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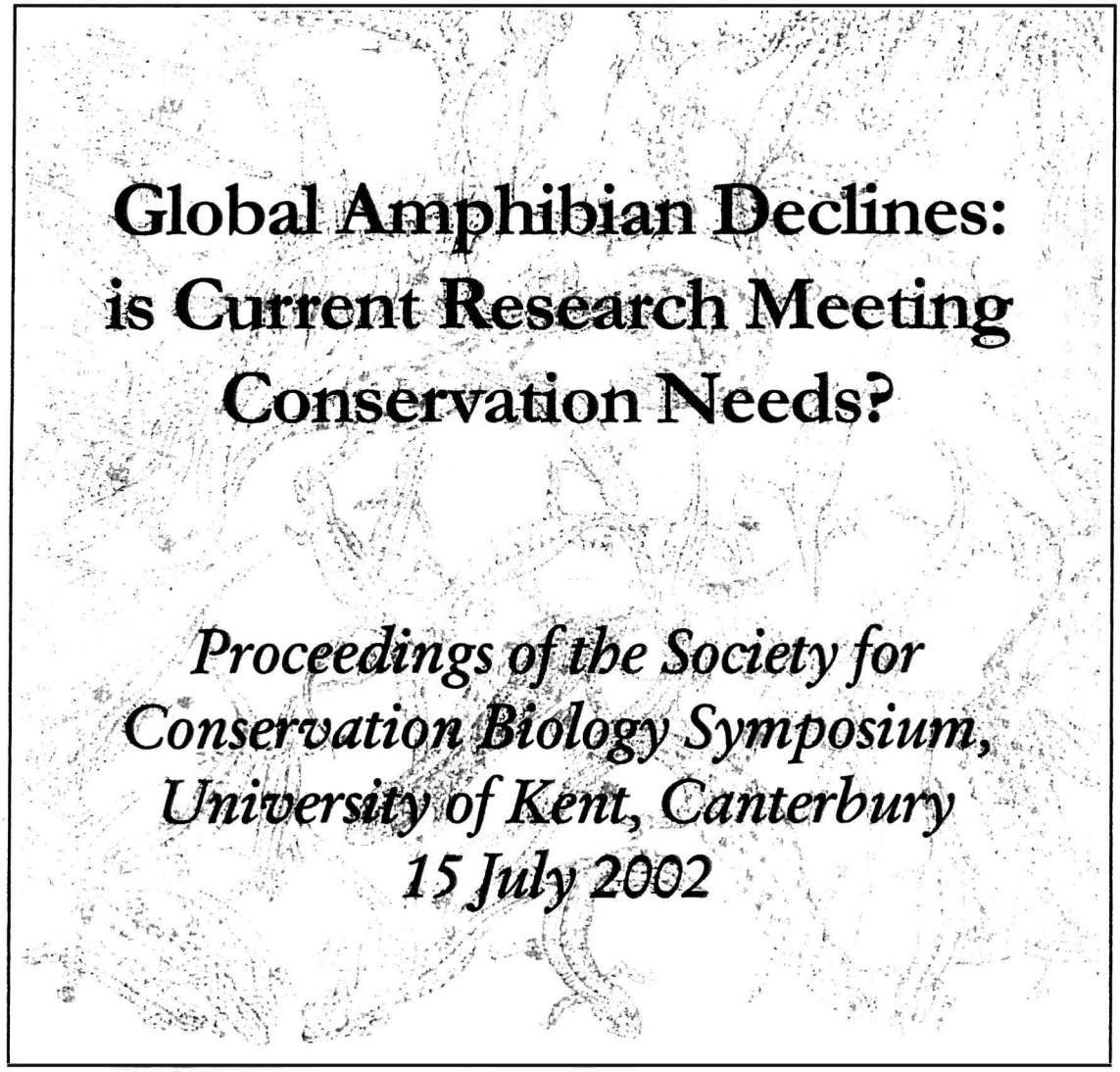

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EDITORIAL

GLOBAL AMPHIBIAN DECLINES: IS CURRENT RESEARCH MEETING CONSERVATION NEEDS?

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How much research is needed to do effective amphibian conservation? Although most herpetologists would hold the view that sound biological research should lay the foundations for conservation action, in the real world this is frequently not the case. There are probably several reasons for this. First, the direction that conservation research takes is driven by several agendas, of which conservation needs is just one. Resolving a conservation problem may require long-term, repetitive, and sometimes arcane research of local rather than international importance. Such work may not be attractive to either funding agencies or scientists, who both require a quick return on their investments. Second, many of the personnel charged with implementing conservation action are not scientists. Such people may not have wide access to the relevant scientific literature, and if they do, they may lack the time or expertise to ponder on the relevance of the latest population model or genetic technique to their problems. Thirdly, conservation research – and conservation science – usually operate within the confines of traditional academic disciplines (e.g. zoology, ecology, genetics, evolutionary biology), whereas conservation problems are multifaceted. Consequently, elegant models to manage endangered species are doomed to fail in practice unless they embrace the legal, political, cultural and socioeconomic frameworks within which the threats to the species have arisen. Because of these issues, it is hardly surprising that most conservation management is rooted in traditional practices, personal experience and word-of-mouth communication rather than evidence-based approaches (Sutherland *et al.*, 2004). Equally, many conservation practitioners take a dim view of scientists and believe that they are not carrying out work that is relevant to their needs (Cummins & Griffiths, 2000). Such is the current wider perception of science, we know of at least one international conservation organization that has advertised the fact that it does not fund research as one of the selling points of its campaigns.

Concern over these issues led to the organization of this symposium on 15 July 2002 at the Society of Conservation Biology meeting in Canterbury. It is particularly appropriate that this symposium was held at the same location as the First World Congress of Herpetology some 13 years on, as it was this seminal meeting that precipitated the increasinging interest in declining amphibians. Although there have been several subsequent symposia on various topics associated with the amphibian decline phenomenon, most of these have been hosted within herpetological meetings. We hoped that the SCB symposium would raise the profile of amphibian declines within the wider conservation community, and encourage feedback and debate on research-related issues. Given the constraints of time-slots within a wider programme, our choice of speakers and topics was, perhaps, not broad enough to encompass all of the complex problems that amphibians are facing. Rather ambitiously, however, we approached researchers who we considered to be leading workers in their fields, and were delighted when they all accepted our invitation to attend and contribute. All of the speakers subsequently agreed to papers based on their presented work being submitted to this special issue of the *Herpetological Journal*. We are grateful to all the contributors for their patience during the review process, and also to the various referees who reviewed the submitted manuscripts.

Fundamental to all amphibian conservation are sound data on population status. Benedikt Schmidt's paper challenges the value of many of the widely used methods based on simple counts of animals, and makes a plea for better methods of population assessment that account for detection probabilities. This raises the whole issue of how much data are needed to arrive at regional conservation assessments. Jean-Marc Hero and Clare Morrsion review the status of – and threats to – Australian frogs and further highlight some of the problems involved. With many species of amphibians breeding in patchily distributed ponds, metapopulation theory has provided a very convenient framework for examining how such populations function and persist. Indeed, Per Sjögren-Gulve's pioneering work on the pool frogs of Sweden remains one of the benchmark studies in metapopulation ecology. Building on this earlier work, Telgström and Sjögren-Gulve compare the genetic differentiation both within and between pool frog populations in northern Europe, and discuss how such data can be used to assign conservation value to different populations. Of all the topics currently being pursued within amphibian decline research, emerging infectious diseases is one that is being closely followed by conservation scientists and practitioners alike. Two papers by Jim Collins and colleagues, and Peter Daszak and colleagues, deal with the twin spectres of chytridiomycosis and ranaviruses respectively, and

highlight the importance of collaborative research in these areas. All conservation research and action operates within limits imposed by political boundaries. Unfortunately the problems facing amphibian populations transcend such boundaries, and variable research effort in different parts of a species' range can sometimes appear to frustrate wider conservation efforts. Jim Foster and Trevor Beebee describe how conservation policy has been translated into research and action for amphibians within the UK. The wider impact that such local conservation initiatives can have may be limited, however, and there are important aspects of the amphibian decline phenomenon for which the answer to the question posed in our title must be 'no'. The priorities for action on global factors that are negatively affecting amphibians – such as climate change and elevated UV-B – lie within the social and political arena, rather than within conservation biology or herpetology.

We therefore admit that this symposium did not provide an unequivocal answer to the question that it posed. Amphibian decline research is often long-haul, and for many of the issues discussed only time will tell how effective the research has been in informing conservation management. However, we hope that the symposium – and indeed these proceedings – may provide a refocus of research directions. If so, it may help us all wrestle with the dilemma posed by McCoy (1994): 'Do ecologists wear their conservationist hats and muster their expertise in defence of life, or do they wear their scientist hats and muster their expertise in defence of truth?'

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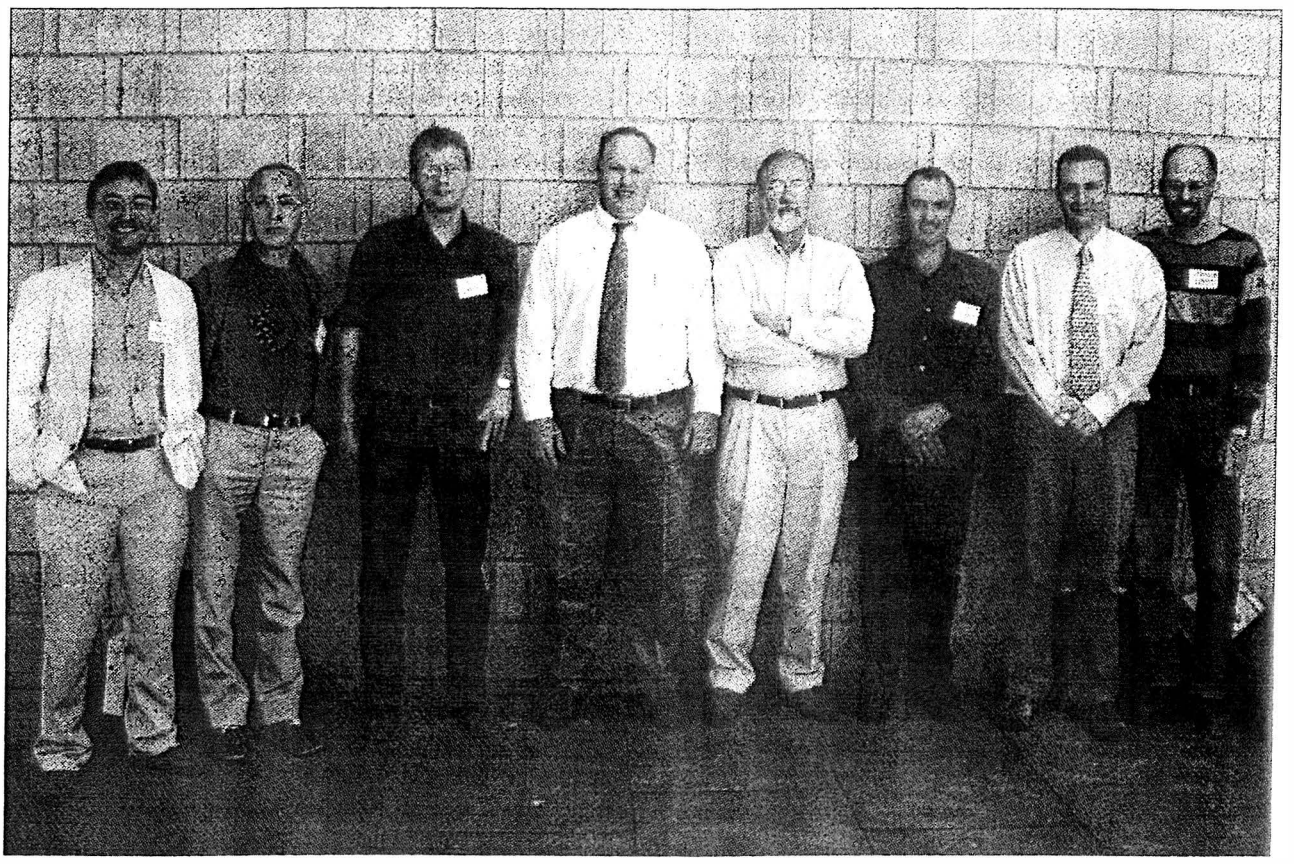

Contributors to the symposium (left to right): Richard Griffiths, Tim Halliday, Benedikt Schmidt, Peter Daszak, Jim Collins, Jean-Marc Hero, Jim Foster, Per Sjögren-Gulve

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DECLINING AMPHIBIAN POPULATIONS: THE PITFALLS OF COUNT DATA IN THE STUDY OF DIVERSITY, DISTRIBUTIONS, DYNAMICS, AND DEMOGRAPHY

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Most data used in the study of the demography, dynamics, distributions, diversity, and declines of amphibians are count data that are not adjusted for detection probabilities, which are generally variable and low. Such unadjusted count data are unreliable for understanding amphibian ecology, amphibian declines, or when developing conservation and management strategies. In the future, detection probabilities should be estimated and counts adjusted accordingly. This could be achieved by using capture-mark-recapture, distance sampling or novel Bayesian methods.

Key words: conservation, detection probability, population census, survey methods

INTRODUCTION

Amphibians are declining locally and globally for a variety of reasons (Cooke, 1972; Beebee, 1973; Blaustein & Wake, 1990; Alford & Richards, 1999; Corn, 2000; Houlahan *et al.*, 2000). If we want to understand why amphibians are declining and how we can halt and reverse the negative trends, then we must improve our understanding of amphibian ecology. In particular, we should strive to better understand the demography and dynamics of amphibian populations and the factors that govern the distribution of species and species diversity at particular sites. Collecting reliable field data is an important step towards understanding these basic elements of amphibian ecology. Here, I argue that most field data on the ecology of amphibians are of limited use because they are unadjusted counts. Next, I suggest estimating detection probabilities to adjust the counts and improve data quality. High-quality field data are needed to complement experimental studies on the causes of amphibian declines, to parameterise population models, and for better quantification of declines.

THE PITFALLS OF UNADJUSTED COUNT DATA

Counts are commonly used in the study of amphibian ecology. Unadjusted counts are not reliable in amphibian ecology because they underestimate the true population parameters of interest because some individuals, populations, or species are not detected. Additionally, variation in detection probabilities generates variation in the counts which obscures true variation in ecological processes (Burnham, 1981; Nichols & Pollock, 1983; Martin *et al.*, 1995; Anderson, 2001). Nichols's (1992) simple formula indicates the relationship between a count, C, and the population parameter of interest, N (which may be a demographic rate, population size, the number of populations in an area, or the number of species at a site):

$$E(C) = Np, \qquad (1)$$

where p is a detection probability and E indicates an expected value. Obviously, it is impossible to detect all individuals, populations, or species in a given area (e.g. Preston, 1979); therefore, p is < 1 and C always underestimates N to an unknown degree (Nichols & Pollock, 1983). The detection probability will depend on what is being counted. For example, counts of egg masses are probably more reliable (i.e. on average higher – and more importantly – less variable p) than counts of adult newts within a pond. The count C is commonly known as 'return rate' when dealing with demographic rates and as a 'population index' or 'relative abundance' when dealing with population size, density, or abundance (Martin *et al.*, 1995; Anderson, 2001).

A comparison of two (or more) counts is problematic because one must assume that the detection probabilities are constant. A comparison of two counts, a trend, is given by:

$$E(C_1/C_2) = N_1p_1/N_2p_2 \qquad (2)$$

$E(C_1/C_2)$ equals N_1/N_2 only if $p_1 = p_2$ (Yoccoz *et al.*, 2001; Pollock *et al.*, 2002). This is unlikely. Anholt *et al.* (2003), for instance, showed that detection probabilities of two species of frogs were sex-, site-, and time-, but not species-specific. Bailey *et al.* (2004*a,b*) provide evidence for strong variation in detection probabilities in salamanders. If $p_1 \neq p_2$, then the comparison (the trend) is biased to an unknown degree and even the direction of bias is unknown. If $C_1 \neq C_2$, it is not known whether $N_1 \neq N_2$, $p_1 \neq p_2$, or both are different (i.e. are variable). With unadjusted counts, it cannot be determined which elements of the equation are variable.

Data from a capture-mark-recapture study on green turtles (*Chelonia mydas*; Chaloupka and Limpus, 2001)

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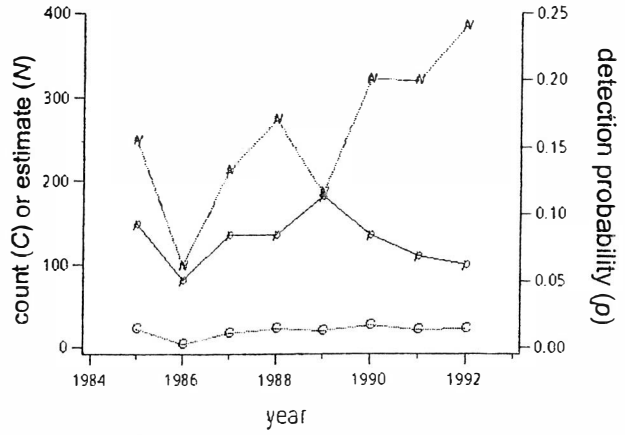

FIG. 1. C, $\hat{N}$, and $\hat{p}$ of male green turtles (*Chelonia mydas*) living in Great Barrier Reef, Australia, taken from the capture-mark-recapture study of Chaloupka and Limpus (2001). $\hat{N}$ was calculated as $C/\hat{p}$.

where C, N, and p were estimated serve to illustrate problems associated with unadjusted counts (Fig. 1): the unadjusted counts C seriously underestimate true population size (N) and suggest that the population is stable (i.e. stationary) while it is in fact increasing (the number of turtles fluctuates greatly because there are many transients). This happens because p is low, variable, and declines towards the end of the study period. Throughout the study period p remains between ca. 0.05 to 0.1. This suggests that even seemingly small fluctuations in p may lead to large errors when using C as an index for N (note that in fact there is a two-fold difference in detection probabilities). Temporal trends in detection probabilities may not be uncommon (e.g. Funk & Mills, 2003).

Herpetologists are well aware of the fact that it is impossible to capture all individuals or detect all populations or species. For instance, Hairston & Wiley (1993) argue that fluctuations in the apparent abundance of terrestrial salamanders are due to variation in student motivation to search for salamanders, i.e. variation in detection probabilities, rather than true variation in salamander abundance. Nevertheless, few amphibian ecologists seem aware of the problems caused by variation in detection probabilities, and often fail to take detection probabilities into account when estimating demographic rates or other population parameters (e.g. Schmidt & Anholt, 1999). Return rates (C) are often treated as if they were survival probabilities (e.g. Berven, 1990; Parris & McCarthy, 2001), 82% and 95% of the time series used by Alford & Richards (1999) and Houlahan *et al.* (2000), respectively, are unadjusted counts, and there are very few studies on the species distribution or diversity of amphibians that take detection probabilities into account (MacKenzie *et al.*, 2002, 2003). The use of unadjusted count data is not unique to herpetology. Most population data of fish, birds, and mammals consists of unadjusted counts (Preston, 1979; Nichols & Pollock, 1983; Martin *et al.*, 1995; Bjørnstad & Grenfell, 2001; Rosenstock *et al.*, 2002).

The most common solution to dealing with variation in detection probabilities is to standardize sampling methods (Heyer *et al.*, 1994). Standardization, it is (tacitly) argued, reduces variability in p such that C becomes a reliable index of N. This may not always be the case. Hyde & Simons (2001) compared several standard methods for sampling terrestrial salamanders. They found that correlations between the counts obtained using different standard methods were generally weak. No method was clearly the best and at least some of the methods are apparently unreliable. Hyde & Simons (2001) concluded that none of the standard methods was suitable for long-term monitoring of salamander populations (see also Bailey *et al.*, 2004*a*,*b*).

In addition to the use of standard methods, environmental variables that may affect p are often measured and used to adjust or calibrate the counts. This may solve the problems associated with unadjusted counts to some extent but is clearly not an easy task. Sauer & Link (1998, 2002) provide examples where environmental variables (in their studies primarily observer effects) are used to adjust counts from the North American Breeding Bird Survey. Standardization and the use of environmental variables are clearly valuable and should be used or collected, respectively, whenever possible; however, it is not possible to control for every factor that may affect detection probabilities. For example, some surveys use the number of calling males as an index of population size. In the natterjack toad (*Bufo calamita*), for instance, some males only call when few males are present and calling. In large populations, some males adopt a satellite strategy (Arak, 1988). Thus, the very focus of the survey – population size – affects the index through the behaviour of individuals. The behaviour of individuals is very difficult to standardize.

USING DETECTION PROBABILITIES TO ADJUST COUNTS

Detection probabilities are needed to adjust counts. Estimation-based methods (such as capture-mark-recapture and distance sampling methods) are the only reliable methods for amphibian population ecology because detection probabilities (p) are estimated and used to adjust C to obtain estimates of N (Buckland *et al.*, 2001; Williams *et al.*, 2002). These methods also accommodate detection probabilities that vary in space and time, for instance because effort is variable. The basic idea underlying all of these methods is simple: first, they estimate a detection probability, $\hat{p}$, and then use it to adjust the count:

$$\hat{N} = C/\hat{p} \qquad (3)$$

(Pearson, 1955; Nichols, 1992; Yoccoz *et al.*, 2001; Pollock *et al.*, 2002; Williams *et al.*, 2002). Currently, capture-mark-recapture methods are available for the estimation of demographic rates, population dynamics, distributions, and species diversity and turnover (e.g. Pollock *et al.*, 1990; Lebreton *et al.*, 1992; Nichols & Conroy, 1996; Schwarz & Arnason, 1996; Nichols *et al.*, 1998; MacKenzie *et al.*, 2002; 2003; see also Preston, 1979; Kéry, 2002). There are many recent

TABLE 1. Results of linear regressions between counts (C), population size estimates ($\hat{N}$), and censuses for various amphibians. R^2 and F tests (PROC GLM in SAS) are from the full model. Asterisks indicate significance at $\alpha = 0.05$. Estimators used are Jolly-Seber for *Cryptobranchus alleganiensis*, various closed population estimators for *Plethodon cinereus*, Lincoln-Peterson for *Scaphiopus holbrooki*, *Scaphiopus couchii*, and *Hyla arenicolor*.

Species	Life stage	Studied in	Intercept	Slope	R^2	F	Reference
			Between count and estimate				
Cryptobranchus alleganiensis	Adults	Natural streams	1.68	0.20	0.09	0.5	Peterson *et al.*, 1988
Plethodon cinereus	Adults	Terrestrial plots	120.62	1.78	0.27	3.0	Jung *et al.*, 2000
Scaphiopus holbrooki	Adults	Terrestrial enclosure	57.71	0.30	0.71	20.2*	Pearson, 1955
Scaphiopus holbrooki	Adults	Terrestrial enclosure	4.69	0.87	0.97	102.4*	Pearson, 1955
Hyla arenicolor	Tadpoles	Natural ponds	-36.98	1.93	0.59	25.1*	Jung *et al.*, 2002
Scaphiopus couchii	Tadpoles	Mesocosms	28.85	1.10	0.95	192.2*	Jung *et al.*, 2002
			Between count and census				
Hyla arenicolor	Tadpoles	Natural ponds	-13.92	1.73	0.66	34.21*	Jung *et al.*, 2002
Scaphiopus couchii	Tadpoles	Mesocosms	12.44	1.17	0.96	283.1*	Jung *et al.*, 2002
			Between estimate and census				
Hyla arenicolor	Tadpoles	Natural ponds	18.35	0.87	0.97	1005.0*	Jung *et al.*, 2002
Scaphiopus couchii	Tadpoles	Mesocosms	15.25	0.94	0.99	1509.0*	Jung *et al.*, 2002

developments in capture-mark-recapture methodology and some may contribute substantially to understanding amphibian ecology. Important examples include the direct estimation of population growth rate and the demographic contributions to it (Pradel, 1996; Nichols *et al.*, 2000; Nichols & Hines, 2002), and population size estimation when not all individuals are present simultaneously at the sampling site (e.g. when the sampling site is a breeding site; Schwarz & Arnason, 1996). Methods are also available for situations in which only a subset of the individuals can be marked or when some members of the population are not available for capture (Pollock, 1982; Lebreton *et al.*, 1999; Dreitz *et al.*, 2002; Kendall & Nichols, 2002). Some recent capture-mark-recapture models were developed specifically to analyse amphibian data (e.g., MacKenzie *et al.*, 2002; 2003; Bailey *et al.*, 2004*c*; Royle, 2004*b*).

Several amphibian studies report C, N (i.e. $\hat{N}$), and sometimes a census (= N because $p = 1$ by definition; Pearson, 1955; Peterson *et al.*, 1988; Jung *et al.*, 2000; 2002). The correlations between C and $\hat{N}$ are often high, but they are also highly variable (Table 1). In these examples, the population estimates $\hat{N}$ are much closer to the census values than the C, suggesting that the capture-mark-recapture estimates are better than the unadjusted counts. These results have two main implications. First, if the only goal is to have a rough idea of N, then the C may be a useful first approximation. C may be sufficient if the purpose is to assign populations to size classes such as 'small', 'medium', or 'large' if one is willing to accept the risk that some large populations are assigned incorrectly to a smaller size class (Corn *et al.*, 2000). However, a strong linear relationship between C and N should not be assumed. The data of Jung *et al.* (2002) suggest that such relationships may be curvilinear rather than linear. Clearly, an index C must be calibrated (see Jung *et al.*, 2000; 2002 for examples). Thus, when using C, researchers should provide evidence that the C is actually a reliable index of N (MacKenzie & Kendall, 2002). If costs prevent the estimation of detection probabilities at all sites in a large-scale monitoring program, then detection probabilities may be estimated and counts calibrated at only a subset of the sites (Pollock *et al.*, 2002).

Capture-mark-recapture methods are often considered not useful because they are labour-intensive and therefore expensive (e.g. Donnelly & Guyer, 1994). This may be true, but it is questionable whether collecting C data means that time and money are better invested. A solution may be to estimate detection probabilities only at a subset of the sites (Pollock *et al.*, 2002). Capture-mark-recapture approaches are unlikely to be more time-consuming or expensive when analysing distributions or patterns of species diversity because all that is required for a capture-mark-recapture analysis is multiple visits to a site or several sites. Populations or species are then treated analogously to individuals in the analysis of demographic parameters (Nichols & Conroy, 1996; Nichols *et al.*, 1998; MacKenzie *et al.*, 2002, 2003).

Recent Bayesian models may allow the estimation of population size and demographic parameters without marking individuals. These models may be especially useful when individuals cannot be marked or when costs of marking are prohibitively high. Royle (2004*a*) developed new models for estimating the size of a closed population based on counts of individuals. Royle's (2004*a*) model uses mixture models to estimate both detection probabilities and population size based on spatially and temporally replicated counts. Dodd & Dorazio (2004) developed these models further and used them successfully to estimate abundance of several

species of salamanders in Great Smoky Mountains National Park, USA. Using a related Bayesian approach, Link *et al.* (2003) describe methods to estimate survival and recruitment from information on age classes such "juveniles" and "adults".

Unadjusted counts are sometimes regarded as better than capture-mark-recapture estimates because the latter often have wide confidence intervals (Alford & Richards, 1999). While this may be true, the width of the confidence interval indicates whether an estimate is good or poor. With unadjusted counts, there is no way of knowing whether the counts are good or poor.

People argue against the use of capture-mark-recapture and related methods because these methods make assumptions whereas counts make no assumptions.The assumptions underlying capture-mark-recapture methods can be tested and the models used for parameter estimation can be adjusted accordingly (Lebreton *et al.*, 1992; for an example, see Schmidt *et al.*, 2002). If assumptions are not met, then the magnitude and direction of bias are known or can be approximated using simulation (e.g., Manly *et al.*, 1999). The assumptions underlying unadjusted counts cannot be tested and the magnitude and direction of bias remain unknown. A single count of, say, a population makes no assumptions. A comparison between two unadjusted counts makes a strong, and untested, assumption: the assumption that detection probabilities are exactly equal (see equation 2). This assumption is probably never met (Anderson, 2001; MacKenzie & Kendall, 2002). Counts are almost always compared and therefore assumptions are made. For example, if an observer counts 20 salamanders in pond A and 50 salamanders in pond B then most people would believe that population B is larger than population A. Such uncritical use of count data is widespread.

DISCUSSION

Amphibian populations are declining for a variety of reasons which we need to understand if we are to halt or reverse the declines (Alford & Richards, 1999; Corn, 2000; Houlahan *et al.* 2000). Most amphibian population data for demography, population dynamics, patterns of distributions and species richness are unadjusted counts (Schmidt *et al.*, 2002; Schmidt, 2003). Such data provide a weak basis for understanding ecological processes because true biological variation is confounded with variation in detection probabilities. Variation in detection probabilities can obscure causal ecological relationships and can generate variability when there is actually none (Pollock *et al.*, 1990; Link & Nichols, 1994; Martin *et al.*, 1995; Anderson, 2001; Cam *et al.*, 2002; Shenk *et al.*, 1998; Yoccoz *et al.*, 2001; Pollock *et al.*, 2002). As Burnham (1981) and Nichols & Pollock (1983) pointed out a long time ago, it is important to use methods that remove variation due to detection probability differences (e.g. capture-mark-recapture or distance sampling methods).

Field data on amphibian populations and communities that take detection probabilities into account will better our understanding of amphibian population declines. Experimental studies have shown that stressors, such as increased UV-B radiation, can affect some life history stages (Blaustein *et al.*, 1994; Kiesecker *et al.*, 2001) but we do not know yet whether these effects translate into population declines. In fact, UV-B may not induce strong mortality in most populations and where it does, UV-B-induced egg mortality may not affect population dynamics at all (Palen *et al.*, 2002; Vonesh & De la Cruz, 2002). Thus, establishing a link between population growth rate and UV-B radiation through time series analysis (e.g. Dennis & Otten, 2000) and estimating the contribution of the larval, juvenile, and adult stage to population growth rate (McPeek & Peckarsky, 1998; Nichols *et al.*, 2000; Biek *et al.*, 2002; Forbes & Calow, 2002) would strengthen the conclusions drawn from experiments. Capture-mark-recapture data and the associated estimation methods are likely the most suitable methods for this kind of research because they contain no variation that is due to variation in detection probabilities and many parameters of interest can be estimated directly from the data. Unadjusted count data probably fail to uncover the subtle differences that may determine whether a population is declining or growing (Fujiwara & Caswell, 2001).

Scaling-up from small-scale experiments to population-level processes is an important task. To fulfil this task and for a more comprehensive understanding of population declines, we need a better general understanding of the demography and dynamics of amphibian populations. Most experiments in amphibian ecology are on tadpoles (Wilbur, 1997), but several recent studies suggest that the juvenile or adult stages are more important determinants of population growth than the tadpole stage (Taylor & Scott, 1997; Biek *et al.*, 2002; Hels & Nachman, 2002; Loman, 2002; Vonesh & De la Cruz, 2002). Despite the fact that all of these studies seem to agree on the importance of the terrestrial stage(s), the relative contribution of different life history stages to population growth requires further study. For instance, both Biek *et al.* (2002) and Loman (2002) analysed population dynamics of the frog *Rana temporaria* and concluded that the terrestrial stages are more important than the tadpole stage. In contrast, Meyer *et al.* (1998) argued that a population of *Rana temporaria* declined because tadpole-eating fish were introduced into the breeding site. There is no consensus yet on which factors and stages are most important for amphibian population dynamics and which dynamical patterns we should expect (e.g. Alford & Richards, 1999; Alford *et al.*, 2001 vs. Houlahan *et al.*, 2001; Green, 2003). Clearly, more studies are needed to resolve these issues and we need reliable methods for the collection of demographic and population dynamic data. Again, capture-mark-recapture methods are probably the most reliable methods.

An explicit focus on detection probabilities would also help when quantifying the extent of amphibian declines. For example, Skelly *et al.* (2003) describe the

effects of survey length on the inferred magnitude of decline, e.g. when resurveying sites where a species was known to occur in the past. In their case study, a (re)survey conducted during one year resulted in an inferred decline of 45% whereas a (re)survey done in two years resulted in an inferred decline of only 28%. If sites are (re)surveyed over a period of five years, then the inferred decline was only 3%. Skelly *et al.* (2003) provided two explanations for this result: a species may be missed at site or species are only present at a site intermittently. Skelly *et al.* (2003) argued that they missed no species that was actually present (i.e., $p = 1.0$) and intermittent presence of species at sites was the best explanation. Skelly *et al.* (2003) discuss the challenges posed by intermittent presence of species. I focus here on the implications of imperfect surveys, where $p < 1.0$. Most surveys are less intense than Skelly *et al.*'s (2003) survey, such that most surveys likely miss species. If one assumes that species are present but imperfectly detected, say, during 60% of the visits to a site, then one would reach a conclusion similar to Skelly *et al.*'s (2003). If sites are visited only once during a survey, then the species is detected at 60% of the sites were it occurs. Thus, the inferred decline after a single visit to each site is 40%. If the sites are visited once per year, then the inferred decline is $0.4^2 = 0.20$ after two years and $0.4^5 = 0.02$ after five years. Obviously, with only one or two visits to a site, researchers would infer a decline although the species is present. This is because in most surveys detection of species is imperfect. The calculations above lead naturally to the approaches for inferring the absence of a species (Preston, 1979; Kéry, 2002; MacKenzie *et al.*, 2002) and the methods for estimating turnover in animal communities (Nichols *et al.*, 1998; MacKenzie *et al.*, 2003). Skelly *et al.* (2003) recommend that "resurveys should extend for long enough to estimate the value of additional data". Knowing detection probabilities allows one to disentangle whether a species is missed or absent from a site and allows investigators to estimate the number of visits (or years) necessary for a resurvey that is "long enough".

Detection probabilities *per se* are uninteresting nuisance parameters. Nevertheless, they are of major importance in the study of the demography, population dynamics, distributions, species diversity, and decline of amphibians. Only if we are aware of the pitfalls of unadjusted count data and use estimates of population and community parameters that are adjusted for detection probabilities, shall we understand the ecology and decline of amphibians.

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FROG DECLINES IN AUSTRALIA: GLOBAL IMPLICATIONS

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Amphibian declines have been reported from around the world. Here we examine life history and distributional characteristics of Australian frogs listed as threatened under the IUCN Global Amphibian Assessment guidelines, and compare these results to available information on threatened amphibians around the world. Forty of 213 Australian frog species (18.8%) are currently recognised as threatened. While eight species are listed as Vulnerable due to small or restricted populations alone (VU D2), the remaining 32 species are associated with population declines. Threatened species are concentrated in upland areas (41% of all upland species are threatened, while only 8% of lowland species are threatened). Twenty-eight of the 40 threatened species (70%) primarily occur in upland areas while only 42 of the 173 non-threatened species (24.3%) occur in upland areas. Restricted geographic range is characteristic of 31 of 40 threatened/declining species (77.5%). However, 41 non-threatened species (23.7%) also have restricted geographic ranges. Latitudinal position is not strongly associated with the degree of threat. Threatened species are strongly associated with specific reproductive habitats: 80% of species occurring in montane wetlands and 58% of species breeding in wet forest streams are threatened. For 22 of the 40 (55%) threatened species, known threats do not adequately explain the extent of decline. Habitat modification is the foremost threatening process associated with declines in 20 of the 40 threatened species (50%), including 11 of 12 threatened lowland species (91.7%). Chytrid fungus is notably associated with declines for five species and a potential contributor for an additional nine species (35% of threatened species). However, the chytrid has also been detected in an additional 33 non-threatened species (19%). Minor threats associated with threatened species include fire and global changes in weather patterns. Phylogenetic relationships of Australian frogs are poorly resolved, and there are no strong associations between phylogeny and declines within known taxonomic groups. A notable exception are frogs of the myobatrachid genus *Taudactylus* where five of the six species are threatened. Global patterns are difficult to assess, however, as declines are strongly associated with species that are primarily distributed in upland areas. Chytrid fungus has been found in both declining and non-declining species throughout Australia, and while its role as an emerging infectious disease is currently under investigation (in Australia, New Zealand, Spain, South Africa, Costa Rica, Ecuador and the USA), little is known about its distribution and prevalence in other countries.

Key words: altitude, amphibians, geographic range, IUCN, life history, status

INTRODUCTION

Amphibian declines have been reported around the world, and Australia is notably affected (Alford & Richards, 1999; Campbell, 1999; Hero & Shoo, 2003). While numerous hypotheses have been proposed to explain Australian frog declines including habitat destruction, diseases, introduced species, climate change (summarized in Campbell, 1999), to date the cause(s) of many of these declines remain unknown.

In many cases the causative mechanisms of extinction are confounded and may be difficult or impossible to detect (Gillespie, 2001; Kiesecker, Blaustein & Belden, 2001). Studying patterns is an important tool for ecologists (Lawton, 1996) and has been used to understand extinctions of species where no obvious cause has been identified (McKinney, 1997). Williams & Hero (1998) examined frog species from the wet tropics region of northern Australia and found that low ovarian clutch size, habitat specialisation and an association with lotic streams, were the primary ecological characteristics that distinguished the declining species from the non-declining species. Examining patterns/trends in the ecological characteristics and geographic distribution shared by threatened species may help determine the specific causes of declines and identify traits that increase the likelihood of extinction or decline.

Here we present known and potential threats to Australian frogs and examine geographic and ecological traits associated with threatened species. Specifically we aimed to (1) collate data on the ecological characteristics (reproductive habitat etc.) and geographic distribution (extent of occurrence, altitude, latitudinal distribution etc) of all frogs in Australia; (2) identify trends/patterns in the threats, and ecological and geographical traits shared by threatened Australian species;

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(3) compare patterns found in Australia to those from other countries based on available geographical information; and (4) predict characteristics of potentially (but as yet unidentified) vulnerable species.

MATERIAL AND METHODS

We examined the conservation status, known and potential threats and geographic and reproductive data for the 213 frog species currently recognised in Australia. The list includes *Litoria daviesiae, Pseudophryne raveni* and the subspecies *L. verreauxii alpina* (three species not included in the IUCN Red List 2002 principally due to taxonomic restrictions and changes). The introduced cane toad (*Bufo marinus*) was excluded. Conservation status and threats were determined from the results of a workshop as part of the International Union for the Conservation of Nature-Global Amphibian Assessment (IUCN-GAA, see Hero, 2001; IUCN, 2002) and currently available on AmphibiaWeb (updated in 2003). Multiple threats were identified for each species and ranked as either foremost (most notable) or potential based on the GAA assessments. Geographic range (extent of occurrence) was calculated using ArcView and distribution maps generated as part of the GAA process (maps are also available on AmphibiaWeb). Altitudinal distribution was defined as either upland (predominantly distributed at elevations over 400 m asl) or lowland (predominantly distributed at elevations below 400 m asl) based on the geographic range and wherever possible published information on each species (e.g. McDonald, 1992). Natural history data was gathered from local field-guides and AmphibiaWeb (2003). The list of Australian species that have been found with chytrid fungus is summarized from Speare & Berger (2000*a*).

For comparative purposes we examined the conservation status of species from 46 countries listed on the IUCN (2002) Red List of Threatened Species (296 listed as threatened), and the AmphibiaWeb (2003) – Watch List (uploaded on 27 March 2002). Natural history data was gathered from AmphibiaWeb (2003) and local field-guides, and verified wherever possible by regional expert herpetologists within each country. While these data are not complete from all countries of the world they represent regions of concern (see results). A thorough analysis of this information will be completed once the IUCN Global Amphibian Assessment has been completed. The global list of species that have been found with chytrid fungus is summarised from Speare & Berger (2000*b*) and updated from individual publications (e.g. Bosch, Martinez-Solano & Garcia-Paris, 2001). It should be noted that limited information on the global distribution of chytrid is available. Statistical analyses are only suitable for a subset of this data and a manuscript examining upland areas of eastern Australia is currently in review (J.-M. Hero, pers. comm.). Herein we present a qualitative analysis of species characteristics associated with threatened status for all Australian frogs.

RESULTS

AUSTRALIAN THREATENED FROGS

Forty of 213 frog species in Australia (18.8%) are currently recognised as threatened and most are associated with population declines (Table 1). Eight species can no longer be found in the wild (*Rheobatrachus silus, R. vitellinus, Taudactylus diurnus, T. acutirostris, Litoria castanea/flavipunctata, L. lorica, L. nyakalensis* and *L. piperata*), an additional three species have almost entirely disappeared throughout their historical range (*Taudactylus rheophilus, Litoria booroolongensis* and *Pseudophryne corroboree*), and at least four species have disappeared from most upland areas throughout their geographic range (*Litoria nannotis, L. rheocola, Nyctimystes dayi* and *Taudactylus eungellensis*).

No species were listed using quantitative analysis (IUCN criterion E). Eight species were listed as Vulnerable due to restricted area of occupancy (IUCN criterion D2), although fragmentation and declines have been associated with these species. The remaining 32 species are associated with population declines, and listed under several IUCN criterion including: A ~ reduction in population size (14 species), B ~ geographic range restricted, fragmented and the population declining (17 species), C ~ small population size and declining (three species), and D ~ population very small or restricted (eight species). As such, the terms threatened and declining are synonymous for these 32 species.

Multiple threats have been identified for each species (Table 2). The principal threats to Australian frogs are currently unknown for 22 species where identified threats do not adequately explain their threatened status. Known threats include: habitat modification (foremost for 18 species and potential contributor for an additional two species), restricted geographic range (foremost for 16 species and potential contributor for an additional 15 species), chytridiomycosis (foremost for five species and potential contributor for an additional nine species), and introduced species (foremost for two species and potential contributor for an additional seven species), fire (potential contributor for six species) and global change in weather patterns/UV-B levels (potential contributor for two species).

Distribution correlates of threatened frogs are presented in Table 3. Restricted geographic range (extent of occurrence less than 20 000 km^2) is a characteristic of 31 threatened species (77.5%). Eight of these (25.8%) are listed as Vulnerable (IUCN criterion D2, having an area of occupancy of less than 20 km^2), however the remaining 23 species were associated with fragmentation and population declines (IUCN criterion A & B). An additional 41 non-threatened species (23.7%) have restricted geographic range (Table 4).

Twenty-eight threatened species (70%) primarily occur in upland areas (Table 3). Of the remaining 12 threatened lowland species, 11 are most notably threatened with habitat loss (Tables 2 and 3). For non-threatened species the inverse pattern is clear with

TABLE 1. IUCN categories from the Australian Global Amphibian Assessment 2001. Asterisks (*) denote species no longer found in the wild. Numbers in brackets and "x" denotes species with those characteristics but not classified using that criterion.

Species	Status	IUCN-GAA Criterion				
	IUCN 2002	A	B	C	D	E
		Reduction in population size	Geographic range restricted fragmented & declining	Population size fewer than 10 000mature individuals	Population very small or restricted	Quantitative analysis
*Rheobatrachus silus**	EX	-	X	X	1	-
*Rheobatrachus vitellinus**	EX	-	X	X	1	-
*Taudactylus diurnus**	EX	-	X	X	1	-
Geocrinia alba	CR	-	2 a b(ii)	-	-	-
Litoria booroolongensis	CR	-	2 a b(i-v)	-	-	-
*Litoria castanea / flavipunctata**	CR	-	X	X	1	-
*Litoria lorica**	CR	2c	1a b(iv)	X	1	-
*Litoria nyakalensis**	CR	2ac	1a b(iv)	X	1	-
*Litoria piperata**	CR	-	X	X	1	-
Litoria spenceri	CR	-	2 a b(i-v)	-	-	-
Litoria verreauxii alpina	CR	1 a, b, c	X	-	-	-
Philoria frosti	CR	2abc	1ab(i,ii,iii) + 2 a b(i,ii,iii,iv,v)	-	-	-
Pseudophryne corroboree	CR	2abc+3abc	2ab(i-v)	1	-	-
Pseudophryne pengilleyi	CR	-	2 a b(ii,iv,v)	-	-	-
*Taudactylus acutirostris**	CR	2ac	1a b(iv,v) + 2 b(v)	2 a(i)	1	-
Taudactylus pleione	CR	-	1ab(iii,v)+ 2 a b(iii,v)	-	-	-
Taudactylus rheophilus	CR	2ac	X	-	-	-
Litoria brevipalmata	EN	-	2 a b(iii)	-	-	-
Litoria nannotis	EN	2 a	X	-	-	-
Litoria raniformis	EN	-	2 a b(iii)	-	-	-
Litoria rheocola	EN	2 a c	X	-	-	-
Mixophyes fleayi	EN	-	2 a b(iii)	-	-	-
Mixophyes iteratus	EN	-	2 a b(iii)	-	-	-
Nyctimystes dayi	EN	2 a c	X	-	-	-
Taudactylus eungellensis	EN	3 c	X	-	-	-
Cophixalus mcdonaldi	VU	-	X	-	2	-
Cophixalus neglectus	VU	-	X	-	2	-
Cophixalus saxatilis	VU	-	X	-	2	-
Crinia tinnula	VU	-	2 a b(ii-v)	-	-	-
Geocrinia vitellina	VU	-	X	-	2	-
Heleioporus australiacus	VU	2 b c	-	-	-	-
Philoria richmondensis	VU	-	X	-	2	-
Litoria andiirrmalin	VU	-	X	-	2	-
Litoria aurea	VU	2 a b c e	-	-	-	-
Litoria freycineti	VU	-	2 a b(ii-v)	-	-	-
Litoria olongburensis	VU	-	2 a b(ii-v)	-	-	-
Mixophyes balbus	VU	-	-	1+2 a(i)	-	-
Pseudophryne australis	VU	2 b c	X	-	-	-
Pseudophryne covacevichae	VU	-	X	-	2	-
Spicospina flammocaerulea	VU	-	X	-	2	-
Threatened Total	40	14	17 (20)	3 (7)	16	0

TABLE 2. Multiple threats identified for each species (GAA assessments), ranked as either Foremost (F) or Potential (P). Totals represent Foremost in bold, with Potential in brackets. X, an unknown threat is suspected as known threats do not adequately explain observed declines. Bold type denotes species found primarily in upland areas (see Table 3).

Species	Status	Foremost & Potential Threats						
	IUCN 2002	Restricted Geographic Range	Chytrid Infection recorded	Fire	Habitat Modification	Introduced Species	Global weather Change	Threat Unknown
Rheobatrachus silus	EX	F	-	-	-	P (pigs)	-	X
R. vitellinus	EX	F	-	P	-	-	-	X
Taudactylus diurnus	EX	F	-	-	-	P (pigs)	-	X
Geocrinia alba	CR	F	-	P	F		-	-
Litoria booroolongensis	CR	-	-	-	F	P (pigs)	-	X
L. castanea / flavipunctata	CR	F	-	-	F	P (fish)	-	X
Litoria lorica	CR	F	-	-	-	-	-	X
L. nyakalensis	CR	F	-	-	-	-	-	X
L. piperata	CR	F	-	-	F	P (fish)	-	X
L. spenceri	CR	F	F	-	F	F (fish)	-	-
L. verreauxii alpina	CR	F	-	-	F	-	-	-
Philoria frosti	CR	F	-	-	-	-	-	X
Pseudophryne corroboree	CR	P	P	P	-	-	P	X
P. pengilleyi	CR	F	P	-	-	-	P	X
Taudactylus acutirostris	CR	P	F	-	-	-	-	X
T. pleione	CR	P	-	-	-	P (pigs)	-	X
T. rheophilus	CR	F	-	-	-	-	-	X
Litoria brevipalmata	EN	-	-	-	F	-	-	-
Litoria nannotis	EN	P	F	-	-	-	-	X
Litoria raniformis	EN	-	P	-	F	-	-	X
Litoria rheocola	EN	P	F	-	-	-	-	X
Mixophyes fleayi	EN	P	P	-	P	P (pigs)	-	X
M. iteratus	EN	-	P	-	F	-	-	-
Nyctimystes dayi	EN	P	F	-	-	-	-	X
Taudactylus eungellensis	EN	F	P	-	-	-	-	X
Cophixalus mcdonaldi	VU	P	-	-	-	-	-	-
Cophixalus neglectus	VU	P	-	-	-	-	-	-
Cophixalus saxatilis	VU	P	-	-	-	-	-	-
Crinia tinnula	VU	-	-	-	F	-	-	-
Eocrinia vitellina	VU	P	P	P	F	-	-	-
Heleioporus australiacus	VU	-	P	-	F	-	-	-
Philoria richmondensis	VU	P	-	-	-	-	-	-
Litoria andiirrmalin	VU	P	-	-	-	-	-	-
Litoria aurea	VU	-	P	-	F	F (fish)	-	X
Litoria freycineti	VU	-	-	-	F	-	-	-
Litoria olongburensis	VU	P	-	-	F	-	-	-
Mixophyes balbus	VU	-	-	-	P	-	-	X
Pseudophryne australis	VU	F	-	P	F	-	-	-
P. covacevichae	VU	F	-	-	F	-	-	-
Spicospina flammocaerulea	VU	P	-	P	F	-	-	-
Threatened total	40	**16** (15)	**5** (9)	**0** (6)	**18** (2)	**2** (7)	**2**	**22**

TABLE 3. Geographic distribution and reproductive habitats of Australia's threatened frogs. Bold type denotes species with a geographic range less than 20 000 km². TT = totally terrestrial reproductive mode.

Species	Status	Geog. range	Altitudinal range		Latitudinal range			Reproductive Habitat		
	IUCN 2002	km²	Low-land	Up-land	Temp.	Sub-tropic	Tropic	Montane wetlands	Wet forest stream	Isolated ponds & swamps
Rheobatrachus vitellinus	EX	131		X			X		X	
Rheobatrachus silus	EX	1394		X		X			X	
Taudactylus diurnus	EX	1417		X		X			X	
Geocrinia alba	CR	164	X		X					X
Litoria booroolongensis	CR	135 674		X	X				X	
Litoria castanea/flavipunctata	CR	8520		X	X					X
Litoria lorica	CR	1172		X			X		X	
Litoria nyakalensis	CR	11 636		X			X		X	
Litoria piperata	CR	5030		X	X				X	
Litoria spenceri	CR	16 578		X	X				X	
Litoria verreauxii alpina	CR	3227		X	X			X		
Philoria frosti	CR	293		X	X			X		
Pseudophryne corroboree	CR	1079		X	X			X		
Pseudophryne pengilleyi	CR	1109		X	X			X		
Taudactylus acutirostris	CR	14 620		X			X		X	
Taudactylus pleione	CR	126		X		X			X	
Taudactylus rheophilus	CR	4716		X			X		X	
Litoria brevipalmata	EN	72 540	X			X				X
Litoria nannotis	EN	19 044		X			X		X	
Litoria raniformis	EN	433 569	X		X					X
Litoria rheocola	EN	15 201		X			X		X	
Mixophyes fleayi	EN	6 985		X		X			X	
Mixophyes iteratus	EN	105 945	X			X			X	
Nyctimystes dayi	EN	18 894		X			X		X	
Taudactylus eungellensis	EN	335		X			X		X	
Cophixalus mcdonaldi	VU	345		X			X	TT		
Cophixalus neglectus	VU	562		X			X	TT		
Cophixalus saxatilis	VU	248		X			X	TT		
Crinia tinnula	VU	30 272	X			X				X
Geocrinia vitellina	VU	32	X		X					X
Heleioporus australiacus	VU	80 013		X	X					X
Philoria richmondensis	VU	967		X			X	TT		
Litoria andiirrmalin	VU	5 669	X				X		X	
Litoria aurea	VU	132 439	X		X					X
Litoria freycineti	VU	58 628	X			X				X
Litoria olongburensis	VU	8368	X			X				X
Mixophyes balbus	VU	110 441		X	X				X	
Pseudophryne australis	VU	17 504	X		X					X
Pseudophryne covacevichae	VU	379		X			X			X
Spicospina flammocaerulea	VU	562	X		X					X
Threatened total	40	31 78%	12 30%	28 70%	16 40%	9 23%	15 37%	4 (4) 10 + 10%	19 47.5%	13 32.5%

TABLE 4. Ecological characteristics of Australian frogs. Numbers represent the number of species within each IUCN category. CR includes the subspecies *L. verreauxi alpina*.

IUCN-GAA Assessment 2001	No. of Species	Geographic Range	Altitudinal Range		Latitudinal Range			Reproductive Habitat				
		Restricted (<20K km²)	<400m asl	>400m asl	Temp-erate	Sub-tropical	Tropical	Montane wetlands	Wet forest stream	Open forest stream	Isolated ponds & swamps	Totally terrest.
EX	3	3		3		2	1		3			
CR EN *	14	13	1	13	9	1	4	4	8		2	
EN	8	5	3	5	1	3	4		6		2	
VU*	15	10	8	7	6	3	6		2		9	4
Threatened sub-tot.	40	31	12	28	16	9	15	4	19	0	13	4
(% of 40 species)		77.5%	30%	70%	40%	22.5%	37.5%	10%	47.5%		32.5%	10%
Near Threatened	4	2	2	2	1	2	1		3		1	
Data Deficient	22	9	19	3	7	1	14		1	2	17	2
Least Concern *	147	30	110	37	50	34	63	1	10	7	109	20
Non-threatened sub-tot.	173	41	131	42	58	37	78	1	14	9	127	22
(% of 173 species)		23.7%	75.7%	24.3%	33.5%	21.4%	45.1%	0.6%	8.1%	5.2%	73.4%	12.7%
All species totals	213	72	143	70	74	46	93	5	33	9	140	26
(% of 213 species)		33.8%	67%	33%	34.7%	21.5%	43.7%	2.3%	15.5%	4.2%	65.7%	11.3%
Threatened sub-total												
All species totals	18.8%	43%	8%	41%	22%	20%	16%	80%	58%	0	9%	15%

only 42 species (24.3%) occurring in upland areas. Subsequently 41% of upland species are threatened while only 8% of lowland species are threatened (Table 4).

Latitudinal position is not strongly associated with the degree of threat. While more threatened species occur in the tropical and temperate areas than in subtropical regions (Table 3) this is primarily due to higher species richness in those regions. The percentage of threatened species in each region was 21.6%, 19.6% and 16.1% for the temperate, subtropical and tropical regions respectively (Table 4).

Threatened species are strongly associated with specific reproductive habitats: 19 threatened species breeding in wet forest streams (47.5%), 13 species breeding in isolated ponds and swamps (32.5%) and the remaining eight species breed in montane wetlands (Table 3). However, considering all species, 80% of all species breeding in montane wetlands and 57.6% of all species breeding in wet forest streams are threatened (Table 4). In contrast only 9.3% of all pond-breeding species are threatned (Table 4).

A preliminary examination of the phylogenetic relationships of Australia's threatened frogs (Table 5) suggests there are no strong associations between taxonomic groups and threatened status. One notable exception is the genus *Taudactylus* (Myobatrachidae) where five of the six species in the genus have a threatened status. Some phylogenetic associations occur within the hylid genus *Litoria* (Tyler & Davies, 1978; Hutchinson & Maxson, 1987) however the relationships among species within this genus require further examination.

GLOBAL THREATENED SPECIES

While comparative information from other countries is scarce, the average proportion of species that are threatened is 10% (Table 6). Several countries exceeding that (with over 15% threatened) include Australia, Fiji, Jamaica, Japan, New Zealand, Philippines, Puerto Rico, Seychelles, Venezuela & Virgin Islands. There is a strong association between threatened status and high altitude in all countries for which data is available (Table 6). Research on the potential impacts of the chytrid fungus has detected numerous species carrying the disease in Australia and the USA, with limited records from five other countries (Table 6).

DISCUSSION

Approximately 18.8% of Australia's frogs are currently listed as threatened. At least eight of these species can no longer be found in the wild, despite intensive searching. This is higher than in most countries (average of 10%) and is justification for concern in Australia. Completion of the Global Amphibian Assessment throughout the world is likely to change the context of these results however concern for countries known to have high levels of threatened species (Australia, Fiji, Jamaica, Japan, New Zealand, Philippines, Puerto Rico,

TABLE 5. Geographic Distribution and Reproductive Habitats of Phylogenetic groups of Australia's frog species. Threatened species groups are highlighted in bold. **Austrochaparina* was *Sphenophryne*.

Phylogeny	No. of Species	Geographic Range	Altitudinal Range		Latitudinal Range			Reproductive Habitat					IUCN GAA classification						
													Threatened				Non-threatened		
		Restricted <20K km²	<400m asl	>400m asl	Temperate	Sub-tropical	Tropical	Montane wetlands	Wet forest stream	Open forest stream	Ponds & swamp	TT	EX	CR	EN	VU	NT	DD	LC
MYOBATRACHIDAE																			
Adelotus	1	-	-	1	-	1	-	-	1	-	-	-					1		
Arenophryne	1	-	1	-	-	1	-	-	-	-	-	1							1
Assa	1	-	1	-	-	1	-	-	-	-	-	1							1
Bryobatrachus	1	1	-	1	1	-	-	-	-	-	-	1							1
Crinia	14	2	12	2	10	1	3	-	-	1	13	-				1		1	12
Geocrinia	7	4	6	1	7	-	-	-	-	3	4	-		1		1			5
Helioporus	6	1	6		6	-	-	-	-	-	6	-				1			5
Philoria/Kyarranus	5	5	-	5	3	2	-	-	-	-	-	5		1		1			3
Lechriodus	1	-	-	1	-	1	-	-	-	-	1	-							1
Limnodynastes	12	1	12	-	5	3	4	-	-	-	12	-							12
Megistolotus	1	-	-	1	-	-	1	-	-	-	1	-							1
Metacrinia	1	-	1	-	1	-	-	-	-	-	-	1							1
Mixophyes	5	2	1	4	1	4	-	-	4	-	1	-			2	1			2
Myobatrachus	1	-	1	-	1	-	-	-	-	-	-	1							1
Neobatrachus	10	1	10	-	5	4	1	-	-	-	10	-							10
Notaden	4	1	4	-	-	1	3	-	-	-	4	-						1	3
Paracrinia	1	-	1	-	1	-	-	-	-	-	1	-							1
Pseudophryne	13	4	10	3	8	4	1	2	-	1	10	-		2		2		3	6
Rheobatrachus	2	2	-	2	-	1	1	-	2	-	-	-	2						
Spicospina	1	1	1	-	1	-	-	-	-	-	1	-				1			
Taudactylus	6	5	-	6	-	1	5	-	6	-	-	-	1	3	1		1		
Uperolia	24	7	20	4	3	4	17	-	-	1	23	-						12	12
HYLIDAE																			
Cyclorana	13	-	13	-	-	5	8	-	-	-	13	-							13
Litoria	62	17	38	24	20	12	30	2	17	3	40	-		7	4	4	2	3	42
Nyctimystes	1	1	-	1	-	-	1	-	1	-	-	-			1				
MICROHYLIDAE																			
*Austrochaparina**	5	4	1	4	-	-	5	-	-	-	-	5							5
Cophixalus	13	13	4	9	-	-	13	-	-	-	-	13				3		2	7
RANIDAE																			
Rana	1	-	1	-	-	-	1	-	-	-	-	-							1

TABLE 6. Conservation status of frog species listed by IUCN 2002. The proportion listed known from upland areas does not include species for which complete information on altitudinal range was unavailable. * denotes countries where altitude data was unavailable.

Country	No. species	No. species with chytrid	No. listed IUCN 2002	No. listed IUCN & Amphibia Web	Proportion listed%	No. listed from upland	Proportion listed from upland %
Afghanistan	7		1	1	14	1	100
Argentina	147		5	5	3	*	*
Australia	213	47	40	40	18.8	28	70
Bolivia	135		1	1	1	*	*
Bosnia Herzegovina	10		1	1	10	1	100
Brazil	700		6	64	3	*	*
Cameroon	165		1	1	1	*	*
Canada	40		1	1	3	1	100
Chile	44		3	3	7	3	100
China	315		1	1	0	1	100
Costa Rica	165	1	1	5	3	5	100
Côte d'Ivoire	48		1	1	2	*	*
Croatia	10		1	1	10	1	100
Dominican Republic	37		1	1	3	*	*
Ecuador	437	2	0	24	5	*	*
Equatorial Guinea	26		1	1	4	*	*
Fiji	3		1	1	33	0	0
France	40		2	2	5	2	100
Georgia	14		1	2	14	2	100
Greece	20		1	1	5	1	100
Guinea	44		1	1	2	*	*
Haiti	47		1	1	2	1	100
Honduras	91		7	7	8	7	100
India	217		3	3	1	*	*
Iran (Islamic Republic)	20		2	2	10	2	100
Italy	43		4	4	9	4	100
Jamaica	24		4	4	17	3	75
Japan	62		10	10	16	4 / 4	100
Kazakstan	9		1	1	11	1	100
Madagascar	181		2	2	1	*	*
Mexico	330		4	6	2	6	100
Namibia	26		1	1	4	*	*
New Zealand	6	2	1	1	17	0	0
Peru	355		1	1	0	*	*
Philippines	93		22	23	25	16	70
Portugal	19		1	1	5	1	100
Puerto Rico	22		3	12	55	*	*
Seychelles	12		4	4	33	4	100
Slovenia	18		1	1	6	1	100
South Africa	107	1	9	9	8	5	56
Spain	31	2	3	3	10	3	100
Turkey	21		3	1	5	1	100
United States	272	13	25	26	10	20	77
Venezuela	291		0	63	22	52 / 52	100
Vietnam	115		1	1	1	1	100
Virgin Islands (British)	4		1	1	25	0	0
Totals	4962		170	296	average 10%	134	average 85%

Seychelles, Venezuela & Virgin Islands) is likely to remain high. Amphibian biodiversity is concentrated in the tropical regions (see Crump, 2003; Hero & Shoo, 2003). However, amphibian declines in tropical countries of Asia, Africa and South America are poorly represented in the IUCN 2002 Little Red Data Book assessments (Hero & Shoo, 2003). The Global Amphibian Assessment is currently addressing this shortfall. Nevertheless, conservation effort should focus on tropical regions where biodiversity is high and threats are not well known (Hero & Shoo, 2003).

Threats to Australian Frogs

While multiple threats were identified for each species, the most alarming result of the Australian (GAA) assessment is that the threats for 23 species are currently listed as unknown. In each case the multiple threatening processes associated with these species do not adequately explain the observed declines. This result is cause for major concern as management actions are hampered by the lack of knowledge on the relative importance of threats for these species. Management actions should place equal effort firstly into mitigating the known threats (e.g. habitat modification) and secondly, continuing research focusing on testing alternative hypotheses for the unexplained declines.

Habitat modification remains a key threat to Australian frogs (associated with the status of 50% of threatened species). Legislative protection is an essential process to ensure the conservation of these species. Species currently listed as Endangered, Critically Endangered or Extinct under the Australian Commonwealth *Environment Protection and Biodiversity Conservation Act* (1999) invoke strict legislative protection. Protection of "Vulnerable" species however is less clear. Of particular concern are 10 of the 15 species currently listed as "Vulnerable" threatened by habitat modification. If habitat modification continues these species will eventually be upgraded to the "Endangered" category. Eight "Vulnerable" species have relatively large geographic ranges (>5000 km^2) and information on the "area of occupancy" (the area within its extent of occurrence which is occupied) is urgently needed to assess their status. While it may not be realistic to monitor populations over such large areas, some species have well known associations with specific habitats/ vegetation types which can be mapped and the loss of area can be mapped over time. As a solution to this, Shoo & Hero (pers. comm.) propose modelling the area of occupancy (using site records in combination with spatial habitat modelling and overlaid with current land use) for each species. This management tool will estimate the predicted area of occupancy for each species and can be updated on a regular basis (e.g. five yearly) providing a dynamic picture on the potential conservation status for each species based on habitat loss and geographic distribution.

In 1998 a chytrid fungus (*Batrachochytrium dendrobatidis*) was found to have lethal effects on amphibians in Australia and in other parts of the world (Berger *et al.*, 1998). In Australia, the chytrid fungus is directly associated with declines for five species and a potential contributor for an additional nine species (35% of threatened species). In 2002 the Australian Government listed chytridiomycosis as a "key threatening process". It should be noted, however, that *B. dendrobatidis* has also been detected in an additional 33 (19%) non-threatened species (Speare & Berger, 2000*b*). Clearly other factors are contributing to declines associated with chytrid infection (e.g. altitudinal distribution, breeding habitat etc.). Hero (1996), and Williams & Hero (1998, 2001) found small clutch size was associated with declining species in some areas and this relationship is currently under further examination (Hero, J.-M. unpublished).

The role of the chytrid fungus as a global threat is slowly emerging (Speare & Berger, 2000*a,b*; Daszak, Cunningham & Hyatt, 2001). Chytrid fungus has now been isolated from declining species in Central and South America (Lips 1999, Young *et al.*, 2001; Lips *et al.*, 2003; Ron et al., 2003), several species in North America (Carey, Cohen & Rollins-Smith, 1999), Germany (Speare & Berger 2000*a,b*), Spain (Bosch, Martinez-Solano & Garcia-Paris, 2001) and South Africa (Lane, Weldon & Bingham, 2003). Continuing research on this emerging infectious disease is an important step in resolving global amphibian declines.

Introduced fish have been notably associated with declines in two Australian species *Litoria spenceri* and *L. aurea* and potentially contribute to the decline of several other species including *L. castanea / flavipunctata* and *L. piperata* (Gillespie & Hines, 1999). Action to mitigate these effects are feasible, e.g. introduce legislation to stop fish stocking and movement of both native and non-native fish species, and promote active management of threatened species by eradicating introduced species from their breeding habitats (Gillespie & Hero, 1999; Gillespie, 2001). Other species that are potential threats to Australian frogs include feral pigs and cattle, however the impacts of these species have not been investigated (Hines, Mahony & McDonald, 2003). Management agencies should investigate these impacts where appropriate and apply mitigation procedures where feasible.

Few studies have investigated the impacts of fire on frogs. Fire has been proposed as a potential threat to several *Geocrinia* species and *Spicospina flammocaerulea* from small isolated populations in Western Australia (Driscoll & Roberts, 1997), and to remaining isolated populations of *Pseudophryne australis* in the Sydney region (AmphibiaWeb, 2003). Following severe declines in the geographic distribution of *Pseudophryne corroboree* in the Australian Alps, wildfires affected the few remaining breeding sites in January 2003 (Will Osborne, pers comm.). Protection measures should be investigated and an experimental approach to examine the impacts of fire on these species implemented.

Changing weather patterns (global warming and increased UV-B radiation) have not been strongly associated with frog declines in Australia. Unusual

weather appears to be an inadequate explanation for the dramatic decline of montane frogs in Queensland (Laurance, 1996). Furthermore, many threatened frog species reproduce in wet forest streams where direct UV-B radiation is limited. For Australia's montane species the effects of global warming are expected to be complex and associated with other factors (Kiesecker, Blaustein & Belden, 2001). Increased UV-B has been proposed as a contributing factor in the decline of *Litoria verreauxi alpina* in the Australian alpine region (Broomhall *et al.*, 2000). While this hypothesis has not been investigated thoroughly, we conclude that increased UV-B is currently not expected to be a major threat to frogs in Australia.

Geographic distribution and Reproductive Ecology of Australian Frogs

Restricted geographic range is characteristic of 31 of 40 threatened species (77.5%) however an additional 41 non-threatened species (23.7%) also have restricted geographic range. These data suggest that restricted geographic range (<20 000 km^2) in isolation is not a threat. We propose that this distributional variable might be correlated with a species characteristic (e.g. fecundity) that would provide a biological explanation for their vulnerability to extinction processes (Smith & Quin, 1996).

Amphibian declines have been recorded from numerous high altitude areas including Australia (Campbell, 1999; this study), South Africa (Lane, Weldon & Bingham, 2003), Spain (Bosch, Martinez-Solano & Garcia-Paris, 2001), Latin America (Young *et al.*, 2001; Ron *et al.*, 2003), and the USA (Kiesecker, Blaustein & Belden, 2001). This global pattern is confirmed in our analysis (Table 6). The strong association between threatened status and high altitude both in Australia and on a global scale is not easily explained. Upland habitats contain areas of high species richness, endemism and a high proportion of geographically restricted species (Hero & Shoo, 2003). Habitat modification is more prevalent in lowland areas and unexplained threats to upland species is a major concern (in eastern Australia 8 upland species can no longer be found in the wild). Our results demonstrate this is a global phenomenon, and conservation agencies should focus on assessing the conservation status of amphibians in high altitude areas.

Morrison & Hero (2003) proposed that declines in upland areas might be associated with intrinsic changes in the life history characteristics of species associated with altitude. Amphibian populations at higher altitudes tend to: (1) have shorter activity periods and hence shorter breeding seasons; (2) have longer larval periods; (3) be larger at all larval stages including metamorphosis; (4) be larger as adults; (5) reach reproductive maturity at older ages; (6) produce fewer clutches per year; (7) produce larger clutches absolutely and smaller clutches relative to body size; and (8) produce larger eggs. The combination of these characteristics suggested that high altitude individuals, and hence populations, may be less resilient to extinction processes (Morrison & Hero, 2003).

Species breeding in Australian montane habitats and wet forest streams are more likely to be threatened than pond breeding or totally terrestrial species. This is similar to the many species of the genus *Atelopus* that have suffered severe declines in the high altitude wet forest streams of Central and South America (Young *et al.*, 2001; Ron *et al.*, 2003). The strong association with altitude may be associated with breeding site attributes that work synergistically with other causes of decline (e.g. chytridiomycosis).

The principal threat to lowland Australian frogs is habitat loss (Table 2) – this is not surprising as land clearing for human activities is generally concentrated in lowland areas (Brooks *et al.*, 1999; Hero & Shoo, 2003). Hence we expect that more lowland species would be threatened worldwide than upland species. In Australia the principal threats to upland species are varied, often associated with restricted geographic range (however as discussed previously this association is not foremost) but more notably associated with the presence of chytrid fungus, habitat modification, introduced species and unknown causes all contributing (Table 2).

Global Threats to Frogs

Clearly much can be learned from the Australian assessment. Frog declines are a complex interaction of threatening processes (unknown, habitat modification, chytridiomycosis, fire and global warming) and species vulnerability (breeding habitat, altitudinal distribution and restricted geographic range). Species ecology may also play a role (e.g. reproductive ecology and population dynamics). The traits of declining frogs in Australia can be used to predict species vulnerability in countries where little is known about the status of their amphibian populations. There is a dearth of information available on the conservation status and threatening processes (e.g. chytridiomycosis) in tropical countries where amphibian biodiversity is high. Hero & Shoo (2003) proposed establishing a global network of "Research Centres for Amphibian Conservation" in biodiversity hotspots with significant species richness, endemism or both. International assistance by the global herpetological community is urgently needed to provide research training, resources and funding to scientists in tropical countries.

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GENETIC DIFFERENTIATION AMONG NORTHERN EUROPEAN POOL FROG (*RANA LESSONAE*) POPULATIONS

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** Deceased: 20.3.04*

The isolated Swedish metapopulation of pool frogs (*Rana lessonae*) was not discovered until the 1940s and is classified as "vulnerable" in conservation terms. Swedish pool frogs are now known from 96 localities along the Baltic coast of east-central Sweden, and differ from Central European conspecifics in terms of coloration and low allozyme heterozygosity. Using mini- and microsatellite DNA fingerprinting and allozyme electrophoresis, we studied genetic differentiation among pool frogs from Poland, Latvia, Russia, and Sweden. Allozyme variability was partitioned equally within and among the populations (F_{ST} = 0.50). Both allozyme and DNA fingerprint analyses indicated that Swedish frogs were most similar to Latvian ones. The average similarity in DNA fingerprints among Swedish populations was of the same order as the similarity within the Polish, Latvian or Russian populations. Pool frogs from opposite ends of the Swedish distribution, however, were as different from one another as they were from continental conspecifics. Our results complement and corroborate the evidence from other studies, suggesting that there is a "northern clade" of Swedish, Norwegian and British pool frogs, and that the Swedish pool frogs constitute a relict population rather than being descendants from a recent introduction by humans.

Key words: DNA fingerprinting, enzyme electrophoresis, green frogs

INTRODUCTION

The pool frog, (*Rana lessonae* Camerano), is a European water frog taxon that occurs from northern Italy, northwards through central Europe, and with the northernmost populations at 59 and 60 °N in Russia and Sweden, respectively (Fig. 1). In Scandinavia it is known from 96 localities along the Baltic coast of east-central Sweden and from two localities in southern Norway (Dolmen, 1997; Edenhamn & Sjögren-Gulve, 2000). The Swedish local populations form a metapopulation on the northern fringe of the species' distribution, isolated from other conspecific populations on the European continent. In Sweden, dispersing individuals connect central local populations, but there are also a number of isolated populations. Isolation-dependent extinction is common at distances > 1 km from the closest local pool frog population, and from the 1960s until 2001, 60 extinctions and 51 new populations have been recorded (Sjögren, 1991*a*; Sjögren-Gulve & Ray, 1996; Sjögren-Gulve, unpubl. data). Annual census size of an average Swedish local breeding population over five years ranged from 79 to 204 adults with an effective breeding population size of 35-60 (Sjögren, 1991*b*) – about 10% of the size of more central European populations (L. Borkin & M. Rybacki, personal communication).

In Sweden, the pool frog is classified as "vulnerable" according to IUCN criteria (Gärdenfors, 2000), mainly due to its restricted geographical occurrence and threats posed by large-scale forestry (Sjögren-Gulve and Ray, 1996). The Swedish population was discovered in the 1940s, and its origin is unknown and debated (Forselius, 1948; 1962; Waldén, 1955). Two main hypotheses have been put forward: (1) pool frogs from Central Europe were introduced by humans in the mid-18th century (Waldén, 1955); (2) the frogs constitute a relict from the warm Ancylus period about 7000-5500 BC (Forselius, 1962) when the average temperature was 2-2.5 °C higher than today. These two scenarios have different implications for the population's conservation value. If introduced by man, its conservation would merit low consideration, whereas if the population is a geographically peripheral and genetically distinct relict it implies a significantly different population history compared to continental populations, and substantial conservation value (e.g. Lesica & Allendorf, 1995). A similar issue concerns British pool frogs from an area in Norfolk that were examined by Zeisset & Beebee (2001) using genetic variation at microsatellite loci. Using six polymorphic loci, they found that pool frogs from Norfolk, Norway and Sweden clustered as a "northern clade", genetically distinct from conspecifics in the Netherlands, France, Poland, Switzerland, Hungary and Italy. Like the frogs from Norfolk and Norway, Swedish pool frogs differ from Central European conspecifics by having brown dorsal colour instead of green or greenish. They also differ by their pronounced sexual colour dimorphism with very dark brown females (Forselius, 1962; Berger, 1977; Sjögren, 1991*b*), which may gain thermal advantages in the northern climate (Gibbson &

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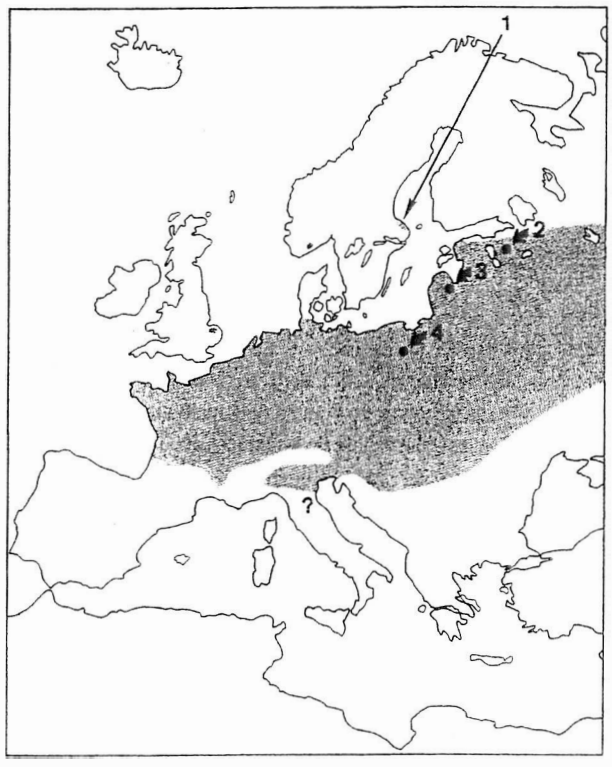

FIG. 1. The distribution of the pool frog (*Rana lessonae*) in Europe (modified from Sjögren 1991*b*). Sampling sites for the present study was: 1. Sweden (see also Fig. 2), 2. Luga (Russia), 3. Tukums (Latvia) and 4. Turew (Poland).

Falls, 1979). The results of Zeisset & Beebee (2001) and the potentially adaptive coloration of the "northern clade" of pool frogs support the relict hypothesis. However, so far, pool frogs from countries north of Poland and east of the Baltic Sea remain unexamined, and these are also potential source areas for introductions.

Fringe populations of widespread taxa often are less genetically variable than central populations (e.g. Lesica & Allendorf, 1995). A previous study (Sjögren, 1991*b*) found that Swedish pool frogs have low allozyme heterozygosity ($\bar{H}_t$ = 0.002, 31 loci) with one or two variable loci in two out of five populations. This low variability seemed more plausibly explained by long-term fluctuations in population size than by anthropogenic introduction in the mid-18th century (Sjögren, 1991*b*). However, because repeated bottlenecks may have the same effect as a single founder event, data on allozyme variability alone do not allow any firm conclusion regarding the population's origin. The process of restoring variation by mutation is slow for neutral genes like most allozymes, in the range of hundreds of thousands of generations (Lande & Barrowclough, 1987). In our present examination of pool frogs from Poland, Latvia, Russia, and Sweden representing "the northern clade", we have therefore used, in addition to allozymes markers, markers with a fast mutation recovery time, which potentially could reveal a higher level of genetic variability and detect a genetic structuring among Swedish local populations. Multilocus DNA fingerprinting using mini- or microsatellites detects many loci at the same time and because of their high mutation rate, in the order of 0.001 to 0.05 mutations per locus and generation (Jeffreys *et al.*, 1985*a*,*b*), the method may discern variability in populations depauperate in allozyme variation and may therefore also be sensitive to recent partitioning of populations.

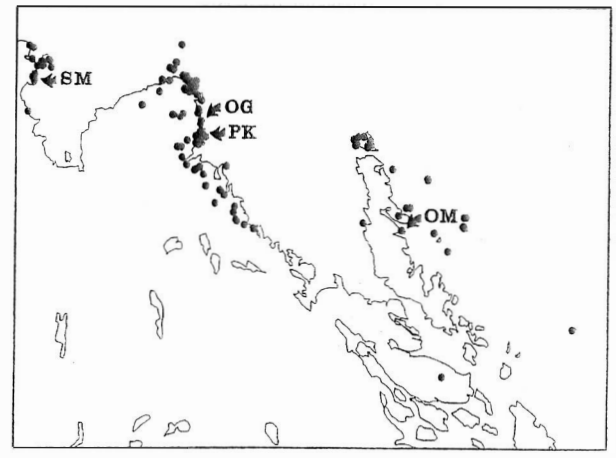

FIG. 2. The regional distribution of pool frog (*Rana lessonae*) localities along the Baltic coast of east-central Sweden (1962 – 1994) and the localities where Swedish samples were collected.

MATERIAL AND METHODS

Biological Material

Frogs were collected from three Swedish localities (Fig. 2, Södra Marörspussen = SM, Prästbäckskärret = PK and Östra Mörtarö = OM). These localities were chosen to represent the distribution of the Swedish population with the easternmost locality (OM) on an island separated from the mainland, one locality (PK) within the main distribution (about 30 km west and east from the other two investigated localities), and finally a western locality (SM). These populations are isolated from each other by sea, geographic distance and effective migration (Sjögren, 1991*a*,*b*). Populations nearby surround population PK. This set represents the centre of the Swedish distribution, whereas OM and SM represent the periphery. Eastern populations on the European continent are represented by one locality in Latvia (Tukums =TK), one locality in Russia (Luga = LU) and central European populations by one locality in Poland (Turew = TU, Fig. 1). The three samples from the European continent each are from one local population situated within a fairly continuous distribution and with population sizes about tenfold larger than the Swedish local populations (L. Borkin & M. Rybacki, personal communication). For the enzyme electrophoresis, one additional Swedish local population was used (Östra Granskärsdammen = OG) which is closely situated to locality PK (about 2 km) and these two localities are probably sparsely interconnected by migration. Some of the results of the enzyme electrophoresis have been published elsewhere (Sjögren, 1991*b*).

Different numbers of individuals have been used for enzyme electrophoresis and DNA fingerprinting. For

enzyme electrophoresis, the average sample size per locus for the four Swedish local populations was SM: 34.0, PK: 9.0, OM: 11.0, OG: 71.1 and for the three remaining eastern and southern localities on the European continent the sample sizes were TK: 23.2, LU: 9.0 and TU: 63.0. For the DNA fingerprinting, sample sizes were considerably lower due to the complexity of the technique, especially because comparisons between gels are not possible and only about 14 individuals could be used on one gel. Thus, comparisons between individuals from the same local population and between individuals from different populations often had to be performed on separate gels. Three Swedish local populations were used for DNA fingerprinting, and sample sizes were SM: 4 , PK: 10 and OM: 11. Sample sizes for the DNA fingerprinting of the populations on the European continent were six individuals each for populations TK, LU and TU.

Enzyme Electrophoresis

Starch-gel enzyme electrophoresis was performed as described by Sjögren (1991*b*) and scored 28 loci: AAT-1, AAT-2, ADH-1, ADH-2, AGP, CPK, DIA-1, DIA-2, EST-1, EST-2, EST-3, FDP-1, FDP-2, GAPDH, GUS, IDH-1, IDH-2, LDH-1, LDH-2, MDH-1, MDH-2, ME-1, ME-2, PGI-1, PGI-2, PGM, PMI and SOD. Analysis of genetic distance was performed using Rogers' modified distance (Rogers, 1972; Wright, 1978) and Nei's unbiased genetic distance (Nei, 1978) with the programme BIOSYS (Swofford & Selander, 1989).

Isolation of DNA

Blood samples were collected from the tarsal vein with a sterile syringe, transferred to Eppendorf tubes with SSC buffer (0.15 M NaCl, 0.15 mM trisodium citrate, 0.5 mM EDTA pH 7.0) and stored at -70°C. Genomic DNA was extracted from approximately 25 µl of blood by addition of 2.5 ml SET-buffer (0.15 M NaCl, 0.05 M Tris-HCl, 1 mM EDTA pH 8.0, autoclaved), 50 µl of 25% SDS w/v and 80 µl of proteinase K (10 mg/ml). The tubes were gently shaken for 2-4 hrs at 37°C, and DNA was purified with two extractions of phenol/chloroform and two with chloroform. DNA was precipitated with 0.1 volume 3 M sodium acetate and 2 volumes 99% -20°C ethanol, removed with a sterile glass hook, washed by dipping in 70% ethanol and dissolved in 0.4-1.5 ml sterile 0.01 M Tris-HCl, pH 8.0 for at least 24 hours. DNA (8-10 µg) was digested with 30 units of restriction enzyme *Alu* I for 4 hours at 37°C, extracted once with phenol/chloroform, once with chloroform and precipitated as above, pelleted at 12 000 g for 20 minutes, washed with 70% ethanol and vacuum dried. The digested DNA was dissolved in 25 µl 0.01 M Tris-HCl, pH 8.0. DNA-fragments were separated in 20 x 30 cm 0.8% agarose gels for about 48 hrs at 1.6 V/cm and transferred to Biodyne Nylon membranes by Southern blotting in 10 × SSC.

DNA Fingerprinting

50-75 ng of the insert of human minisatellite clone 33.15 (Jeffreys *et al.*, 1985*a*,*b*) was ^{32}P labelled by the random primer method (Feinberg & Vogelstein, 1983). Prehybridization and hybridization were performed according to Georges *et al.* (1988) using dried skimmed milk. Membranes were washed 2 × 15 min in 1.5 × SSC, 0.1% SDS at room temperature, 2 × 15 min in 1 × SSC, 0.1% SDS at 60°C and finally 10 min in 1 × SSC at room temperature and autoradiographed at -70°C for 1-6 days using Kodak X-omat AR and intensifying screens. Apart from the 33.15 DNA probe, that was used throughout our investigation, we initially also tested three other DNA probes (and two other restriction enzymes, *Hae III* and *Hinf I*) to improve resolution. The minisatellite probe M13 (Vassart *et al.*, 1987) was isolated directly from the phage and labelled, hybridised and washed from the membranes with the stringency conditions given above. The synthetic dinucleotide microsatellite repeats $(TC)_n$ and $(TG)_n$ (250ng, Pharmacia LKB Biotechnology) were labelled by standard nick translation (Promega), hybridised to the *Alu* I digested DNA according to Ellegren (1991) and membranes were washed in 0.1 × SSC, 0.1% SDS at 60 °C for 40 min and exposed to x-ray film as above. DNA probes were removed from membranes by washing in 0.4 M NaOH and 0.2 M Tris-HCl, pH 7.5. Most membranes were subjected to different exposure times to visualise bands of different intensities. DNA fragments in the range of 2-20 kilobases separated in the same gel were compared using acetate sheet overlays where all bands were marked and subsequently used to calculate genetic similarity. The band sharing statistics according to Wetton et al. (1987) were used to calculate the proportion of shared bands between two individuals (Similarity index $S = 2N_{AB}/(N_A+N_B)$). The significance of differences between average similarities was tested using two-tailed *t*-tests.

RESULTS

Enzyme Electrophoresis

Among the seven populations that were investigated for enzyme variation at 28 loci, there were seven polymorphic loci (AAT-2, EST-2, EST-3, GUS, IDH-2, LDH-2 and PGM). Among the four Swedish local populations, two loci were polymorphic (EST-2 and IDH-2), but two of the local populations (PK and OM with the smallest sample size, 9 and 11, respectively) were monomorphic at all 28 loci. The Russian sample (LU) was variable at one locus (EST-2) and fixed for a unique alternative allele at the GUS locus. The Latvian sample (TK) was polymorphic at two loci (AAT-2 and EST-2) and the Polish population (TU) at five loci (AAT-2, EST-2, EST-3, LDH-2 and PGM). No more than two alleles were found per locus. The average heterozygosity was low in the Swedish populations (0.0-0.5%; SE: 0.0 - 0.4%) and in no case did observed

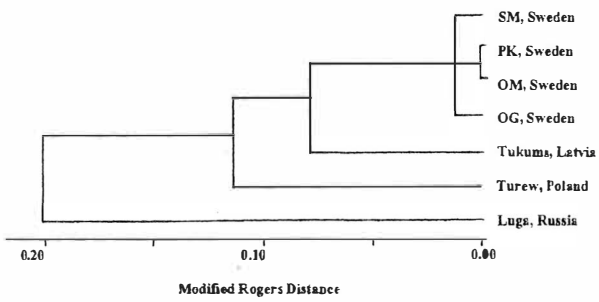

FIG. 3. Genetic similarity in allozymes (28 loci) among Swedish, Latvian, Polish and Russian pool frogs (*Rana lessonae*) illustrated by a UPGMA clustering phenogram based on Rogers (1972) modified distance.

heterozygosity deviate from the Hardy-Weinberg expectations in the Swedish or the other populations. The heterozygosity level was considerably higher in the Polish population (5.3 %±2.7) as well as in the Latvian population (2.3%±1.7) whereas the Russian population showed an average heterozygosity (0.8%±0.8) close to that of the Swedish local populations. Allele frequency data for all populations are available from the second author on request.

In the total sample and over all variable loci, the relative genetic diversity (F_{ST}) calculated according to Nei (1973) showed that approximately 50% of the variability was distributed within the populations, and the remaining 50% of the diversity was distributed between populations. The comparably high level of between-population variability indicates that they have diverged genetically. Based on the modified Rogers distance (Wright, 1978), a phenetic analysis showed that the four Swedish local populations cluster first with small distances (0.000-0.011, Fig. 3). The Latvian population is the genetically most similar continental population (distances of 0.077-0.079 versus the Swedish conspecifics), followed by the Polish population (distances of 0.104-0.105 vs. Swedish conspecifics) and the Russian population (distances of 0.189-0.190). The Russian population was homozygous for an alternative and unique GUS allele. If all loci except GUS are considered, the Russian frogs were most similar to the Swedish ones. The same clustering of populations emerged using Nei's (1978) unbiased distance, but with lower resolution (distances between 0.006 and 0.037 between Swedish and continental populations, and 0.000 among Swedish populations).

DNA FINGERPRINTING

The four different DNA probes we tested showed very different levels of variability within and differentiation between populations. M13 gave the lowest level of variability, sometimes with identical fingerprints for frogs from Swedish local populations (Tegelström & Sjögren, 1990) and fewer bands compared to the other probes tested. The 33.15 probe revealed an intermediate level of variability, a high number of well-resolved bands (Fig. 4a) and was chosen for further analysis.

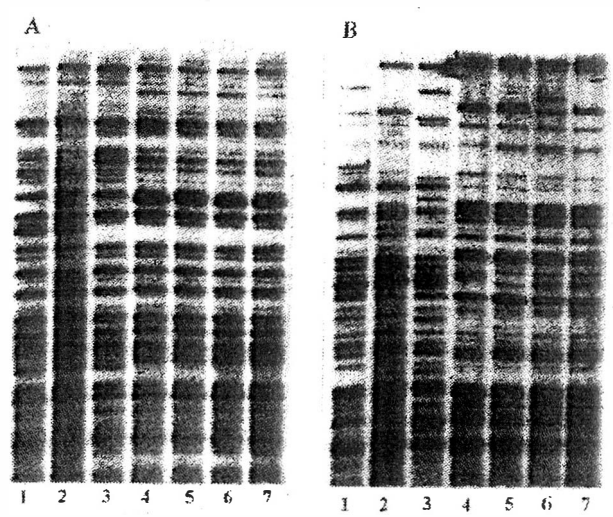

FIG. 4. DNA fingerprints of Swedish pool frogs (*Rana lessonae*) using the minisatellite probe 33.15 (A) and the dinucleotide microsatellite repeat $(TG)_n$ (B) on the same membrane. Fragment sizes are between 20 000 (top) and 2000 base pairs (bottom). Samples 1-3 are from locality PK and samples 4-7 from locality OM. Note the difference in variability and difference between localities revealed by the two DNA probes.

However, the two dinucleotide microsatellite repeats $(TC)_n$ and $(TG)_n$ gave well-resolved fingerprints with a high number of bands and with a potential to detect more variation than the minisatellite probes. Especially the $(TG)_n$ probe gave excellent resolution and detected a higher level of variation than the minisatellite probes (Fig. 4b), even within local Swedish populations.

The average number of bands detected by the $(TG)_n$ probe was 30.2±3.2 (mean±SE; based on 24 Swedish individuals. The level of similarity was consistent within the three Swedish populations (SM: 0.86±0.05, PK: 0.76±0.06, OM: 0.89±0.04, considerably lower than that obtained by DNA probe 33.15 (0.94, $t>100$, $P<0.0001$). When individuals from different Swedish local populations were compared using the $(TG)_n$ probe we found a similarity of 0.50±0.09 (12 comparisons), considerably lower than that found using the 33.15 probe (0.73; $t= 5.6$, $P<0.001$). This level of similarity within the Swedish population obtained by the $(TG)_n$ probe indicates that when more distantly related populations are compared the DNA fingerprinting similarity will approach zero and therefore will be insensitive for such estimates. Therefore, we chose the 33.15 probe for our further investigations to compare the similarities between samples from Sweden and the European continent.

The total number of scorable bands per individual detected by the 33.15 probe ranged from 29 to 43 with an average of 35.6±3.9 over all populations. The band sharing between individuals within the Swedish local populations was 0.94±0.05 (106 comparisons, range 0.84-1.00). The level of similarity was consistent within the three populations (SM: 0.99±0.01, PK: 0.91±0.05, OM: 0.97±0.02). When we compared individuals from

TABLE 1. Band sharing (mean and S.D.) among pool frog (*Rana lessonae*) individuals from the same locality (diagonal) or different localities using DNA probe 33.15. n= number of pairwise comparisons. Locality PK and OM are Swedish, LU is Russian, TK is from Latvia and TU from Poland

	PK	OM	LU	TK	TU
PK	0.81±0.08 *n*=4	0.74±0.07 *n*=6	0.56±0.07 *n*=5	0.62±0.09 *n*=5	0.47±0.07 *n*=5
OM		0.98±0.01 *n*=22	0.50±0.03 *n*=5	0.50±0.05 *n*=5	0.33±0.02 *n*=5
LU			0.74±0.08 *n*=5	0.54±0.05 *n*=8	0.37±0.08 *n*=7
TK				0.75±0.03 *n*=5	0.47±0.05 *n*=10
TU					0.71±0.07 *n*=3

the different Swedish local populations we found a mean band sharing similarity of 0.73±0.10 (11 comparisons, range 0.56-0.79) which is considerably lower than within the local populations (t=6.95, P<0.001). In no case did a value for a comparison of individuals from different local populations exceed the lowest value found for comparisons of individuals from the same population.

The average similarity found between individuals from the three Swedish populations was almost identical to that found within the other three European populations (0.74±0.06, range 0.71-0.75, Table 1). A lower similarity was found when we compared individuals from the Swedish local populations with individuals from Russia, Latvia and Poland (0.48±0.09). These between-population similarities were significantly lower than found for the within-population comparisons (t=11.10, P<0.001). The band sharing similarity for comparisons between the four geographical populations (Table 1) shows that the two Swedish local populations are most closely related to the Latvian (0.56±0.09) and the Russian population (0.53±0.07) and least similar to the Polish population (0.4±0.09). The Polish population compared to the Russian and Latvian population showed a low level of similarity (0.43±0.08) indicating that the sample of Polish pool frogs is the most dissimilar among the four geographical populations.

DISCUSSION

Genetic Variability in the Swedish Population

A previous investigation of allozyme variability of Swedish pool frogs (Sjögren, 1991*b*) showed a low level of variability, which was attributed to long-term strong fluctuations in effective population size. Of the 31 loci that were scored in five local populations, only two (EST-2 and IDH-2) showed variation with one common allele (frequency ≥0.95) and one rare allele. This variation was found in the population OG, while population SM was variable at one locus only (EST-2) and the other three populations (not investigated in the present report) were monomorphic at all 31 loci. These results were confirmed in the present study where two of the Swedish local populations (PK and OM) were monomorphic at all 28 loci and the other two were variable at one locus (EST-2, population SM) and two loci (EST-2 and IDH-2, population OG) respectively. Thus, the allozyme analyses show a very similar genetic constitution of the Swedish local populations.

In a pilot study, Tegelström & Sjögren (1990) used DNA fingerprinting (with the M13 and 33.15 DNA probes) to examine seven juvenile frogs and one adult male from the OG population and found no variation. The present investigation shows that the variability we can detect in single local populations is dependent on the characteristics of that population and which DNA probe is used. The most variable of the local Swedish populations (PK) showed an average similarity of 0.81 using the 33.15 probe and 0.76 with the $(TG)_n$ probe, corresponding to seven bands that differed between individuals, a high level of variation compared to the results using allozymes. Obviously, a single local population may harbour significant micro- or minisatellite variation, indicating that other parts of the genome that may be important for local adaptation also may contain a significant level of genetic variation. Among the three Swedish populations, the two most peripheral ones (SM and OM) showed the lowest level of variation and the most central (PK) the highest. This is an expected result considering both the effective size of the peripheral populations, that is less than that of the central population, and the level of gene flow which is higher among the more central populations (Sjögren, 1988, 1991*b*). By both these mechanisms, peripheral populations are more exposed to genetic drift than central populations and will lose alleles at higher rates than the more central populations.

Even though pool frogs from the same pond or local population are comparably similar in DNA fingerprints, the geographically separated Swedish local populations are genetically different. Using the most variable microsatellite DNA probe $(TG)_n$ we found an average similarity of 0.50 among Swedish populations. Thus, more than 50% of the bands were different comparing

individuals from different populations. Although we have investigated only one central and two peripheral populations, spanning the Swedish range, the large difference in the DNA fingerprints between individuals from different populations, and the unique IDH-2 allele of the OG population, indicate that the Swedish population has gone through a differentiation process. A significant part of the microsatellite variability has become distributed between populations. Pool frogs from the NW and SE ends of the Swedish distribution are as different from one another as they are from Latvian and Russian conspecifics more than 380 km away. Assuming that the neutral genetic markers we have used in our investigation are also good indicators for genetic differences in loci and characters exposed to selection, our results have significant conservation implications. Populations have their own genetic characteristics, rendering a marked genetic conservation value. In cases where translocation of individuals is necessary for rescue actions, actions should be preceded by a genetic survey of the population characteristics.

The Origin of the Swedish Pool Frogs

Two main hypotheses have been suggested for the occurrence of the Swedish pool frogs, isolated from their Baltic and Central-European conspecifics. Either the frogs were introduced from Central Europe by humans in the mid-18th century (Waldén, 1955) or they colonized part of present-day Sweden during the warm Ancylus period about 7000-5500 BC (Forselius, 1962). The importance of distinguishing between these two alternatives could be unimportant, were it not for arguments concerning their conservation value and costs or efforts involved to save these marginal populations. If they were introduced – possibly with a small founder population size – they would be just a sample of the original population and would have lost much of the original genetic variation

Zeisset & Beebee's (2001) study showed that Swedish pool frogs belong to the "northern clade", which also includes English (now extinct) and Norwegian conspecifics, and which is genetically dissimilar to Central European populations. At fingerprint loci, we found that frogs from opposite ends of the Swedish range differ as much from one another as they do from continental conspecifics. Considering both allozymes and microsatellites, we argue that there is sufficient genetic divergence to assign a high conservation value to the Swedish frogs, and frogs representing the "northern clade" (Zeisset & Beebee, 2001) – whether introduced by humans or being relicts. Both allozymes and microsatellite loci clearly show that Swedish pool frogs are more closely related to conspecifics from north-eastern Europe than from Poland indicating a closer ancestry among these marginal populations than to Central European populations. The distribution of the diversity among Swedish local populations could be explained by an introduction 250 years ago, but because of the unique GUS genotype of Russian frogs, we would have to assume that frogs were collected at a location in Latvia, Estonia or Lithuania. The number of introduced frogs would also have to be fairly high, and not just introduced at one Swedish locality, but at several. This scenario is inconsistent with what would be required to achieve the present-day allozyme constitution of the Swedish frogs (see Sjögren, 1991*b*). Inclusion of the alternative AAT-2 allele among founders would also be highly likely, and the Swedish IDH-2 polymorphism must have arisen since the hypothetical introduction. We conclude that the differentiation pattern demonstrated here and in Zeisset & Beebee (2001), together with the brown coloration of the "northern clade" of pool frogs, indicate that the relict hypothesis is the most likely to explain the unusual presence of the peripheral Swedish pool frog population.

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A MODEL HOST-PATHOGEN SYSTEM FOR STUDYING INFECTIOUS DISEASE DYNAMICS IN AMPHIBIANS: TIGER SALAMANDERS (*AMBYSTOMA TIGRINUM*) AND *AMBYSTOMA TIGRINUM* VIRUS

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Pathogens are among the suspected causes of declining amphibian populations, but studying infectious diseases in small, threatened populations is ethically and experimentally questionable. Progress on understanding amphibian diseases requires model host-pathogen systems with populations large enough for robust experimental designs that do not threaten the amphibian host with extinction. We report on viral genomics, persistence, and host-pathogen dynamics of a model system we are using for studying an amphibian disease: tiger salamanders (*Ambystoma tigrinum*) and *Ambystoma tigrinum* virus (ATV). ATV is a large, cytoplasmic, double-stranded DNA virus that causes systemic infections in individuals and recurrent epidemics in tiger salamander populations in western North America. The ATV genome is now completely sequenced, which is an important step toward understanding viral pathogenesis. Further, because tiger salamanders and the closely related axolotl have a long history as model organisms for developmental genetics, the genetics, development, and physiology of these species are known at levels that can support detailed studies of the host-virus interaction. Salamanders become infected with ATV via direct contact, feeding on infected tissues, and by immersion in water containing virus particles. There is no evidence of long-term persistence of ATV in the environment outside of salamanders: the virus becomes quickly undetectable in pond water and dry mud, and no other syntopic hosts are known. ATV is usually lethal within 2-3 weeks of infection, although some salamanders lose overt symptoms of infection, including papules and lesions, and survive. In one laboratory experiment ATV was re-isolated from 40% of these survivors, which then transmitted the disease to uninfected salamanders. Chronic infections also occur in field populations and appear to be the means by which ATV persists between epidemics. The tiger salamander – ATV system offers us a model for studying the host-pathogen interactions thought to be threatening some amphibian populations with extinction.

Key words: amphibian declines, conservation, ATV, ranaviruses,

INTRODUCTION

The leading explanations for amphibian declines include land use change, exotic species, commercial exploitation, global change, toxins, and pathogens (Collins & Storfer, 2003). Ecologists and conservation biologists are only just beginning to appreciate the pervasive role that pathogens play in structuring wildlife populations and communities. But more to the point relative to amphibian declines, we are just beginning to understand the conditions under which infectious disease can lead to extinction, and there are only a handful of cases illustrating this mechanism. Disease may have caused the extinction of the Australian marsupial wolf early in the 20th century (McCallum & Dobson, 1995), and clearly caused the extinction of the snail *Partula turgida* in 1996, but the latter is a special case. The last individuals of *P. turgida* in the wild were brought into captivity to avoid extinction from predation by an introduced land snail species, but all died from infection by a lethal microsporidean parasite (Cunningham & Daszak, 1998). Hawaiian honeycreepers seem to offer a case of extinction facilitated by pathogens under field conditions. The avian malarial parasite was probably introduced intermittently into Hawaii for thousands of years, and avianpox virus, potentially fatal but slow acting, was introduced with colonists' poultry. Endemic bird populations were unaffected, however, since neither parasite had a vector in the archipelago. Everything changed early in the 19th century when the mosquito *Culex quinquefasciatus* was inadvertently introduced into Hawaii and began transmitting parasites to native birds. Avian malaria has diminished the sizes and ranges of many native honeycreepers, and is the suspected cause of extinction for several species (Warner, 1968; Benning *et al.*, 2002). The infectious disease chytridiomycosis is also thought to have played a key role in the extinctions of frog species in the Americas and Australia. If this proves to be the case, and the lethal amphibian pathogen continues spreading worldwide, amphibian species losses facilitated by disease will be our most compelling example to date of how a pathogen can act globally and rapidly to cause widespread extinctions.

A combination of forces can propel a parasite – the general epidemiological term for any infectious organism – from being apparently benign to becoming

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dangerous (Lafferty & Gerber, 2002). Exotic species may introduce novel parasites into susceptible populations, or a parasite may already be present in a population, but something in the environment (e.g. toxins, climate change, added predation) makes individual hosts more susceptible. Crowding hosts and high population densities also facilitate transmission of parasites. And of course parasites are not static, but evolve in response to their selective environment. Based on theory we would predict that when transmission rates are high more virulent parasites would be favoured (Ebert, 1999). Virulence traits can help the parasite defeat a host's immune system, improving the likelihood of within-host persistence and eventual transmission. In contrast, high virulence may reduce host activity and possibly also longevity, thereby reducing the period of infectiousness and decreasing the likelihood of transmission; highly virulent pathogens may also "burn out" their host populations and become locally extinct (Rand *et al.*, 1995).

What tips the balance from host-parasite (H-P) coexistence to host extinction? Amphibians and their pathogens offer ideal, if unfortunate, cases for studying these forces because study species span a continuum from H-P coexistence, to – we suspect – population declines and extinction. An interdisciplinary research program at the intersection of virulence of parasites in amphibian populations, susceptibility of hosts to infection, and population dynamics of host and pathogen is an effective way to study the forces balancing coexistence and extinction because individually and collectively these elements control H-P systems (Fig. 1; Collins *et al.*, 2003).

TIGER SALAMANDER – VIRUS SYSTEM

Model organisms have played a key role in advancing the life sciences (Kohler, 1994), from biomedicine (Koshland, 1988) to evolutionary biology (Kellogg & Shaffer, 1993). Progress on understanding the contribution of diseases to decline and extinction of amphibians will require model H-P systems with populations large enough to support robust experimental designs. For bioethical reasons we do not want to initiate experiments or observations that might threaten with extinction already imperiled amphibian hosts. Several features of its biology suggest that the tiger salamander (*Ambystoma tigrinum*) and one of its viral pathogens have the qualities of a comprehensive model system for studying how diseases, alone or interacting with other factors, affect amphibian population dynamics, perhaps threatening some populations with extinction.

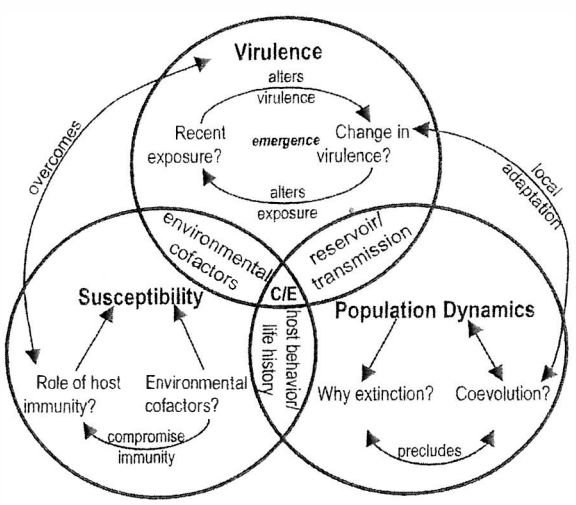

FIG. 1. Venn diagram outlining some of the interactions among virulence, susceptibility, and population dynamics. Ultimately, these three elements of host-pathogen systems determine where species fall on the coexistence-extinction (C/E; center) continuum.

GLOBAL AMPHIBIAN PATHOGENS

Ranaviruses are global amphibian pathogens (Daszak *et al.* 1999; Carey *et al.* 2003; Collins *et al.*, 2003; Daszak *et al.* 2003). Two ranavirus strains were isolated independently from tiger salamander epizootics in North America: *Ambystoma tigrinum* virus from Arizona, USA (Jancovich *et al.*, 1997), and Regina ranavirus from Saskatchewan, Canada (Bollinger *et al.*, 1999). The two research groups have collaborated in isolating and characterizing ranaviruses from tiger salamander epizootics in six states in the USA, and two Canadian provinces in western North America (Jancovich *et al.*, 2003). The isolates had similar genomes, and are now recognized as one widespread species, *Ambystoma tigrinum* virus (ATV) in the genus *Ranavirus* (family *Iridoviridae*).

ATV is a large, cytoplasmic double stranded DNA virus that causes systemic infections. The genome is completely sequenced, and is 106 332 base pairs with 96 putative open reading frames (ORFs) that sort into four functional classes: genes with homology to putative viral/cellular replicative proteins (n=24); genes possibly involved with immune modulation/pathogenesis (n=3); genes with homology to other iridovirus ORFs, but of unknown function (n=61); and genes of unknown function with no homology (n=8) (Jancovich *et al.*, 2003). Chromosome 7 of the human genome has nearly 158 million nucleotides of DNA, and some 1917 gene structures (known genes, novel genes, partial genes, predicted genes, putative and noncoding RNA genes) (Scherer, 2003). By comparison, ATV is a much more tractable genome for analysis via functional genomics.

The ATV sequence allows us to do several things. First, functional genomic techniques can yield important clues for fighting disease through an analysis of what all of the genes do. In the recent case of severe acute respiratory syndrome (SARS), the virus sequence allowed researchers to develop the probable structure for a key protein involved in replication, a step in developing a possible drug target (Vogel, 2003). Sequence data can hasten the search for a pathogen's weak spots; in the case of ATV, a starting point would be the three ORFs coding for immune modulation/pathogenesis. We can also use the variability among gene sequences, particularly those involved in pathogenesis, from various

geographic sites to test hypotheses regarding the spatial and temporal spread and emergence of disease; we are doing just that (Jancovich, *et al.* in prep.). Tiger salamanders have a wide geographic range and well-known systematic relationships, making these spatial and temporal analyses robust. Further, tiger salamanders are easily manipulated in the field and laboratory, and occur in large population sizes allowing experimental work on virulence, resistance, and susceptibility in this H-P system.

Having the gene sequence of the host as well as the pathogen would be especially valuable for analyzing interactions between the two species and their co-evolution. *Ambystoma tigrinum* offers the possibility for such a dual analysis of the functional genomics of host and pathogen because of its close relationship to the axolotl, *Ambystoma mexicanum* (Shaffer, 1993). The axolotl has been a leading model in developmental biology throughout much of the 20th century (Armstrong & Malacinski, 1989), making it the object of an extraordinary amount of research (Smith & Smith, 1971). Furthermore, we now know that axolotls carry lethal ranaviruses closely related to ATV (Davidson *et al.*, 2003). Voss *et al.* (2001) used genetic linkage analysis to identify chromosome segments homologous between ambystomatid salamanders and distantly related vertebrate models. Their study animals were the axolotl and *Ambystoma tigrinum tigrinum* because of their long history as research models. They concluded that, "comparative gene mapping appears to be an efficient strategy for identifying orthologous loci between ambystomatid salamanders and genomically well characterized vertebrate model organisms." There is a distinctive opportunity for studying the H-P biology of an infectious disease in amphibians by uniting the functional genomics of ATV with the bridge between *Ambystoma tigrinum* and what we know about the genetics and developmental biology of *Ambystoma mexicanum*. But even though host and virus genome sequences can yield insights into the intimate biology of ATV in tiger salamanders, a pathogen's effect on its host population is also a function of the environment, including host population dynamics.

Host-pathogen Population Dynamics

Not all infectious diseases are density-dependent, and not all amphibian H-P systems fit a susceptible-infectious-resistant (SIR) paradigm, but this basic theory affords a useful background for framing the problem. The extinctions of several amphibian species are thought to be a result of infectious disease, but "most simple epidemiological models indicate that there is a host-threshold density below which a pathogen cannot invade a host population, suggesting that rare or depleted populations should be less subject to invasion..." (Lafferty & Gerber, 2002). When coupled with the density-dependent nature of transmission, basic epidemiological theory suggests that pathogens are unlikely to cause extinction (Dobson & May, 1986). How can the particular details of host-pathogen systems explain this apparent contradiction? What conditions increase the risk of extinction from chytridiomycosis? Answering these questions will explain how pathogens could be responsible for the decline and extinction of some amphibian populations.

The first thing to note is that disease may not act alone in eliminating a species. Pathogens could reduce population density to levels where stochastic or deterministic factors cause extinction. Additionally, a host and pathogen are usually part of a community where one or more alternate hosts could act as reservoirs, relaxing the dependence of transmission rate on single-host density. Reservoirs are a powerful means by which one host species may suffer little disease while harboring a parasite that might drive an alternate host species to extinction (Woodroffe, 1999); conversely, multiple reservoirs could dilute a pathogen's effectiveness and reduce the chance of an epizootic (Schmidt & Ostfeld, 2001). These divergent outcomes depend on the number of subsequent infections produced by the first infected individual to appear in the population (Anderson & May, 1982). Under these circumstances we must know transmission rates within- and between-species (Dobson & Foufopoulos, 2001), making disease dynamics a property of the entire community. The complexities of understanding community disease dynamics, however, are staggering. A model system would ideally be simple enough to be tractable, yet incorporate the essential features of other disease systems. ATV in tiger salamanders appears to be just such a system.

ATV is transmitted between animals by direct contact or, at least in the laboratory, via water previously holding an infected animal; there are no vectors. Nor are there reservoir species, at least not within the regions of our studies. ATV infects other salamander species, but none of the several frog species we have experimentally challenged maintain virus replication (Jancovich *et al.*, 2001). Fish may permit replication (Schock, unpublished data), but fish and tiger salamanders do not coexist for long. There is no evidence of long-term persistence of ATV in the environment outside of salamanders: within weeks the virus becomes undetectable in pond water and dry mud. Thus, within our study areas we have a single host-parasite system.

Larval and metamorphosed animals infected with ATV usually die within weeks of infection (Jancovich *et al.*, 1997; 2001). ATV causes annual epidemics that can decimate a salamander population (Collins *et al.*, 1988). These epidemics are observed within the aquatic, primarily larval segment of the population. After the larvae metamorphose and disperse, host densities are quite low for many months. Thus we face, in a slightly different guise, the classic question of how a virulent disease that apparently drives its host population to very low densities is maintained. We suspected that in hosts with a complex life cycle, such as amphibians, one life history stage could be an intraspecific reservoir for another stage, which led to the hypothesis that relative to the

evolution of viral virulence larvae may be the "host" and the metamorphosed adults the "reservoir" (Brunner *et al.*, 2003). As far as the parasite is concerned, each amphibian life stage is effectively a different organism: larvae are abundant in dense populations that are short-lived and spatially restricted, while metamorphosed animals are much less dense and scattered in diffuse populations that are long-lived and vagile.

To study the salamander-virus interaction, we first used an infection experiment to determine the relative susceptibility of the two phenotypes (Brunner *et al.*, 2003). Sibling larvae and metamorphosed animals (to minimize genetic differences in susceptibility) were exposed to a viral dose sufficient to kill half of the sample. The results showed that metamorphosed animals, our putative "reservoir," were quite sensitive to the virus. More importantly, of those that survived the infection about 30% harboured chronic, sublethal infections for over five months. We then tested if these sublethal infections were the means of viral persistence. Surviving, sublethally infected animals were housed with naïve animals to determine if their infections were transmissible – they were in four of ten cases.

Salamander-to-salamander transmission was a means of viral persistence at least in the laboratory. In the field drift fences captured young-of-the-year as they dispersed from a study pond after an epidemic. Over a nine-day sampling period the prevalence of infection varied from 46-100% (n=77 animals); 25% displayed signs of infection and 78% tested positive for virus demonstrating that asymptomatic animals may still be infectious. As in the laboratory, metamorphosed animals were highly susceptible to ATV infection, but were metamorphosed salamanders returning to the pond sublethally infected? In spring 2002 the same study site was surrounded completely with a drift fence to capture breeding, metamorphosed animals before they entered the pond. Two of 30 animals collected were infected. Later in summer 2002 there was an epidemic presumably initiated by sublethally infected animals that returned to the pond.

Conditions for the virus appear to be good in summer, but bad in winter. Little transmission likely occurs in winter when ATV persists in chronic, but transmissible infections of metamorphosed adults and juveniles. This mode of persistence is different from the "classic" definition of a reservoir since both larval and metamorphosed life history stages are required for ATV's long term persistence: larvae amplify viral prevalence, and metamorphosed animals maintain the virus between epidemics. More generally, the result raises the possibility that intraspecific reservoirs may explain the persistence of parasites in, and the declines of small, isolated amphibian populations.

DISCUSSION

An amphibian iridovirus, *Ambystoma tigrinum* virus, is completely sequenced making possible a functional genomic analysis of pathogenesis in the tiger salamander – ATV system. Coupling the ATV analysis with the impressive (for wildlife) knowledge of genetics and development available for ambystomatid salamanders will lead to a much fuller understanding of the H-P interactions that lead to infection, virulence, and transmission. Since tiger salamanders are widespread, abundant, and easily maintained in the laboratory, experiments manipulating aspects of host and parasite are possible.

Studies of ATV in tiger salamanders in the wild have uncovered some novel insights into infectious disease in amphibian populations. In theory, virulent parasites need a reservoir host to persist in infected populations that are small, which raises the question: in hosts with complex life cycles like amphibians, can one life history stage be an intraspecific reservoir for another stage? Larval and adult life stages of amphibians can act as two different "organisms" when it comes to the evolution of a pathogen's virulence. High larval densities in ephemeral sites and low densities of metamorphosed adults and juveniles suggest virulent parasite persistence is unlikely in either stage alone, but transmission between stages could maintain virulent parasites in populations. We hypothesized that larval epidemics amplify virus prevalence and sublethally infected metamorphosed animals (re)introduce virus into uninfected larval populations. Our evidence supports this hypothesis.

Ongoing studies using the molecular tools available from a fully sequenced parasite and a host with a well-studied genome and phylogeny are beginning to tease apart the various hypotheses relating to the ecology and evolution of an infectious disease in amphibians. In the future we may be able to pinpoint the gene or genes that have allowed ATV to spread in tiger salamander populations, and not into populations of frogs and other vertebrates.

There is substantial overlap between the three factors that determine H-P dynamics (Fig. 1). Except for some examples involving human diseases (e.g. cholera, malaria) and some economically important agricultural models, few H-P interactions have been dissected from molecular biology to population dynamics, and even fewer in non-game wildlife. We propose that the tiger salamander – ATV system holds this potential and can be an important model for understanding how emerging infectious diseases contribute to amphibian declines, a key example of the general loss of biodiversity.

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EXPERIMENTAL EVIDENCE THAT THE BULLFROG (*RANA CATESBEIANA*) IS A POTENTIAL CARRIER OF CHYTRIDIOMYCOSIS, AN EMERGING FUNGAL DISEASE OF AMPHIBIANS

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To test the susceptibility of bullfrogs (*Rana catesbeiana*) to amphibian chytridiomycosis, groups of captive bred, recently metamorphosed bullfrogs were inoculated with zoospores of *Batrachochytrium dendrobatidis*, the causative agent of chytridiomycosis, and assayed for clinical and pathological signs of infection. A novel technique for counting *B. dendrobatidis* zoospore inocula is described. Inoculation regimes varied from single exposures of 1-10 million zoospores per animal to inocula of 10 million zoospores per animal per day for a 31 day period. Twenty-five to fifty percent of each inoculated cohort was histologically positive for *B. dendrobatidis* on necropsy. However, lesions were focal, small with relatively little thickening of the keratinized epidermis and no clinical signs of chytridiomycosis were observed. Only one animal died during the experiment, due to cardiac puncture procedure. A fungal isolate used in these experiments was inoculated onto four metamorphosed poison dart frogs (*Dendrobates tinctorius*) to test whether *B. dendrobatidis* had become attenuated following repeated passage in culture. All four animals died within 30 days with severe chytridiomycosis, whereas two uninfected controls survived, demonstrating that the fungus had not become attenuated. These results provide the first experimental evidence that bullfrogs can be infected by *B. dendrobatidis*, but are relatively resistant to the disease chytridiomycosis, which is lethal to many other amphibian species. By demonstrating that *R. catesbeiana* is likely to be an efficient carrier of this pathogen, our experimental data add weight to the hypothesis that this host species is important in the spread of chytridiomycosis, particularly by commercial activities.

Key words: amphibian decline, *Batrachochytrium dendrobatidis*, chytrid fungus, frog

INTRODUCTION

Chytridiomycosis is an emerging fungal disease of amphibians that was first reported causing mass mortality associated with population declines in Central America and Australia (Berger *et al.*, 1998). The causative agent, a non-hyphal zoosporic fungus, *Batrachochytrium dendrobatidis*, was first isolated from captive Central American frogs (Pessier *et al.*, 1999) and Koch's postulates have since been fulfilled (Longcore, Pessier & Nichols, 1999). Chytridiomycosis has since been reported as the cause of mass mortalities and population declines in North America (e.g. Muths *et al.*, 2003) and Europe (Bosch, Martinez-Solano & Garcia-Paris, 2001), and as the cause of at least one, and possibly several, species extinctions (Daszak *et al.*, 2003; Cunningham *et al.*, in press). It is emerging in south-western Australia (Alpin & Kilpatrick, 2000) and New Zealand (Waldman *et al.* 2001) and has recently been reported from wild and museum specimens of amphibians from Ecuador (Ron & Merino, 2000), Venezuela (Bonaccorso *et al.*, 2003) and Africa (Lane *et al.*, 2003). Outbreaks of chytridiomycosis are often characterized by simultaneous die-offs of multiple amphibian species at affected sites (Berger *et al.*, 1998). Experimental infections and field data demonstrate that a range of frogs, toads and salamanders are susceptible to chytridiomycosis (Berger *et al.*, 1998; Speare *et al.*, 2001). However, the pattern of amphibian declines in the tropics is characterized by the presence of sympatric declining and non-declining species (Williams & Hero, 1998). It is unknown which, if any, amphibian species are resistant to infection, or to the disease chytridiomycosis.

Most emerging diseases of wildlife are driven by anthropogenic environmental changes such as altered land use, introduction of alien species, deforestation and others (Daszak, Cunningham & Hyatt, 2000; Daszak, Cunningham & Hyatt, 2001; Cunningham *et al.*, in press). Climate change, anthropogenic introduction of

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Batrachochytridum dendrobatidis and other factors have been hypothesized as drivers of chytridiomycosis emergence (Daszak *et al.*, 1999; Daszak *et al.*, 2003). The pattern of amphibian declines (Daszak *et al.*, 1999) and DNA sequence phylogeny of *B. dendrobatidis* isolates (Morehouse *et al.* 2003; Daszak, Cunningham & Hyatt, 2003) support the hypothesis that chytridriomycosis has been recently introduced into naïve populations in some regions. The presence of *B. dendrobatidis* in the national and international trades of amphibians for pets (Mutschmann *et al.*, 2000), amphibians destined for outdoor pond stocking (Groff *et al.*, 1991), laboratory animals (Reed *et al.*, 2000), zoo animals (Pessier *et al.*, 1999) and food (Mazzoni *et al.*, 2003) provides potential mechanisms for the anthropogenic introduction of this pathogen.

In this paper, we report the results of a series of experimental infections of bullfrogs (*Rana catesbeiana*) with *B. dendrobatidis*. Our data suggest that *R. catesbeiana* is susceptible to infection by *B. dendrobatidis*, but resistant to the clinical effects of the disease chytridiomycosis, with no evidence of the severe lesions that are typical of chytridiomycosis, no evidence of behavioural changes associated with this disease and no mortality following infection. We discuss the results with reference to the ability of *R. catesbeiana* to act as a carrier of chytridiomycosis, and its potential involvement in the anthropogenic introduction of *B. dendrobatidis* to new regions.

MATERIALS AND METHODS

ANIMALS

Four experiments were conducted with recently metamorphosed, captive-bred bullfrogs (*Rana catesbeiana*), purchased from a commercial breeder (Rana Ranch Bullfrog Farm, Twin Falls, Idaho). All individuals were shipped less than three weeks after loss of final portions of tail bud. Frogs were purchased during July of 2000 (n=45), August of 2000 (n=20), November of 2000 (n=25) and March of 2001 (n=30). Because animals were captive-bred metamorphs, concerns about changes in immunological function at different seasons were considered irrelevant to the current study. To determine whether experimental groups were pathogen-free at the start of the experiment, five to ten frogs from each shipment were killed by soaking in a bath of 0.15% aqueous tricaine methane sulfonate (MS-222) buffered to pH 7.0 with sodium bicarbonate ($NaHCO_3$) for 10 minutes after cessation of respiratory activity, followed by pithing (Fowler, 1993). Three toes and webbing from each hind foot and approximately 3 cm^2 of skin from the ventral groin were fixed in neutral-buffered 10% formalin, embedded in paraffin, sectioned and stained with haemotoxylin and eosin and examined histologically for the presence of *Batrachochytrium dendrobatidis* zoosporangia (Pessier *et al.*, 1999). These representative samples from each experimental group were negative for *B. dendrobatidis*.

All experimental animals were captive bred and were acclimatized for one week prior to inoculation. Animals were individually housed in autoclaved plastic aquaria (29.8 cm length × 19.7 cm width × 20.3 cm depth) (Kritter-Keepers) within an environmental chamber with regulated thermal (20°C) and light conditions (fluorescent lighting for 14 hours per day) throughout the period of study. Deionized water (450 ml) was added to a bed of 200 ml of autoclaved gravel and a plastic shelter placed in the containers to provide a hiding place for the amphibians. Water was changed every three days. Frogs were fed commercially-bred crickets every two days. Tanks were covered with a mesh lids that prevented escape of crickets. All discarded fomites and water were sterilized with a 1% aqueous solution of sodium hypochlorite (diluted Chlorox) and containers were autoclaved prior to re-use. Used water was treated for 10 min with Chlorox to make a final 1% solution.

Six dendrobatid frogs of a species (*Dendrobates tinctorius*) known to be susceptible to chytridiomycosis (Nichols *et al.*, 2001) were purchased from a captive breeder. Four were inoculated with *B. dendrobatidis* to test if the pathogen had become attenuated by repeated passage in culture and two served as negative controls. The numbers used were small due to the difficulty in obtaining suitable animals, and for ethical (conservation) reasons.

PATHOGEN CULTURES AND ZOOSPORE COUNTS

Cultures of *B. dendrobatidis* were maintained on TGhL agar plates (Longcore *et al.*, 1999) at 17°C. Zoospores were harvested by flooding plates with a dilute salt (DS) solution used by mycologists to mimic pond water in pH and salt concentration (Fuller & Jaworski, 1987) and incubating at 15°C for 24 hrs. We determined zoospore concentration in the zoospore-rich supernatant as follows: First, we added 29 µl of the zoospore-rich supernatant to 1 µl of Lugols Iodine, which killed zoospores and stained them light brown. Using a Neueberg counting chamber and phase contrast microscopy, we counted flagellated zoospores and calculated the number of zoospores per ml of supernatant. Cells without flagella were not counted.

INOCULATIONS

To maintain uniformity, inocula were prepared by adding DS to provide the desired number of zoospores for each frog. We pipetted a 5 ml inoculum over each frog individually while it sat in a small, sterile container. The containers were shaped such that the only position the frogs could maintain was to sit with their groins, abdomens and hindlegs submerged in the inoculum. After an initial inoculation period, each frog was returned to its individual aquarium. The inoculation period was varied from 1 hr to overnight (Table 1), so as to provide a range of intensity of exposures. To maintain an environment that resembled as natural a situation as possible, i.e. where *Batrachochytrium dendrobatidis* may persist as a saprobe in the water and on the substrate (Daszak *et*

al., 1999; Johnson & Speare, 2003), the inoculum that had been pipetted over each frog was added to the respective aquarium and mixed with the water already present.

HISTOLOGICAL ANALYSIS

From each frog necropsied, three toes and webbing from each hind foot and approximately 3 cm^2 of skin from the ventral groin were sampled and fixed in neutral-buffered 10% formalin, embedded in paraffin, sectioned and stained with haemotoxylin and eosin and examined histologically for the presence of *Batrachochytrium dendrobatidis* zoosporangia (Pessier *et al.*, 1999). Individuals were considered uninfected if 25 fields of view at low power (25×) were examined without finding *B. dendrobatidis* developmental stages. All carcass remains were autoclaved and discarded. All post mortem equipment was autoclaved after use.

RATIONALE FOR EXPERIMENTS 1-4

The aim of these experiments was to test whether bullfrogs could be infected in the laboratory with *B. dendrobatidis* and whether these infections would produce clinical signs of the disease chytridiomycosis and death (Berger *et al.*, 1998; Pessier et al 1999; Daszak *et al.*, 1999).

Experiment 1. This involved an initial high inoculum (20 million zoospores for one hour), mimicking an individual moving into a body of water during an epizootic, where infected individuals are hypothesized to produce large numbers of zoospores. This was followed by a repeat exposure around three weeks later, to boost infection rates. The temperature selected (21°C) was within the optimal range for maximum growth of *B. dendrobatidis* in culture (Piotrowski *et al.*, 2004). The isolates used were from North American ranid frogs (no. 216, *Rana muscosa*, California; no. 228, *R. yavapaiensis*, Arizona). The 33-day period of the experiment corresponded to the three week time course of chytridiomycosis observed previously (Berger *et al.*, 1998). Within this and other experimental groups, control frogs were housed individually within tanks that were placed between rows of inoculated frogs.

Experiment 2. Following the failure to produce severe infections or clinical disease in experiment 1, infections were conducted that followed the protocols of Nichols *et al.* (2001) by adopting a lower temperature of 15°C, using an isolate known to be lethal to a range of frogs in captivity (no. 198, *Dendrobates auratus*, captive National Zoo) and increasing the number of inoculations (5 million for 2 hours on days 1, 3, 5, 10, 15 & 20). The experiment was terminated on day 28.

Experiment 3. Following the failure of the Nichols *et al.* (2001) protocol to produce severe infections or clinical disease in bullfrogs, the protocol used in experiment 1 was repeated, using North American ranid frog isolates (no. 217, *Rana muscosa*, California; no. 270, *R. catesbeiana*, California; no. 260, *R. catesbeiana*, Quebec) but with inoculations of 5 million zoospores for 2 hours daily for 28 days at a temperature of 21°C.

Experiment 4. This was set up to test if the *B. dendrobatidis* isolates we were using had become attenuated during repeated passage in culture, i.e. in the absence of the amphibian host, or keratin. The isolate used in experiment 4 (no. 197, *Dendrobates auratus*, captive National Zoo) was collected during an outbreak of chytridiomycosis in multiple species of captive frogs, and was known to be lethal to dendrobatids and other species (Longcore *et al.*, 1999; Pessier *et al.*, 1999). It was one of the first two isolates of *B. dendrobatidis* collected and had been passaged approximately every 10-14 days for over two years, therefore was most likely to have become attenuated. Dendrobatid frogs of a known susceptible species were inoculated with 10 million zoospores for 2 hours daily for 31 days. The temperature selected (21°C) was within the optimal range for growth of *B. dendrobatidis* in culture.

Experiment 5. Following the production of lethal infections in experiment 4, 1-10 million of the same isolate (no. 197, *Dendrobates auratus*, captive National Zoo) was inoculated for 2 hrs daily onto bullfrogs for 29 days at 21°C to cross check whether this proven virulent strain, known to cause death in multiple frog species, would cause clinical chytridiomycosis or mortality in *R. catesbeiana*.

RESULTS

EXPERIMENTAL INFECTION OF BULLFROGS

Histological evidence for the presence of *B. dendrobatidis* was found in 25-50% of the bullfrogs inoculated in experiments 2, 3 and 5. We saw no significant differences in the degree of infection or percentage infected between cohorts kept at 15°C and 21°C (χ^2=0.274, P>0.5). Despite the presence of *B. denrobatidis* (i.e. infection by this parasite), the lesions were not consistent with the disease chytridiomycosis, which is characterized by extensive thickening of the keratinized epidermal cell layer (hyperkeratosis and hyperplasia). Experimentally infected frogs positive for *B. dendrobatidis* in experiments 2, 3 and 5 showed only infrequent lesions, which were focal, small (approximately 100-300 μm surface diameter on histological section) and consisted of developing and mature zoosporangia within areas of minimal thickening of the keratinized layer of the epidermis (up to a thickness of 3 cells maximum). No clinical signs of chytridiomycosis (inappetance, behavioural abnormalities) were observed in the exposed bullfrogs. Bullfrogs from the inoculated cohorts fed at the same rate as those in controls and gained weight during the experimental periods (data not shown). Only one infected bullfrog (animal no. 6 in experiment 1, on day 25) died during the experiments, shortly after a cardiac puncture procedure conducted as part of another experiment. One control animal was found infected in experiment 3. This animal had a low-level of infection with *B. dendrobatidis*.

TABLE 1. Experimental infection of bullfrogs (*Rana catesbeiana*) and dendrobatid frogs (*Dendrobates tinctorius*) with *Batrachochytrium dendrobatidis*, the agent of amphibian chytridiomycosis. *Clinical signs of inappetance and extensive sloughing of skin were observed prior to death.

Experiment number	Frog identification number	Isolate used	Number positive for *B. dendrobatidis* on necropsy
Expt. 1 *Rana catesbeiana*	1-5, 21-25 (controls)	-	0/10
	6-20	216	4/15
	26-40	228	0/15
Expt. 2 *R. catesbeiana*	1-5 (controls)	-	0/5
	6-20	198	4/15
Expt. 3 *R. catesbeiana*	1-3 (controls)	-	0/3
	4-7	270	2/4
	8-11	260	1/4
Expt. 4 *Dendrobates tinctorius*	1,2 (controls)	-	0/2
	3-6	197	4/4*
Expt. 5 *R. catesbeiana*	2,8,13 (controls)	-	1/3
	3-7, 9-12,14-22	197	4/11

EXPERIMENTAL INFECTION OF DENDROBATID FROGS

The inoculated frogs showed clinical signs of chytridiomycosis (inappetance, increased sloughing of their skin, listlessness). Histological evidence of intense *B. dendrobatidis* infection was found in all four inoculated *Dendrobates tinctorius*. No evidence of infection was found in the two controls. Lesions were numerous, extensive (70-100% of skin surface on histological sections) and revealed sporangia within areas of marked thickening of the keratinaceous layer of the epidermis (up to 10 cells thick). Two of the inoculated animals died (days 25 and 29) before the end of the experiment.

DISCUSSION

The experimental data presented here show that bullfrogs (*Rana catesbeiana*) are able to be infected by *B. dendrobatidis*, without progression to clinical chytridiomycosis or death. The distinction between infection by *B. dendrobatidis* and presence of the disease chytridiomycosis is based on the presence or absence of clinical and pathological signs of chytridiomycosis (Berger *et al.*, 1998; Pessier *et al.*, 1999; Nichols *et al.*, 2001). These include (1) The presence of a substantial area of hyperplasia and hyperkeratosis (between 5 and 10 keratinaceous cell layers thick) of the ventral skin containing substantial numbers of *B. dendrobatidis* developmental stages; (2) clinical signs such as inappetance, loss of righting reflex and increased epidermal sloughing; and (3) rapid progression to death. The absence of any of these signs of chytridiomycosis in the bullfrogs inoculated with zoospores in our experiments (including those that became infected) suggests that they are resistant to the disease, even though they can be infected by *B. dendrobatidis*. Bullfrogs showed no signs of chytridiomycosis, remained healthy, and did not die, even when inoculated with up to 10 million zoospores daily for over four weeks. This contrasts with experimental infections of highly susceptible species such as the Australian frog *Myxophyes fasciolatus*, individuals of which died 35 days after a single inoculation of 100 zoospores (Berger, pers. comm.). Our findings suggest that bullfrogs are relatively resistant to clinical chytridiomycosis, even though they are susceptible to *B. dendrobatidis* infection.

Alternative explanations of the data can be ruled out. Firstly, repeated passage in culture of a range of pathogens (viruses, bacteria and others) often leads to attenuation, i.e. loss of virulence when inoculated onto susceptible hosts (Ford *et al.*, 2002). Our finding (in experiment 4) that an isolate of *B. dendrobatidis* produced clinical and pathological signs of severe chytridiomycosis and death in dendrobatid frogs, but only very mild, focal lesions, no clinical signs of chytridiomycosis and no mortality in bullfrogs, demonstrates that our cultures of *B. dendrobatidis* had not become attenuated despite repeated passage outside the host. It is important to note that this isolate originated from dendrobatid frogs during an outbreak that killed multiple frog species, therefore is proven to be lethal to a range of amphibians. Secondly, some pathogens exist as a series of strains that are species-specific, or specific to a geographical region. It is conceivable that the isolates of *B. dendrobatidis* are lethal only in amphibians from which they are isolated, therefore non-bullfrog isolates would not kill or cause disease in bullfrogs. However, in the current experiments, we used fungal isolates that are known to be lethal to a range of frogs (e.g. isolate nos. 197 and 198) and others isolated spe-

cifically from North American ranid frogs, including bullfrogs. None of these caused chytridiomycosis or death of bullfrogs. Furthermore, molecular data suggest that isolates of *B. dendrobatidis* collected from a range of sites and hosts (including some of those used in this study) are a single clonal strain (Morehouse *et al.*, 2003) and there are no significant morphological or physiological differences between isolates in culture (Longcore *et al.*, 1999; Piotrowski *et al.*, 2004). Finally, experimental infection of Australian frogs using isolates collected from other species and geographical sites produced lethal infections (Berger *et al.*, 1998; Berger pers. comm.).

The finding of an infected control frog in experiment 4, and other infected animals from the same supplier, suggests that it is possible frogs within the supply facility may have been contaminated with *B. dendrobatidis*. It could be hypothesized that the animals we inoculated with zoospores may have been partially immune to the pathogen, explaining the lack of disease or mortality. We doubt that this is a valid hypothesis, because the animals used in our studies were purchased less than three weeks (i.e. less than the clinical course of chytridiomycosis) after resorption of the tail bud. Only post-metamorphic amphibians have cutaneous chytridiomycosis, therefore if animals were infected at the beginning of the post-metamorphic period at the breeders, they should have been at a late stage of progression to chytridiomycosis by the time of arrival, if the species were susceptible. Instead, in each experiment, the animals we examined prior to the experiment were negative for *B. dendrobatidis* and no signs of chytridiomycosis were found in those used for experiments. The infection of this control animal may, therefore more likely be due to experimental error or inadvertent transmission of *B. dendrobatidis* between enclosures. It is unknown whether tadpoles infected at the suppliers would develop immunity to chytridiomycosis, or be able to carry immunity through metamorphosis.

In the current paper, we describe for the first time a simple, reliable protocol for counting *B. dendrobatidis* zoospores in inocula. Because chytrid pathogens are normally transmitted to new hosts via flagellated zoospores, this technique provides an accurate way to assess the number of zoospores (and hence the degree of challenge) in experimental inocula, while excluding non-motile (non-infectious) zoospores. We also describe a series of detailed techniques for inoculating, handling and housing experimental animals for future laboratory studies of chytridiomycosis.

The findings reported here support a simple population model of chytridiomyocsis in which individual hosts, host populations and species vary in susceptibility to *B. dendrobatidis* (Daszak *et al.*, 1999; Daszak *et al.*, 2003). In this model, only a small proportion of biologically and ecologically predisposed populations undergo local extinctions and declines due to chytridiomycosis, whereas the majority of other species and populations remain relatively unaffected. Our data suggest that *B. dendrobatidis* would cause little, if any, mortality in populations of bullfrogs, and that the majority of individuals would be able to grow and breed normally. Our data are supported by reports of similar low intensity infections in farmed (Mazzoni *et al.*, 2003), feral (Hanselmann *et al.*, 2004) and wild-caught (Daszak *et al.*, 2003) bullfrogs. No evidence in any of these reports indicated that infection by *B. dendrobatidis* in bullfrogs caused the abnormal behavioural syndromes associated with chytridiomycosis or skin lesions consistent with those found in frogs that had died of chytridiomycosis (Berger *et al.*, 1998).

Our data have conservation significance for two reasons. Firstly, they suggest bullfrogs may be an efficient reservoir or alternative host of this emerging pathogen because they are able to harbour the fungus, but do not suffer clinical signs of infection. Although it is so far unknown whether the light *B. dendrobatidis* infections in bullfrogs are sufficient for transmission of the agent from one individual to another, the presence of fully formed sporangia in many of the positive animals suggests this is likely. Other authors have recently highlighted the role of such reservoir or alternative hosts in "apparent" or "parasite-mediated" competition (Holt and Lawton, 1994; McCallum & Dobson, 1995; Hudson & Greenman, 1998). Parasite-mediated competition occurs when two sympatric host species share a common parasite and the least susceptible host uses the differential impact of the parasite as a competitive edge. In some cases, parasite-mediated competition allows the less susceptible host to drive the more susceptible host to extinction. In areas where susceptible endemic amphibians occur sympatrically with introduced, *Batrachochytrium*-positive *R. catesbeiana*, apparent competition mediated by chytridiomycosis may be an outcome.

Secondly, the ability of *R. catesbeiana* to become infected by *B. dendrobatidis* – but not to suffer clinical signs of infection – suggests that introduced individuals could act as efficient carriers of this pathogen. *Batrachochytrium dendrobatidis* has been reported from bullfrogs that are part of an increasingly centralized and expanding trade in live animals for food within South American countries and between South America and the USA. (Mazzoni *et al.*, 2003). In Venezuela, bullfrogs introduced as a farmed food source have escaped, expanded their population locally, and support a high prevalence of *B. dendrobatidis* infection (Hanselmann *et al.*, 2004). Our findings of infected, but otherwise healthy, *R. catesbeiana* suggest that the trade in ranid frogs for laboratory or educational uses is yet another source for the introduction of chytridiomycosis. Bullfrogs have been introduced into Europe, Asia and the western USA. (Kupferberg, 1997). The western USA populations are descended from individuals introduced as a food source in the late 19th and early 20th centuries and have successfully colonized large areas, competing with endemic species and perturbing community structure (Kupferberg, 1997; Kiesecker &

Blaustein, 1997). The potential of *R. catesbeiana* to act as an efficient carrier of chytridiomycosis adds to its capacity as an invasive species threat to amphibian populations (Kats & Ferrer, 2003).

Previous authors have commented on the likely role of introduced species in the spread or emergence of chytridiomycosis (Berger *et al.*, 1998; Daszak *et al.*, 1999; Cunningham *et al.*, 2003). Molecular evidence also suggests anthropogenic introduction is involved in the spread or emergence of chytridiomycosis (Morehouse *et al.*, 2003; Daszak *et al.*, 2003). Amphibian chytridiomycosis is a key example of an emerging infectious disease of wildlife which, as a group, is responsible for a series of significant population declines and extinctions of wildlife species (Daszak *et al.*, 2000, 2001; Dobson & Foufopoulos, 2001). The most commonly cited driver of these diseases is the anthropogenic introduction of pathogens or hosts to new regions, or 'pathogen pollution' (Cunningham *et al.*, 2003). Our current paper provides evidence that *R. catesbeiana* is a potentially efficient carrier of chytridiomycosis and adds weight to its hypothesized role in pathogen pollution-mediated spread of chytridiomycosis. We urge that strong measures be taken to combat this threat to amphibian biodiversity. These should include quarantine regulations (Cunningham *et al.*, 2001; Johnson & Speare, 2003) and efforts to curtail the legal and illegal trade in amphibian species.

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RESEARCH AS A TOOL TO INFORM AMPHIBIAN CONSERVATION POLICY IN THE UK

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In the UK, research has improved our understanding of amphibian populations, their habitats, threats and the effectiveness of conservation measures. The greatest research effort has been directed to the protected and declining species, notably *Triturus cristatus*, *Bufo calamita* and *Rana lessonae*. However, several challenges arise when attempting to employ research findings as a tool to shape policy. Wild populations and threats to them are not often simple systems that invite straightforward investigation. Extrapolating from small studies to more comprehensive application can also generate problems, especially with widespread species. The standards of confidence commonly used in science may not be directly transferable to conservation policy, as in conservation it is often desirable to apply the precautionary principle. When constructing policies, it is important to be realistic about the constraints that may be imposed due to factors beyond the control of conservation agencies and researchers, notably those of a legislative or socioeconomic nature. There is a need for conservation practitioners to engage more closely with scientists, with a view to identifying the current knowledge gaps that hinder the achievement of conservation gains. The increasing success of *B. calamita* reintroductions provides an excellent illustration of such an application of scientific knowledge.

Key words: crested newt, natterjack toad, population ecology, research

INTRODUCTION

It is widely recognised that activities and policies aimed at conserving biodiversity should be based on sound ecological information (Semlitsch, 2002; IUCN, 1998). Whilst it is self-evident that conservation will not be achieved without an understanding of the species in question, the paradigm that links research with conservation policy may take various shapes according to the prevailing conservation status, mechanisms for achieving conservation and the state of scientific knowledge. This paper examines the relationship between ecological research and conservation policy in the UK, and makes recommendations aimed at maximizing biodiversity gain.

The United Kingdom has a depauperate amphibian fauna, with only three urodeles (*Triturus vulgaris*, *T. helveticus* and *T. cristatus*) and three anurans (*Rana temporaria*, *Bufo bufo*, and *B. calamita*) widely recognised as native species. Recent evidence strongly suggests that some populations of a further species, *R. lessonae* – previously assumed to be introduced – were also native prior to extinction in the 1990s (Beebee & Griffiths, 2000). Though small, the UK amphibian fauna is an instructive group with which to examine the relationship between research and conservation given that (1) the species exhibit a range in conservation status from widespread and locally abundant to extremely localized and rare (Beebee & Griffiths, 2000); (2) the ecology, distribution and conservation status of all species are well understood in comparison to the situation pertaining in most countries (Arnold, 1996; Swan & Oldham, 1993; Beebee & Buckley, 2001); (3) the species historically have been influenced by – and are currently vulnerable to – a wide range of threats; and (4) there is a comparatively well-documented history of amphibian conservation in the UK involving a range of government and non-government sectors (e.g. Hilton-Brown & Oldham, 1991; Banks *et al.*, 1994).

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RESEARCH ON UK AMPHIBIANS

Prior to the middle of the 20th century, published studies concentrated on species descriptions, basic natural history, taxonomy and anatomy (e.g. Bell, 1869). Although rarely referred to now because of their lack of detail in ecological terms, these early studies may be valuable from a conservation perspective as they contain qualitative descriptions of species and habitats prior to major human interference. Later studies progressed to descriptive ecological work, such as the Savage's (1961) monograph on *R. temporaria*. Such studies provided the baseline information for further more detailed investigation, including demography and migration (e.g. Gittins *et al.*, 1980), behaviour (Halliday, 1974), and habitat preferences (Beebee, 1979). Experimental studies (i.e. those in which manipulation occurred) were initiated mainly from the 1980s and provided further useful insights into ecological processes, for instance work on competition (Griffiths *et al.*, 1991). Although some amphibian studies at this stage touched on conservation issues, most were primarily aimed at enhancing

ecological knowledge. The last decade however has witnessed a major increase in the publication of studies directed towards, or highly relevant to, key conservation issues (e.g. Cooke, 1997; Baker & Halliday, 1999; Hitchings & Beebee, 1997; for a general account see Lambert, 1997). This proliferation has been stimulated by growing concern about amphibian conservation status, and has been assisted by the development and increased accessibility of various techniques, notably genetic analyses. This later work has focused on the protected and more threatened species, *Triturus cristatus*, *Bufo calamita* and *Rana lessonae*. All of the UK amphibians also occur in mainland Europe, and a considerable amount of scientific study undertaken there has complemented domestic studies (e.g. Arntzen & Teunis, 1993; Miaud *et al.*, 1993).

RESEARCH AS A CONSERVATION TOOL

Research can perform the following functions in helping to inform conservation policy:

(1) Providing baseline ecological, taxonomic, distributional and status information. UK amphibian conservation is well served in this field, which covers a wide range of studies providing fundamental information required for species conservation. The areas covered include (i) ecology and behaviour, required for planning monitoring programmes and habitat management (e.g. studies of *B. bufo* breeding migrations, Slater *et al.*, 1985); (ii) genetic studies to clarify status and to inform reintroductions (e.g. relationships between pool frog populations, Zeisset & Beebee, 2001; genetic variation across UK *B. calamita* populations, Rowe *et al.*, 1999; consequences of isolation, Rowe & Beebee, 2003); (iii) collated and interpreted site records, which allow an assessment of conservation status and the setting of recovery targets (e.g. amphibian inventories, Swan & Oldham, 1993; Beebee & Buckley, 2001).

(2) Providing information on threats, and the responses of populations and habitats to them. Fewer studies have been published in this area, but some progress has been made in recent years. For instance, mapping work has provided an insight into the magnitude and timing of loss of breeding ponds (Boothby, 1997), and long-term monitoring has revealed the extent of development impacts (Cooke, 1997).

(3) Providing information on conservation measures, and the responses of populations and habitats to them. There remains much scope for published research in this area, but again there has been a recent trend for targeted investigations, such as the effects of pond management regimes on breeding success in *B. calamita* (Phillips *et al.*, 2002).

(4) Providing information on techniques critical to conservation. A key example is the comparative study of monitoring methods (e.g. Cooke, 1995; Griffiths *et al.*, 1996), where investigations of effectiveness are essential to inform surveillance programmes.

(5) Investigating findings from anecdotal reports, to assess whether there are real effects. The UK is fortunate in having a large complement of volunteers with an interest in amphibians, and suggestions of potential conservation issues from this group are worthy of consideration (for instance the reports of decline among lowland *B. bufo* populations investigated by Carrier & Beebee, 2003).

THE NATTERJACK TOAD: A CASE STUDY

An examination of the success of reintroductions of *B. calamita* serves as a composite example of the above functions, and demonstrates that research is often critical to achieving a real conservation benefit. *B. calamita* is restricted to three main habitat types in the UK (lowland heathland, sand dunes and upper saltmarsh) and has suffered considerable decline in the 20th century, largely through habitat destruction and successional changes. Approximately 50 populations currently remain, representing a significant loss in terms of the number of occupied sites and the geographic range (Banks *et al.* 1994). One strategy to address this decline has been to reintroduce *B. calamita* to suitably prepared sites, either from captive bred stock or by translocation from wild populations. Reintroductions were started in the late 1960s, and still form an important part of the national conservation strategy for this species (The UK Biodiversity Steering Group, 1995). The success of the reintroductions, as determined by the establishment of a breeding population, has improved considerably over this period (Fig. 1). Ecological research along the lines outlined in (1)-(5) above has played a key role in ensuring more favourable outcomes from reintroduction attempts. In particular there is now a better understanding of the preferred pond characteristics, the importance of terrestrial habitat structure, the importance of predators and competitors, and the significance of different founder stock characteristics (Beebee, 1983; Banks *et al.* 1994). This knowledge has informed improvements to conservation practice, such as the design of reintroduction ponds, selection of appropriate reintroduction sites, defining sympathetic habitat management regimes, and refining procedures for releases. Indeed, before research about the profile of preferred breeding ponds was initiated, the species actually suffered due to competitors invading inappropriately created or managed ponds. The failure of some recent reintroductions still gives cause for concern, but these seem to be restricted to one particular habitat type, indicating that further targeted research on this subject is desirable.

CHALLENGES IN TRANSLATING RESEARCH INTO POLICY

Conservation policy can operate at various levels, from prescribing the nature of land use at a broad scale, to recommending specific strategies or methods for habitat management and reintroductions. Perhaps the biggest challenge when using research to inform policy is the fact that natural ecosystems are so complex. This means that investigating conservation problems in truly natural situations can be fraught with difficulties. The

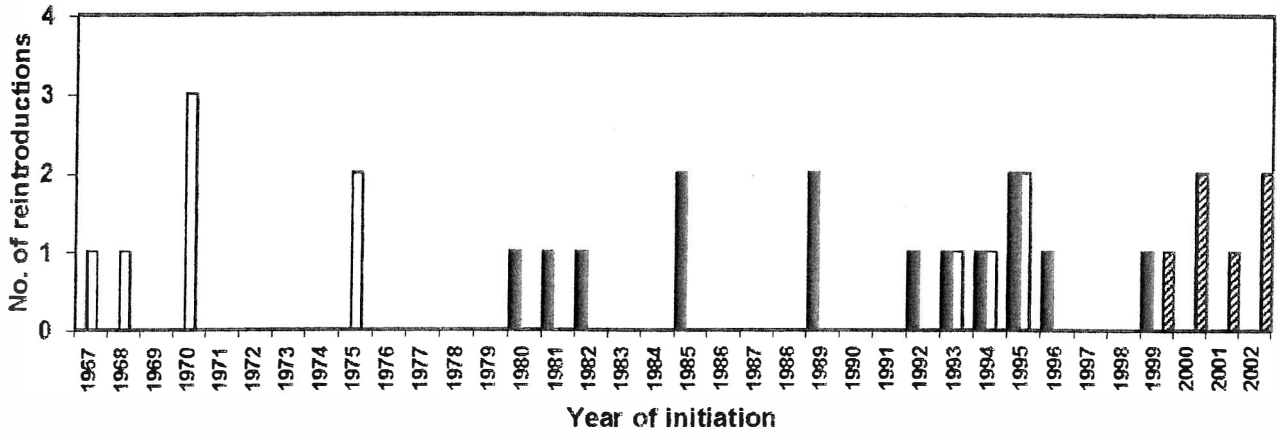

FIG. 1. The outcome of *B. calamita* reintroduction attempts in the UK, 1967-2002. Key to bars: open, failure; solid, success; hatched, as yet unproven.

problems arise because populations vary across many dimensions (e.g. habitats present, population size and demography, links to other populations, presence of competitors, climate, positive and negative management factors applying). Sample sizes required for sound hypothesis testing are often very large. Hence, establishing clear-cut relationships is problematic if not impossible. An example is a recent study seeking to recommend optimal fish control methods in amphibian ponds (Watson, 2002). The study concluded that selecting the appropriate technique must be based on an examination of a wide range of interacting site characteristics, such as substrate type, pond size, weather conditions, vegetation type and fish species. Undertaking observations in captive or semi-natural conditions can help to reduce variability, but at the same time often lessens the applicability of the results.

Linked to this point is the risk inherent in generalizing or extrapolating from single studies to more comprehensive application. Due to the variation across sites and the factors affecting them, the findings from a given detailed study (or small numbers of unrepresentative studies) may be misleading if applied across a wider range of situations. The study of amphibian dispersal provides a good example of this situation. Dispersal distances are important to inform decisions on, for example, the definition of protected area boundaries and the assessment of impacts by damaging activities. Field studies on the UK's most protected amphibian, *T. cristatus*, have revealed a range of observed maximum dispersal distances, from 95 m (Jehle, 2000) to 1290 m (Kupfer, 1998). Both studies are excellent investigations of newt ecology, but the results may be misinterpreted, and arguably misused, by policy makers and conservation workers if not set in context. The former study may be cited to contend that *T. cristatus* disperses over short distances and therefore more distant landuse changes will not affect populations. The latter study may be invoked to contradict this. In fact, such debates (which have occurred amongst those involved in site safeguard for this species in the UK) demonstrate the difficulty in using results from a given investigation to apply to other situations. Examination of the aims and methods of these studies clearly demonstrates that a simplistic inference of their maximum dispersal estimates to other populations is questionable; for instance Jehle (2000) specifically investigated post-breeding emigration of a sample of adults, thus other dispersal (for instance autumnal juvenile migration) was not examined. It should be stressed that these comments are in no sense a criticism of the individual published results; on the contrary they are designed to highlight the potential for misuse of the data by others. An overview of such studies, bringing together the common points, indicating the pitfalls of wider interpretation and placing them in a conservation context, may sometimes be required for them to be translated soundly into practical guidance (e.g. English Nature, 2001). It is suggested that the ease with which research can be used to inform policy varies by species, with for instance habitat specialists lending themselves more readily to direct application of research results (Fig. 2). This is not to suggest that where research reveals a complex situation it will be less likely to inform conservation policy, rather that those formulating the resulting policy will need to be especially careful to avoid pitfalls of over-simplification.

Traditional statistical standards of confidence may not always be appropriate when shaping conservation policy. This is because the standard hypothesis testing model may not be applicable and it is often difficult to establish a high degree of confidence in results of field studies, given the complexity of the systems being examined. Partially for such reasons, the application of the precautionary principle is often advised in conservation practice (United Nations, 1992; Department of Environment, 1994), and Bayesian statistical inference is better able to handle the associated statistical tests. Bayesian methods can incorporate inherent uncertainty and a consideration of conservation context, and permit the simultaneous assessment of several different hypotheses, giving relative probabilities for each (Wade, 2000). An example of Bayesian methods in amphibian conservation research is the investigation of the biogeographic range of *R. lessonae*, in which multiple

Ease of translating research into policy	Habitat specificity	Vagility	Species distribution	Threats to conservation status (number/ complexity)
Complex	Generalist	High	Widespread	Many/ complex
↓	↓	↓	↓	↓
Simple	Specialist	Low	Localised	Few/ specific

FIG. 2. Factors influencing the prospects for research on amphibian ecology to be translated into conservation policy.

populations were simultaneously assessed to give the most probable closest relatives of the native UK population (Zeisset & Beebee, 2001).

When dealing with potential threats, it is often simply not possible to empirically test the likely impacts, and so a balanced view based on the (often limited) evidence is required. The task for policy makers is most difficult when dealing with new situations that may have potentially devastating impacts, and which may demand drastic intervention, as with the discovery of novel pathogens and invasive species (Daszak *et al.*, 1999; Mazzoni *et al.*, 2003). When a non-native species is introduced, there needs to be a rapid judgment as to its likely impact on native ecosystems. Conducting a study of the actual impacts as the species establishes would provide interesting scientific evidence to support a view on the best course of action, but it would often be unwise as the conservation losses accruing during the study could be considerable. Where significant impacts are predicted, preventative action should be initiated and this in itself can result in useful data. Such a situation has occurred recently in the UK with the introduced North American bullfrog *Rana catesbeiana*, where useful information on the efficiency of control methods have been collected, though the outcome of these measures will not be known for several years (Banks *et al.*, 2000).

Conservation practitioners are often presented with anecdotal or conflicting evidence, and whilst the precautionary principle may assist in dealing with such situations it does create problems of its own. If a particular threat to conservation status is implicated in one study but not supported by others, it often requires a meticulous examination of the circumstances combined with a degree of professional judgment in order to clarify whether a real issue has been revealed and if so, what relevance it has to wider conservation.

When assessing the implications of ecological research for policy, it is important to consider the context in which the resulting guidance will be employed. Research findings are (quite rightly) often presented without reference to the pragmatic constraints that accompany practical application. Foremost among such considerations are resource and financial limits on implementation, and the prevailing socioeconomic situation in which the policy will be employed. The former constraint is, for instance, a significant determinant of the design of amphibian monitoring programmes. Whilst sophisticated survey techniques as recommended by research findings are desirable in terms of data quality and resolution (see Schmidt, this volume), in practice there are limitations on the human resource for undertaking surveys (meaning that overall effort is constrained); the funding available will also place controls on implementation. In these circumstances, there is a need for a consideration of the trade-off between the power of the monitoring programme to detect conservation-relevant trends, and the resources required for proper implementation. This assessment is greatly aided where there are sound data on the performance of the available monitoring techniques, as is now the case for *B. calamita* in the UK (Beebee & Buckley, 2001; Herpetological Conservation Trust, 2002).

SUGGESTIONS FOR ENHANCING THE CONSERVATION VALUE OF FUTURE RESEARCH

Conservation practitioners are advised to engage more closely with researchers, and vice versa, so that mutually beneficial investigations can be undertaken. Priorities for pure research do not always coincide with conservation priorities, and given the complexity of natural systems there may be problems with designing programmes to deliver the answers to the precise questions required by conservation workers. This situation arises partly because university research priorities are led, to a large extent, by funding opportunities for international or over-arching studies. Thus, studies of local or even national conservation issues may not receive as much attention. Resulting high-level studies are obviously important but may not produce information that is useful at a local scale. However, there remain some interesting areas for conservation research in the UK that would also produce scientifically valuable results. A key first step, already progressed to some extent in the UK, is for those involved in designing and implementing policy to identify the main gaps in knowledge that frustrate progress towards conservation gain. Presently, we suggest, these areas include: the relationship between survey count data and actual population size; definitions of population status; viability and genetic impoverishment of small or isolated populations; impacts of extensive habitat management regimes used in the agricultural landscape; impacts of development and value of mitigation measures; factors affecting reintroduction outcome.

In order to advance towards this goal, the authorities that direct and fund ecological research need to foster, and more readily accept, proposals based on conservation priorities. Indeed, the discipline of conservation biology urgently requires wider recognition and its own financial support system if it is to provide an adequate science base. The recent initiation of the Biodiversity Research Working Group in the UK should help to address these issues.

Taking conservation-relevant research to its "end user" (land managers, surveyors, the public, etc.) will normally involve conversion into a different format from the original research publication. This often takes the form of published guidance, e.g. landowner-oriented habitat management advice based on published research (Langton *et al.*, 2001), but other channels include training courses, news releases for the media, or web-based advice. The co-operation of researchers and conservation practitioners at this stage will ensure that the accuracy and precision of the advice is maintained. Joint meetings, such as the symposium on *T. cristatus* hosted by the British Herpetological Society in 1998 (Cummins & Griffiths, 2000), can be beneficial in bringing together otherwise separate groups.

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Global Amphibian Declines: is Current Research Meeting Conservation Needs?

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CONTENTS

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