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Investigating Fine-Scale Breeding Habitat Use by Amphibians in a Continuous Wetland Using Environmental DNA

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ABSTRACT

Designing effective conservation plans to protect species from extinction requires a comprehensive understanding of their ecology. Conventional methods used to investigate habitat use are time-consuming, and the detectability of cryptic species is often insufficient. Environmental DNA (eDNA)-based approaches provide a complementary tool to traditional monitoring methods for ecosystem monitoring and assessment. Nevertheless, to our knowledge, such methods have rarely been applied to investigate habitat use at a fine scale in a continuous wetland environment. Here, we used an eDNA metabarcoding approach to characterize the breeding habitat use of local amphibian species in a wet meadow expanse along the southern shore of Lake Neuchâtel, Switzerland. We retrieved DNA from six out of the seven species expected to be present. We tested the influence of six abiotic environmental variables on overall species assemblages and individual species occurrences. We showed that the main factor structuring species assemblages was water temperature and that the distribution of three amphibian species was associated with several environmental variables. Our results indicate that the eDNA detection approaches are promising tools to study species' ecology at a small scale in continuous wetland habitats.

1 | Introduction

Amphibian populations have been collapsing for decades (Houlahan et al. 2000; Zedan 2004). A major contributor to this decline is habitat loss, fragmentation, and degradation (Arntzen et al. 2017; Becker et al. 2010). While most amphibians live in freshwater wetlands or humid habitats, 87% of global wetland surface has been destroyed during the last three centuries (Davidson 2014). Conservation measures to preserve the remaining natural habitats are therefore crucial to reverse current population declines.

Designing sound conservation measures requires a deep understanding of species ecology, population trends, and distributions (Cristescu and Boyce 2013). Thus, reliable and efficient monitoring methods are needed. However, standard amphibian surveys relying on acoustic and/or visual detection and trapping are time consuming and often prove to be inefficient in monitoring

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cryptic species, such as most salamander species (Rödel and Ernst 2004). Hence, effective survey tools must be developed to increase the efficiency and detectability of such species.

Recently developed environmental DNA (eDNA)-based survey methods offer new tools for monitoring wildlife communities (Garlapati et al. 2019; Ruppert, Kline, and Rahman 2019; Taberlet et al. 2018). The eDNA approach is a non-invasive survey method that relies on the collection and analysis of DNA released by individuals through, for example, dead cells, hair, feces, gametes, or carcasses into the environment (e.g., soil or water; Taberlet et al. 2012). This approach, combined with DNA metabarcoding (i.e., the amplification and sequencing of short species-diagnostic DNA sequences with universal primers; Taberlet et al. 2012) has been used for present and past biodiversity monitoring (e.g., Lodge et al. 2012; Willerslev et al. 2003), to survey endangered (e.g., Thomsen et al. 2012) and invasive species (e.g., Dejean et al. 2012; Ficetola et al. 2008; Smart et al. 2015), in diet analyses (e.g., De Barba et al. 2014; Shehzad et al. 2012), but also to study trophic relationships and niche partitioning (e.g., Calderón-Sanou et al. 2021; Chalmandrier et al. 2019; Martinez-Almoyna et al. 2019) and habitat use (e.g., Perl et al. 2022; Sakata et al. 2017).

eDNA-based methods are generally more sensitive and efficient, and often more cost-effective than standard monitoring methods (Biggs et al. 2015; Lopes et al. 2017) and have often been used to monitor amphibian species (reviewed in Ficetola, Manenti, and Taberlet 2019). Nevertheless, for these taxa, eDNA metabarcoding approaches have so far mainly been used in discrete environments such as distinct water bodies (e.g., Biggs et al. 2015; Dufresnes et al. 2019; Eiler et al. 2018) or streams (e.g., Li et al. 2021a; Pilliod et al. 2013, 2014) to monitor species occurrences, but rarely in continuous natural wetlands to study habitat use at a fine scale. Some studies have, however, extended the use of eDNA metabarcoding techniques, demonstrating their efficacy in assessing finer ecological dynamics within continuous natural wetlands by assessing the reproductive phenology of an endangered frog species (Chen et al. 2023) and examining the impact of freshwater connectivity on spatially integrated biodiversity signals (Littlefair et al. 2023).

In this study, we used eDNA metabarcoding to study the fine-scale breeding habitat use of the amphibian species present in a continuous wetland complex in Switzerland. We tested the influence of six abiotic environmental variables (average mud depth, percentage of emerged and submerged vegetation, percentage of emerged land, average water temperature, and the distance to the wintering habitat) on species assemblages and individual species distribution. Finally, we investigated whether biotic interactions could influence amphibian distribution by examining co-occurrences of the different species in the studied area.

2 | Methods

2.1 | Study Area

La Grande Cariçaie, located along the southern shore of Lake Neuchâtel, is the largest remaining continuous wetland area in Switzerland. The area is protected by eight natural reserves, totaling about 660 ha. The habitat consists of three main vegetation types (after Delarze, Gonseth, and Galland 1998): sedge meadows dominated by *Carex elata* and *Cladium mariscus* (*Magnocaricion*); reedbeds dominated by *Phragmites australis* (*Phragmition*); and open water bodies (ponds, ruts) dominated by *Nymphaea alba* (*Nymphaeion*). We focused on two of the eight natural reserves: Yverdon and Gletterens (Figure 1).

Seven amphibian species are present in La Grande Cariçaie: the smooth newt (*Lissotriton vulgaris*), the palmate newt (*L. helveticus*), the common toad (*Bufo bufo*), the common frog (*Rana temporaria*), the European tree frog (*Hyla arborea*), and the complex of pool frogs of the genus *Pelophylax* comprising two mitochondrial haplotypes: *P. ridibundus* and *P. bergeri* (Dubey, Leuenberger, and Perrin 2014; Dufresnes et al. 2017).

To ensure the presence and distribution of amphibian species in the study areaand to obtain an estimate of their densities, we monitored the number of individuals per species during prenuptial migration using interception barriers (Table S1). Local amphibian species, except for most *Pelophylax*, winter in forests and migrate to wetlands to breed. Wintering habitats in Yverdon are separated from the breeding habitat by a road. To reduce mortality due to traffic, barriers leading to tunnels allowing amphibians to cross below the road were built. These tunnels are on average 58.28 m apart (min = 35 m; max = 105 m) over 408 m, that is, totaling seven tunnels. We placed net traps at the exit of the tunnels to capture and count migrating individuals every morning from January 23 to April 4, 2018 (with some interruptions when migration was stopped by low temperatures). In Gletterens, we installed a 420-m long barrier between the forest and the wetlands to intercept migrating individuals, which then fell into buckets buried every approx. 25 m. We then counted them and released them on the other side of the barrier where they had been captured. For logistical reasons, the barrier was set only between March 1 and March 31, 2018, which can result in an underestimation of the number of individuals during the prenuptial migration compared to the other location. However, it still provides a reliable estimation of the relative density of amphibians along the sampled area, given that the peak migratory activity of amphibian individuals was between March 10 and March 31, 2018 (Info Fauna CSCF&karch 2018).

We defined a sampling point as a 5-m diameter circle within which we collected water samples and measured the environmental variables that were used to characterize the habitat. At both locations, we randomly assigned 25 sampling points stratified across the three vegetation types (*Magnocaricion*, *Phragmition*, and *Nymphaeion*; Figure 1). These are referenced in a vegetation map produced between 2012 and 2014 using a combination of aerial photographs and field identification of the different habitat types (based on Delarze, Gonseth, and Galland 1998). To ensure independence of our samples, the centers of all sampling points were located at least 20 m apart. Sampling points are considered independent given that during water sampling in May (see below) we mainly expect to encounter aquatic amphibian larvae rather than adult individuals, which minimizes the potential movement of terrestrial adults between sampling points, and that DNA transport in such environments has been estimated to be five meters for a newt and up to 10 meters for frogs at high densities (Brys et al. 2021). In

FIGURE 1 | Legend on next page.

FIGURE 1 | Location of the study areas. Top left: Map of Switzerland with the study area delineated by a red square. (A) Map of Yverdon. (B) Map of Gletterens. Habitat types (sensu Delarze, Gonseth, and Galland 1998) are shown by colored polygons (red: *Magnocaricion*; green: *Phragmition*; blue: *Nymphaeion*). Yellow dots represent sampling points. Green lines represent amphibian barriers used for the prenuptial migration study. The background map was extracted from Google Map using QGIS.

addition, the transport of genetic material in the wet meadows of La Grande Cariçaie is probably limited by stretches of emerged land. Due to the relatively small water surfaces of *Phragmition* and *Nymphaeion*, fewer samples could be collected in these habitats than in *Magnocaricion* (final number of sampling points: 27 for *Magnocaricion*, 16 for *Nymphaeion*, and 7 for *Phragmition*; Figure 1).

2.2 | Water Sampling

We collected water samples from May 21 to 28, 2018, which corresponds to the breeding season for local amphibians. We collected two liters of water at each sampling point using the VigiDNA kit (Spygen, Le Bourget du Lac, France) following the manufacturer's protocol. We were interested in recent amphibian presence (i.e., individuals present during the breeding season). In the water column, DNA becomes undetectable within approximately 2 weeks (Dejean et al. 2011; Thomsen et al. 2012), but it can be preserved for much longer in the sediments (Barnes and Turner 2016; Nielsen et al. 2007). To avoid resuspending sediments while walking near the sampling points, we attached the spoon to collect water provided by the kit to a 4-m fishing rod (Figure S1). To avoid cross-contamination between sampling points, the spoon was replaced at each sampling point, and we ensured the fishing rod did not enter the water and rinsed it with bottled water after sampling. Right after the water was filtered, we filled the VigiDNA capsules with 80 mL of CL1 conservation buffer provided by the kit. Filtration capsules were stored at room temperature and in the dark for 2 months until DNA extraction.

2.3 | Environmental Variables

To characterize the habitat at each sampling point, we measured five environmental variables: average mud depth, percentage of emerged and submerged vegetation, percentage of emerged land, and average water temperature. In addition, we computed the distance to the wintering habitat (nearest forest) using QGIS (version 3.0.1).

We estimated average mud depth by averaging measures taken at the center and at 2.5 m from the center at the four cardinal points and at the four edges of the sampling point (Figure S2). The same observer estimated coverage of emerged and submerged vegetation and emerged land for all points. We measured these environmental variables from April 21 to 23, 2018, that is, 1 month prior to water sample collection to avoid contamination with amphibian DNA from sediments released during this measurement procedure. We measured the water temperature every hour at each point simultaneously during the sampling period (April 21 to May 28), which also corresponds to the time of egg laying and development of amphibian larvae (Meyer et al. 2009). At each sampling point, we placed thermologgers (1-Wire/iButton) in Falcon tubes and sealed them with parafilm (Appendix S1). We then calculated the average temperature for each sample point over the entire measurement period.

2.4 | Laboratory Procedures

We extracted the DNA from the VigiDNA capsules in a room dedicated to low DNA-content sample extraction and pre-PCR setup. We followed the DNA extraction protocol as described in Mas-Carrió et al. (2022). We eluted DNA by adding 100 μL of SE buffer and centrifuging twice.

Before performing the amplification of the extracted DNA, we tested eight samples chosen randomly among the 50 samples for inhibitors using RT-qPCR (Biggs et al. 2015), carried out on a QuantStudio 3 Real-Time PCR instrument (ThermoFisher). We amplified a fragment of the 12S mitochondrial ribosomal DNA using the BATR01 primers (Valentini et al. 2016). The qPCR mixture contained 1× AmpliTaq Gold 360 mix (Applied Biosystem), 0.5 μM of BATR01 primers, and 10,000 times diluted stock SyberGreen (ThermoFischer Scientific). As BATR01 primers amplify other vertebrate species including *Homo sapiens*, we added 2 μM of human-blocking primer to the qPCR mix (batra_blk; Valentini et al. 2016). The final volume was 20 μL including 2 μL of DNA template. We diluted the eight samples 1×, 0.5×, or 0.1× to reduce the impact of inhibitors and replicated each concentration three times. We included four PCR and four extraction negative controls in the qPCR plate and a dilution series (from 1× to $10^{-5}\times$ of *R. iberica* DNA extracted from tissues with an initial concentration of 47.2 ng/μL) replicated three times. Thermocycling conditions were the following: denaturation at 95°C for 10 min, followed by 55 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, followed by a melting curve analysis of the PCR products by heating from 55°C to 95°C and continuous measurement of the fluorescence. The samples showed no inhibition; hence, we did not dilute the 50 samples for further metabarcoding amplifications.

We then amplified the extracted DNA from the 50 water samples. The PCR mix included 1× AmpliTaq Gold 360 mix (Applied Biosystem), 0.5 μM of each dual combinatorial indexing tagged forward and reverse BATR01 primers (i.e., primers with eight variable nucleotides added to their 5′ end, allowing further sample identification) and 2 μM of human-blocking primer. The final volume was 20 μL including 2 μL of DNA template. We replicated each sample amplification 12 times in 12 separate PCR plates. In each PCR plate, we set 12 blanks corresponding to empty wells, and this allowed us to estimate the proportion of sequence leaking and tag switches (i.e., false combination of tags, generating chimeric sequences occurring during the sequencing process,

c.f. Sequence processing section), seven negative extraction controls (extraction performed on Milli-Q$^{(R)}$ water), seven negative PCR controls (water) and seven positive controls containing an equimolar assembly of DNA from three exotic species (*P. nigromaculatus*, *Pseudacris maculata*, and *R. arvalis*), with a similar DNA concentration to that of eDNA samples estimated with the qPCR (Appendix S2; see Taberlet et al. 2018 for plate layout). Thermocycling conditions were denaturation at 95°C for 10 min, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, with a final elongation step of 7 min at 72°C. One each out of the seven positive and negative PCR controls per replicate plate was visualized on a 1.5% agarose gel stained by ethidium bromide. We excluded one of the twelve replicate PCR plates from further analyses since no amplification was detected. We then pooled the PCR products from the eleven remaining plates and purified amplicons using a MinElute PCR purification kit (Qiagen).

We size-selected BATR01 amplicons on a 2% agarose gel and purified them using the MinElute Gel Extraction kit (Qiagen). Library preparation was performed using the TruSeq DNA PCR-Free Library Prep (Illumina) with the following modifications to ensure a maximal yield of small-size DNA (amplicon size is on average 110 bp including primers): (i) we skipped the "Remove large fragments" step; (ii) we added 100 μL of undiluted Sample Purification Beads (SPB) to the 100 μL of end-repaired sample; and (iii) we followed the protocol starting from step three of the "Remove small fragments" step. The final library was quantified by qPCR using the KAPA Library Quantification Kit (Roche). Sequencing of the library was carried out at the Genomic Technologies Facility (University of Lausanne, Switzerland). A 100-cycle paired-end sequencing was performed on a single Illumina HiSeq 2500 lane.

2.5 | Sequence Processing

We compiled a reference database by recovering the set of vertebrate DNA sequences from the EMBL-European Nucleotide Archive (release 138) and by downloading Taxonomy from NCBI. We converted those files into ecoPCR format using *obiconvert* (OBITools; Boyer et al. 2016) and performed an *in silico* PCR using ecoPCR (Gentile F. Ficetola et al. 2010). We retained all sequences that (i) matched BATR01 primers with up to three mismatches, except in the last two nucleotides of the primer, and (ii) had a minimum and a maximum amplicon length between 15 and 101 bp, respectively (Bellemain et al. 2010; Valentini et al. 2016).

The metabarcode sequence used in the present study was missing for *L. helveticus* and for *P. bergeri* in the reference database. Thus, we Sanger-sequenced a fragment of the mitochondrial 12S gene covering the whole length of our metabarcode using DNA extracted from a tissue sample for both species and amplified using primers L2519 and H3296 (Wang et al. 2017) (accession numbers: *L. helveticus* OK032503 and *P. bergeri* OQ508898). We then added the sequences manually to the reference database.

Sequence reads were processed using OBITools (Boyer et al. 2016). We assembled forward and reverse reads using *illuminapairedend* with a minimal quality score set to 40 and discarded unaligned sequences (i.e., labeled as joined sequences) using *obigrep*. We assigned each sequence to samples (i.e., we demultiplexed them) using *ngsfilter*. We dereplicated reads by clustering strictly identical sequences into a unique sequence using *obiuniq*. We removed singletons and assigned molecular operational taxonomic units (MOTUs) to a taxon using *ecotag* with the reference database. We cleaned the taxonomically assigned sequences from PCR and sequencing errors using *obiclean* with a minimum ratio between counts of two sequence records set at 0.25.

All downstream analyses were conducted in R version 4.0.3 (R core Team and R Foundation for Statistical Computing 2020). We first removed unassigned sequences and sequences with an identity lower than 98% as described in Valentini et al. (2016). We then used several approaches to filter contaminations and chimeric sequences resulting from sequence leaking. To account for contaminations stemming from laboratory manipulation, we retrieved the maximum number of reads across all negative controls and used it as a minimum threshold for presence in a sample. That is, a MOTU was considered present in a sample only if the number of reads was higher than the highest number of reads across all negative controls. Since we retrieved reads corresponding to two exotic species (*Xenopus tropicalis* and *Rhinella* sp.) after these cleaning steps, we removed the number of reads corresponding to these species from all other MOTUs in each sample. We then accounted for sequence leaking by computing the maximum abundance of MOTUs in blanks (i.e., sequences retrieved in the empty wells set in each PCR plate) and plotting the effect of different proportions of leaking removal on the numbers of MOTUs and sequences (Figure S3). Based on these graphs, we concluded that leaking accounted for 60% of sequences retrieved in samples. Hence, we removed 60% of total reads per MOTU in each sample.

To consider a species as present, no consensus threshold is set in the literature (Goldberg et al. 2016; Harper et al. 2018). In the present study, we attempted to be conservative to limit the occurrence of false positives. We considered a species present if at least three out of the eleven PCR replicates contained a positive number of reads after all cleaning steps for a given MOTU.

2.6 | Statistical Analyses

We first asked whether the species were randomly distributed or whether they were aggregated in the study area. We performed a distance decay analysis in zeta diversity (ζ), which measures the number of species shared by multiple sites and provides insight into changes in species composition related to spatial distribution and species community composition across the landscape (Hui and McGeoch 2014). As we wanted to determine whether sampling points close together had more similar species assemblages than sampling points far apart, and the two reserves are more than 20 km apart, we examined zeta diversity decay for order two (ζ2), three (ζ3), and five (ζ5) (i.e., the number of species shared between two, three and five sampling points) for each reserve separately. We used the *Zeta.ddecay* function in the "zetadiv" package (Latombe et al. 2018). The number of samples from which zeta diversity was calculated was set to a maximum of 1000 for each distance decomposition analysis.

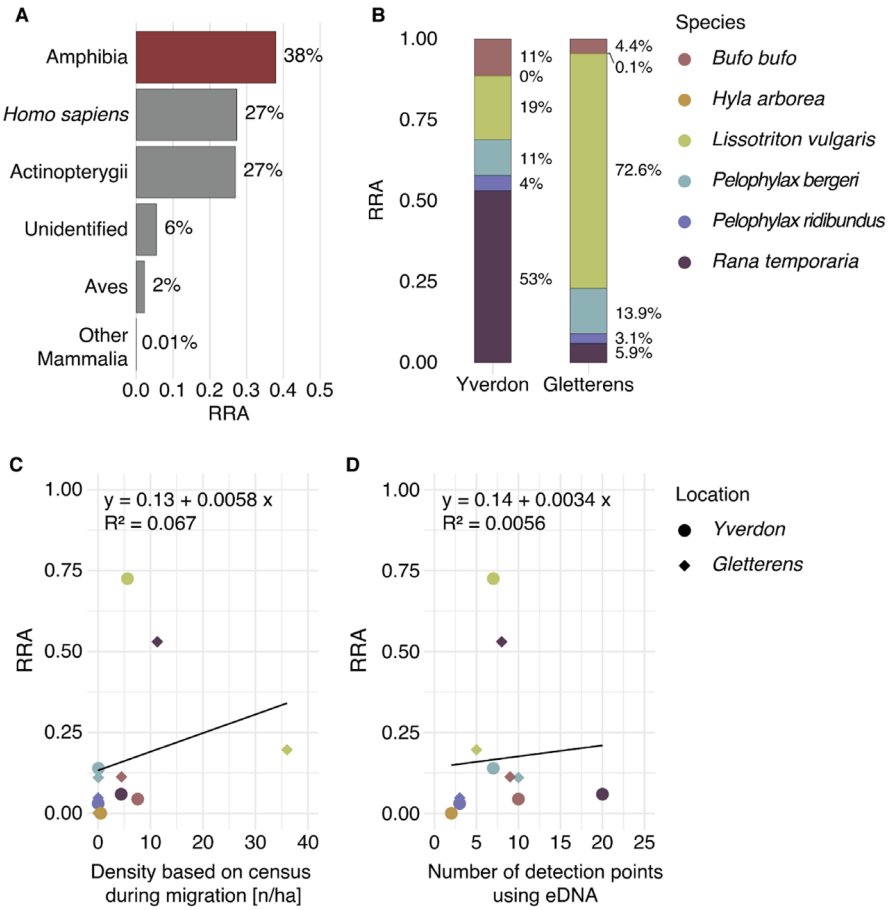

FIGURE 2 | Barplots representing the relative read abundance (RRA) of (A) each retrieved class and (B) of amphibian species for each of the two locations. (C) Relation between the RRA of local amphibian species and their estimated densities calculated using the prenuptial migration survey. (D) Relation between the RRA of local ampibian species and the number of points where they were detected using eDNA approach. Total number of reads after processing sequences with OBITools software was 182,672,348. The total number of Amphibia reads was 50,015,762.

Then, we investigated if the species assemblages could be explained by habitat characteristics. We performed a distance-based redundancy analysis (db-RDA; Legendre and Andersson 1999), which detects linear relationships between a matrix of explanatory variables and a dissimilarity matrix of response variables generated by measures that are non-linear. We used the function *capscale* from the package "vegan" to perform the db-RDA (Oksanen et al. 2020). We added 0.001 to each species value prior to the analysis. We used the *jaccard* method to compute dissimilarity matrices, and this was found to be the most suitable according to the *rankindex* method from the package "vegan." We tested whether the influence of the environmental variables and their quadratic effects on the species assemblages was significant by performing a permanova (i.e., permutational multivariate analysis of variance) using the function *adonis2* from the package "vegan."

Finally, we aimed at assessing the association between environmental variables characterizing the breeding habitat and the presence of each amphibian species. To this end, we tested the effect of the recorded environmental variables and the effect of other amphibian species on the probability of the presence of each amphibian species. We performed separate binomial generalized linear models (GLMs) for each species, using the presence-absence of the focal species as the response variable and the environmental variables. The full model contained each environmental variable. To test the effect of other amphibian species on the probability of the focal species, we performed a PCA on the distribution of all other species and used the first axis as an additional explanatory variable. Backward model selection was then performed using the *drop1* function from the package "lme4" (Bates et al. 2015). Specifically, models were compared based on the Akaike information criterion (AIC), and the model with the lowest AIC was selected. The "test" argument in the *drop1* function was set to "none" to ensure that the selection was purely based on AIC values. The set of models included various combinations of the recorded environmental variables and the first PCA axis representing the distribution of other species. Justifications for the inclusion of each variable were based on their ecological relevance and findings from prior studies. The delta AIC and Akaike weights for each model are provided in Table S3. GLM assumptions were checked for each model using the "DHARMa R" package (version 0.3.3.0; Hartig 2020).

3 | Results

After sequencing the DNA extracted from water samples, we obtained 182,672,348 raw reads. After processing them using OBITools, we retained a total of 131,883,594 reads, of which

50,015,762 were attributed to Amphibia, corresponding to a relative read abundance (i.e., proportion of reads, hereafter RRA) of 0.38, which represents the highest RRA among the identified taxa. We identified three other vertebrate classes: Mammalia (RRA = 0.27, of which 99.99% were attributed to *Homo sapiens*), Actinopterygii (RRA = 0.27), and Aves (RRA = 0.02). A small proportion (RRA = 0.06) of sequences was not assigned using our reference database (Figure 2A).

We detected DNA from six out of the seven species expected in La Grande Cariçaie (*H. arborea*, *B. bufo*, *L. vulgaris*, *R. temporaria*, *P. ridibundus*, and *P. bergeri*). No DNA from *L. helveticus* was recovered despite 97 individuals entering the area during the prenuptial migration. In contrast, we detected DNA of *H. arborea* in Yverdon, although no individuals were caught during the prenuptial migration survey (Figure 2B and Table S1).

Then, we asked whether the density of individuals could be estimated by the number of DNA sequences recovered for a particular species. We found no correlation between the RRA of each amphibian species and its estimated density calculated from the prenuptial migration survey ($R^2 = 0.067$, $p = 0.42$; Figure 2C) or the number of sampling points where the species was detected ($R^2 = 0.0056$, $p = 0.82$; Figure 2D).

We then investigated which factors explained the distribution of our target species within the wetlands. Examining the distance decay plots of ζ2, ζ3, and ζ5 (Figure S4), we found that the sample points furthest apart shared as many species as the sample points closest together in both reserves, suggesting that the species assemblage is randomly distributed with regard to geographic location. We then tested whether the species assemblages were associated with the environmental variables. The db-RDA analysis showed that 27.4% of the variance in species assemblages could be explained by the recorded environmental variables (Total Inertia Eigenvalue $\lambda_{Total} = 16.67$; Constrained Inertia Eigenvalue $\lambda_{Constrained} = 4.57$; adjusted $R^2 = 0.057$). The first axis (CAP1, Figure 3) explained 12.77% ($\lambda_1 = 2.13$), the second axis (CAP2, Figure 3) explained 8.04% ($\lambda_2 = 1.34$), and the six remaining constrained axes explained 6.59% of the total variance. The PERMANOVA performed on the db-RDA indicated that the quadratic effect of water temperature seemed to affect the species assemblages (PERMANOVA: $R^2 = 0.054$, $p = 0.045$, $F = 2.76$; Table S2). The proportion of emerged vegetation and the quadratic effect of the proportion of emerged land tended to affect the species assemblages, but these tendencies were borderline none significant (PERMANOVA: $R^2 = 0.056$, $p = 0.06$, $F = 2.81$, and $R^2 = 0.046$, $p = 0.084$, $F = 2.35$, respectively; Table S2).

We then investigated the breeding habitat use of each of the local species. The distribution of three out of the six detected species was correlated with some of the environmental variables we recorded (Figure 4). The distribution of *P. bergeri* was positively affected by the proportion of emerged vegetation (GLM: df = 49, $Z = 2.052$, $p = 0.04$) and tended to be affected negatively by the presence of other anuran species, proxied in the model by the first axis of the PCA on other amphibian species represented in Figure S5 (GLM: df = 49, $Z = -1.663$, $p = 0.096$). The distribution of *P. ridibundus* was positively associated with high mud depth (GLM: df = 49, $Z = 2.059$, $p = 0.04$). Finally, the distribution of *R. temporaria* was positively affected by the distance to the wintering habitats (GLM: df = 49, $Z = 2.24$, $p = 0.025$). The recorded environmental variables did not seem to affect the distribution of *B. bufo*, *L. vulgaris*, and *H. arborea*, though the distributions of *L. vulgaris* and *H. arborea* tended to be negatively affected by the distance to the wintering habitat (GLM: df = 49, $Z = -1.79$, $p = 0.074$) and vegetation cover (GLM: df = 49, $Z = -1.15$, $p = 0.071$), respectively.

4 | Discussion

Designing efficient conservation measures for endangered species requires a good knowledge of their distribution and ecology. Yet, for inconspicuous species, traditional survey methods often

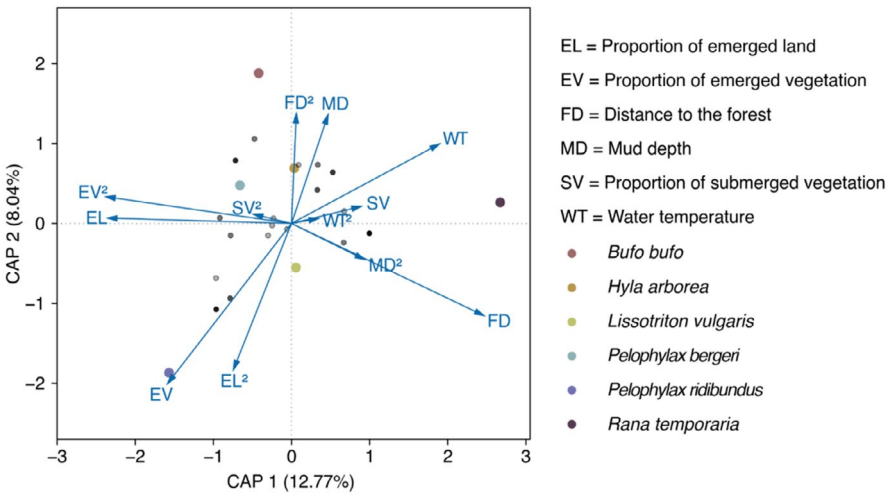

FIGURE 3 | Ordination plot of the distance based-redundancy analysis (db-RDA) on the amphibian species composition. Colored dots represent each species, small transparent gray dots represent the 50 sampling points, blue arrows represent the environmental variables ("²" designates the quadratic effect of a variable). 27.4% of the variance in species composition could be explained by the recorded environmental variables (Total Inertia Eigenvalue $\lambda_{Total} = 16.671$ and Constrained Inertia Eigenvalue $\lambda_{Constrained} = 4.568$). The first axis (CAP1) explained 12.77% of the total variance ($\lambda_1 = 2.13$), the second axis (CAP2) explained 8.04% ($\lambda_2 = 1.34$), and the six constrained axes explained 6.59% of the total variance.

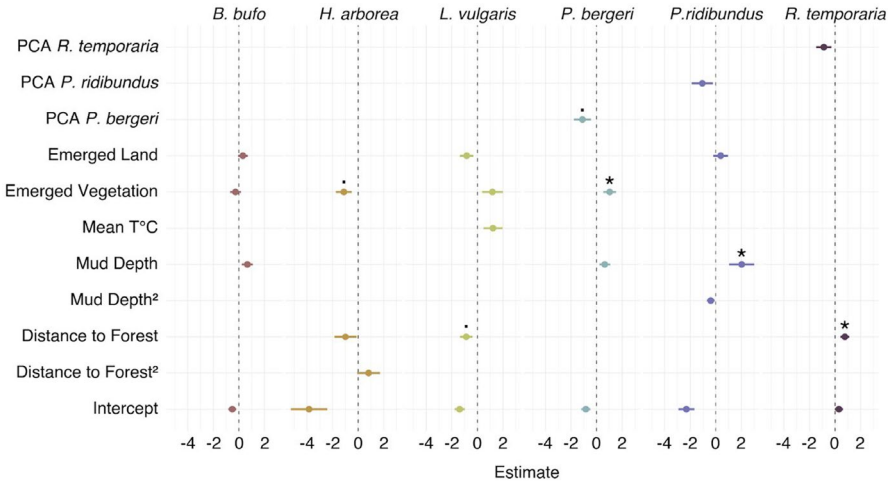

FIGURE 4 | Coefficient plot of the generalized linear models testing the effect of recorded environmental variables on the probability of presence of each of the amphibian species detected using environmental DNA approach. The dots represent the estimate and lines correspond to the standard error. PCA *R. temporaria*, PCA *P. ridibundus* and PCA *P. bergeri*: The effect of other amphibian species on the probability of the focal species, represented by the first axis of the PCA on the distribution of the other species. "2" designates the quadratic effect of a variable. A star on top of the estimate indicates significance ($p < 0.05$); a dot indicates a trend ($0.10 > p > 0.05$).

fail to provide reliable distribution and ecological data. Here, we studied the fine-scale distribution and breeding habitat associations of amphibians in a continuous wetland expanse by means of eDNA metabarcoding. We recovered DNA from six out of seven local amphibian species, that is, four native species, *L. vulgaris*, *R. temporaria*, *B. bufo*, *H. arborea*, and two non-native species from the *Pelophylax* complex, *P. ridibundus* and *P. bergeri*. However, we did not detect DNA from one local species, *L. helveticus*. We found significant positive and negative associations between several environmental variables and some of the detected taxa. These associations underscore the potential of eDNA detection approaches to enhance our understanding of species ecology in these natural systems, providing valuable data for reliable long-term monitoring.

In particular, our multivariate analysis suggested that an important factor structuring species assemblages was water temperature. In addition, while the proportion of emerged land and emerged vegetation both had a marginal statistical influence on communities, they may still hold biological significance despite lacking statistical power. These findings highlight the importance of preserving diverse microhabitat features, such as natural variations in water temperature and diverse vegetation structures, to support amphibian community diversity in wetland ecosystems.

We then investigated the specific habitat characteristics of each of our focal species. The fact that *L. helveticus* was not detected in areas where *L. vulgaris* is present suggests a potential habitat segregation between these related species. This contrasts with previous findings of co-occurrence and similar niches at other locations (Griffiths 1986, 1987). This could be explained by *L. vulgaris* being an opportunistic species that can occupy a wide range of habitats when available (Ćirović, Radović, and Vukov 2008; Dolmen 1988). Accordingly, it was only marginally more likely to be found closer to the wintering habitat, in line with previous findings that it does not migrate over large distances (Kovar et al. 2009), and other environmental variables did not seem to be associated with its distribution. Interestingly, studies elsewhere suggested that vegetation cover might be associated with the presence of *L. vulgaris*. Some studies found that a high coverage of vegetation is required for *L. vulgaris* to breed (Babik and Rafiński 2001; Cooke and Frazer 1976; Ildos and Ancona 1994), while Marnell (1998) found that the presence of *L. vulgaris* was associated with open water bodies with less floating vegetation, although he acknowledged that submerged grasses were more common in ponds where this species was present. Other environmental parameters, such as the chemical composition of the water, might affect habitat use by these species. Indeed, Cooke and Frazer (1976) suggested that *L. vulgaris* and *L. helveticus* tend to occupy water bodies with different chemical properties. While the latter is associated with soft and acidic water, *L. vulgaris* might occur more in hard and neutral water. Taken together, these findings suggest that *L. vulgaris* is a generalist species that can use different breeding habitat types and migrate for up to 300 m from the forest (Table S1), while *L. helveticus* may be restricted to wetlands located closer to the wintering habitats.

The only environmental factor associated with the distribution of *R. temporaria* was the distance to the wintering habitats. *Rana temporaria* is a generalist species that can breed in a wide range of temporal and permanent water bodies (Babik and Rafiński 2001; Beebee 1981; Marnell 1998), in agreement with the results from our eDNA approach. In addition, this species breeds early in the season, and some froglets might already have metamorphosed and dispersed from their natal area where they hatched at the time of sampling.

The distribution of *P. ridibundus* was associated with mud depth. This is consistent with the fact that most *Pelophylax* species often overwinter in the mud (Kovar et al. 2009; Tattersall and Ultsch 2008). Consequently, the low densities measured during the prenuptial migration survey might be underestimated, and a large proportion of individuals might have overwintered downstream from the barriers.

The distributions of *P. ridibundus* and the related *P. bergeri* were not related to the same environmental factors. *P. bergeri* was found in areas with high vegetation cover and tended to be found in areas occupied by fewer other anurans. Whether this reflects competitive exclusion or niche segregation is unclear. This invasive species has replaced the native *P. lessonae* in our study area and throughout a large part of its range (Dufresnes et al. 2017), but its impact on other species is unknown. It is important to note that *P. ridibundus* can produce F_1 hybrids with *P. lessonae* or *P. bergeri*. Because we targeted a mitochondrial marker, it was not possible to identify hybrids (Dufresnes et al. 2018). Consequently, some individuals labeled as *P. ridibundus* or *P. bergeri* might be hybrids, which might have increased apparent habitat heterogeneity within these two species, thus decreasing our ability to detect significant associations.

We did not detect a significant association between the presence of either *B. bufo* or *H. arborea* and any of the environmental variables recorded. However, *H. arborea* tended to be found mainly in habitats with lower emerged vegetation. Higher statistical power is required to confirm this trend, as *H. arborea* was found at only four sampling points.

We did not find a positive correlation between the RRA and the estimated amphibian species densities proxied by the number of individuals of each species captured during the migratory survey, contrary to what has been shown in multiple studies (Di Muri et al. 2020; Handley et al. 2019; Hänfling et al. 2016; Li et al. 2021b). However, standard survey methods have their own biases (Di Muri et al. 2020). Indeed, the migration barriers used in this study were not installed continuously during the migration period, which could have led to an underestimation of species densities. In addition, this survey method is ineffective for *P. ridibundus*, which overwinters primarily underwater, and for *H. arborea*, which is able to climb the barriers. The use of eDNA thus appears to be a good complement to traditional migration surveys, providing a more comprehensive assessment of fine-scale distribution by integrating eDNA data with observations from migration barriers.

Three main hypotheses could explain the absence of *L. helveticus* in our water samples. First, it could simply be absent from the two reserves we sampled. This species is indeed known to be absent from the wetlands of Gletterens; however, it is expected to be present in Yverdon, where we detected 97 individuals during the prenuptial migratory survey. Second, the density of *L. helveticus* individuals in Yverdon might be too low to enable its detection using eDNA approaches. This would be unlikely, as we detected other local amphibian species from which fewer individuals were recorded during the prenuptial migration (Table S1). In addition, it has been repeatedly demonstrated that eDNA is a sensitive method for detecting organisms at low densities, as is the case for the detection of alien species in the initial phase of invasion (Cowart et al. 2018; Dejean et al. 2012; Jerde et al. 2011). Finally, the absence of *L. helveticus* DNA in our water samples could be the result of this species breeding near the wintering habitats in places not covered by our sample points. In this study, we targeted three main vegetation types that are considered most suitable for breeding for most amphibian species. However, other temporarily flooded vegetation types such as *Nanocyperion*, *Cladietum*, and *Caricion davallianae* (as described by Delarze, Gonseth, and Galland 1998) are found directly downstream of the migration barriers and could be used as breeding habitats by *L. helveticus*. This hypothesis is in line with the literature, as this species has been previously observed to winter near its breeding habitat (Diego-Rasilla and Luengo 2007).

In contrast, we detected *H. arborea* DNA in two sampling points in Yverdon. This result is surprising, as this species has not been observed in this natural reserve since 2000 (data from the Swiss Centre for Wildlife Mapping; https://lepus.unine.ch/carto/70120) and no individual was captured during the prenuptial migration survey at this location. It is unlikely that this occurrence can be attributed to cross-contaminations, given that none of the samples were collected or their DNA extracted at the same time as the Gletterens samples containing *H. arborea* DNA. Cross-contaminations during the PCR amplification also seem unlikely, as the samples where this species was detected were spread throughout the plate layout. It is thus likely that DNA from this species was indeed present in Yverdon. It is possible, though unlikely, that this species is still present at a low density in the area but has been undetected for decades. Alternatively, the detected DNA could originate from older eDNA resuspended from the sediments by other animals (e.g., Turner, Uy, and Everhart 2015).

The application of eDNA-based approaches to study fine-scale habitat use in a continuous wet meadow expanse poses new challenges due to the biological and environmental conditions that influence eDNA dynamics. For instance, DNA persistence must be considered to determine the temporal and spatial scales at which environmental variables must be measured to describe the species habitat. Previous studies have investigated the persistence of DNA in water in laboratory or mesocosm conditions and established that DNA remains detectable approximately up to 2 weeks in the water column (Thomsen et al. 2012), while its persistence can extend over several years in sediments (Corinaldesi, Beolchini, and Dell'Anno 2008) or soil (Foucher et al. 2020). In the present study, we aimed to study the habitat use of our focal amphibian species during reproduction. Thus, to avoid contamination from older DNA, we recorded the environmental variables one month before the collection of water samples to ensure DNA resuspended from the sediments had sufficient time to degrade, and we were careful not to resuspend the sediments during sampling. Another crucial parameter to design eDNA surveys is the diffusion potential of DNA in the environment caused by hydrodynamic processes affecting water currents. Brys et al. (2021) determined that the distance to detect amphibians using eDNA methods ranges from five meters for a newt to 10 m for frogs at high density. In La Grande Cariçaie, the water bodies are interspersed with stretches of emerged land and dense vegetation, which reduces DNA diffusion in the environment. We, therefore, considered that the 5 m radius we used to characterize the habitat was a realistic estimate.

Our study highlighted the potential of an eDNA-based approach to investigate species ecology at a fine scale in a continuous wet meadow expanse. While eDNA metabarcoding has proved effective in investigating the association of habitat features with species presence and improving our understanding of their ecology, several challenges remain. Future research should address

issues such as detecting patterns of habitat use in relatively continuous environments and refining methodologies to overcome limitations associated with environmental heterogeneity and eDNA degradation. These efforts will enable us to better estimate small-scale habitat use. Increased knowledge of species ecology is crucial for designing effective conservation policies to protect endangered species by conserving and restoring threatened environments.

Author Contributions

Conception or design of the study and manuscript editing: J.M.G., A.G., G.L., and L.F. Acquisition, analysis, or interpretation of the data and first draft: J.M.G. and G.L.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The DNA metabarcoding data generated for this study are available on SRA (BioProject ID: PRJNA938759, availability upon acceptance of the manuscript). Sanger sequences for *L. helveticus* and *P. bergeri* generated for this study were deposited in GenBank under accession no. OK032503 and OQ508898, respectively. All data, OBITools script, and R script underlying the study can be downloaded from the public repository: https://github.com/JulieGuenat/Amphibian_habitat_Characterization_eDNA.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.