A genetic assessment of the two remnant populations of the natterjack toad (*Bufo calamita*) in Luxembourg

A.C. Frantz[1], R. Proess[2], T. Burke[1] & L. Schley[3]

[1]*Department of Animal and Plant Sciences, University of Sheffield, UK*
[2]*ECOTOP – Bureau d'Études Écologiques et Environnementales, Luxembourg*
[3]*Service de la Conservation de la Nature, Direction des Eaux et Forêts, Luxembourg*

The natterjack toad (*Bufo calamita*) has experienced a dramatic decline in Luxembourg over the last 100 years. Today, only two remnant populations are known. Here, we examine their genetic constitution at 10 microsatellite loci in order to assess the genetic risk from isolation and inbreeding to the species' long-term survival in the country. Genetic diversity in both populations was relatively high, and we did not find evidence for inbreeding. However, the natterjack toads have experienced a recent reduction in their effective population sizes, and there was no evidence of recent gene flow between the two localities. The main short-term objective of conservation measures should be to increase population sizes by continuous safeguarding and management of the two sites.

Key words: bottleneck, conservation genetics, inbreeding, microsatellites

INTRODUCTION

The natterjack toad (*Bufo calamita*) has experienced a dramatic decline in Luxembourg over the past 100 years. At the end of the 19th century and during the first quarter of the 20th, the species was generally distributed throughout the country, and even locally abundant (Junck et al., 2003). Currently, only two remnant populations are known. The population near Steinfort, located in the west of Luxembourg in a disused sandstone quarry, has been known since the mid 1980s. The second population in the centre of the country, near Ernzen, was discovered about ten years later (Fig. 1; Junck et al., 2003). The Ernzen population is also located in a (still exploited) sandstone quarry, and both remnant populations are thus confined to secondary habitat. Irrespective of the intrinsic value of protecting the species, Luxembourg has a legal obligation to do so, as the European Commission included natterjack toads in Appendix IV of the EU Habitats Directive 92/43/CEE as a species of community interest requiring strict protection (Conseil des Communautés Européennes, 1992).

Individuals in small and isolated populations can suffer from genetic impoverishment and inbreeding depression, issues that cause concern among conservation biologists in relation to a wide range of endangered species (Lynch et al., 1995a,b; Bataillon & Kirkpatrick, 2000), including the natterjack toad (Rowe & Beebee, 2003, 2005). Given the demography of the species, Luxembourg natterjack toads might suffer low genetic variability and reduced fitness, potentially negating mitigating conservation measures and threatening the long-term survival of the population in Luxembourg. Here, therefore, we aimed to assess the remaining extent of the genetic variability of natterjack toad populations in Luxembourg and the evidence for inbreeding and recent genetic bottlenecks. Finally, we also investigated whether there has been recent gene flow between the two populations.

MATERIALS AND METHODS

The two Luxembourg populations are separated by a straight-line distance of about 25 km. The nearest foreign population is separated from Steinfort not only by a distance of about 10 km, but also by a number of busy roads (Fig. 1). Given what is known about dispersal distances of amphibians in general, and considering that the furthest dispersal distance recorded for natterjack toads was 5 km (Jehle & Sinsch, 2007), it appears likely that the two Luxembourg populations are isolated. In addition, surveys of the Steinfort population between 1997 and 2002 showed the population to be in decline (Junck et al., 2003). Natterjack spawn was regularly observed to become desiccated as it was deposited in small ephemeral pools, perhaps in response to the presence of frogs and newts (*Rana* spp., *Triturus* spp.) in the main body of water on the site (Banks & Beebee, 1987a,b). In 2002, eight small, shallow artificial ponds were created, that have since allowed the toad population to resume successful reproduction (Junck et al., 2003).

Both populations were visited and sampled on separate occasions between 10 May and 3 August 2007 (Table 1). Since female natterjacks generally lay one single undivided clutch each year (Denton & Beebee, 1993), and since one clutch is generally fertilized by a single male (Banks et al., 1994), we attempted to minimize the probability of collecting full siblings by sampling at different time intervals and/or in separate ponds. Larvae of different size classes were collected when distinctive ages coexisted within one pond. However, given the relatively large number of samples collected from some individual ponds, we cannot confidently exclude the possibility that we did include a small number of full siblings (Table 1). We also

Correspondence: Alain Frantz, Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK.
E-mail: alainfrantz@yahoo.co.uk

counted spawn strings and used the accumulated total as a minimum estimate of the number of adult females.

Laboratory work

DNA was extracted using an ammonium acetate precipitation method (Richardson et al., 2001) and re-suspended in 30 μl low TE (10 mM Tris-HCl, 0.1 mM EDTA). Genotyping was performed using 10 microsatellite loci: *Bcalμ1*, *Bcalμ3*, *Bcalμ4*, *Bcalμ5*, *Bcalμ6*, *Bcalμ8* (Rowe et al., 1997), *Bcalμ9*, *Bcalμ11* (Rowe et al., 2000), *Buca2*, *Buca6* (Rogell et al., 2005). No linkage disequilibrium was detected among loci *Bcalμ1* to *Bcalμ8* in an earlier study (Rowe et al. 1999). We chose these specific loci because, due to non-overlapping allele size ranges, nine loci could be included in a single multiplex PCR (see below). In order to avoid noise from variable non-templated adenylation during the polymerase chain reaction (PCR), the "pigtail" sequence GTTTCTT was added to the 5'-end of each reverse primer (Brownstein et al., 1996). While locus *Bcalμ8* was amplified individually, the remaining nine loci were amplified in a multiplex PCR. Loci *Bcalμ1*, *Bcalμ4*, *Bcalμ8*, *Bcalμ9* and *Buca6* were labelled with HEX at the 5'-end of the forward primer, while the 6-FAM label was used for the remaining loci. Every locus was initially amplified individually in twelve samples to ensure that the various size ranges did not overlap. The QIAGEN Multiplex PCR Kit was used for both reactions, which contained 1 x QIAGEN Multiplex Master Mix and 0.2 μM of each primer.

After drying 1 μl of each DNA extract (non-quantified) for 15 min at 55 °C in a 384-well plate, multiplex reactions were performed in a total volume of 2 μl (Kenta et al., 2008). Reaction times were as described in the manual, using an annealing temperature of 62 °C in both cases. Reactions were performed using a DNA Engine Tetrad thermocycler (MJ Research). Fragments were separated using an ABI 3730 automated DNA sequencer (Applied Biosystems) and the data were analysed using GENEMAPPER v.3.5. (Applied Biosystems). In order to assess genotyping errors (e.g. Bonin et al., 2004), 10 samples (out of a total of 100) were chosen randomly from the database, re-extracted and re-genotyped. Allelic mismatches were identified by comparing these 10 duplicate genotypes to the corresponding initial ones.

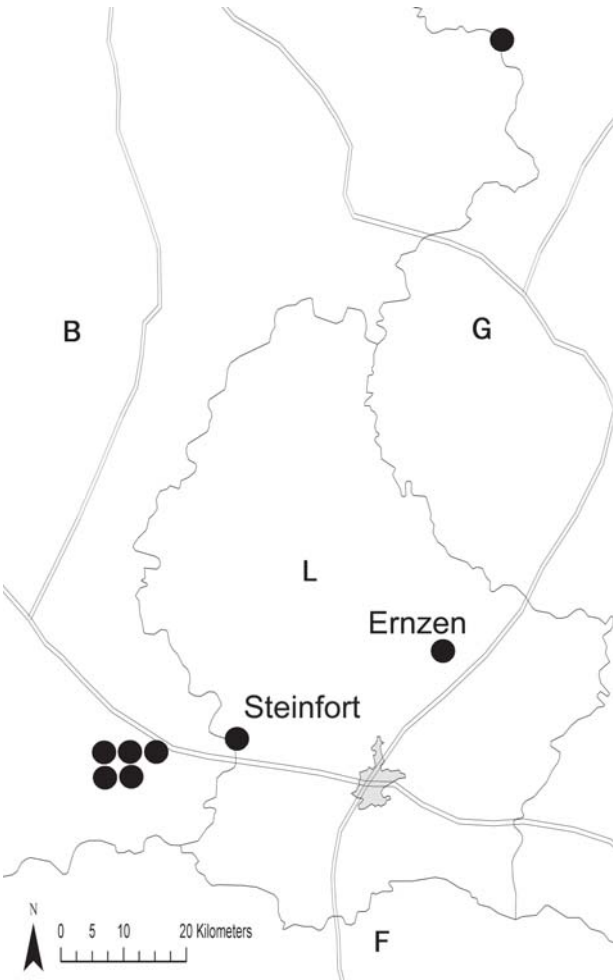

Fig. 1. Locations of remnant populations of natterjack toads (*B. calamita)* in Luxembourg (at Steinford and Ernzen) and neighbouring regions in Belgium. B=Belgium, F=France, L=Luxembourg, D=Germany. Data taken from Junck et al. (2003) and Jacob et al. (2007).

Genetic diversity

Genetic diversity was measured as the mean number of alleles per locus (*A*), observed (H_o), and expected (H_e) heterozygosities (Nei, 1978), using GENETIX 4.05.2 (Belkhir, 2004). We tested for the significance of heterozygote deficiency or excess with the Markov-chain method in GENEPOP 3.4 (Raymond & Rousset, 1995), using the default values suggested by the program. Populations were tested for linkage disequilibria among loci using an exact test based on a Markov-chain method as implemented in GENEPOP 3.4. The sequential Bonferroni technique was used to eliminate false assignment of significance by chance (Rice, 1989). For each population and locus, F_{IS}-values (Weir & Cockerham, 1984) were calculated using SPAGEDI 1.2 (Hardy & Vekemans, 2002) and significance was tested with 10,000 permutations of individual genotypes within populations.

Bottleneck

To test for evidence of recent bottlenecks, allele frequency data were tested for evidence of "heterozygosity excess" (*HE*) using the program BOTTLENECK (Cornuet & Luikart, 1996). This methodology correctly identified genetic bottlenecks in natterjack populations known to have undergone recent demographic declines (Beebee & Rowe, 2001). We computed deviations from expected heterozygosity for every polymorphic locus in both populations using the two-phased model of mutation (TPM), as this model is most appropriate for microsatellite loci (Di Rienzo et al., 1994). We used a variance of 30 for the TPM and assumed 30% of changes to be multi-step. Three statistical tests (sign test, standardized differences test and Wilcoxon signed-ranks tests) were conducted to test for significant *HE*, which may indicate that a recent bottleneck has occurred. We performed the tests both in-

Table 1. Information on sample collection.

Population	Collection date	Location	No. of larvae collected	Spawn strings counted in previous visits
Steinfort	03/06/2007	Schwaarzenhaff Pond 1	5	10–15
Steinfort	03/06/2007	Schwaarzenhaff Pond 2	5	6
Steinfort	03/06/2007	Schwaarzenhaff Pond 4	5	9
Steinfort	03/06/2007	Schwaarzenhaff Pond 5	5	4
Steinfort	03/06/2007	Jongebësch	5	No previous visit
Steinfort	19/07/2007	Jongebësch	15	12
Steinfort	03/08/2007	Jongebësch	10	
Ernzen	04/06/2007	Puddle in Area D	5	No spawn strings observed on previous visit
Ernzen	04/06/2007	Track in Area D	5	
Ernzen	04/06/2007	Track in Area D	5	
Ernzen	04/06/2007	Track in Area D	10	
Ernzen	24/06/2007	Track in Area A	5	4
Ernzen	24/06/2007	Track in Area D	5	10–15
Ernzen	24/06/2007	Track in Area D	5	
Ernzen	17/07/2007	Track in Area D	15	5

cluding and excluding loci that deviated from Hardy–Weinberg proportions.

Population differentiation and migration

The level of genetic differentiation between the two populations was quantified using F_{ST} (Weir & Cockerham, 1984) in SPAGeDi 1.2 (Hardy & Vekemans, 2002) and significance was tested with 10,000 permutations of individual genotypes between populations. We then used the Bayesian method implemented in program BayesAssNM (Wilson & Rannala, 2003; Jehle et al., 2005), to test for evidence of recent gene flow between the two populations. The method tests specifically for the presence of offspring produced by matings between a migrant and a resident, and estimates migration rates from these estimates. It requires loci to be unlinked, but these do not need to be in Hardy–Weinberg equilibrium. The software allows the user to change parameters affecting the proposed distributions, namely *deltap*, *deltam*, and *deltaf*. As different values for these did not change our results, the results presented here are based on the default values proposed by the program. We ran a total of 3×10^6 MCMC iterations, discarding the first 10^6 iterations as a burn-in.

RESULTS

The minimum number of adult female natterjack toads was estimated to be 59 in Steinfort and 33 in Ernzen. We collected tail-tips of 46 larvae from the former and of 54 larvae from the latter population, and genotyped the DNA extracts at 10 microsatellite loci. In the Ernzen population, we did not observe any spawn strings on our first visit (Table 1), which explains the discrepancy between the number of larvae collected and the minimum estimate of adult females. We assessed the reliability of 10 of these genetic profiles by re-extraction and re-analysis. One case of allelic dropout was observed at locus *Bcalμ8* in the replicated genotypes (the error was confirmed by re-amplification), translating into a genotyping error rate of 0.005 per allele. We consider this error rate low enough not to affect our results significantly.

Genetic diversity

All the 10 loci used in this study were polymorphic, with between two and five alleles per locus across both populations (Table 2). The mean number of alleles per locus was 3.9 in Ernzen and 3.8 in Steinfort. Mean H_e values (Ernzen: 0.585; Steinfort: 0.571) were lower than mean H_o values (Ernzen: 0.631; Steinfort: 0.584), generating a significantly negative F_{IS} value in the case of Ernzen (F_{IS}=–0.070, P=0.02), but not Steinfort (F_{IS}=–0.012, P=0.76). In Ernzen, three loci deviated from Hardy–Weinberg proportions before Bonferroni correction (Table 2), but only one afterwards (*Bcalμ3*, P<0.006). In Steinfort two loci deviated significantly from Hardy–Weinberg proportions after Bonferroni correction (*Bcalμ6*, *Bcalμ9*, P<0.0063), as compared to five beforehand (Table 2).

In Steinfort, loci *Bcalμ3* and *Bcalμ9*, as well as loci *Buca6* and *Bcalμ9*, were in linkage disequilibrium after Bonferroni correction (P<0.0012). In Ernzen, five pairs of loci were in linkage disequilibrium after Bonferroni correction (P<0.0013; *Bcalμ1* & *Bcalμ6*, *Bcalμ1* & *Bcalμ3*, *Bcalμ3* & *Bcalμ5*, *Bcalμ5* & *Bcalμ11*, *Buca6* & *Bcalμ9*). The only linkage pair common to both populations was *Buca6* and *Bcalμ9*. With this possible exception, the linkage disequilibrium observed could be the result of the demographic history of both populations, including genetic drift and the admixture of different populations, rather than physical linkage. The most likely explanation, however, is that it resulted from the inclusion of some full siblings in the analysis. The fact that the population in Ernzen exhibited a significantly negative overall F_{IS} value is consistent with this interpretation (see Discussion).

Bottlenecks

We found evidence for heterozygosity excess in both the Ernzen and Steinfort populations. As in both populations only one of the 10 loci analysed exhibited a heterozygosity deficit (Ernzen: *Bcalμ11*; Steinfort: *Bcalμ5*), all three statistical tests gave evidence of significant heterozygosity excess when all the loci were included in the analysis (Table 3). The same result was obtained for the Ernzen

Table 2. Number of alleles (*A*), allele size range, observed (H_o) and expected (H_e) heterozygosities and departures from Hardy–Weinberg proportions (F_{IS}) for all loci in both populations. NS = non-significant, * P<0.05, ** P<0.01, *** P<0.001.

		Population				Population	
Locus		Ernzen	Steinfort	Locus		Ernzen	Steinfort
Bcalμ1	*n*	54	46	*Bcalμ8*	*n*	53	46
	A	4	4		*A*	5	5
	range	131–149	131–164		range	169–192	169–202
	H_e	0.552	0.585		H_e	0.706	0.617
	H_o	0.556	0.630		H_o	0.830	0.783
	F_{IS}	0.002	–0.066		F_{IS}	–0.166	–0.257
		NS	NS			NS	*
Bcalμ3	*n*	54	46	*Bcalμ9*	*n*	54	46
	A	5	5		*A*	4	3
	range	132–143	132–143		range	114–126	118–126
	H_e	0.663	0.765		H_e	0.717	0.571
	H_o	0.796	0.804		H_o	0.796	0.652
	F_{IS}	–0.192	–0.041		F_{IS}	–0.101	–0.131
		**	NS			*	**
Bcalμ4	*n*	54	46	*Bcalμ11*	*n*	54	45
	A	3	2		*A*	2	4
	range	203–229	203–213		range	205–209	203–211
	H_e	0.527	0.406		H_e	0.168	0.609
	H_o	0.611	0.348		H_o	0.185	0.556
	F_{IS}	–0.151	0.153		F_{IS}	–0.093	0.099
		NS	NS			NS	*
Bcalμ5	*n*	54	46	*Buca2*	*n*	54	46
	A	5	4		*A*	5	3
	range	235–252	235–256		range	297–307	299–307
	H_e	0.762	0.413		H_e	0.690	0.414
	H_o	0.704	0.348		H_o	0.778	0.348
	F_{IS}	0.085	0.168		F_{IS}	–0.118	0.17
		*	*			NS	NS
Bcalμ6	*n*	54	46	*Buca6*	*n*	54	46
	A	2	4		*A*	4	4
	range	176–178	172–178		range	231–241	231–239
	H_e	0.479	0.713		H_e	0.586	0.616
	H_o	0.389	0.717		H_o	0.667	0.652
	F_{IS}	0.198	0.005		F_{IS}	–0.129	–0.048
		NS	**			NS	NS

population when only loci in Hardy–Weinberg equilibrium were considered. When analysing the Steinfort population with the two loci deviating from Hardy–Weinberg proportions omitted, the Wilcoxon test was significant, but not the other two tests (Table 3). Given the loss of power of both the sign and standard differences test when reducing the number of loci in an analysis, we believe that our results support the conclusion that there has been a recent genetic bottleneck in both remnant populations of *B. calamita* in Luxembourg.

Population differentiation and migration

The genetic differentiation between the two populations was high and significant (F_{ST}=0.143; P<0.0001). Locus *Bcalμ9* was omitted from the BayesAssNM analysis, as it was in linkage disequilibrium with *Buca6* in both populations. The two populations appear to have been isolated over the last generation. The proportion of individuals (± standard deviation) estimated to have originated from the focal population was 0.989 (±0.010) for Ernzen and 0.988 (±0.011) for Steinfort. When repeating the analysis with loci *Bcalμ1*, *Bcalμ5* and *Bcalμ9* omitted to avoid loci in linkage disequilibrium, the values obtained were virtually unchanged: 0.986 (±0.013) for Ernzen and 0.987 (±0.013) for Steinfort.

DISCUSSION

The Luxembourg populations of natterjack toads in Steinfort and Ernzen were estimated to contain a minimum of 59 and 33 adult females, respectively. Given the small number of visits to the two sites, these numbers will be underestimates. Nevertheless, both populations are likely to be very small. The adult male:female ratio is typically

Table 3. Genetic analysis of population bottlenecking in the two remnant Luxembourg populations of *B. calamita*. Tests were performed both including and excluding loci that deviated from Hardy–Weinberg genotypic proportions. *N* = number of loci used, *HE* = number of loci showing excess heterozygosity. The standardized differences test consists of comparing the T_2 statistic to a normal distribution $N(0,1)$: for further explanations see Cornuet & Luikart (1996).

Population	*N*	*HE*	Sign test	Standardized differences	Wilcoxon[b]
Steinfort	10	9	P=0.030	T_2=2.216; P=0.013	P=0.007
Ernzen	10	9	P=0.025	T_2=2.720; P=0.003	P=0.002
Steinfort[a]	8	7	P=0.074	T_2=1.550; P=0.061	P=0.027
Ernzen[a]	9	8	P=0.038	T_2=2.692; P=0.004	P=0.004

[a]These tests exclude loci deviating from Hardy–Weinberg proportions.
[b]Probability is two-tailed.

close to unity in natterjack populations, but can vary from 0.7 to greater than 2.0 (Tejedo, 1992; Denton & Beebee, 1993; Sinsch, 1998). The minimum viable population size is not known for this species, although colonies of less than 100 adults have persisted for many generations (Denton et al., 1997).

The aim of the present paper was to assess the genetic make-up of the two remnant populations of the natterjack toad in Luxembourg in order to assess the genetic risks to their long-term survival. In Britain, where the species is at the northern edge of its biogeographical range (Beebee & Rowe, 2000), critical fitness attributes of toad populations were shown to vary in relation to their degree of genomic heterozygosity at microsatellite loci and to be substantially higher in a large, outbred population than in a small isolated one. Larvae from the smallest and most isolated populations exhibited particularly low fitness (Rowe et al., 1999; Rowe & Beebee, 2003).

The genetic variability of the Luxembourg study populations, which both exhibited greater than 57% expected heterozygosity (averaged over all loci), however, compared very favourably with values reported from Britain, where not one population had maintained similar levels of diversity (all H_e<0.39 in Britain). While populations in western Belgium exhibited comparable levels of variability to Luxembourg (Stevens et al., 2006), Spanish populations have been shown to be much more genetically diverse (Beebee & Rowe, 2000; Gomez-Mestre & Tejedo, 2004). The genetic diversity observed in Luxembourg does not support the hypothesis of genetic impoverishment but fits into an overall picture of a decline in variability with increasing latitude, consistent with a post-glacial recolonization from a presumed refugium in Iberia (Beebee & Rowe, 2000). We should add the caveat that the different studies used different, though partially overlapping, sets of loci, and that this comparison assumes that the different sets of loci used have identical mutation rates.

Apart from genetic drift in small populations, inbreeding is also generally considered to affect the demography and persistence of natural populations (e.g. Keller & Waller, 2002). However, the F_{IS} values did not provide evidence for inbreeding in Luxembourg natterjack toads. Similarly, Rowe et al. (1999) found that only a single population in Britain, located on an island off mainland Britain, had significant positive F_{IS}-values across more than one locus. Both Luxembourg populations had negative F_{IS}-values, one of which was significantly different from zero. Indeed, there is a tendency for there to be an excess of heterozygotes in small populations of animals with separate sexes, caused by different allele frequencies in the two sexes (Allendorf & Luikart, 2007). It is possible that this phenomenon was exacerbated through stochastic sampling and the sampling of siblings that were the product of individuals that were homogygous for different alleles at various loci. We note that the sampling of siblings should not affect the main conclusions, as we obtained virtually identical results in analyses where we excluded, as appropriate, loci that deviated from Hardy–Weinberg proportions or contributed to linkage disequilibria.

The estimator of genetic differentiation revealed significant differences between the two Luxembourg populations that are consistent with isolation and we did not find any evidence for recent gene flow. The two populations thus clearly require management as separate units. However, they retain comparable or greater levels of genetic diversity than other European populations. Translocation of individuals between the two Luxembourg populations is thus not necessary, especially considering the risks associated with such practices (e.g. Woodford & Rossiter, 1993). However, the genetic bottleneck tests found evidence for substantial recent declines in the effective sizes of both populations. This independent assessment accords with what is known about the history of the Steinfort population, which declined between 1997 and 2002 due to reproductive failures (Junck et al., 2003). Also, this finding does not contradict the conclusion that the Luxembourg populations are not genetically impoverished, as recently bottlenecked populations are likely to have lost rare alleles, but may still contain substantial heterozygosity and quantitative genetic variation that will be lost more slowly than allelic variation at specific loci, and which will influence fitness in current environments more (Leberg, 1992; Luikart et al., 1998; Hedrick, 2001). However, while strongly deleterious alleles are likely to be purged during a bottleneck event, mildly deleterious alleles can become fixed, increasing the

genetic load of the population (Bataillon & Kirkpatrick, 2000; Hedrick, 2001).

In order to alleviate all the stochastic threats to the Luxembourg populations (not only genetic, but also demographic, environmental and catastrophic), the main objective of conservation measures has to be to increase the sizes of the populations. Most important in this aspect is the continuous safeguarding and management of the two sites in Steinfort and Ernzen. While conservation measures have helped the Steinfort population to recover (Junck et al., 2003), the Ernzen population is located in an actively exploited sandstone quarry where the habitat is subject to abrupt changes. While natterjacks prefer spawning in ephemeral pools, even avoiding deeper ones when choice is available (Beebee, 1979), it is very important to continuously monitor this population. At the end of active exploitation, the quarry habitat will need to be maintained, rather than restored to agricultural use. More detailed suggestions for the conservation of the species in Luxembourg in the longer term are given in Junck et al. (2003).

Overall, there can be little doubt that Luxembourg's natterjack toads have responded negatively to the extensive loss of their habitat. Therefore, conservation measures including the development of a species action plan (SAP) and its implementation within the framework of the National Plan for the Protection of Nature as well as of Natura 2000, as recently decided by the Luxembourg government (Juncker et al., 2007), seem fully justified. The results of the present study should be considered when drawing up the SAP.

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