Landscape-genetic analysis of Natterjack toad (*Epidalea calamita*) populations in the Suhre valley (Switzerland)

Master thesis

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Abstract

Understanding the influence of landscape elements on dispersal and connectivity of potentially isolated populations is essential for the successful conservation management, especially in agricultural landscapes. Here, we assessed the migration pattern and genetic structure of the endangered Natterjack toad (*Epidalea calamita*) in the Suhre valley (Switzerland), a homogeneous agriculture area with a comparatively large occurrence of this toad. We further analysed the population dynamics in the spatial and temporal dimension. We also studied the influence of land-use on population size and migration. Finally, we compared the performance of radio-tracking with genetic analyses to identify migration. Our results showed that none of the distinguished populations were genetically isolated. Accordingly, we found that migration takes place regularly. Most of the uncovered migration events were found by genetic analyses and not by radio-tracking. The network analysis revealed that the largest populations were the most important for the population network, confirmed by the source–sink analysis. Among land-use elements, forests seemed to have a negative effect on gene flow as well as on population size. We conclude that neither main roads nor the canalised Suhre are migration barriers for the Natterjack toad. It seems that this species can survive in agriculture landscapes, if enough breeding ponds exist. Nevertheless, conservation activities should focus on the large populations since they are sources and therefore essential for the continuance of a viable Natterjack toad population. We recommend using genetic analyses to detect migration if one is not interested in the exact migration route of individuals.

Introduction

Through the continuous modification of landscapes by humans, natural habitats have been degraded, fragmented or even lost (Lindenmayer and Fischer 2006). Not surprisingly, destruction and fragmentation of natural habitats are considered as the main cause of the loss of biodiversity (Cushman 2006). Many conservation areas have been set up in the past decades, but they are often small islands in otherwise intensively used landscapes, often isolated through migration barriers. This is a major challenge to nature conservation (Lindenmayer and Fischer 2006).

Barriers do not only impede migration between populations, they also hamper the colonisation of new suitable habitats (Bowne and Bowers 2004). Even more, the fragmentation of habitats may also result in an increased genetic separation of populations, higher inbreeding rates and in reduced genetic diversity within populations due to stochastic processes such as genetic drift (Allendorf et al. 2012; Frankham 1995; Keyghobadi 2007).

Pond-breeding amphibians are especially affected by the loss of habitats and fragmentation, because they rely on breeding ponds, are often philopatric, and they have to migrate between their aquatic breeding habitats and their terrestrial foraging sites (Blaustein et al. 1994; Hamer and McDonnell 2008). Even more so, amphibians are often relatively poor dispersers, and the possibility to occupy new habitats and breeding ponds is rather low (Blaustein et al. 1994; Hamer and McDonnell 2008).

This is also the case in the densely populated Swiss lowlands, where large areas are highly fragmented through intensive agriculture, urban sprawl and dense traffic infrastructures (Jaeger et al. 2008). In these highly managed and homogenised landscapes, structured habitats are largely missing and the natural dynamics of wildlife movement is impeded. Particularly wetlands have been drained and most rivers have been canalised in the past (Jaeger et al. 2008). Many amphibian species have strongly declined in the Swiss lowlands during the last 30 years (Schmidt and Zumbach 2005). In particular, amphibian species of pioneer habitats as the Natterjack toad (*Epidalea calamita*), naturally living in dynamic pioneer habitats such as floodplains or in wetlands, are affected by the loss of their natural habitats through canalisation, regulation of the flood dynamic and drainage (Schmidt and Zumbach 2005; Sinsch 1998). Many populations substituted the loss of their traditional habitats with secondary habitats such as gravel pits. Through the intensification of gravel mining or abandonment of mining activities and subsequent restoration of the original landscape, many populations are now also losing their secondary habitats (Schmidt and Zumbach 2005; Sinsch 1998).

However, amphibian populations can survive in agriculture areas if breeding ponds exist and if the areas are not isolated by migration barriers (Berger et al. 2011; Sinsch 1998). Besides the protection of intact habitats, the most important measures to improve the situation for amphibians in the Swiss lowlands are to restore degraded habitats and create new breeding ponds (Berger et al. 2011; Moilanen et al. 2005; Schmidt and Zumbach 2005), as well as to ensure and improve the connectivity between habitat patches (Bennet 1999; Berger et al. 2011; Schmidt and Zumbach 2005). But to implement conservation measures successfully and efficient, a detailed understanding of dispersal patterns among the studied population is required (Ricketts 2001). Nevertheless, control measures in nature conservation are often restricted to the monitoring of population sizes. But with this approach only population trends can be analysed and it is not possible to gain insights about migration patterns or connectivity of populations.

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Traditionally, the number of migrants and movement patterns are assessed by direct monitoring through telemetry or GPS collars (Jaquiéry et al. 2011). These methods provide the most detailed data on individual dispersal trajectories (e.g. Riley et al. 2006). Another possibility to estimate dispersal rates are mark–recapture studies (e.g. Rouquette and Thompson 2007). Both methods are costly and laborious (Bowne and Bowers 2004). Furthermore, without genetic analyses, it is often unclear whether individuals in artificial ponds can successfully reproduce or whether they are immigrants from other populations. If they cannot reproduce, or more exactly, if young individuals die before they are able to reproduce, temporarily flooded ponds in agriculture areas cannot support a population. In this case, temporarily flooded ponds might even act as "ecological traps" (Griffin and Case 2001) because reproductive effort is lost and the potential reproductive capacity of the population network is reduced.

Favoured by the immense development of genetic analyses in the past years, the upcoming use of landscape genetics provides promising tools to assess migration and connectivity (Manel and Holderegger 2013; Storfer 2013). Landscape genetics explains observed spatial genetic patterns among populations with patterns in the landscape (Holderegger and Wagner 2008; Manel and Holderegger 2013; Manel et al. 2003). The advantages of landscape-genetic analyses are that effective connectivity and barriers to gene flow can be detected through genetic analyses (Sork and Waits 2010; Storfer 2013). In addition, the method helps gaining insight into basic biological processes, for example metapopulation dynamics or distribution limits of species or speciation (Storfer 2013). A disadvantage of genetic approaches is, however, that exact movement paths of individuals cannot be measured. Due to limitations in time and money, nature conservation often cannot apply both direct monitoring and genetic analyses but has to choose one of them.

Here, in this study, we had the unique possibility to assess a Natterjack toad population with genetic and traditional monitoring analyses (radio-telemetry and long-time amphibian monitoring). The goals of this study were (i) to quantify the movement among Natterjack toad populations in an intensely human-dominated landscape, (ii) to analyse the population dynamics using a network analysis, (iii) to study the influence of land use on population size and migration, and (iv) to compare the performance of radio-tracking with genetic analyses to identify migration.

Materials and methods

Study species

Natterjack toads (*Epidalea calamita*, formerly *Bufo calamita*) are medium-sized anurans distributed across Western Europe. The toads breed in ephemeral ponds surrounded by bare ground or open vegetation (Beebee 1983; Sinsch 1998). The loss of floodplains through channelling of watercourses, drainage and intensification of agriculture increasingly threatens the species (Schmidt and Zumbach 2005; Sinsch 1998). In Switzerland, more than 60 % of the known Natterjack toad occurrences disappeared during the last 30 years. The Natterjack toad is therefore listed as endangered (EN) on the Swiss Red List (Schmidt and Zumbach 2005).
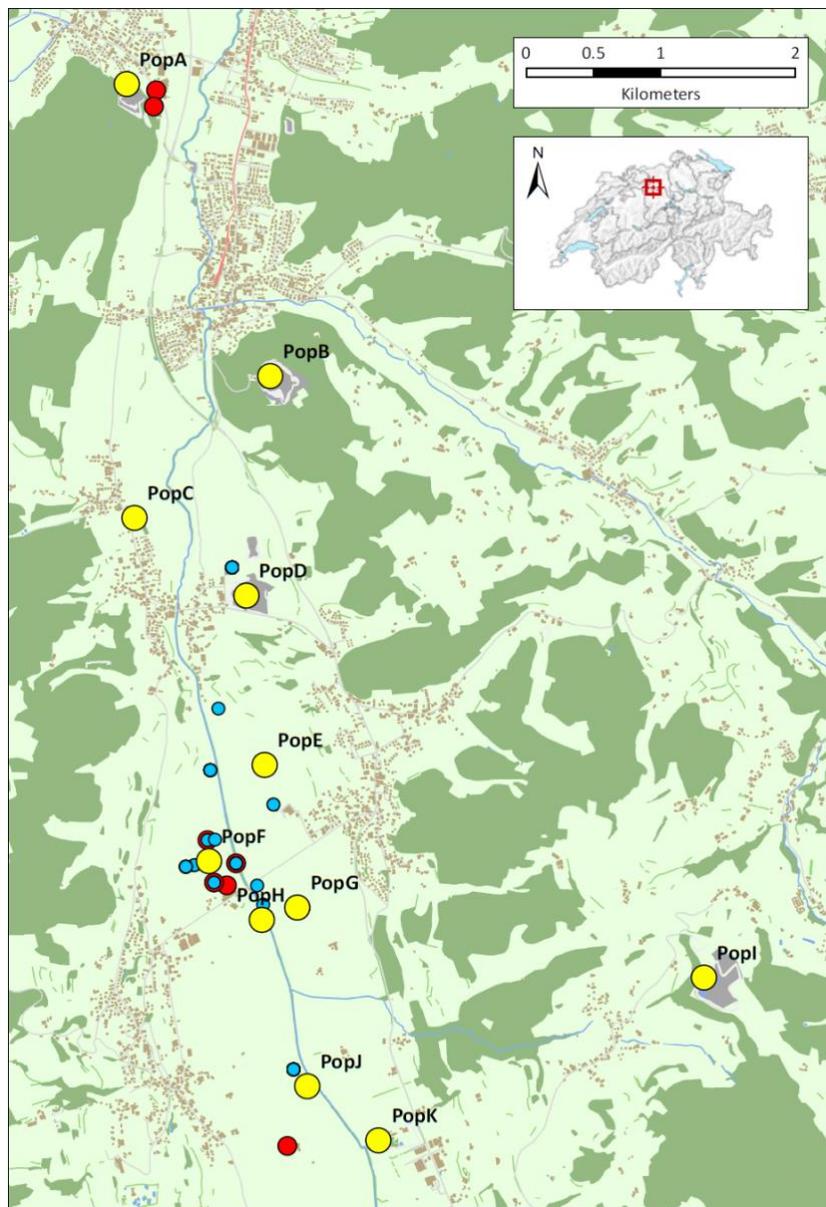

Fig. 1: Locations of the studied Natterjack toad populations (yellow points), breeding ponds (red points) and sites of genetic sampling (blue points) in the upper Suhre valley, Switzerland. Dark green areas mark forests and hedges, light green areas show open lands. Water bodies are blue, roads are grey and railway lines are red. Grey areas indicate gravel pits, light grey areas show slopes, and buildings are coloured in brown.

Study area

The study area covered around 25 km^2 of the upper Suhre valley, cantons of Aargau and Lucerne, on the central Plateau of Switzerland (Fig. 1). The valley bottom is located at 480 m a.s.l., encompassing a moraine with an elevation difference of ca. 50 m. Whereas the valley bottom is dominated by intensive agriculture, the valley slopes are mostly covered by forest (Fig. 1). Several settlements are located along the valley slopes. Seven gravel pits are distributed over the study area. The canalised Suhre river, which is about 8 m wide, divides the study area into a western and an eastern part, and three frequented main roads (but no fenced motorway) cross the centre of the study area (Fig. 1).

Around 20 clusters of breeding ponds of the Natterjack toad are known in the upper Suhre valley. Thereof, 17 are located on the valley bottom and five are situated in gravel pits (Fig. 1 and Table 1). To measure migration and analyse barriers among the patchily distributed breeding ponds, distinct population units were required. Based on the expected migration barriers (main roads, Suhre river, moraine) and distances between habitats, we arranged the breeding ponds into eleven geographically distinct population units (PopA-PopK, see Fig. 1 and Table 1). Migration barriers were thereby located between populations. These artificially delineated populations were used for further analysis. Straight-line distances (Euclidean distances, *ED*) between these populations ranged from 281 to 8121 m (Table 2).

Table 1: Coordinates (according to the Swiss national grid), sample size, allelic richness (A_R), expected (H_E) and observed heterozygosity (H_O), inbreeding coefficient (F_{IS}) of the studied Natterjack toad populations in the Suhre valley, Switzerland. Additionally, means and trends of population sizes are listed, and it is stated in which population individuals were radio-tracked.

Population units	E	N	Genetic data						Population size		Radio-tracking data available
			Sample size	A_R	H_E	H_O	F_{IS}	Private alleles	Mean[3]	Trend[4]	
PopA[1]	645'532	240'996	3*	1.46	0.21	0.31	-0.48	0	2	↘	-
PopB[1]	646'598	238'811	-	-	-	-	-	-	3	↘	-
PopC	645'589	237'750	-	-	-	-	-	-	7	→	-
PopD[1]	646'421	237'169	68	2.51	0.58	0.59	-0.02	6	149	→	x
PopE	646'556	235'902	24	2.37	0.54	0.55	-0.02	0	-	-	x
PopF	646'143	235'182	36	2.45	0.57	0.56	0.02	0	20	→	x
PopG	646'800	234'835	16	2.46	0.58	0.56	0.03	1	-	-	x
PopH[2]	646'535	234'742	41	2.40	0.55	0.55	0.00	0	32	↗	x
PopI[1]	649'820	234'310	9*	2.39	0.54	0.56	-0.04	0	-	-	-
PopJ[2]	646'874	233'497	21	2.43	0.55	0.61	-0.11	0	14	↗	x
PopK[1]	647'402	233'093	48	2.45	0.56	0.58	-0.04	0	-	-	-

*Sampled in 2014; [1]Located in gravel pits; [2]Artificial ponds built in 2009; [3]Mean of 2009-2013; [4]Development of population sizes during the last 15 years: ↗ increasing, → constant, ↘ decreasing

Table 2: Euclidean distances (*ED*) between the studied Natterjack toad populations in the Suhre valley (Switzerland) are listed below and pairwise F_{ST}-values above the diagonal.

	PopA	PopD	PopE	PopF	PopG	PopH	PopI	PopJ	PopK
PopA		0.1549	0.1830	0.1662	0.1561	0.1560	0.1999	0.1907	0.1733
PopD	3929		0.0103	0.0122	0.0000	0.0132	0.0339	0.0131	0.0139
PopE	5196	1274		0.0038	0.0111	0.0031	0.0627	0.0163	0.0159
PopF	5846	2006	830		0.0154	0.0060	0.0484	0.0027	0.0086
PopG	6290	2365	1095	743		0.0041	0.0279	0.0000	0.0125
PopH	6334	2430	1160	589	281		0.0421	0.0006	0.0099
PopI	7943	4442	3632	3779	3065	3313		0.0461	0.0270
PopJ	7618	3700	2426	1837	1340	1290	3056		0.0065
PopK	8121	4192	2934	2439	1843	1863	2707	665	

Data

Three data sets were available for this project: (i) various measures derived from genetic data sampled for this study; (ii) radio-tracking data (Schweizer 2014); and (iii) census data of population sizes collected within a long-term amphibian monitoring (unpublished data, provided by C. Bühler, Hintermann & Weber AG).

Genetic data

In total, 266 adult individuals were genetically sampled using buccal swabs. Buccal swabbing is a widely used, minimally invasive method that has been shown to be an efficient approach to gain amphibian DNA (Angelone and Holderegger 2009; Broquet et al. 2007; Pidancier et al. 2003). In 2013, 254 individuals were sampled, supplemented with 12 individuals in 2014 (Table 1). By chance, 16 individuals sampled in 2013 were sampled twice and one even three times. The samples were genotyped at 13 nuclear microsatellite loci with markers that were available for this species (Bcalµ1-Bcalµ8: Rogell et al. 2005; Bcalµ10: Rowe et al. 2000; Buca1, Buca2, Buca5, Buca6: Rowe et al. 1997) and that have been successfully applied several times (e.g. Allentoft et al. 2009; Frantz et al. 2009; Oromi et al. 2012; Stevens et al. 2006b).

We extracted genomic DNA using the Qiagen QIAmp 96 Blood Kit. Buccal swabs were first transferred to collection tubes (96), and we added 320 µl PBS buffer (phosphate buffered saline, pH 7.4), 16 µl protease (included in the kit) and 320 µl AL buffer (included in the kit) per sample. Samples were then placed in a shaking incubator (Heidolph) for 3 min at 56 °C. We then added 320 µl of ethanol (SIGMA, puriss.), transferred the samples to the QIAmp plate and followed the manufacturer's protocol. DNA was eluted with 120 µl AE buffer (included in the kit).

We amplified 13 nSSRs in two multiplex mixes. The primers used were originally described by Rowe et al. (2000); Rowe et al. (1997) and Rogell et al. (2005). Reactions (10 µl) contained 4 µl (0.8 x conc.) of Type-it Mastermix PCR Kit and 50-70 ng of DNA. Multiplex 1 contained 0.1 µM of primer pairs Bcalµ1(FAM), Bcalµ2(FAM), Bcalµ3(ATTO532), Bcalµ6(ATTO565), Bcalµ7(ATTO550) and Bcalµ8(ATTO532), 0.25 µM of Bcalµ4(ATTO550) and 0.2 µM of Bcalµ5(FAM). Multiplex 2 contained 0.075 µM of Bcalµ10(ATTO532), 0.3 µM of Buca1(ATTO532), 0.2 µM of Buca2(FAM) and Buca6(FAM) and 0.1 µM of Buca5(ATTO565). Each forward primer was labelled with the fluorescent dye indicated

in brackets. Polymerase chain reactions (PCRs) were performed with the following thermal profile. Multiplex 1: 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 90 s at 58 °C, 30 s at 72 °C and a final extension step at 30 min at 60 °C. Mulitplex 2: 5 min at 95 °C, 32 cycles of 30 s at 95 °C, 90 s at 55 °C, 30 s at 72 °C and a final extension step at 30 min at 60 °C. The ramp was 1.2 °C/s for both multiplexes.

PCR fragments were sized on a 4-capillary LifeTechnologies sequencer (3100Avant) with LIZ500 as size standard. Alleles were scored using GeneMapper 5.0 (Life Technologies).

Radio-tracking data

Also in 2013, 50 males were tracked by a radio-telemetric study (Schweizer 2014). The time span of observation was different for each male and varied from two days up to four months. On average, the positions were measured less than once per day (Schweizer 2014).

Amphibian inventory

Since 1999, population sizes of amphibian breeding ponds have been measured in a long-term amphibian monitoring ordered by the canton of Aargau (Table S1, data provided by C. Bühler, Hintermann & Weber AG). A subset of the sites to be visited each year is defined through a partially randomized process for every year anew. For more details see Bühler (2014). Each selected site is visited three times per season. The maximum visiting time is given by the pond size. The largest number of individuals counted during the three visits is taken as an estimate of the population size (Bühler 2014).

Landscape data

The landscape and land-use data characterising the Suhre valley were derived from the SwissTLM3D and SwissBUILDINGS3D 1.0 data sets (swisstopo, Switzerland). For the landscape analysis, we categorised the landscape of the study area into ten land-use classes (buildings, roads, railway lines, standing waters, flowing waters, hedges, slopes, gravel pits, open lands and forests; Fig. 1). Because the raw data of SwissTLM3D are sometimes given by simple lines or points, we buffered roads according to their width, hedges with 3 m, the Suhre river with 4 m and other named rivers with 3 m on both sides of respective elements using ArcGIS 10.1 (ESRI, USA). Finally, to bring all land-use classes together on one layer, we combined the land-use classes into one raster map with a spatial resolution of 0.5 m.

Genetic diversity

To measure genetic diversity, we calculated allelic richness (A_R), expected heterozygosity (H_E), observed heterozygosity (H_O) after Nei (1973) and an inbreeding coefficient (F_{IS}) after Wright (1965) with R 3.1.1 (R Development Core Team 2014) using the package diveRsity (Keenan et al. 2013). Genetic differentiation between populations was determined through pairwise fixation index (F_{ST}) after Weir and Cockerham (1984). We also calculated the F_{ST}-values with the diveRsity package on R 3.1.1 (Keenan et al. 2013; R Development Core Team 2014).

Genetic structure

We estimated the effect of geographic distance on the genetic structure by isolation-by-distance (IBD) tests (Wright 1943) between log-transformed distances and standardised genetic differentiation among populations ($F_{ST}/1-F_{ST}$). We further conducted a Bayesian cluster analysis to

describe the spatial genetic structure and past gene flow between the populations using STRUCTURE 2.3.2.1 (Falush et al. 2003; Pritchard et al. 2000). Sample group information of the individuals was taken into account by the LocPrior model (Hubisz et al. 2009). Ten independent runs were calculated for each predefined cluster number of K = 1-10 performed with a burn-in length of 100'000 and a Markov Chain Monte Carlo (MCMC) of 1'000'000 repeats. We defined the optimal cluster number (K_{max}) by following the STRUCTURE 2.3.2.1 guidelines (Pritchard et al. 2000) and carried out its visualisation by HARVESTER (Earl and Vonholdt 2012). The STRUCTURE 2.3.2.1 output was edited in CLUMPP 2.1.1 to correct discrepancies between the ten runs with K_{max} (Jakobsson and Rosenberg 2007). The figure, finally, was created with DISTRUCT 1.1 (Rosenberg 2004).

Migration

Contemporary gene flow was estimated with two Bayesian assignment tests. First, we conducted a first-generation migration test with GENECLASS 2 (Piry et al. 2004). This assignment test identifies migrants as individuals that were sampled in another site (population) than the one where it was born (Piry et al. 2004). Because all known breeding ponds were sampled, we used the ratio $L = L_{home}/L_{max}$ as the statistical criterion for the likelihood computation (Paetkau et al. 2004). The partial Bayesian method of Rannala and Mountain (1997) together with the MCMC resampling algorithm of Paetkau et al. (2004) were used, and 1000 individuals were simulated at a threshold value of 0.01 (p-value).

Second, we calculated an assignment test as implemented in BIMR (Faubet and Gaggiotti 2008; Faubet et al. 2007). BIMR defines posterior estimates of first-generation migration rates using MCMC and Reversible Jump MCMC methods (Faubet and Gaggiotti 2008; Faubet et al. 2007). We tested several burn-ins between 10'000 and several millions, but it had no effect on the general pattern of migration rates. A burn-in period of 100'000 runs and a sample size of 10'000 runs with ten replicates per run were chosen. Mean and standard deviations for posterior regression model probabilities of the ten replicates per run were calculated for all ten land-use variables individually. The migration rates were related to land-use variables to test to what degree the environment determines movement of the Natterjack toad. BIMR relies on a permuted generalized linear model (GLM). The pairwise correlation between land-use variables entering the GLM was maximally 0.6. The final full model contained all ten land-use variables as well as geographic distance (*ED*).

Besides the indirect measurements of migration, we were also able to observe movements directly through the data of the 50 radio-tracked males (Schweizer 2014) and the multiple genetic sampling of 17 individuals.

Network analysis

To assess the importance of a single population (= node) in the population network of Natterjack toads, we performed network analyses using CONEFOR SENSINODE 2.6 (Pascual-Hortal and Saura 2006; Saura and Pascual-Hortal 2007; Saura and Torné 2009). We measured the node importance by the graph-based index probability of connectivity (*PC*) (Pascual-Hortal and Saura 2006; Saura and Rubio 2010; Saura and Torné 2009). To calculate *PC*, it is necessary to define a threshold value for the distance and its corresponding probability that dispersal can cover it between two nodes. Based on studies assessing the terrestrial movement range of Natterjack toads, we set the threshold value at 2200 m with a probability of 0.5 (Jehle and Sinsch 2007; Leskovar and Sinsch 2005; Miaud et al. 2000; Sinsch 1988; Sinsch 1992; Sinsch 1997; Sinsch 1998; Sinsch et al. 2012; Smith and Green 2005). Nodes

of the network were characterised by population sizes or A_R, and links either by *ED* or F_{ST}-values. All four possible combinations (nodes x links) were conducted. Because historical data of population sizes were available from the amphibian inventory, we also assessed the node importance of the past by using five-year-means, three-year-means and single years of population sizes as nodes and *ED* as link measure.

Landscape analysis

We tested the effects of land-use types on the movement and on the genetic characteristics of Natterjack toads between the defined populations (corridors) and at each single population (sites). For this purpose, we buffered the straight-line corridors (= *ED*) between the populations with 200 m in ArcGIS 10.1. We also buffered the sites themselves with 200 m to be able to assess the site conditions. This buffer size was chosen to have a size as large as possible on one hand and to have only few overlaps on the other hand, as the smallest distance between populations was 281 m (Table 2). For each buffered feature - corridors and sites - we calculated the proportion for all land-use classes. Besides the ten land-use parameters, we also used ecological parameters to enable the comparison between land-use type and ecological measures. To characterize the sites genetically and ecologically, we used A_R, F_{IS}, mean of population sizes of the last five years, population trend and number of neighbour populations closer than 3 km. The number of neighbour populations served as a connectivity measure. For the corridor measure, we used F_{ST}, *ED* and the number of migrants additionally to the land-use parameters.

The landscape analysis was done by calculating the correlations between all parameters and principal component analyses (PCA). We conducted both in R 3.1.1 (R Development Core Team 2014), for PCA using the Lattice package (Sarkar 2008). To avoid that land-use and ecological parameters influence each other, we performed the PCA separately for land-use and ecological parameters.

Results

Genetic diversity

The measures for genetic diversity (A_R, H_E, H_O, F_{IS}) were similar for all delineated populations, except for PopA, where only three individuals were sampled (Table 1). Allelic richness (A_R), expected heterozygosity (H_E) and observed heterozygosity (H_O) differed only slightly from each other. The inbreeding coefficient (F_{IS}) was low in all populations (< 0.03) or even slightly negative (except for PopA owing to its small sample size), indicating a low degree of inbreeding at the intrapopulation level.

Small pairwise F_{ST}-values (< 0.06) indicated a weak genetic structure among populations and thus no genetic isolation of the delineated populations (Table 2). Only PopA showed higher but still small values of interpopulation genetic differentiations (0.15 - 0.20). According to this result, private alleles could only be found in PopD and PopG, also showing that gene flow among populations is taking place regularly (Table 1).

Genetic structure

The STRUCTURE-HARVESTER analysis resulted in an optimal cluster number $K_{max} = 3$. The analysis of spatial genetic structure (and past gene flow) by STRUCTURE with K_{max} revealed no discrete genetic pattern. All individuals were admixed and could not be assigned to one of the three K (= to a specific population). Rather, a gradient of genetic structure could be observed from north (PopA) to south (PopK, Fig. 2). Thus, most of the populations as defined in this study had no clear genetic structure in the central part of the study area. According to this, there was no strong isolation-by-distance pattern (results not shown). However, separate IBD-analyses, where either distances from the southernmost population (PopK) or the northern population (PopD) to all other populations were plotted against F_{ST}-values to all other populations showed correlations (Fig. 3). This result indicates that Natterjack toads migrate from PopD southward and from PopK northward leading to genetically heterogeneous populations in the centre (Fig. 2).
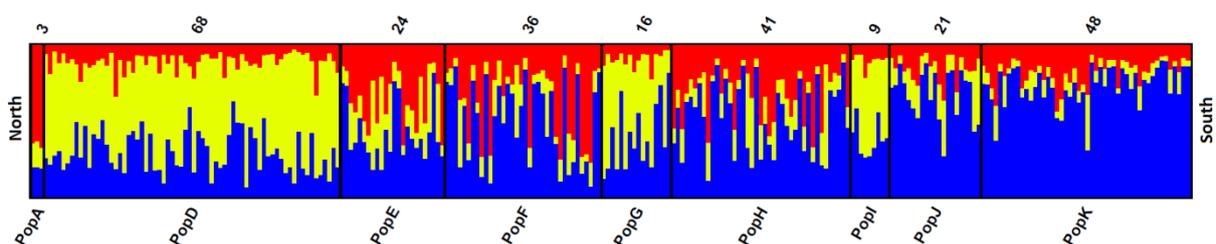

Fig. 2: STRUCTURE output (K_{max} = 3 clusters) of the studied Natterjack toad populations in the Suhre valley, Switzerland. Each vertical bar represents an individual, and the colour composition displays the probability to belong to one of the three clusters defined by STRUCTURE. Black vertical lines delineate pre-defined populations, and the numbers of sampled individuals per population are shown above the illustration.
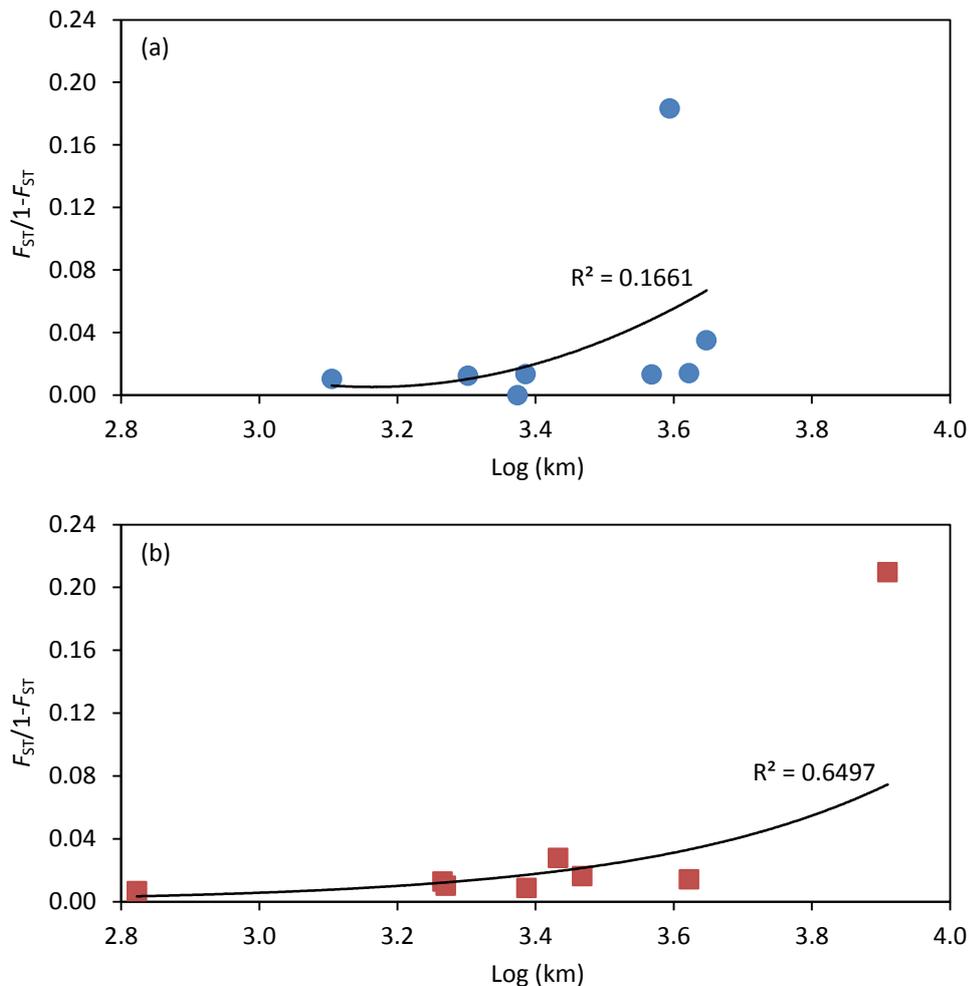
Fig. 3: Isolation-by-distance of the studied Natterjack toad populations in the Suhre valley (Switzerland), measured **a** from PopD and **b** from PopK to all other populations.

Migration

The assessment of first-generation migrants from assignment tests in GENECLASS uncovered five migrants with an error type I probability of $p < 0.01$ (Fig. 4). Two of them migrated between PopD and PopK, i.e. much further than only to the next population.

Six migrants could be detected through the multiple genetic sampling of 17 Natterjack toads, as they were found in different populations (Fig. 4). In contrast to the assignment tests, these migrants were observed by chance and give an incomplete overview of migration events. On the other hand, only two of the 50 radio-tracked males were observed to migrate between populations (Fig. 4). The radio-telemetric study was therefore less successful in detecting migration than the assignment test of first-generation migrants or a genetic "mark–recapture" study.

Altogether, 13 migration events were observed indicating a high amount of exchange between populations. At least five migrants passed the canalised Suhre river and four individuals crossed main roads (Fig. 4). Most of the found migration took place between neighbouring populations.

The BIMR analysis revealed that PopD, PopF, PopH, PopJ and PopK were source populations as they had probabilities > 0.01 that individuals emigrate into all other populations, while the smaller populations were detected to be sinks (Fig. 4). The revealed migration pattern confirmed the source–sink dynamics by BIMR (Fig. 4). The observed migration rates were independent of any landscape structural or thematic features as none of the features considered explained the migration rates (results not shown).
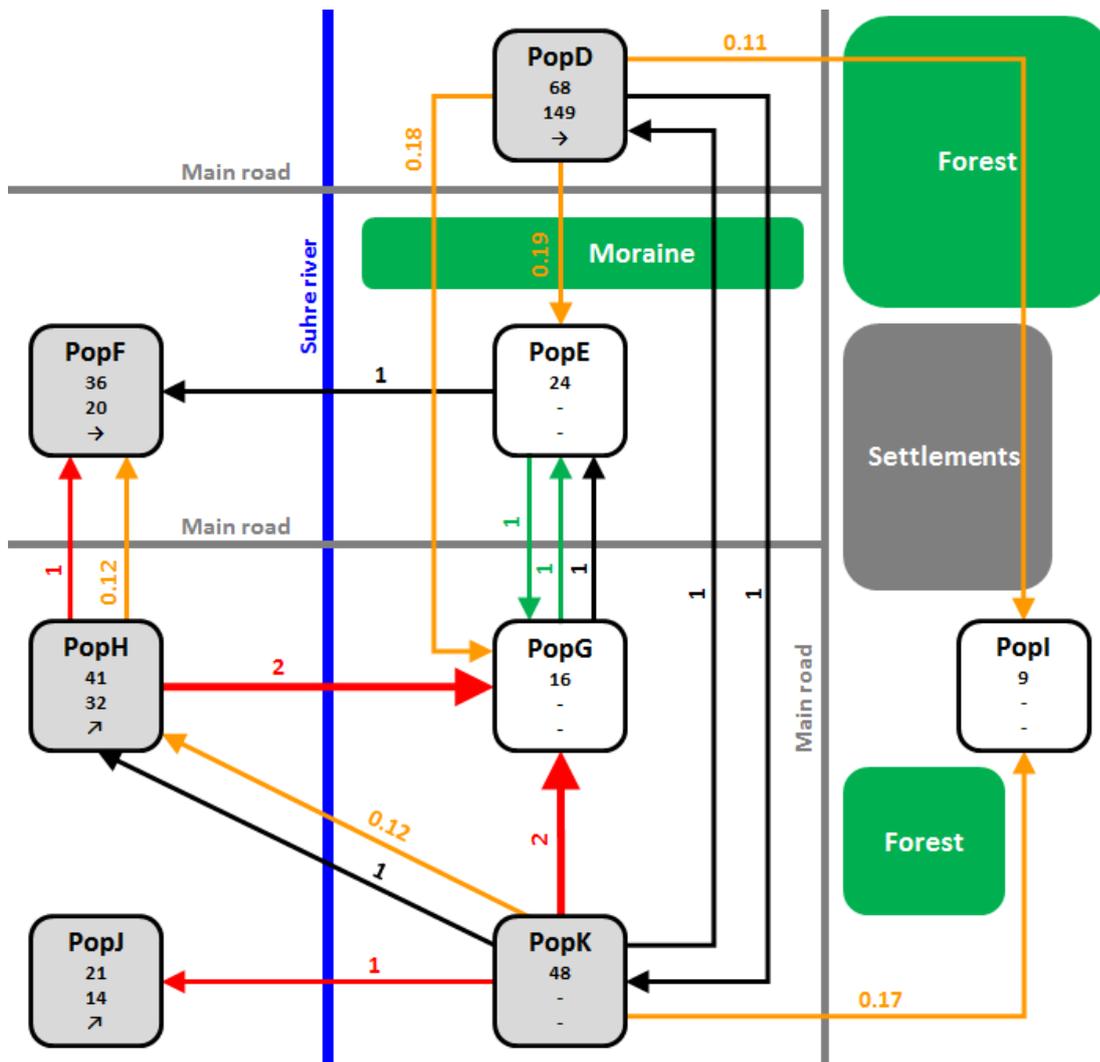

Fig. 4: Schematic illustration of the Natterjack toad populations and expected migration barriers in the Suhre valley, Switzerland. Arrows denote directly observed and indirectly detected migration events between populations, and the numbers of migrants are shown on each arrow. Green arrows show migration observed by radio-tracking, red arrows show detected migration through multiple genetic sampling of the same individuals, and black arrows show detected migration events by the analysis of first-generation migrants. Orange arrows display migration probabilities detected by BIMR (only probabilities > 0.1 are shown). Sources detected by BIMR are highlighted through grey background. The first number below the population name is the sample size, the second is the mean population size of the years 2009-2013 and the arrows indicate the population trend.

Network analysis

The importance of processes within a population was higher in larger populations, while connectivity was more important for smaller populations. However, in most cases the importance of internal processes was much lower compared to the importance of connectivity. Because of that only the total node importance (dPC) is shown in Table 3 without to split up in internal processes and connectivity. If population size was taken as the node measure, the assessment of node importance (dPC) revealed primarily the importance of population size, independent if Euclidean distances (ED) or F_{ST} were used as link measure (Table 3). Accordingly, the by far largest population (= PopD, see Table 1) turned out to be by far the most important one in 2013, but also by assessing the past population sizes (Table 3, results of the past not shown). The pattern differed when A_R was used as node measure, as the differences of importance were much smaller between populations (Table 3). In the case of F_{ST} as link measure, the importance of all populations was at the same level, only PopA had a smaller node importance according to its lower A_R (Tables 1 and 3). When ED was used as link measure, the importance of populations differed more corresponding to the variation of ED, indicating that ED overestimates the relevance for migration (Table 3).

Table 3: Node importance (dPC) of the studied Natterjack toad populations in the Suhre valley (Switzerland) for all four combinations (node x links) measured by CONEFOR SENSINODE. The higher the value, the more important is a population for the population network.

Population units	Node:	Population size[1]		Allelic richness[2]	
	Link:	ED	F_{ST}	ED	F_{ST}
PopA		0.68	2.06	4.45	13.47
PopB		1.51	-	-	-
PopC		5.40	-	-	-
PopD		88.80	90.08	18.92	22.56
PopE		-	-	22.82	21.37
PopF		13.65	17.55	24.58	22.05
PopG		-	-	25.79	22.14
PopH		19.62	27.26	25.17	21.63
PopI		-	-	14.63	21.54
PopJ		6.72	12.67	22.18	21.88
PopK		-	-	20.41	22.05

[1]Mean of 2009-2013; [2]2013

Landscape analysis

Remarkable are the high amount of variation that is explained by the first and second axes (= principal components) of the PCA, both for landscape and ecological parameters (Fig. 5). For both measures - sites and corridors - the proportions of roads and buildings, flowing waters and hedges as well as slopes and gravel pits were positively correlated (> 0.7), while open lands were negatively correlated with forests (< -0.7, Fig. 5 and Table S2). At the site measure, A_R was positively correlated with F_{IS}, and forests were negatively correlated with the population trend (Table S2). The correlation

analysis for the corridors revealed positive correlations between buildings, roads, distance and F_{ST}, and between roads and distance (Fig. 5 and Table S2).
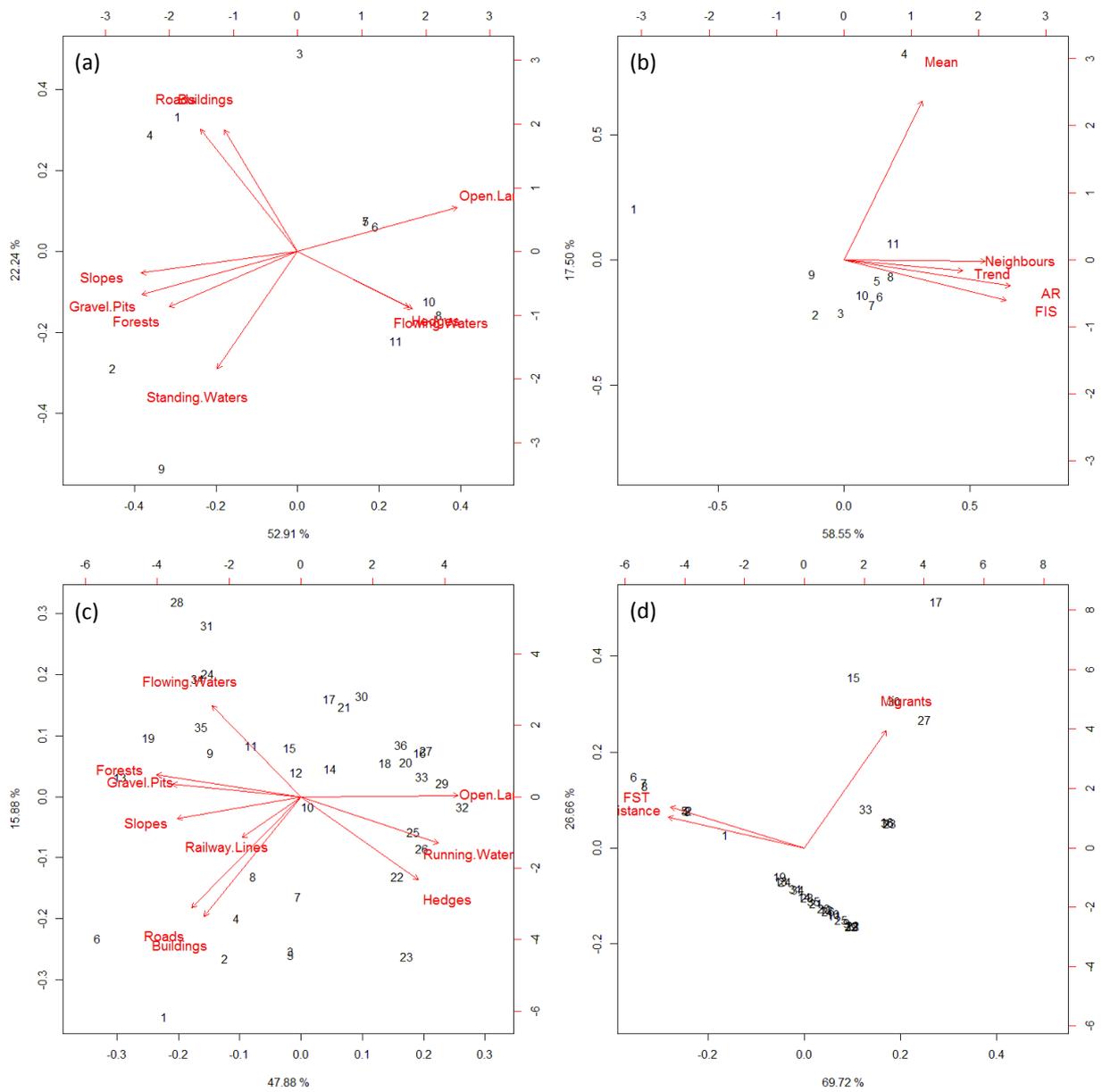

Fig. 5: Biplots of the PCA of the Natterjack toad in the Suhre valley, Switzerland. **a** and **b** are the results of the PCA for the site measures, **c** and **d** for the corridor measures. The percentages on the axes show the amount of variation that is explained by the first (x-axis) and second principal component (y-axis).

Discussion

Studies on terrestrial movements of amphibians among ponds concluded that spatial units larger than single ponds are necessary for the persistence of amphibian populations (Marsh and Trenham 2001; Semlitsch 2003). Even more so, pond populations closer than some hundred meters are not demographically independent, which is why they should be treated as the same monitoring unit (Petranka 2007; Petranka et al. 2004). Because of these findings, Petranka and Holbrook (2006) concluded that amphibian populations should be considered as patchy populations rather than metapopulations. As Natterjack toads have been recognised to be amphibians with a relative large ability to move (e.g. Jehle and Sinsch 2007; Miaud et al. 2000; Sinsch et al. 2012), Smith and Green (2005) hypothesised that if Natterjack toad populations are closer than 10 km to each other, a metapopulation should be expected. As the largest distance between the studied populations was 8121 m and thereby smaller than 10 km (Table 1), the breeding ponds in the Suhre valley likely belong to a population network.

According to the expectation of Smith and Green (2005), the STRUCTURE analysis did not reveal any distinct populations (Fig. 2). Even more, none of the individuals could be assigned completely to one of the three K, also indicating that no separated population existed. The estimation of genetic parameters also did not indicate that the a priori spatially delimited populations are isolated from each other nor that they are inbred, as the pairwise F_{ST}-values were small (Table 2) and F_{IS}-values were around zero or slightly negative (Table 1). All populations, irrespective of their sizes, had similar values at each genetic parameter. Only PopA differed from the others, very likely due to the small sampling number of only three individuals (Table 1). Genetic variability (heterozygosity) is thereby at the same level as in non-isolated populations in Belgium (Stevens et al. 2006b) and Luxembourg (Frantz et al. 2009), but higher than in Denmark (Allentoft et al. 2009) or Britain where the studied populations tend to be isolated (Beebee and Rowe 2000; Rowe et al. 1998; Rowe et al. 1999).

However, the analysis in STRUCTURE showed a continuous gradient of genetic assignment from north to south (Fig. 2). The populations in the north and south (PopD and PopK, Fig. 1) had a relatively homogeneous genetic structure, while the central populations were highly heterogeneous (Fig. 2). An explanation for this pattern could be that individuals from PopD and PopK emigrate into the centre, leading to genetically heterogeneous pond populations consisting of immigrants from both source populations. In line with this hypothesis is the result of isolation-by-distance (IBD) measures, because the analysis among all populations did not reveal a clear pattern, whereas the genetic differentiation increased with distance from PopD as well as from PopK, respectively (Fig. 3). Hence, it seems that the large populations PopD and PopK are source populations. This conforms to the results of the BIMR analysis, which revealed that indeed PopD and PopK were sources, at least for the year 2013 (Fig. 4).

The analysis of node importance, i.e. the relevance of single populations for the population network, confirmed that larger populations are generally more important for the persistence of a population network than smaller ones. PopD was therefore always the most important population if population sizes were taken as node measure (Table 3). As PopD is the most import population for the network, the results of the network analysis also fit the assumption that PopD and PopK are source populations from where Natterjack toads migrate into the centre of the study area.

Many studies identified transportation infrastructures such as roads and railway lines as significant barriers for migrating amphibians (e.g. Elzanowski et al. 2009; Fahrig et al. 1995; Mazerolle et al. 2005). Additionally, Schweizer (2014) and nature conservationists (pers. comm. C. Bühler, Hintermann & Weber AG) supposed that also the canalised Suhre river acts as a barrier. However, due to the weak genetic structure among populations, one would expect that frequent migration and thereby gene flow takes place between the populations despite landscape elements presumed to be barriers. Indeed, the analyses revealed a high rate of migration (Fig. 4). In contrast to the conclusions from the literature, neither the main roads nor the Suhre river could be identified as barriers to migration in this present study. Because populations were defined due to the expected migration barriers, so that the barriers are situated between populations, we claim that individuals have crossed barriers (Fig. 4). However, we do not know the exact pathway.

Radio-tracking of individuals is the traditional, widely used method to observe migration of individuals (Jaquiéry et al. 2011; Storfer 2013). This method was already used several times to estimate the movements of Natterjack toads (e.g. Husté et al. 2006; Miaud et al. 2000; Sinsch 1988). Surprisingly, the use of genetic methods was much more successful to detect migration between the populations in the Suhre valley than the radio-tracking observation study of Schweizer (2014). Only two individuals could be observed by radio-tracking to change a population, while totally 13 individuals were detected as migrants.

The advantage of radio-tracking over genetic analyses is the possibility to measure the movements precisely (Storfer 2013). But because the positions were measured less than once per day on average in the study of Schweizer (2014), the results cannot provide a better insight on the precise migration routes. With this data at hand, it is therefore not possible to state where exactly Natterjack toads have crossed e.g. the Suhre river, if they use bridges or swim across the river. It was also not possible to see how Natterjack toads move in agricultural areas, e.g. if they migrate along dirt roads or across meadows and arable fields.

Miaud et al. (2000) observed adult Natterjack toads staying in intensive agricultural fields, and Miaud and Sanuy (2005) showed that toads in fact forage in agricultural fields, but much less frequently than expected due to the total amount of agricultural area. In contrast to these outcomes, Stevens et al. (2006a) and Stevens et al. (2006b) found, contrary to their expectations, that forested areas and bare grounds were preferred by toadlets, whereas agricultural environments were avoided. While it is plausible that a species of pioneer habitats prefers bare ground, as shown by Stevens et al. (2004), it is not obvious why they should prefer forests and avoid agriculture areas. The reason for the differences in land-use preferences between adult toads and toadlets is not clear (Stevens et al. 2006a).

However, the radio-tracking study of Schweizer (2014) showed that the Natterjack toads use agriculture areas as summer habitats in the Suhre valley. The analysis of the influence of land-use parameters further revealed that forests are negatively correlated with population trends (Fig. 5 and Table S2). This could indicate the negative influence of forested areas on population sizes and could be a reason for the decrease in population size of the northern populations that are surrounded by forested areas (Table 1 and Fig. 1). Nevertheless, most of the correlations found are given by the composition of the landscape (e.g. the negative correlation between forests and open lands). Except for the correlation mentioned above, the analysed land-use parameters do not seem to have a major impact on population size or migration rates compared to the importance of population size itself.

Also the BIMR analysis indicated that the land-use types are not relevant to explain the migration pattern.

Although no migration barrier was identified, the analysis of land-use and ecological parameters on migration corridors revealed that the amount of roads and buildings are positively correlated with pairwise F_{ST}-values. This result is an indication for the negative effect of infrastructure on migration, even though they are not strong barriers. Quite interesting is the fact that F_{ST} and *ED* were positively correlated on the PCA, suggesting that isolation-by-distance is present, although the IBD test over all connections did not result in a clear pattern.

Conclusions

The outcomes of this study are generally positive from the perspective of nature conservation at the regional scale considered here. It seems that the Natterjack toad is able to build a viable patchy population in the Suhre valley, an intensely managed agricultural area. None of the predefined populations were isolated, and no strong migration barrier could be detected as Natterjack toads seem to frequently cross the main roads and the canalised Suhre river.

Furthermore, agricultural land use does not seem to have a negative effect on the dispersal of Natterjack toad per se, at least when ephemeral breeding ponds exist and migration is not suppressed by migration barriers. On the basis of our results it is not likely that artificial ponds could be ecological traps and therefore counterproductive for the conservation of this species. However, the population network is presumably dependent on the two large source populations in the north and south of the study range (PopD and PopK). It should therefore be of high interest to protect these two large populations.

It is further worth to mention that genetic analyses were more successful and efficient regarding time and money in this study compared to the traditional radio-tracking method to measure migration. On the basis of our results, we recommend for future studies on migration to use genetic analyses if only migration between populations (gene flow) should be detected. If one is interested in spatially exact movements, we recommend conducting a radio-tracking analysis. But the interval of data recording have to be short (e.g. every 10 min by GPS transmitter), otherwise the spatial resolution will be too low to get the expected data.

Acknowledgments

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Supplementary materials

Table S1: Census data of the Natterjack toad populations in the upper Suhre valley, Switzerland (unpublished data, provided by C. Bühler, Hintermann & Weber AG).

Population	Single Years															Means		
	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	1999-2003	2004-2008	2009-2013
PopA	22	-	20	-	-	10	15	-	8	-	6	-	0	1	2	21	11	2
PopB	20	-	35	14	-	-	41	19	-	-	4	-	-	1	-	23	30	3
PopC	15	-	3	0	-	0	0	-	4	10	12	2	-	8	-	6	4	7
PopD	200	-	80	120	-	94	62	-	-	-	220	-	-	78	-	133	78	149
PopF	47	-	3	10	27	-	32	26	-	-	27	0	6	17	50	22	29	20
PopH	-	-	-	-	-	-	-	-	-	-	-	18	8	51	51	-	-	32
PopJ	5	-	0	-	-	-	-	-	-	-	-	11	4	23	19	3	-	14

Table S2: Correlation matrix of the studied Natterjack toad populations in the Suhre valley (Switzerland). **a** for population site measures and **b** for migration corridor measures. Correlations (> |0.7|) are marked in bold.

(a)

	Buildings	Roads	Standing Waters	Flowing Waters	Hedges	Slopes	Gravel Pits	Open Lands	Forests	A_R	F_{IS}	Neighbours	Mean	Trend
Buildings	1.0000	**0.8596**	-0.1759	-0.3316	-0.2376	0.2817	0.1914	-0.2769	0.1790	-0.6715	-0.6966	-0.4526	0.1524	-0.4403
Roads	**0.8596**	1.0000	-0.2015	-0.4341	-0.4132	0.5258	0.4083	-0.3885	0.1305	-0.3101	-0.3571	-0.3047	0.4169	-0.4477
Standing Waters	-0.1759	-0.2015	1.0000	-0.1696	-0.0701	0.4368	0.5814	-0.5551	0.4643	0.0317	0.0120	-0.6046	-0.0206	-0.0942
Flowing Waters	-0.3316	-0.4341	-0.1696	1.0000	0.9089	-0.4408	-0.4377	0.4210	-0.3602	0.1839	0.0772	0.2330	-0.0208	0.8292
Hedges	-0.2376	-0.4132	-0.0701	0.9089	1.0000	-0.4269	-0.4452	0.4191	-0.3672	0.2127	0.1258	0.2655	0.0264	0.8215
Slopes	0.2817	0.5258	0.4368	-0.4408	-0.4269	1.0000	0.9755	-0.9316	0.6391	-0.0485	-0.1090	-0.4288	0.4679	-0.5468
Gravel Pits	0.1914	0.4083	0.5814	-0.4377	-0.4452	0.9755	1.0000	-0.9387	0.6498	-0.0692	-0.1212	-0.5038	0.4309	-0.5200
Open Lands	-0.2769	-0.3885	-0.5551	0.4210	0.4191	-0.9316	-0.9387	1.0000	-0.8618	0.2669	0.3037	0.6451	-0.1537	0.6550
Forests	0.1790	0.1305	0.4643	-0.3602	-0.3672	0.6391	0.6498	-0.8618	1.0000	-0.4327	-0.4167	-0.6778	-0.3433	-0.7321
A_R	-0.6715	-0.3101	0.0317	0.1839	0.2127	-0.0485	-0.0692	0.2669	-0.4327	1.0000	0.9639	0.5870	0.3073	0.5054
F_{IS}	-0.6966	-0.3571	0.0120	0.0772	0.1258	-0.1090	-0.1212	0.3037	-0.4167	0.9639	1.0000	0.6225	0.2252	0.4133
Neighbours	-0.4526	-0.3047	-0.6046	0.2330	0.2655	-0.4288	-0.5038	0.6451	-0.6778	0.5870	0.6225	1.0000	0.2882	0.4485
Mean	0.1524	0.4169	-0.0206	-0.0208	0.0264	0.4679	0.4309	-0.1537	-0.3433	0.3073	0.2252	0.2882	1.0000	0.2020
Trend	-0.4403	-0.4477	-0.0942	0.8292	0.8215	-0.5468	-0.5200	0.6550	-0.7321	0.5054	0.4133	0.4485	0.2020	1.0000

(b)

	Buildings	Roads	Railway Lines	Standing Waters	Flowing Waters	Hedges	Slopes	Gravel Pits	Open Lands	Forests	Distance	F_{ST}	Migrants
Buildings	1.0000	0.7672	0.3208	0.0829	-0.2281	-0.0611	0.3171	0.1977	-0.5583	0.4535	0.6404	0.7291	-0.2767
Roads	0.7672	1.0000	0.1800	-0.0506	-0.4368	-0.2034	0.5288	0.3814	-0.5147	0.3824	0.7398	0.8548	-0.3029
Railway Lines	0.3208	0.1800	1.0000	0.0327	-0.1335	-0.2159	0.0623	0.0440	-0.3659	0.3791	0.3828	0.3763	-0.0813
Standing Waters	0.0829	-0.0506	0.0327	1.0000	-0.3235	-0.1335	0.2163	0.2949	-0.5677	0.6077	0.0796	-0.0533	-0.0727
Flowing Waters	-0.2281	-0.4368	-0.1335	-0.3235	1.0000	0.8747	-0.5572	-0.6135	0.5520	-0.5291	-0.3736	-0.2391	0.0083
Hedges	-0.0611	-0.2034	-0.2159	-0.1335	0.8747	1.0000	-0.2691	-0.3864	0.5193	-0.5052	-0.2628	0.3069	-0.0678
Slopes	0.3171	0.5288	0.0623	0.2163	-0.5572	-0.2691	1.0000	0.9646	-0.5636	0.4124	0.3213	0.3563	-0.3353
Gravel Pits	0.1977	0.3814	0.0440	0.2949	-0.6135	-0.3864	0.9646	1.0000	-0.5052	0.3563	0.2469	0.1489	-0.3405
Open Lands	-0.5583	-0.5147	-0.3659	-0.5677	0.5520	0.5193	-0.5636	-0.5052	1.0000	-0.9731	-0.5593	-0.5085	0.4209
Forests	0.4535	0.3824	0.3791	0.6077	-0.5291	-0.5052	0.4124	0.3563	-0.9731	1.0000	0.4949	0.4265	-0.3496
Distance	0.6404	0.7398	0.3828	0.0796	-0.3736	-0.2628	0.3213	0.2469	-0.5593	0.4949	1.0000	0.8891	-0.3030
F_{ST}	0.7291	0.8548	0.3763	-0.0533	-0.2391	0.3069	0.3563	0.1489	-0.5085	0.4265	0.8891	1.0000	-0.3615
Migrants	-0.2767	-0.3029	-0.0813	-0.0727	0.0083	-0.0678	-0.3353	-0.3405	0.4209	-0.3496	-0.3030	-0.3615	1.0000