



Pond area and availability safeguard amphibian genetic diversity across Iberia's largest protected wetland

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Abstract

1. Wetland ecosystems worldwide are threatened by habitat alteration, climate change and the introduction of invasive species, even within protected areas. Unravelling the reliance of sensitive wetland-dwelling species, such as amphibians, on habitat characteristics is thus essential to identify conservation targets.
2. Here we assess the distribution of genetic diversity of two strongly aquatic amphibians (*Pelophylax perezi* and *Pleurodeles waltl*) in association with habitat features across the most extensive, protected wetland of the Iberian Peninsula: Doñana National Park.
3. Despite inhabiting a protected area free from anthropogenic barriers, the genetic diversity of *P. perezi* and *P. waltl* is not homogeneously distributed across the wetland, but instead concentrates in core areas, mainly in the northern zone. Both genetic diversity and connectivity (as opposed to genetic differentiation) showed significant positive associations with the area of the breeding sites and the flooded area surrounding the breeding sites within the dispersal potential of either species, that is nearby pond availability.
4. Large water bodies connected to abundant temporary ponds are key for the maintenance of amphibian genetic diversity. Nevertheless, the core populations of our target species, which show markedly aquatic habits, are concentrated in areas colonised by invasive species, which could compromise their long-term viability.
5. Our results highlight that maintaining widely connected arrays of ponds of different hydroperiods, including large breeding sites free from invasive predators and competitors, is paramount for amphibian conservation in Mediterranean wetlands.

KEYWORDS

functional connectivity, genetic differentiation, genetic structure, *Pelophylax perezi*, *Pleurodeles waltl*

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1 | INTRODUCTION

Wetland ecosystems are among the most affected by human destruction and fragmentation worldwide, because of their combined dependence on terrestrial and freshwater environments (Reid et al., 2019; Reis et al., 2017). At the same time, wetlands provide essential habitats for an ample variety of taxa, including a large proportion of endemic species, many of which depend on aquatic sites for reproduction (Díaz-Paniagua et al., 2015; Dudgeon et al., 2006; Florencio et al., 2009). Amphibians, which are facing dramatic declines globally (Catenazzi, 2015; Howard & Bickford, 2014), are specially associated with wetlands, since a large fraction of species use aquatic sites for oviposition and larval development (Gomez-Mestre et al., 2012). Furthermore, as a result of their relatively limited capacity for dispersal and high philopatry, amphibians are also strongly linked to their breeding habitats, and the genetic structure of their populations usually reflects the current and past structural connectivity of the landscape with high fidelity (Cayuela et al., 2020; Smith & Green, 2005).

Amphibian persistence is critically dependent on the availability of networks of adequate aquatic sites capable of harbouring sufficiently large populations (Fortuna et al., 2006; Heard et al., 2015; Smith & Green, 2005). Direct destruction and degradation of flooded areas and water bodies are thereby causing local extinctions of amphibians worldwide (Alroy, 2015; Sodhi et al., 2008; Stuart et al., 2004). In preserved wetlands, the decreasing availability and suitability of ponds for amphibian reproduction is a serious concern (Cayuela, Arsovski, Bonnaire, et al., 2016; Hamer & McDonnell, 2008; Zylstra et al., 2019). Two of the main factors contributing to this trend are (i) the reduction of flooded areas and shortening of pond hydroperiod by climate aridification and/or the unsustainable use of aquifers which ultimately leads to the complete loss of ponds; and (ii) the introduction of invasive aquatic species which, based on their capacity for habitat modification and/or their role as predators, competitors and/or vectors of infectious diseases, are capable of rendering entire wetlands unsuitable for most amphibians (Falaschi et al., 2021; Gallardo et al., 2016; Hamer & McDonnell, 2008).

On the one hand, stochasticity in pond hydroperiod strongly influences the breeding success of amphibians, which show wide differences in their requirements for successful larval development (Cayuela et al., 2012; Fortuna et al., 2006; Gómez-Rodríguez, Díaz-Paniagua, et al., 2010). Alterations in the availability of ponds and hydroperiod shortening can also induce developmental acceleration in amphibians, with concomitant consequences for postmetamorphic survivorship, time to sexual maturity and dispersal rates (Burraco, Díaz-Paniagua, et al., 2017; Burraco, Valdés, et al., 2017; Cayuela, Arsovski, Thirion, et al., 2016; Cayuela, Boualit, Arsovski, et al., 2016; Liedtke et al., 2021). On the other hand, non-native fish and crayfish, which have been introduced in wetlands worldwide, exert strong predation pressure on amphibian eggs and tadpoles, often causing local population extinctions (Cruz et al., 2008; Díaz-Paniagua et al., 2014; Falaschi et al., 2020). Unlike other recognised causes of amphibian declines (e.g. disease outbreaks), the effects of pond hydroperiod reduction and invasive species often pass unnoticed while paving the

way for local extinctions. In this scenario, genetic assessments of wild amphibian populations provide key information to identify units of conservation value, unravel the drivers of species persistence in wetland ecosystems, test their susceptibility to pond availability and quality, and design and evaluate conservation practices (González-Pliego et al., 2019; Howard & Bickford, 2014).

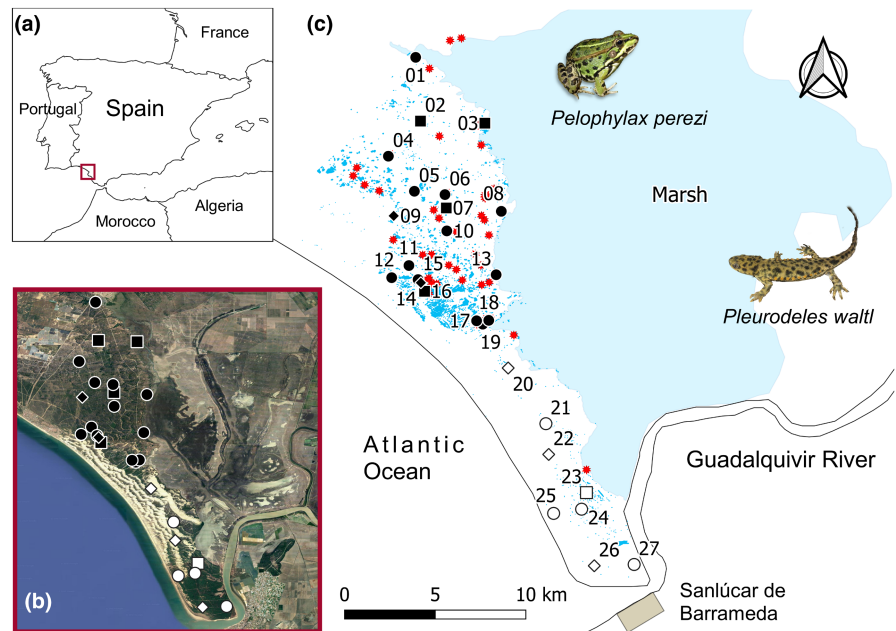
Here we assess the genetic diversity and structure of the Iberian common frog *Pelophylax perezi* (López Seoane, 1885) and the Iberian ribbed newt *Pleurodeles waltl* Michahelles, 1830, across a large pond network in southern Spain (Doñana National Park, DNP). Both species are typically associated with long-lasting or permanent water bodies, although they also successfully exploit temporary ponds during the inundation period, and display strongly aquatic lifestyles. Despite being located within a strictly protected area, Doñana wetlands are not free of threats, of which perhaps the two most important ones are (i) groundwater overexploitation, and (ii) invasive species, particularly invasive predators and competitors. Groundwater overexploitation is causing the loss of many temporary ponds and the progressive reduction of the flooded area, and a shortening of the hydroperiod of the few remaining ponds (Bustamante et al., 2016; de Felipe et al., 2023; Díaz-Paniagua & Aragonés, 2015; Gómez-Rodríguez, Bustamante, et al., 2010). Doñana wetlands are affected by various invasive species harmful to amphibians, such as floating fern mats (e.g. *Azolla filiculoides* Lamarck 1783) (Pinero-Rodríguez et al., 2021) or, especially, invasive predators and competitors such as red swamp crayfish *Procambarus clarkii* (Girard, 1852) and mosquitofish *Gambusia holbrooki* Girard, 1859 (Cabrera-Guzmán et al., 2017; Díaz-Paniagua et al., 2014). We assessed variation in 15 and 17 microsatellite markers to assess the genetic diversity and structure of *P. perezi* and *P. waltl*, respectively, across the pond network of DNP, and tested the role of pond availability and presence of invasive species as potential determinants of the distribution of their genetic diversity. We expected a high degree of connectivity within the Park given the absence of major geographical barriers. However, populations breeding in smaller and more isolated ponds are likely to show reduced genetic diversity and greater differentiation, owing to smaller population sizes and limited gene flow with neighbouring populations. Additionally, populations coexisting with invasive species could also be subjected to reduced population sizes as a result of direct predation and competition exerted by exotic crayfish and mosquitofish, and thus might show signs of genetic impoverishment and differentiation. We discuss the implications of the ongoing deterioration of the pond network for the persistence of *P. perezi* and *P. waltl* in this protected wetland.

2 | MATERIALS AND METHODS

2.1 | Study system, sampling and laboratory procedures

The study area is the pond network of DNP, located at the right bank of the Guadalquivir River estuary in southwestern Spain (Figure 1). The geomorphology of Doñana is composed of three main types of

FIGURE 1 Map of the study area showing the location of the 27 sampled breeding sites of *Pelophylax perezi* (dots), *Pleurodeles waltl* (squares) and both species (diamonds) in the northern (black figures) and southern (white figures) zones of Doñana. (a) Location of Doñana in the Iberian Peninsula. (b) Orthophoto showing the position of the sand stripes dividing northern and southern zones. (c) Vectorial map showing the sampling sites (numbered figures) over the layer of temporary ponds (blue polygons, note that ponds are scarcer in the southern zone), the maximum extension of the marsh flooding area (light blue) and the aquatic sites with records of invasive species, either *Procambarus clarkii* and/or *Gambusia holbrooki* (red stars).



systems: a vast but temporary marsh, two areas of stabilised dunes with different ages and soil composition at the northwest and southern areas (Díaz-Paniagua et al., 2015; Siljeström et al., 1994), and a mobile dune strip composed of three to four fronts running parallel to the coast (Siljeström & Clemente, 1990). In the dune areas, more than 3,000 temporary ponds may be filled in rainy years. Permanent water bodies are scarce, including only three natural large permanent ponds, around 15 intermittent streams with flowing waters from the sandy to the marsh areas (locally named "caños") and about 200 ponds artificially deepened to maintain water throughout the year (locally called "zacallones"). As a consequence of the high variability of timing and quantity of rains among years in Mediterranean areas, the time of pond-filling (and thus their hydroperiod) shows high interannual variation.

The Doñana pond network can be divided into two areas: in the north, temporary ponds are abundant and geographically close to one another; in the south, temporary ponds are not abundant, and most water bodies consist of small and isolated permanent "zacallones" (Figure 1). These differences between north and south of the Doñana wetland have already been associated with restricted gene flow in one amphibian species (Albert & García-Navas, 2022). In rainy years, more temporary ponds may be formed, increasing the connectivity in the northern and southern areas, and extending the pond network to the border of the marsh. The seasonal marsh, when flooded, may also be considered a connecting path between northern and southern areas (Florencio et al., 2014).

The Doñana wetland system is an essential habitat for the persistence of complex floral and faunal communities, and provides breeding sites for 11 amphibian species (Díaz-Paniagua et al., 2006, 2015; Gómez-Rodríguez et al., 2009; Gómez-Rodríguez, Díaz-Paniagua, et al., 2010). Most of these species reproduce in temporary ponds which, as a result of their high interannual variability in hydroperiod, also differ in their habitat characteristics, thus

providing optimal breeding habitats for different species in different years (Gómez-Rodríguez, Díaz-Paniagua, et al., 2010). Among the local amphibians, *P. perezi* and *P. waltl* are widespread, often syntopic inhabitants of permanent and long-lasting temporary aquatic sites, either natural or artificial, including the marsh. Nonetheless, both species can have high breeding success in all kinds of temporary ponds. Larval development in both species is usually limited by the seasonal drying up of temporary ponds in the early summer, although it may extend longer in permanent water bodies such as the "zacallones" (Díaz-Paniagua, 1992).

Owing to their preference for temporary ponds with long hydroperiod or permanent aquatic habitats, *P. perezi* and *P. waltl* show a high degree of overlap with invasive species such as the red swamp crayfish (Gomez-Mestre & Díaz-Paniagua, 2011). This invasive species not only has an important direct predatory effect on amphibian eggs and larvae (Cruz & Rebelo, 2005; Portheault et al., 2007), but also reduces survival of amphibian larvae by impoverishing habitat structure and quality (Arribas et al., 2014). However, notwithstanding >35 years of exposure to the invasive crayfish, *P. perezi* is unable to detect its chemical cues and consequently fails to activate corresponding behavioural and morphological inducible defences (Gomez-Mestre & Díaz-Paniagua, 2011). Exotic mosquitofish are also frequent in permanent waters, where they compete with urodeles for feeding resources (Cabrera-Guzmán et al., 2017) and reduce amphibian reproductive success (Cabrera-Guzmán et al., 2019). Direct predation of mosquitofish on amphibian larvae has also been documented in other areas (Remon et al., 2016; Vannini et al., 2018).

Between 2011 and 2016 we collected 543 *P. perezi* and 278 *P. waltl* tissue samples from 22 and 10 breeding sites, respectively, across DNP, covering both the northern and southern areas, and including sites in the transition between the sandy area and the marsh (Table 1; Figure 1). Sampling consisted in active capture of tadpoles and recently metamorphosed individuals located at each

TABLE 1 Breeding sites of *Pelophylax perezi* (*Pper*) and *Pleurodeles waltl* (*Pwaltl*) sampled in the study area.

ID	Locality	Zone	Area (m ²)	Around (m ²)	D. Marsh (m)	Pc	Gh	AR (<i>Pper</i>)	AR (<i>Pwaltl</i>)
01	Manecorro	N	1,470,188	26,648	0	X	X	8.40	
02	Espajosa	N	6821	4174	2617	X			7.18
03	Casas Viejas	N	2042	42,900	94	X			11.65
04	Mata Los Domínguez	N	37,733	33,695	5236	X		8.67	
05	Membrilla	N	5330	14,804	4692			6.93	
06	Hilillo Rosado	N	1955	8586	3379	X		8.80	
07	Madroñas Norte	N	1634	23,945	3159				8
08	Caño Martinazo	N	5900	3346	57	X	X	10.80	
09	Moral	N	7502	18,695	5795	X		4.33	9.76
10	Alto Zalagalano	N	1681	45,005	2648			5.60	
11	Raposo	N	293	17,404	4558			8.27	
12	Zahillo	N	35,032	31,467	5632			9.00	
13	Lucio Bolín	N	37,308	41,307	0	X	X	9.80	
14	Sanguijuela	N	1383	179,050	4065	X		8.20	11.18
15	Pino Muerto	N	661	123,582	4164			9.07	
16	Laguna Dulce	N	75,909	223,231	3835	X	X		11.71
17	Rincón del Guerrero	N	126	37,499	741	X	X	5.20	
18	Galápago	N	2347	32,072	189	X	X	8.13	
19	Sopetón	N	29,419	33,374	222	X		10.93	
20	Corral de Félix	S	80	2018	894			6.87	6.53
21	Rincón Sotillos	S	107	300	2252			8.13	
22	Punta de Zalabar	S	159	150	1752			6.93	7.18
23	Cerro de los Junqueros	S	310	3020	884				6.41
24	Polluelas	S	171	1743	1810			7.47	
25	Máquinas del Inglesillo	S	48	100	3143			5.47	
26	El Cuerno	S	80	299	4786			4.73	4.65
27	Faginado	S	7502	18,695	5795			7.40	

Note: For each site, the name of the locality, zone of Doñana (north -N- or south -S-), area of the breeding site, area of temporary ponds located within a 760-m radius (Around), distance to the marsh, and mean allelic richness (AR) for each species is shown. The presence of invasive species at the sampling sites is marked with an 'X' in the corresponding column (Pc, *Procambarus clarkii*; Gh, *Gambusia holbrooki*).

breeding area using dip nets. At each sampling site, we homogeneously surveyed the shores and the flooded area of the ponds up to 1 m deep to obtain a representative sample of the offspring cohort of the year and reduce the chances of overrepresenting related individuals in the sample. We clipped tail tips from tadpoles and two toes (two to three phalanges each) from recently metamorphosed individuals and stored them in absolute ethanol, obtaining between 19 and 31 samples per breeding site (Tables S1 and S2). Individuals were immediately released back in the place of capture after tissue sampling.

For DNA isolation, each tissue sample was left overnight at 56°C in a solution containing 315 µl of digestion buffer (100 mM NaCl; 50 mM Tris pH 7.5; 50 mM EDTA pH 8.0; SDS 1%) and 25 µl of proteinase K (20 mg/ml). Samples were inspected for complete digestion before implementation of a DNA isolation protocol in a robotic Freedom EVO platform (Tecan) using magnetic beads. In the last

step, genomic DNA was eluted in 100 µl of TLE buffer (10 mM Tris pH 8.0; 0.1 mM EDTA pH 8.0) and stored at -20°C until genotyped.

All DNA samples were genotyped with microsatellite markers optimised for each species (Gutiérrez-Rodríguez et al., 2014; Sánchez-Montes et al., 2016; Sánchez-Montes, Ariño, et al., 2017; Van de Vliet et al., 2009). Markers of each species were combined in three multiplex reactions by dye-labelling forward primers (Tables S3 and S4). Microsatellites were amplified for each sample in PCR reactions composed of 7.5 µl of Type-it Master Mix (Qiagen), 1.2 µl of primer mix, 5.3 µl of RNase-free water and 1 µl of sample DNA, for a total volume of 15 µl. Thermocycling temperatures consisted in an initial denaturation step (95°C, 5 min), followed by 30 cycles of denaturation (95°C, 30 s), annealing (60°C, 90 s) and extension (72°C, 30 s), with a final extension step (60°C, 30 min). An ABI PRISM 3130 sequencer was used for DNA fragment analysis of PCR products using the GeneScan 500 LIZ (GS500) standard (Applied Biosystems). We

used software GENEMAPPER version 4.0 (Applied Biosystems) to manually assign alleles from the peaks observed in the chromatograms, thus obtaining the multilocus genotype for each sampled individual.

2.2 | Genetic diversity

We tested the concordance between observed genotypic frequencies and Hardy–Weinberg proportions (HWP) by implementing exact tests with 10,000 Monte Carlo replicates for each marker in each breeding site using the "hw.test" function in the R package *pegas* (Paradis, 2010). We used the R package *genepop* (Rousset, 2008) to test for linkage disequilibrium (LD) between all pairs of loci in each breeding site setting a 10,000 length dememorization step for the Markov chain algorithm and 1000 batches with 10,000 iterations per batch. We adjusted for multiple testing by applying the sequential Bonferroni correction (Rice, 1989) to assess the significance of both HWP and LD tests.

We used the R package *adegenet* (Jombart, 2008) to estimate the genetic diversity for each locus and breeding site. Specifically, we calculated the observed number of alleles per locus and estimated the average allelic richness (AR) and the observed (H_o) and expected heterozygosity (H_e) across all loci for each site. We estimated the inbreeding coefficient F for each individual from a 1000 sample from a likelihood function computed with the "inbreeding" function in *adegenet*. We also estimated the effective number of breeders (N_b) (Waples & Antao, 2014) at each breeding site using the sibship frequency method (Wang, 2009) with the software COLONY (Jones & Wang, 2010), assuming the possibility of polygamy for both sexes, no inbreeding, and genotyping error rate priors=0.05 for all markers (Sánchez-Montes, Ariño, et al., 2017). We ran two independent analyses with different random seeds for each breeding site using all available genotypes at the corresponding site as offspring sample, and implementing the full likelihood method with maximum run length and precision, weak paternal and maternal sibship size priors at unity (Sánchez-Montes, Wang, et al., 2017), full sibship scaling, and without updating the allelic frequency.

2.3 | Genetic structure

We used the R package *diveRsity* (Keenan et al., 2013) to estimate pairwise genetic differentiation among all breeding sites for each species according to Hedrick's G_{ST} (Hedrick, 2005) and Jost's D (Jost, 2008), to control for the effect of high heterozygosity typical of microsatellite markers (Edelaar et al., 2011). We calculated the significance of both indexes by estimating their 95% confidence intervals from a 1000 iterations bias-corrected bootstrap. We then tested for isolation-by-distance (IBD) across the study area for each species separately by performing a 9999 permutation Mantel test between pairwise genetic and geographical distances among all breeding sites using package *ad4* (Dray & Dufour, 2007). Genetic distances were

calculated as Edwards' genetic distances (Edwards, 1971) with package *adegenet*, whereas Euclidean geographic distances were calculated from geographical coordinates with package *stats* in R (R Core Team, 2019).

We inspected the distribution of genetic variation of each species across the study area by implementing Bayesian clustering analyses with software STRUCTURE (Pritchard et al., 2000). We assessed genetic clustering according to a priori defined values of K (the number of clusters) from one to either 25 or 12 for *P. perezi* and *P. waltl*, respectively (thus the maximum K exceeding the number of sampled sites for each species). We ran all analyses using an admixture model with correlated allele frequencies (Falush et al., 2003), with 500,000 burn-in and 500,000 post burn-in iterations, and 10 replicates for each value of K . The likelihood of different K values to parametrise the genetic variation of the dataset was checked with STRUCTURE HARVESTER (Earl & vonHoldt, 2012), attending to both the original (Pritchard et al., 2000) and the ΔK (Evanno et al., 2005) methods. We used CLUMPAK (Kopelman et al., 2015) to summarise results and generate individual assignment plots.

On account of the bimodal (north–south) genetic–spatial variation observed in the pygmy marbled newt *Triturus pygmaeus* (Wolterstorff, 1905) in Doñana (Albert & García-Navas, 2022), we tested for a biogeographical scenario of possible vicariance, in which more or less deeply differentiated lineages might have merged to form a secondary contact zone. The best fitting geographical clines for Structure Q-scores under $K=2$ were estimated with HZAR software under a protocol used previously (Arntzen et al., 2017; Derryberry et al., 2014). The reference point for distance in this analysis is the northern bank of the Guadalquivir River, with the coordinates 36.7956 N, 6.3700 W.

2.4 | Environmental features associated to genetic diversity and structure

We tested the association between five characteristics of the sampled breeding sites and observed patterns of genetic diversity and structure. These environmental features were: (i) area of the breeding site, (ii) presence of invasive crayfish (*P. clarkii*) or mosquitofish (*G. holbrooki*), (iii) availability of temporary ponds around the breeding site, (iv) distance to the marsh, and (v) location within the Doñana pond network (northern vs. southern area). We manually delineated the perimeter of the flooded area of each of the sampled breeding sites using QGIS version 3.6.0 (QGIS Development Team, 2014) and calculated its area using the R package *raster*, and its shortest distance to the marsh using the function "st_distance" from package *sf* (R Core Team, 2019). We gathered geographical information on the temporary pond network and the presence of *P. clarkii* and *G. holbrooki* from previous publications (Díaz-Paniagua et al., 2014; Gómez-Rodríguez et al., 2011) and data provided by the Doñana Monitoring and Survey Service. Finally, we set a buffer of 760m radius from each breeding site, which corresponds to the maximum recorded distance for adult individuals of both species across

terrestrial landscape (Capellà-Marzo et al., 2020; Fernández de Larrea et al., 2021). Considering that buffer zone, we intersected the resulting polygons with the pond network layer (Gómez-Rodríguez et al., 2011) to identify the ponds located within the dispersal capacity of both species for each sampling site. The availability of other ponds around each breeding site was calculated as the sum of the areas of all the ponds included (partly or completely) within this distance.

We constructed univariate linear regression models in R to test the significance of the five explanatory variables on the observed genetic diversity (AR and H_E indexes) and structure (mean G_{ST} and D of each breeding site with respect to the remaining sites). In the latter case, we tested for spatial autocorrelation using marginal modelling via generalised least square analysis (Pekár & Brabec, 2016) to account for the non-independence of pairwise genetic differentiation measures. Given that the area of the breeding sites and the area of the ponds in their surroundings showed highly positive skewed distributions, we log-transformed both variables. Some explanatory variables showed moderate levels of collinearity according to their variable inflation factors (VIFs between 1.18 and 7.56), so we constructed partial least square (PLS) regression models to test their contributions in a multivariate framework. This procedure is based on the reduction of the dimensionality in multivariate components while accounting at the same time for the association of each explanatory variable with the response (Carrascal et al., 2009). We tested the significance of each multivariate component with the response variable and checked the normality of residuals of the final models by qq plots and skewness, kurtosis and Shapiro–Wilk tests.

3 | RESULTS

3.1 | Genetic diversity and structure

Allelic richness across the study area ranged between 4.33 (locality 09: Moral) and 10.93 (19: Sopotón) in *P. perezi* and between 4.65 (26: El Cuerno) and 11.71 (16: Laguna Dulce) in *P. waltl* (Table 1). Most southern breeding sites of both species, as well as some *P. perezi* northern sites such as 05: Membrilla, 09: Moral, 10: Alto Zalagalano and 17: Rincón del Guerrero showed strong genetic differentiation (Figure 2). Both genetic differentiation indexes and clustering analyses concordantly showed wide connectivity among some northern breeding sites, either geographically close or located near the marsh, and marked structure across the southern

zone (Figure 2). Locus- and population-specific results are detailed in an extended population genetics report in the Supporting Information (Data S1).

For *P. waltl*, the one-but-best supported partition scheme obtained by *structure* analysis was $K=2$ (Figure S2). This setting yielded a geographical cline with a sharp transition in the south of the Doñana peninsula. The most relevant features of the cline were a central position at 13.3 km north of the Guadalquivir (95% credibility interval, CI 9.8–16.1 km) and a width of 440 m (CI 0.0–5.1 km) (Figure 2b; Table S5). For *P. perezi*, $K=2$ was less strongly supported (fourth position; see Figure S1), yet this partition resulted in a sharp cline similar to that in *P. waltl*. This cline had a central position at 9.3 km north of the Guadalquivir (CI 6.3–9.6 km) and a width of 780 m (CI 0.1–5.8 km) (Figure 2a; Table S5).

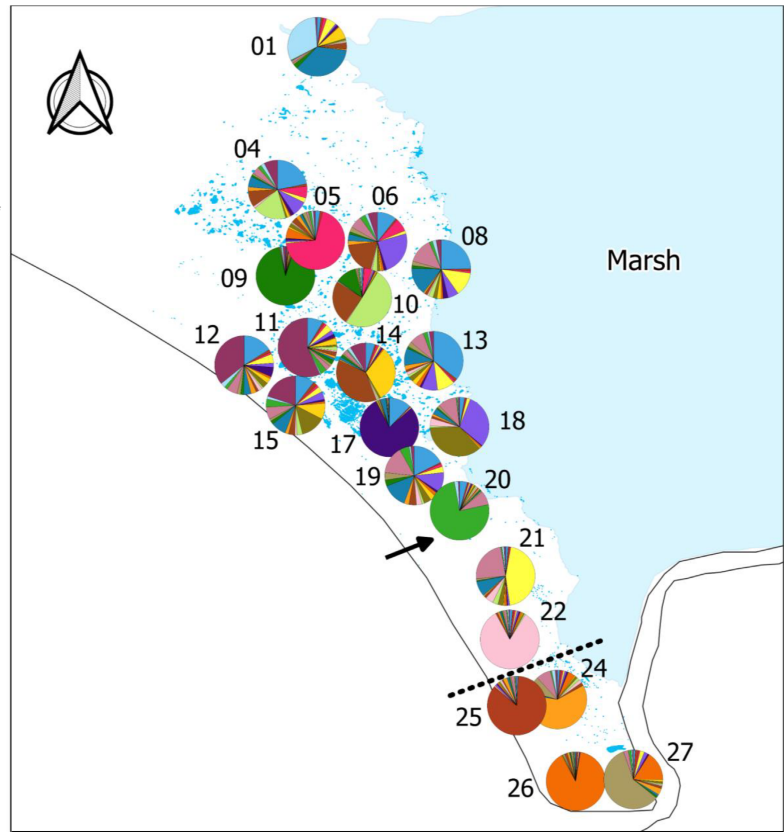
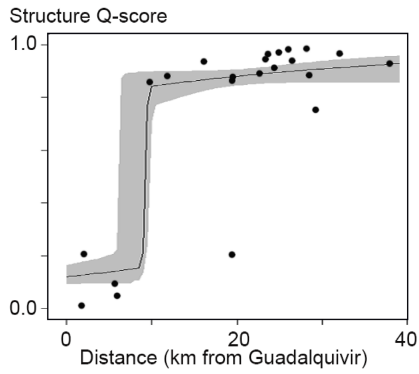
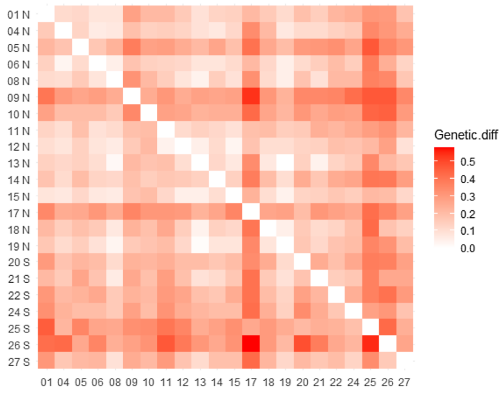
3.2 | Environmental features associated to genetic diversity and structure

Breeding sites of the northern area of Doñana showed overall higher values and more variance across explanatory variables than southern sites. In particular, northern breeding sites were larger (median pond size: north=5330 m², south=133 m², $p=0.002$) and more densely surrounded by temporary ponds (median of flooded area in the surroundings: north=32,072 m², south=1022 m², $p<0.001$). Also, invasive species affected only northern sites, because neither *P. clarkii* nor *G. holbrooki* were present in any of the sampled southern breeding sites.

Accordingly, the geographical zone (north vs. south) was a significant factor in all of the PLS regression models (Table 2). Only the first component of each PLS analysis showed a significant association with the corresponding response variable, and the contributions of each explanatory variable to this component (measured as weights and square weights; see Table 2), resembled the trends observed in the univariate analyses (Figures 3 and 4; Table S6). Both the area of the breeding site and the area of temporary ponds in the surroundings showed significant positive associations with genetic diversity (both with AR and H_E) and negative associations with genetic differentiation (both with mean G_{ST} and D indexes, Table 2). The PLS regression models showed better explanatory power for *P. waltl* (R^2 between 0.51 and 0.79) than for *P. perezi* ($R^2=0.22$ to 0.24, Table 2). Only for *P. waltl* did the presence of invasive species show a significant association with genetic diversity and structure, although, contrary to expectation, the relationship was positive with genetic diversity and negative with genetic differentiation.

FIGURE 2 Genetic structure in *Pelophylax perezi* (a) and *Pleurodeles waltl* (b). Heatmaps represent pairwise genetic differentiation (upper diagonal: Hedrick's G_{ST} ; lower diagonal: Jost's D) across all pairs of breeding sites (codes as in Table 1; labels in the vertical axis indicate north -N- or south -S- zones for each site). Maps show clustering results for $K=20$ (*P. perezi*) and $K=10$ (*P. waltl*). Note that charts of some breeding sites have been slightly displaced to avoid overlapping; see Figures S1 and S2 in Supporting Information for the complete genetic assignment barplots. Cline graphs show geographical clines reconstructed under $K=2$; the solid round symbols represent populations, and the grey area shows the 95% confidence interval. Both transitions are indicated on the respective maps by dashed lines. The arrow indicates the approximate position of a similar genetic transition observed in *Triturus pygmaeus* (Albert & García-Navas, 2022).

(a) *Pelophylax perezi*



(b) *Pleurodeles waltl*

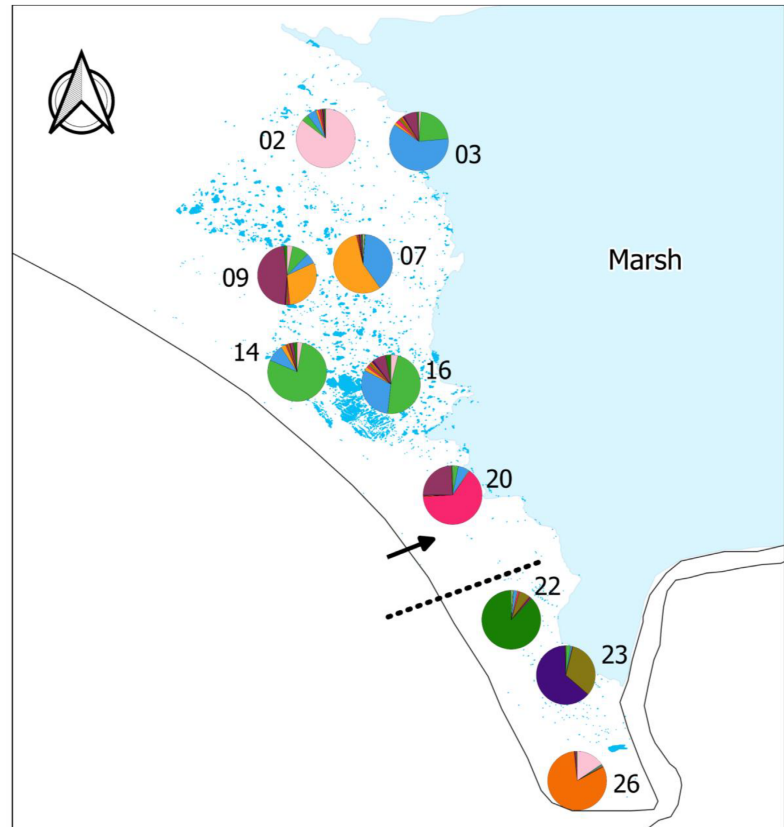
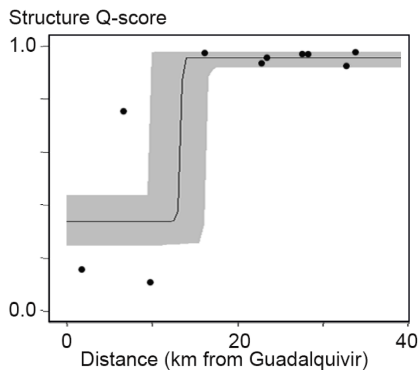
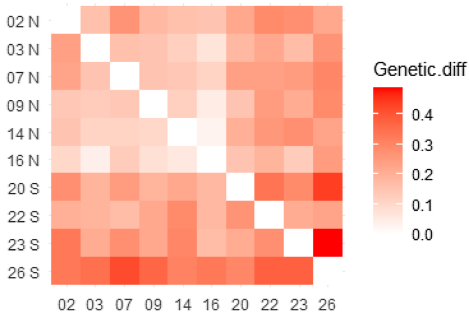


TABLE 2 Results of partial least square (PLS) regression analyses on genetic diversity, measured as allelic richness (AR) and expected heterozygosity (H_E), and genetic structure, measured as Hedrick's G_{ST} and Jost's D .

Response	Explanatory variable	<i>Pelophylax perezi</i>		<i>Pleurodeles waltl</i>	
		Weight (95% CI min, max)	Weight ²	Weight (95% CI min, max)	Weight ²
AR <i>Pp</i> : $p=0.016$, $R^2=0.22$ <i>Pw</i> : $p=0.001$, $R^2=0.71$	Log(area)	0.583 (0.327, 0.855)	0.340	0.461 (0.257, 0.550)	0.212
	Invasive species	0.388 (-0.206, 0.615)	0.151	0.499 (0.356, 0.558)	0.249
	Log(temporary ponds around)	0.387 (-0.049, 0.583)	0.150	0.548 (0.435, 0.686)	0.300
	Distance to the marsh	-0.404 (-0.832, 0.226)	0.163	0.053 (-0.363, 0.514)	0.003
	Group (North/South)	-0.443 (-0.565, -0.061)	0.196	-0.486 (-0.522, -0.359)	0.236
H_E <i>Pp</i> : $p=0.013$, $R^2=0.23$ <i>Pw</i> : $p=0.013$, $R^2=0.51$	Log(area)	0.573 (0.346, 0.863)	0.328	0.436 (0.006, 0.531)	0.190
	Invasive species	0.341 (-0.228, 0.537)	0.116	0.435 (0.093, 0.540)	0.190
	Log(temporary ponds around)	0.488 (0.003, 0.614)	0.238	0.619 (0.486, 0.846)	0.383
	Distance to the marsh	-0.239 (-0.727, 0.347)	0.057	-0.112 (-0.511, 0.528)	0.013
	Group (North/South)	-0.511 (-0.576, -0.129)	0.261	-0.475 (-0.526, -0.202)	0.225
G_{ST} <i>Pp</i> : $p=0.013$, $R^2=0.23$ <i>Pw</i> : $p=0.001$, $R^2=0.73$	Log(area)	-0.586 (-0.902, -0.319)	0.343	-0.501 (-0.541, -0.351)	0.251
	Invasive species	-0.339 (-0.570, 0.217)	0.115	-0.460 (-0.508, -0.317)	0.211
	Log(temporary ponds around)	-0.498 (-0.640, -0.031)	0.248	-0.540 (-0.622, -0.442)	0.292
	Distance to the marsh	0.221 (-0.332, 0.638)	0.049	-0.032 (-0.496, 0.357)	0.001
	Group (North/South)	0.496 (0.126, 0.593)	0.246	0.495 (0.395, 0.529)	0.245
D <i>Pp</i> : $p=0.012$, $R^2=0.24$ <i>Pw</i> : $p<0.001$, $R^2=0.79$	Log(area)	-0.592 (-0.882, -0.329)	0.351	-0.519 (-0.556, -0.404)	0.269
	Invasive species	-0.355 (-0.598, 0.190)	0.126	-0.477 (-0.522, -0.391)	0.227
	Log(temporary ponds around)	-0.491 (-0.629, -0.071)	0.241	-0.507 (-0.561, -0.400)	0.257
	Distance to the marsh	0.199 (-0.346, 0.662)	0.040	-0.080 (-0.492, 0.308)	0.006
	Group (North/South)	0.492 (0.167, 0.585)	0.242	0.491 (0.392, 0.534)	0.241

Note: Only the first component of each PLS was significant. The p -value of the component and the R^2 for each analysis of each species are shown below each response variable (*Pp*, *Pelophylax perezi*; *Pw*, *Pleurodeles waltl*). Explanatory variables showing significant weights (i.e., with bootstrapped 95% confidence intervals [CI] not including zero) are marked in bold.

The distance to the marsh was the only explanatory variable that showed non-significant associations in all analyses. Similar results were obtained using either G_{ST} or D as proxies of genetic differentiation (neither showed evidence of spatial autocorrelation according to GLS analyses, results not shown), and either AR or H_E for genetic diversity (Table 2). For this reason, only graphical plots for AR and G_{ST} are shown for visualisation of the univariate effects of the main explanatory variables in Figures 3 and 4.

4 | DISCUSSION

4.1 | Genetic structure within a highly conserved area with a dense pond network

Despite occupying a relatively small and localized area, the genetic diversity of *P.perezi* and *P.waltl* across the Doñana pond network is strongly structured. The density and extent of permanent and temporary ponds is larger in the northern than in the southern areas of Doñana (Flores et al., 2014), and the observed genetic structure

largely corresponds to this configuration. Our results suggest that the greater pond density of the northern zone of the park increases the connectivity among breeding sites, with genetic clusters displaying wide genetic admixture among nearby ponds, but also in some more distant ones. The influence of the extensive (yet temporary) marsh was also apparent in the northern zone. Although the distance to the marsh was not a statistically significant factor in our models, genetic admixture was common among breeding sites located near the edge of the marsh, even across long geographical distances (e.g., >7.5 km between the widely connected *P.perezi* breeding sites 08: Caño Martinazo and 19: Sopotón). The high genetic connectivity in the northern clusters sharply contrasts with the strong genetic structure observed in northwestern breeding sites distant from the well-connected clusters (mainly in *P.perezi*), and in the isolated breeding sites of the southern zone (both species). Most of these sites showed lower genetic diversity and a very strong differentiation, suggesting that each of them may represent a different isolated population.

The dissimilar genetic structure patterns observed in the north-south zonation of the study area may have an ancient origin, as Doñana falls within the Mediterranean biodiversity hotspot, a region

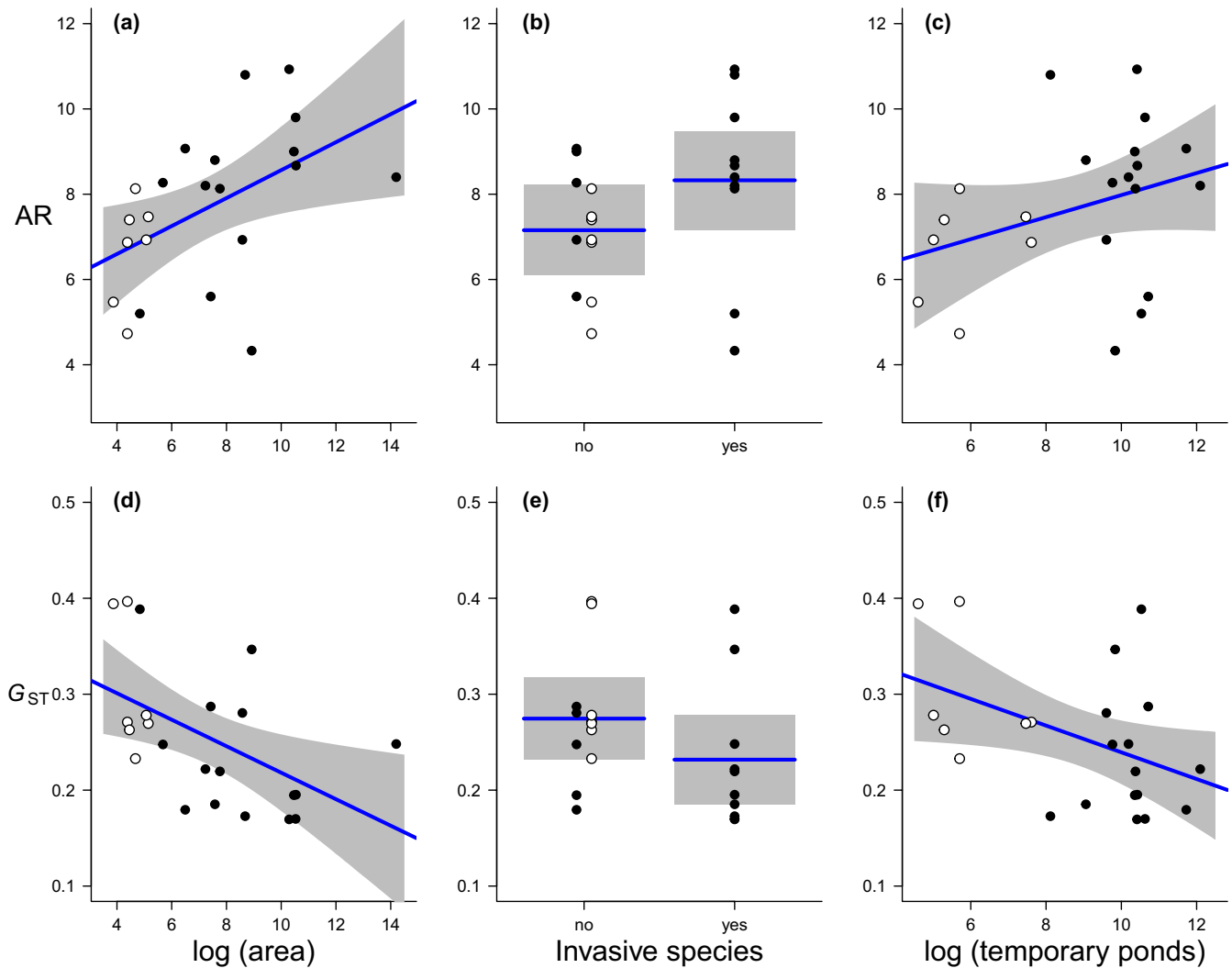


FIGURE 3 Results of univariate regression models (according to Table S6) on observed genetic diversity (measured as allelic richness, AR) and structure (measured as Hedrick's G_{ST}) of *Pelophylax perezi* throughout Doñana National Park. Plots represent three of the tested explanatory variables: area of the breeding site (in log scale, panels a and d), presence of invasive species (crayfish and/or mosquitofish) on the breeding site (panels b and e) and total area of temporary ponds located within 760m distance from the breeding sites (in log scale, panels c and f). Blue lines and shaded areas represent predicted values and 95% confidence intervals of each response variable according to univariate regressions. Dots represent the breeding sites of the northern (black) and southern (white) zones of Doñana.

harbouring a wide variety of recognised and possibly unrecognised species and phylogeographic lineages (Laikre et al., 2016; Sauquet et al., 2009). Accordingly, we observed sharp, yet near-coincident and near-concordant geographical clines for the two studied species. The genetic transition is positioned at the southern fringe of the Doñana peninsula. In the absence of a strong present-day environmental barrier between the northern and southern sections, a historical explanation might also be considered. We suggest that the genetic transition coincides with the former position of the Guadalquivir River and that the southern Doñana population groups may form separate lineages that, with a redirection in the course of the river, became disconnected from their main, southern distributions, to eventually connect to the northern stock. Archaeological studies acknowledge that the Guadalquivir previously discharged into an inland sea, the Gulf of Tartessos, before reaching the Atlantic Ocean. Bathymetric reconstructions for the 4,000–2,200 year BP

period suggest the past presence of a spit of land protruding northward from Sanlúcar de Barrameda into the Tartessian gulf (Abril et al., 2013). This peninsula may have been populated by several of the amphibian species in the area. Indeed, a similar genetic transition is discernible from a study on the newt *T. pygmaeus* (Albert & Garcia-Navas, 2022), strengthening the support for a wide applicability of this historical biogeographical scenario (see Arntzen, 2024).

4.2 | Environmental features associated to genetic variation and structure

The current genetic structure of *P. perezi* and *P. waltl* across Doñana shows that the most widely connected breeding sites are located in the northern area, coincident with high pond abundance. Concordantly, we obtained significant relationships of the availability

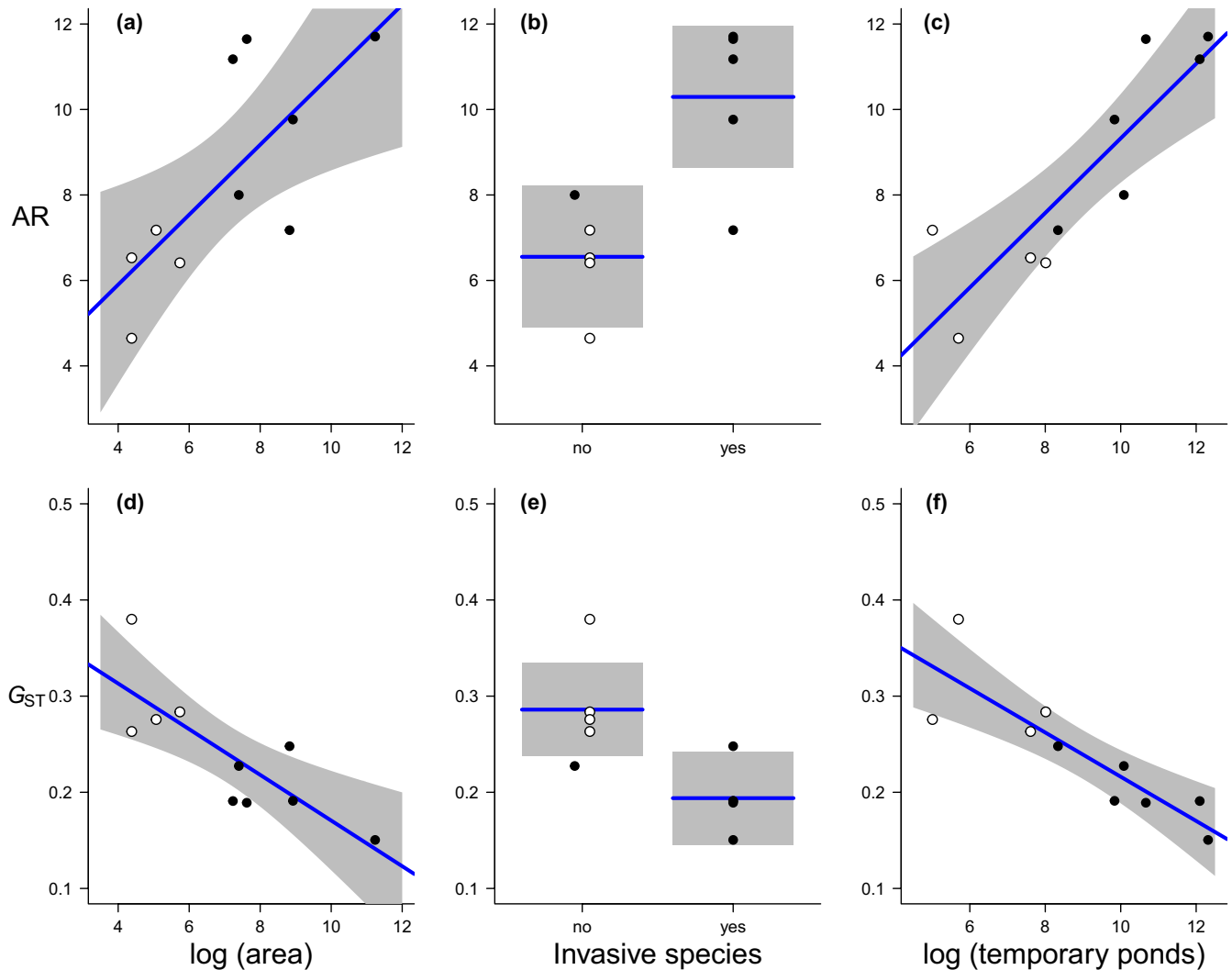


FIGURE 4 Results of univariate regression models (according to Table S6) on observed genetic diversity (measured as allelic richness, AR) and structure (measured as Hedrick's G_{ST}) of *Pleurodeles waltl* throughout Doñana National Park. Plots represent three of the tested explanatory variables: area of the breeding site (in log scale, panels a and d), presence of invasive species (crayfish and/or mosquitofish) on the breeding site (panels b and e) and total area of temporary ponds located within 760m distance from the breeding sites (in log scale, panels c and f). Blue lines and shaded areas represent predicted values and 95% confidence intervals of each response variable according to univariate regressions. Dots represent the breeding sites of the northern (black) and southern (white) zones of Doñana.

of ponds in the surroundings of each sampled breeding site with genetic diversity (positive) and differentiation (negative), which hold true after controlling for collinearity among explanatory variables in PLS analyses. In the same way, the area of the breeding site was a predominant predictor in our models, and illustrated that larger ponds are associated with higher genetic diversity and lower differentiation with the remainder of the network. This is an expected result, in concordance with previous studies (Falaschi et al., 2021; Lourenço et al., 2017), and clearly shows that extensive permanent, semipermanent, and temporarily flooded areas constitute irreplaceable assets for amphibian populations. Large breeding ponds are required for amphibian populations to reach sufficient abundance to prevent the risk of fitness depression caused by inbreeding and genetic depletion (Frankham et al., 2014). Even in a well-preserved wetland such as Doñana, larger breeding sites, both in the north and in the south, showed the highest genetic diversity and the lowest

overall differentiation with the rest of the network, thus demonstrating their preeminent role for maintaining genetic diversity and gene flow across the entire (meta)populations of *P. perezi* and *P. waltl*.

Contrary to our expectations, the presence of invasive predators and competitors such as red swamp crayfish and mosquitofish in the northern part of the Park is not yet associated with reduced genetic diversity or increased genetic differentiation in either *P. perezi* or *P. waltl*. In the case of *P. waltl*, we found a positive association between the presence of invasive species and genetic diversity or the degree of connectivity among its populations. This result is due to the presence of invasive predators and competitors in the large, permanent or long-lasting temporary ponds that also function as the main genetic reservoirs for both *P. perezi* and *P. waltl*. The extent and heterogeneity of the pond network in Doñana, which provides widespread availability of temporary ponds (free from mosquitofish) in the northern area, may allow *P. perezi* and *P. waltl* to recolonise breeding sites where invasive

predators cause local extinctions, via demographic rescue from close neighbouring populations (Díaz-Paniagua et al., 2014; Falaschi et al., 2021). Furthermore, strongly aquatic and ecologically resilient species such as *P. perezi* and *P. waltl* may indirectly benefit from reduced competition with other amphibians, especially at larval stages, which might be more severely affected by both invasive species (Ficetola et al., 2011). Nevertheless, both red swamp crayfish and mosquito-fish pose serious threats to amphibians (Cabrera-Guzmán et al., 2017; Falaschi et al., 2020; Hamer et al., 2002; Kats & Ferrer, 2003), and red swamp crayfish in particular have been directly responsible for local extinction of amphibian guilds in other regions (Cruz et al., 2008; Cruz & Rebelo, 2005; Ficetola et al., 2011), so their overall negative impact on amphibian communities should not be overlooked, even when no clear signs of genetic impoverishment are yet evident.

In this regard, we obtained low N_b estimates for most breeding sites, well below the minimum threshold values to prevent negative fitness effects from genetic erosion (100–1,000 according to Frankham et al., 2014). It must be noted that our N_b estimates are based on single-cohort samples, and therefore correspond to the effective number of breeders during a single season (N_b) and not to the more comprehensive parameter N_e , the effective population size across an entire generation (Wang et al., 2016; Waples & Antao, 2014). Also, single cohort samples are more prone to oversampling related individuals, which could be the cause of the extremely low N_b and strong genetic differentiation observed for *P. perezi* in Rincón del Guerrero (17), a surprising result given its close proximity to the genetically diverse and well-connected sites of Sopotón (19) and Galápago (18). At any rate, the low N_b estimates observed in many breeding sites raise concern about the resilience of *P. perezi* and *P. waltl* populations in the current scenario of accelerated reduction of the number and hydroperiod of ponds, even within a highly protected area such as DNP. The role of artificially deepened "zacalones" providing permanent water bodies seems to be essential for the persistence of these populations, especially in the southern zone.

4.3 | The risk of pond network deterioration

The availability of temporary and permanent ponds in Doñana is conditioned by rainfall, the sandy substrate, and the ground waters that sustain these wetlands. Climate change and aquifer overexploitation are jointly drying up ponds in Doñana, reducing the extent and hydroperiod even of permanent or semipermanent ponds (Bustamante et al., 2016; Camacho et al., 2022; de Felipe et al., 2023; Gómez-Rodríguez, Bustamante, et al., 2010). Large areas that used to become flooded every year are drying up (de Felipe et al., 2023; Díaz-Paniagua & Aragonés, 2015), to the point that terrestrial vegetation (e.g., pines, heath, rockroses) is colonising what used to be pond basins just a few years ago (de Felipe et al., 2023; Díaz-Paniagua et al., 2023). Indeed, some of the breeding sites sampled in the present study have already lost considerable amounts of flooding area in the 6–11 years following our tissue sampling. This trend poses a serious threat not only for the persistence of amphibians like *P. perezi* and *P. waltl*, but also, arguably,

for all wetland-associated fauna and flora, with consequences that will likely affect the genetic heritage of populations before census declines become apparent. In the event that aquifer overexploitation, climate change and invasive species ended up causing a population decline in the northern breeding sites which constitute the core of the *P. perezi* and *P. waltl* populations, the low genetic diversity and connectivity of southern and peripheral populations may be insufficient to reconstitute viable population networks. Against that scenario, protecting the persistence of large, well-connected ponds (including permanent, semipermanent and temporary ones) is the best safeguard to preserve amphibian biodiversity in this area, for which an environmentally sensitive control of water extractions from the aquifer is necessary (Camacho et al., 2022; de Felipe et al., 2023).

Given the widespread regression of wetlands worldwide, it is not surprising that habitat loss and deterioration are the main factors causing global amphibian declines (Sodhi et al., 2008; Stuart et al., 2004). This study highlights that even exceptionally protected areas are not free from these threats. Efforts must be made to preserve pond network dynamics and maintain natural hydric regimes, essential for aquatic ecosystems. The large-scale deterioration of amphibian breeding sites is likely to be causing population-level gene pools to become depauperate (as shown in our two target species), limiting their adaptive potential against the joint challenges posed by climate change, human exploitation, invasive predators and infectious disease outbreaks. The effectiveness of aquatic habitat conservation policies and actions in tackling these threats will determine the fate of amphibians and their associated biological communities in wetland ecosystems.

AUTHOR CONTRIBUTIONS

Conceptualisation and developing methods: GSM, IMS, CDP, IGM. Data analysis and data interpretation: GSM, HMG, JWA. Preparation of figures and tables: GSM, JWA. Conducting the research and writing: GSM, IMS, CDP, HMG, JWA, IGM.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

Individual genotypes of *Pelophylax perezi* and *Pleurodeles waltl* generated and analysed in this work are available from the authors upon reasonable request.

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