

Disease risk analysis and post-release health surveillance for a reintroduction programme: the pool frog *Pelophylax lessonae*

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Running Head: Disease risk analysis for a reintroduction programme

Summary

There are risks from disease in undertaking wild animal reintroduction programmes. Methods of disease risk analysis have been advocated to assess and mitigate these risks, and, post-release health and disease surveillance can be used to assess the effectiveness of the disease risk analysis but results for a reintroduction programme have not to date been recorded. We carried out a disease risk analysis for the reintroduction of pool frogs (*Pelophylax lessonae*) to England, using information gained from the literature and from diagnostic testing of Swedish pool frogs and native amphibians. Ranavirus and *Batrachochytrium dendrobatidis* were considered high risk disease threats for pool frogs at the destination site. Quarantine was used to manage risks from disease due to these two agents at the reintroduction site: the quarantine barrier surrounded the reintroduced pool frogs. Post-release health surveillance was carried out through regular health examinations of amphibians in the field at the reintroduction site and collection and examination of dead amphibians. No significant health or disease problems were detected but the detection rate of dead amphibians was very low. Methods to detect a higher proportion of dead reintroduced animals and closely related species are required to better assess the effects of reintroduction on health and disease.

Key words: translocation, amphibian, biosecurity, quarantine, ranavirus, chytridiomycosis, *Batrachochytrium dendrobatidis*, disease risk management

Introduction

Reintroduction programmes undertaken for conservation purposes present a risk of disease to the reintroduced and recipient populations due to changes in the parasite (viral, bacterial, fungal, protozoal, helminth and ectoparasite) complement of the reintroduced and recipient hosts, stressors on these animals and exposure to non-infectious disease agents (Sainsbury et al 2012). For example reintroduction of Mallorcan midwife toads (*Alytes muletensis*) using captive-bred animals led to the introduction of the fungal pathogen (a pathogenic parasite) *Batrachochytrium dendrobatidis* to free-living populations (Walker et al 2008) and this pathogen has been associated with amphibian extinctions (Berger et al 1998); reintroduced cirl buntings (*Emeriza cirlus*) succumbed to stress-induced isosporosis (McGill et al 2010), and reintroduced Californian condors (*Gymnogyps californianus*) were threatened by toxins (Green et al 2008). Despite these hazards, reintroduction programmes continue to be considered important for conserving species (Ewen et al 2012) and translocations are expected to be increasingly used as conservation tools to counter the adverse effects of climate and other anthropogenic changes to the environment (McLachlan et al 2007; Hunter 2007).

The risk of disease outbreaks associated with reintroductions has been recognised by the International Union for the Conservation of Nature (IUCN) and consequently the IUCN recommended disease monitoring of reintroduction programmes in their Guidelines on Reintroductions (IUCN 2012) and more recently in a specific guideline for amphibian reintroduction programmes (Pessier and Mendelson 2010). Appropriate methods for carrying out disease risk analysis for wild animal translocation programmes have been set out (Armstrong et al 2003; Davidson and Nettles 1992; Leighton 2002; Miller 2007; Sainsbury and Vaughan-Higgins 2012; World Organization for Animal Health 2014; Jakob-Hoff et al 2014; World Organization for Animal Health 2015) in order that the probability of a disease outbreak and the magnitude of its effects can be evaluated prior to reintroduction, and mitigation measures can be devised and implemented. A key objective of these disease risk analyses is to prevent the introduction of parasites alien to the reintroduction site (source hazards) (Sainsbury and Vaughan-Higgins 2012) because there is observed evidence that alien parasites, introduced through translocations, are associated

with major epidemics of disease (Bobadilla et al 2016), for example squirrelpox virus introduced to the UK lead to catastrophic disease in red squirrels (Sainsbury et al 2008) and therefore Sainsbury and Vaughan-Higgins (2012) assessed all infectious agents novel to the destination as hazards. Other objectives of disease risk analysis include evaluation of the impact of parasites at the reintroduction site on the health of the reintroduced animals, the assessment of the effect of the stress of reintroduction on the health of the translocated animals, and analysis of non-infectious agents, for example toxins, at the reintroduction site on the newly arrived population (Sainsbury et al 2012). Monitoring the health of reintroduced and recipient populations following the reintroduction provides important information on the fate of animals, the reasons for the success or failure of the reintroduction and effectiveness of the disease risk analysis (Sainsbury et al 2012), and the need to monitor reintroduced populations rigorously and using standard methods has been advocated by Sutherland et al (2010).

There are some examples in the literature of disease risk analyses carried out for avian reintroduction programmes (Neimanis and Leighton 2004; Sainsbury and Vaughan-Higgins 2012) but neither of these publications reported on the post-release health monitoring that was carried out, and therefore the effects of reintroduction on the health of reintroduced and recipient populations has not, to date, been recorded, or the effectiveness of the disease risk analysis evaluated. Despite records showing that 58 species of amphibians have been reintroduced for conservation purposes (Griffiths and Pavajeau 2008), and that diseases are known to threaten amphibian populations, no disease risk analyses have been reported for amphibian reintroductions or translocations. In this paper we describe the disease risk analysis (including disease risk management) and post-release health surveillance undertaken for the reintroduction of the pool frog (*Pelophylax lessonae*) to the UK from Sweden, and evaluate the results.

The pool frog is a European species, a member of the green or water frog group, which became extinct in the UK in 1994 (Buckley and Foster 2004). In 2003 a proposal was made by Natural England (formerly English Nature) and the Herpetological Conservation Trust to reintroduce pool frogs to England from Sweden, and Buckley and Foster (2004) explained the rationale for reintroduction. There was good evidence that habitat loss and degradation were the key factors in the decline of the pool frog in England (Buckley and Foster 2004) and a suitably protected and managed site for the reintroduction was selected and prepared within the species historic home range. At the same time that these reintroduction plans were being prepared, considerable evidence was becoming apparent that two infectious diseases, chytridiomycosis and ranaviral disease, were associated with disease outbreaks and the decline of amphibian populations, and that the disease outbreaks had probably been precipitated by the transfer of pathogens between geographic areas (Cunningham et al. 1996, Berger et al. 1998, Daszak et al. 2003). Indeed, the danger of the spread of these infectious agents through the amphibian trade has subsequently been recognised and both agents are now listed by the World Organization for Animal Health (OIE) (World Organization for Animal Health 2015). As a consequence, the team which set out to reintroduce the pool frog was concerned that the risk of disease to pool frogs and native UK amphibians should be evaluated prior to reintroduction and appropriate mitigation measures put in place. At this stage, it was decided to translocate free-living pool frogs directly from Sweden to England, rather than undertake a captive breeding programme because the risks from disease were perceived to be lower.

Between 2003 and 2005 a detailed disease risk analysis was undertaken, disease risk management recommendations were made and, after release of the first pool frogs in 2005, post-release health surveillance was implemented. The aim of this work was to investigate (i) the risk of disease to the pool frogs to be translocated from Sweden, and to native amphibians in the UK, from changes in parasite complement of populations, (ii) exposure to non-infectious disease agents at the reintroduction site and (iii) stressors throughout the reintroduction process. Additional aims included communicating these risks of disease to stakeholders in the reintroduction, and, where possible, proposing and implementing mitigation measures for disease and welfare threats and monitoring the consequences of reintroduction to the health and welfare of all the populations involved. The results of welfare monitoring during procedures and post-

release will be reported in a separate paper. The results of the investigations on risk of disease and health are reported here to explain how we set out to achieve the aims, the difficulties encountered in analysing the risk from disease, and the consequences of reintroduction to amphibian population health so that methods in monitoring the health of reintroduced populations can be improved in future.

Methods

Disease risk analysis

The disease risk analysis commenced in 2003 and we used the literature available, at that time, to guide our method on conducting an assessment of the risks of disease to translocations: we defined the hazards (agents of disease) similarly to Leighton (2002) and we carried out a disease risk assessment using a method adapted from that reported by Davidson and Nettles (1992). The disease risk analysis terminology used is as described by Murray et al (2004). The team selected by the Zoological Society of London and Natural England to conduct the disease risk analysis was composed of two wildlife veterinarians and an amphibian ecologist and they received support from a pathology technician, diagnostic microbiologists and parasitologists, Masters students, ecology and veterinary experts and two wildlife veterinarians in Sweden.

Hazard identification. We identified the parasites present in pool frogs in the source environment, Sweden, and in native amphibians (common frog, *Rana temporaria*, common toad, *Bufo bufo*, smooth newt, *Lissotriton vulgaris* and great crested newt, *Triturus cristatus*) present at the reintroduction site through literature review and screening with diagnostic tests. Literature was sought from Web of Science and Zoological Record using the keywords *disease*, *amphibian*, *parasite*, *infectious* and *pathology*.

Sample sizes of amphibians selected for screening for parasites were based on the estimated prevalence of the specific parasite to be tested in the population. Where no data were available to predict the prevalence, it was assumed to be 1% on the basis that a pathogenic parasite prevalence as low as this approximate magnitude of prevalence is predicted to be able to regulate population numbers (Tompkins et al 2001). The sample size was calculated using the methods described by DiGiacomo and Koepsell (1986) and Thrusfield (1995) in order to detect at least one infected animal in the sample at 95% confidence.

Screening of adult pool frogs, adult great crested newts and adult smooth newts. A toe clip was taken using surgical scissors together with a sample of in contact water, for a polymerase chain reaction test (PCR) for chytrid fungi (Aguilar Sanchez et al. 2004). Cloacal and oral swabs were collected for viral culture and stored at -70°C. A skin swab was taken for fungal culture (newts only). A blood smear was taken from the cut surface of the toe clip. Small volumes of blood were available by this method and so the blood was dabbed onto the middle of a slide and a second slide placed at 90 degrees to the first and the two slides pressed together. The skin was examined for ectoparasites. Faecal samples were taken (newts only) and cultured for bacteria, and examined for protozoa and helminths as described below.

Screening of adult common frogs. For PCR for chytrid fungi, skin samples were taken from the dorsal skin, inguinal region and the axillary region and stored in bijoux containers at -70°C. The skin was examined for ectoparasites and a skin swab was taken for fungal culture. Samples of liver, kidney and intestine were taken for viral culture and stored at -70°C. Samples of liver, kidney, spleen and heart were also taken where available, for ranavirus PCR, and stored at -70°C. The large intestine was cultured for bacteria and examined for protozoa and helminths. A blood smear for haemoparasitology was made from heart blood.

Screening of larvae (all species). Mouthparts were sampled for chytrid fungi PCR. A skin swab was taken for fungal culture. Bacterial culture was undertaken on the intestine. Half the remaining body was taken for viral isolation, and the other half of the body was taken for ranavirus PCR, both samples being stored at -70°C.

Unless otherwise stated screening was undertaken at the Institute of Zoology. The protocols for ranavirus PCR were undertaken using the methods of Mao *et al* (1996). Viral culture samples were passaged three times in chicken embryo fibroblast cultures and once in BHK 21 and frog embryo fibroblast (ICR-2A) cultures at the Animal and Plant Health Agency, Weybridge, Surrey (Umo *et al* 2004). A standard protocol was used for chytrid fungus PCR (Boyle *et al* 2004). Swabs taken for fungal culture were placed on Sabourauds agar including chloramphenicol (QCM Laboratories, Unit 206 Greenheath Business Centre Three Colts Lane, London, UK), incubated aerobically at 25°C and observed for fungal elements at 1, 2, 5, 7 and 14 days. Fungal isolates were either identified using API biochemical tests (bioMerieux Ltd, Marcy-l'Etiole, France) or sent to the Mycology department of CABI Bioscience (UK Centre, Egham, Surrey) for identification. Samples from the intestine/ large intestine for bacteriology were plated onto Columbia blood agar including 5% horse blood (QCM Laboratories), incubated aerobically at 25°C and observed at 1, 2 and 5 days. Bacterial isolates were tentatively identified using API biochemical tests (bioMerieux Ltd, Marcy-l'Etiole, France). A wet preparation of intestinal contents was examined at magnifications of x10 and x100 for protozoa and helminths. Where protozoa or helminths were detected, half the samples were placed into 2% aqueous potassium dichromate, and half into 70% ethanol for subsequent identification. The helminths were identified by Dr Eileen Harris at the Natural History Museum, London.

If a species of parasite was present in both the source and destination environments this parasite was not identified as a hazard. A parasite present in the source environment and not the destination was identified as a source hazard and a parasite present at the destination but not the source as a destination hazard.

Disease risk assessment. We used an adaptation of the method proposed by Davidson and Nettles (1992) to assess the risk that parasites, introduced to the destination with translocated animals, would cause disease in either the translocated animals or in recipient populations. Each parasite identified as a hazard was assessed by Davidson and Nettles's two-tiered, reasoned, logical process. In the first step, an assessment of the probability that the parasite would become established at the release location was made, and this was followed, in the second step, by an assessment of the parasite's pathogenic capabilities. Probability of establishment at the release location was assessed to be increased if (a) the parasite had a widespread geographic distribution and therefore was well adapted to different environments, (b) the parasite had a direct transmission cycle, or the parasite's transmission was indirect and the vectors / intermediate hosts were known to be present at the destination, (c) the prevalence of infection in the translocated animals was high and (d) the parasite was infective for other species at the destination. The parasite's pathogenic capabilities were assessed for both the translocated animals or other species at the destination. Once the two steps had been completed, the risk that the parasite would cause disease was assessed as high, medium or low based on a combination of the establishment and pathogenicity rating, where 'high' is 'extending above the normal level', 'medium' is 'the normal level' and

'low' is 'less than the normal level'. If there was uncertainty in making this assessment but the risk was assessed greater than 'negligible' then 'non-negligible' was used.

We devised a similar process to assess the risk of disease in the translocated animals from parasites present in animals at the destination but not present at the source. Probability of establishment of the parasite in the translocated pool frogs was assessed to be increased if (a) the parasite had a widespread geographic distribution, (b) the parasite had a direct transmission cycle, (c) the prevalence of infection in animals at the destination was high and (d) the parasite was infective for the translocated species. The probability that the parasite would be pathogenic in the translocated animals was evaluated using information from the literature. Overall the risk that the parasite would cause disease was assessed as high, medium or low based on a combination of the establishment and pathogenicity rating where 'high' is 'extending above the normal level', 'medium' is 'the normal level' and 'low' is 'less than the normal level'.

Davidson and Nettles (1992) considered the possibility that the translocation of animals into a destination site would increase the potential number of hosts and therefore lead to an 'artificial intensification' of a previously endemic disease and we examined whether this might occur in this translocation.

Disease Risk Management. A detailed protocol of disease risk management was devised based on the results of the disease risk assessment and our knowledge of captive and free-living wild animal epidemiology and preventive medicine. A quarantine barrier was established between Swedish pool frogs and the UK environment to prevent transfer of infectious agents to other areas in the UK apart from the reintroduction site. Tools, boots, clothing, nets and all other equipment used to capture pool frogs in Sweden were dedicated to the project. Boots were either new or, cleaned and disinfected prior to use, and latex gloves were worn.

Health examination of juveniles and adult pool frogs prior to reintroduction included measurements of (i) body weight (Pesola scales, Switzerland); (ii) estimation of body condition (poor, good, fat) was made using a combination of the qualitative assessment of the thickness of the femoral musculature and fat cover upon palpation and an assessment of the lumbar musculature; poor - concave lumbar musculature; good - level lumbar musculature; fat - rounded muscle and fat cover; (iii) physical examination of eyes, ears, oral cavity (Sleek tape® (BSN Medical, UK) was used to open and examine the oral cavity) and skin; (iv) palpation of the musculoskeletal system; (v) auscultation of the thorax to detect abnormalities of respiratory and cardiovascular sounds; (vi) coelomic palpation for abnormalities of texture, shape, and consistency of coelomic organs; and (vii) physical examination of the vent. Photographs were taken of juvenile and adult pool frogs for identification purposes (to record notable stripes and markings). Larvae and egg masses were visually inspected for abnormalities of shape, size and colour. Results of examinations were recorded onto standard forms and by digital photography. Throughout the clinical examination the amphibians were kept moist with the pond water of origin. Immediately after examination amphibians were returned to transport containers. Tools, boots and nets were cleaned and disinfected before travelling to the UK. During transport, husbandry was designed with the aim of reducing the stress of the pool frogs to a minimum. Post-metamorphic frogs were housed as individuals in transparent, rigid, smooth sided, plastic transport boxes containing damp moss and approximately 3mm deep pond water. These boxes were placed in ventilated cool boxes in compliance with International Air Transport Association (IATA) guidelines. Spawn was placed in plastic bags filled with pond water to approximately quarter depth and tied to enclose as much air as possible.

Risk communication. Once the disease risk analysis had been completed, the results were submitted in a report to the steering committee of the reintroduction programme which subsequently submitted it to the Department for Environment, Food and Rural Affairs (Defra).

Post-release health surveillance

Post-release health surveillance was achieved through health examinations of live amphibians and detailed pathological examinations of amphibians found dead at the reintroduction site. Juvenile and adult amphibians in the UK were captured using nets either from the banks of the pond, or from wading into the pond, and transferred into plastic transporting boxes approximately 150mm x 100mm x 80mm which had previously had holes of approximately 5mm diameter drilled in the lids to provide ventilation. These boxes contained a small amount of water and pond weed and were of an appropriate size to prevent jumping amphibians from damaging themselves. Health examinations of frogs and toads were carried out using the protocol described above. The welfare of the pool frogs post-release was assessed both through the health examinations, behavioural observation (by an observer and using digital video in 2006) and faecal cortisol measures (Wardley 2006) but the results of behavioural observations and cortisol changes are not reported here. Binary logistic regression analysis was employed to identify predictors of whether pool-frogs were seen post-release and odds ratio and its 95% confidence intervals were reported.

Juvenile and adult great crested and smooth newts were examined in the same manner as for pool frogs, with the exception that body condition scoring was assessed by examination of the lumbar musculature, which was concave either side of the lumbar vertebrae in those in poor body condition (score 1), in line with the spinous processes in those in good body condition (score 2) and prominent of the spinous processes in those in fat body condition (score 3). Examination of larval forms included measurement of body length and body weight (unless animals were less than approximately 20mm in length, with consequent health risks if handled in which case these measurements were not taken), visual examination of eyes, gills and skin; visual estimation of body condition.

In carrying out health examinations of pool frogs in the first months post-release the objective was to examine the effect of the reintroduction on their health and welfare. In health examinations of native amphibians and, in later years of the programme of pool frogs, the objective was to detect an epidemic disease outbreak, either in native amphibians due to a novel parasite introduced by pool frogs, or in pool frogs which encountered a novel parasite at the reintroduction site. The sample size of each species chosen for health examinations was based on the proportion of each species which might be affected by an infectious disease outbreak and therefore the probability of detecting sick individuals at 95% confidence limits.

This study was reviewed by the Zoological Society of London Ethical Review Committee.

Results

Translocation pathway

A decision was taken to translocate pool frogs directly from the environment in Sweden to the release site in England, because no hazards with a high risk of causing disease in native English amphibians had been detected in Swedish pool frogs (see below) and a 'wild-to-wild' translocation was assessed as preferable to a translocation with an intermediate captive stage for the management of the pool frogs. Pool frogs of all life stages were captured by hand and using dip nets from ponds in eastern Sweden as described in more detail by Foster et al (in press). They were transferred to transport containers (see below), transported by car to the airport at Stockholm, flown to southern England and transferred to the reintroduction site close to Thetford, in Norfolk, UK by car. The maximum duration between capture in Sweden and release in England was 7 days. Between 2005 and 2008, inclusive, 90 adults, 88 juveniles, and 3605 larvae were released.

Disease risk analysis

Hazard identification. Three infectious agents associated with disease in amphibians were identified in the literature search in 2004: a ranavirus-like agent had been identified in England in the common frog *Rana temporaria* in association with mortality outbreaks (Drury et al 1995; Cunningham et al 1996); *Batrachochytridium dendrobatidis*, the chytrid fungus, was detected in bull frogs (Cunningham et al 2005) in England in 2004 and *Dermocystidium ranae* (now *Amphibiocystidium ranae*) had been detected in Italy in association with population declines of pool frogs (Pascolini et al 2003). No infectious agents associated with disease in Swedish amphibians were found in the literature but several apparently commensal infectious agents were recorded in both Sweden and the UK (Jaenson 1990, Cedhagen 1988, McCarthy 1999 and Jackson and Tinsley 2001).

In view of the limited information on the parasites of pool frogs in Sweden, and, in particular, the presence or absence of ranavirus and chytrid fungus, it was decided that screening of pool frogs in Sweden for parasites was warranted to gather better information to assess the risk of the disease to the proposed reintroduction. At the same time we elected to screen UK amphibians in the reintroduction area for infectious agents to improve our understanding of the parasites present in these species such that assessments could be made on the risk of disease in reintroduced pool frogs and the effect of reintroduction on the parasite community at the reintroduction site. Considering the detection of ranavirus in the UK, we assumed this virus was present at the reintroduction site, or would likely spread there, because of an absence of barriers to amphibian movements, and therefore we did not test for it in UK amphibians. Table 1 includes data on the number of pool frogs from Sweden and amphibians from the UK which were screened for parasites, the samples tested, and the parasites screened for in those samples.

Table 1. The numbers of pool frogs and amphibians in the UK sampled for infectious agents for the disease risk analysis including the samples collected, the diagnostic tests chosen and the infectious agents targeted for detection, and those infectious agents detected.

Species	Life stage	No screened	Sample tested	Infectious agent target	Diagnostic test	Infectious agents detected
Pool frog	larval	70	faeces	bacteria	bacterial culture	see text
		73	body	viruses	viral culture	none

		70	skin swab	fungi	fungal culture	none
		72	mouthparts	<i>Batrachochytrium dendrobatidis</i>	PCR	negative
	juvenile	29	faeces	bacteria, protozoa, helminths	bacterial culture, microscopic examination	see text
			skin	<i>Batrachochytrium dendrobatidis</i> ; other fungi; ectoparasites	PCR; fungal culture; clinical examination	<i>Penicillium expansum</i>
		28	liver, kidney, spleen, heart	ranavirus	PCR	negative
		29	liver, kidney, intestine	other viruses	viral culture	none
			blood smear	haemoparasites	microscopic examination	none
	Adult	5	faeces	bacteria, protozoa, helminths	bacterial culture, microscopic examination	free living rhabditid nematodes
		36	skin	<i>Batrachochytrium dendrobatidis</i> ; other fungi; ectoparasites	PCR; fungal culture; clinical examination	<i>Penicillium expansum</i>
		4	liver, kidney, spleen, heart	ranavirus	PCR	negative
		31	cloacal swabs; oral swabs;	other viruses	viral culture	none
		5	liver, kidney, intestine	other viruses	viral culture	none
		36	blood smear	haemoparasites	microscopic examination	resembling <i>Trypanosoma rotatorium</i>
	Smooth newt	Adult	12	faeces	bacteria, protozoa, helminths	bacterial culture, microscopic examination
oral swabs				viruses	viral culture	none
4			cloacal swabs	viruses	viral culture	none
12			skin	fungi; <i>Batrachochytrium dendrobatidis</i>	fungal culture; PCR	none; PCR negative
			blood smear	haemoparasites	microscopic examination	reddish intraerythrocytic inclusions
larvae		31	faeces	bacteria	bacterial culture	see text
		26	body	viruses	viral culture	none
		31	skin	fungi	fungal culture	<i>Candida kefyr</i>
		24	mouthparts	<i>Batrachochytrium dendrobatidis</i>	PCR	negative
Great-crested		Adult	11	faeces	bacteria, protozoa, helminths	bacterial culture, microscopic examination

newt		12	cloacal swabs	viruses	viral culture	none
		10	oral swabs	viruses	viral culture	none
		12	skin	fungi; <i>Batrachochytrium dendrobatidis</i>	fungal culture; PCR	none; PCR negative
			blood smear	haemoparasites	microscopic examination	none
	larvae	29	faeces	bacteria	bacterial culture	see text
		30	body	viruses	viral culture	none
		29	skin	fungi	fungal culture	<i>Penicillium expansum</i>
	49	mouthparts	<i>Batrachochytrium dendrobatidis</i>	PCR	negative	
Common frog	Adult	6	faeces	bacteria, protozoa, helminths	bacterial culture, microscopic examination	see text
			liver, kidney, intestine	viruses	viral culture	none
			skin	fungi; <i>Batrachochytrium dendrobatidis</i>	fungal culture; PCR	none; PCR negative
			blood smear	haemoparasites	microscopic examination	none
	larvae	30	faeces	bacteria	bacterial culture	see text
			body	viruses	viral culture	none
			skin swab	fungi	fungal culture	none
		68	mouthparts	<i>Batrachochytrium dendrobatidis</i>	PCR	negative
Common toad	larvae	30	faeces	bacteria	bacterial culture	see text
			body	viruses	viral culture	none
			skin	fungi	fungal culture	none
		159	mouthparts	<i>Batrachochytrium dendrobatidis</i>	PCR	negative

N.B. PCR = polymerase chain reaction.

Culture of all cloacal and oral swabs and tissues collected from Swedish pool frogs and UK native species was negative for signs of viral growth. All pool frogs tested negative for ranavirus by PCR. Numerous bacterial species were detected on faecal bacteriology (McGill et al 2004), for example *Aeromonas hydrophila* and *Hafnia alvei*. The majority of these species were present in both Sweden and the UK, and a search on Web of Science showed that all the species detected were globally widespread. Helminths detected in the intestines of seven pool frogs were identified as free living rhabditid nematodes (E Harris) and assessed as non-pathogenic to amphibians. Protozoa were found in the intestines of 29 pool frogs and identification to a species level was not

carried out. Flagellate, ciliate and cyst forms of protozoa were detected in UK amphibians. No external parasites were detected in any species. Haemoparasitological study demonstrated that one pool frog had parasites morphologically resembling *Trypanosoma rotatorium* (M Peirce) and two smooth newts demonstrated reddish intraerythrocytic inclusions similar to those seen in ranavirus infections in other amphibian species (Gray et al 2009). Mycological isolates included *Penicillium expansum* cultured from two pool frogs and three UK amphibians, and *Candida kefyr* cultured from one UK amphibian. A search on Web of Science showed that *Candida kefyr* is widespread in the Palearctic. PCR for chytrid fungus was negative for all UK native amphibian species examined and pool frogs.

Parasites identified as either source or destination hazards are shown in Tables 2 and 3. Those parasites which were present in both the UK and Sweden were not assessed as hazards. No non-infectious hazards were identified from the literature.

Table 2 Disease risk assessment for hazards recorded or detected in Swedish amphibians and not detected in amphibians in the UK (see the text for the literature used to provide evidence for the assessment)

Hazard	Probability of establishment at the release site					Parasite's pathogenic capability		Total disease risk	
	Widespread geographic distribution?	Method of transmission	Intermediate hosts / vectors present?	Prevalence of infection in translocated animals	Probability that other species at the destination will become infected	Overall assessment	Pathogenic for the translocated animals		Pathogenic for other species at the destination
<i>Trypanosoma rotatorium</i>	reported from the Americas and Asia in amphibians but not from UK	indirect	probably leeches of genus <i>Batracobdella</i> and <i>Helobdella</i>	1.5% (n=65)	high	medium	no reports of disease in the literature	unknown	non-negligible
Unidentified intestinal Opalinid cysts	unknown	direct	-	85% (n=34)	unknown	high	no reports of pathogenicity	unknown	non-negligible

Table 3 Disease risk assessment for hazards detected in amphibians native to the UK and not in Swedish amphibians (see the text for the literature used to provide evidence for the assessment)

Hazard	Probability of establishment in the translocated population					Parasite's pathogenic capability	Total disease risk
	Widespread	Method of	Prevalence of	Probability that	Overall	Probability pathogenic	

	geographic distribution?	transmission	infection in animals at destination	translocated animals will be infected	assessment	for the translocated animals	
Ranavirus	widespread	probably direct	unknown	high	high	high	high
<i>Batrachochytridium dendrobatidis</i>	widespread	direct	unknown	high	high	high	high
<i>Amphibiocystidium ranae</i>	widespread	probably direct	unknown	high	high	medium	medium
Unidentified intestinal protozoa – cysts in common frogs; flagellates and ciliates in smooth newts; flagellates and cysts in great crested newts	unknown	direct	in common frogs 50%(n = 6); in smooth newts flagellates 33% (n = 12) and ciliates 8% (n = 12); in great crested newts flagellates 42% (n = 12) and cysts 25% (n=12)	unknown	unknown	cysts and ciliates probably commensal; flagellates believed to be pathogenic when host is under stress	non-negligible

Disease risk assessment. The results of the disease risk assessment are shown in Tables 2 and 3. A search on Web of Science showed that *Trypanosoma rotatorium* is reportedly widespread in frogs and is probably transmitted through leeches (Desser 1976; Ray and Choudhury 1984), including *Helobdella* spp. which are present in the UK (Spelling and Young 1986). There are no reports of disease in amphibians associated with this parasite, although other trypanosomes are pathogenic in amphibians (Poynton and Whitaker 2001) and disease might occur in immunologically naïve amphibians. Opalinid protozoa are considered commensals (Poynton and Whitaker 2001) and are transmitted directly and therefore those detected in pool frogs are likely to become established at the release site but were predicted to represent a low risk of disease. Of the protozoa detected in native amphibians, ciliates are believed to be commensal (Poynton and Whitaker 2001), the cysts detected are likely to be opalinid commensal cysts and flagellates have been recorded to be associated with a ‘failure to thrive’ in captive amphibians in association with stress such as shipping (Poynton and Whitaker 2001).

Two parasites were assessed as of high risk to the reintroduction: chytrid fungus and ranavirus. In both cases these were destination hazards which were not detected in pool frogs from Sweden and therefore it was expected that pool frogs would be immunologically naïve to these infectious agents and therefore susceptible to disease. In 2004 the mechanism of transmission of ranaviruses was unclear but it now appears that ranaviruses are persistent in the pond environment (Nazir et al 2012) and several routes of direct (without vectors or intermediate hosts) transmission occur (Gray et al 2009). The prevalence of infection in common frog populations in the UK is probably variable depending on whether disease is transient, endemic or epidemic (Teacher et al 2010) and epidemiological studies continue to suggest that infection of pool frogs with ranaviruses from native species cannot be discounted (Miller et al 2011). Indeed a mortality outbreak associated with ranavirus infection has been reported in edible frogs *Pelophylax* kl. *esculentus* in Denmark (Ariel et al 2009) and a common midwife toad-like virus (CMTV-like) was associated with epidemic disease in *Pelophylax* spp in the Netherlands (Kik et al 2011) and therefore the pathogenicity rating in the assessment was evaluated as ‘high’. The inclusions found in smooth newt erythrocytes may represent a sign of ranaviral infection.

The susceptibility of frogs in the genus *Pelophylax* to *Bd* was difficult to predict, and remains so despite further research since our disease risk assessment was made because species differences in susceptibility are known to occur (Stockwell et al 2010). Since *Bd* has been pathogenic and led to the extinction of other amphibian species (Stockwell et al 2010) the pathogenicity rating was made 'high'. *Bd* is now known to infect natterjack toads (*Epidalea calamita*) in the UK and a current survey has shown that the fungus is widespread in this country (ZSL/Defra/ARG-UK 2012). The taxonomic nomenclature / position of *Amphibocystidium ranae* is unclear but it appears to be widespread in anuran and caudate species in Europe (Gonzalez-Hernandez et al 2010; Pereira et al 2005) and of variable pathogenicity (Gonzalez-Hernandez 2010) and therefore the pathogenicity rating in the assessment was rated as medium.

Risk communication. The results of the disease risk assessment were reported to English Nature and Defra's ACRE Committee and a recommendation made by the authors that further sampling of pool frogs for ranavirus and *Bd* should be conducted prior to reintroduction proceeding, on the basis that sample sizes were not sufficiently high to detect infectious agents of lower than approximately 10% prevalence. The report concluded that, although ranavirus or *Bd* had not been detected in Swedish pool frogs their presence could not be excluded and, since these were known to have potentially high pathogenicity and strains from Sweden might represent alien parasites in England, these agents therefore presented great risk of precipitating a disease outbreak, with potential effects on large numbers of free-living amphibians associated with the reintroduction (Leighton 2002; Sainsbury and Vaughan-Higgins 2012). The report also emphasized the difficulty of detecting all the parasites which might be harboured by amphibians because our understanding of their parasite complement is relatively poor and tests to detect some parasites may not be available. The report was considered by ACRE, without external review as far as the authors understand, and approval given for reintroduction to proceed from 2005, without recommendations for disease risk management.

Disease risk management. Health examinations were performed on all pool frogs captured in Sweden (Table 4) and detailed results are described under post-release health surveillance below and in Table 5. Frogs showing signs of disease were not transported to the UK but following an assessment by the wildlife veterinarian conducting the examinations, frogs from the same pond were allowed to travel.

A quarantine barrier was imposed on the reintroduction site and all staff (ecologists, veterinarians) and visitors were required to follow a strict biosecurity protocol. Staff in contact with captive amphibians and infectious disease laboratories were requested to shower and change their clothes before visiting the reintroduction site. Tools, boots, clothing, nets and all other equipment were dedicated to the site. Persons entering the reintroduction site cleaned and disinfected their boots prior to use and wore latex gloves while on site. Any equipment which needed to be taken off site was disinfected prior to departure. The disinfectant used was sodium hypochlorite at 200mg/L water with a contact time of 15 minutes conforming to the principles in the Aquatic Animal Health Code (World Organization for Animal Health 2015). Entry to the reintroduction site was restricted and a locked barrier prevented vehicular access. The ponds chosen for reintroductions were fenced in 2005 and 2006 to prevent predator incursion and to enhance the ability to monitor the pool frogs post-release.

Health examinations were carried out on pool frogs within 24 hours of arrival in the UK using the same methods as above, and, immediately following the examination, juvenile and adult pool frogs were released at the reintroduction site. Adult pool frogs above a snout-vent length (SVL) of approximately 40mm had microchips (AVID2028; AVID, USA) inserted subcutaneously in the left coelomic area in 2005 and 2006. As described above, microchip insertion ceased from 2007, because it was found that photographs of back patterns could be effectively used for identification purposes, which thus reduced the probability of

infectious agent entry or exit through microchip wounds. Following examination larvae were either released into tadpole cages or reared in captivity for release. Tadpole cages were composed of wood and wire mesh, and were of approximate dimensions 100mm x 300mm x 500mm. The cages had removable lids of the same construction, and open bases which were pressed into the pond mud to form a base.

For skin, oral or musculo-skeletal lesions, dry swabs were taken for the detection of chytrid fungus (*Batrachochytrium dendrobatidis*) by real time PCR (Boyle et al. 2004), and swabs in transport medium were screened for bacteria and fungi using the culture methods described above under methods.

Post-release health surveillance

The health of amphibians at the reintroduction site was monitored between 2006 and 2012. Table 4 lists the number of pool frogs examined clinically between 2006 and 2012 at the reintroduction site. Health examinations of pool frogs, and native amphibians, were carried out at monthly intervals at the reintroduction site between May and September in 2006 and 2007. In 2008 the first examinations of native amphibians were conducted in March, and no examinations were conducted in August. Native amphibians (common frogs, great crested newts, and smooth newts) were examined in May, July, August and September in 2006 and 2007, and in March, May, July and September in 2008. From 2009 examinations were conducted on these three species plus common toads; in 2009 examinations were conducted in March, May and September; in 2010 in March and September; and thereafter in 2011 and 2012 examinations were only carried out in March. The mean and range, in brackets, of native amphibians examined per annum was as follows: mean 25 (range 0 and 62) smooth newts; 23 (0 and 42) great crested newts; 9 (1-20) common frogs; 31 (29-34) common toads. Immediately after examination the juvenile and adult amphibians were returned to their pond of origin. Following examination, larvae were either i) re-released into tadpole cages or ii) re-released directly into a pond.

Our sample size of pool frogs examined was chosen on the basis of the proportion of the population likely to be affected by a disease outbreak and the probability of detecting sick individuals. Our disease risk assessment suggested that *Bd* and ranavirus were the most likely agents to cause disease in the reintroduced population of pool frogs. In both ranaviral disease and chytridiomycosis epidemics reported in the USA, mortality has been known to exceed 90% at affected sites (Green, Converse and Schrader 2002) probably dependent on species susceptibility (Blaustein et al 2005; Brunner et al 2005). We were unable to predict the susceptibility of pool frogs based on any evidence in 2005, and subsequent research suggested susceptibility is variable within a species (Padgett-Flohr and Hayes 2011; Woodhams et al 2011) and consequently we made a decision to attempt to detect a disease outbreak affecting 10% of the population because this degree of mortality would probably be significant for population viability. In order to detect a single diseased frog with 95% confidence for a disease causing 10% mortality in a population of 250 frogs, 29 frogs are predicted to require examination (DiGiacomo and Koepsell 1986) and therefore we decided to attempt to examine at least 29 pool frogs on each visit. We had no information in our disease risk assessment with which to predict the number of native amphibians requiring examination, and probably the greatest disease threat to these species was posed by any undetected agents of disease of unknown pathogenicity, and therefore, in the absence of a better guide, we chose to examine approximately 30 animals of each native species on each visit.

By the autumn of 2009 our results indicated that the reintroduced pool frog population was healthy following release, and signs of breeding had been detected: as early as 2006 released pool frogs had spawned (Foster et al in press). Given these findings, and constraints on resources, we chose to dedicate

health monitoring activities to native amphibians. By the end of 2010, no diseases assessed as of risk to the populations of smooth newts or great-crested newts had been detected and therefore we focused our health examinations on common frogs and common toads because these species have a closer phylogenetic relationship to pool frogs and therefore were assessed as more likely to contract a novel parasite. At the same time, we closely followed the results of population monitoring being conducted by Foster et al (in press) in readiness to alter the focus of our health monitoring should any of the amphibian populations show a decline.

No abnormalities of health were detected in the majority of pool frogs and native amphibians. The body weight of adult and juvenile pool frogs at the time of capture, either three (in 2006 and 2008) or four (in 2007) days later (at the time of release) and 31 days (2008), 37 days (2007) or 38 days (2006) after capture is illustrated in Figures 1 and 2. The figure shows that although pool frogs consistently exhibited a reduction in body weight in the 3 or 4 days between capture in Sweden and release in the UK (the reduction was significant: paired t-test; n = 123; p < 0.001), those examined over the following month regained and showed a significant increase in body weight between capture and either day 31, day 37 or day 38 post-capture (paired t-test; n = 22; p < 0.001).

Adult frogs (male or female) were more likely to be seen post-release than juvenile pool frogs (of unknown sex) (male odds ratio= 1.40; 95% confidence interval (CI) = 1.02 – 1.91; p = 0.035 ; female odds ratio 1.05 ; 95% CI = 0.81 – 1.36; p = 0.70).

Table 4. Numbers of pool frogs examined clinically before and after reintroduction between 2006 and 2012

Adult and juvenile Pool frogs examined before reintroduction (in Sweden and the UK)			Pool frogs examined after reintroduction						
2006	2007	2008	2006	2007	2008	2009	2010	2011	2012
27 (including 2 juveniles)	47 (including 30 juveniles) plus between approx 2000 and 4000 eggs	55 (including 30 juveniles) and approx 3000 eggs	47 (including 22 metamorphs)	30 (including 12 juveniles)	55 (including one juvenile)	38 (including 6 juveniles)	1	1	1

Table 5 describes the diseases (defined as any abnormality of an animal's structure or function) detected in pool frogs and native amphibian species between 2006 and 2012 in Sweden (pool frogs) or at the reintroduction site (pool frogs and native amphibians). As a consequence of the detection of the *Saprolegnia*-like infection in the egg mass in Sweden, and a wound in an adult pool frog, neither the egg mass nor the adult frog were translocated to the UK. None of the other diseases detected were assessed as sufficiently serious to prevent translocation to the UK or re-release at the reintroduction site by the experienced wild animal veterinarians conducting the examinations; nor were these diseases assessed as warranting treatment. The lesions noted in Table 5 were confined to restricted areas of the body. The wounds on the tongue observed on six pool frogs were suspected to have been associated with feeding on prey because these pool frogs were seen eating adult dragon flies prior to capture. The following bacteria were grown in pure culture: *Burkholderia cepacia* from the erythematous skin lesions from two pool frogs before release; *Aeromonas hydrophila* from one pool frog with a minor skin wound post-release; *Pseudomonas fluorescens* (0157557) from minor skin lesions on three pool frogs post-release; *Burkholderia cepacia* from a superficial ulcer on a great crested newt. The following bacteria were cultured as predominant growths: *Pasteurella aerogenes* from the punctate ulcers found on one of the common frogs with these lesions; *Ralstonia pickettii* (0041455) from a male common frog with yellow thickened epidermis on the ventrum and medial hindlimbs. These were apparently the first recorded isolates of *Ralstonia pickettii* and *Burkholderia cepacia* from native amphibians in the UK (and *Ralstonia pickettii* was also isolated from three pool frogs in mixed culture) but both bacteria have been widely reported from the UK (Muhdi et al. 1996, Sousa et al. 2011; Kimura et al. 2005; Maroye et al. 2000; Ryan et al. 2006; Weidmann et al. 2008, The Environment Agency 2002; Health Protection agency 2008, 2009). In 2006 and 2007 dry swabs collected from all lesions on all species examined at the reintroduction site and examined by PCR for *Batrachochytrium dendrobatidis* were negative for the fungus. From 2008 dry swabs collected from the inguinal and hindlimb skin of all frogs and toads (those with and without lesions) and from lesions on newts were negative for *Batrachochytrium dendrobatidis* on PCR. PCR for ranavirus was carried out on skin swabs from any animals with skin lesions examined at the reintroduction site from 2011 and no virus detected. The punctate ulcers described on two common frogs were consistent in appearance with *Amphibocystidium ranae* infection but this fungus was not detected. A single leech was detected on each of two common frogs, without signs of disease, and one of these leeches was identified as *Helobdella stagnali*.

Table 5. The number of cases of disease detected during clinical examination of pool frogs and native amphibians examined at the reintroduction site (all data collected between 2006 and 2012 combined).

Clinical findings		Smooth newt	Great-crested newt	Pool frog		Common frog	Common toad
				Before reintroduction	After reintroduction		
Infectious, or suspected infectious, diseases	Erythematous skin	2		4	3	2	
	Superficial ulceration of the skin		1		1	1	1
	Other minor skin lesions	3	2	3	20	3	3

	Multiple punctate ulcers on the dorsum					2
	Yellow thickened epidermis on ventrum and medial hindlimbs					2
	Off-white cotton-wool growth - infection with <i>Saprolegnia</i> – like fungus			1 (egg mass)		
	Swelling of, and excessive mobility in, the mandibular articulation				1	
Non-infectious diseases - traumatic wounds	Loss of a part of a limb		4	2	1	3
	Fresh minor skin wounding	4	2	1	5	2
	Small (<1mm) reddish wounds on tongue				6	
	Minor trauma to the oral mucosa					3
Miscellaneous diseases	Poor body condition; flaccid coelom				2	

N.B. More than one clinical finding may have been recorded in a single animal; the location of lesions on the animal's body was varied if not stated; all animals were active and alert.

During ecological and health monitoring visits to the site personnel checked each pond and the surrounding land at the reintroduction site for dead amphibians. Table 6 shows the results from pathological examinations on two pool frogs found dead.

Foster et al (in press) used capture-mark-recapture data to show that the pool frog population at the reintroduction site was stable by 2012, with an estimated maximum adult population of 7, and, from a low point in 2009 there was possible evidence of growth. The project should not yet be considered a success.

Table 6. Post-mortem examination findings for pool frogs found dead at the reintroduction site as a component of post-release health surveillance.

History	Age	Sex	Date found dead	Pathological findings
Found in shallow water at the edge of a pond	Adult	M	10 March 2007	Two areas of erythema were present over the ventral aspect of both shoulders, of approximate diameter 7mm. The central inguinal area was also erythematous. Wounds without bruising in coelom suggested scavenging post mortem. The heart, mid and caudal gastro-intestinal tract, liver and spleen had probably been removed by a scavenger. No other abnormalities detected.
Found in the shallows of a pond at the reintroduction site	Adult	F	7 March 2010	Good body condition with coelomic fat deposits; spawn present in the caudal coelom; white, cotton-wool like growth covered the body surface; congested liver, kidneys and gastrointestinal tract; congested lumbar spine at the level of the urostyle. A moderate mixed growth of <i>Aeromonas hydrophila/caviae</i> was isolated from the skin, oral cavity, heart and intestine and <i>Ochrobacterium anthropi</i> from the oral cavity and intestine. No fungi isolated. PCR for chytrid fungus and ranavirus negative. Body weight 14g.

Discussion

Between 2003 and 2005 a disease risk analysis was carried out on the proposed reintroduction of pool frogs using information from the literature and diagnostic testing of pool frogs and native amphibians in the UK, the first time a disease risk analysis has been reported for an amphibian reintroduction. The disease risk analysis identified *Batrachochytrium dendrobatidis* and ranavirus as hazards for the reintroduced pool frogs, assessed them as of high risk, and recommended further diagnostic testing of pool frogs to fully evaluate the infectious agents of highest risk to native amphibians. Prior to the first reintroduction of pool frogs in 2005 a detailed disease risk management protocol was enacted which included quarantine measures, attention to hygiene, husbandry practices to reduce the stress to translocated amphibians and health examinations of all pool frogs before export from Sweden. Quarantine was implemented at a natural reintroduction site, the first record of such a scheme. Following the first reintroduction a post-release health surveillance protocol was put into action, involving clinical and pathological examinations, and this protocol was continually modified to target available surveillance resource at the population which we believed was most at risk from disease. Pool frogs suffered a significant loss of body weight between capture in Sweden and release in England but apparently regained this body weight within approximately one month at the release site suggesting that the transport methods did not harm the pool frogs irrevocably. Adult pool frogs were more likely to be seen post-release than juveniles and this finding should be considered in selecting animals for future reintroductions. No disease outbreaks of significance have been detected at the release site and the majority of pool frogs and native amphibians showed signs of good health. A small population of pool frogs remains on site and health surveillance continues. The population would have been expected to have grown, given apparently good resources, to carrying capacity and the project cannot yet be assessed as a success.

The method of disease risk analysis employed here utilised the best practice for wild animal translocations published and available in 2003. Since 2003 several more publications have been produced (for example, Armstrong et al 2003, Miller 2007, Sainsbury and Vaughan-Higgins 2012, World Organization for Animal Health 2014; Jakob-Hoff et al 2014) which have built on the work of Davidson and Nettles (1992) and Leighton (2002), and we are using the method described by Sainsbury and Vaughan-Higgins (2012) to conduct current disease risk analyses for reintroduction programmes. Davidson and Nettles (1992) recognized the uncertainty in making their disease risk assessment because alien parasites could have unpredictable pathogenicity and the evaluation of the establishment rating or the pathogenic potential is very difficult in the absence of information. We did not attempt to make individual assessments of uncertainty probabilities for each hazard but recent advances in uncertainty analysis (EFSA in press) should be considered in future disease risk analyses for conservation translocations. No independent studies have been carried out to determine whether disease risk analysis is effective in reducing the threat of disease to reintroduced and recipient free-living wild animal populations and there are no independent recommendations on the most effective method. In this reintroduction programme, our disease risk analysis did not detect any parasites at high risk of causing disease in native amphibian populations but we cannot rule out their presence because (i) the literature on parasites and disease in amphibians in Sweden was limited, (ii) the sample numbers of pool frogs screened were insufficient to detect agents of low prevalence and (iii) we may not have used appropriate tests for some unknown, undetected parasites. Our methods of disease risk analysis generally conform well with the recommendations of the Aquatic Animal Health Code (World Organization for Animal Health 2015), into which amphibians were written after this reintroduction commenced. The Code recommended the assessment of hazards of known harm to amphibians and considers just two disease agents in detail, while we would recommend that all parasites novel to the destination environment (source hazards) are assessed in wild animal translocations (Sainsbury and Vaughan-Higgins 2012), because previously unknown novel infectious agents have been associated with major outbreaks of disease in wild animals (Bobadilla et al 2015).

Our disease risk analysis recognised *Bd* and ranavirus as the known hazards of highest risk of precipitating disease in the translocated pool frogs. No cases of disease associated with these agents have been detected at the reintroduction site, and screening for *Bd* and ranavirus has not detected these agents on site. It is possible that *Bd* and ranavirus are present but not to date detected, perhaps because infected animals die and are scavenged and therefore have not been found. The presence of undetected *Bd*-associated or ranaviral-associated disease is possible given (i) positive survey results for *Bd* and ranavirus from native amphibians in the other parts of the UK (Cunningham and Minting 2008; ZSL/Defra/ARG-UK 2011), (ii) amphibians at the reintroduction site may not have yet encountered these agents and (iii) in the case of ranavirus it has been reported associated with disease in *Pelophylax* spp in Denmark and the Netherlands (Ariel et al 2009; Kik et al 2011). The absence of disease due to *Bd* and ranavirus at the reintroduction site, if confirmed, would provide some reassurance that our quarantine methods have maintained this site *Bd* and ranavirus-free but there are no apparent ecological or geographical barriers to the spread of *Bd* or ranavirus into the reintroduction site which implies that natural spread will occur and these agents remain a hazard to the reintroduced pool frogs. The quarantine barrier may serve a useful purpose if it allows pool frog numbers to reach capacity for the site before they face a potential challenge from *Bd* and / or ranavirus incursion.

Quarantine of a natural habitat to prevent incursion of infectious agents to protect a reintroduced population has not previously been reported. Pessier and Mendelson (2010) set out biosecurity and disinfection recommendations for field sites in amphibian reintroduction programmes. Although there was a public right of way through the pool frog reintroduction site, and therefore the quarantine barrier was probably broken by the public walking through the site, infectious agents from other native amphibian populations might more likely have been introduced by the ecologists and veterinarians who work on other projects involving amphibians at other sites. The quarantine method was set out to prevent the risk from these ecologists and veterinarians. If a novel

infectious agent has been reintroduced with the pool frogs, quarantine will also potentially have slowed its spread from the reintroduction site, and if there had been signs of a disease outbreak in native amphibians at the reintroduction site, may have allowed time for mitigation measures to be put in place.

Criteria for the success of reintroduction programmes have rarely been reported and there are no established guidelines on this facet of reintroduction management. Buckley and Foster (2004) set out detailed criteria for the success of the pool frog reintroduction programme, the majority of which have been met (see the more detailed discussion in Foster et al in press). Positive indicators of pool frog reintroduction success have included; (i) an increase in numbers of pool frogs on site, (ii) an increase in the number of ponds where frogs have been found, and (iii) a great deal of calling activity from male frogs. Negative indicators have included; (i) the rapid decrease in the numbers of adults and immature frogs during the second half of summer 2008, (ii) limited evidence of spawning (12 spawn clumps found in 2008) and (iii) low counts of metamorphs in 2008 (Baker 2009). Seddon (1999) argued for reintroduction managers to look for measures of population persistence and to assess how regularly the population should be monitored to assure managers that persistence had been accomplished. Guidelines on the resources that should ideally be devoted to post-release health surveillance are also scarce. In other reintroduction programmes carried out for English Nature's (now Natural England's) Species Recovery Programme, health and disease monitoring has been continued indefinitely, but resources are focused on the basis of our understanding of the risk to the health of the reintroduced population from disease. For example post-release health surveillance of red kites (*Milvus milvus*) has been continuing since 1989 in the context of threats to this species from misuse and abuse of pesticides. The pool frog population at the reintroduction site in Norfolk has not reached its carrying capacity and as the population of pool frogs changes the transmission dynamics of parasites and the risk from disease will change. For example, many parasites require a threshold density or critical community size before transmission occurs (Dobson and Hudson 1995; Swinton et al 2001) and this density or size may not have been reached at the reintroduction site. Therefore the current intention is to continue to monitor the health of amphibians at the reintroduction site in tandem with measures of population dynamics because although, no disease outbreaks have yet been detected, the risk of disease to the populations remains.

In order for our post-release health surveillance to detect an epidemic of disease our visits to the reintroduction site would need to coincide with an epidemic because sick individuals are likely to disappear from view because they may hide and dead individuals are likely to be quickly scavenged or decompose (Wobeser 2006). For these reasons although the annual mortality rate of the adult amphibians at the reintroduction site is likely to be approximately 50-80% (the adult survival of common toads *Bufo Bufo* was between 42 and 63% (Lornan and Madsen 2010) and adult *Pelophylax lessonae* between 72 and 84% (Peter 2001) only two dead animals were found at the reintroduction site over a 7 year period. This result is an example of the low probability of identifying disease in the reintroduced or resident populations of amphibians using current methods. More intensive post-release health and disease monitoring is resource hungry and therefore we are using the results of population monitoring of amphibians at the release site to decide when, or if, more resources should be dedicated to this activity. Post-release disease surveillance, through detection and examination of dead amphibians did not detect any significant diseases. *Aeromonas hydrophila/caviae* and *Ochrobacterium anthropi*, are ubiquitous organisms in the UK and since they were identified in mixed culture they were not believed to be significant in the death of one of the pool frogs. Considering the number of amphibians at the reintroduction site which would have died during these years, improved methods of post-release disease surveillance would clearly improve our ability to monitor the disease impact of a reintroduction.

In this paper we have reported, for the first time, the results of a disease risk analysis for an amphibian reintroduction and the post-release health surveillance guided by the disease risk analysis. Two high risk pathogens, hazardous to pool frogs at the destination, were noted by the disease risk analysis, ranavirus and *Batrachochytrium dendrobatidis*, but have not to date been recorded and disease risk management may have protected the reintroduced pool frogs

from these hazards to date, the first record of the use of quarantine at a natural site to protect a reintroduced species. The health of pool frogs and native amphibians at the destination site has been good but as the pool frog population has not grown to expected carrying capacity we can expect some changes to the pool frog population and the transmission of parasites within it and with other amphibian populations. The project cannot yet be considered a success and health and welfare monitoring will be continued. Post-release disease surveillance has been hampered by the difficulty of detecting dead amphibians and better methods to achieve detection are needed in future reintroduction projects, for their success to be more easily determined.

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