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Summary

The Critically Endangered Hooded Grebe *Podiceps gallardoi* has suffered a population decline of 80% since the 1980s. The evolutionary history and its critical conservation status place it 20th in the EDGE of Existence Bird List (EDGE-ZSL) among the more than 10,000 bird species of the world. The identification of demographically independent units ("management units") is essential to address appropriate conservation and management strategies for threatened species. Genetic markers can be used to infer isolated populations without the need for logistically expensive banding and recapture. We used blood samples of 71 Hooded Grebes (c.10% of the global population) from three reproductive populations located at different plateaus that hold over 90% of the species' global population. We analysed genetic population structure using a 353-bp fragment of mtDNA control region and 1,886 RAD loci to study whether Hooded Grebes are philopatric or not. We did not find differences in genetic structure of populations between plateaus indicating that Hooded Grebes do not consistently return to their plateau of origin. Our results are critical to understanding the connection of populations throughout the full annual movement cycle and propose management actions accordingly.

Introduction

The Hooded Grebe *Podiceps gallardoi* has suffered a drastic 80% decline in its population since the 1980s, with a global population now amounting to only c.700 adult individuals (Roesler et al. 2012a, 2025). It is classified as "Critically Endangered" (BirdLife International 2023), and because of its acute and steep decline and evolutionary history, it is classified as 20th among the more than 10,000 species of birds in the EDGE of Existence Bird List (EDGE-ZSL 2023). The Hooded Grebe is an endemic breeder in southernmost continental Patagonia, Argentina (Roesler et al. 2025), with regular – but scattered – records in Chile, where no reproduction has yet been recorded (Roesler 2015).

Several threats have been identified at the Hooded Grebe's reproductive grounds in the lakes of highland basaltic plateaus of western Santa Cruz Province, Argentina. Population viability is affected by alien invasive species, such as American mink *Neogale vison* (Fasola and Roesler 2018; Roesler et al. 2012b) and rainbow trout *Oncorynchus mykiss* (Lancelotti et al. 2016; Porcel et al. 2022), and by the neo-native Kelp Gull *Larus dominicanus* (Roesler et al. 2016), as well as by abiotic factors arising from global climate change (Lancelotti et al. 2020; Roesler et al. 2012a). Also, projected infrastructure constructions, such as hydroelectric dams and wind farms, will affect its wintering grounds (Roesler et al. 2025).

The Hooded Grebe was only discovered and formally named 50 years ago (Rumboll 1974). One of the reasons for this late discovery is that its main reproductive habitat is restricted to 11 isolated, remote, and almost inaccessible, highland basaltic plateaus, in western Santa Cruz Province, southern Argentina, and only a few lakes in the Magallanes Region, in extreme southern Chile (Roesler et al. 2025). These plateaus are island-like habitats, surrounded by deep, glacial valleys (Mazzoni and Rabassa 2018). For similar reasons, remoteness and the extreme conditions of the area, its migratory behaviour towards wintering grounds in estuaries of the

Atlantic Ocean was only described 20 years after the discovery of the species (Johnson and Serret 1994). In winter, Hooded Grebes flock in the estuaries of four rivers (i.e. Coyle, Gallegos, and Santa Cruz-Chico) that flow into the Atlantic Ocean in eastern Santa Cruz Province, where groups of several hundred – up to 97% of the population – have been recorded (Roesler et al. 2025).

Both reproductive and wintering grounds are clearly defined and non-overlapping, which led to the idea of isolated populations (Roesler 2016). Furthermore, Fjeldså (1986) suggested the probable existence of philopatry (site fidelity) in breeding populations. This behaviour seems to be supported by a relatively constant number of individuals at each plateau in different years and by regular re-observation of banded individuals (Roesler et al. 2025).

Genetic markers can be used to infer the connectivity among populations without the need for the logistically expensive capture, banding, and recapture (DeSaix et al. 2019; Ruegg et al. 2014). In migratory species, populations are more difficult to define because individuals may share common areas at some period of their annual movements cycle (Møller et al. 2014). However, the subdivision into reproductively isolated populations can occur despite the high mobility when individuals are philopatric and consistently return to a certain area. In some species site fidelity can be maintained both in reproductive and wintering grounds, while in others that separation is only maintained during a certain time in the annual cycle, with individuals of different populations coexisting during the rest of the year (Esler 2000).

Migratory species are particularly susceptible to environmental changes as they depend on both wintering and reproductive areas, along with stop-over sites during migration (Runge et al. 2014). Understanding the role of seasonal migration in mediating gene flow among populations is important to identify distinct evolutionary and demographic units relevant to conservation and management (Battey et al. 2018), and to identify the impact of local stressors on population declines as disturbances at one stage of the annual cycle can have carry-over effects to other phases of the annual cycle (Kramer et al. 2018; Norris and Taylor 2006). Furthermore, restricted gene flow can accelerate the evolution of adaptive divergence (Rolshausen et al. 2009). This is particularly relevant in a context of climate change and high biodiversity loss (Díaz et al. 2019).

Here we investigated if Hooded Grebes are philopatric towards their breeding plateaus by studying their genetic population structure. This work seeks to understand populations throughout the full annual cycle, which is critical for understanding the ecology and evolutionary biology (Faaborg et al. 2010; Marra et al. 2015; Sherry 2018), as well as detecting the potential impact of local extinctions at individual plateaus for the conservation of this Critically Endangered species. The identification of demographically independent units (Palsbøll et al. 2007), also called "management units" or "conservation units", is essential to maintain the adaptive potential of threatened species and address appropriate conservation and management strategies (Allendorf and Luikart 2007; Fraser and Bernatchez 2001). The results obtained provide key information to define and optimise present and future conservation actions of the Hooded Grebe's Conservation Project (led by the Programa Patagonia of Aves Argentinas/BirdLife International).

Methods

Study site and species

Study site

A series of 11 highland plateaus run parallel to the Andes mountain ridge in central-western Santa Cruz Province, Argentina. Seven of those plateaus were used by the Hooded Grebe during the last 15 breeding seasons (Roesler et al. 2025; Figure 1A). These plateaus comprise a vast area, covering approximately 24,000 km^2. The origin of the plateaus is recent from a geological point of view, since they originated during the Miocene to Pliocene geological epochs, with recent lava records of just over 100,000 years, during the Pleistocene (Brown et al. 2004; Lancelotti 2009; Mazzoni and Rabassa 2018). All plateaus are similar, although they vary in size (from 1,706 km^2 to 2,983 km^2). Their height ranges from 500 m to 1,500 m a.s.l., with peaks such as Mount Zeballos at 2,700 m a.s.l. (Roesler 2016). Most of the remaining populations of Hooded Grebes are concentrated on three of these plateaus (Roesler et al. 2012a): Lake Buenos Aires Plateau (BAP) 47°09'20S 71°16'32W; Lake Strobel Plateau (ST) 48°28'40S 71°22'46W); La Siberia – or Lake San Martín – Plateau (SIB) 49°01'31S 71°43'59W) (Figure 1A).

Study species

Although the reproductive strategy of the Hooded Grebe is somewhat similar to that of other grebes in terms of colonial behaviour, it is more extreme, forming larger, denser colonies of up to 100 pairs on just a few lakes per plateau (Beltrán et al. 1992; Fjeldså 2004; Johnson 1997; Roesler et al. 2012a, 2025). This strategy facilitates the annual location of most individuals at each plateau (Roesler et al. 2012a, 2025) during the reproductive season (October–March). Regular censuses showed relatively constant numbers at each important reproductive plateau (BAP, ST, and SIB) leading to the hypothesis that those numbers might be a consequence of a strong philopatric behaviour (Roesler 2016, 2025) with grebes returning to the same plateau after wintering in the Atlantic estuaries.

Sampling methods

Samples were obtained from a total of 71 individuals BAP ($n = 27$), ST ($n = 24$), and SIB ($n = 20$) (see Supplementary material Table S1). They were mostly taken during banding captures that took place in the reproductive season at night using a small net, flashlights, and an inflatable paddle boat, as described in Roesler (2016) (Permit: Consejo Agrario provincial N°31/2012-2016). A blood sample of approximately 50 µl was taken from the brachial vein and stored in lysis buffer (100 mM TRIS, 100 mM EDTA, 10 mM NaCl, 2% SDS) at room temperature until genetic analysis. Some samples were obtained from dead individuals found at the reproductive plateaus. Pectoral muscle samples of these individuals were stored in DMSO buffer (20% v/v DMSO, 250 mM EDTA, NaCl).

DNA sequencing

We purified genomic DNA from the blood samples using a DNA blood and tissue extraction kit (Qiagen, Valencia, CA, USA). A 353-bp fragment of the mtDNA control region was amplified using primers CR3F (5' GCCYCTTATGTCGCCATGC 3') and CR550R (5' GGTGTAGGGGGAAAGAATGATCC 3') (Ogawa et al. 2015). Amplification products were sequenced in an ABI 3130xl (Applied Biosystems, Foster City, CA, USA) sequencer using ABI Big DyeTM Terminator Chemistry at the Faculty of Exact and Natural Sciences, University of Buenos Aires. Sequences were edited and aligned using Clustal W (Thompson et al. 1994) with Bioedit v.7.2.5 (Hall 1999).

We also generated restriction site-associated DNA markers using the 3RADseq procedure following Hoffberg et al. (2016). The procedure was divided into two phases. First, we sequenced three individuals from different localities using 300-bp paired-end reads on the Illumina MiSeq to generate high-quality reference libraries. This followed our approach for constructing single-copy

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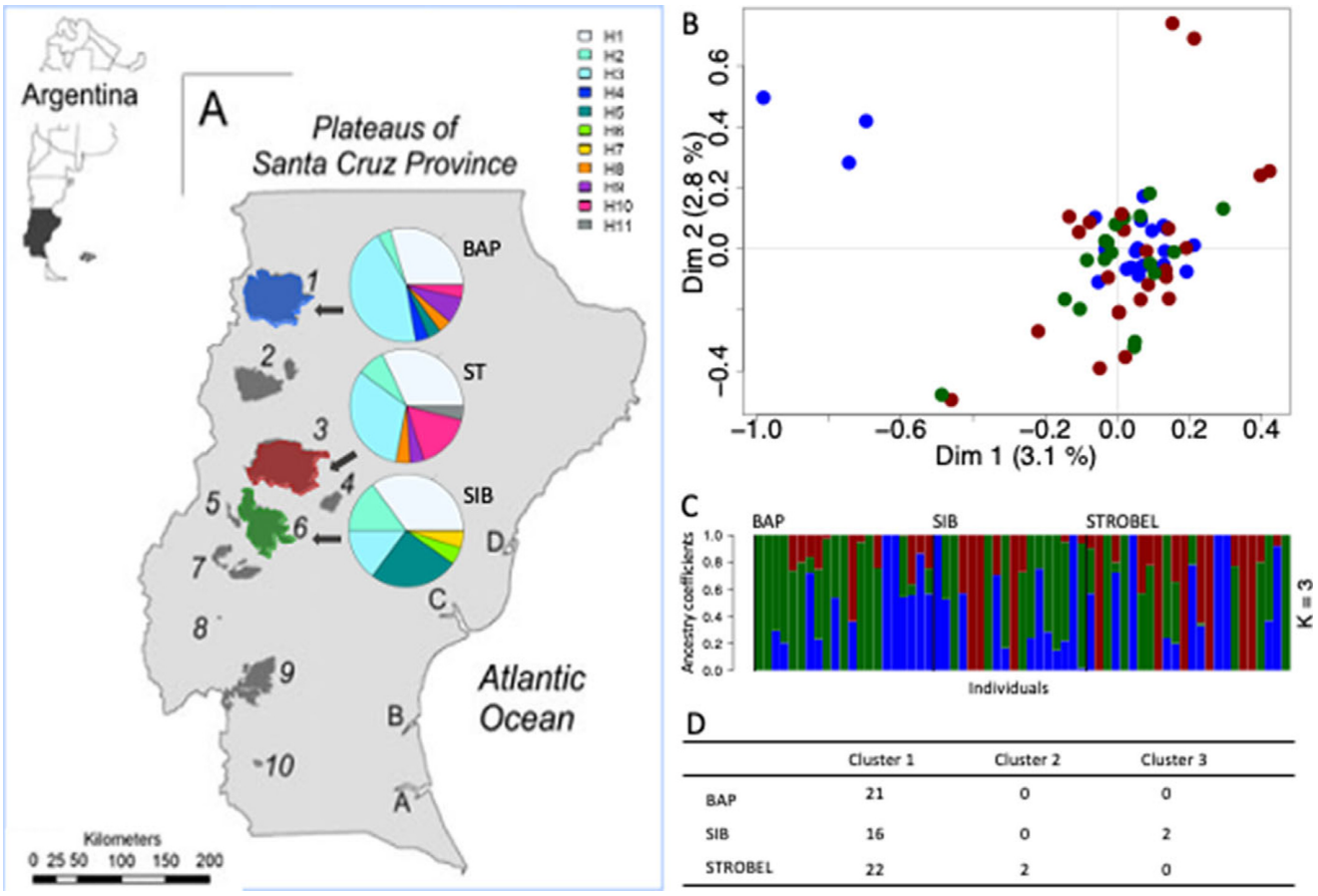

Figure 1. Molecular marker distribution among plateaus: (A) mtDNA data (353 bp control region fragment); (B–D), genomic data (1,738 SNPs). (A) Circles show haplotype (H1–H11) frequency distribution among plateaus. Numbers indicate plateaus where Hooded Grebes breed (studied plateaus are coloured). 1: Buenos Aires (BAP; blue; $n = 27$); 2: Asador; 3: Strobel (ST; red; $n = 24$); 4: Ventana; 5: Moro; 6: La Siberia (SIB; green; $n = 20$); 7: Viedma; 8: La Gringa; 9: Vizachas; 10: La Torre. Letters indicate the estuaries where Hooded Grebes winter. A: Gallegos; B: Coyle; C: Santa Cruz; D: San Julián. (B) Principal Component Analysis; (C) Bayesian clustering; (D) Discriminant Analysis for 63 individuals (BAP, blue, $n = 21$; ST, red, $n = 24$; SIB, green; $n = 18$).

orthologs without a high-quality reference genome (Driller et al. 2021; Vilaça et al. 2023). Next, we prepared libraries of individual pools, calibrated via a spike-in on the Illumina MiSeq (Arantes et al. 2023). In short, genomic DNA of each sample was standardised to 20 ng/µl and digested using three restriction enzymes, XbaI, EcoRI, and NheI (New England Biolabs, Ipswich, MA, USA). Fragments were then ligated to P1 and P2 adapters (with unique barcode combinations) to 5' and 3' ends, respectively. We pooled all samples and size-selected the DNA library using BluePippin (Sage Science), retaining fragments between 290 bp and 590 bp. We then performed a single-cycle polymerase chain reaction (PCR) to add an 8N fragment at 5' for duplicate detection and finally amplified the DNA fragments by performing eight PCR cycles with an outer P5 and an indexing P7 primer. To avoid bad sequencing outputs, we divided the library into six aliquots before performing the indexing PCR (i.e. we added six different P7 indexes). We finally pooled the six amplification products in equimolar ratios to create a single library for sequencing. The library containing 65 individuals was sequenced on one lane of an Illumina NextSeq 500 at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv), producing 140 million 150-bp paired-end reads.

Bioinformatics and assembly of RAD loci

To generate the single-copy orthologs reference library based on three samples, we first improved the quality average of the 300-bp paired-end reads. Quality was assessed using FastQC version 0.11.6 and quality improvements were performed as follows. First, we cleaned fragments containing the cutting side of the third enzyme (NheI); we then trimmed Nextera Adapter Sequences with Trim Galore (Krueger 2021); afterwards, reads containing an average Phred quality score below 20 were removed; finally, we trimmed sequences to 220 bp using fastX_trimmer (Gordon and Hannon 2010) removing shorter sequences. We demultiplexed the reads using the process_radtags module from the Stacks pipeline ver. 1.44 (Catchen et al. 2013) to obtain files containing sequences that were specific to each individual. PCR duplicates were removed using clone_filter. The single-copy orthologs reference library was built using the denovo_map.pl script from Stacks with parameters m = 3, M = 3, $n = 3$, and $N = 5$ after Paris et al. (2017). With the populations module, we retained all loci present in the three individuals ($N = 20.852$).

Reads obtained from the NextSeq run containing all individuals were demultiplexed using process_radtags and quality was improved as described above, with reads trimmed to 125 bp. Reads were aligned

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to the single-copy orthologs reference library with Bowtie2 version 2.2.8 (Langmead and Slazberg 2012) with an average alignment rate of 80%. Aligned sequences were converted with SAMtools (Li et al. 2009) and assembled into RAD loci using the ref_map.pl script from the Stacks pipeline (Catchen et al. 2013). Single nucleotide polymorphisms (SNPs) were then exported using the populations module, retaining loci present in at least 75% of the individuals of each plateau (*r* parameter) and shared among the three plateaus. We applied a minor allele frequency filter of at least 0.05 (--min_maf) and a maximum heterozygosity of 0.5 (--max_obs_het) retaining SNPs with a minimum stack depth of 10. SNPs were exported in different data sets as variant call format (.vcf).

Population genetic statistics

For mtDNA sequences, we calculated basic genetic variability parameters using the pegas package (Paradis 2010) in R environment (R Core Team 2017). The same package was used to analyse population structure calculating pairwise ΦST among sites.

Population genetic statistics (major allele frequency, percent polymorphic loci, nucleotide diversity (π), and Wright's F statistics FIS) were calculated for every SNP using the populations package in Stacks (Catchen et al. 2013). We also assessed the overall level of differentiation among populations at plateaus by calculating individual (per SNP) and general (across all SNPs) differentiation (F_{ST}; Wright 1943).

We used the R package SambaR (de Jong et al. 2021) to analyse population structure and population differentiation. For these analyses we exported one SNP per locus (write_single_SNP) with the populations module of Stacks. The SNP data set was converted from variant call format to the final input format with PGDSpider v.2.1.1.5 (Lischer and Excoffier 2012) and PLINK 1.9 (Chang et al. 2015). SambaR performs diverse clustering methods to analyse SNP data: Principal Component Analysis (PCA), Discriminant Analysis (DA), and Bayesian clustering. To run the analyses, we set a maximum of missing data per individual of 25% (indmiss = 0.25) and per SNP of 10% (snpmiss = 0.1). We also performed Bayesian clustering algorithms in STRUCTURE (Pritchard et al. 2000) using the admixture ancestry model and correlated allele frequencies. Markov Chain Monte Carlo (MCMC) models were fixed at 750,000 iterations, discarding the initial 250,000 as burn in, and *K* varying from 1 to 3 (conducting 10 replicates per *K* value with a different random seed). We used structure harvester v0.6.94 (Earl and von Holdt 2012) and clumpp v1.1.2 (Jakobsson and Rosenberg 2007) to combine results from replicate runs and calculate the average membership coefficients of each individual to each cluster.

Results

MtDNA sequences of the 71 Hooded Grebe individuals showed seven variable sites defining 11 haplotypes (GenBank Accession Numbers OR760039–OR760049). Two haplotypes were frequent (H1, H3), four were less frequent (H2, H5, H9, H10), and five were rare (Figure 1A). Diversity values for the entire population were h = 0.77 and π = 0.003. Pairwise ΦSTs were low and only significantly different between the southernmost SIB and the northernmost BAP (SIB-BAP ΦST= 0,09, *P* = 0,03). ST was not significantly different from either of the other two plateaus (ST-BAP ΦST= 0,00, *P* = 0,75; ST-SIB ΦST= 0,06, *P* = 0,06; Figure 1).

We recovered a total of 2,120 SNPs in 1,886 RAD loci across 63 individuals after completing all filtering processes. Diversity values were similar among populations (Table 1). Pairwise differentiation among populations was not significant (*P* >0.5). The average FST value between pairs of populations was FST= 0.01 (0.0137–0.0157) and consistently low among all SNPs.

Different analyses for population structure showed consistent results grouping all individuals in one cluster, independently of the plateau of origin. The proportion of missing data was low for individuals (mean = 0.02) and for SNPs, of which 148 were discarded. PCA based on 1,738 retained SNPs did not separate individuals by plateaus but grouped them in one cluster with only a few individuals from different plateaus showing some genetic distance from the majority (Figure 1B). Bayesian clustering algorithms also showed the highest log-likelihood for one cluster (K = 1). Clustering in three groups (K = 3) did not assign individuals to plateaus (Figure 1C). Finally, the DA including three clusters also assigned almost all individuals to the same group (Figure 1D).

Discussion

We did not find differences between plateaus in population structure of Hooded Grebes. Nuclear markers did not separate individuals by plateaus but pooled them into one cluster containing all individuals belonging to three different reproductive populations. For mtDNA, we found a low differentiation between individuals belonging to the northernmost and southernmost plateaus included in the study, BAP and SIB, respectively. Thus, genetic markers do not show the pattern of differentiation expected for a long-lasting and strict philopatric behaviour towards the breeding plateaus. Our results, stemming from c.10% of the global population, also show that genetic diversity indices are within the range of the mean values found in birds (Ellegren 2013), indicating that no significant loss of genetic variability has yet occurred, despite the dramatic population decline the species has suffered in the last years. Since the reduction in population size occurred between the 1980s and early 2000s and the

Table 1. Summary genetic statistics across 2,120 variable RAD sites for the three populations (BAP = Lake Buenos Aires Plateau, ST = Strobel Plateau, SIB = La Siberia Plateau). These statistics include the average number of individuals genotyped at each locus (*N*), the number of polymorphic nucleotide sites across the data set (Poly_sites), percentage of polymorphic loci (%poly), the average frequency of the major allele (P), the average observed heterozygosity per locus (Hobs), the average nucleotide diversity (pi), and the average Wright's inbreeding coefficient (FIS)

	N	Poly_sites	%poly	P	Hobs	pi	FIS
BAP	20.92	2,094	0.045	0.78779	0.29014	0.30698	0.03818
ST	23.89	2,108	0.045	0.78716	0.29338	0.30712	0.03121
SIB	17.94	2,083	0.045	0.78550	0.29548	0.30980	0.03335

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generation time is supposed to be relatively long (Fjeldså 1986), it is possible that individuals still retain ancestral genetic variability.

The hypothesis of site fidelity was based mostly on field observation. For 10 consecutive breeding seasons (2012–2022), approximately the same number of individuals was observed at different plateaus (Roesler et al. 2025). A plausible explanation for the absence of a genetic pattern despite observational evidence is that Hooded Grebes exhibit reproductive but not natal philopatry. In this scenario, after the first migration towards the estuaries, first-year individuals return to any of the plateaus without selecting the one where they were raised, returning to that last plateau during the following reproductive attempts. In this scenario, a genetic differentiation among plateaus is not expected.

Differences between natal and reproductive philopatry might be related to the strength of parent–offspring associations. It has been shown that migratory behaviour has a genetic basis (Berthold and Querner 1981; Helbig 1991; Merlin and Liedvogel 2019), but is also culturally transmitted in many species, where the bond between parental individuals and their offspring determines the first migration of juveniles (Sutherland 1998). Roesler et al. (2025) mentioned an association between adults and juveniles in autumn migration, but not a strict association, since some juveniles remain at different wintering grounds than the adults. In European grebes, it has been found that fidelity to the breeding site is more pronounced in adult individuals than in juveniles, suggesting that natal philopatry might not be as strong as reproductive philopatry (Konter and Konter 2006), with breeding dispersal being less pronounced than natal dispersal (Paradis et al. 1998).

A second explanation is that Hooded Grebe has both natal and reproductive philopatric behaviour, but that this behaviour is not fixed. A relaxed philopatry, with eventual movements between lakes and/or plateaus, might arise from unstable conditions of certain lakes/plateaus. This kind of dispersion between nearby lakes within the same plateaus was mentioned in the past when the conditions in a year were unfavourable (Lange 1981). The same situation was also detected between plateaus, when lakes dry up completely, as is the case of Cerro Ventana Plateau in 2010, where the populations disappeared (Roesler 2016). Although the fate of Cerro Ventana's individuals is unknown, it could be linked to dispersal or, alternatively, simply to populations becoming locally extinct.

Migration and movement features can change with global warming. Visser et al. (2009) showed that global warming induced declines in migration distance in many bird species in north-west Europe. Climate change may thus not only result in strong shifts in phenology and species breeding ranges (Parmesan and Yohe 2003; Root et al. 2003; Visser and Both 2005), but also in changes in migration timing, passage, and wintering areas, and consequently the entire annual cycle of migratory birds (Berthold 2001). A thorough study on Hooded Grebes' habitat availability at ST (Lancelotti et al. 2020) showed a marked reduction of lakes during the last four decades due to climatic changes. Banding information suggests that during dry periods, like 2022–2023 (IR), the proportion of marked individuals relocated outside their "plateaus of origin" increases (i.e. individuals banded in one plateau and reobserved in a different one; Roesler et al. 2025). Banded individuals from SIB were found in ST, and individuals from BAP were also found in ST. However, no individuals banded in BAP or in SIB were found south or north of ST, respectively (Roesler et al. 2025). These findings relate to the mtDNA differences found between individuals of the more distant plateaus. This might well fit with the pattern suggested above, that under unfavourable lake/plateau conditions, individuals disperse but within close areas, not dispersing randomly to any of the other plateaus. Therefore, a "relaxed" philopatry, conditioned by external conditions might be operating in Hooded Grebes.

A slight differentiation between the northernmost (BAP) and the southernmost (SIB) plateaus in mtDNA, but not nuclear DNA, might be related to dispersal differences between sexes (Prugnolle and de Meeus 2002). There is no information on sex-biased dispersal in grebes. A relevant implication of the existence of this different rate of dispersal between sexes is that conservation efforts might focus on supplementing depressed populations with females since males have higher chances of colonisation.

The identification of management units or conservation units (Moritz 1994; Palsbøll et al. 2007) is essential to maintain the adaptive potential of threatened species and address appropriate conservation and management strategies (Allendorf and Luikart 2007; Fraser and Bernatchez 2001). We were unable to identify management units for the Hooded Grebe. The genetic patterns we observed do not support a strict philopatric behaviour towards reproductive plateaus. On the contrary, our results suggest that the Hooded Grebe is adapted to a variable, rather than a stable, environment, requiring large areas where individuals can search for proper breeding habitats during different climate conditions. Evidence shows that migratory traditions are not static and that migration routes, stop-over sites, and migration phenology are dynamic systems evolving over time (Jonker et al. 2013). A philopatric behaviour intended to maximise fitness outcomes is probably an interplay of fixed and plastic movements that optimise the use of known habitats but also allows flexible responses to varying environments (Clausen et al. 2018). The plasticity of some birds where individuals switch between strategies in response to previous experience, suggests that those individuals explore environmental conditions to adopt an optimal migration strategy (Madsen 2001). These changes can be magnified manifold by the influence of these individuals on conspecifics copying their choice. Such cultural transmission between individuals is probably a common phenomenon among migrating social birds (Jonker et al. 2013; Mueller et al. 2013). Thus, Hooded Grebes might have responded to environmental changes in the past, as well as the present, moving towards suitable breeding and wintering sites, showing only a recent use of the plateaus in evolutionary terms and a current switch of plateaus due to unfavourable conditions. Further studies on marked individuals will give us more information about natal and reproductive philopatry and the tendency for individuals to return to their reproductive plateaus depending on sex.

Relevant actions to protect the species need to switch from strategies focused on conserving spatially limited restricted areas (i.e. only focused on nature reserve creation) to collaborative, large-scale, mixed land-management strategies. These should include the creation of protected areas, as well as the restoration and protection of private lakes (i.e. collaborative work with fish farmers and sport fishermen). Moreover, our results are highly relevant for a current programme that actively recovers ecologically lost eggs from the wild to produce juveniles by hand rearing to reinforce and maximise annual population recruitment (Gabarain et al. in prep.). The lack of genetic structure allows us to use those individuals to reinforce all plateaus' populations, regardless of their origin. This is especially important since there has been a strong reduction in some of those plateaus with almost no reproduction in over a decade (Roesler et al. 2025).

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