Conservation genetics of an island toad: *Bufo bufo* in Jersey

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On Jersey (British Channel Islands), common toads often reproduce in small, urban ponds. This atypical breeding strategy has implications for their persistence and they have declined on the island in recent times. We used polymorphic microsatellite markers to compare genetic diversity in *Bufo bufo* from five different ponds in Jersey with two populations from north-west France. Genetic diversity of Jersey toads was comparable with that of populations elsewhere in Europe. Numbers of breeding female toads in Jersey were correlated with pond area but estimators of genetic diversity were unrelated to pond area or female numbers. F_{st} estimates and isolation by distance tests indicated that there is little gene flow between breeding sites on the island. Jersey populations last shared a common ancestor with those of north-west France long before the island's physical separation about 6000 years ago. Toads have a long history in Jersey and were once probably very numerous there. The average effective historical population size of Jersey toads is estimated to be 15,000–16,000. Although genetic diversity of Jersey *B. bufo* is currently quite high, recent developments on the island may threaten this situation in the near future.

Key words: common toad, conservation, genetic diversity, small populations

INTRODUCTION

At least one-third of the world's amphibian species are now regarded as "Threatened" under IUCN criteria (Stuart et al., 2004) and amphibian declines are recognized as a global phenomenon (e.g. Alford & Richards, 1999). Many causes have been proposed for such declines but it is apparent that, whatever the reasons, some declines have been occurring for several decades or more (Houlahan et al., 2000). Declines of the European common toad, *Bufo bufo*, have been noted for at least 40 years on the British Channel Island of Jersey (Le Sueur, 1968, 1976; Buley, 1995; Beebee & Griffiths, 2000), where it was once reportedly very common.

Jersey (Fig. 1) is approximately 50°N, 2°W, some 22 km west of Normandy, France and 160 km south of Great Britain. Toads have a cultural significance for Jersey islanders who sometimes refer to themselves as *crapauds* (toads) and to the toads as "Jerseymen" (Le Sueur, 1968) – a consequence of the species' former abundance on the island. *B. bufo* remains widespread in Europe as a whole (Gasc, 1997) but declines in the species have also been documented in Norway (Semb-Johansson, 1992), the Iberian peninsula (Lizana & Pedraza, 1998) and lowland England (Carrier & Beebee, 2003).

Identification of toad breeding sites in Jersey for a wider study into the ecology of the species there revealed that most of the (<200) extant sites were restricted to the periphery of the island (JWW, unpublished data), and that many of these were small ornamental ponds in private gardens. This is in contrast to the "classical" toad breeding pond, typical of mainland Britain, the average size of which is reported to be about 1000 m^2 (Beebee & Griffiths, 2000). Hitchings & Beebee (1998) found that measures of genetic diversity were significantly lower in such small, urban populations of *B. bufo* than in larger, rural ones in Britain and that this correlated with reduced fitness of larvae. Small populations like these are also susceptible to genetic depletion through drift and inbreeding with potentially adverse consequences for viability (Beebee, 2005).

At many Jersey garden sites, only a few (<10) female toads reproduce each year (JWW, unpublished data). The population persistence model generated by Halley et al. (1996) predicts that *B. bufo* ponds supporting less than 30 breeding females per year have a more than 50% extinction probability within 20 generations, and that extinction probability of populations in ponds with less than three

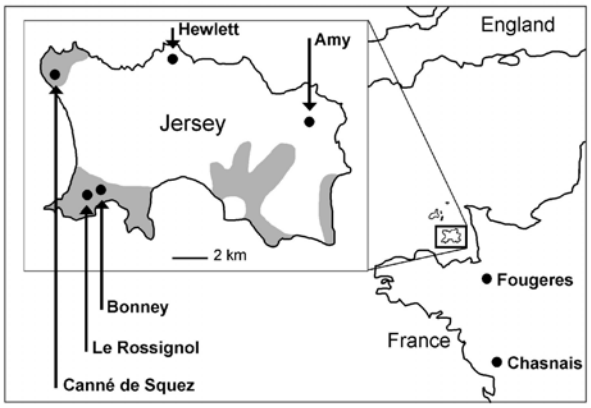

Fig. 1. The position of Jersey and locations of the sample sites. The shading shows areas of the island where most toad ponds are found.

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Table 1. Characteristics of four Jersey toad breeding sites.

Pond	Type	Total surface area (m^2)	Number of female toads 2006
Canné de Squez	"Wild" maritime heath site in protected area (2 adjacent ponds)	51*	80
Le Rossignol	Garden fishpond in rich urban pond cluster	28	22
Amy	Isolated garden fishponds (2 small adjacent ponds)	13	4
Hewlett	Isolated garden fishpond	5	2

*During toad breeding: pond dries up in summer.

Table 2. Mean number of alleles per locus, mean expected (H_e) and mean observed (H_o) heterozygosities in seven toad populations, with overall values for Jersey and NW France.

Population	Mean no. alleles per locus	Heterozygosity	
		Mean H_e	Mean H_o
Canné de Squez	3.50	0.556	0.556
Le Rossignol	4.00	0.457	0.535
Amy	3.75	0.609	0.666
Hewlett	2.25	0.490	0.669
Bonney	3.25	0.565	0.457
Fougères	5.25	0.516	0.451
Chasnais	4.75	0.605	0.420
Jersey	4.75	0.536	0.577
NW France	6.00	0.560	0.435

breeding females per year is greater than 95%. These findings have implications for the long-term persistence of toad populations in small, urban ponds in Jersey that are becoming increasingly isolated by continuing development, and which constitute the majority of available breeding habitat.

The use of DNA-based markers such as microsatellites is an appropriate method for investigating the conservation genetics of temperate anurans such as *B. bufo* (Beebee, 2005) and offers several advantages over alternative techniques (e.g. allozyme methods – for a review see Jehle & Arntzen, 2002). This paper describes a comparison of the genetics of *B. bufo*, using neutral microsatellite markers, from a range of Jersey breeding sites with those of two populations from north-west France, the most appropriate for a direct comparison because of their geographical proximity. Toads are not found on any of the other Channel Islands. We consider these findings with respect to the species' long-term conservation in Jersey.

MATERIALS AND METHODS

Sampling and surveys

Genetic samples of *B. bufo* were obtained from a total of seven breeding sites during 2005 and 2006 (Fig. 1): five from widespread locations in Jersey and two from north-west France (Fougères, Ille-et-Vilaine and Chasnais, Vendée). Tissue was obtained from tadpoles at approximately stage 30 (Gosner, 1960) that had either been reared from small samples of spawn from several strings or had attained that stage in nature. Spawn samples were collected from up to 10 widely-spaced strings per site (as available) and tadpoles were randomly netted to reduce the probability of relatedness of the individuals sampled. Small (2–3 mm) sections of tail tip were removed from 40 tadpoles from each site and stored in 90% ethanol as a source of DNA for analysis. The tadpoles were later released.

Estimates of the number of breeding female toads were made in 2006 at four of the Jersey sites (Canné de Squez, Le Rossignol, Amy and Hewlett) by conducting cumulative counts of fresh spawn strings on regular visits. This is made possible in Jersey by the toads' utilization of small, shallow breeding sites where it is usually possible to locate all spawn strings. Estimates of pond surface area were also made at this time. Breeding female numbers were not estimated at the Bonney site as hatching had occurred before sampling was possible.

Genetic techniques and microsatellite analyses

DNA was extracted from tadpole tail tips using the Chelex 100 protocol (Walsh et al., 1991). Microsatellite loci were amplified by PCR in the presence of locus-specific primers for *B. bufo* (Brede et al., 2001) and [α^{33} P]-dATP. PCR products were electrophoresed on standard 6% w/v polyacrylamide sequencing gels with an M13 marker and the alleles scored after visualization by autoradiography. Main analyses ultimately employed four microsatellite loci (*Bbuf*μ24, 39, 47 and 54) out of a total of nine for which amplification was attempted (one, *Bbuf*μ63, amplified but was excluded and the remainder did not amplify successfully; see Results and Discussion, respectively, below).

Possible genotypic disequilibrium across loci (unbiased Markov chain estimation method) was assessed with GENEPOP 3.1 (Raymond & Rousset, 1995). Tests for Hardy-Weinberg equilibrium and measures of genetic diversity (locus polymorphism, mean numbers of alleles, observed and expected heterozygosities) were performed with BIOSYS-1 (Swofford & Selander, 1981). Mean number of alleles rather than allelic richness was appropriate because sample sizes were identical for all populations. Pairwise values of F_{st} (index of fixation among populations; Wright, 1965) and their significance levels were estimated using GENEPOP and FSTAT 1.2 (Goudet, 1995). Isolation by distance (historical gene

Table 3. Pairwise F_{st} values among all seven toad populations.

Population	Canné de Squez	Le Rossignol	Amy	Hewlett	Bonney	Fougères
Le Rossignol	0.0725					
Amy	0.0346	0.0745				
Hewlett	0.0534	0.1325	0.0258			
Bonney	0.0539	0.1055	0.0519	0.104		
Fougères	0.1047	0.1505	0.1309	0.1911	0.1007	
Chasnais	0.0504	0.1154	0.0559	0.104	0.0793	0.0663

flow) for Jersey sites (using $F_{st}/(1- F_{st})$ and ln distance) was also investigated using the GENEPOP subprogram ISOLDE. Geographical distances (in metres) between Jersey sample sites were measured using Cadcorp SIS software (Cadcorp Ltd, Stevenage, UK). Group level mixture analysis (Bayesian inference of population genetic structure) was performed with BAPS 3 (Corander & Marttinen, 2004). This program treats allele frequencies and the number of genetically diverged groups as random variables and infers group genetic structure based on user-denoted theoretical divisions. In this case, these were the seven sample sites in this study, which were assigned as putative population origins for BAPS analysis.

Phylogenetic relationships between the Jersey and NW France sample sites were inferred using PHYLIP 3.5 (Felsenstein, 1993) to generate unrooted consensus neighbour-joining (NJ) and maximum likelihood (ML) trees based on Cavalli-Sforza chord distances (Cavalli-Sforza & Edwards, 1967). Historical divergence times between Jersey and French populations were estimated using IM (Hey & Nielsen, 2004). This method incorporates a maximum likelihood approach to estimate the divergence times, interpopulation migration rates and effective sizes of population pairs. We used the stepwise mutation model for the four microsatellite loci in linkage equilibrium (see Results) and two randomly selected sets of 40 individuals, one from among the two French populations and one from among the five Jersey populations. We ran the analysis in duplicate, each with a burn-in of 10^5 steps followed by 10^7 iterations. There were five Markov chains and ten chain swaps for each run. Values of scalars for θ_1 (= $4N_e\mu$ of population 1) maximum and maximum times of population splitting were determined empirically by a short preliminary run, as suggested by the program authors. We set the inter-site migration rates in both directions to zero because population divergence was generated by sea level rise. *B. bufo* shows poor salt-tolerance (e.g. Banks & Beebee, 1987) so sea level rises normally preclude subsequent gene flow in this species.

Additional statistical tests were performed using SPSS (SPSS Inc., Chicago, USA).

RESULTS

Jersey toad ponds

The pond surface area and number of breeding female toads in 2006 at four Jersey sites are presented in Table 1. The number of breeding females was very low at Amy and Hewlett. *Ad hoc* observations in 2005 and data from other reliable observers (Jersey Environment Division staff, pond owners) confirmed that these female numbers were not unusual at any of the four sites. Amy and Hewlett seem typical of other garden breeding sites in Jersey, the relatively high number at Le Rossignol being the exception. The number of breeding female toads at the sites was directly correlated with pond surface area (Pearson correlation, r=0.97, df=2, P=0.016).

Genetic diversity

Tests for genotypic disequilibrium on the five successfully-amplified loci revealed that *Bbuf*μ47 and *Bbuf*μ63 were in linkage disequilibrium (i.e. not independent) in all pairwise comparisons. Of these two loci, *Bbuf*μ63 had been most difficult to score accurately due to the presence of other PCR products within its allele size-range (in all populations), so this locus was excluded from other analyses. The remaining four loci were polymorphic in all populations and had 2–18 alleles (mean 7) overall. Loci were considered to be out of Hardy-Weinberg equilibrium (HWE) in a population when P<0.0018 (adjusted nominal 5% level for multiple tests; chi-square goodness of fit test) for both pooled and unpooled allele frequencies. The "pooling" of all but the commonest alleles removes the potentially confounding effect of the presence of few, rare ones (the software treats each locus as biallelic). Only *Bbuf*μ24 from Canné de Squez, *Bbuf*μ24 and 39 from Hewlett and *Bbuf*μ54 from Chasnais deviated significantly from HWE.

The mean number of alleles per locus, mean expected (H_e) and mean observed (H_o) heterozygosities are shown for each population in Table 2. Values for Jersey and NW France overall are also given. Mean number of alleles per locus was lowest in the Jersey population with fewest breeding females (Hewlett) and highest in the two French populations. The overall mean number of alleles per locus in Jersey was lower, but not significantly so, than that of NW France (Fisher's Exact Test, P=0.93). Heterozygosity differences between populations were not significant for either H_e or H_o (ANOVAs on arcsin-transformed data; H_e: $F_{6,21}$=0.322, P=0.918; H_o: $F_{6,21}$=0.749, P=0.617). There was no correlation between the mean number of alleles per locus and heterozygosity (H_e: Spearman rank correlation, r_s=0.071, n=7, P=0.879), possibly as a result of very small effective population sizes. There were no correlations between any measure of genetic diversity in Jersey populations and either pond area or number of female toads in 2006 (from Table 1; Spearman rank correlation, values of r_s = –0.8 to 0.50, n=4 in each case, all values of P>0.10).

Table 4. Maximum likelihood estimates (MLE) of demographic parameters.

	θ (Jersey)	N_e (Jersey)	*t*	Divergence time (years BP)
IM run 1				
MLE	0.6339	15,848	0.0433	12,990
95% CI L	0.1831	4,578	0.0133	3,990
95% CI U	1.2645	31,613	0.1378	41,340
IM run 2				
MLE	0.6551	16,378	0.0457	13,710
95% CI L	0.1937	4,845	0.0118	3,540
95% CI U	1.2632	31,580	0.1487	44,610

CI L and CI U are 95% lower and upper confidence limits of the MLE for each run. BP = before present.

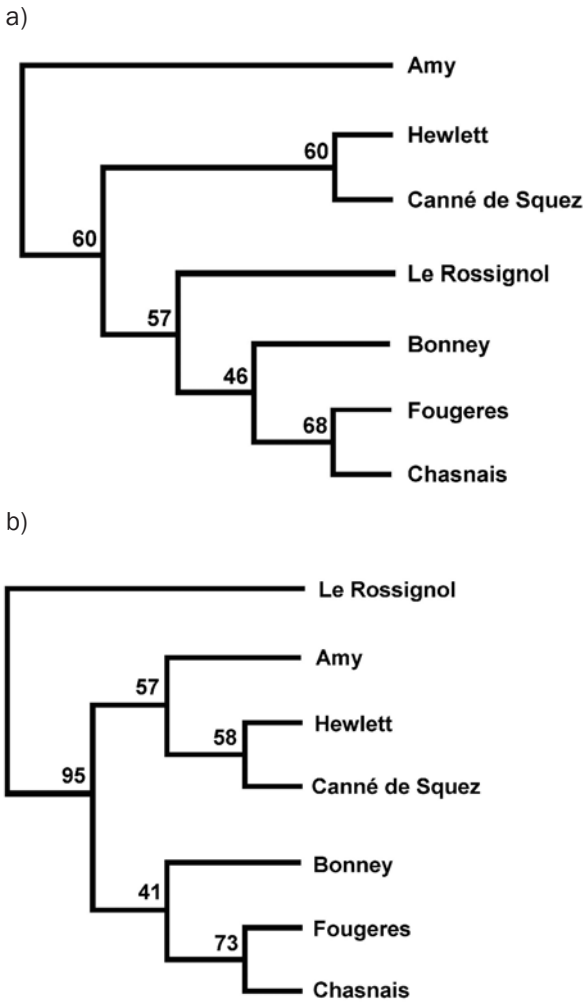

Fig. 2. Unrooted phylogenetic trees of relationships between *B. bufo* populations from Jersey and NW France based on Cavalli-Sforza chord distances. a) Neighbour-Joining tree. b) Maximum Likelihood tree. Bootstrap values (%) over 1000 pseudoreplicates are shown.

Population structure

Pairwise F_{st} estimates among the populations are given in Table 3. All values were significantly different from zero (P<0.0024, adjusted nominal 5% level for multiple comparisons). The averaged F_{st} estimates for within-Jersey comparisons and for the comparisons between Jersey and French populations were 0.0752 and 0.1083 respectively, compatible with more recent gene flow between the Jersey populations than between Jersey and French populations. No significant correlation of genetic and geographic distance was found for toad populations within Jersey (Spearman rank correlation; 10,000 permutations, r_s=–0.024, n=10, P=0.776), i.e. populations in ponds that were close to each other were not necessarily genetically similar. BAPS analysis indicated the presence in our dataset of five "real" population entities in Jersey, and two from NW France, corresponding exactly to the seven sampled ponds.

In phylogenetic analyses, both NJ and ML consensus trees generated similar results (Fig. 2). Two sites from the north of Jersey (Hewlett and Canné de Squez; see Fig. 1) clustered together in both trees. The French sites also clustered together and with one of the southern Jersey sites (Bonney, though with lower bootstrap values) in both trees. The relationship of the other Jersey sites (Amy and Le Rossignol) was ambiguous.

The results of IM analyses, with ML estimates of population parameters for the toad populations from NW France and Jersey, are shown in Table 4. From the *t* estimates we can obtain estimates of divergence time (T), in generations, from the relationship $t = T\mu$, where μ is the average mutation rate per generation of the four loci. Similarly, mean effective population size (N_e) over the intervening time period since divergence can be estimated from $N_e = \theta/4\mu$. The generation time of *B. bufo* has been estimated as around three years (Halley et al., 1996). The average mutation rate of *B. calamita* microsatellites was previously estimated at around 10^{-5} per generation (Rowe et al., 2006), and here we assume a similar rate for *B. bufo*. The results indicate an average historical effective population size of Jersey common toads between 15,000 and 16,000, and a last shared common ancestor with French populations around 13,000 years ago.

DISCUSSION

Microsatellite loci

The findings of this study are partly limited by the number of microsatellite loci successfully amplified in the PCR. For example, it was not possible to confidently assess past bottlenecking events in our study populations as use of a greater number of loci is recommended (Cornuet & Luikart, 1996). Some of the loci we tested either amplified very poorly (*Bbuf*μ14, *Bbuf*μ15) or not at all (*Bbuf*μ46, *Bbuf*μ62) in the Jersey samples, despite repeated attempts with new reagents on different gels, so it was not possible to utilize these four loci in comparative analyses. This lack of successful amplification probably indicates the presence of null or low amplification alleles

resulting from point mutations in the microsatellite flanking sequences in Jersey populations, leading to poor primer annealing (Dakin & Avise, 2004), rather than failure of the technique. Genetic diversity in an anuran using just four microsatellite loci has, however, previously been assessed in Burns et al.'s (2004) study of *Litoria aurea* in Australia. Our results seem credible in the context of the wider ecological information about Jersey's mostly small and isolated toad breeding assemblies.

Jersey toad genetics

Heterozygosity values from all seven sites in this study were comparable to those found by Scribner et al. (1994) and Brede & Beebee (2006a) in English toad populations, and to those from rural toad populations in Hitchings and Beebee's (1998) (minisatellite-based) study. H_e values for urban populations in the latter study were somewhat lower, a pattern not yet apparent in Jersey's garden breeding sites: all measures of genetic diversity in Jersey toads were unrelated to either pond area or number of breeding females in 2006. The fact that samples at one of our sites (Hewlett) originated from only two spawn strings makes this all the more remarkable. Our measures of H_e in Jersey and NW France also fell within the range of values given by Brede & Beebee (2006b) (0.411–0.748) for 14 *B. bufo* populations from across the species' European range.

The lack of isolation by distance effects between populations in Jersey is similar to the observations of Brede & Beebee (2004), who found high differentiation between *B. bufo* populations over comparable distances in Sussex, England. Pairwise F_{st} estimates in that study were, however, much higher, possibly indicating that the Jersey populations have become separated more recently. The Brighton area of Sussex shares similarities with Jersey in that toads are known to breed routinely in urban ponds, possibly (in both cases) as a result of geological histories that have not favoured the persistence of large water bodies. The lack of substantive gene flow within Jersey is also reflected in the relatively high F_{st} estimate (>0.1) for the pairwise interaction between Le Rossignol and Bonney, two sites separated by less than 1 km and in an area rich with garden toad ponds in Jersey's second largest conurbation (Red Houses). Remarkably, F_{st} estimates an order of magnitude lower were found for *B. bufo* in Seppä & Laurila's (1999) allozyme marker study of small island populations in Finland. The Baltic Sea in this region has very low salinity and evidently this water was a less effective barrier to toad movements than the terrestrial landscapes of Jersey and Sussex. Evidence for substantial isolation of Jersey's toad populations is further supported by the population structuring revealed by BAPS, and by our phylogenetic analyses.

Jersey has been separated from France by the English Channel for around 6000 years (Johnston, 1981), a period of time equivalent to roughly 2000 generations of toads. Results of the IM analyses strongly suggest that Jersey toads last shared a common ancestor with French populations long before that time, probably just before the Younger Dryas cold period of around 11,000 years BP. Although the 95% confidence intervals on the ML estimates were quite broad, the two independent estimates were strikingly similar and certainly confirm that *B. bufo* has a long history on Jersey.

Considering that the ratio of census:effective population size in this species is probably greater than 20 (Brede & Beebee, 2006a), the ML estimates of N_e infer an average historical census size of some 300,000 toads in Jersey or around 26 per hectare. This density is similar to that given for *B. bufo* by Beebee (1996) (>20 per hectare), yet lower than in some studies on other temperate anurans (e.g. >40 per hectare for *Bufo calamita*, Beebee et al., 1996; 100 per hectare for *Rana pretiosa*, Cuellar, 1994). Such an estimate is therefore realistic and supports historical accounts from Jersey of "scarce credible" numbers of toads "walking over the rocks like an army" (citations in Le Sueur, 1968) and the origins of the *crapaud* nickname of islanders. The original toad colonizers of Jersey apparently adapted well enough to differences in local conditions to become abundant and persist over a long period, but today toads exist at a much lower density over most of the island.

This study does not suggest that Jersey's extant *B. bufo* populations are genetically any less diverse than might be expected following their separation on an island for a period of several thousand years. This differs from the results of Rowe et al.'s (1999) work on mainland Britain's *Bufo calamita*. British *B. calamita* populations are significantly less diverse than those in mainland Europe (Rowe et al., 2006), probably because in Britain this toad is confined to relatively few, small and isolated localities. It is much more widespread on the European mainland. The situation of *B. bufo* in Jersey is, however, now similar to that of *B. calamita* in mainland Britain – both breed in specialized (or unusual), largely peripheral habitats isolated by unsuitable habitat. In the case of *B. bufo* in Jersey, this appears to be the predominantly agricultural land in the centre of the island and, increasingly, new housing and other developments that will further reduce connectivity between breeding populations. Our results show that Jersey toad populations are already highly differentiated. It is probably because this situation is relatively new for Jersey toads that there is still a high level of genetic diversity on the island. The remaining toad populations there appear to represent the remnants of a single and island-wide, diverse, but now collapsed, metapopulation.

Perspectives for conservation strategies

Residential areas in Jersey favour toad breeding because of the coincident presence of apparently suitable (if atypical) ponds (i.e. small ornamental ponds), but degrees of isolation are inherent in these habitats because of the presence of roads and vehicle traffic that cause mortality and increase fragmentation (Hels & Buchwald, 2001; Cooke & Sparks, 2004). Such mortality can be particularly damaging to Jersey populations in which breeding female numbers are typically very low (less than 10, often less than 5) and where it is conceivable that all breeding females could be killed in any given year. This study has shown that such low female numbers are correlated with the size (small surface area) of breeding ponds.

The high site philopatry and relatively low vagility of *B. bufo* (e.g. Scribner et al., 2001) make it unlikely that individual adult toads in Jersey will use several adjacent breeding sites, especially when these are separated by roads. Migration is probably conducted largely by dispersal of juveniles post-metamorphosis, and in urban areas dispersing juveniles are liable to the same mortality risks as adults. There is no incentive, moreover, to undertake long-distance movements to and from suitable hibernacula as extended periods of frost occur in Jersey only during very severe winters (Le Sueur, 1976). Detrimental genetic impoverishment, therefore, is increasingly likely to occur on the island as toad breeding populations are further fragmented by ongoing developments.

All Jersey's amphibians are now legally protected by the Conservation of Wildlife (Jersey) Law 2000 (as amended), but the halting of toad declines there may depend upon increasing the connectivity (geographic and genetic) both between garden populations and between these and the few remaining semi-natural breeding ponds. Though they are small in area, at least two of the latter (including Canné de Squez) support relatively large numbers of breeding female toads (approximately 80 at both sites) per year. The protection of these semi-natural sites is, of course, critical. The most important source populations in urban pond clusters should also be identified and conserved and there may be a need for legal or voluntary pond protection structures in order to achieve this. The location of toad breeding ponds should also be considered with regard to planning applications and such consideration incorporated into the standard Jersey planning process.

Though genetic diversity in Jersey toad populations does not yet appear to have reached critically low levels, the nature of the species' reproductive ecology on the island, its differentiated population structure and increasing development pressures suggest the immediate need for measures to prevent further population declines. The crapaud's long history on Jersey, and its historical association with Jersey's islanders, surely make its persistence and recovery a laudable and attainable goal.

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