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RESEARCH ARTICLE

No distinct barrier effects of highways and a wide river on the genetic structure of the Alpine newt (*Ichthyosaura alpestris*) in densely settled landscapes

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Abstract
Linear landscape elements such as roads, railways and rivers have been shown to act as barriers to dispersal and gene flow, hence impeding functional connectivity and increasing genetic differentiation between individuals or populations on opposite sides of the barrier. Such putative barriers act through a confluence of mechanisms, including crossing mortality, barrier avoidance and modifications to organisms' effective dispersal patterns. Small, terrestrial animals such as amphibians are predicted to be vulnerable to the effects of such barriers given their limited locomotive performance and their dependence on spatially distinct breeding habitats. Here, we examined the effects of highways and a wide river on *Ichthyosaura alpestris* in three regions of northern Switzerland by measuring the genetic differentiation between local populations and describing the spatial genetic structure. Moreover, we estimated effective population sizes as an indicator for the susceptibility of populations to random genetic drift. Based on genetic differentiation, we found evidence to suggest that the highways and river acted as barriers to gene flow for the newt in the study regions, but results were inconsistent when ignoring breeding ponds with low samples sizes. Admixture-based genetic clustering suggested the delineation of the genotypes to rough regional clusters, with only weak structure inferred within these clusters. Thus, results suggest that at present, highways and rivers do not substantially affect the genetic structure of *I. alpestris* within northern Switzerland in a negative manner. Alternatively, the lack of a distinct genetic structure in regional newt populations may be explained by, e.g., large effective population sizes.

Keywords Amphibians · Barrier effect · Functional connectivity · Gene flow · Landscape genetics

Introduction

The detection of genetic boundaries can elucidate patterns of population connectivity, identify barriers to gene flow and help resolve population structure (Safner et al. 2011). Correlation may be inferred between genetic boundaries, gene flow and the composition and spatial configuration of the landscape elements (Holderegger and Wagner 2006; Manel et al. 2003; Storfer et al. 2007). It has been shown that landscape elements such as rivers and roads can act as barriers to dispersal and gene flow in natural populations, impede functional connectivity and increase genetic differentiation between individuals or populations on opposite sides of the barrier (Balkenhol and Waits 2009; Burkart et al. 2016; Hepenstrick et al. 2012; Holderegger and Di Giulio 2010). Such putative barriers act through a confluence of mechanisms, including crossing mortality (e.g. road collisions), barrier avoidance (behavioural response), physical impasse and a modification to the organisms' effective dispersal patterns (Corlatti et al. 2009 and references therein). Conversely, barrier effects may be alleviated by infrastructure that span the barrier such as bridges, underpasses and other structures that facilitate barrier crossing (Corlatti et al. 2009; Lesbarrères et al. 2004; Lesbarrères and Fahrig 2012). Barrier effects may also be relieved by high dispersal ability and high effective population size, N_e, which mitigate genetic drift (Gauffre et al. 2008). Yet, it is often unclear how different landscape elements influence gene flow in

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natural populations, which often necessitate direct investigations into population genetic structure.

It is vital to form predictions of the effects of landscape elements on gene flow and functional connectivity as transportation infrastructures such as roads, railroads and canals have become increasingly ubiquitous features in contemporary landscapes (Balkenhol and Waits 2009). Small, terrestrial animals such as amphibians are predicted to be negatively affected by such barriers by virtue of their often limited locomotive performance and often discrete habitats, particularly so in regard to breeding site fidelity. Landscape elements and land cover defining the landscape matrix are expected to influence the dispersal between discrete (breeding) sites of amphibians (Van Buskirk 2012), as is the spatial distribution of the populations (Vaupel et al. unpublished results). Nascent effects of genetic differentiation and isolation, however, take time to build up and be detectable. It has been estimated that around 15–20 generations are needed for signals of genetic effects to be observable in the genetic structure (Holderegger and Di Giulio 2010; Landguth et al. 2010; but see; Murphy et al. 2008). A caveat is that the genetic structure inferred is a snapshot of response to past landscape configuration and composition rather than to contemporary ones, due to the time lag between causal processes and genetic response. Given that a fair period of time has passed since the construction of many major transport infrastructure, signals may now be expected in the genetic structure of populations that reflect effects from these elements, contingent upon the target species' generation time.

The Alpine newt (*Ichthyosaura alpestris*) is a common amphibian with frequent but limited dispersal behaviour, with short generation times (2–3 years) and that breeds annually in discrete breeding ponds, making it a promising species for which to test the effects of different landscape elements on genetic structure. Previous studies have shown mixed evidence for a genetic barrier effect from highways on this species. Van Buskirk (2012) detected a significant reduction in gene flow in populations of *I. alpestris* in the presence of roads and divided highways in north-eastern Switzerland (though no effect from an intervening river), whereas Prunier et al. (2014) found no such negative genetic effect in eastern central France. Similar mixed evidence has been found for a number of other animal species, including amphibians, voles and larger carnivores with equivocal effects of putative barriers on genetic differentiation (reviewed in Holderegger and Di Giulio 2010). Given this lack of consensus, little can be presently predicted of the genetic and population effects of roads and rivers on *I. alpestris*.

Within a conservation context, determining the effect these landscape elements may have on gene flow is critical for understanding population connectivity and viability, as well as for identifying key habitats for preserving genetic variation. Gene flow maintains population connectivity and evolutionary potential, which buffer against the negative effects of isolation and inbreeding, and allow for future response to environmental change. Understanding how landscape elements such as roads and rivers influence the genetics of populations can thus lead to more effective conservation and management strategies, and inform planners whether mitigation measures such as bridges and underpasses effectively facilitate gene flow across assumed barriers.

In this study, we examined the genetic effects of highways and a river on populations of *I. alpestris* in three regions of northern Switzerland (hereafter considered as meta-populations). This was achieved via (i) estimating the genetic variation among populations, as well as estimating and comparing population differentiation between population pairs situated on adjacent sides of the putative barriers against those situated on opposite sides of the putative barriers; (ii) assessing the spatial genetic structure (SGS) of the meta-populations to discern whether the spatial distribution of genotypes follows patterns of isolation by distance; (iii) inferring the genetic structure of the meta-populations by admixture-based Bayesian clustering methods, and (iv) estimating the effective population size N_e of the meta-populations.

Materials and methods

Study species

The widespread Alpine newt, *I. alpestris* (Laurenti 1768; syn. *Triturus alpestris, Mesotriton alpestris*), inhabits a large part of Central Europe and the Balkans, as well as isolated regions in the Iberian, Apennine and Balkan peninsulas. Within Switzerland, *I. alpestris* is considered an ecological generalist that occurs in a variety of habitats ranging from gardens to forests as well as pioneer sites (Emaresi et al. 2011), and it is commonly found both in the lowland and mountain regions of the northern side of the Alps (Meyer et al. 2009). In the Swiss lowland areas, *I. alpestris* generally reaches sexual maturity at around 2–3 years of age and has a life expectancy of 7–10 years, though with considerable variability in life history traits according to the local environment (e.g. elevation; Jacob et al. 2007). Two distinct, annually repeated seasons characterise adulthood in this species; the breeding season in spring and summer when newts occupy aquatic habitats, and the dormant season in autumn and winter when newts occupy terrestrial habitats for hibernation. Seasonal transitions between these two habitats are characterised by regular migrations. Average migration distances between aquatic and terrestrial habitats have been estimated to be around 400 m for related species of newt (*Lissotriton vulgaris, Triturus cristatus;* Cooke 1986;

Dolmen 1981; Griffiths 1984), and a similar figure has been applied for *I. alpestris* (Joly et al. 2001). *Ichthyosaura alpestris* moves relatively frequently between aquatic breeding sites, with more than one-third of newts found changing their aquatic habitats during the breeding season (Kopecký et al. 2010). Nevertheless, the majority of Alpine newts demonstrate relatively high fidelity to their breeding site of origin (Joly and Miaud 1989), but instances of migration > 1 km have been observed (Jehle and Sinsch 2007).

Study area and sampling

The study area was located in the densely settled Swiss Plateau in northern Switzerland and covered approximately 180 km^2 (Fig. 1). The area comprised of a mixture of forests, fertile plains, agricultural lands and settlements. Sampling was conducted in three regions: Aargau (105 km^2), Thurgau (36 km^2) and Zurich (36 km^2). Minimal distances between populations from different regions were 48.9 km (AG–TG), 44.9 km (AG–ZH) and 9.7 km (TG–ZH). Newts were sampled from artificial and natural ponds as well as from small streams, using buccal swabs (FLOQSwabs, Copan Diagnostics, Brescia, Italy; Appendix A1), in April–June 2014. Sampling sites within regions were chosen so as to be situated on either side of, and in relatively close proximity (< 4 km) to potential barriers to migration and gene flow. These barriers were: (1) a highway section (A1, opened May 1967) and the river Aare in the canton of Aargau, (2) a highway section in the canton of Thurgau (A1, opened November 1970) and (3) a highway section in the canton of Zurich (A4, opened October 1973; information from Swiss Federal Roads Office). Highways were ca 20–30 m wide, while the width of the river Aare in Aargau varied between 50 and 150 m. Other putative barriers occurring in the study regions were not specifically assessed. In a total of 102 locations, 20 newts per site were sampled where possible; in sites with < 20 newts, sample number was determined by the site's availability of newts (Table S1). Of the final sample set, we excluded all

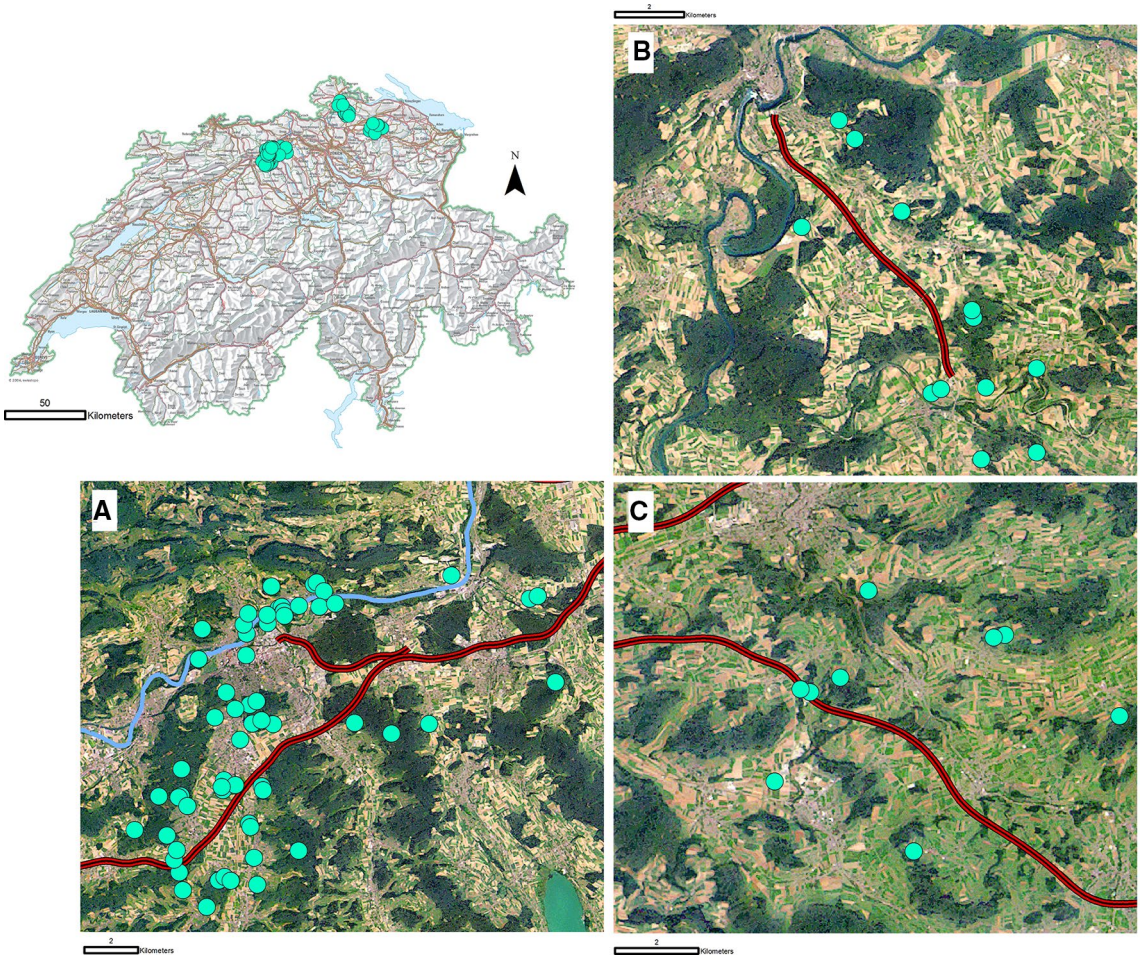

Fig. 1 Aerial photos of the study areas in northern Switzerland (top left). Samples of *I. alpestris* were obtained from discrete breeding sites (light green dots) which were situated in close proximity to a putative barrier, namely a highway (highlighted in red) or a river (coloured light blue). **a** Aargau, **b** Thurgau, **c** Zurich

sites with < 5 individuals from the analyses except in STRUCTURE and TESS (see also below). Notably, our sampling did not consider all occurrences in a given region, owing to the high abundance of available breeding sites.

Genetic analysis

DNA extraction and genotyping

DNA was extracted from all samples using QIAGEN QIAmp 96 DNA Blood Kit (QIAGEN, Hilden, Germany) according to Frei et al. (2016). Spectrophotometric analysis (BioPhotometer, Eppendorf, Hamburg, Germany) on a sub-sample ensured quantity of eluted DNAs. DNA was amplified at 12 di- and tetra-nucleotide nuclear microsatellite loci in two sets of multiplex PCRs with fluorescently labelled primers (Table S2; Appendix A2). To check for correct amplification and for quality control, a positive and a negative control were added to each PCR plate. PCR fragments were run through an ABI3130 (Applied Biosystems) automated capillary sequencer, and alleles were visually scored using GeneMapper 5.0 (Applied Biosystems).

Genetic structure

To assess the effect of the highways and the river on the genetic structure of *I. alpestris*, we considered each (breeding) pond to represent a population. With two exceptions, all populations were separated by a minimum distance of 100 m. Prior to analyses of population differentiation and structure, we tested our loci for deviation from Hardy–Weinberg equilibrium (HWE; exact test with 1,000,000 Markov chain steps and 100,000 dememorisation steps) and for linkage disequilibrium (LD) between all possible pairs of microsatellite loci (likelihood-ratio test with 16,000 permutations and five random initial conditions for the EM algorithm runs) in Arlequin 3.5 (Excoffier and Lischer 2010). Indices of genetic diversity were calculated in SPAGeDi 1.5 (Hardy and Vekemans 2002) and Arlequin 3.5. Analysis of molecular variance (AMOVA) was performed with Arlequin 3.5. Global as well as locus-by-locus estimates were calculated and tested for significance (p value to within $\delta = 0.01$ with 99% confidence) via 16,000 permutations. F-statistics (inbreeding coefficient F_{IS} and F'_{ST}) (standardized F_{ST} via AMOVA; hereafter referred to simply as F_{ST}) were calculated in GenAlEx 6.501 (Peakall and Smouse 2012). F_{ST} significance was tested against 9999 permutations of the individuals between populations. To test if pairwise F_{ST} values of sites located on the same side of a putative barrier (mean $F_{ST-ADJACENT}$) were smaller than pairwise F_{ST} values of sites located on opposite sides of the putative barrier (mean $F_{ST-OPPOSITE}$), we employed a generalized linear mixed-effects model fitted with Markov chain Monte Carlo (MCMC) techniques. Tests were run in R 3.3.2 (R Core Team) using the MCMCglmm package 2.24 (Hadfield 2010) with 500,000 burnins, 2,000,000 MCMC iterations and a thinning interval of 500. We included with/without barrier (1/0) as a fixed effect, as well as pairwise geographic (Euclidean) distance as a second predictive variable to factor out the effect of isolation by distance. As a random effect, we integrated a matrix in the model that described the structure of non-independence in the data. In other words, this random effect matrix accounts for a possible bias in the data because each population out of the n populations affects all $n-1$ pairwise F_{ST} values in which it is involved. The code for the analyses is available in Appendix A3.

These analyses were performed in order to test for the effect of the highway as a barrier separately in all three regions (to avoid pairwise comparisons across regions) and for the effect of river Aare in Aargau. In the latter case, we excluded populations south and east of the highway so as to avoid the confounding effect of the regional highway. Moreover, we removed two populations located on island-like areas surrounded by river arms on both sides. We ran additional analyses including only breeding ponds with ≥ 9 and ≥ 15 individuals to see if our results were robust even with low sample numbers per breeding pond. These subsets left us with 54/39 of 63 sites in Aargau, 7/7 of nine sites in Thurgau and 9/6 of 12 sites in Zurich; for the test on the effect of river Aare, we remained with 37/24 of 44 sites.

To account for potential confounding distance effects in the clustering results (François and Durand 2010; Guillot et al. 2009), IBD was assessed prior to cluster analyses. To avoid population hierarchical effects in our IBD and SGS analyses (Meirmans 2012), we performed pairwise comparisons separately for every region. While this approach reduces the scale and statistical power for identifying SGS, it avoids having the signal for IBD being confused with the signal for large-scale hierarchical population structure.

Spatial genetic structure was investigated using (1) permuted regression analyses of pairwise genetic differentiation on geographic distance and (2) spatial autocorrelation analyses, performed between all pairs of populations within region, both implemented in SPAGeDi. To test the significance of the regression analyses, the regression slope of pairwise differentiation (F_{ST}) on distance (or *log* distance) was tested against the null hypothesis that F_{ST} and distance (or *log* distance) were uncorrelated (i.e. no SGS). This was achieved through the creation of a null distribution formed by randomly permuting populations' spatial location 9999 times, to obtain a 0.1% significance level. For spatial autocorrelation analysis, F_{ST} estimates were averaged over a set of distance classes, d, and plotted against geographical distance (or *log* distance). The 19 distance classes were determined to ensure a roughly equal number of pairwise comparisons within each distance

class. The significance of the spatial autocorrelation was calculated via 9999 randomised permutations of population locations to obtain a 0.1% significance level. Standard errors for multilocus estimates of F_{ST} and regression slopes were obtained in SPAGeDi by jack-knifing the data over loci. In addition to using standard F_{ST}, IBD was also calculated using the linearized F_{ST} of Slatkin (1995), $F_{ST}/(1-F_{ST})$. This estimator for pairwise differentiation was used for it is expected to vary linearly with *log* distance under IBD patterns in two-dimensional habitats (and with distance in one-dimensional linear habitats; Rousset 1997).

To define the genetic structure and infer genetic boundaries, we employed STRUCTURE 2.3.4 (Pritchard et al. 2000) and TESS 2.3 (Chen et al. 2007; Durand et al. 2009), the latter incorporating exact spatial coordinates in the Bayesian prior distribution. Admixture-based models were used for both approaches.

STRUCTURE was implemented for a range of $K=1$–10 (20 runs; 1,000,000 steps; 100,000 steps burn-in). Run convergence was assessed by examining α, F and likelihood values. Independent and correlated allele frequency models were tested with and without sampling locations set as prior information (LOCPRIOR; Hubisz et al. 2009). CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) was used to permute and match cluster outputs from independent runs. Estimation of K was achieved (1) by selecting the $ln\Pr(D|K)$ with the highest value (Pritchard et al. 2000), and (2) by examining the bar plot of admixture proportions across values of K. Measure (1) was assessed using STRUCTURE HARVESTER (Earl and vonHoldt 2012), while measure (2) was achieved via DISTRUCT (Rosenberg 2004).

TESS was implemented for K_{max}=2–10 (20 runs; 50,000 sweeps; 10,000 sweeps burn-in). TESS does not allow for assessing a K_{max}=1 and additionally implements a value called K_{max} rather than K; this refers to the assumed maximum number of clusters which may be equal or larger than the true number of clusters K. Convergence was assessed on the basis of likelihood values and individual admixture proportions. The spatial interaction parameter ψ, which defines the strength of the spatial autocorrelation, was iteratively revised by the model, with a default initial starting value of 0.6. Both the CAR and BYM models were tested, as well as models with varying degrees of trend surfaces (0-nonspatial and 1-spatial). For estimation of K_{max}, we considered (1) the K_{max} for which the deviance information criterion (DIC) starts to plateau in a plot of DIC against K_{max} (Durand et al. 2009), (2) the K_{max} value with the highest associated DIC value (François et al. 2008), and (3) the bar plot of admixture proportions across values of K_{max}. Outputs of admixture proportions from independent runs were permuted and matched with CLUMPP before being visualised as bar plots in DISTRUCT.

Estimation of effective population size

Effective population size was estimated using three different single-sample estimators: (1) a LD method (Waples and Do 2008), (2) a heterozygote-excess method (Zhdanova and Pudovkin 2008) and (3) a molecular coancestry method (Nomura 2008). All three of these methods estimate the current or short-term effective population size N_e, or more specifically, the effective number of breeders N_{eb} of a cohort from which the sample was produced for a particular period. Estimations were performed via NeEstimator 2.01 (Do et al. 2014) and calculated with parametric chi-squared and/or jackknifed 95% confidence intervals. To balance the precision–bias trade-off of the methods, we excluded rare alleles with frequency p_{crit} <0.02 (Waples and Do 2010). The LD method was implemented with the assumption of random mating; all other options were left as default. In addition to calculating N_e using data from all individuals, N_e was also calculated based on a restricted random subset of 100 individuals per meta-population to assess the effects of sample size on the N_e estimates.

Results

Basic population genetic analyses

Buccal swabbing generally provided large amounts of DNA per sample (mean: 127.6 ng/µL; range 12.5–358 ng/µL). Positive controls in the PCR plates were used to calculate an estimated allele call error rate of 1.04%. Negative controls returned negative calls in 100% of cases.

All loci across all populations conformed to HWE, with the exception of three instances out of 1224 comparisons (12 markers × 102 populations) that showed significant departure from HWE ($p<0.05$ β; β = Bonferroni correction factor); all markers were thus retained in further analyses. Significant linkage disequilibria ($p<0.05$) were identified in 4.71% of 6732 possible population-specific linkage pairs, with the CopTa14–CopTa9 and CopTa2–CopTa3 marker pairs having the highest incidences of significant LD at 12.75 and 9.8% incidence, respectively.

Genetic diversity and population differentiation

All regions showed negligible evidence for non-random mating, F_{IS}, and similar levels of allelic richness and gene diversity (Table 1). H_e was marginally higher than H_o in all three regions. Global F_{ST} values for all three regions were low (0.016–0.022). Pairwise F_{ST} between regions was an order of magnitude higher for Aargau–Zurich ($F_{ST}=0.037$; $p<0.001$) and Aargau–Thurgau ($F_{ST}=0.049$; $p<0.001$) than for Zurich–Thurgau ($F_{ST}=0.005$; $p<0.001$). Means

Table 1 Gene diversity indices for regional samples of *I. alpestris*

	n	*NA*	NA_E	A_R	H_E	H_O	F_{ST}	F_{IT}	F_{IS}
Aargau	982	12.75	4.95	3.65	0.62	0.57	0.022***	0.082***	0.061***
Zurich	171	9.67	5.20	3.75	0.63	0.59	0.014***	0.069***	0.056***
Thurgau	146	10.17	5.91	3.82	0.64	0.59	0.016***	0.082***	0.067***

n Sample size (individuals), *NA* number of alleles, NA_E effective number of alleles, A_R allelic richness (expected number of alleles among 10 gene copies), H_E gene diversity (corrected for sample size), H_O observed heterozygosity, F_{ST} fixation index, F_{IT} overall fixation index, F_{IS} inbreeding coefficient

p Values are of the form *p*(2-sided test, H1: observed <> expected): ***$p < 0.001$

of all pairwise F_{ST} between sites located on opposite sides of a putative barrier (highway and river), $F_{ST-OPPOSITE}$, was slightly but significantly ($p < 0.05$) higher than mean pairwise F_{ST} between sites located on the same side of the putative barrier, $F_{ST-ADJACENT}$, when considering the highways in Aargau and Zurich (not significant for Thurgau), and also when considering the river Aare (Table 2). The distance effect was highly significant ($p < 0.001$) in all analyses except for Thurgau (Table 2). However, results on the barrier effect partly changed when including reduced data sets (only breeding ponds with ≥ 9 or ≥ 15 individuals), while distance effects consistently remained significant (Table 2): In Aargau, the effect of the highway as a barrier was non-significant at the medium sample size, but highly significant with only large sample sizes ≥ 15. In Thurgau, the non-significant highway effect remained for the reduced data set. The effect of the highway on F_{ST} in Zurich was highly significant for the medium-size sample set, but marginally significant when including only sites with largest sample sizes. Finally, river Aare did not significantly affect pairwise F_{ST} when testing fewer sites with higher sample numbers.

AMOVA indicated that 89% of the genetic variance was explained by within-individual variance, with only 4% variance attributed to among-region, 2% to among-population within region and 5% to among-individual within population components. Ignoring the within-individual level, 94% of total genetic variance was attributed to within-population variance. AMOVA performed for the Zurich and Thurgau dataset (Aargau excluded) indicated a much lower proportion of genetic variance explained by among-region variance (0.35%) compared to the three-region AMOVA.

Spatial genetic structure and IBD

From the tests of linear regressions with spatial permutations, we observed that for all three regions, the regression slopes approached zero and the coefficients of determination were low (Fig. 2a–c). In addition, the results from Aargau and Thurgau lacked significance. Plots and regression parameter values were almost identical for regressions calculated with F_{ST} to those calculated with $F_{ST}/(1 - F_{ST})$, a consequence of the low F_{ST} values (data not shown). Similar

Table 2 Comparisons of mean F_{ST} for population pairs of *I. alpestris* situated on opposite sides of putative barriers ($F_{ST-OPPOSITE}$) with those situated on adjacent sides ($F_{ST-ADJACENT}$)

Region	Effect	All samples (n > 5)		Reduced data sets			
				n ≥ 9		n ≥ 15	
		Post mean	*p* value (MCMC)	Post mean	*p* value (MCMC)	Post mean	*p* value (MCMC)
Aargau	Highway	0.00136	0.043*	0.00080	0.295	0.00282	< 0.001
	Distance	9.898×10^{-7}	< 0.001	1.202×10^{-7}	< 0.001	6.791×10^{-7}	< 0.001
	River Aare	0.00287	0.022*	0.00185	0.212	0.000953	0.627
	Distance	1.055×10^{-6}	< 0.001	1.431×10^{-6}	< 0.001	1.120×10^{-6}	< 0.001
Thurgau	Highway	−0.00181	0.713 ns	−0.00290	0.629 ns	(Same populations/results as for n ≥ 9)	
	Distance	1.721×10^{-6}	0.159 ns	2.381×10^{-6}	0.127 ns		
Zurich	Highway	0.00883	0.022*	0.0149	< 0.001	0.01028	0.094
	Distance	2.306×10^{-6}	< 0.001	1.257×10^{-6}	< 0.001	2.620×10^{-6}	0.039

Models included the presence of a putative barrier and geographic distance as well as the structure on non-independence in the data as explanatory variables to explain their effects on $F_{ST-OPPOSITE}$ vs. $F_{ST-ADJACENT}$. Results are given for all samples as well as for reduced data sets comprising populations with n ≥ 9 and n ≥ 15. Marginal posterior means of the Bayesian models (post.mean) and levels of significance from generalized linear mixed-effects models with Markov chain Monte Carlo (MCMC) techniques are given

ns not significant

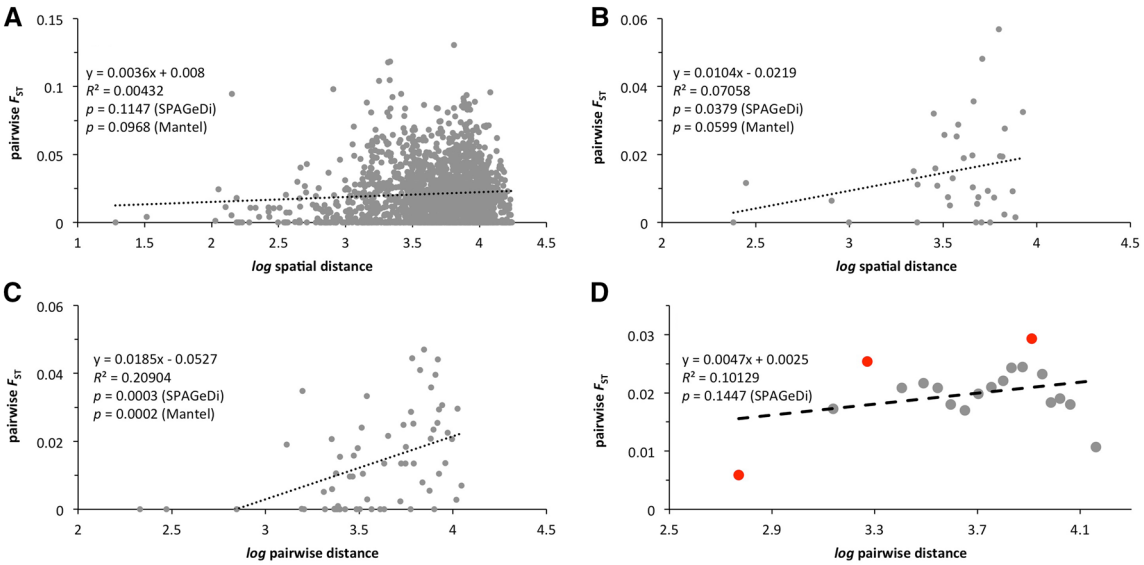

Fig. 2 Plots of *log* distance against F_{ST} in the Alpine newt (*I. alpestris*) for the regions of **a** Aargau, **b** Thurgau and **c** Zurich. Significance estimates from spatial permutations (SPAGeDi; Hardy and Vekemans 2002) are indicated in the plot. **d** Correlogram expressing the autocorrelation of mean F_{ST} vs. *log* pairwise distance (mean over distance class), in Aargau. Red dots mark significant mean F_{ST} values; grey dots mark non-significant mean F_{ST} values. Regression slope and R^2 values are indicated for all plots. This analysis was only done for Aargau because of low sample numbers in Thurgau and Zurich

to the results from above, the regression slope of the autocorrelation analysis in Aargau approached 0 and exhibited a low R^2 (Fig. 2d). Mean values of F_{ST} were significant only for the 1st (0.00–1.05 km), 3rd (1.59–2.16 km) and 14th (7.87–8.49 km) distance classes; the other distance classes together with the overall (positive) regression slope were non-significant (Table S3). Spatial autocorrelation analysis was not run for the Thurgau and Zurich datasets because of too few samples.

Of the models tested in STRUCTURE, only the correlated allele frequency model with LOCPRIOR (*CorLOC*) was sensitive enough to recover a signal of within-region genetic structure. Of those tested, the $K=3$ model had the highest lnPr(D|K) (Fig. S1). Assessing the bar plot of admixture proportions for $K=2$–4, we found that whilst a third cluster added structure to the Aargau region, a fourth cluster did not contribute much further signal. We thus considered $K=3$ to be the best estimate for our overall dataset, with a clear signal separating the Aargau meta-population from the Thurgau and Zurich meta-populations, and a much weaker signal separating the Aargau meta-population into two smaller (sub-)clusters (Fig. S2a). The Thurgau and Zurich meta-populations were recovered as one homogenous cluster in this analysis.

To assess finer (sub-)structure within the study regions, we performed STRUCTURE on the regions separately (Aargau and Zurich + Thurgau). Cluster analysis (*CorLOC* model) on the Aargau dataset produced results similar to that from the full dataset analysis, with the $K=2$ model producing the highest lnPr(D|K) (Figs. 3a, S2b), suggesting no finer sub-structure was discernable. Notably, cluster assignment in this region followed a North–South gradient across the entire sampling range (Fig. 3a). However, cluster analysis for the Thurgau + Zurich dataset uncovered additional sub-structure at $K=2$, supported by the estimation that this model ($K=2$) was the model with the highest lnPr(D|K) for this dataset (Figs. 3b, c, S2c).

Of all algorithms and parameter combinations tested in TESS, only the BYM model with trend of degree = 1 recovered any signal of genetic structure (for the full dataset). Additional (within-region) structure was nonetheless recovered when TESS was run for the Aargau region and Thurgau + Zurich region separately. The bar plot of admixture proportions for Aargau ($K=2$; Fig. S2d) was similar to that inferred under the *CorLOC* model by STRUCTURE (Fig. S2b). The results for the Thurgau + Zurich analysis (Fig. S2e) were however characteristically different from those inferred via STRUCTURE (Fig. S2c). DIC values for the spatial (trend of degree = 1) and non-spatial (trend of degree = 0) models were more or less identical (Figs. S3b, c) for the regional datasets and offered no hint at which models or K values were optimal. Similarly, no clue could be garnered from the plots of DIC vs. K_{max}, which showed no distinct plateaus in the curves (Figs. S3b, c). We therefore conferred to qualitative inspection of the bar plot of admixture proportions across values of K to inform our estimation of K. Given that no significant additional structure was recovered with $K=3$, we

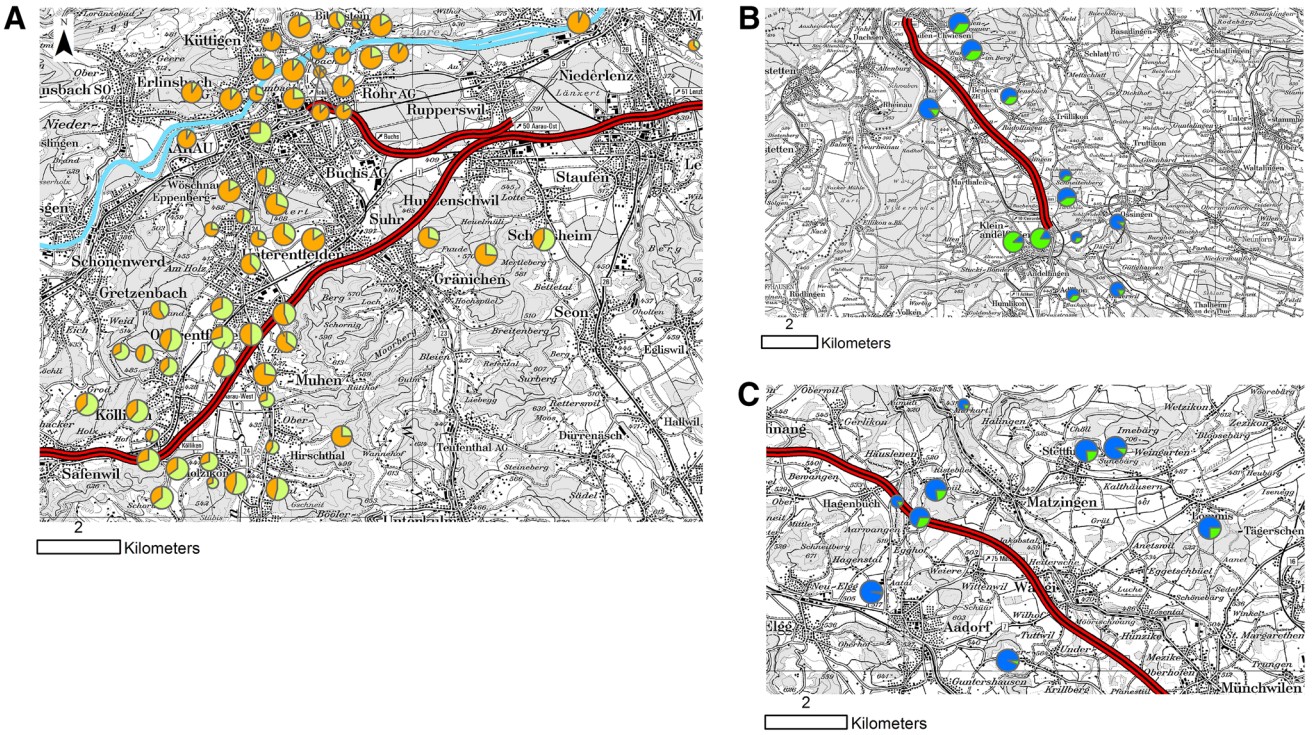

Fig. 3 Spatial distribution of ancestry proportions in populations of the Alpine newt (*I. alpestris*) in Aargau (**a**), Thurgau (**b**) and Zurich (**c**). Each pie chart represents a breeding site. The sizes of the pie charts are proportional to the number of individuals sampled in that population (note that only populations with sample sizes n ≥ 5 are displayed). The colours of the clusters follow from Fig. S2. Highways are highlighted in dark red and the river Aare is coloured light blue. Light grey indicates forests and white is open land, while black represents settlements, minor roads and railways

suggest $K = 2$ to be the best estimate for both the Aargau and Thurgau + Zurich meta-populations.

Effective population size

The effective population size N_e for Aargau was estimated to be either very large (> 1000) or infinite (signal approaches 0) according to all three N_e estimation methods tested, both when estimated using all individuals and a smaller random subset of 100 individuals (Table 3). For Thurgau + Zurich (considered one meta-population given results above), N_e was also estimated to be very large (> 1000) or infinite according to the tested methods, with the exception of the molecular co-ancestry method, which estimated a finite and moderately sized N_e of 435 (when estimated using all individuals) and 141 (when estimated using a subset of 100 individuals; Table 3). Confidence intervals were infinite in most cases, but where finite (and bounded), describing large ranges suggesting a lack of precision.

Discussion

We found no strong evidence to support the hypotheses that highways and a wide river acted as significant barriers to gene flow for *I. alpestris* in our three study regions. The assessed meta-populations seemed well-mixed within their regional boundaries and possessed large effective population sizes. Genetic structure suggested little or no restriction in genetic exchange, or increase in functional isolation, between the populations within the assessed regions. Alternatively, the large effective population sizes of the newt's meta-population may have hitherto buffered substantial genetic differentiation due to barrier effects of the highways since their establishment, so that the result of reduced gene flow and consequently the effects of random genetic drift are not yet apparent.

Results from our complementary analyses suggested the presence of two main clusters that each characterise a regional meta-population: Aargau and Thurgau + Zurich. Zurich and Thurgau genotypes were found to be virtually

Table 3 Estimates of effective population size N_e, calculated according to the linkage disequilibrium, heterozygote excess and molecular co-ancestry methods

	Linkage disequilibrium	Heterozygote excess	Molecular co-ancestry
All individuals			
Aargau			
Number of individuals	1019	1019	1019
Harmonic mean sample size	1007	1012	1012
Estimated N_e (confidence interval; 95%)	2222 (1455–4252)	Infinite (infinite–infinite)	Infinite (infinite–infinite)
Zurich/Thurgau			
Number of individuals	321	321	321
Harmonic mean sample size	316	319	319
Estimated N_e (confidence interval; 95%)	Infinite (3635–infinite)	Infinite (infinite–infinite)	435 (0.4–2184)
100-Individual subset			
Aargau			
Number of individuals	100	100	100
Harmonic mean sample size	99	99	99
Estimated N_e (95% confidence interval)	Infinite (307–infinite)	Infinite (infinite–infinite)	Infinite (infinite–infinite)
Zurich/Thurgau			
Number of individuals	100	100	100
Harmonic mean sample size	98	99	99
Estimated N_e (confidence interval; 95%)	5924 (461–infinite)	Infinite (infinite–infinite)	141.2 (0.1–709)

N_e (More specifically N_{eb}) was estimated from the mean harmonic sample size, which is the weighted mean sample size across loci whose weights are based on the number of alleles (Peel et al. 2013), to account for missing data. Top table displays N_e estimates calculated using all sampled individuals; bottom table on the right displays N_e estimates calculated using a restricted random subset of 100 individuals. All estimates were calculated using a p_{crit} value of 0.02

indistinguishable according to these analyses. Each of the two main clusters could be further decomposed into two sub-clusters, which described weak within-region sub-structuring of genotypes. The recovery of genetic structure only with the inclusion of spatial priors (LOCPrior) in STRUCTURE and a spatial trend surface (trend of degree = 1) in TESS suggest that the spatial signal of the data was informative. Inferences of population structure by TESS and STRUCTURE were similar between regions and for Aargau (Fig. S2), however noticeably different for the Zurich and Thurgau regions, which we suggest may be due to the weak signal of structure contained within these sampling populations. STRUCTURE seemed overall more sensitive towards discriminating signals of genetic structure than TESS in our analyses.

Similar ancestry proportions on either sides of the highways and river for the Aargau meta-population (Fig. 3a) reflect that neither the river nor the highway seemed to observably affect the ancestry proportions, suggesting that neither element exhibited a perceivable barrier effect. The only spatial pattern that could be evoked was a weak North–South cline in the cluster composition, which could not be confidently associated with any of the assessed putative barriers. We propose this cline may be an effect of urban settlement density, which appeared to follow a similar North–South gradient in Aargau. Urban settlement areas (density) have been shown to exhibit negative effects on gene flow in *I. alpestris* (Emaresi et al. 2011; Van Buskirk 2012), but a similar gradient has been observed in the common toad in an agriculture-dominated study area (Frei et al. 2016). While no support could be made for the highways or river acting as a strong barrier to dispersal and gene flow, there was some evidence to suggest a minor barrier effect. In the Zurich meta-population, a perceivable difference appeared in the ancestry proportions for populations on opposite sides of the highways (Fig. 3c), potentially indicative of a barrier effect. A barrier effect, after factoring out the geographic distance and non-independence of data, was also alluded to by the comparison of pairwise F_{ST} values which showed that pairs of populations on the same side of a highway in Aargau and Zurich were genetically less differentiated than if they occurred on opposite sides (not significant for Thurgau) and the same holds for the river Aare. Our confidence in these results was however compromised due to the partly inconsistent outcomes when analysing reduced data sets (excluding breeding sites with low sample sizes; Table 2). There are several reasons for these partly conflicting results. Considering only breeding ponds with large sample sizes yields more precise estimates of allele frequencies. In turn, removing sites from the data, coupled with an uneven distribution of sites relative to the putative landscape barrier, reduces statistical power. Noteworthy, the region that was most densely

sampled in our study, Aargau, showed the most prominent changes in how we evaluated the effect of the highway as a landscape barrier. Moreover, a study system with a generally low level of genetic differentiation, as found in the Alpine newt in the three study regions, is likely to be sensitive to sampling issues. We conclude that such effects as described above, among other reasons, may have caused inconsistencies among previously published studies, as reviewed in Holderegger and Di Giulio (2010). In consequence, we see this mixed evidence, coupled with other results presented here, as indicative of rather minor barrier effects of highways and the river Aare on the genetic structure of Alpine newt in our study areas.

The lack of a clear signal reflecting a barrier effect does not imply that no significant barrier effect was present; merely that we failed to well substantiate it. We attribute this outcome to one or a combination of the following, as detailed below: large effective population size, N_e; insufficient time for build-up of genetic signal, possibly due to higher than expected generation time; a genetic effect that was too weak to be detected by our employed methods/tools of analysis; higher than expected dispersal ability or tendency, and permeability of the assessed landscape elements to movement and gene flow, via e.g. connecting structures such as culverts, bridges and underpasses, or simply via the surface.

Estimates of effective population size N_e were very high to infinite for both of the assessed meta-populations (Aargau and Thurgau + Zurich). This result supported the general observation that the Alpine newt is common and abundant in northern Switzerland. Large N_e buffers against genetic drift and thus may explain the absence of a strong barrier effect from the highways and river. Distinguishing large populations from indeterminably large (aka infinite) ones has been shown to be particularly difficult (Waples and Do 2010) for single sample N_e estimation methods, as these methods all depend on a signal whose strength is inversely proportional to N_e. This means that these methods inevitably have high power and precision when working with small populations, but low power and precision when working with large populations. We considered the LD method (and estimates) to be more appropriate than the heterozygote excess and molecular coancestry methods as the latter two had been previously shown to exhibit greater bias and less precision than the LD method for cases when the true N_e is moderate or large in size (Do et al. 2014). All three of these N_e estimation methods share the fundamental assumption that of the four evolutionary forces (genetic drift, migration, mutation and selection), only genetic drift affects changes in allele frequency over time. This consequently assumes use of neutral markers, negligible (or very low) mutation in the time scale of the estimated N_e, and that immigration from other populations is absent. This last assumption of a single isolated population without immigration may be the least tenable of the assumptions here, for our study system and for natural populations in general.

The detection of barrier effects for a given species is also highly contingent on its life history traits (e.g. lifespan, generation time). By corollary, an observed lack of barrier effect does not necessarily imply no negative genetic consequence, but can simply be a result of insufficient time for a signal to be apparent (Hoffman et al. 2017). The highways assessed in our study were constructed between 42 and 48 years ago. At low elevations (where the study sites were located), Alpine newts have been suggested to reach sexual maturity at 2–3 years of age (approximate generation time; Jacob et al. 2007; Thiesmeier and Schulte 2010), though this was highly variable depending on geographic location and altitude (Miaud et al. 2000). Fifteen generations, as taken in this study as the approximate time after which a genetic signal for a barrier effect may be expected, corresponds to between 30 and 45 years, which overlaps the ages of the assessed highways. If the approximate time needed is beyond 42–48 years (i.e. > than age of the highways), we may not expect any detectable effect as not enough time has passed. This may be the case if the generation time of the newts is more than 3 years, or if 15 generations underestimate the time needed to acquire a genetic signal. Note, however, that Murphy et al. (2008), using a simulation study and generating genetic surfaces, were able to detect a significant effect of a landscape barrier after ≥ 5 generations, despite low levels of genetic structure. Nevertheless, we note that Van Buskirk (2012) observed a barrier effect from a highway in a similar area of northern Switzerland in his study of *I. alpestris*, though he did not indicate the exact age or section of the assessed highway, only that it had been present since the 1970s. Prunier et al. (2014) did not observe a barrier effect from a similarly aged (40 years old) highway in their study of *I. alpestris* in eastern central France, although they noted that such an effect should be detectable after 40 years based on their simulation study. Inherently related to this issue of time lag is the sensitivity of the methods and tools used in this study. A signal may have been present but not detectable given our sampling design, sample size, number and type of markers, and statistical approach used, as these factors all affect the ability and power to detect signals of genetic structure (Fogelqvist et al. 2010; Patterson et al. 2006). Evidently, it is difficult to apply a stringent, consistent sampling design in a landscape genetic study, because every study area is unique and many (landscape) features vary among regions, including the spatial arrangement of sampling sites. Hence, single studies are inherently prone to yielding individualistic results, so that only effect sizes determined via meta-analysis over a large set of independent studies may reveal general trends.

Notably, the river Aare also did not evoke a clear barrier effect in our analyses (only significant when including all sampling locations with n > 5; Table 2), even though this river has existed for millennia and hence appears as a structural landscape barrier. However, we presume that the potential barrier effect could have established over the past roughly 150 years since the river has been canalized for flooding protection and electricity production, whereas previously it was widely meandering with several smaller river arms that occasionally fell dry. Hence, the river basin may have served as breeding habitat rather than landscape barrier in this pre-industrial period. To see that even dozens of newt generations were insufficient for inducing a clear signal of genetic differentiation supports our interpretation that various factors counteracting the effects of random genetic drift were stronger than that exerted by river Aare.

Finally, an absence of a signal for a barrier effect may imply that highways and large rivers do not impede the movement and gene flow of *I. alpestris* to a detectable extent. It may be that the newts are more mobile, more capable of crossing, and have a greater tendency to utilise bridges, underpasses and culverts, than expected. It is also possible that few successful crossing events of individuals may suffice to warrant gene flow in the long term. To explicitly determine this, we suggest conducting direct observations of newt dispersal, e.g. via capture–mark–recapture, camera traps and tagging and tracking, as a measure to assess the frequency of barrier crossing. Assessing recent or contemporary gene flow, e.g. through parentage analysis or assignment tests, seems less promising because of the low level of genetic structure in the study system. If animal movement across the putative barrier is observed and is frequent, it may indicate the mechanism of gene flow across the putative barrier. If however little or no movement across the putative barrier is observed yet low genetic differentiation is still maintained, a lack of genetic differentiation may be due to the large population sizes or large, non-differentiated populations in the respective hinterland.

In addition to the effects of landscape elements such as highways and rivers, dispersal and gene flow between populations is also influenced by different land-use and land-cover types, and by the spatial distribution of the breeding sites themselves. For our study system, the spatial distribution of breeding ponds and habitable winter shelters may greatly affect how and to what extent intervening landscape elements affect gene flow. We believe that incorporating landscape configuration and composition, together with the spatial distribution of populations and also demographic descriptors, may lead to a greater ability in predicting and understanding patterns of gene flow in amphibians.

Conclusions

Our findings suggest that at present, populations of *I. alpestris* within Aargau, Thurgau and Zurich are not negatively affected by the assessed highways and a large river. It is unclear whether this result reflects a lack of barrier effect, or simply a barrier effect that is too weak to detect. The indication of a low barrier effect in Zurich may imply that for this region it is the latter, however these results were inconclusively supported. We cannot preclude the possibility that a barrier effect will emerge from these landscape elements in the future, even without further modification to these structures. Currently however, the outlook seems favourable for the Alpine newt in these regions, and there does not appear to be cause for urgent conservation concern. Nonetheless, the maintenance of networks of well (inter-)connected ponds remains crucial as a way to mitigate potential isolation. Maintaining connectivity measures such as bridges, culverts and underground passes is equally important, and should be performed concurrently with assessment measures to determine whether and to what extent these crossing structures facilitate dispersal and minimize the genetic isolation effect of highways and rivers.

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