









Spatio-temporal monitoring of lake fish spawning activity using environmental DNA metabarcoding

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Abstract

Determining the timing and location of fish reproductive events is crucial for the implementation of correct management and conservation schemes. Conventional methods used to monitor these events are often unable to assess the spawning activity directly or can be invasive and therefore problematic. This is especially the case when threatened fish populations are the study subject, such as the Arctic charr (*Salvelinus alpinus* L.) populations in Windermere (Cumbria, UK). Arctic charr populations have been studied in this lake since the 1940s, and the locations and characteristics of spawning grounds have been described in detail using techniques such as hydroacoustics, as well as physical and visual surveys of the lake bottom. Here, in conjunction with established netting surveys, we added an environmental DNA (eDNA) metabarcoding approach to assess the spatial distribution of Arctic charr in the lake throughout the year to test whether this tool could allow us to identify spawning locations and activity. Sampling was carried out between October 2017 and July 2018 at three locations in the lake, covering putative and known spawning sites. eDNA metabarcoding provided accurate spatial and temporal characterization of Arctic charr spawning events. Peaks of Arctic charr relative read counts from eDNA metabarcoding were observed during the spawning season and at specific locations of both putative and known spawning sites. Net catches of mature Arctic charr individuals confirmed the association between the Arctic charr spawning activity and the peaks of eDNA metabarcoding relative read counts. This study demonstrates the ability of eDNA metabarcoding to effectively and efficiently characterize the spatial and temporal nature of fish spawning in lentic systems.

KEYWORDS

Arctic charr, breeding grounds, conservation, freshwaters, lentic systems, *Salvelinus alpinus*

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1 | INTRODUCTION

Anthropogenic pressures are threatening freshwater fish populations worldwide (Díaz et al., 2019), and conservation biologists and environmental managers are striving to preserve such diversity as it provides ecosystem services to humans and holds intrinsic evolutionary and ecological value (Lynch et al., 2016; Piccolo, 2017). Arctic charr (*Salvelinus alpinus* L.) has the most northerly distribution of all anadromous freshwater teleosts (Hansen et al., 2019), ranging from the temperate areas of eastern North America and the European Alps, to the most northern points of the Eurasian and North American continents (Johnson, 1980). The species is adapted to cold, highly oxygenated waters and is therefore especially vulnerable to climate change and eutrophication (Winfield et al., 2008). As a consequence, many local populations of Arctic charr have already become extinct (Kelly et al., 2020; Winfield et al., 2010). Despite the observed extinctions and population declines, the level of national and international protection is low. Globally, Arctic charr are not considered to be threatened (Freyhof & Kottelat, 2008), but within Great Britain and Ireland, where the species are at the western edge of their European range, their presence is considered to be rare. While this species does not appear in the annexes of the European Union Habitats and Species Directive (Adams et al., 2007), Arctic charr has been included as a priority conservation taxon within the UK Biodiversity Action Plan and a high conservation value species because of its limited distribution, past extirpations, and current concerns over the conservation status of many populations (Bean et al., 2018; Maitland et al., 2007).

Interest in Arctic charr conservation is primarily driven by the often-unique characteristics of individual populations (Jonsson & Jonsson, 2001; Klemetsen, 2010). The high polymorphism and plasticity of Arctic charr populations encompasses life-history tactics (e.g., anadromous and nonmigratory forms; Klemetsen et al., 2003), specialization in diet and habitat preferences (Adams et al., 2003; Klemetsen et al., 2006), and reproductive strategies (Frost, 1965; Smalås et al., 2013; Telnes & Saegrov, 2004). For example, in lakes where sympatric populations of Arctic charr exist, differences in the spatial and temporal separation of spawning grounds can occur, leading to the emergence of phenotypic variation and genetic divergence between populations (Garduño-Paz et al., 2012).

Arctic charr typically spawn for a limited period at shallow gravel banks where the females dig depressions in which eggs are incubated (Esteve, 2005). Here, a clean substrate with low amounts of fine sediment and a well-oxygenated interstitial zone, is required to ensure successful reproduction (Sterneckner et al., 2014). These stringent requirements mean that only a small fraction of available lake habitat is typically used for spawning. Low et al. (2011) estimated that in Irish lakes, Arctic charr spawning substrate comprised between just 0.4% and 0.7% of the littoral habitat, and that egg numbers are significantly correlated with gradient and spawning site width. Furthermore, a number of studies have recognized that siltation and sedimentation on the spawning gravels are major causes of reproductive failure (Franssen et al., 2012; Levasseur et al., 2006;

Winfield et al., 2010). These variables, but often very specific, temporal and spatial characteristics of Arctic charr spawning mean that detailed local knowledge is invaluable for conservation efforts, to ensure protection of high-quality habitats for reproduction and to identify management units.

Arctic charr spawning areas and spawning behavior in Windermere (Cumbria, UK) have been extensively studied over more than 50 years (Frost, 1965; Miller et al., 2015). Research on breeding habitats of Windermere's Arctic charr has described the presence of two sympatric populations with autumn- and spring-spawning events (Frost, 1965), and documented their patterns of genetic and phenotypic divergence (Corrigan et al., 2011; Partington & Mills, 1988). Outside the spawning season, Arctic charr in Windermere are exclusively restricted to offshore areas of the lake (Lawson Handley et al., 2019; Winfield et al., 2008). Autumn-spawners release their gametes at depths of around 2 m between November and December, whereas spring-spawners mature between February and March and spawn at deeper sites between 15 and 20 m. Both the mesotrophic north basin and eutrophic south basins of Windermere sustain autumn- and spring-spawners, and a variety of putative and known spawning locations have been described in the lake (Frost, 1965; Miller et al., 2015; Winfield et al., 2015). However, these Windermere Arctic charr populations have declined markedly over the last few decades (Winfield et al., 2019) in parallel with increased eutrophication and the associated decrease in spawning habitat quality (Miller et al., 2015; Winfield et al., 2015). Suitable spawning habitats are now limited to the shallowest areas of the lake at depths below 5 m where clean, hard substrates still occur (Miller et al., 2015; Winfield et al., 2015). This poses important conservation concerns for spring-spawning Arctic charr populations with deeper breeding grounds (Winfield et al., 2008). Importantly, extensive monitoring of spawning activity at depths <5 m is challenging using established noninvasive survey methods such as hydroacoustic applications (Miller et al., 2015), and netting surveys cannot be deployed widely due to the conservation status of target species. Noninvasive and broadly applicable monitoring approaches are therefore required to characterize times and locations of fish spawning activities.

The analysis of environmental DNA (eDNA) has been recently applied to the study of riverine fish spawning activity whereby the isolation of genetic material from water samples, eDNA, coupled with species-specific quantitative PCR assays, allowed spawning aggregation and migration patterns of riverine fish to be identified (e.g., Bracken et al., 2019; Bylemans et al., 2017; Tillotson et al., 2018). Temporally and spatially constrained changes in eDNA concentration of target fish species were found to be associated with spawning activity and release of gametes. However, this approach has not yet been applied to fish populations in standing water.

Recent studies have demonstrated that eDNA metabarcoding, which is used to describe the entire fish community as opposed to single target species, is able to detect seasonal variation in community composition, heterogeneity in the use of habitat, and even estimates of population biomass and abundance (Di Muri et al., 2020;

Lawson Handley et al., 2019). Here, we apply eDNA metabarcoding to investigate the spawning activity of Arctic charr in Windermere. Based on evidence provided by previous eDNA research, we hypothesize that (1) eDNA metabarcoding analyses, using extensive spatio-temporal water sampling, can detect lake fish spawning activity via the temporal and spatial variation in the proportion of eDNA read counts during the breeding season, and that (2) species-specific peaks in relative read counts from eDNA metabarcoding reflect the sites and times where spawning events are expected. To test our hypotheses, we focused on the shallowest breeding grounds of the autumn-spawning Arctic charr population in Windermere's north basin that have recently been assessed as being suitable to support spawning activity (Miller et al., 2015; Winfield et al., 2015). Specifically, we targeted putative and known spawning grounds, with the latter monitored by annual netting since 1940 (Winfield et al., 2008). Gill-netting survey results were compared with Arctic charr relative read counts associated with spawning individuals caught at these sites. Additionally, in autumn, we expected an eDNA signal of similar strength at the putative spawning grounds if any spawning activity was occurring.

2 | MATERIALS AND METHODS

2.1 | Water sample collection

Water sampling was carried out in Windermere (UK, Figure 1) on 12 dates between October 2017 and July 2018, with a higher sampling effort in late autumn when Arctic charr are expected to be reproductively active (Figure 2). Water samples were collected at the known autumn-spawning site of North Thompson Holme island (NTH, three locations), and along two transects located at the putative spawning site on the west shore at Red Nab (RN, eight locations) and at offshore locations approximately in the middle of the lake (OF, five locations) that are deep-water feeding habitats (Figure 1). Due to logistic reasons (i.e., suitable boat not available) we were unable to collect water samples at NTH on the 01, 14, and 16 of November, and RN water samples were not collected on the 08 of November (Figure 2). Site coordinates were recorded during the first eDNA sampling event (18 October 2017) using a hand-held Geographic Positioning System (GPS) (Garmin eTrex 10, Kansas, USA; Table S1) and these coordinates were used to navigate to the collection sites during all subsequent events.

At each of the eight sites in the onshore location (RN; <0.5 m depth), five subsamples (5 × 400 ml taken across 50 m) were collected at the surface water layer from the shoreline and merged into a single 2 L sterile plastic bottle (Gosselin™ Square HDPE, Fisher Scientific UK Ltd, UK). One sampling blank, consisting of a 2 L sterile bottle filled with ultra-purified water (Milli-Q), was used and opened once in the field. In the middle-lake sites, NTH and OF, samples were collected at different depths (2–40 m; Figure 1) from a boat using a 1.5 L, Friedinger-like water sampler left semi-open to fill up during the descent. The water sampler was lowered three times at each

sampling site (3 × ~650 ml) in order to collect subsamples that were subsequently merged into a single 2 L sterile plastic bottle. The water sampler was sterilized, while moving between sites, by soaking in a 10% v/v chlorine-based commercial bleach solution (Elliott Hygiene Ltd, UK) for 10 min, followed by rinsing with 5% v/v MicroSol detergent (Anachem, UK) and purified water. At each site, after sterilization, the sampler was also quickly lowered and washed with the lake's water before collection occurred. Two sampling blanks were used during the offshore sample collection (beginning and end of water sampling) to account for potential contamination introduced by the use of a water sampler. After bleaching, the Milli-Q water of each blank was used to rinse the water sampler before pouring it back into the 2 L bottles. To further minimize contamination risk, nitrile sterile gloves (STARLAB, UK) were worn and changed between collection sites.

2.2 | Netting surveys and Arctic charr maturity assessment

At NTH, between October and December 2017, spawning activity at the time of water sampling was verified by catches of Arctic charr individuals from 12 gill-netting surveys. The procedures adopted for the netting surveys at NTH are described in detail in Winfield et al. (2008). In brief, a gill net c. 28 m long and 1.8 m deep with a mesh size of 32 mm was used. The net was set overnight at a depth of c. 2 m and all fish caught were identified and measured (fork length in cm) and returned alive. The maturity of Arctic charr individuals was assessed via the morphological evaluation of body shape and coloration as well as the ease with which gametes could be expressed.

At RN, Arctic charr spawning activity has not been recently monitored or demonstrated through catches of spawning individuals. However, the area has been identified as a putative spawning ground based on anecdotal historical records, presence of suitable substrate, and passive acoustic monitoring (PAM) data which identified noises connected to Arctic charr spawning activities (gravel displacement or sounds associated with air exchange with swimbladder regulation; Bolgan et al., 2018).

2.3 | Water sample processing and sequencing library preparation

Water samples were kept in cool boxes covered with ice packs and filtered within 6 h after water collection. Water was filtered using vacuum-pumps coupled with Nalgene™ units and DNA was captured onto 0.45 µm mixed cellulose ester filters (47 mm diameter, Whatman, GE Healthcare). Generally, two filters were used per 2 L of water collected from the shoreline sites and one filter was used for water samples collected offshore with a few exceptions. A filtration blank was run during each filtration round ($n = 21$), where 2 L of ultra-purified water was filtered alongside water samples ($n = 160$)

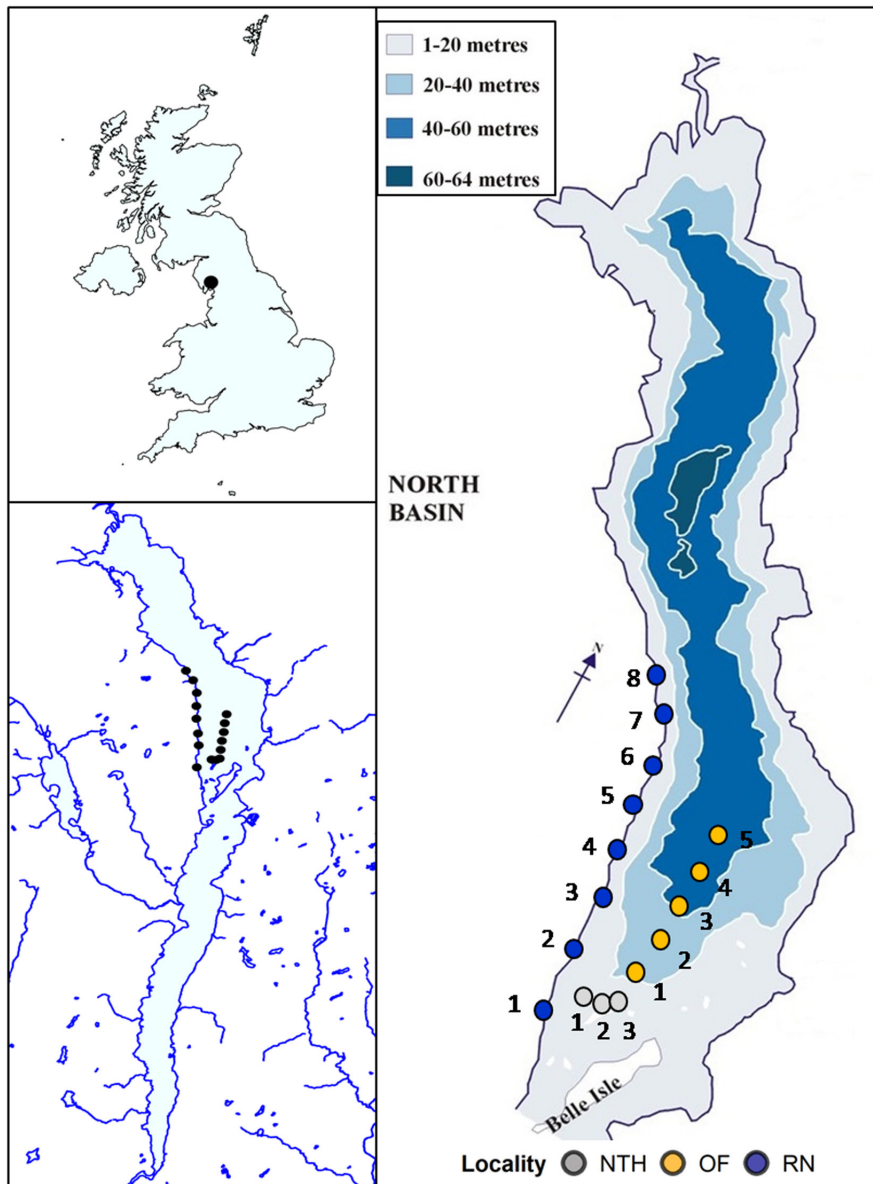


FIGURE 1 Map of Windermere and eDNA collection sites. Windermere's location in Cumbria, UK and location of eDNA sampling sites in Windermere's north basin (left side panels). Detailed bathymetric map of Windermere's north basin with sites and localities sampled during our eDNA surveys (right side panel). In the bathymetric map, "OF" are offshore sites (Arctic charr feeding grounds), "NTH" are inshore sites located at the shore of North Thompson Holme island (Arctic charr monitored spawning grounds) and "RN" are shoreline sites on the west side of the lake at Red Nab (Arctic charr putative spawning grounds). The bathymetric map was edited from Ramsbottom (1976) and used with permission of the Freshwater Biological Association.

and sampling blanks ($n = 29$). Filters were stored in sterile 50 mm Petri dishes (Fisher Scientific UK Ltd, UK), sealed with parafilm (Bemis™, Fisher Scientific UK Ltd, UK), at -20°C until extraction.

The mu-DNA water protocol (Sellers et al., 2018) was used for DNA extraction from filters, and filters from the same sample were lysed together in the same tube. Samples, sampling blanks, and filtration blanks belonging to different sampling dates were extracted in separate batches. An extraction blank, consisting only of extraction reagents, was included for each extraction round ($n = 13$). DNA yield and purity were checked using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific).

The sequencing library was built using a custom library preparation protocol, which includes tagged primers in two rounds of PCR (sensu Li et al., 2019). For the first PCR, indexed primers amplifying a ~ 106 bp region of the mitochondrial 12S ribosomal RNA (rRNA) in fish were used (Kelly et al., 2014; Riaz et al., 2011). PCR was performed with a final reaction volume of $25\mu\text{l}$, including $12.5\mu\text{l}$ of Q5® Hot-Start

High-Fidelity 2X Master Mix (New England Biolabs® Inc., MA, USA), $1.5\mu\text{l}$ of each indexed primer ($10\mu\text{M}$; Integrated DNA Technologies, Belgium), $0.5\mu\text{l}$ of the Thermo Scientific™ Bovine Serum Albumin (Fisher Scientific UK Ltd, UK), $7\mu\text{l}$ of molecular grade water (Fisher Scientific UK Ltd, UK) and $2\mu\text{l}$ of DNA template at the original sample concentration. To avoid cross-contamination between samples, reactions were prepared in 8-strip tubes with individually attached caps and covered with a drop of mineral oil (Sigma-Aldrich Company Ltd, UK). Amplifications were performed on Applied Biosystems® Veriti thermal cyclers (Life Technologies, CA, USA) with the following conditions: initial denaturation at 98°C for 5 min; 35 cycles of 98°C for 10 s, 58°C for 20s, and 72°C for 15s; final elongation at 72°C for 7 min. Samples, blanks, PCR negative controls (molecular grade water, $n = 11$), and PCR positive controls (genomic DNA [$0.05\text{ng}/\mu\text{l}$] from *Maylandia zebra*, a cichlid from Lake Malawi not present in UK, $n = 11$) were amplified in triplicate. Amplicons were checked on 2% agarose gels stained with 10,000× GelRed Nucleic Acid Gel Stain (Cambridge

Bioscience, UK). Gels were imaged using Image Lab Software (Bio-Rad Laboratories Ltd, UK) to visually check for contamination in blanks/PCR negative controls, presence of target band, and consistency of results among replicates. In case of PCR failure, reaction preparation and amplification would have been repeated. After visualization, PCR replicates were combined and samples belonging to the same collection date were pooled into sub-libraries using different volumes based on strength of PCR products on gels (no visible band = 20 μ l, very faint or faint band = 15 μ l, visible band = 10 μ l, bright band = 5 μ l; Alberdi et al., 2018). For each sub-library, 1 μ l of the PCR positive controls and 10 μ l of blanks/PCR negative controls were used. Sub-libraries were cleaned using a double-size selection magnetic bead protocol (Bronner et al., 2013) with a ratio of 0.9 \times and 0.15 \times of magnetic beads (Mag-Bind® RXNPure Plus, Omega Bio-tek Inc, GA, USA) to sub-library. Two replicates of bead clean-up were performed per sub-library and replicates were individually checked on a 2% agarose gel before pooling.

A second PCR was used to add Illumina tags to each sub-library. Second PCRs were run in duplicate in a final reaction volume of 50 μ l using 25 μ l of Q5® Hot-Start High-Fidelity 2X Master Mix (New England Biolabs® Inc., MA, USA), 3 μ l of each Illumina tag (10 μ M; Integrated DNA Technologies, Belgium), 14 μ l of molecular grade water (Fisher Scientific UK Ltd, UK), and 5 μ l of cleaned sub-library. Second PCR thermal cycling conditions consisted of: initial denaturation at 95°C for 3 min; 8 cycles of 98°C for 20 s, and 72°C for 1 min; final elongation at 72°C for 5 min. Second-round PCR products were checked on a 2% agarose gel alongside their non-tagged, cleaned counterparts to check for size differences after the addition of tags. A second double-size selection bead purification was carried out with a ratio of 0.7 \times and 0.15 \times of magnetic beads to PCR products. Tagged, cleaned sub-libraries were quantified using the Qubit™ 3.0 fluorometer and a Qubit™ dsDNA HS Assay Kit (Invitrogen, UK) before being pooled at equimolar concentrations into a single final library. The final library, comprised of 181 eDNA samples and 85 controls, was checked for size and integrity using the Agilent 2200 TapeStation and High Sensitivity D1000 ScreenTape (Agilent Technologies, CA, USA), then quantified with the NEBNext® Library Quant Kit for Illumina® using qPCR on a StepOne Plus real-time PCR platform (New England Biolabs® Inc., MA, USA). Following qPCR, a final dilution to 4 nM was performed and 13 pM of the final denatured library was loaded onto the Illumina MiSeq® with 10% PhiX using 2 \times 300 bp v3 chemistry (Illumina Inc., CA, USA).

2.4 | Bioinformatics and statistical analyses

Raw sequence reads were demultiplexed using a custom Python script and then processed using metaBEAT (metaBarcoding and Environmental Analysis Tool) v0.97.11 (<https://github.com/HullU-ni-bioinformatics/metaBEAT>), a custom bioinformatics pipeline incorporating commonly used open source software. Briefly, Trimmomatic v0.32 (Bolger et al., 2014) was used for read quality trimming (phred score Q30). During the trimming step, reads

were also cropped to a maximum length of 110 bp and reads shorter than 90 bp were discarded. Additionally, the first 18 bp of remaining reads were trimmed to ensure removal of the locus primers. FLASH v1.2.11 (Magoč & Salzberg, 2011) was then used to merge read pairs into single reads. For subsequent processing, merged reads and high-quality forward reads of sequences that failed to merge were kept. A final length filter (106 bp \pm 20%) was applied to ensure sequences reflected the expected fragment size (106 bp). Remaining reads were screened for detection of chimeric sequences against our custom reference database for UK fish (Hänfling et al., 2016) using the uchime algorithm (Edgar et al., 2011), as implemented in vsearch v1.1 (Rognes et al., 2016). Clustering at 100% identity in vsearch v1.1 (Rognes et al., 2016) was used to remove redundant sequences and possible sequencing errors, and clusters represented by less than three sequences were omitted from downstream processing. Finally, the retained reads were compared against a UK fish reference database (Hänfling et al., 2016) using BLAST (Zhang et al., 2000) and a lowest common ancestor (LCA) approach based on the top 10% BLAST matches for any query that matched a reference sequence across more than 95% of its length at minimum identity of 100%. Unassigned sequences from this comparison were subjected to a separate BLAST search against the complete NCBI nucleotide (nt) database using the same query and identity parameters.

Final metaBEAT results were summarized as the number of reads assigned to each OTU in each sample screened. We defined the proportion of reads assigned to each fish OTU over the total read counts on a sample-by-sample basis and used the proportion of Arctic charr reads in each sample (relative read count hereafter) to run downstream analyses in R v4.1.1 (R Core Team, 2021). A low-frequency noise threshold of 0.001 (0.1%) was applied to the dataset and the choice of the threshold level was guided by the analysis of the relative read counts in PCR positive controls that were not assigned to *M. zebra* (De Barba et al., 2014; Hänfling et al., 2016). For a higher stringency, species-specific thresholds were also applied and the highest number of reads assigned to fish species in PCR negatives and blanks was removed from those species across the entire dataset.

Maps with circles proportional to Arctic charr relative read counts were used to visualize temporal and spatial patterns at the sites monitored. Maps were created using shape files downloaded from EDINA Digimap® Ordinance Survey service (<http://edina.ac.uk/digimap>). Shape files were read into R using the package *rgdal* v1.5–25 (Bivand et al., 2019) and the *fortify* function together with the package *ggpoly* v0.1.0 (Sumner, 2016) were used to build the maps. All graphs were plotted using *ggplot2* v3.3.5 (Wickham, 2016). The relative read counts assigned to Arctic charr on different eDNA sampling dates (all water samples collected) were compared using the nonparametric Kruskal–Wallis test followed by pairwise comparisons between sampling dates using a Wilcoxon rank sum test. The Benjamini and Hochberg method was used to adjust the *p*-value for multiple comparison (Benjamini &

Hochberg, 1995) and the minimum level of significance was set at $p = 0.05$.

3 | RESULTS

3.1 | Sequencing outputs and bioinformatics

The raw number of sequences generated was 41,160,110. Across all samples/controls, 73% sequences survived the quality trimming step, of which 98% were successfully merged. Following removal of chimera sequences and clustering, the total number of sequences for the library was 16,357,422. After taxonomic assignment against the 12S UK fish database (Hänfling et al., 2016), 5,421,189 sequences matched 21 fish OTUs. After application of the thresholds, 18 OTUs were retained in the final dataset (Figure S1).

3.2 | Variation in Arctic charr eDNA signal

The relative read counts assigned to Arctic charr varied significantly across sampling dates (Kruskal–Wallis rank sum test; $\chi^2 = 25.504$, $p < 0.001$).

In the prespawning period (October 2017), we did not find any reads assigned to Arctic charr at the spawning sites of RN, NTH, or OF (Figure 2).

During the spawning period (between 8 November and 7 December 2017), reads were assigned to Arctic charr for all sampling events at NTH, RN, and OF (Figure 2), showing a marked contrast to the prespawning period and the postspawning period (Figure 2; Table S2). In addition, there was no significant difference between Arctic charr relative read counts of sampling dates belonging to the spawning season (Table S2). The relative read counts assigned to Arctic charr in the spawning period ranged from 0.18 to 0.005 at NTH shore (known spawning grounds) and from 0.22 to 0.001 at RN (shoreline, putative spawning grounds). Arctic charr reads were also detected in the deepest waters along the offshore transect during the spawning period with the relative read counts ranging from 0.41 to 0.011 (Figure 2).

At the known spawning grounds of NTH shore (NTH1, NTH2, NTH3; Figure 1), the highest relative read counts of Arctic charr was observed at site NTH2 on 13 November 2017 (Figure 2). All samples collected in the spawning period at NTH shore showed positive detection of Arctic charr with the exception of sampling site NTH1 on 17 November (Figure 2). The highest relative read count for Arctic charr at the putative spawning locality of RN was observed at site RN4 in December 2017 (Figure 2) and 23 of 48 samples collected at RN during the spawning period were positive for Arctic charr. At RN, the highest occupancy and relative read counts were found in December (7/8 sites, with the highest relative read counts at site RN4 and RN8; Figure 2). At the deepest

sites along the offshore transect (site OF1 to OF5; Figure 1), the highest relative read count matching Arctic charr was found on 8 November 2017 at site OF3 (Figure 2). Overall, at the OF 18 samples out of 25 collected showed positive Arctic charr detection over the spawning period and the highest number of detections occurred on 17 November 2017 (Figure 2).

In January (after spawning), Arctic charr was only detected at two sites at RN (RN5 and RN6; Figure 2) and at two sites at OF (OF2 and OF5; Figure 2). The Arctic charr relative read count in January was significantly lower than in the spawning dates of 13 November and 7 December (Wilcoxon rank sum test: 13 November 2017, $p = 0.0153$; 7 December 2017, $p = 0.0025$; Table S2), but not significantly lower than in the spawning dates of 15 and 17 November (Wilcoxon rank sum test: 15 November 2017, $p = 0.1154$; 17 December 2017, $p = 0.1449$; Table S2). In spring (April 2018), Arctic charr was only detected in one sample from the offshore transect (OF3; Figure 2) with a relative read count significantly lower than the relative read counts of any sampling events carried out during the spawning period (Wilcoxon rank sum test: 13 November, $p = 0.0022$; 15 November, $p = 0.0145$; 17 November, $p = 0.0209$; 7 December, $p = 0.0012$; Table S2). Arctic charr were not detected at any sites during the final sampling event in July 2018 (Figure 2).

3.3 | Gill-netting survey

Arctic charr spawning individuals were caught and measured on eight out of 12 gill-netting surveys performed in autumn 2017 (Figure 3). Twelve spawning Arctic charr were caught including one ripe female, four spent or partially spent females, six running males, and one spent male (Figure 3). Males ranged from 25.3 to 31.5 cm in fork length, whereas females ranged from 22.7 to 31.5 cm. On the 8 and 15 of November 2017, the two netting dates overlapping with the eDNA samplings at NTH, one ripe female (30.6 cm) and one spent female (22.7 cm) were caught, respectively (Figure 3).

4 | DISCUSSION

This study revealed the ability of eDNA metabarcoding to monitor the reproductive activity of Arctic charr autumn-spawning individuals in a lacustrine system. Such molecular observations were supported by catches of mature specimens at the monitored breeding sites where peaks of Arctic charr reads were detected. In addition, the temporal gradient of the genetic signal observed in autumn 2017 was a further indication of the species' spawning activity. In this study, we have characterized the times and locations of Arctic charr spawning events, revealing key information on putative breeding localities where spawning has not been monitored or observed for over 50 years.

4.1 | Arctic charr eDNA is absent from the monitored sites outside the spawning season

In line with our initial hypotheses, we observed seasonally limited eDNA detections of Arctic charr at the localities monitored, and Arctic charr eDNA was not found in water samples collected prespawning (October 2017; Figure 2) and postspawning (January–April–July 2018; Figure 2) at the shoreline locations of RN and NTH

(putative and known breeding grounds, respectively). These results confirm previous observations from Windermere showing that Arctic charr is only detected in deep waters outside the species' spawning season (Hänfling et al., 2016; Lawson Handley et al., 2019). Such localized distribution of the organisms' genetic signal is common in lentic systems where the spatial and temporal distribution of eDNA reflects the sites occupied by a species in the water at a given time (Brys et al., 2021; Li et al., 2019; Zhang et al., 2020).

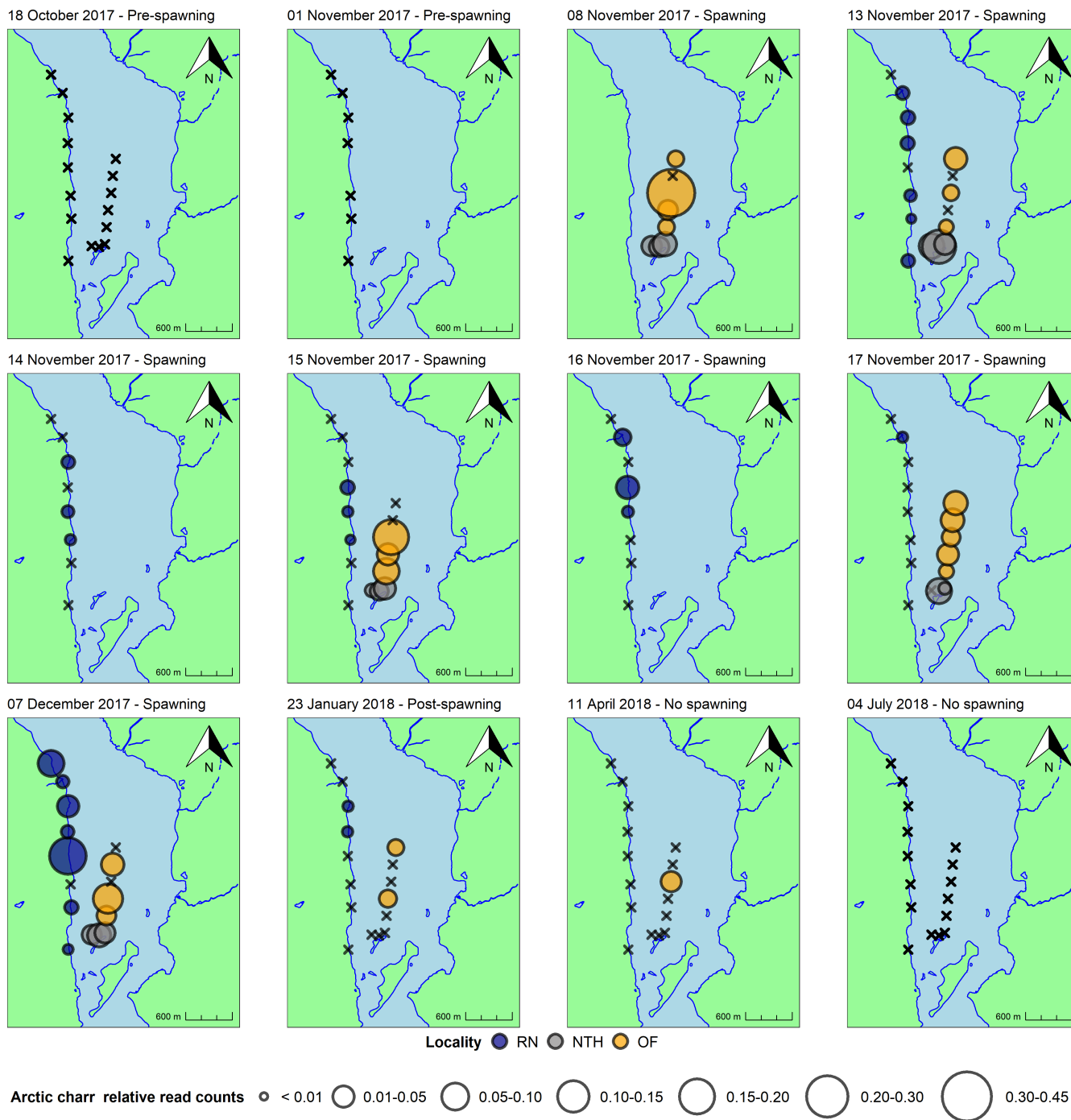


FIGURE 2 Spatio-temporal variation of Arctic charr eDNA signal in the north basin of Windermere. Bubble size is proportional to relative read counts assigned to Arctic charr, whereas black crosses indicate sites where samples were collected but the species was not detected, and the absence of any symbol indicates that water samples were not collected.

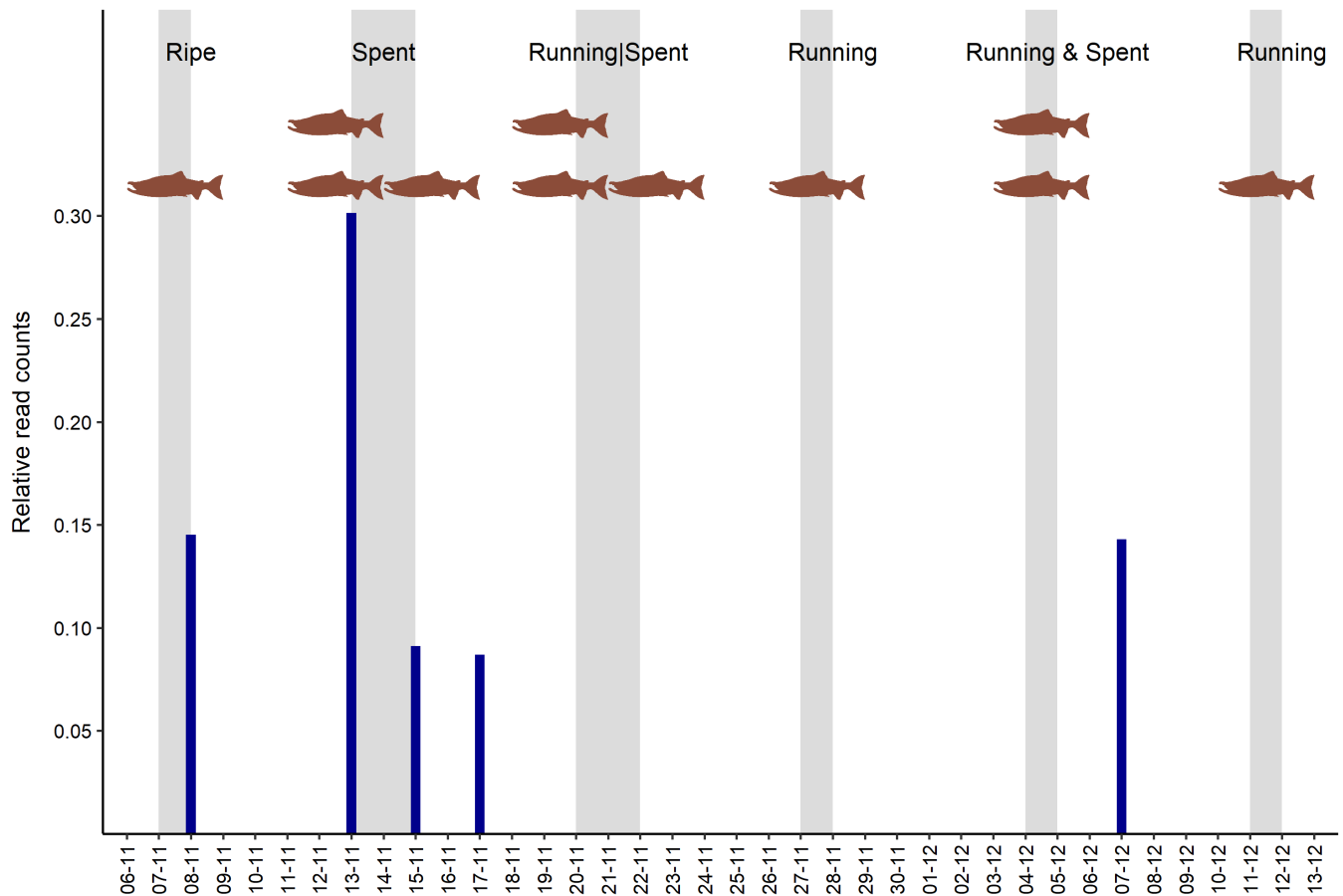


FIGURE 3 Arctic charr catches and eDNA relative read counts at the autumn-breeding grounds of NTH. Gray bars indicate the gill-netting dates (net lowered/net lifted) and the number and status of Arctic charr specimens caught. Dark blue bars show the Arctic charr eDNA total relative read counts from the five water sampling events.

Arctic charr feeding grounds are located in the offshore areas of Windermere (Frost, 1977; Mills et al., 1990); however, the water collection sites along the depth transect (OF; Figure 1) sampled outside the breeding season showed no detection of Arctic charr (October, April, and July; Figure 2). The limited sampling effort carried out in the deep waters of the lake combined with the aggregated distribution of the species (Jørgensen et al., 1993) may have hindered the detection of Arctic charr at the species' feeding grounds. A more comprehensive sampling effort along the lake midline would likely have found the species in the deepest areas of the lake beyond its spawning season as shown in Lawson Handley et al. (2019). In temperate lentic systems, water-mixing regimes influence the spatial distribution of eDNA, with eDNA more vertically structured when the water column is stratified (warm seasons) and evenly dispersed when the water column is mixed (cold seasons) (Hänfling et al., 2016; Lawson Handley et al., 2019; Littlefair et al., 2021). In our previous work, we demonstrated that Arctic charr is only detected in mid-depth and lake bottom samples during the summer months (Lawson Handley et al., 2019). It is likely that water stratification also contributed to the nondetection of Arctic charr in the deep offshore sites, at least in October 2017 and in July 2018, although possibly also

in April 2018 when the thermocline started to develop (ST and EM pers. comm.).

4.2 | Arctic charr eDNA is detected consistently at the monitored sites in late autumn

During the Arctic charr spawning season, between November and December 2017, Arctic charr eDNA was consistently detected at offshore (OF) and shallow water sites (RN and NTH; Figure 2), and peaks in signal strength were recorded at few sampling sites and at specific dates within the spawning period (Figure 2).

The high proportion of Arctic charr read counts found at two shallow breeding habitats (NTH2 and RN4) and at one deep water site (OF3), respectively, on the 13 November and 7 December and on the 8 and 15 November (Figure 2), exceeded Arctic charr reads reported otherwise by orders of magnitude. Here, we found an increase in Arctic charr relative read counts of about 15- and 40-fold compared to other locations and dates within the spawning season (Figure 2). Similar peaks of eDNA were observed during the spawning activity of Japanese eel (*Anguilla japonica*, Temminck and Schlegel,

1846) in a mesocosm experiment when eDNA concentrations were 10–100 times higher after the release of gametes (Takeuchi et al., 2019). Additionally, Tsuji and Shibata (2021), used a manipulative field experiment, and found that the release of sperm is the main factor explaining peaks in eDNA concentration during fish spawning events. The authors observed 3–25 times higher eDNA concentrations during the spawning activity of medaka species (*Oryzias latipes*, Temminck and Schlegel, 1846; *Oryzias sakaizumii*, Asai, Senou and Hosoya, 2012), suggesting that such species-specific peaks in eDNA can be used to identify localities and timings of true spawning events and distinguish them from those sites where other spawning-associated activities occur (Tsuji & Shibata, 2021). Similarly, in our study, the eDNA peak found at NTH on the 13 November coincided with the capture of spent Arctic charr specimens (Figure 3), whereas the less intense Arctic charr eDNA signal detected consistently at the shoreline breeding sites (RN-NTH; Figure 2) in autumn could be associated with several reproductive features of the species (i.e., redd-building females, courting and/or competing males, aggregation of mature individuals).

The detection of Arctic charr eDNA at the offshore sites during the breeding season (Figure 2) might be explained by migratory mature individuals moving from the offshore feeding grounds to the shallow breeding habitats. Time-limited and localized variation in eDNA concentrations have been used to infer fish movements associated to the spawning season in lotic systems whereby visual surveys, egg collection, or telemetry have been used to confirm that spatio-temporal variation in eDNA reflects fish migration to the spawning grounds during the reproductive season (Antognazza et al., 2019; Erickson et al., 2016; Thalinger et al., 2019). At one offshore site (OF3), remarkable peaks in Arctic charr relative read counts were observed during the spawning season, especially on 8 and 15 November 2017 (Figure 2). Such eDNA peaks found in deep water sites could reveal the presence of offshore aggregation areas of mature Arctic charr individuals (Bracken et al., 2019; Takeuchi et al., 2019). Interestingly, the same deep water site was also the only one to be positive for Arctic charr in April in parallel with the breeding period of the spring-spawning Arctic charr population in Windermere (Frost, 1965). These observations could indicate the existence of specific offshore habitats (deeper than 5 m) within the lake which are still suitable to sustain Arctic charr spawning events. Historical records of confirmed and putative deep-water spawning sites exist, but no recent Arctic charr spawning activity could be verified at these sites using conventional methods (Miller et al., 2015; Winfield et al., 2015). eDNA-based approaches provide the opportunity to monitor these critical habitats for Arctic charr spawning more broadly in lentic systems.

Overall and in agreement with previous conventional surveys (i.e., net catches, visual surveys, hydroacoustic, PAM; Bolgan et al., 2018; Miller et al., 2015; Winfield et al., 2015), our results confirmed the suitability of the NTH breeding grounds to support Arctic charr reproductive activity, accounting for (i) the autumn-limited Arctic charr eDNA detections, (ii) the peak of Arctic charr relative

read counts on the 13 November, and (iii) the catches of spent Arctic charr specimens when the eDNA peak was observed (Figures 2, 3). In addition, given that the same or even higher Arctic charr eDNA was detected at the putative spawning grounds of RN only during the spawning season (Figure 2), we infer that spawning activity was occurring at this locality even though mature individuals have not been caught at these sites in the last 50 years. Ethical implications of destructive established methods (i.e., gill-netting) restrict the application of these “traditional” monitoring approaches, especially when the target species are threatened and of conservation concern, such as Arctic charr in Windermere (Winfield et al., 2009). Therefore, we have demonstrated the suitability of eDNA metabarcoding as a broadly applicable, noninvasive molecular tool to infer spawning activity through the temporal and spatial localization of the Arctic charr genetic signal in a large lake.

5 | CONCLUSION

In this study, the temporal water sampling coupled with eDNA metabarcoding analysis characterized the Arctic charr spawning activity in a lacustrine ecosystem. We demonstrated that this approach can be used to accurately describe fish spawning locations and timings, to determine the intensity of the spawning effort, and to identify true spawning locations where gametes are released.

As opposed to targeted molecular techniques, the use of eDNA metabarcoding provides an array of “by-catch” community information that can support a more comprehensive evaluation of the status of fish population and ecosystem dynamics. For example, the decline of Arctic charr in Windermere has been linked to the degradation of the species' spawning grounds as well as to a number of other factors including the establishment of nonnative species such as roach (*Rutilus rutilus*, L. 1758) and common bream (*Abramis brama*, L. 1758) (Winfield et al., 2008). Such species, facilitated by the changing environmental conditions and eutrophication, have now become abundant or dominant and they are indeed the most commonly detected fish within the lake (Figure S1; Hänfling et al., 2016; Lawson Handley et al., 2019). For these reasons, we suggest that the periodic use of eDNA metabarcoding in lacustrine ecosystems can assist the monitoring of fish spawning activity and, simultaneously, evaluate changes in fish community composition. The characterization of these two aspects is essential for an accurate assessment of native fish populations as well as to predict changes in their conservation status.

AUTHOR CONTRIBUTIONS

C.D.M., B.H., L.L.H., C.W.B., J.B., and I.J.W. conceived the study. C.D.M., L.R.H., M.B., and J.L. carried out the fieldwork. C.D.M. performed laboratory experiments, bioinformatics, and statistical analyses supported by L.R.H., M.B., and J.L. C.D.M. wrote the first draft of the manuscript and all authors critically contributed to the manuscript revisions and gave the final approval for publication.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw sequence reads are deposited in the SRA (BioProject: PRJNA770176; Accession numbers: SAMN22210989-SAMN22211238).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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