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

2 metabarcoding

3 Di Muri C.^{1,2}, Lawson Handley L.², Bean C. W.³, Benucci M.^{2,4}, Harper L. R.⁵, James B.⁶, Li J.^{7,8},
4 Winfield I. J.⁶, Hänfling B.²

5 ¹National Research Council (CNR), Institute of Research on Terrestrial Ecosystems (IRET),
6 Università del Salento, Lecce, 73100, Italy

7 ²Evolutionary Biology Group (@EvoHull), Department of Biological and Marine Sciences,
8 University of Hull (UoH), Cottingham Road, Hull, HU6 7RX, UK

9 ³NatureScot, Caspian House, Mariner Court, Clydebank Business Park, Clydebank G81 2NR, UK

10 ⁴Fera Science Ltd, York Biotech Campus, Sand Hutton, York, YO41 1LZ, UK

11 ⁵NatureMetrics Ltd, CABI Site, Bakeham Lane, Egham, Surrey, TW20 9TY, UK

12 ⁶Lake Ecosystems Group, UK Centre for Ecology & Hydrology, Lancaster Environment Centre,
13 Library Avenue, Bailrigg, Lancaster LA1 4AP, UK

14 ⁷State Key Laboratory of Marine Resources Utilization in South China Sea, Hainan University,
15 Hainan 570228, China

16 ⁸College of Marine Sciences, Hainan University, Haikou, Hainan 570228, China

17 Abstract

18 Determining the timing and location of fish reproductive events is crucial for the implementation of
19 correct management and conservation schemes. Conventional methods used to monitor these
20 events are often unable to assess the spawning activity directly or can be invasive and therefore
21 problematic. This is especially the case when threatened fish populations are the study subject,
22 such as the Arctic charr (*Salvelinus alpinus* L.) populations in Windermere (Cumbria, UK). Arctic
23 charr populations have been studied in this lake since the 1940s, and the locations and
24 characteristics of spawning grounds have been described in detail using techniques such as
25 hydroacoustics, as well as physical and visual surveys of the lake bottom. Here, in conjunction with
26 established netting surveys, we added an environmental DNA (eDNA) metabarcoding approach to
27 assess the spatial distribution of Arctic charr in the lake throughout the year to test whether this
28 tool could allow us to identify spawning locations and activity. Sampling was carried out between
29 October 2017 and July 2018 at three locations in the lake, covering putative and known spawning

sites. eDNA metabarcoding provided accurate spatial and temporal characterisation of Arctic charr spawning events. Peaks of Arctic charr 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 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: breeding grounds, Arctic charr, *Salvelinus alpinus*, freshwaters, lentic systems, conservation

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 and 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. Whilst 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 (Maitland *et al.*, 2007; Bean *et al.*, 2018).

Interest in Arctic charr conservation is primarily driven by the often-unique characteristics of individual populations. At the onset of the most recent post-glacial era, the isolation of many Arctic charr populations in post-glacial lakes led to high polymorphism and plasticity among and within local populations (Jonsson and Jonsson, 2001; Klemetsen, 2010; Skúlason *et al.*, 2019). This high diversity reflects the ability of Arctic charr populations to adapt to cold, nutrient impoverished and species-poor lacustrine habitats with a variety of niches available (Jonsson and Jonsson, 2001; Reist *et al.*, 2013). Arctic charr polymorphism encompasses life-history tactics (e.g. anadromous and non-migratory forms; Klemetsen *et al.*, 2003), specialisation in diet and habitat preferences associated to morphological variation (Adams *et al.*, 2003; Klemetsen *et al.*, 2006), and reproductive strategies (Frost, 1965; Smalås, Amundsen and Knudsen, 2013; Telnes and 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 differences 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 (Sternecker, Denic and Geist, 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 0.4-0.7% 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

83 conservation efforts, to ensure protection of high-quality habitats for reproduction and to identify
84 management units.

85 Arctic charr spawning areas and spawning behavior in Windermere (Cumbria, UK) have been
86 extensively studied over more than 50 years (Frost, 1965; Miller *et al.*, 2015). Research on
87 breeding habitats of Windermere's Arctic charr has described the presence of two sympatric
88 populations with autumn and spring spawning events (Frost, 1965), and documented their patterns
89 of genetic and phenotypic divergence (Corrigan *et al.*, 2011; Partington and Mills, 1988). Outside
90 the spawning season, Arctic charr in Windermere are exclusively restricted to offshore areas of the
91 lake (Lawson Handley *et al.*, 2019; Winfield *et al.*, 2008). Autumn-spawners release their gametes
92 at depths of around 2 m between November and December, whereas spring-spawners mature
93 between February and March and spawn at deeper sites between 15 and 20 m. Both the
94 mesotrophic north basin and eutrophic south basins of Windermere sustain autumn and spring-
95 spawners, and a variety of putative and known spawning locations have been described in the lake
96 (Frost 1965; Miller *et al.*, 2015; Winfield *et al.*, 2015). However, these Windermere Arctic charr
97 populations have declined markedly over the last few decades (Winfield *et al.*, 2019) in parallel
98 with increased eutrophication and the associated decrease in spawning habitat quality (Miller *et al.*,
99 2015; Winfield *et al.*, 2015). Suitable spawning habitats are now limited to the shallowest areas of
100 the lake at depths below 5 m where clean, hard substrates still occur (Miller *et al.*, 2015, Winfield *et*
101 *al.*, 2015). This poses important conservation concerns for spring-spawning Arctic charr
102 populations with deeper breeding grounds (Winfield *et al.*, 2008). Importantly, extensive monitoring
103 of spawning activity at depths less than 5 m is challenging using established non-invasive survey
104 methods such as hydroacoustic applications (Miller *et al.*, 2015), and netting surveys cannot be
105 deployed widely due to the conservation status of target species. Non-invasive and broadly
106 applicable monitoring approaches are therefore required to characterise times and locations of fish
107 spawning activities.

108 The analysis of environmental DNA (eDNA) has been recently applied to the study of riverine fish
109 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. Antognazza *et al.*, 2019; Bracken *et al.*, 2019; Thalinger *et al.*, 2019). 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 a 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 hypothesise that (1) eDNA metabarcoding analyses, using extensive spatio-temporal water sampling, can detect lake fish spawning activity via the temporal and spatial variation in eDNA read counts during the breeding season, and that (2) species-specific peaks in 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 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.

Materials and methods

Water sample collection, filtration, and DNA extraction

Water sampling was carried out in Windermere (UK, Fig. 1) on twelve dates between October 2017 and July 2018, with a higher sampling effort in late autumn when Arctic charr are expected to be

136 reproductively active (Fig. 2). Water samples were collected at the known autumn spawning site of
 137 North Thompson Holme island (NTH, three locations), and along two transects located at the
 138 putative spawning site on the west shore at Red Nab (RN, eight locations) and at offshore
 139 locations approximately in the middle of the lake (OF, five locations) that are deep-water feeding
 140 habitats (Fig. 1). Due to logistic reasons (i.e. suitable boat not available) we were unable to collect
 141 water samples at NTH on the 01st, 14th and 16th of November, and RN water samples were not
 142 collected on the 08th of November (Fig. 2). Site coordinates were recorded during the first eDNA
 143 sampling event (18th October 2017) using a hand-held Geographic Positioning System (GPS)
 144 (Garmin eTrex 10, Kansas, USA; Table S1) and these coordinates were used to navigate to the
 145 collection sites during all subsequent events. At NTH between October and December 2017,
 146 spawning activity at the time of sampling was verified by catches of Arctic charr individuals from
 147 twelve gill-netting surveys. The maturity of Arctic charr individuals caught at NTH was assessed via
 148 the morphological evaluation of body shape and coloration as well as the ease with which gametes
 149 could be expressed. At RN, Arctic charr spawning activity has not been recently monitored or
 150 demonstrated through catches of spawning individuals. However, the area has been identified as a
 151 putative spawning ground based on anecdotal historical records, presence of suitable substrate,
 152 and passive acoustic monitoring (PAM) data which identified noises connected to Arctic charr
 153 spawning activities (gravel displacement or sounds associated with air exchange with swimbladder
 154 regulation; Bolgan *et al.*, 2018).

155 At each of the eight sites in the onshore location (RN; less than 0.5 m depth), five subsamples (5 x
 156 400 mL taken across 50 m) were collected at the surface water layer from the shoreline and
 157 merged into a single 2 L sterile plastic bottle (Gosselin™ Square HDPE, Fisher Scientific UK Ltd,
 158 UK). One sampling blank, consisting of a 2 L sterile bottle filled with ultra-purified water (Milli-Q),
 159 was used and opened once in the field. In the middle-lake sites, NTH and OF, samples were
 160 collected at different depths (2 m to 40 m; Fig. 1) from a boat using a 1.5 L, Friedinger-like water
 161 sampler left semi-open to fill up during the descent. The water sampler was lowered three times at
 162 each sampling site (3 x ~650 mL) in order to collect subsamples that were subsequently merged
 163 into a single 2 L sterile plastic bottle. The water sampler was sterilised, while moving between

164 sites, by soaking in a 10% v/v chlorine-based commercial bleach solution (Elliott Hygiene Ltd, UK)
 165 for 10 minutes, followed by rinsing with 5% v/v MicroSol detergent (Anachem, UK) and purified
 166 water. At each site, after sterilisation, the sampler was also quickly lowered and washed with the
 167 lake's water before collection occurred. Two sampling blanks were used during the offshore
 168 sample collection (beginning and end of water sampling) to account for potential contamination
 169 introduced by the use of a water sampler. After bleaching, the Milli-Q water of each blank was used
 170 to rinse the water sampler before pouring it back into the 2 L bottles. To further minimise
 171 contamination risk, nitrile sterile gloves (STARLAB, UK) were worn and changed between
 172 collection sites.

173 Water samples were kept in cool boxes covered with ice packs and filtered within six hours after
 174 water collection. Water was filtered using vacuum-pumps coupled with Nalgene™ units and DNA
 175 was captured onto 0.45 µm mixed cellulose ester filters (47 mm diameter, Whatman, GE
 176 Healthcare). Generally, two filters were used per 2 L of water collected from the shoreline sites and
 177 one filter was used for water samples collected offshore with a few exceptions. A filtration blank
 178 was run during each filtration round ($n = 21$), where 2 L of ultra-purified water was filtered
 179 alongside water samples ($n = 160$) and sampling blanks ($n = 29$). Filters were stored in sterile 50
 180 mm Petri dishes (Fisher Scientific UK Ltd, UK), sealed with parafilm (Bemis™, Fisher Scientific UK
 181 Ltd, UK), at -20° C until extraction.

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

188 ***Sequencing library preparation***

189 The sequencing library was built using a custom library preparation protocol which includes tagged
190 primers in two rounds of PCR (*sensu* Li *et al.*, 2019). For the first PCR, indexed primers amplifying
191 a ~106 bp region of the mitochondrial 12S ribosomal RNA (rRNA) in fish were used (Kelly *et al.*
192 2014; Riaz *et al.* 2011). PCR was performed with a final reaction volume of 25 μ L, including 12.5
193 μ L of Q5® Hot-Start High-Fidelity 2X Master Mix (New England Biolabs® Inc., MA, USA), 1.5 μ L of
194 each indexed primer (10 μ M; Integrated DNA Technologies, Belgium), 0.5 μ L of the Thermo
195 Scientific™ Bovine Serum Albumin (Fisher Scientific UK Ltd, UK), 7 μ L of molecular grade water
196 (Fisher Scientific UK Ltd, UK) and 2 μ L of DNA template at the original sample concentration. To
197 avoid cross-contamination between samples, reactions were prepared in 8-strip tubes with
198 individually attached caps and covered with a drop of mineral oil (Sigma-Aldrich Company Ltd,
199 UK). Amplifications were performed on Applied Biosystems® Veriti thermal cyclers (Life
200 Technologies, CA, USA) with the following conditions: initial denaturation at 98°C for 5 min; 35
201 cycles of 98°C for 10 sec, 58°C for 20 sec and 72°C for 15 sec; final elongation at 72°C for 7 min.
202 Samples, blanks, PCR negative controls (molecular grade water, $n = 11$), and PCR positive
203 controls (genomic DNA [0.05 ng/ μ L] from *Maylandia zebra*, a cichlid from Lake Malawi not present
204 in UK, $n = 11$) were amplified in triplicate. Amplicons were checked on 2% agarose gels stained
205 with 10,000X GelRed Nucleic Acid Gel Stain (Cambridge Bioscience, UK). Gels were imaged using
206 Image Lab Software (Bio-Rad Laboratories Ltd, UK) to visually check for contamination in
207 blanks/PCR negative controls, presence of target band, and consistency of results among
208 replicates. In case of PCR failure, reaction preparation and amplification would have been
209 repeated. After visualisation, PCR replicates were combined and samples belonging to the same
210 collection date were pooled into sub-libraries using different volumes based on strength of PCR
211 products on gels (no visible band = 20 μ L, very faint or faint band = 15 μ L, visible band = 10 μ L,
212 bright band = 5 μ L) (Alberdi *et al.* 2018). For each sub-library, 1 μ L of the PCR positive controls
213 and 10 μ L of blanks/PCR negative controls were used. Sub-libraries were cleaned using a double-
214 size selection magnetic bead protocol (Bronner *et al.*, 2013) with a ratio of 0.9X and 0.15X of
215 magnetic beads (Mag-Bind® RXNPure Plus, Omega Bio-tek Inc, GA, USA) to sub-library. Two

216 replicates of bead clean-up were performed per sub-library and replicates were individually
217 checked on a 2% agarose gel before pooling.

218 A second PCR was used to add Illumina tags to each sub-library. Second PCRs were run in
219 duplicate in a final reaction volume of 50 μ L using 25 μ L of Q5® Hot-Start High-Fidelity 2X Master
220 Mix (New England Biolabs® Inc., MA, USA), 3 μ L of each Illumina tag (10 μ M; Integrated DNA
221 Technologies, Belgium), 14 μ L of molecular grade water (Fisher Scientific UK Ltd, UK), and 5 μ L of
222 cleaned sub-library. Second PCR thermal cycling conditions consisted of: initial denaturation at
223 95°C for 3 min; 8 cycles of 98°C for 20 sec and 72°C for 1 min; final elongation at 72°C for 5 min.
224 Second-round PCR products were checked on a 2% agarose gel alongside their non-tagged,
225 cleaned counterparts to check for size differences after the addition of tags. A second double-size
226 selection bead purification was carried out with a ratio of 0.7X and 0.15X of magnetic beads to
227 PCR products. Tagged, cleaned sub-libraries were quantified using the Qubit™ 3.0 fluorometer
228 and a Qubit™ dsDNA HS Assay Kit (Invitrogen, UK) before being pooled at equimolar
229 concentrations into a single final library. The final library, comprised of 181 eDNA samples and 85
230 controls, was checked for size and integrity using the Agilent 2200 TapeStation and High
231 Sensitivity D1000 ScreenTape (Agilent Technologies, CA, USA), then quantified with the
232 NEBNext® Library Quant Kit for Illumina® using qPCR on a StepOne Plus real-time PCR platform
233 (New England Biolabs® Inc., MA, USA). Following qPCR, a final dilution to 4nM was performed and
234 13 pM of the final denaturated library was loaded onto the Illumina MiSeq® with 10% PhiX using 2
235 x 300 bp v3 chemistry (Illumina Inc., CA, USA).

236 ***Bioinformatics and statistical analyses***

237 Raw sequence reads were demultiplexed using a custom Python script and then processed using
238 metaBEAT (metaBarcoding and Environmental Analysis Tool) v0.97.11 ([https://github.com/HullUni-](https://github.com/HullUni-bioinformatics/metaBEAT)
239 [bioinformatics/metaBEAT](https://github.com/HullUni-bioinformatics/metaBEAT)), a custom bioinformatics pipeline incorporating commonly used open
240 source software. Briefly, Trimmomatic v0.32 (Bolger *et al.*, 2014) was used for read quality
241 trimming (phred score Q30). During the trimming step, reads were also cropped to a maximum
242 length of 110 bp and reads shorter than 90 bp were discarded. Additionally, the first 18 bp of

243 remaining reads were trimmed to ensure removal of the locus primers. FLASH v1.2.11 (Magoč and
244 Salzberg, 2011) was then used to merge read pairs into single reads. For subsequent processing,
245 merged reads and high quality forward reads of sequences that failed to merge were kept. A final
246 length filter (106 bp $\pm$ 20%) was applied to ensure sequences reflected the expected fragment size
247 (106 bp). Remaining reads were screened for detection of chimeric sequences against our custom
248 reference database for UK fish (Hänfling *et al.*, 2016) using the uchime algorithm (Edgar *et al.*,
249 2011), as implemented in vsearch v1.1 (Rognes *et al.*, 2016). Clustering at 100% identity in
250 vsearch v1.1 (Rognes *et al.*, 2016) was used to remove redundant sequences and possible
251 sequencing errors, and clusters represented by less than three sequences were omitted from
252 downstream processing. Finally, the retained reads were compared against a UK fish reference
253 database (Hänfling *et al.*, 2016) using BLAST (Zhang *et al.* 2000) and a lowest common ancestor
254 (LCA) approach based on the top 10% BLAST matches for any query that matched a reference
255 sequence across more than 95% of its length at minimum identity of 100%. Unassigned sequences
256 from this comparison were subjected to a separate BLAST search against the complete NCBI
257 nucleotide (nt) database using the same query and identity parameters.

258 Final metaBEAT results were summarised as the number of reads assigned to each OTU in each
259 sample screened, henceforth referred to as read counts. The final dataset with the complete fish
260 OTU x read counts matrix was used to run downstream analyses within R v4.1.1 (R Core Team,
261 2021). A low-frequency noise threshold of 0.001 (0.1%) was applied to the dataset and calculated
262 as the proportion of reads assigned to each fish OTU over the total read counts on a sample-by-
263 sample basis (De Barba *et al.*, 2014; Hänfling *et al.*, 2016). The choice of the threshold level was
264 guided by the analysis of reads in PCR positive controls that were not assigned to *M. zebra*. For a
265 higher stringency, species-specific thresholds were also applied and the highest number of reads
266 assigned to fish species in PCR negatives and blanks was removed from those species across the
267 entire dataset.

268 Maps with circles proportional to Arctic charr read counts were used to visualise temporal and
269 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 *ggplot2* v3.3.5 (Wickham, 2016). The read counts assigned to Arctic charr on different eDNA sampling dates (all water samples collected) were compared using the non-parametric Kruskal-Wallis test followed by pairwise comparisons between sampling dates using a Wilcoxon rank sum test, where the minimum level of significance was set at $p = 0.05$.

Results

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 (Fig. S1).

Variation in Arctic charr eDNA signal

Overall, 155,678 reads were assigned to Arctic charr with significant variation across sampling dates (Kruskal-Wallis rank sum test; $\chi^2 = 26.233$, $p < 0.001$).

In the pre-spawning period (one sampling event in October 2017), we did not find any reads assigned to Arctic charr at the spawning sites of RN and NTH nor at the sites at OF (Fig. 2).

During the spawning period (between November and December 2017), reads were assigned to Arctic charr for seven sampling events at both NTH and RN (Fig. 2), showing a marked contrast to the pre-spawning period and the post-spawning period (Fig. 2; Table S2). In addition, there was no significant difference between Arctic charr read counts of sampling dates belonging to the

294 spawning season (Table S2). The number of reads assigned to Arctic charr in the spawning period
295 ranged from 30,729 to 182 at RN (shoreline, putative spawning grounds) and from 15,257 to 413 at
296 NTH shore (known spawning grounds). Arctic charr reads were also detected in the deepest
297 waters along the offshore transect during the spawning period (15,689 to 290 at OF1 to OF5; Fig.
298 2).

299 The highest read counts for Arctic charr were found at the putative spawning site RN4 in
300 December 2017 (30,729 reads; Fig. 2) and 23 of 55 samples collected at RN during the spawning
301 period were positive for Arctic charr. The highest occupancy and read counts were found in
302 December (7/8 sites, with the highest read counts 30,729 and 13,943 reads at site RN4 and RN8
303 respectively; Fig. 2). At the known spawning grounds of NTH shore (NTH1, NTH2, NTH3; Fig. 1),
304 the highest read counts of Arctic charr (15,257 reads) were observed at site NTH2 on 13th
305 November 2017 (Fig. 2). All samples collected in autumn showed positive detection of Arctic charr
306 with the exception of sampling site NTH1 on 17th November (Fig. 2). At the deepest sites along the
307 offshore transect (site OF1 to OF5; Fig. 1), the highest read counts matching Arctic charr were
308 found on 8th November 2017 at site OF3 (15,689 reads; Fig. 2). Across offshore sites, 18 samples
309 out of 25 collected showed positive Arctic charr detection across the spawning period (Fig. 2).

310 In January (after spawning), Arctic charr was only detected at two sites at RN (RN5 = 578 reads,
311 and RN6 = 483 reads; Fig. 2) and at two sites at OF (OF2 = 910 reads, OF5 = 997 reads; Fig. 2).
312 This read count was significantly lower than in the spawning dates of 13th November and 7th
313 December (Wilcoxon rank sum test: 13th November 2017, $p = 0.0115$; 7th December 2017, $p =$
314 0.0016 ; Table S2), but not significantly lower than in the spawning dates of 15th and 17th November
315 (Wilcoxon rank sum test: 15th November 2017, $p = 0.0854$; 17th December 2017, $p = 0.1562$; Table
316 S2). In spring (April 2018), Arctic charr was only detected in one sample from the offshore transect
317 (OF3 = 1838 reads; Fig. 2). This read count was significantly lower than the read counts of any
318 sampling events carried out during the spawning period (Wilcoxon rank sum test: 13th November, p
319 $= 0.0016$; 15th November, $p = 0.0178$; 17th November, $p = 0.0275$; 7th December, $p = 0.0016$; Table

320 S2). Arctic charr were not detected at any sites sampled during the final sampling event in July
321 2018 (Fig. 2).

322 ***Gill-netting survey***

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

330 **Discussion**

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

339 ***Arctic charr eDNA is absent from the monitored sites outside the spawning season***

340 In line with our initial hypotheses, we observed seasonally-limited eDNA detections of Arctic charr
341 at the localities monitored, and Arctic charr eDNA was not found in water samples collected pre-
342 spawning (October 2017; Fig. 2) and post-spawning (January-April-July 2018; Fig. 2) at the
343 shoreline locations of RN and NTH (putative and known breeding grounds respectively). These
344 results confirm previous observations from Windermere showing that Arctic charr is only detected

345 in deep waters outside the species' spawning season (Hänfling *et al.*, 2016; Lawson Handley *et al.*,
346 2019). Such localised distribution of the organisms' genetic signal is common in lentic systems
347 where the spatial and temporal distribution of eDNA reflects the sites occupied by a species in the
348 water at a given time (Brys *et al.*, 2021; Li *et al.* 2019; Zhang *et al.*, 2020).

349 Arctic charr feeding grounds are located in the offshore areas of Windermere (Frost, 1977; Mills,
350 1990); however, the water collection sites along the depth transect (OF; Fig. 1) sampled outside
351 the breeding season showed no detection of Arctic charr (October, April and July; Fig. 2). The
352 limited sampling effort carried out in the deep waters of the lake combined with the aggregated
353 distribution of the species (Jørgensen *et al.*, 1993) may have hindered the detection of Arctic charr
354 at the species' feeding grounds. A more comprehensive sampling effort along the lake midline
355 would likely have found the species in the deepest areas of the lake beyond its spawning season
356 as shown in Lawson Handley *et al.*, (2019). In temperate lentic systems, water mixing regimes
357 influence the spatial distribution of eDNA, with eDNA more vertically structured when the water
358 column is stratified (warm seasons) and evenly dispersed when the water column is mixed (cold
359 seasons) (Hänfling *et al.*, 2016; Lawson Handley *et al.*, 2019). In our previous work we
360 demonstrated that Arctic charr is only detected in mid-depth and lake bottom samples during the
361 summer months (Lawson Handley *et al.*, 2019). It is likely that water stratification also contributed
362 to the lower detection of Arctic charr in the deep offshore sites, at least in October 2017 and in July
363 2018, although possibly also in April 2018 when the thermocline started to develop (ST and EM
364 pers. comm.).

365 ***Arctic charr eDNA is detected consistently at the monitored sites in late autumn***

366 During the Arctic charr spawning season, between November and December 2017, Arctic charr
367 eDNA was consistently detected at offshore (OF) and shallow water sites (RN and NTH; Fig. 2),
368 but exceptional peaks in signal strength were only recorded at shallow water sites (Fig. 2).

369 The high number of Arctic charr reads found at the shallow breeding habitats of RN and NTH,
370 especially on the 13th November at NTH and 7th December at RN (Fig. 2), exceeded read counts

371 reported otherwise by orders of magnitude. Here, we found an increase in Arctic charr read counts
 372 of about 15 and 30-fold compared to other locations and dates within the spawning season (Fig.2).
 373 Similar peaks of eDNA were observed during the spawning activity of Japanese eel (*Anguilla*
 374 *japonica*, Temminck and Schlegel, 1846) in a mesocosm experiment when eDNA concentrations
 375 were 10 to 100 times higher after the release of gametes (Takeuchi *et al.*, 2019). Additionally, Tsuji
 376 and Shibata (2021), used a manipulative field experiment, and found that the release of sperm is
 377 the main factor explaining peaks in eDNA concentration during fish spawning events. The authors
 378 observed 3 to 25 times higher eDNA concentrations during the spawning activity of medaka
 379 species (*Oryzias latipes*, Temminck and Schlegel, 1846; *Oryzias sakaizumii*, Asai, Senou and
 380 Hosoya, 2012), suggesting that such species-specific peaks in eDNA can be used to identify
 381 localities and timings of true spawning events and distinguish them from those sites where other
 382 spawning-associated activities occur (Tsuji and Shibata, 2021). Similarly, in our study, the eDNA
 383 peak found at NTH on the 13th November coincided with the capture of spent Arctic charr
 384 specimens (Fig. 3), whereas the less intense Arctic charr eDNA signal detected consistently at the
 385 shoreline breeding sites (RN-NTH; Fig. 2) in autumn could be associated with several reproductive
 386 features of the species (i.e. redd-building females, courting and/or competing males, aggregation
 387 of mature individuals).

388 The detection of Arctic charr eDNA at the offshore sites during the breeding season (Fig. 2) might
 389 be explained by migratory mature individuals moving from the offshore feeding grounds to the
 390 shallow breeding habitats. Time-limited and localised variation in eDNA concentrations have been
 391 used to infer fish movements associated to the spawning season in lotic systems whereby visual
 392 surveys, egg collection or telemetry have been used to confirm that spatio-temporal variation in
 393 eDNA reflects fish migration to the spawning grounds during the reproductive season (Antognazza
 394 *et al.*, 2019; Erickson *et al.*, 2016; Thalinger *et al.*, 2019).

395 Overall and in agreement with previous conventional surveys (i.e. net catches, visual surveys,
 396 hydroacoustic, PAM; Bolgan *et al.* 2018; Miller *et al.*, 2015; Winfield *et al.*, 2015), our results
 397 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 read counts on the 13th November, and (iii) the catches of spent Arctic charr specimens when the eDNA peak was observed (Figs. 2, 3). In addition, given that the same or even higher Arctic charr eDNA read counts were detected at the putative spawning grounds of RN only during the spawning season (Fig. 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, non-invasive molecular tool to infer spawning activity through the temporal and spatial localisation of the Arctic charr genetic signal in a large lake.

410 **Conclusion**

In the present study the temporal water sampling coupled with eDNA metabarcoding analysis characterised 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 non-native 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 (Fig. S1; Hänfling *et al.*, 2016; Lawson Handley *et al.*, 2019). For such reason, we suggest

425 that the periodic use of eDNA metabarcoding in lacustrine ecosystems can assist the monitoring of
426 fish spawning activity and, simultaneously, evaluate changes in fish community composition. The
427 characterisation of these two aspects is essential for an accurate assessment of native fish
428 populations as well as to predict changes in their conservation status.

429 This research study entails novel applications of eDNA metabarcoding, enhancing the tool's
430 capabilities far beyond its descriptive and semi-quantitative capacities. Our observations showed
431 that this non-invasive approach provides reliable information on species' reproductive events, and
432 therefore, eDNA metabarcoding could contribute to the long-term monitoring of fish populations
433 through the spatio-temporal assessment of fish spawning events, an essential aspect for the
434 conservation and management of threatened populations.

435

436

References

- Adams C., Fraser D., McCarthy I., Shields S., Waldron S., Alexander G. (2003). Stable isotope analysis reveals ecological segregation in a bimodal size polymorphism in Arctic charr from Loch Tay, Scotland. *Journal of Fish Biology*, **62**(2), pp.474-481. <https://doi.org/10.1046/j.1095-8649.2003.00044.x>
- Adams C.E., Bean C.W., Fraser D., Maitland P.S. (2007). Conservation and management of the Arctic charr: a forward view. *Ecology of Freshwater Fish*, **16**(1), 2-5. <https://doi.org/10.1111/j.1600-0633.2006.00180.x>
- Alberdi A., Aizpurua O., Gilbert M.T.P., Bohmann K. (2018). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, **9**(1), 134-147. <https://doi.org/10.1111/2041-210X.12849>
- Antognazza C.M., Britton J.R., Potter C., Franklin E., Hardouin E.A., Gutmann Roberts C., Aprahamian M., Andreou D. (2019). Environmental DNA as a non-invasive sampling tool to detect the spawning distribution of European anadromous shads (*Alosa* spp.). *Aquatic Conservation: Marine and Freshwater Ecosystems*, **29**(1), 148-152. <https://doi.org/10.1002/aqc.3010>.
- Bean C.W., Mainstone C.P., Hall R.A., Hatton-Ellis T.W., Lee A.S.L., Boon P.J. (2018). Guidelines for the Selection of Biological SSSIs. Part 2: Detailed Guidelines for Habitats and Species Groups. Chapter 19 Freshwater Fish. JNCC, Peterborough. <https://data.jncc.gov.uk/data/18f27480-a7e1-498c-9cca-f9eaced2324/SSSI-Guidelines-19-Freshwaterfish-2018.pdf>
- Bivand R., Keitt T., Rowlingson B. (2019). rgdal: Bindings for the 'Geospatial' Data Abstraction Library. R package version 1.4-8. <https://CRAN.R-project.org/package=rgdal>
- Bolgan M., O'Brien J., Chorazyczewska E., Winfield I.J., McCullough P., Gammell M. (2018). The soundscape of Arctic Charr spawning grounds in lotic and lentic environments: can passive acoustic monitoring be used to detect spawning activities?. *Bioacoustics*, **27**(1), 57-85. <https://doi.org/10.1080/09524622.2017.1286262>
- Bolger A.M., Lohse M., Usadel B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, **30**(15), 2114-2120. <https://doi.org/10.1093/bioinformatics/btu170>
- Bracken F.S., Rooney S.M., Kelly-Quinn M., King J.J., Carlsson J. (2019). Identifying spawning sites and other critical habitat in lotic systems using eDNA "snapshots": A case study using the sea lamprey *Petromyzon marinus* L. *Ecology and Evolution*, **9**(1), 553-567. <https://doi.org/10.1002/ece3.4777>
- Bronner I.F., Quail M.A., Turner D.J., Swerdlow H. (2013). Improved protocols for Illumina sequencing. *Current Protocols in Human Genetics*, **79**(1), 18.2.1-18.2.42. <https://doi.org/10.1002/0471142905.hg1802s79>
- Brys R., Haegeman A., Halfmaerten D., Neyrinck S., Staelens A., Auwerx J., Ruttink T. (2021). Monitoring of spatiotemporal occupancy patterns of fish and amphibian species in a lentic aquatic system using environmental DNA. *Molecular ecology*, **30**(13), 3097-3110. <https://doi.org/10.1111/mec.15742>

475 Corrigan L.J., Lucas M.C., Winfield I.J., Hoelzel A.R., (2011). Environmental factors associated
476 with genetic and phenotypic divergence among sympatric populations of Arctic charr (*Salvelinus*
477 *alpinus*). *Journal of Evolutionary Biology*, **24**(9), 1906-1917. [https://doi.org/10.1111/j.1420-](https://doi.org/10.1111/j.1420-9101.2011.02327.x)
478 [9101.2011.02327.x](https://doi.org/10.1111/j.1420-9101.2011.02327.x)

479 De Barba M., Miquel C., Boyer F., Mercier C., Rioux D., Coissac E., Taberlet P. (2014). DNA
480 metabarcoding multiplexing and validation of data accuracy for diet assessment: application to
481 omnivorous diet. *Molecular Ecology Resources*, **14**(2), 306-323. DOI: 10.1111/1755-0998.12188

482 Díaz S., Settele J., Brondízio E., Ngo H.T., Guèze M., Agard J., Arneth A., Balvanera P., Brauman
483 K., Butchart S., Chan K., Garibaldi L., Ichii K., Liu J., Subramanian S.M., Midgley G., Miloslavich
484 P., Molnár Z., Obura D., Pfaff A., Polasky S., Purvis A., Razzaque J., Reyers B., Chowdhury R.R.,
485 Shin Y.-J., Visseren-Hamakers I., Willis K., Zayas C. (2019). Summary for policymakers of the
486 global assessment report on biodiversity and ecosystem services of the Intergovernmental
487 Science-Policy Platform on Biodiversity and Ecosystem Services.
488 [https://ipbes.net/sites/default/files/inline/files/ipbes_global_assessment_report_summary_for_polic](https://ipbes.net/sites/default/files/inline/files/ipbes_global_assessment_report_summary_for_policymakers.pdf)
489 [ymakers.pdf](https://ipbes.net/sites/default/files/inline/files/ipbes_global_assessment_report_summary_for_policymakers.pdf)

490 Di Muri C., Lawson Handley L., Bean C.W., Li J., Peirson G., Sellers G.S., Walsh K., Watson H.V.,
491 Winfield I.J., Hänfling B. (2020). Read counts from environmental DNA (eDNA) metabarcoding
492 reflect fish abundance and biomass in drained ponds. *Metabarcoding and Metagenomics*, **4**,
493 e56959. <https://doi.org/10.3897/mbmq.4.56959>

494 Edgar R.C., Haas B.J., Clemente J.C., Quince C., Knight R. (2011). UCHIME improves sensitivity
495 and speed of chimera detection. *Bioinformatics*, **27**(16), 2194-2200.
496 <https://doi.org/10.1093/bioinformatics/btr381>

497 Erickson R.A., Rees C.B., Coulter A.A., Merkes C.M., McCalla S.G., Touzinsky K.F., Walleiser L.,
498 Goforth R.R., Amberg J.J. (2016). Detecting the movement and spawning activity of bigheaded
499 carps with environmental DNA. *Molecular Ecology Resources*, **16**(4), 957-965. DOI: 10.1111/1755-
500 0998.12533

501 Esteve M. (2005). Observations of spawning behaviour in Salmoninae: *Salmo*, *Oncorhynchus* and
502 *Salvelinus*. *Reviews in Fish Biology and Fisheries*, **15**(1-2), 1-21. [https://doi.org/10.1007/s11160-](https://doi.org/10.1007/s11160-005-7434-7)
503 [005-7434-7](https://doi.org/10.1007/s11160-005-7434-7)

504 Franssen J., Blais C., Lapointe M., Bérubé F., Bergeron N., Magnan P. (2012). Asphyxiation and
505 entombment mechanisms in fines rich spawning substrates: experimental evidence with brook
506 trout (*Salvelinus fontinalis*) embryos. *Canadian Journal of Fisheries and Aquatic Sciences*, **69**(3),
507 587-599. <https://doi.org/10.1139/f2011-168>

508 Freyhof J. and Kottelat M. (2008). *Salvelinus alpinus*. The IUCN Red List of Threatened Species
509 2008: e.T19877A9102572. <https://dx.doi.org/10.2305/IUCN.UK.2008.RLTS.T19877A9102572.en>.
510 Downloaded on 18 October 2021

511 Frost W.E. (1965). Breeding habits of Windermere charr, *Salvelinus willughbii* (Günther), and their
512 bearing on speciation of these fish. *Proceedings of the Royal Society of London. Series B.*
513 *Biological Sciences*, **163**(991), 232-284. <https://doi.org/10.1098/rspb.1965.0070>

514 Frost W.E. (1977). The food of Charr,**Salvelinus willughbii* (Günther), in Windermere. *Journal of*
515 *Fish Biology*, **11**(6), 531-547. <https://doi.org/10.1111/j.1095-8649.1977.tb05710.x>

516 Garduño-Paz M.V., Adams C.E., Verspoor E., Knox D., Harrod C. (2012). Convergent evolutionary
517 processes driven by foraging opportunity in two sympatric morph pairs of Arctic charr with
518 contrasting post-glacial origins. *Biological Journal of the Linnean Society*, **106**(4), 794–806.
519 <https://doi.org/10.1111/j.1095-8312.2012.01906.x>

520 Hänfling B., Lawson Handley L., Read D.S., Hahn C., Li J., Nichols P., Blackman R.C., Oliver A.,
521 Winfield I.J. (2016). Environmental DNA metabarcoding of lake fish communities reflects long-term
522 data from established survey methods. *Molecular Ecology*, **25**(13), 3101-3119.
523 <https://doi.org/10.1111/mec.13660>

524 Hansen M.J., Krueger C.C., Muir A.M., Klemetsen A., Power M. (2019). Assessing the impact of
525 charr research past, present, and future. *Hydrobiologia*, **840**(1), 1-10.
526 <https://doi.org/10.1007/s10750-019-04012-3>

527 Johnson L. (1980). The Arctic charr, *Salvelinus alpinus*. In: Balon, E.K. (ed.) *Charrs, salmonid*
528 *fishes of the genus Salvelinus*. The Hague: Dr. W. Junk Publishers. pp. 15-88.

529 Jonsson B. and Jonsson N. (2001). Polymorphism and speciation in Arctic charr. *Journal of Fish*
530 *Biology*, **58**(3), 605-638. <https://doi.org/10.1111/j.1095-8649.2001.tb00518.x>

531 Jørgensen E.H., Christiansen J.S., Jobling M. (1993). Effects of stocking density on food intake,
532 growth performance and oxygen consumption in Arctic charr (*Salvelinus alpinus*). *Aquaculture*,
533 **110**(2), 191-204. [https://doi.org/10.1016/0044-8486\(93\)90272-Z](https://doi.org/10.1016/0044-8486(93)90272-Z)

534 Kelly R.P., Port J.A., Yamahara K.M., Crowder L.B. (2014). Using environmental DNA to census
535 marine fishes in a large mesocosm. *PloS One*, **9**(1), p.e86175.
536 <https://doi.org/10.1371/journal.pone.0086175>

537 Kelly S., Moore T.N., de Eyto E., Dillane M., Goulon C., Guillard J., Lasne E., McGinnity P., Poole
538 R., Winfield I.J., Woolway R.I., Jennings E. (2020). Warming winters threaten peripheral Arctic
539 charr populations of Europe. *Climatic Change*, **163**(1), 599-618. [https://doi.org/10.1007/s10584-](https://doi.org/10.1007/s10584-020-02887-z)
540 [020-02887-z](https://doi.org/10.1007/s10584-020-02887-z)

541 Klemetsen A., Amundsen P.A., Dempson J.B., Jonsson B., Jonsson N., O'connell M.F., Mortensen
542 E. (2003). Atlantic salmon *Salmo salar* L., brown trout *Salmo trutta* L. and Arctic charr *Salvelinus*
543 *alpinus* (L.): a review of aspects of their life histories. *Ecology of freshwater fish*, **12**(1), pp.1-59.
544 <https://doi.org/10.1034/j.1600-0633.2003.00010.x>

545 Klemetsen A., Knudsen R., Primicerio R., Amundsen P. A. (2006). Divergent, genetically based
546 feeding behaviour of two sympatric Arctic charr, *Salvelinus alpinus* (L.), morphs. *Ecology of*
547 *Freshwater Fish*, **15**(3), pp.350-355. <https://doi.org/10.1111/j.1600-0633.2006.00128.x>

548 Klemetsen A. (2010). The charr problem revisited: exceptional phenotypic plasticity promotes
549 ecological speciation in postglacial lakes. *Freshwater Reviews*, **3**(1), 49-74.
550 <https://doi.org/10.1608/FRJ-3.1.3>

551 Lawson Handley L., Read D.S., Winfield I.J., Kimbell H., Johnson H., Li J., Hahn C., Blackman R.,
552 Wilcox R., Donnelly R., Szitenberg A. (2019). Temporal and spatial variation in distribution of fish
553 environmental DNA in England's largest lake. *Environmental DNA*, **1**(1), 26-39.
554 <https://doi.org/10.1002/edn3.5>

555 Levasseur M., Bergeron N. E., Lapointe M. F., Bérubé F. (2006). Effects of silt and very fine sand
556 dynamics in Atlantic salmon (*Salmo salar*) redds on embryo hatching success. *Canadian Journal of*
557 *Fisheries and Aquatic Sciences*, **63**(7), 1450-1459. <https://doi.org/10.1139/f06-050>

558 Li J., Hatton-Ellis T.W., Lawson Handley L.J., Kimbell H.S., Benucci M., Peirson G., Hänfling B.
559 (2019). Ground-truthing of a fish-based environmental DNA metabarcoding method for assessing
560 the quality of lakes. *Journal of Applied Ecology*, **56**(5), 1232-1244. <https://doi.org/10.1111/1365-2664.13352>

562 Low J.J., Igoe F., Davenport J., Harrison S.S.C. (2011). Littoral spawning habitats of three
563 southern Arctic charr (*Salvelinus alpinus* L.) populations. *Ecology of Freshwater Fish*, **20**(4), 537-
564 547. <https://doi.org/10.1111/j.1600-0633.2011.00502.x>

565 Lynch A.J., Cooke S.J., Deines A.M., Bower S.D., Bunnell D.B., Cowx I.G., Nguyen V.M., Nohner
566 J., Phouthavong K., Riley B., Rogers M.W. (2016). The social, economic, and environmental
567 importance of inland fish and fisheries. *Environmental Reviews*, **24**(2), 115-121.
568 <https://doi.org/10.1139/er-2015-0064>

569 Magoč T. and Salzberg S.L. (2011). FLASH: fast length adjustment of short reads to improve
570 genome assemblies. *Bioinformatics*, **27**(21), 2957-2963.
571 <https://doi.org/10.1093/bioinformatics/btr507>

572 Maitland P.S., Winfield I.J., McCarthy I.D., Igoe F. (2007). The status of Arctic charr *Salvelinus*
573 *alpinus* in Britain and Ireland. *Ecology of Freshwater Fish*, **16**(1), 6-19.
574 <https://doi.org/10.1111/j.1600-0633.2006.00167.x>

575 Miller H., Winfield I.J., Fletcher J.M., James J.B., Van Rijn J., Bull J.M., Cotterill C.J. (2015).
576 Distribution, characteristics and condition of Arctic charr (*Salvelinus alpinus*) spawning grounds in
577 a differentially eutrophicated twin-basin lake. *Ecology of Freshwater Fish*, **24**(1), 32-43.
578 <https://doi.org/10.1111/eff.12122>

579 Mills C.A., Heaney S.I., Butterwick C., Corry J.E., Elliott J.M. (1990). Lake enrichment and the
580 status of Windermere charr, *Salvelinus alpinus* (L.). *Journal of Fish Biology*, **37**(sA), 167-174.
581 <https://doi.org/10.1111/j.1095-8649.1990.tb05032.x>

582 Partington J.D. and Mills C.A. (1988). An electrophoretic and biometric study of Arctic charr,
583 *Salvelinus alpinus* (L.), from ten British lakes. *Journal of Fish Biology*, **33**, 791-814.
584 <https://doi.org/10.1111/j.1095-8649.1988.tb05524.x>

585 Piccolo J.J. (2017). The Land Ethic and conservation of native salmonids. *Ecology of Freshwater*
586 *Fish*, **26**(1), 160-164. <https://doi.org/10.1111/eff.12263>

587 R Core Team (2021). R: A language and environment for statistical computing. R Foundation for
588 Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>

589 Reist J. D., Power M., Dempson J. B. (2013). Arctic charr (*Salvelinus alpinus*): a case study of the
590 importance of understanding biodiversity and taxonomic issues in northern fishes. *Biodiversity*,
591 **14**(1), 45-56. <https://doi.org/10.1080/14888386.2012.725338>

592 Riaz T., Shehzad W., Viari A., Pompanon F., Taberlet P., Coissac E. (2011). ecoPrimers: inference
593 of new DNA barcode markers from whole genome sequence analysis. *Nucleic Acids Research*,
594 **39**(21), pp.e145-e145. <https://doi.org/10.1093/nar/gkr732>

595 Rognes T., Flouri T., Nichols B., Quince C., Mahé F. (2016). VSEARCH: a versatile open source
596 tool for metagenomics. *PeerJ*, **4**, p.e2584. <https://doi.org/10.7717/peerj.2584>

597 Sellers G.S., Di Muri C., Gómez A., Hänfling B. (2018). Mu-DNA: a modular universal DNA
598 extraction method adaptable for a wide range of sample types. *Metabarcoding and Metagenomics*,
599 **2**, p.e24556. <https://doi.org/10.3897/mbmq.2.24556>

600 Skúlason S., Parsons K., Svanbäck R., Räsänen K., Ferguson M., Adams C., Amundsen P-A.,
601 Bartels P., Bean C.W., Boughman J., Englund G., Gudbrandsson J., Hooker O., Hudson A.,
602 Kahilainen K., Knudsen R., Kristjánsson B., Leblanc C., Jónsson Z., Öhlund G., Smith C.,
603 Snorrason S. (2019). A way forward with eco-evo-devo: an extended theory of resource
604 polymorphism with postglacial fishes as model systems. *Biological Reviews*, **94**, 1786-1808.
605 <https://doi.org/10.1111/brv.12534>

606 Smalås A., Amundsen P. A., Knudsen R. (2013). Contrasting life history strategies of sympatric
607 Arctic charr morphs, *Salvelinus alpinus*. *Journal of Ichthyology*, **53**(10), 856-866.
608 <https://doi.org/10.1134/S0032945213100111>

609 Sternecker K., Denic M., Geist J. (2014). Timing matters: species-specific interactions between
610 spawning time, substrate quality, and recruitment success in three salmonid species. *Ecology and*
611 *Evolution*, **4**(13), 2749-2758. <https://doi.org/10.1002/ece3.1128>

612 Sumner M.D. (2016). ggpolypath: Polygons with Holes for the Grammar of Graphics. R package
613 version 0.1.0. <https://CRAN.R-project.org/package=ggpolypath>

614 Takeuchi A., Iijima T., Kakuzen W., Watanabe S., Yamada Y., Okamura A., Horie N., Mikawa N.,
615 Miller M.J., Kojima T., Tsukamoto K. (2019). Release of eDNA by different life history stages and
616 during spawning activities of laboratory-reared Japanese eels for interpretation of oceanic survey
617 data. *Scientific Reports*, **9**(1), 1-9. <https://doi.org/10.1038/s41598-019-42641-9>

618 Telnes T. and Saegrov H. (2004). Reproductive strategies in two sympatric morphotypes of Arctic
619 charr in Kalandvatnet, west Norway. *Journal of fish biology*, **65**(2), 574-579.
620 <https://doi.org/10.1111/j.0022-1112.2004.00432.x>

621 Thalinger B., Wolf E., Traugott M., Wanzenböck J. (2019). Monitoring spawning migrations of
622 potamodromous fish species via eDNA. *Scientific Reports*, **9**(1), 1-11.
623 <https://doi.org/10.1038/s41598-019-51398-0>

624 Tsuji S. and Shibata N. (2021). Identifying spawning events in fish by observing a spike in
625 environmental DNA concentration after spawning. *Environmental DNA*, **3**(1), 190-199.
626 <https://doi.org/10.1002/edn3.153>

Wickham H. (2016). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York
260pp.

Winfield I.J., Fletcher J.M., James J.B. (2008). The Arctic charr (*Salvelinus alpinus*) populations of
Windermere, UK: population trends associated with eutrophication, climate change and increased
abundance of roach (*Rutilus rutilus*). *Environmental Biology of Fishes*, **83**(1), 25-35.
<https://doi.org/10.1007/s10641-007-9235-4>

Winfield I.J., Fletcher J.M., James J.B., Bean C.W. (2009). Assessment of fish populations in still
waters using hydroacoustics and survey gill netting: experiences with Arctic charr (*Salvelinus*
alpinus) in the UK. *Fisheries Research*, **96**(1), 30-38. <https://doi.org/10.1016/j.fishres.2008.09.013>

Winfield I.J., Hateley J., Fletcher J.M., James J.B., Bean C.W., Clabburn P. (2010). Population
trends of Arctic charr (*Salvelinus alpinus*) in the UK: assessing the evidence for a widespread
decline in response to climate change. *Hydrobiologia*, **650**(1), 55-65.
<https://doi.org/10.1007/s10750-009-0078-1>

Winfield I.J., van Rijn J., Valley R.D. (2015). Hydroacoustic quantification and assessment of
spawning grounds of a lake salmonid in a eutrophicated water body. *Ecological Informatics*, **30**,
235-240. <https://doi.org/10.1016/j.ecoinf.2015.05.009>

Winfield I.J., Berry R., Iddon H. (2019). The cultural importance and international recognition of the
Arctic charr *Salvelinus alpinus* populations of Windermere, UK. *Hydrobiologia*, **840**(1), 11-19.
<https://doi.org/10.1007/s10750-018-3814-6>

Zhang Z., Schwartz S., Wagner L., Miller W. (2000). A greedy algorithm for aligning DNA
sequences. *Journal of Computational Biology*, **7**(1-2), 203-214.
<https://doi.org/10.1089/10665270050081478>

Zhang S., Lu Q., Wang Y., Wang X., Zhao J., Yao M. (2020). Assessment of fish communities
using environmental DNA: effect of spatial sampling design in lentic systems of different sizes.
Molecular Ecology Resources, **20**(1), 242-255. <https://doi.org/10.1111/1755-0998.13105>

Figures

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.

Arctic charr read counts ○ < 500 ○ 500-1,000 ○ 1,000-3,000 ○ 3,000-6,000 ○ 6,000-10,000 ○ 10,000-15,000 ○ 15,000-30,000

Figure 2. Spatio-temporal variation of Arctic charr eDNA signal in the north basin of Windermere. Bubble size is proportional to 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.

668

669 **Figure 3.** Arctic charr catches and eDNA read counts at the autumn-breeding grounds of NTH.
670 Grey bars indicate the gill-netting dates (net lowered / net lifted) and the number and status of
671 Arctic charr specimens caught. Darkblue bars show the Arctic charr eDNA total read counts from
672 the five water sampling events.