Patterns of gene flow across multiple anthropogenic infrastructures: insights from a multi-species approach

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Abstract

Large-scale Transportation Infrastructures (LTIs; roads, railways, *etc.*) are among the main determinants of landscape fragmentation, with strong impacts on animal dispersal movements and functioning of metapopulations. Although the detection of the impacts of LTIs is now facilitated by landscape genetic tools, studies are often conducted on a single specie, while it is acknowledged that different species might react differently to the same obstacle. Multi-specific approaches are thus required to get a better overview of the impacts of human-induced fragmentation, especially in landscapes crossed by multiple LTIs whose impacts can accumulate. We surveyed two vertebrates species (the grass snake *Natrix helvetica* and the midwife toad *Alytes obstetricans*) and two insect species (the butterfly *Maniola jurtina* and the ground-beetle *Abax parallelepipedus*) in a landscape fragmented by six LTIs: a motorway, a railway, a country road, a gas pipeline, a power line and a secondary road network. Using multiple linear regressions and commonality analyses on both classical and hierarchical genetic distances computed over reduced spatial scales, we showed that 38% of the overall explained genetic variability across all species was due to LTIs. While the butterfly was seemingly not impacted by any LTI, the genetic structure of the three ground-dwelling species was mostly influenced by roads, motorway and railway. LTIs, and especially roads, mostly acted as barriers to gene flow, barrier effects accounting for 85% of the overall variance in genetic distances explained by LTIs across species. Although the power line did not affect any studied species and the

gas pipeline only impacted gene flow in the ground-beetle through forest fragmentation, other LTIs systematically affected at least two species. Importantly, we showed that some LTIs could somehow promote gene flow, embankments probably providing favourable habitats for vertebrate species. Considering the high variability in species response to LTIs, we argue that drawing general conclusions on landscape connectivity from the study of a single species may lead to counterproductive mitigation measures and that multi-species approaches are to be more systematically considered.

Key-words: connectivity; fragmentation; dispersal; individual-based; hierarchical genetic distance; commonality analysis; spatial scale; conservation

INTRODUCTION

The human-induced fragmentation of natural habitats is one of the main determinants of the global biodiversity collapse (Fahrig, 2003; Haddad et al., 2015). The most ubiquitous form of habitat fragmentation is due to large-scale transportation infrastructures (LTIs) (Forman and Alexander, 1998; Trombulak and Frissell, 2000; Balkenhol and Waits, 2009). LTIs are linear infrastructures allowing the transportation of goods, vehicles or energy, such as roads, motorways, railways, power lines, pipelines and canals. They are expending considerably, creating dense transportation networks with profound impacts on natural ecosystems (Dulac, 2013; Laurance et al., 2014). It notably deeply affects metapopulation dynamics through a reduction in population sizes in response to direct habitat degradation but also through a reduction in demographic and genetic exchanges between populations in response to a decrease in the permeability of the landscape matrix to dispersal (Balkenhol et al., 2009b). As populations become smaller and isolated, they might exhibit higher rates of inbreeding through genetic drift, resulting in an increased risk of population extinction (McCauley, 1991; Legendre et al., 1999; Fagan and Holmes, 2006). Understanding the influence of LTIs on wildlife dispersal patterns is thus of crude importance to fuel conservation policies.

The most obvious detrimental effects of LTIs on dispersal success are direct collisions with vehicles and physical crossing impediment when infrastructures are for instance fenced (Forman and Alexander, 1998; Trombulak and Frissell, 2000; Hels and Buchwald, 2001; Beebee, 2013; Barrientos et al., 2019). Most animals are affected, from small invertebrates to large mammals (Balkenhol and Waits, 2009; Fahrig and Rytwinski, 2009; Borda-de Agua et al., 2017). LTIs may also induce behavioral alterations that further affect nearby populations (Trombulak and Frissell, 2000; Ascensao et al., 2016). For example, both breeding migrations and reproductive success of anurans can be perturbed by main roads due to sound interference with males mating calls (Bee and Swanson, 2007; Troïanowski et al., 2017), in turn possibly impacting effective dispersal and thus gene flow (Ronce, 2007).

Over the past fifteen years, "molecular road ecology" has emerged as a fully-fledged discipline to thoroughly estimate landscape functional connectivity (Holderegger and Di Giulio, 2010). Building on

population genetics, landscape ecology and spatial statistic tools (Manel et al., 2003; Holderegger and Wagner, 2008; Manel and Holderegger, 2013), its objective is to elucidate how the genetic variability is influenced by LTIs and other anthropogenic obstacles, with numerous applications in species management and conservation (Segelbacher et al., 2010). However, one major limitation of such studies is that they generally focus on a single species (Balkenhol and Waits, 2009; Keller et al., 2015), while different species may actually respond differently to the same type of infrastructure. Furthermore, they also often focus on a single LTI, while multiple LTIs are commonly built next to each other because of technical and economic constraints, notably within valleys or along coastlines: although the impacts of LTIs are then expected to add up and result in a "cumulative" barrier effect, some LTIs might actually be neutral to movement or even create corridors to dispersal (Penone et al., 2012; Vandevelde et al., 2012; Bartzke et al., 2015), these antagonistic effects making the whole picture even more complex. For example, Paquet and Callagan (1996) showed that a motorway strongly impeded crossing events in wolves but that a railway and power lines located within the same study area together redirected wolves movements and thus rather acted as corridors. In the same vein, Latch et al. (2011) found that gene flow in the desert tortoise *Gopherus agassizii* was affected by roads but not by power lines. In highly fragmented landscapes, it is thus highly advisable to assess the concomitant influence of all existing LTIs using a multi-species, and ideally a multi-taxa, approach to adopt efficient conservation policies (Balkenhol et al., 2009a; Keller et al., 2015; Richardson et al., 2016; EEA, 2015).

In this study, We used recent molecular and statistical tools to assess the respective and cumulative impacts of six LTIs in four parapatric terrestrial species with contrasted life history traits (two vertebrates and two insects including a flying species, sampled in south-western France). We hypothesized that flying species would be less affected by LTIs than ground-dwelling ones and that large infrastructures carrying vehicles (roads, motorways, railways) would overall be more impactful than infrastructures carrying energy (power lines, gas pipelines). We also hypothesized that the impacts of some LTIs might accumulate to shape spatial patterns of gene flow in studied species.

MATERIAL AND METHODS

Study area and biological models

The study was carried out in the 'Périgord' region in South-Western France between Brive-La-Gaillarde and Périgueux (45°07'31.8"N; 0°58'56.9"E; Fig. 1). It is a $300km^2$ rural landscape composed of limestone plateaus including crops, mowed meadows, deciduous forests and small villages. The hydrology is limited to small streams and ponds. Altitude ranges from 91 to 294 m above sea level. Six types of LTIs are present in this study area (from the widest to the narrowest): a fenced motorway ("A89") commissioned in 2004; a low traffic single-track railway built in the 19th century; a high traffic country road ("D6089")

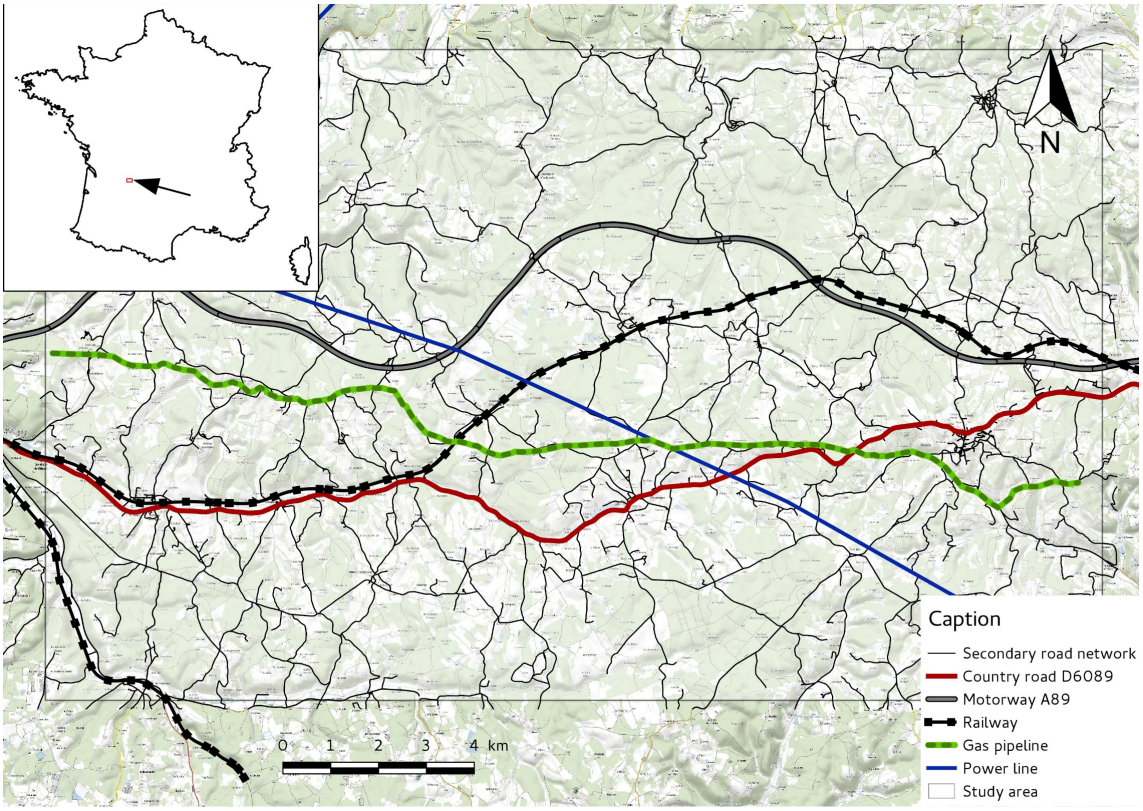

Figure 1: Study area in south-western France

present since the 18th century; a power line and a gas pipeline constructed in 1962 and 1955, respectively, both associated with breaches in forest cover; a 1370 km dense network of low traffic secondary roads (Fig. 1).

We considered four species with various life history traits in order to span a large amount of biological variability: two vertebrates (the snake *Natrix helvetica* and the midwife toad *Alytes obstetricans*) and two insects (the butterfly *Maniola jurtina* and the ground-beetle *Abax parallelepipedus*). *Alytes obstetricans* is a small toad widely distributed in Western Europe. It is highly sensitive to fragmentation because local populations are known to function as relatively independent entities with strong genetic structuring (Tobler et al., 2013; Maia-Carvalho et al., 2014; Albert et al., 2015). *Natrix helvetica* is also widely distributed in Western Europe (Kindler et al., 2017). This snake species is considered to exhibit good dispersal abilities, with individuals travelling over more than 1 km in less than a month (Pettersson, 2014). A previous study did not detect any genetic structure in this species in a intensively used agricultural landscape, indeed suggesting good dispersal ability in fragmented environments (Meister et al., 2010). *Maniola jurtina* is an univoltine butterfly which is very common in Europe with locally very high densities. It shows medium dispersal capacity with mean dispersal distances ranging from about 50 to 300 m (Schneider et al., 2003; Ouin et al., 2008; Stevens et al., 2013). Previous studies revealed that both land cover (arable lands and forests) and LTIs (motorway and railway) could affect

its dispersal (Villemey et al., 2016; Remon et al., 2018). Finally, *Abax parallelepipedus* is an opportunistic carnivorous ground-beetle (Loreau, 1983) that inhabits the upper layer of litter in forest environments (Loreau, 1987). It typically shows limited dispersal capacity with an avoidance behaviour face to open habitats (Charrier et al., 1997) and a high sensitivity to fragmentation due to roads (Keller et al., 2004).

Sampling and genotyping

For all species, tissues were collected between April and September in 2015 and 2016. For the two vertebrate species (*N. helvetica* and *A. obstetricans*), we followed an individual-based sampling design due to low abundances in the field. Individual-based sampling design has been proved to be a good alternative method to population-based sampling design as less individuals are required per sampling location (1 to 4) and more geographical locations can be sampled over the landscape (Prunier et al., 2013, 2014). Accordingly, the entire study area was prospected to collect toads and snakes, at night and at day time, respectively. We mainly focused on sampling locations with high probability of presence such as wetlands, ponds, rivers, woodland edges and small villages. To attract snakes and facilitate data collection, 108 artificial shelters were laid across the study area. When an individual was detected, it was hand-captured and manipulated directly in the field. A GPS location (Garmin Etrex20, USA) was recorded for each captured individual (see Fig. 2 and 3 for sampling locations). Each individual was sexed, measured, weighted, marked (to avoid sampling individual twice) and a genetic sample was collected. Captured toads were marked using 7x1.35 mm FDX-B Passive Integrated Transponder (PIT) tags (Loligo Systems, Denmark) and a non-destructive genetic sample was collected by gently opening mouth with a little metal spoon and swabbing mouth cavity for about 10 seconds. We used ventral scales clipping following Brown and Parker (1976) to both mark snakes and collect DNA. We also opportunistically collected genetic samples from snakes and amphibians found dead (road kill or predation) and from snake shed skins. The two insect species (*M. jurtina* and *A. parallelepipedus*) were sampled within 30 sites using a classical population-based sampling design. Site locations were obtained by dividing the study area into 30 sectors using a 5x6 regular grid in QGIS (V. 2.8). In each sector and each species, a single sampling site was chosen according to the presence of suitable habitats (woodlands for beetles and grasslands for butterflies). At each sampling location, 30 individuals were sampled, resulting in 900 genetic samples per species (see Fig. 2 and 3 for sampling locations). Butterflies were captured during day time with nets. Beetles were trapped using non-lethal dry pitfalls. Pitfalls were 20 cm in diameter and 15 cm in depth and were arranged in circles at regular intervals of 5 m. They were emptied every day until 30 individuals were captured. For both insect species, we collected the middle right leg of each captured individual, as both a source of DNA and a way to avoid sampling the same individual twice.

All genetic samples were stored in 70 % EtoH until DNA extraction. All material for marking animals and collecting genetic samples was washed and disinfected using absolute ethanol between each individual

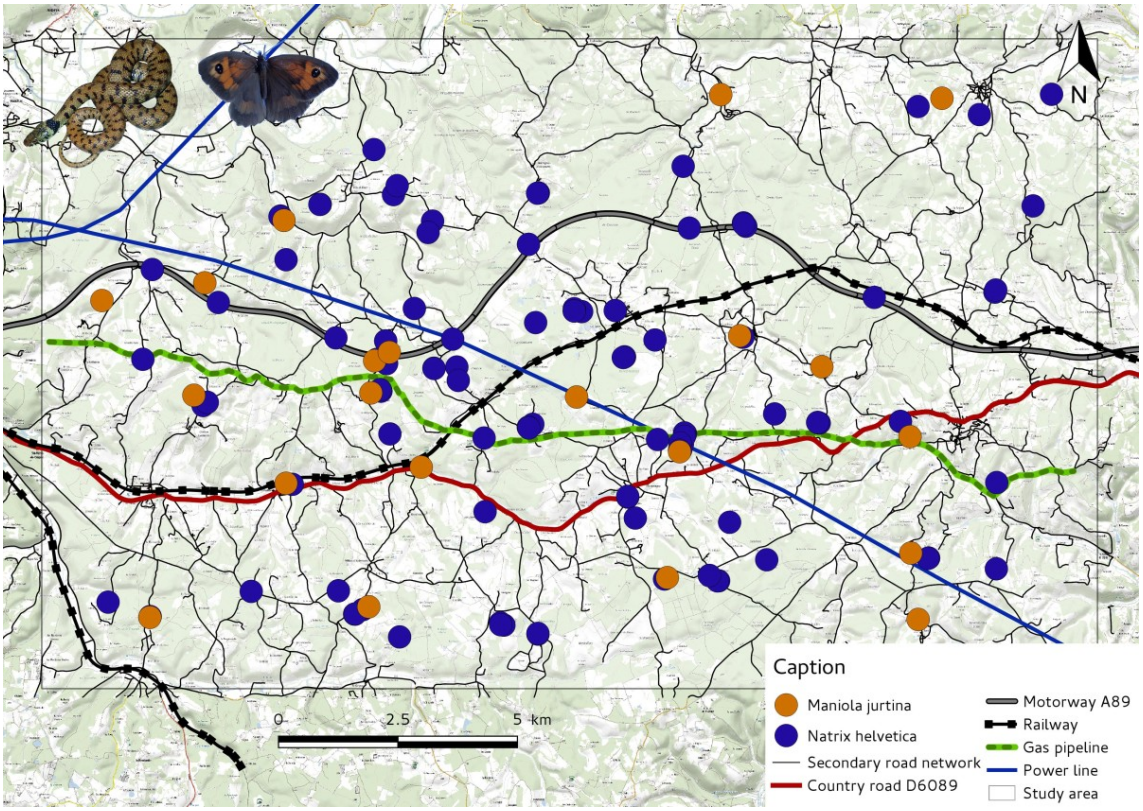

Figure 2: Sampling locations of the snake *Natrix helvetica* and the butterfly *Maniola jurtina* within the study area. Each snake location represents an individual and each butterfly location represents a sampled population (about 30 individuals per population). For these two species, no genetic structure was identified (see result section).

sampling. Care was taken to minimise animal handling and stress and all individuals were rapidly released at the place of capture after manipulation. We amplified 13 (Pokrant et al., 2016), 14 (Tobler et al., 2013; Maia-Carvalho et al., 2014), 15 (Richard et al., 2015) and 14 (Marcus et al., 2013) polymorphic microsatellite loci in *N. helvetica*, *A. obstetricans*, *M. jurtina* and *A. parallelepipedus*, respectively. For a detailed procedure of DNA extraction, amplification and genotyping, see Appendix 1. Some individuals could not be correctly genotyped because of insufficient amounts of DNA: genotypes with more than 2 loci presenting missing values were discarded to allow robust subsequent genetic analyses. We used Genepop 4.2 (Rousset, 2008) to test for linkage disequilibrium among pairs of loci and deviation from Hardy-Weinberg Equilibrium after sequential Bonferroni correction to account for multiple related tests (Rice, 1989). The presence of null alleles was tested using MICROCHECKER 2.2.3 (Van Oosterhout et al., 2004). Loci with null alleles and/or in linkage disequilibrium were discarded, resulting in the final selection of 13, 10, 6 and 10 microsatellite loci in toads, snakes, butterflies and beetles, respectively (Appendix 1).

Classical and hierarchical genetic distances

The presence of related individuals in data sets may lead to an over-estimate of the number of clusters when assessing population structure (Anderson and Dunham, 2008) and thus bias subsequent genetic analyses. We therefore used COLONY2 (Jones and Wang, 2010) to identify and discard siblings within our individual-based data sets (*N. helvetica* and *A. obstetricans*, Appendix 2). In addition, because sites were unevenly sampled for snakes and toads, we only retained a maximum of three randomly picked genotypes per sampling location (Prunier et al., 2013). In the population data sets, we only retained populations for which at least 15 genotypes were available. The final data sets comprised 848 genotypes (30 populations) in *A. parallelepipedus*, 508 genotypes (21 populations) in *M. jurtina*, 115 genotypes in *N. helvetica* (68 sampling locations) and 132 genotypes in *A. obstetricans* (56 sampling locations).

For each of the four final data sets (either individual- or population-based data sets), genetic structure was investigated using STRUCTURE 2.3.4 (Pritchard et al., 2000) with the admixture and the correlated allele frequency models and prior sampling location information when structure in the data was too weak. We followed a hierarchical genetic clustering procedure (Coulon et al., 2008). At each hierarchical level, we tested the number K of clusters from 1 to 10 and repeated analyses for each value 5 times. Runs were performed with a burn-in period of 50 000 and 50 000 subsequent Markov chain Monte Carlo (MCMC) repetitions. We also checked that the alpha value had stabilised before the end of the burn-in period to ensure algorithm convergence. If convergence was not reached, we used a burn-in period of 100 000 and 100 000 MCMC repetitions. We then used STRUCTURE HARVESTER (Earl and VonHoldt, 2012) to obtain deltaK statistics to infer the optimal K-value. We used this optimal K-value to perform 20 runs with a burn-in period of 200 000 and 200 000 MCMC repetitions. We finally compiled the ten best runs

using CLUMPP (Jakobsson and Rosenberg, 2007) to obtain individual or population Q-values. Each individual or population was assigned to the cluster for which the Q-value was higher than 0.6 (Prunier et al., 2017). We then repeated the analysis for each inferred cluster separately until no more structure was found in the data. For each hierarchical level, we used Q-values to compute pairwise matrices (among individuals or populations depending on the sampling design) of ancestry-based hierarchical genetic distances (HGD) (Balkenhol et al., 2014; Prunier et al., 2017). HGD were only calculated for species displaying a significant genetic structure. When more than one hierarchical level was detected, each hierarchical level (HGD1, HGD2...) was considered separately. We also computed classical genetic distances (GD), using the Bray-Curtis (bc) percentage dissimilarity index (Legendre and Legendre, 1998) for the individual-based data sets (*N. helvetica* and *A. obstetricans*) and Fst for the population-based data sets (*M. jurtina* and *A. parallelepipedus*). While these classical genetic distances are well suited to detect surface elements affecting gene flow at a regional scale, HGD have been shown to allow a better detection of sharp genetic variations caused by linear elements such as LTIs (Prunier et al., 2017).

Multiple linear regressions and commonality analyses

Both classical and hierarchical genetic distances were tested against the six types of LTIs present in our study area, along with a number of covariates likely to affect patterns of genetic differentiation (isolation-by-distance IBD, difference in altitude and the following landcover features: water, crops, woodlands, grasslands and urban areas), although assessing the respective influence of these non-LTIs features was not the main scope of this study. Five LTIs (motorway, railway, D6089 country road, gas pipeline and power line) were coded into binary pairwise matrices, with 0 indicated that individuals/populations of each pair were on the same side of the LTI and 1 indicated that they were on either side of the LTI. Because of the density of the secondary road network in the study area, this sixth LTI was treated as other landcover features. Landcover features were defined by digitalizing the entire study area in QGIS (V. 2.8) using national maps and aerial photographs (National Geographic Institute, France). Every elements of the landscape was classified into 49 habitat types of the EUNIS Habitat Classification System (Davies and Moss, 1999). Botanic field surveys were also performed in 2015 to confirm the affiliation of certain habitat types. We combined these 49 elements into six main landcover predictors (Appendix 3): Water (stagnant water bodies, streams and rivers), Crops (intensive and non intensive cultures), Woodlands (all types of forests), Grasslands (uncultivated open lands), Urban (villages, industrial sites, *etc.*) and Secondary road network (all roads excluding small trails, motorway and D6089 country road). These six landcover classes were each rasterised at a 1 m resolution using ARCGIS 10.2.2 and its SPATIAL ANALYST extension. Each raster was then used to create a resistance surface based on the spatial density of the corresponding element in the landscape. To do so, we overlaid a 20 m grid on each spatial class and calculated the percentage of the element in each grid (Balkenhol et al., 2014; Prunier et al.,

2017). For each resistance surface, we rescaled pixel resistance values to range from 1 (null or extremely low densities) to 100 (the element covers the entire pixel) and the final rescaled resistance surface was used in CIRCUITSCAPE 4.0 (McRae, 2006; McRae et al., 2013) to compute pairwise effective distances between sampling locations (individuals or populations). The IBD pairwise matrix was similarly obtained by running CIRCUITSCAPE on a uniform resistance surface only composed of pixels of value 1. Finally, altitude pairwise matrices were computed as the absolute values of pairwise differences in altitude between sampling locations.

The local influence of landscape features may go unnoticed if all pairs of genetic distances are retained, as isolation-by-distance might take over the influence of isolation-by-barriers or isolation-by-resistance (Anderson et al., 2010), with strong implications in terms of biological interpretation of results (Keller et al., 2013; Schregel et al., 2018). We thus considered subsets of pairwise data by defining a maximum euclidean distance threshold between sampling locations. Following Cayuela et al. (2019), this distance threshold was selected for each species and each metric of genetic distances (GD or HGD) as the neighbouring distance maximizing the model fit of a classical multiple linear model including all predictors (see Appendix 4 for details). For each species, we then explored the relationship between subsets of each type of genetic distances (GD or HGD) and the corresponding 13 predictors using standard multiple linear regressions. The contributions of predictors to the dependent variables were assessed using commonality analyses (CA). Commonality analysis is a variance partitioning procedure allowing the detection and the withdrawal of statistical suppressors that are responsible for a distortion of model estimates (beta weights and confidence intervals), thus providing decisive support when trying to assess the reliability of model parameters in face of multicollinearity (for more details about CA, see Appendix 5 and Ray-mukherjee et al. (2014); Prunier et al. (2015, 2017)). We performed model simplification by discarding predictors identified as statistical suppressors in an iterative way, following Prunier et al. (2017) and Cayuela et al. (2019) (see Appendix 6 for details).

In each final simplified model, we assessed final levels of collinearity among predictors using Variance Inflation Factors VIF (Dormann et al., 2013). Because pairwise data are not independent, the p-values inferred from simplified models could not be interpreted (Legendre and Legendre, 1998): we thus computed 95 % confidence intervals around regression estimates using a jackknife procedure, with 1000 replicates based on a random removal of 10 % of individuals without replacement (Peterman et al., 2014; Prunier et al., 2015). These confidence intervals were used to assess the significance of the predictors contributions to the variance in the corresponding genetic distances. We considered that a predictor was a robust contributor to the variance in the response variable as soon as the confidence interval about the corresponding β value did not include 0. A predictor with a positive β value was associated with an increase in the genetic distances and was interpreted as impeding gene flow . On the contrary, a predictor with a negative β was associated to a reduction in genetic distances and was thus interpreted

as promoting gene flow (Jacquot et al., 2017).

In order to summarise model results across species and predictors, we built three 100 % stacked barplots. In the first plot, averaged unique contributions of predictors to the variance in genetic variables were presented per species and across all species with contributions combined into two main classes: those associated with LTIs and those associated with non-LTI predictors (IBD, Altitude, Woodlands, Grasslands, Water, Crops and Urban). In the two last plots, averaged unique contributions of LTIs were either presented per species (and across all species) or per LTI (and across LTIs), with contributions combined into two main classes: those associated with an increase and those associated with a decrease in genetic distances. Predictors that were absent from final simplified models were given a unique contribution of 0.

RESULTS

Genetic structures

Structure outputs indicated a single genetic cluster in both the grass snake *N. helvetica* and the butterfly *M. jurtina*, suggesting high gene flow across the study area in these species. On the contrary, we found strong hierarchical genetic clustering in the toad *A. obstetricans* and in the beetle *A. parallelepipedus* (Fig. 3). In toads, we identified two hierarchical genetic clusters. At the first level, one cluster (B) was surrounded by a second cluster (A) with no clear geographical boundaries explaining this pattern (Fig. 3). Ten individuals could not be assigned to any of these two clusters (cross-assigned individuals), suggesting some exchanges between these two clusters. At the second hierarchical level, only cluster A was further divided into three sub-clusters: A1, A2 and A3. These three sub-clusters were not separated by clear geographical patterns and a high number of individuals (21) were cross-assigned, again suggesting frequent exchanges among them. We similarly identified two hierarchical clustering levels in beetles (Fig. 3). At the first level, 19 populations were assigned to cluster A and ten were assigned to cluster B. Cluster A included populations sampled mostly in the western part of the study area and north of the road D6089 (Fig. 3). One population at the extreme south-west could not be assigned to any of these two clusters (cross-assigned). Cluster B, was further divided into two sub-clusters at the second hierarchical level. Clusters B1 and B2 were separated by the D6089 and the gas pipeline, with B1 in the north comprising five populations and B2 in the south comprising four populations. At the second hierarchical level, only one population could not be assigned to any of these two clusters (cross-assigned). This population was located between the road “D6089” and the gas pipeline, exactly in-between clusters B1 and B2.

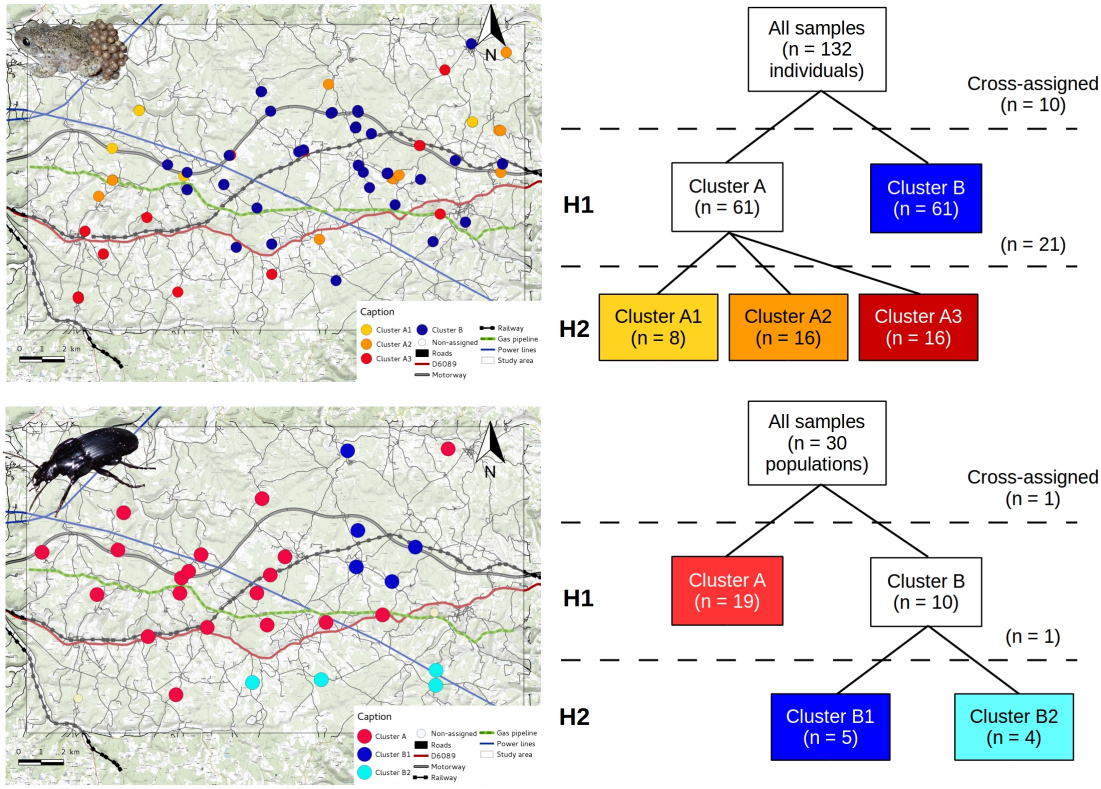

Figure 3: Left panels: STRUCTURE outputs for *A. obstetricans* (132 individuals in 56 sampling locations) and *A. parallelepipedus* (30 populations of about 30 individuals each) plotted over the study area. Right panels: hierarchical splits of inferred clusters from the first to the second hierarchical level. Each box represents a cluster, with n the number of samples (individuals for *A. obstetricans* and populations for *A. parallelepipedus*) assigned to it. The number of cross-assigned samples at each hierarchical level (Q-values < 0.6) is also indicated.

Table 1: Outputs of multiple linear regressions and additional parameters from commonality analyses (CA) for each species and for each type of data set. DV represents the dependent variable: classical genetic distances (GD) calculated either with the Bray-Curtis dissimilarity index (bc) or with Fst and hierarchical genetic distances (HGD1 and HGD2 for first and second level of hierarchy, respectively). For each model, the model fit (Multiple R^2) was estimated from reduced scale analyses, with a maximum distance threshold between pairs of individuals or populations (Distance) ranging from 2800 to 18500m. In each model and for each retained predictor, we estimated the structure coefficient (rs), the beta weight (β), as well as unique (U), common (C) and total (T) contributions. Significance of a predictor's contribution to the dependent variable was estimated using confidence intervals (CI-inf and CI-sup). A CI that included 0 was considered as a non-informative predictor (indicated in bold). Grey colour indicates predictors with negative relationship with the dependent variable (negative β) that may thus be considered as promoting gene flow.

DV	Species	Multiple R^2	Distance	Predictor	rs	B	CI-inf	CI-sup	U	C	T
GD(bc)	*A. obstetricans*	11.82%	3000 m	IBD	0.823	0.126	0.066	0.198	0.009	0.071	0.080
				Altitude	0.618	0.098	0.052	0.140	0.008	0.037	0.045
				Woodlands	0.554	0.145	0.091	0.191	0.018	0.018	0.036
				Roads	0.623	0.113	0.062	0.153	0.009	0.037	0.046
				D6089	0.320	0.091	0.043	0.142	0.008	0.004	0.012
HGD1	A. obstetricans	10.76%	2400 m	Woodlands	0.461	0.100	0.037	0.172	0.010	0.013	0.023
				Crops	0.687	0.185	0.099	0.254	0.032	0.018	0.051
				Roads	0.675	0.159	0.100	0.203	0.024	0.025	0.049
				Railway	0.442	0.108	0.048	0.178	0.011	0.010	0.021
HGD2	A. obstetricans	19.92%	2500 m	Woodlands	0.538	0.188	0.134	0.240	0.031	0.026	0.058
				Urban	-0.465	-0.241	-0.276	-0.203	0.047	-0.004	0.043
				Roads	0.448	0.184	0.134	0.238	0.033	0.006	0.039
				D6089	0.440	0.196	0.145	0.250	0.037	0.001	0.039
				Motorway	-0.278	-0.120	-0.159	-0.076	0.014	0.002	0.016
GD(bc)	*N. helvetica*	4.15%	2800 m	Roads	-0.533	-0.125	-0.193	-0.062	0.015	-0.003	0.012
				Motorway	0.616	0.148	0.078	0.221	0.021	-0.005	0.016
				Railway	-0.520	-0.088	-0.155	-0.022	0.008	0.004	0.011
GD(Fst)	*M. jurtina*	19.91%	5500 m	IBD	0.468	0.264	0.001	0.490	0.066	-0.023	0.044
				Woodlands	0.685	0.315	0.077	0.519	0.089	0.004	0.093
				Power line	-0.595	-0.180	**-0.388**	**0.046**	0.030	0.040	0.071
GD(Fst)	*A. parallelepipedus*	25.87%	6500 m	Altitude	0.203	0.121	**-0.023**	**0.251**	0.015	-0.004	0.011
				Grasslands	0.971	0.498	0.372	0.610	0.248	-0.004	0.244
HGD1	*A. parallelepipedus*	17.22%	18500 m	Roads	0.812	0.262	0.170	0.350	0.063	0.051	0.114
				D6089	0.797	0.254	0.159	0.338	0.059	0.051	0.110
HGD2	*A. parallelepipedus*	26.76%	4500 m	Altitude	0.445	0.223	0.056	0.397	0.049	0.004	0.053
				D6089	0.759	0.350	0.184	0.500	0.114	0.040	0.154
				Motorway	-0.316	-0.114	**-0.273**	**0.041**	0.012	0.015	0.027
				Gas pipeline	0.518	0.225	0.070	0.368	0.049	0.022	0.071

Multiple linear regression and commonality analyses

The maximum euclidean distances between sampling locations that optimized the amount of variance in classical and hierarchical genetic distances (variance explained by full regression models) ranged from 2800 to 3500m in individual-based data sets (vertebrate species) and from 4500 to 18500m in population-based data sets (insect species; Table 1; Appendix 4). After simplification (Appendix 6) and whatever the model, Variance Inflation Factors (VIF) ranged from 1.00 to 1.70 (Appendix 7), suggesting little collinearity among retained variables (Dormann et al., 2013; Prunier et al., 2015).

When considering classical genetic distances in toads, the multiple linear regression explained 11.8 % of variance (Table 1). Two infrastructures (the road D6089 and the secondary road network) were associated with an increase in genetic distances in this model, thus suggesting barrier effects. Both explained about 33 % of the averaged unique contribution and similarly contributed to the dependent variable (U = 0.009 and 0.008, respectively). When considering the first level of hierarchical genetic distance (HGD1), the linear regression explained 10.76 % of the variance. In the final model, the secondary road network (U = 0.024) and the railway (U = 0.011) were both associated with an increase

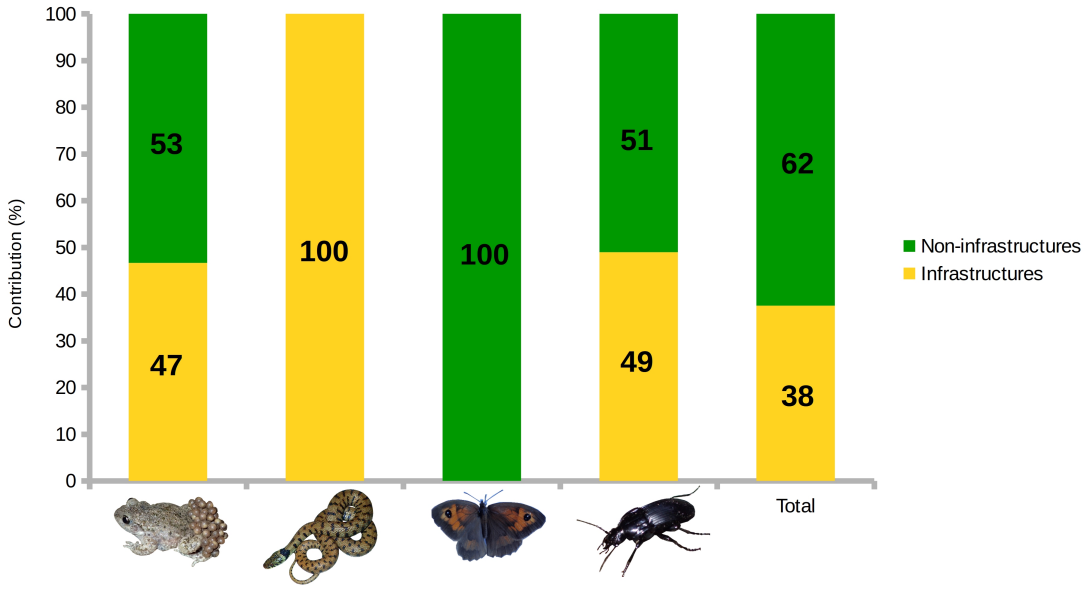

Figure 4: For each each species and for all species combined (Total), 100 % stacked barplots indicating the averaged unique contributions of non-LTI predictors (IBD, Altitude, Woodlands, Water, Grasslands, Crops and Urban) and LTIs (all infrastructures combined) to the overall variance explained by simplified models.

in genetic distances (positive β values), here again indicating barrier effects. At the second level of the hierarchy, our model explained 20 % of variance in HGD2. The secondary road network and the road D6089 were, again, associated with an increase in genetic distances (positive β values) but the motorway was also detected has having a weak but significant positive effect on toads effective dispersal (U = 0.047). Other features such as IBD, altitude, crops and woodlands were found to increase genetic distances whereas urbanisation was detected has having a positive effect on dispersal (see Appendix 8 for more details). When unique contributions were averaged across genetic distances, LTIs accounted for 47 % of overall explained variance (Fig. 4). Infrastructures were mostly associated with an increase in genetic distances, with 90 % of overall explained variance accounted for barrier effects of the road D6089 and of the secondary road network (82%) and, to a smaller extent, of railway (8%; Fig. 5). The 10 % left were explained by a reduction in genetic distances across the motorway at the second level of the hierarchy (HGD2; Fig. 5).

In snakes, the simplified model explained a small amount (4.15%) of variance in the dependent variable (Table 1) but only comprised LTIs predictors (Fig. 4). The motorway was associated with an increase in genetic distances (positive β value) and accounted for 49% of explained variance (U = 0.021; Fig. 5). The two other types of infrastructures (the secondary road network and the railway) had unique

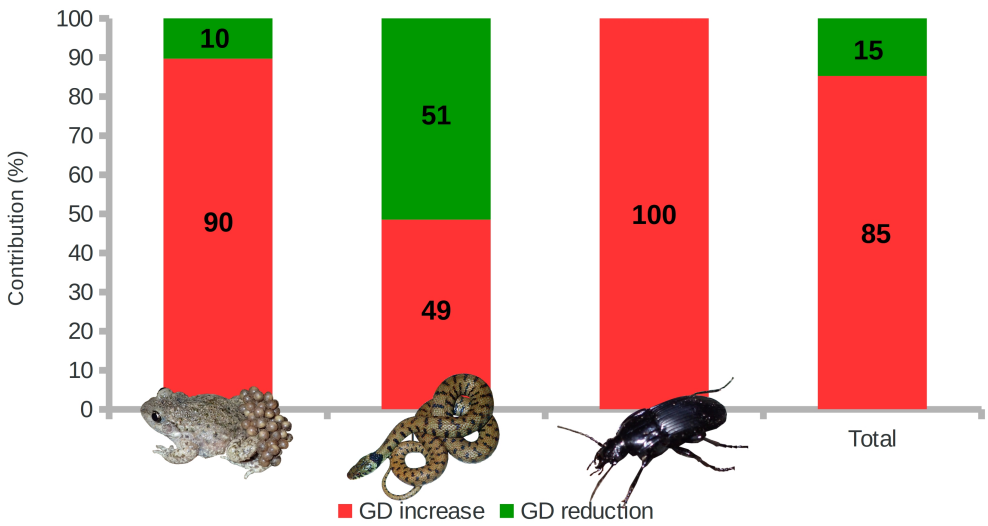

Figure 5: For each each species (except for *M. jurtina*, see main text for details) and for all species combined (Total), 100 % stacked barplots indicating the averaged unique contributions of LTIs associated with an increase (barrier effect) or with a decrease (corridor effect) in genetic distances, expressed as a % of the overall variance explained by simplified models.

contribution of 0.015 and 0.008, respectively, and both had negative β values, indicating that they were associated with a reduction in genetic distances in this species, together accounting for 51% of explained variance (Roads = 34% and Railway = 17.7%; Fig. 5).

In butterflies, the simplified model explained 20 % of variance in Fst values (Table 1). The only LTI that remained in the final model was the power line but it did not significantly contribute to the model predictive power (95% confidence interval including 0). The entire genetic variability in this species was thus explained by isolation by distance and woodlands, both impeding gene flow (Fig. 4 and Appendix 8).

In the ground-beetle, the simplified model explained 26% of the variance in Fst values (Table 1). The entire genetic variability was yet here explained by non-LTIs elements, open areas notably strongly affecting gene flow in this species (see Appendix 8 for more details). When considering the first and the second level of the inferred hierarchical genetic structure, simplified models explained 17% and 27% of the variance in HGD1 and HGD2, respectively. In both cases, the road D6089 was associated with an increase in genetic distances, indicating a consistent barrier effect (U = 0.059 in HGD1 and 0.114 in HGD2). In addition, the secondary road network (HGD1) and the gas pipeline (HGD2) were also detected as having negative effects on gene flow (U = 0.063 and 0.049, respectively). In HGD2, the motorway did not significantly contribute to the model predictive power. Overall, explained variance in genetic distances was accounted for by both LTIs (49 %) and non-LTIs elements (51 %) (Fig. 4), with all LTIs being associated with an increase in genetic distances (Fig. 5).

Assessment of infrastructure effects

Overall, 38 % of the explained variance in genetic distances across all species was due to LTIs (Fig. 4), of which 85% was associated with an increase in genetic distances, that is, with a barrier effect (Fig. 5). The only LTI that did not contribute to genetic distances in any species was the power line. On the contrary, the road D6089 and the gas pipeline were both systematically associated with barrier effects, in toads and beetles for the former and in beetles only for the latter. Other LTIs however showed more nuanced impacts, with corridor effects detected in some species (15% of explained variance by LTIs). While 82 % of the overall genetic variability explained by the motorway across genetic distances and species corresponded to a barrier effect in snakes, the remaining 18 % corresponded to a reduction in genetic distances in toads (Fig. 6). It was the opposite in the case of the railway, with 33 % corresponding to a barrier effect in toads but 67 % to a reduction in genetic distances in snakes. Finally, the secondary road network acted as a barrier to gene flow in both toads and beetles (74 %) but as a corridor in snakes (26%; Fig. 6).

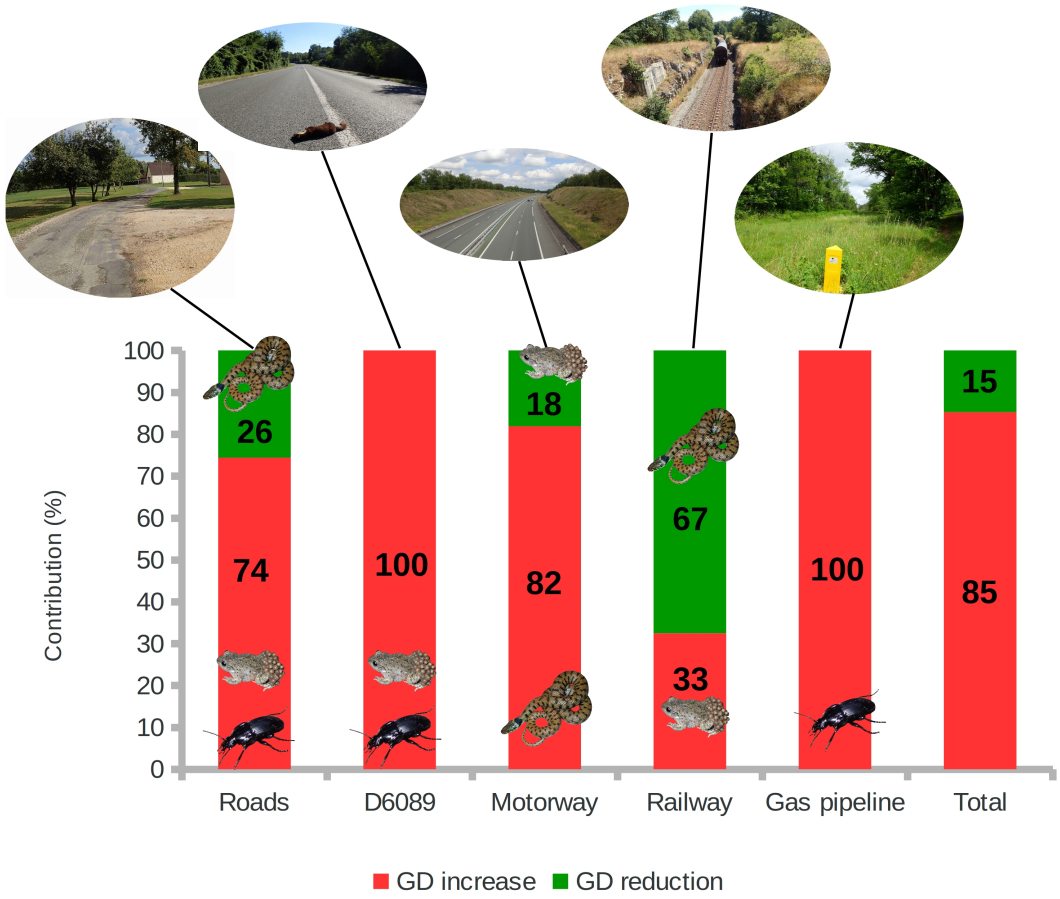

Figure 6: For each LTI and for all LTIs combined (Total), 100 % stacked barplots indicating the averaged unique contributions of LTIs associated with an increase (barrier effect) or in a decrease (corridor effect) in genetic distances, expressed as a % of the overall variance explained by simplified models across all datasets. The power line is not represented as no species was affected by this infrastructure (see results).

DISCUSSION

The goal of this study was to assess landscape functional connectivity in four parapatric species occupying a landscape fragmented by multiple large-scale transportation infrastructures. We were particularly interested in the potential cumulative or on the contrary the antagonistic effects of six LTIs. We used individual- and population-based regression analyses along with commonality analyses over restricted spatial scales to thoroughly evaluate the relative contribution of various landscape predictors to the variance in both classical and hierarchical genetic distances. We notably showed that LTIs were overall responsible for a significant amount of genetic variability across species but that the response of organisms to these LTIs was highly species-dependant. Most importantly, we found that LTIs did not only act as barriers to gene flow but might on the contrary promote gene flow, with some antagonistic effects across species.

Overall, LTIs were found to have a strong influence (either positive or negative) on gene flow, accounting for about 38% of the total explained genetic variability across species and genetic distances, as against 62% explained by other non-LTI features (Fig. 5). All ground-dwelling species were affected by LTIs, with contributions to the variance by LTIs ranging from about 50% in toads and beetles to 100% in snakes , contrary to the flying species *M. jurtina* whose genetic variability was only negatively affected by distance and woodlands, as expected from a previous study (Villemey et al., 2016). Although butterflies have a lower probability to be impacted by vehicles than ground-dwelling species, previous studies showed that roads (Polic et al., 2014) and motorways (Remon et al., 2018) could hinder crossing events in this species. A direct Mark-Release-Recapture survey conducted in the same study area notably found that the motorway was responsible for a six-fold decrease in crossing events when compared to adjacent habitats (Remon et al., 2018). It is possible that large population sizes in *M. jurtina* are responsible for a temporal inertia in the setting-up of genetic differentiation since the creation, in 2004, of the motorway (Gauffre et al., 2008; Landguth et al., 2010), but Remon et al. (2018) also showed that some butterflies were able to cross it, thus possibly ensuring sufficient gene exchange across the landscape (Munguira and Thomas, 1992). Although we could not ascertain the negative aftermaths of human-induced fragmentation in *M. jurtina* from our genetic data, our study highlights the potential benefits of combining landscape genetics and Mark-Release-Recapture surveys (Safner et al., 2011; Cayuela et al., 2018).

As expected, LTIs were mainly associated with a reduction in gene flow, barrier effects accounting for 85% of the variance explained by LTIs across ground-dwelling species (Fig. 4). LTIs carrying vehicles (roads, motorway and railway) were more impacting than infrastructures carrying energy (gas pipeline and power line). Roads in particular (secondary road network and road D6089) were responsible for most of inferred barrier effects in this landscape, with negative effects on gene flow in all ground-dwelling species (Fig. 6). The motorway and the railway also accounted for non-negligible amounts of explained genetic variability but to a lesser extent than roads, only negatively affecting snakes and toads, respectively.

In contrast, the contributions of LTIs carrying energy were less important. The gas pipeline negatively affecting gene flow in the ground-beetle only, probably in response to associated breaches in forest cover (Charrier et al., 1997), and the power line did not affect any studied species. These results indicate that conservation measures should primarily focus on infrastructures carrying vehicles rather than on infrastructures carrying energy (Shepard et al., 2008; Bartzke et al., 2015), although we acknowledge that some taxa were not considered in this study, for instance birds, might be negatively affected by LTIs such as power lines (Loss et al., 2014, 2015).

Despite these general negative impacts of LTIs on gene flow, we found that species showed very different responses to the same LTI, which perfectly highlights the importance of considering functional rather than just structural landscape connectivity in empirical studies (Taylor et al., 2006). Three of the six studied LTIs were associated with an increase in genetic distances in toads (Table 1), these barrier effects together accounting for 90% of genetic variance explained by LTIs (Fig. 4). The secondary road network and the country road D6089 were the main barriers to dispersal in *A. obstetricans*, affecting both classical (GD) and second-order hierarchical genetic distances (HGD2). In addition, the secondary road network also impeded gene flow at the first level of the hierarchy (HGD1). Garcia-Gonzalez et al. (2012) similarly found that all roads, including small secondary roads, acted as barriers to gene flow in *A. obstetricans* in northern Spain. Amphibians are particularly vulnerable to road kills (Fahrig and Rytwinski, 2009) because of their numerous movements during dispersal but also during seasonal migrations between breeding water bodies and shelters. Although these results advocate for effective mitigation measures to limit road kills of amphibians (Beebee, 2013) such as tunnels (Ottburg and Van Der Grift, 2019), it is important to keep in mind that other road features such as traffic noise may also affect amphibians population dynamics (Bee and Swanson, 2007; Troïanowski et al., 2017).

In addition to toads, we found that roads also deeply impacted the ground-beetle *A. parallelepipedus*. The country road D6089 and the secondary road network explained the whole genetic variance at the first hierarchical level (HGD1) resulting in clusters A and B (Fig. 3). At the second hierarchical level (HGD2), the country road D6089 (but also the gas pipeline) further impacted gene flow and was responsible for the split of cluster B into two sub-clusters (Fig. 3). Our results are congruent with Keller et al. (2004) who found that roads are barriers to dispersal in *A. parallelepipedus* but also in other ground-beetle species (e.g. Keller and Largiader, 2003). Roads may act as barrier to gene flow because of road kills but also because ground-beetles may be reluctant to cross roads due to behavioural changes (Holderegger and Di Giulio, 2010).

Contrary to roads, we found that the motorway and the railway showed limited barrier effects. The only species that was negatively affected by the motorway was the snake *N. helvetica*. Genetic studies estimating gene flow of reptiles across LTIs are lacking (Holderegger and Di Giulio, 2010) (but see Clark et al., 2010) and we here revealed that half of the explained genetic variability in snakes resulted from the

negative impacts of the motorway. Because it is fenced with fine mesh, snakes can only reach the other side by using crossing structures (bridges, underpasses, culverts, etc.). These crossing structures may yet be seldom used by snakes due to inadequate placement, architectural design and snakes' behaviour (Woltz et al., 2008). Thermoregulatory behaviour of reptiles is probably the main reason why individuals would not use underpasses (Rodriguez et al., 1996), as a 50 m-length underpass would provide inadequate thermal conditions due to the absence of sunlight. In addition, Baxter-Gilbert et al. (2015) evaluated the effectiveness of different mitigation measures implemented to reduce reptile road mortality (including underneath culverts) and found that these structures were seldom used by reptiles. Underpasses may yet be used by other taxa such as amphibians (Prunier et al., 2014) and insects (Georgii et al., 2011), which may explain why the motorway was only found as acting as a barrier in a single species. Similarly, only one species was negatively affected by the railway. At the first level of the hierarchy (HGD1), we found that a positive relationship between the presence of the railway and genetic distances in toads (Table 1), although clusters A and B were not clearly separated by this LTI (Fig. 3), suggesting a modest barrier effect. Railways are known to restrict gene flow in some amphibian species such as frogs or salamanders (e.g. Reh et al., 1990; Bartoszek and Greenwald, 2009) and many studies on train collision with wildlife reported a high abundance of amphibian kills (Borda-de Agua et al., 2017), representing up to 47 % of all vertebrate records (Heske, 2015). However, the railway in our study area had a low traffic density with approximately 10 trains/day, and train collisions may not be the only driver of the observed reduction in gene flow in *A. obstetricans*. The physical features of the railway are more likely to explain this pattern (Barrientos et al., 2019). Amphibians indeed have a high probability to be trapped between or along rail tracks, making them more vulnerable to both collisions and desiccation than other vertebrates (Budzik and Budzik, 2014). The studied railway was more than 150 years-old, which seems to be of sufficient duration for the detection of a barrier effect from genetic data (Cushman and Landguth, 2010; Prunier et al., 2014; Epps and Keyghobadi, 2015) and suggests that this LTI was actually permeable to the movement of other species.

Our most striking finding is that, instead of acting as barriers, some LTIs might somehow promote dispersal. This "corridor effect" accounted for 15% of the overall genetic variance explained by LTIs across species (Fig. 4) and concerned both vertebrates. We first found that, at the second level of the hierarchy (that is, at a more local scale), gene flow in toads was promoted by the motorway (Table 1). This counter-intuitive genetic pattern (Van Buskirk, 2012) could stem from the availability of new habitats provided by the LTI. Adults and tadpoles of *A. obstetricans* were indeed detected in eight out of the ten storm-water retention ponds present along the studied motorway (data not shown). These ponds may provide favorable breeding habitats, free of predatory fish and surrounded by sand or gravel, the ideal substrates to build their burrows. furthermore, the motorway is crossed by underneath culverts and tracks which are good dispersal corridors for amphibians (Georgii et al., 2011), especially when

their are filled with water (Veenbaas and Brandjes, 1999; Lesbarrères et al., 2004). This is not the first study showing a potential positive effect of a motorway on amphibian gene flow. Prunier et al. (2014) revealed that a 40-years old motorway was not a barrier for the alpine newt (*Ichthyosaura alpestris*) and could even serve as a longitudinal dispersal corridor when the surrounding landscape matrix is highly unfavourable. Interestingly, they even found negative relationships between genetic distances and presence of the motorway, indicating that, as in our study, gene flow across the motorway was probably enhanced; but because they analysed their data using one-tailed Mantel tests, they did not discuss this possibility (Prunier et al., 2014). These results might yet be interpreted with caution due to the recent age of the motorway ($<$ 15 years old): this genetic pattern could stem from ancestral landscape configurations and direct monitoring surveys are now necessary to confirm that the motorway is indeed not an obstacle for toads.

Despite limited explained variance in snakes, we also identified two LTIs acting as corridors in this species, together accounting for 51% of genetic variance explained by LTIs (Fig. 4): the secondary road network and the railway. Roads are known to be responsible for a high mortality in snakes (Rosen and Lowe, 1994): they bask on road surfaces to absorb radiant heat but this behaviour increases the probability of collisions (Rosen and Lowe, 1994) and can result in a reduction in gene flow across roads (Clark et al., 2010). However, we found the exact reverse pattern, with the secondary road network associated with a reduction in genetic distances in *N. helvetica*. This conflicting result can be explained by an attractive effect of roads and road verges that provide basking surfaces, reinforced by a limited traffic volume in our study area. In addition, the distribution of grass snakes being strongly dependent on wetlands for foraging, water-filled ditches often found alongside secondary roads may provide rich feeding areas (Matos et al., 2012), resulting in a local increase in snake abundance that favours road crossings and gene flow. A similar explanation was proposed by Johansson et al. (2005) who found a positive effect of gravel roads and associated ditches in the common frog (*Rana arvalis*). The railway was probably as attractive as the secondary road network for snakes, which may similarly explain gene flow enhancement observed in snakes. Railway embankments provide important alternative habitats for reptiles with optimal thermal conditions for basking (Graitson, 2006; Stoll, 2013; Borda-de Agua et al., 2017). Even active lines can harbour particularly high diversity in reptiles species (Graitson, 2006), notably because human presence is scarce and because reptiles can perceive vibrations transmitted through the rail tracks and the ballast when a train approaches, allowing them to reach a shelter before collision (Borda-de Agua et al., 2017).

Conclusion

The accumulation of LTIs within landscapes is emerging as an important concern and local conservation policies are to be fueled by a thorough assessment of landscape functional connectivity. Although focusing on a single species may help corridor planning (Baguette et al., 2013), we here illustrated how important it is to assess landscape connectivity from a multi-species perspective. Overall, we did not find consistent evidence of a cumulative barrier effect of the six LTIs across species: indeed, butterflies were not influenced by any LTI and snakes were only negatively impacted by the motorway. The case of toads and beetles was yet much more compelling. These two species were the most heavily impacted, with patterns of gene flow affected by various LTIs at different spatial scales. Roads were critical determinants of gene flow across all hierarchical levels in both species, but the railway and the gas pipeline respectively reinforced these impacts in *A. obstetricans* at the first hierarchical level and in *A. parallelepipedus* at the second one. In these two species, the impact of the accumulation of LTIs was thus more a question of a hierarchical than of a cumulative effect of barriers (Bélisle and St. Clair, 2001; Connelly, 2011). Importantly, we also showed that some LTIs, acting as barrier for some species, could somehow promote gene flow in some others, leading to antagonistic LTIs effects: the motorway affected snakes but provided favourable habitats for toads, while the railway affected toads but provided favourable habitats for snakes. Considering the high variability in species response to LTIs, we argue that considering a single species may lead to counterproductive mitigation measures and that integrative approaches based on multiple species are to be more systematically considered. As it obviously seems impossible to assess functional connectivity in all existing species in a given landscape, it is also necessary to determine the extent to which species-specific mitigation measures (Jaarsma and Willems, 2002; Woltz et al., 2008; Glista et al., 2009) can benefit the largest number of species, and, more generally, to investigate which life-history traits are driving organisms response to the presence of LTIs at a taxonomic level (Blanchet et al., 2017).

Authors' contributions

JR, JGP, MB and SM contributed to the conception and design of the study. JR, SM and JHC collected the data. LG digitalized the soil occupancy of the study area. JR and MR performed laboratory analyses and genotyping. JR and JGP performed data analyses and wrote the manuscript. All authors participated in critical revisions of the manuscript.

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