free-phibs), along with increased communication between researchers and stakeholders, would help reduce the dissemination of pathogens into wild populations (Perry and Farmer, 2011).

Reducing the risk pathogens pose to both captive and wild amphibian populations through trade, should be a priority for stakeholders, conservationists, researchers and regulatory decision makers alike. Substantial data deficiencies have precluded the objective assessment of the risks and benefits of the amphibian trade, and filling the voids in information available will become more important as the pressure on biodiversity increases. This study goes some way to addressing the deficiencies in the understanding of the amphibian pet trade in the UK, and advocates the instigation of similar studies in other countries where amphibians are widely traded.

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Appendix 1: Example of amphibian swab record sheet.

Amphibian Bd Swab Record Sheet

Date	
HARC Shipping Ref	
Importer	
No. and species of amphibian in consignment	
No. boxes	
Import/Transit	
Origin	
Destination (if transit)	

Swab No.	Box No.	Species	No. in box	Notes	+/-*
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					

Please use additional sheets if necessary.

* For lab purposes only

Appendix 2: Amphibian retailer questionnaire

Amphibians as Pets:

A Survey of Pet Shops in the UK

This survey is being conducted as part of a three year PhD thesis focusing on the international amphibian trade, specifically the economic importance of and sustainability of the trade. With disease becoming ever more relevant in the trade sector, I hope to develop realistic mitigation strategies, which will improve not only the health of amphibians but also long term viability of the trade.

Your participation in this study will greatly help to gain a clearer understanding of the trade sector and issues facing amphibian retailers.

As well as a summary of the final report, a free and confidential disease screening service will be offered to participating establishments.

All information in given in this questionnaire will be treated with the upmost confidentiality, and neither persons or establishments will be identifiable in any reporting of the study, unless acknowledgement is specifically requested.

This study is being undertaken at The Durrell Institute of Conservation and Ecology, University of Kent and Institute of Zoology, Zoological Society of London.

Research funded by the Natural Environment Research Council and the Economic and Social Research Council. NERC-ESRC Interdisciplinary Research Studentship.

Thank you for your time in completing this survey.

I understand that the information given in this questionnaire will remain strictly confidential. Persons and establishments will not be made in anyway identifiable in the final report or any publications resulting from this research.

Signed: ______

Date: ______

I would like a summary of the final report ☐

I am interested in the free disease screening service ☐
If yes please give details of who to contact below.

Name of Establishment: ______

Contact Name: ______

Contact Telephone No.: ______

Convenient time to call: ______

Please return completed questionnaires to:

Emma Wombwell, Ph.D. Candidate.
Wellcome Building, Institute of Zoology, Zoological Society of London, Regent's Park, London. NW1 4RY

If you have any questions regarding the questionnaire or disease screening, please contact me by email or phone:
EMAIL: Emma.Wombwell@ioz.ac.uk TEL: 0207 449 6698 MOB: 07779 637 232

Appendix 2: Amphibian retailer questionnaire

SECTION 1: Background Information

Q1: How long have you been selling amphibians? ☐ Years

Q2: How many people are employed? ☐ Full-time ☐ Part-time

Q3: In your experience, would you say:

- Amphibians are becoming **less** popular as pets ☐
- Popularity of amphibians has **remained the same** ☐
- Amphibians are becoming **more** popular as pets ☐

Please tick one box

Q4: Approximately what proportion of your annual turnover (including non-livestock, dry goods, equipment etc) derives from amphibian sales?

<5% ☐ 6-25% ☐ 26-50% ☐ 51-75% ☐ >75% ☐

Please tick one box

Q5: Which of these categories best describes your establishment?

- Garden Centre ☐
- Reptile Specialist ☐
- Aquatic Specialist ☐
- Non-specialist Pet Shop ☐
- Other (please specify) ☐

Additional Comments:

SECTION 5: Husbandry

Q1: Are the amphibians in your establishments housed in:

- Single species groups? ☐
- Mixed species groups? ☐
- Some mixed/some single groups? ☐

Please tick one box

Q2: In the box below please briefly describe your routine husbandry procedures. *E.g. frequency of enclosure cleaning, use of gloves etc.*

Q3: Are newly acquired amphibians most frequently housed:

- With existing stock of the same species? ☐
- With existing stock of other species? ☐
- In separate set-ups to existing stock? ☐

Please tick one box

Additional Comments:

Appendix 2: Amphibian retailer questionnaire

SECTION 4: Stock Acquisition

Q1: How many suppliers do you use to source amphibians? ☐

Q2: Briefly explain the factors you consider when purchasing both wild-caught (WC) and captive bred (CB) amphibians (*e.g. price, availability etc*), indicating your preference for either WC or CB stock.

Captive Bred ☐	
Wild Caught ☐	

Please complete the table, then tick either the CB or WC box

Q3: In general, how would you describe the condition of newly acquired ...

A) Captive bred amphibians?

Poor 1 2 3 4 5 Excellent 6 N/A

B) Wild caught amphbians?

Poor 1 2 3 4 5 Excellent 6 N/A

Please circle a number from 1-6

Additional Comments:

SECTION 2: Amphibian Health

Q1: What, if any, diseases do you think are the biggest threat to:

Wild amphibians?

Captive amphibians?

Q2: What is/would be your primary source for amphibian disease information?

Please tick one box

Internet ☐ Suppliers ☐

Veterinarian ☐ None ☐

Other retailers ☐ Other (*please specify*)

Q3: In the last two years, have you experienced any mortality in your amphibian collection?

Please tick one box

No ☐

Yes ☐

If you answered 'yes', briefly explain the event(s) in the box below. *Please include details such as: the species affected, number of individuals, causes (if known), and frequency of occurrence.*

Additional Comments:

Appendix 2: Amphibian retailer questionnaire

SECTION 3: Amphibian Species Stocklist

- Please complete the following table, listing all the amphibian species you sell.
- For species not regularly stocked but able to order, please indicated by writing 'OT' in the 'No. In stock' column.
- For species acquired sporadically, please estimate number per year. E.g. two per year = 2/12 as shown in the example.
- If more space is required please continue on a separate sheet.

	Species Name (Latin name if possible)	No. In Stock	No. Acquired per Month	Retail Price per individual (£)	CB/WC/ CF/LTC/ unknown	Country of Origin	Acquired from: e.g. local breeder, UK supplier, direct import etc.
E.g.	Dendrobates tinctorius	OT	2/12	35.99	WC	Nicaragua	USA Supplier
E.g.	Xenopus laevis	18	10	12.00	CB	UK	UK breeder

Appendix 3: Relative occurrence of amphibian taxa in the pet trade.

e.g. Amphibians of the family Bominatoridae were found in 48.6% of pet shops, and *Bombina variegata* in 2.6% of reptile shops.

FAMILY	Aqu	Pet	Rep	GENUS	Aqu	Pet	Rep	SPECIES		Aqu	Pet	Rep
ALYTIDAE	-	-	2.6	*Alytes*	-	-	2.6	*obstetricans*		-	-	2.6
ARTHROLEPTIDAE	1.6	2.7	10.3	*Leptopelis*	1.6	2.7	10.3	*flavomaculatus*		-	-	1.3
								macrotis	NT	-	-	1.3
								rufus		-	-	1.3
								vermiculatus	VU	1.6	2.7	7.7
				Trichobatrachus	-	-	1.3	*robustus*		-	-	1.3
BOMBINATORIDAE	22.6	48.6	53.8	*Bombina*	22.6	48.6	53.8	*bombina*		-	-	2.6
								orientalis		22.6	43.2	52.6
								variegata		-	8.1	2.6
BREVICIPITIDAE	-	-	1.3	*Breviceps*	-	-	1.3	*adspersus*		-	-	1.3
BUFONIDAE	6.5	2.7	23.1	*Amietophrynus*	-	2.7	5.1	*rangeri*		-	-	2.6
								regularis		-	2.7	2.6
				Anaxyrus	-	-	10.3	*cognatus*		-	-	2.6
								debilis		-	-	7.7
				Incilius	-	-	1.3	*alvarius*		-	-	1.3
				Melanophryniscus	-	-	2.6	*stelzneri*		-	-	2.6
				Pseudepidalea	-	-	6.4	*viridis*		-	-	6.4
				Rhinella	6.5	-	10.3	*marinus*		6.5	-	10.3
CALYPTOCEPHALELLIDAE	-	-	3.8	*Calyptocephalella*	-	-	3.9	*gayi*	VU	-	-	3.9
CERATOPHRYIDAE	25.8	29.7	60.3	*Ceratophrys*	25.8	27.0	60.3	*cornuta*		-	5.4	1.3
								cornuta x cranwelli		3.2	-	-
								cranwelli		14.5	16.2	37.2
								cranwelli albino		-	5.4	11.5
								ornata	NT	9.7	-	24.4
								sp.		3.2	2.7	9.0
				Lepidobatrachus	-	2.7	2.6	*laevis*		-	2.7	2.6
CRAUGASTORIDAE	-	2.7	-	*Craugastor*	-	2.7	-	*augusti*		-	2.7	-
DENDROBATIDAE	4.8	13.5	28.2	*Adelphobates*	-	-	3.9	*galactonus*		-	-	3.9
				Dendrobates	4.8	13.5	23.1	*auratus*		1.6	5.4	10.3
								azureus		-	-	5.1
								leucomelas		-	-	3.9
								sp.		1.6	8.1	10.3
								tinctorius		4.8	5.4	11.5
				Epipedobates	1.6	-	6.4	*anthonyi*	NT	-	-	1.3
								tricolor	EN	1.6	-	5.1
				Excidobates	-	2.7	1.3	*mysteriosus*	EN	-	2.7	1.3
				Hyloxalus	-	-	1.3	*azureiventris*	EN	-	-	1.3
				Oophaga	-	2.7	1.3	*pumilio*		-	2.7	1.3
				Phyllobates	-	-	6.4	*bicolor*	NT	-	-	2.6
								terribilis	EN	-	-	2.6
								vittatus	EN	-	-	2.6
				Ranitomeya	-	2.7	1.3	*benedicta*	VU	-	-	1.3
								imitator		-	2.7	-
								vanzolinii		-	-	1.3
HYLIDAE	30.6	48.6	80.8	*Agalychnis*	1.6	5.4	26.9	*callidryas*		1.6	5.4	26.9
				Hyla	17.7	21.6	41.0	*arborea*		3.2	-	9.0
								cinerea		16.1	18.9	25.6
								gratiosa		-	-	1.3
								squirrella		-	-	2.6
								versicolor		1.6	8.1	10.3
				Litoria	22.6	45.9	59.0	*caerulea*		22.6	45.9	59.0
								infrafrenata		-	-	1.3
								tyleri		-	2.7	-
				Osteopilus	-	2.7	6.4	*septentrionalis*		-	2.7	6.4
				Phyllomedusa	1.6	-	7.7	*hypochondrialis*		-	-	5.1
								sauvagii		1.6	-	2.6
				Trachycephalus	6.5	2.7	20.5	*resinifictrix*		6.5	2.7	20.5

Appendix 3: Relative occurrence of amphibian taxa in the pet trade.

HYPEROLIIDAE	1.6	5.4	17.9	*Afrixalus*	-	-	2.6	*fornasini*		-	-	1.3
								paradorsalis		-	-	1.3
				Heterixalus	-	-	1.3	*madagascariensis*		-	-	1.3
				Hyperolius	-	2.7	9.0	*argus*		-	-	2.6
								mitchelli		-	-	1.3
								parkeri		-	2.7	2.6
								riggenbachi	VU	-	-	1.3
								sp.		-	-	1.3
				Kassina	1.6	2.7	9.0	*maculata*		-	-	2.6
								senegalensis		1.6	2.7	6.4
MANTELLIDAE	-	-	1.3	*Mantella*	-	-	1.3	*betsileo*		-	-	1.3
								expectata	EN	-	-	1.3
MEGOPHRYIDAE	-	2.7	3.8	*Megophrys*	-	2.7	3.8	*montana*		-	-	1.3
								nasuta		-	2.7	2.6
MICROHYLIDAE	11.3	16.2	33.3	*Calluella*	-	-	2.6	*guttulata*		-	-	2.6
				Dyscophus	9.7	16.2	25.6	*antongilii*	NT	3.2	-	1.3
								guineti		6.5	16.2	24.4
				Kaloula	1.6	5.4	9.0	*pulchra*		1.6	5.4	9.0
				Phrynomantis	-	-	3.9	*bifasciatus*		-	-	3.9
				Scaphiophyrne	-	-	2.6	*marmorata*	VU	-	-	2.6
PIPIDAE	69.4	35.1	19.2	*Hymenochirus*	48.4	21.6	7.7	*boettgeri*		48.4	21.6	7.7
				Pipa	-	-	1.3	*pipa*		-	-	1.3
				Xenopus	41.9	16.2	11.5	*laevis*		38.7	16.2	10.3
								laevis albino		3.2	-	1.3
PYXICEPHALIDAE	3.2	16.2	26.9	*Pyxicephalus*	3.2	16.2	26.9	*adspersus*		1.6	13.5	25.6
								edulis		1.6	2.7	1.3
RANIDAE	1.6	-	-	*Pelophylax*	1.6	-	-	*esculentus*		1.6	-	-
RHACOPHORIDAE	-	-	25.6	*Chiromantis*	-	-	3.9	*xerampelina*		-	-	3.9
				Polypedates	-	-	17.9	*leucomystax*		-	-	16.7
								otilophus		-	-	1.3
				Rhacophorus	-	-	2.6	*annamensis*	VU	-	-	1.3
								dennysi		-	-	1.3
				Theloderma	-	-	3.9	*corticale*		-	-	3.9
AMBYSTOMATIDAE	46.8	37.8	39.7	*Ambystoma*	46.8	37.8	39.7	*andersoni*		1.6	-	-
								maculatum		3.2	2.7	1.3
								mexicanum	CR	45.2	35.1	29.5
								opacum		1.6	-	10.3
								tigrinum		1.6	-	2.6
PLETHODONTIDAE	1.6	-	-	*Aneides*	1.6	-	-	*lugubris*		1.6	-	-
				Pseudotriton	1.6	-	-	*ruber*		1.6	-	-
SALAMANDRIDAE	30.6	18.9	26.9	*Cynops*	14.5	13.5	7.7	*orientalis*		14.5	8.1	7.7
								pyrrhogaster		1.6	5.4	-
				Lissotriton	1.6	-	-	*montadoni*		1.6	-	-
				Mesotriton	3.2	-	2.6	*alpestris*		3.2	-	2.6
				Notophthalmus	1.6	2.7	2.6	*viridescens*		1.6	2.7	2.6
				Ommatotriton	1.6	-	-	*ophryticus*	NT	1.6	-	-
				Pachytriton	8.1	-	-	*brevipes*		1.6	-	-
								labiatus		6.5	-	-
				Paramesotriton	3.2	-	1.3	*hongkongensis*	NT	3.2	-	1.3
				Pleurodeles	9.7	5.4	2.6	*waltl*	NT	9.7	5.4	2.6
				Salamandra	6.5	2.7	14.1	*salamandra*		6.5	2.7	14.1
				Taricha	6.5	-	-	*rivularis*		6.5	-	-
				Triturus	1.6	-	1.3	*dobrogicus*	NT	1.6	-	-
								karelini		1.6	-	-
								marmoratus		1.6	-	1.3
								pygmaeus	NT	1.6	-	-
				Tylototriton	1.6	-	2.6	*asperrimus*	NT	1.6	-	-
								kweichowensis	VU	1.6	-	-
								shanjing	NT	-	-	2.6
DERMOPHIIDAE	-	-	1.3	*Geotrypetes*	-	-	1.3	*seraphini*		-	-	1.3

Appendix 4: Sampling intensity and Bd prevalence by country division and county.

County	Licences issued	Amphibian retailers	Target No. shops	Actual No. shops	No. of shops infected	Total No. samples	No Samples Positive
NORTH							
Aberdeenshire	20	5	1	0		0	
Angus Council	12	4*	1	0		0	
Clackmannanshire	2	1*	0	0		0	
Dumfries and Galloway	9	2	0	0		0	
Dundee	8	2	0	2	0 (0%)	78	0 (0%)
East Dunbartonshire	7	2	0	0		0	
East Lothian	5	5	1	0		0	
East Renfrewshire	4	4	1	1	0 (0%)	29	0 (0%)
Edinburgh	10	4	1	0		0	
Fife	16	10	2	3	0 (0%)	72	0 (0%)
Glasgow	28	6	1	1	0 (0%)	10	0 (0%)
Highland	9	3	1	0		0	
Lanarckshire	16	9	2	0		0	
Midlothian	3	2	0	0		0	
Moray	5	5	1	0		0	
North Ayrshire	4	1*	0	0		0	
Renfrewshire	8	8	2	0		0	
South Glamorgan	40	18	4	0		0	
Stirling	4	1	0	0		0	
Vale of Glamorgan	9	2	0	0		0	
West Dunbartonshire	3	2	0	0		0	
West Lothian	6	4	1	0		0	
Total	228	100	19	7	0 (0%)	189	0 (0%)
NORTH EAST							
County Durham	79	27	5	6	2 (33%)	87	12 (14%)
East Yorkshire	41	10	2	2	0 (0%)	6	0 (0%)
North Yorkshire	73	19*	4	3	0 (0%)	49	0 (0%)
Northumberland	20	1	0	1	0 (0%)	28	0 (0%)
Tyne and Wear	39	15	3	3	0 (0%)	30	0 (0%)
Total	252	72	14	15	2 (13%)	200	12 (6%)
NORTH WEST							
Cheshire	89	23	5	0		0	
Cumbria	43	12	2	3	0 (0%)	27	0 (0%)
Greater Manchester	78	28*	6	5	0 (0%)	45	0 (0%)
Lancashire	153	37	7	7	3 (43%)	68	11 (16%)
Merseyside	67	9	2	2	0 (0%)	14	0 (0%)
West Yorkshire	109	49	10	3	0 (0%)	43	0 (0%)
Total	539	158	32	20	3 (15%)	197	11 (6%)
EAST							
Cambridgeshire	60	21	4	4	0 (0%)	38	0 (0%)
Essex	102	31	6	4	2 (50%)	49	4 (8%)
Lincolnshire	88	29*	6	6	1 (17%)	91	6 (7%)
Norfolk	69	17	3	7	1 (14%)	117	4 (3%)
Suffolk	36	8	2	3	2 (0%)	35	0 (0%)
Total	355	106	21	24	4 (17%)	330	14 (4%)
CENTRAL							
Derbyshire	61	17	3	4	1 (25%)	69	3 (4%)
Herefordshire	49	2	0	0		0	
Leicestershire	48	18	4	3	2 (67%)	32	5 (16%)
Northamptonshire	29	8*	2	2	0 (0%)	8	0 (0%)
Nottinghamshire	77	22	4	5	1 (20%)	126	3 (2%)
Rutland	2	2	0	0		0	
Shropshire	21	3	1	0		0	
South Yorkshire	59	29*	6	1	0 (0%)	5	0 (0%)
Staffordshire	87	36	7	0		0	
Warwickshire	93	37	7	7	2 (29%)	88	13 (15%)
West Midlands	46	20	4	1	0 (0%)	9	0 (0%)
Worcestershire	32	15	3	2	0 (0%)	16	0 (0%)

Appendix 4: Sampling intensity and Bd prevalence by country division and county.

Total	604	209	41	20	6 (30%)	353	24 (7%)

County	Licences issued	Amphibian retailers	Target No. shops	Actual No. shops	No. of shops infected	Total No. samples	No Samples Positive
WEST							
Anglesey	8	2	0	0		0	
Bridgend County	12	3	1	0		0	
Caerphilly County	4	1	1	0		0	
Ceredigion	13	1	1	0		0	
Denbighshire	8	2	0	0		0	
Flintshire	8	1	0	0		0	
Monmouthshire	7	1	0	0		0	
Neath Port Talbot	0	0	0	1	1 (100%)	15	5 (33%)
Newport	9	5	1	1	0 (0%)	5	0 (0%)
Pembrokeshire	13	2	0	0		0	
Rhondda Cynon Taff	13	3	1	1	1 (100%)	15	2 (13%)
Swansea	15	2	0	0		0	
Torfaen	3	1	0	0		0	
Wrexham	6	2	0	0		0	
Total	119	26	5	3	2 (67%)	35	7 (20%)
SOUTH EAST							
East Sussex	32	12*	2	0		0	
Greater London	213	60	12	3	1 (33%)	47	15 (32%)
Hertfordshire	38	15	3	3	0 (0%)	69	0 (0%)
Kent	100	23*	5	3	1 (33%)	31	4 (13%)
Surrey	57	16	3	4	0 (0%)	55	0 (0%)
West Sussex	67	13	3	2	1 (50%)	80	4 (5%)
Total	507	139	28	15	3 (20%)	282	23 (8%)
SOUTH WEST							
Cornwall	35	9	2	3	0 (0%)	48	0 (0%)
Devon	88	29*	6	6	0 (0%)	67	0 (0%)
Dorset	48	17	3	4	2 (50%)	92	13 (14%)
Somerset	77	21*	4	5	1 (20%)	90	3 (3%)
Total	248	76	15	18	3 (17%)	297	16 (5%)
SOUTH							
Bedfordshire	29	13	3	3	0 (0%)	53	0 (0%)
Berkshire	41	15	3	3	0 (0%)	54	0 (0%)
Buckinghamshire	25	4	1	0		0	
Gloucestershire	55	13	3	3	3 (33%)	44	13 (30%)
Hampshire	95	21	4	5	0 (0%)	118	0 (0%)
Isle of Wight	12	7	1	0		0	
Oxfordshire	39	13	3	5	3 (60%)	52	8 (15%)
Wiltshire	47	10	2	1	0 (0%)	6	0 (0%)
Total	370	96	20	20	6 (30%)	327	21 (6%)
TOTAL	3195	982	195	147	27 (18%)	2210	128 (6%)

16 counties reporting no amphibian permitted amphibian retail excluded.

* includes an estimation of amphibian retailer numbers due to one or more local councils being unable to provide a precise figure.

Appendix 5: Results of a literature review conducted to inform the risk analysis.

Results of Literature search of records pertaining to *Batrachochytrium dendrobatidis* infection in countries from which exports to the UK are known to occur. All refer to sampling of wild amphibians, unless stated. Bd-Maps: Information taken directly from www.bd-maps.net database, where original text could not be accessed.

Source reference	**Details**	**Assessment**	**Likelihood of consignment infection.**
Madagascar			
Kolby 2014	3/565 *Bd* positives detected in exported consignment, sampled in USA.	All samples taken in-situ have returned negative results (n = 942). Three positive samples were taken post-export, and could therefore have been contaminated during transport. No positive results have been reported since the one unconfirmed report in-situ in 2011.	Very unlikely.
Crottini et al 2014	0/59 samples analysed using RT-PCR.		
Vredenburg et al 2012	0/300 samples analysed using qPCR.		
Crottini et al 2011	0/56 samples analysed using PCR.		
Rabemananjara et la 2011	Unconfirmed report of *Bd* detection, sample size unknown.		
Weldon et al 2008	0/527 samples examined histologically.		
Tanzania			
Zancolli et al 2013	Approx' 40% prevalence (n = 17) Tadpole mouthparts sampled.	According to Bd-Maps *Bd* prevalence is approximately 5% overall. The high volume and diversity of native species exported, increases the probability of *Bd* export. *Bd* has been described as widespread (31.5% prevalence) in neighbouring Kenya (Kielgast 2010), potentially an additional source of amphibian stock.	Likely
Gower et al 2013	7/44 caecilian sp. Analysed using qPCR.		
Vredenberg et al 2013	0/10 museum specimens.		
Peel et al 2012	Bd detected in consignments exported from Tanzania, in two species: *Hyperolius argus* and *H. tuberlinguis*.		
Bd-Maps: Moyer and Weldon 2006	36/576 samples from Udzungwa Mountains, collected 2004.		
Weldon &Du Preez 2004	*Bd* detected in three individuals.		
Bd-Maps: Garner 2003 unpublished data	0/199 East Usambara region. Analysed using PCR		
Cameroon			
Ghose et al 2014	38.5% prevalence, field data in 2011.	Recent studies indicate that *Bd* is widespread, with a high prevalence. Exported amphibians are likely to be native wild caught specimens.	Very likely
Gower et al 2013	51/83 caecilian sp. Analysed using qPCR		
Doherty-Bone et al 2013	124/1137 anuran and caecilian sp. Analysed using RT-PCR.		
Balaz et al 2012	1/70 mixed species. Analysed using RT-PCR.		
Blackburn et al 2010	0/26 single sp. Analysed using RT-PCR.		
Soto-Azat et al 2010	1 archived sample from larger study.		
Doherty-Bone et al 2008	0/284 mixed species in highlands. Analysed using RT-PCR.		
Ghana			
Penner et al 2013	0/292 samples of various origins. Analysed using RT-PCR.	*Bd* has not been detected in field surveys of Ghanain amphibians, although sampling has not occurred to the same extent as in Madagascar.	Unlikely
Bd-Maps: Roedel 2010 Unpublished data	0/7 various species		
Egypt			
	No records found	Assessment not possible	
Russia			Unlikely
Civis et al 2013	0/180 wild amphibians, analysed by RT-PCR.	Lack of sampling leads to low confidence in assessement.	
Ukraine			
	No records found	Assessment not possible	

Appendix 5: Results of a literature review conducted to inform the risk analysis.

South Africa			
Tarrant et al 2014	14.8% prevalence, field samples (n = 392). Threatened species. Analysed using RT-PCR. 26.6% prevalence in wider study (n = 1,577). Analysed using histopathology and PCR.	Bd has been described as "endemic and widespread" in South Africa (Tarrant et al 2013). This is statement supported by other studies. It is unclear whether South Africa represents a trade 'hub' where re-exports from other countries occur, but given the possibility that stock could be mixed in holding facilities, or come into contact during transit, the risk of infection can be considered high. It is also likely that wild caught *Xenopus sp*. are exported for laboratory purposes.	Very likely
Vredenberg et al 2013	0/4 museum specimens.		
Conradie et al 2011	18/235 tadpoles sampled.		
Soto-Azat et al 2010	2 archived samples from larger study.		
Smith et al 2007	62.5% & 38.6% prevalence in two species of anuran tadpole.		
Weldon at al 2004	19/679 museum specimens. Analysed using histopathology.		
Bd-Maps: Hopkins & Channing 2003	36/74 mixed sp. Analysed using histopathology.		
Lane et al 2003	1 individual, found dead.		
Switzerland			
Tobler et al 2012	128/766 mixed species. Analysed using RT-PCR.	Bd appears to be widespread in Switzerland, with some areas infection free. Exports of native species would therefore present a risk. It is not possible to assess infection likelihood of captive-bred or re-exported consignments.	Likely (native species)
Lotters et al 2012	0/109 Salamander and *Bufo* sp.		
Schmidt et al 2010	Widespread distribution of Bd in NW Switerland.		
Garner et al 2005	63/252 Analysed using RT-PCR.		
Oevermann et al 2005	Describe outbreak of chytridiomycosis in Switzerland.		
Hong Kong			
Kolby et al 2014 Captive amphibians in trade.	31/265 samples from consignments at point of import in USA. Bd+ species: *Bombina orientalis* and *Xenopus laevis*.	Infection status in-country has not been well established. However, documented evidence of Bd detection in exports originating Hong Kong suggests infection is likely. Overall prevalence of 5%.	Likely
Rowley et al 2007	0/274 mixed species		
Rowley et al 2007 Captive amphibians in trade.	0/137 three species from pet shops and food markets.		
China			
Zhu et al 2014	60/1,007 archived specimens of mixed wild caught species (dates 1933-2009).	A series of studies have identified *Bd* in native amphibians from China. Whilst, is represents a large geographic range, Bd has also been confirmed in trade amphibians. Overall >7% prevalence. Unconfirmed reports of large scale breeding of amphibians (mainly aquatic species) requires investigation, as high turnover and close confinement of animals increase the risk of infection.	Very Likely
Bai et al 2012	157/2075 across 112 sites. Analysed using nested PCR.		
Bai et al 2012 Captive amphibians in trade.	89/659 samples collected from pet shops and food markets. Analysed using nested PCR.		
Bai et al 2012b	0/120 tadpoles sampled in Northern China.		
Swei et al 2011	0/257 mixed species. Analysed using histopathology and qPCR.		
Wei et al 2010	0/191 *Rana dybowskii*. Analysed using PCR		
Bai et la 2010	35/259 Native species and *L. catesbeianus*.		
Bai et la 2010	4/37 *L. catesbeianus* from food market.		
Ouellet et al 2005	0/2 Rana sp.		
Malaysia			
Swei et al 2011	2/111 mixed species. Analysed using RT-PCR.	*Bd* has been detected in wild amphibians at an overall prevalence of <3%. Further sampling is required, therefore assessment confidence is low.	Unlikely
Savage et al 2011	10/127 mixed species. Analysed using RT-PCR.		
Kaiser & Grafe 2012	0/6 Analysed using RT-PCR		
Bd-Maps: source unknown	1/255 mixed species. Analysed using qPCR.		

Appendix 5: Results of a literature review conducted to inform the risk analysis.

Indonesia			
Swei et al 2011	11/797 mixed species. Analysed using RT-PCR.	Overall prevalence <2%, however assessment confidence is low due to small sample size.	Unlikely
Kusrini et al 2008	4/147 mixed species. Analysed using RT-PCR.		
Suriname			
	No records found for Suriname.	Lack of sampling in Suriname prevents direct assessment of Bd risk. However, the widespread distribution of *Bd* in neighbouring Brazil and demonstration that *Bd* has disseminated across large areas of South America, suggests Suriname should be considered a potential area for *Bd* infection.	Likely
Rodriques et al 2014	24% prevalence (n=2799), archived samples. Multiple species and habitats.		
Ramalho et al 2013	3/5 &5/7 Two tadpole sp. Analysed histologically. Also provide extensive literature review detailing the identification of *Bd* in 400 individuals of 48 species, covering a wide geographic area.		
Luger et al 2008	A study in neighbouring Guyana failed to detect *Bd* in 24 samples (Luger et al 2008)		
Canada			
Voordouw et al 2010	42/320 *Rana pipiens*, across multiple sites.	Bd detected in native amphibians from a range of geographic areas. The extent to which exports from Canada consist of native species is unknown. Canada also exports non-native species and the captive bred or wild caught status is unknown. Restrictions on *Lithobates catesbeianus* introductions reduce the likelihood of infection introduction from Canada.	Likely
Slough et al 2009	13/70 mixed species. Analysed using RT-PCR		
Schock et al 2009	7/556 mixed species. Analysed using qPCR.		
Deguise & Richardson 2009	9/32 *Bufo boreas*. Analysed using PCR.		
Adams et al 2007	2/51 mixed species. Analysed using PCR.		
Garner et al 2006	13/52 *Lithobates catesbeianus*. Analysed using RT-PCR.		
Bd-Maps: Charbonneau 2006	5/43 *Lithobates catesbeianus*. Analysed using PCR.		
Ouellet et al 2005	146/798 mixed species. Analysed using histopathology.		
USA			
Bd-Maps: references therein.	1381/11593 Mainly wild amphibians. 154 species.	There are too many studies to report individually. Studies have been performed across large areas of the USA, and revealed an overall prevalence of 12%. Whilst there are some geographical and species biases (see Olsen et al 2013), it is suspected Bd has a widespread distribution in the USA. The USA is a known hub in the global trade network, exporting native, captive-bred and re-exported amphibians; making determination of the consignments initial country of origin problematic. Due to the high native prevalence, and the opportunity for multiple species to come into contact at holding facilities or during transit, consignments disembarking from USA are considered at risk of containing infected animals.	Very likely
Peel et al 2012	*Bd* detected arriving in consignments from USA. Two species: *Pyxicephalus adspersus* and *Pseudacris crucifer*.		

Appendix 6: Papers co-authored during Ph.D.

Spitzen-van der Sluijs, A., Martel, A., **Wombwell, E**., Van Rooij, P., Zollinger, R.,Woeltjes, T., Rendle, M., Haesebrouck, F., Pasmans,F. (2011) Clinically healthy amphibians in captive collections and at pet fairs: A reservoir of *Batrachochytrium dendrobatidis*. *Amphibia-Reptilia.* 32(30): 419-423.

Penner, J., Adum, G.G., McElroy, M.T., Doherty-Bone, T., Hirschfield, M., Sandberger, L., Weldon, C., Cunningham, A.A., Ohst, T., **Wombwell, E**., Portik, D.M., Reid, D., Hillers, A., Ofori-boateng, C., Oduro, W., Plotner, J., Ohler, A., Leache, A.D., Rodel, MO. (2013) West-Africa – A safe haven for frogs? A sub-continental assessment of the chytrid fungus (*Batrachochytrium dendrobatidis*). *Plos One.* 8 (2) e56236

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M.C., Farrer, R.A., Schmidt, B.R., Tobler, U., Goka, K., Lips, K.R., Muletz, C., Zamudio, K., Bosch, J., Lotters, S., **Wombwell, E**., Garner, T.W.J., Cunningham, A.A., Spitzen-van der Sluijs, A., Salvidio, S., Ducatelle, R., Nishskawa, K., Nguyen, T.T., Kolby, J.E., Van Bocxlaer, I., Bossuyt, F., Pasmans, F. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science.*346 (6209), 630-631.