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LIMNOLOGY AND OCEANOGRAPHY
Letters

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Open Access

Limnology and Oceanography Letters 7, 2022, 443–449

doi: 10.1002/lol2.10271

LETTER

Reproduction influences seasonal eDNA variation in a temperate marine fish community

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Scientific Significance Statement

Environmental DNA (eDNA) represents a powerful and novel source of high-resolution data for ecological inference, with the potential to become a standard survey technique in marine conservation and fisheries science. However, before this goal can be realized, the fundamental biological processes driving its natural variation must be understood. Here, we reveal that fish reproductive activity makes a substantial contribution to seasonal eDNA variation in a temperate marine ecosystem. These findings will improve the predictive power of ecological monitoring with eDNA, and have the potential to open exciting new avenues into phenological biology and organismal responses to environmental change.

Abstract

Many factors influence how environmental DNA (eDNA) abundance varies in natural environments. One of the least studied contributors to eDNA variation is that of reproduction. Marine organisms that broadcast spawn are expected to shed increased quantities of DNA in association with the release of gametes and the elevated levels of activity associated with reproduction. To test this hypothesis at the community level, we present a year-long eDNA time-series of a temperate sea-shelf fish assemblage combined with adult fish and ichthyoplankton abundance data. Our results show that eDNA is associated with species abundance estimated by conventional fish surveys at all life stages (adult, larval, and egg), and was on average 2.3-fold more abundant during predicted fish reproductive periods.

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Associate editor: Sara Beier

Stefano Mariani and Martin J. Genner contributed equally to this study.

Author Contribution Statement: MJG, SM, and DWS obtained funding. RAC, CB, NCH, and SR collected samples and generated data. RAC and MJG analyzed the data. RAC wrote the manuscript. All authors edited the manuscript and contributed ideas.

Data Availability Statement: Data are available in the NCBI Sequence Read Archive (SRA) repository at https://www.ncbi.nlm.nih.gov/sra/PRJNA725897. Code and metadata are available in the Zenodo repository at https://doi.org/10.5281/zenodo.4723276.

Additional Supporting Information may be found in the online version of this article.

The DNA molecules ubiquitous in our natural environments represent a powerful insight into the diversity, abundance, and interactions among organisms, and we can now access this resource at unprecedented scales to answer increasingly complex ecological questions (Bálint et al. 2018). Environmental metagenetic sampling can be particularly valuable in aquatic systems, where observing biodiversity can be expensive, difficult, and often destructive. In marine environments, eDNA has revealed the capacity to provide information on fish biomass (Salter et al. 2019), seasonal diversity (Sigsgaard et al. 2017), habitat diversity (Jeunen et al. 2018), broad spatial patterns (Fraija-Fernández et al. 2020), behavioral ecology (Canals et al. 2021), anthropogenic pressures (DiBattista et al. 2020), and population genetic differentiation (Sigsgaard et al. 2016). However, as eDNA data collection accelerates, biological interpretation of the observed patterns remains challenging (Cristescu and Hebert 2018). A pattern common to aquatic eDNA metabarcoding studies is an often positive but generally weak relationship between eDNA abundance and the density of organisms measured in conventional survey data or mock communities (Lamb et al. 2019). Much of this discordance arises from sampling errors and systematic biases inherent to particular methods. For example, stochastic processes in early PCR cycles and low template abundances increase false-negative detection rates (Ficetola et al. 2015), while priming site mismatches reduce amplification efficiency and introduce species-specific biases (Piñol et al. 2019). Physiological and behavioral differences among species can also account for the observed variation in eDNA detectability (Thalinger et al. 2021).

One underlying biological process that has so far been underexplored is that of reproductive activity as a predictor of environmental DNA variation. During reproduction, organisms such as fishes will often migrate, increase in activity, form aggregations, and in marine environments, many will release large numbers of gametes directly into the water column as part of broadcast spawning. This genetic material can be readily detected in the water, corresponding to an elevated eDNA concentration during periods of spawning activity. This association has been demonstrated in freshwaters under experimental conditions for *Oryzias* ricefishes (Tsuji and Shibata 2021) and *Macquaria* perch (Bylemans et al. 2017). In marine environments, eDNA has been used to identify spawning grounds of *Anguilla* eels (Takeuchi et al. 2019) and detect species occurrences associated with spawning sites in the Celtic Sea (Ratcliffe et al. 2021). Typically, studies have used quantitative PCR (qPCR) assays to detect single species, and it remains to be thoroughly tested whether community-level metabarcode assays are similarly able to detect elevated quantities of eDNA resulting from reproductive events across multiple species over seasonal time scales. Here, we present a year-long time-series of fish community metabarcode data together with conventional survey data for adult, larval, and egg life stages, to test whether eDNA abundances can be predicted by: (1) seasonal changes in the fish community; (2) reproductive period of each species; (3) organism abundance at different life stages (egg, larval, adult); and (4) fish life-history traits.

Materials and methods

Study location

The study was conducted at three long-term sampling stations in the Western English Channel between 15 and 40 km southwest of Plymouth (Devon, UK; Fig. S1), representing open-shelf (E1; 50.03°N, 4.37°W; depth 70 m), reef (L5; 50.18°N, 4.3°W; depth 60 m), and shallow shelf (L4; 50.25°N, 4.22°W; depth 50 m) environments.

Adult and ichthyoplankton surveys

Adult demersal fish surveys were carried out approximately every 2 weeks (bi-weekly) at Sta. L4 by otter trawl with a 50-mm cod-end mesh, with two hauls of approximately 40 min per survey event; values are reported as number of individuals per haul. Ichthyoplankton samples were taken from Sta. L4 (bi-weekly), L5 (monthly), and E1 (monthly), and carried out using a 700-μm knitted terelene multifilament mesh net; values are reported as number of individuals per 1000 m^3 water. Taxonomic resolution of the adult trawl survey results was to species level, except for small gobiids, which were identified to genus level. Larval resolution was to species level, except for species of Ammodytidae, Blenniidae, Clupeidae, Gobiidae, Lotidae, and Syngnathidae, which were identified to family level. For eggs, only those of European pilchard (*Sardina pilchardus*) were identified to species level.

eDNA collection protocols

The sampling of eDNA was carried out at Sta. L4, L5, and E1 at the same frequency as the adult and ichthyoplankton surveys. On each survey event, triplicate 2 L water samples were taken from surface and bottom using a Niskin bottle, and strained through a 250-μm mesh pre-filter before being transferred to Nalgene HDPE collection bottles and placed on ice. Around 4 h later, each sample was filtered through a 0.22-μm Sterivex-GP PES (Merck Millipore) filter using a peristaltic pump, and the filter immediately frozen at −20°C. Reusable equipment was decontaminated with a 10% bleach solution between samples, and field blanks using bottled drinking water were taken at 10% of the sampling events. DNA was isolated from the Sterivex filters using the DNeasy PowerWater Kit (Qiagen), together with extraction blanks. Pre-PCR and post-PCR procedures were carried out in dedicated and isolated laboratories.

Metabarcoding protocols

Metabarcode data were obtained by amplifying an approximately 167-bp fragment of mitochondrial 12S using the

Tele02 primer set (Taberlet et al. 2018). For a total of 96 unique combinations, the forward and reverse PCR primers were adapted with matching unique 8-mer sample identification tags differing across pairs by at least three nucleotides and including variable 5′ random heterogeneity of 2–4 bp. PCRs were conducted in triplicate 20-μL reactions using a single uniquely tagged primer pair, and then pooled; the following reagents were used: 10 μL AmpliTaq Gold 360 Mastermix (ThermoFisher), 0.16 μL BSA, 5.84 μL water, 2 μL forward and reverse primer (5 μM), and 2 μL extracted template DNA (water was used for PCR blanks). Cycling conditions comprised: denaturation at 95°C for 10 min; 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 60 s; final extension of 72°C for 7 min. Amplicons were pooled at equal volume, purified and concentrated with MinElute columns (Qiagen), and double size-selected with AMPure XP paramagnetic beads (Beckman Coulter). Four libraries were prepared using the PCR-free Kapa HyperPrep Kit (Roche) following the manufacturer's instructions, quantified by qPCR, and each sequenced separately on a MiSeq instrument (Illumina) with a v2 (2 × 150-bp paired-end) chemistry and 1% PhiX spike-in.

Bioinformatic processing

Bioinformatic processing followed Collins et al. (2019), and comprised: (1) sample demultiplexing using *cutadapt* v3.4 (Martin 2011); (2) sequence denoising and dereplication using *dada2* v1.20.0 (Callahan et al. 2016); (3) homology filtering of amplicon sequence variants (ASVs) using hidden Markov models in *hmmer* v3.3 (http://hmmer.org/; *E*-value 0.01); (4) first pass taxonomic assignment to exclude non-fish ASVs using *sintax* (Edgar 2016) and NCBI RefSeq v208 (https://www.ncbi.nlm.nih.gov/refseq/); and (5) exhaustive assignment using combined phylogenetic placement (*epa-ng* v0.3.8; Barbera et al. 2019) and sequence similarity (*blastn* v2.11.0), using a curated British Isles fishes reference library (MetaFishLib v245; Collins et al. 2021). Reference library sequences were used as priors during *dada2* denoising to avoid erroneously discarding rare sequences, and an exclusion list of sequences generated from concurrent unrelated laboratory projects was used to control for potential cross-contamination. The *decipher* v2.22.0 package (Wright 2016) was used to predict PCR amplification efficiencies for each species using default settings and amplification parameters (annealing temperature, primer molarity) as outlined above. Only reads assigned to marine and estuarine fishes were retained for analyses (i.e., reads from freshwater fishes and non-fish species were removed).

Statistical analyses

To explore seasonal structure in the eDNA read abundance data (all stations) and adult demersal trawl data (Sta. L4) we employed principal coordinates analysis (PCoA) to reflect dissimilarities between samples taken at different time points along a linear axis of variation. We rescaled both datasets using a $\log_e(n+1)$ transformation to reduce skew before converting them to Euclidean distances. The seasonal trends along the resulting principal coordinates axes were highlighted using generalized additive model fits (smoothing parameter value $k = 4$).

To demonstrate the association between fish reproductive period and eDNA read abundance (all stations), we used a logistic regression model (binomial family, logit link). Data for the fish reproductive period was obtained for each species from FishBase (https://www.fishbase.org/), and refined for the study area following Heessen et al. (2015). The response variable in the model was binary (breeding in each month or not breeding), while the predictor variable was eDNA read abundance (untransformed). To illustrate the association between eDNA read abundance and conventional surveys we used linear regression, including data at the adult (Sta. L4), egg (all stations), and larval (all stations) life stages. Reads were converted to proportions and then 4th root transformed to satisfy the assumptions of the linear model. The linear regression residuals were then summarized for different adult life histories (pelagic, benthopelagic, benthic).

To develop a more complete picture of the factors contributing to eDNA read abundance, additional variables that may co-influence were accounted for using negative binomial regression in a generalized linear mixed-effects model (GLMM) framework. The response variable was untransformed eDNA read abundance, offset to account for sample read depth variation. The read count data had an overrepresentation of zero values, as is typical in eDNA metabarcode data, and therefore the negative binomial model family with a zero-inflated component was considered most appropriate. Fixed predictor variables included demersal trawl catch per unit effort (CPUE), fish reproductive period, ecological life history (pelagic, benthopelagic, benthic), and PCR amplification efficiency. Because of the contrasting magnitudes of the demersal trawl CPUE and PCR amplification efficiency variables, these were standardized by subtracting the mean and dividing by the standard deviation. To control for sample non-independence, water sample replicate (nested within location and event) and species (nested within family and order) were treated as random effects.

For the logistic regression, negative binomial regression, and linear regression analyses, species that were represented across fewer than 1% of the samples were removed in order to minimize noise from rare or spuriously detected species and ensure the dataset was indicative of the typical fish community present. Where taxa could not be identified down to species level in either the conventional or eDNA datasets, both were collapsed down to their lowest common resolutions to ensure appropriate comparison (e.g., *Hyperoplus* and *Ammodytes* combined as Ammodytidae in the adult dataset; Table S1). All statistical procedures were performed in R v4.1.1 (https://cran.r-project.org/); the negative binomial regression model was fitted using the *glmmTMB*

v1.1.2.3 package (Brooks et al. 2017); marginal means of the fixed effects were estimated with the *emmeans* v1.7.0 package (https://cran.r-project.org/package=emmeans); and data transformations and model fits were compared using the *performance* v0.8.0 package (https://cran.r-project.org/package=performance).

Results

Over 27 months (January 2016 to April 2018), we carried out 62 demersal fish survey trawls at Sta. L4. Over 15 months (February 2017 to April 2018), we carried out 200 eDNA sampling events at Sta. L4, L5, and E1, alongside 59 ichthyoplankton surveys. High throughput sequencing of the Tele02 mitochondrial 12S metabarcode marker resulted in a total of 8.6 million reads after bioinformatic processing and taxonomic assignment (Figs. S2, S3; Tables S2, S4); negative controls comprised a total of 16,575 reads over seven field blanks, 10 extraction blanks, six PCR blanks, and 70 unused tag blanks across the four libraries (Table S5). A total of 94 species were recovered from the eDNA data over the survey period (all stations; Fig. S4), while the demersal trawl surveys (Sta. L4) reported 49 species, with 43 (88%) of these also present in eDNA (Fig. S5; Table S1). Summaries of community-wide patterns within the monthly eDNA read and demersal trawl data show that eDNA captured the same seasonally dynamic and cyclical structure as the demersal trawls (Figs. 1, S6), and exploratory ordinations indicated little difference across stations and sampling depths (Fig. S7). Linear regressions demonstrate that all developmental stages were associated with environmental DNA, with relationships shown between eDNA read proportions and organism abundance (catch per unit effort) at the adult ($\beta = 0.08$; $p < 0.001$; $R^2 = 0.43$), larval ($\beta = 0.327$; $p < 0.001$; $R^2 = 0.46$), and egg ($\beta = 0.0013$; $p = 0.008$; $R^2 = 0.2$) life stages (Fig. 2). We further reveal that the relationship between eDNA read and demersal trawl abundance is influenced by fish life history, with pelagic species typically being overrepresented in eDNA read data (Figs. 3, S5). Across eDNA data from all stations, we found a relationship between read abundance and predicted reproductive period, with an odds ratio (OR) increase of 1.061 per 1000 reads (95% CI = 1.048, 1.075; $p < 0.001$; Fig. 4). By applying a zero-inflated negative-binomial GLMM to the Sta. L4 data, we show that reproductive month (OR = 2.34; 95% CI = 1.82, 3.01; $p < 0.001$), demersal trawl CPUE (OR = 1.23; 95% CI = 1.09, 1.40; $p < 0.001$), pelagic life history (OR = 3.94; 95% CI = 1.29, 12.0; $p = 0.016$), and estimated PCR efficiency (OR = 1.34; 95% CI = 1.17, 1.53; $p < 0.001$) are all predictors of increases in eDNA read abundance (Fig. 5; Table S3); a null model excluding reproductive month was a poorer fit (ΔAICc = 44.1; $R^2 = 0.40$) than our model including this predictor ($R^2 = 0.43$).

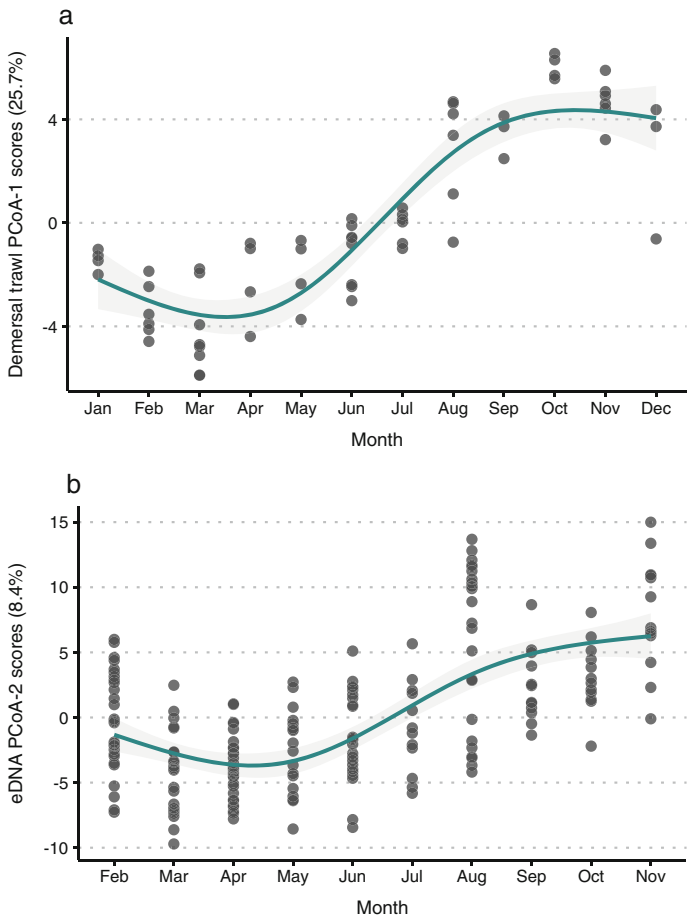

Fig. 1. Principal coordinates (PCoA) derived from **(a)** $\log_e(n+1)$ transformed demersal trawl abundance (L4; sample $n = 62$; species $n = 70$; Jan 2016 to Nov 2017) and **(b)** $\log_e(n+1)$ transformed eDNA read abundance (E1, L5, L4; sample $n = 200$; species $n = 94$; Feb 2017 to Apr 2018). Seasonal trends are highlighted using a generalized additive model fit (smoothing parameter value $k = 4$); shaded areas represent 95% confidence intervals of regression. Seasonal structure in the eDNA dataset is reflected in the second PCoA axis.

Discussion

Most marine teleost fishes display a strategy of broadcast spawning and planktonic larval development at specific times of year, and taken together, our results reveal that the signal of this reproductive activity can be captured by community eDNA metabarcoding. Extending previous studies (e.g., Bylemans et al. 2017; Ratcliffe et al. 2021; Tsuji and Shibata 2021) we show that at the community level, eDNA reads are associated with both egg and larval ichthyoplankton abundances, and the breeding periods of species. This result was supported even after controlling for PCR amplification efficiency and adult biomass as estimated by demersal trawl survey. However, these conclusions rely on the assumption that a quantifiable relationship between metabarcode read abundance and eDNA quantity exists (Piñol et al. 2019). While positive associations between metabarcode read abundance and organism biomass have only

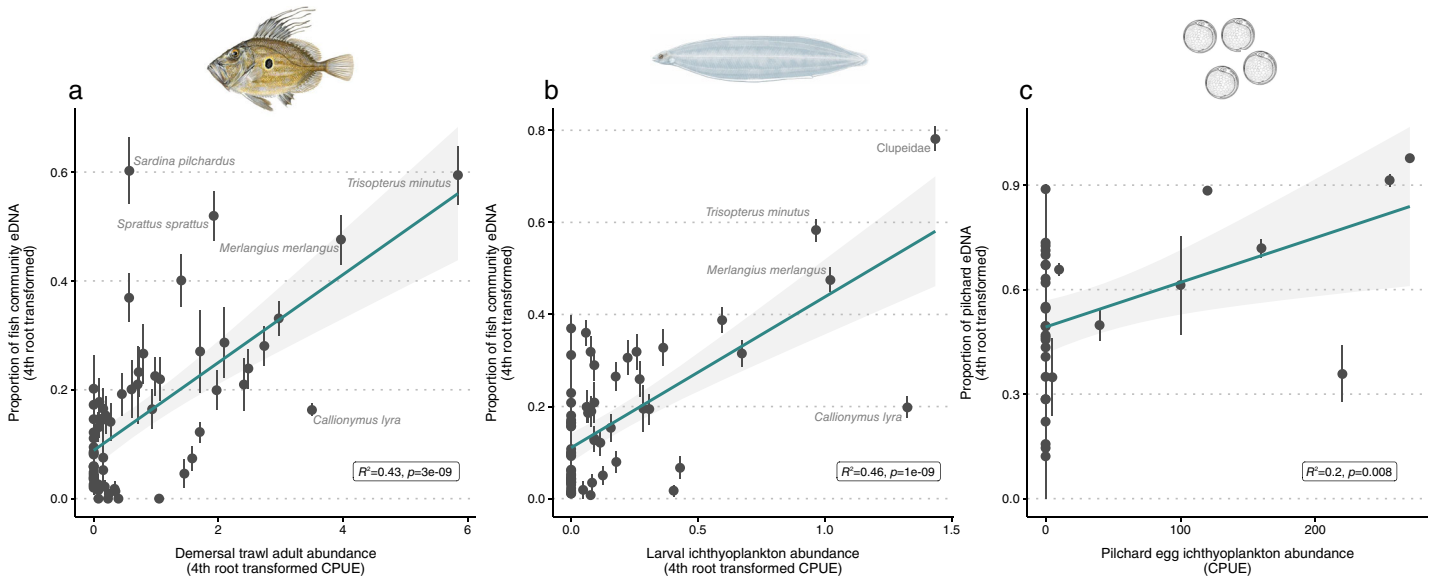

Fig. 2. Linear regressions of mean across-sample eDNA read proportions against CPUE of: **(a)** demersal fish trawl adult abundance (L4; eDNA sample $n = 66$; adult sample $n = 22$; species $n = 65$; average over day); **(b)** ichthyoplankton larvae (L4, L5, E1; eDNA sample $n = 200$; larvae sample $n = 59$; species $n = 64$; average over month); and **(c)** European pilchard (*Sardina pilchardus*) eggs (L4, L5, E1; eDNA sample $n = 200$; egg sample $n = 62$; average over month). Shaded areas represent 95% confidence intervals of regression; bars represent standard error of the mean.

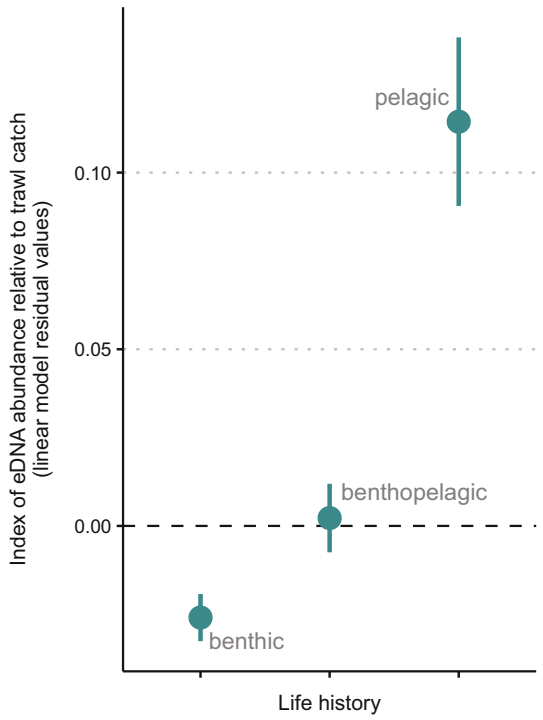

Fig. 3. Metabarcode eDNA read abundance of species classified by ecological life history grouping, after correcting for species CPUE observed in demersal trawl (L4; eDNA sample $n = 66$; adult sample $n = 22$; species $n = 65$; average over day). Points represent mean linear regression residuals; bars represent standard error of the mean.

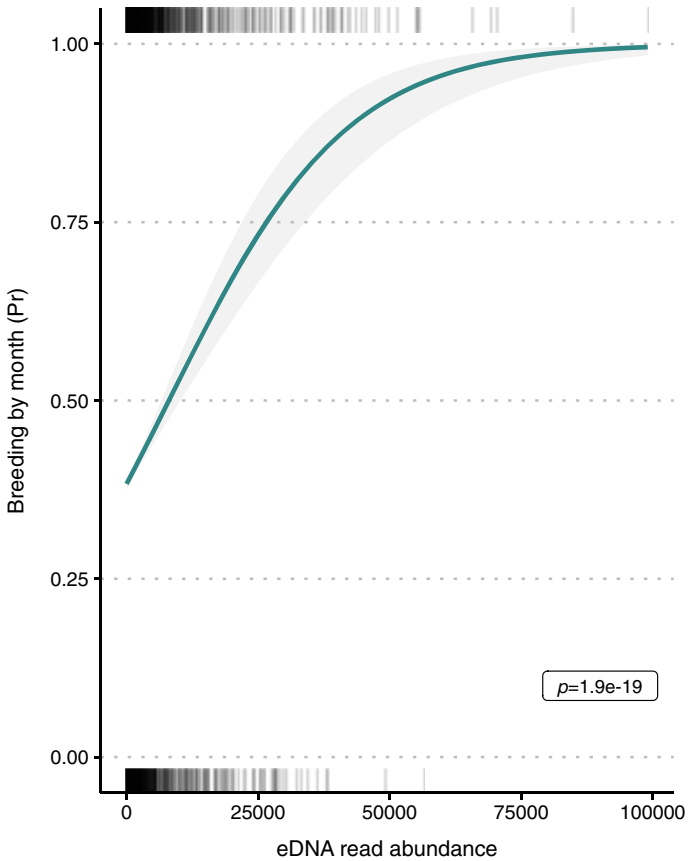

Fig. 4. Logistic regression (binomial family, logit link) of eDNA read abundance against fish reproductive month (sample $n = 200$; species $n = 72$). Shaded area represents 95% confidence interval of regression.

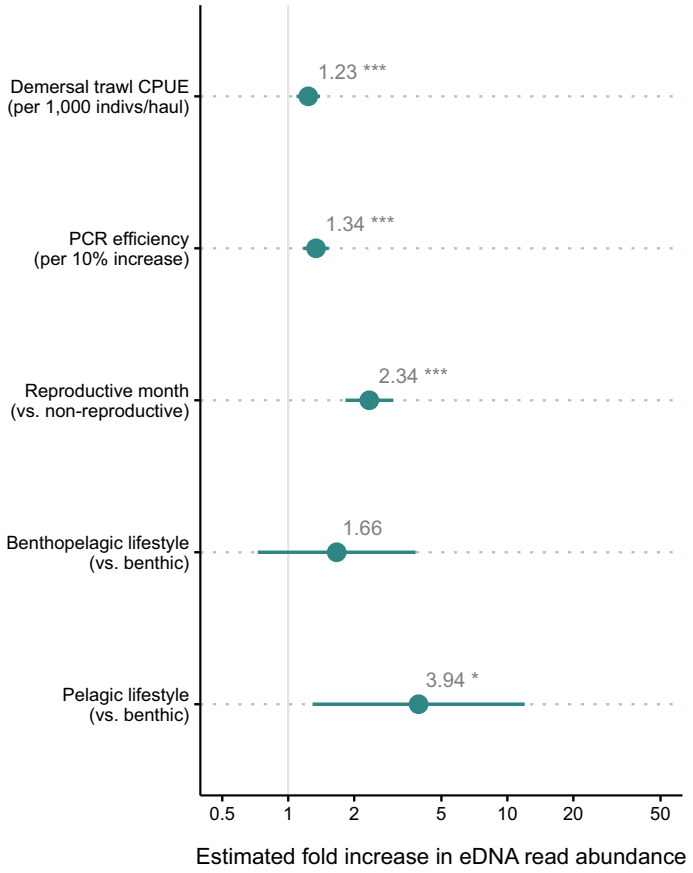

Fig. 5. Odds ratios (ORs) of fixed effects estimates from the zero-inflated negative-binomial GLMM (L4; sample $n = 66$; species $n = 65$). Asterisks represent p values at the *0.05, **0.01, and ***0.001 levels, respectively. Bars represent 95% confidence intervals. Full model output is given in Table S3 and Fig. S8.

been weakly supported by meta-analysis across a broad range of taxa and markers (Lamb et al. 2019), targeted studies of fishes in natural environments using 12S eDNA assays and unbiased biomass sampling methods have shown a strong relationship (Di Muri et al. 2020). Our results additionally show that a pelagic lifestyle is associated with an increase in eDNA read abundance when compared to benthopelagic and benthic species. This overrepresentation of pelagic species such as European pilchard (*Sardina pilchardus*) likely reflects the inefficiency of the demersal trawls in capturing species that forage higher in the water column, as well as any potential differences in eDNA shedding rates between species (Thalinger et al. 2021).

While nucleic acid molecules have a relatively short life in the ocean (Collins et al. 2018), we show that concentrations can rise substantially over the period that a species is reproductively active. This environmental DNA, therefore, represents an overlooked source of information in marine eco-genetic studies, and has implications for wider inferences. Seasonal peaks observed in eDNA may not solely reflect abundance of adult or subadult organisms, but may additionally represent the presence of gametes, zygotes, or larvae in the water column, or reflect changes in activity of reproductively active individuals. Therefore, to account for this source of variation it may be necessary to include reproductive period when modeling eDNA. Conversely, the eDNA spikes could themselves be used as reliable indicators of reproduction, and may become a particularly valuable tool for understanding species that are poorly represented in conventional surveys, cannot reliably be distinguished as ichthyoplankton, or are commercially important. Therefore, routine DNA sequencing of seawater could be a highly sensitive indicator of shifts in timing and intensity of breeding activity, data which could then be associated with climatic, oceanographic, or biological variables. The value of long-term phenological time series in marine ecology is evident (Poloczanska et al. 2016), and synthesis with high-throughput metagenetic data has the potential to open further windows of insight into the drivers of biodiversity change and adaptation in the Anthropocene.

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Acknowledgments

The authors would like to thank Aisling Smith and the captain and crew of the RV MBA *Sepia* at the Marine Biological Association for assistance in collecting the samples, and Chris Brodie for help in the lab. This work was supported by NERC Highlight Topic grant NE/N005937/1, NE/N005759/1 awarded to Martin J. Genner, Stefano Mariani, and David W. Sims.

Submitted 17 December 2021
Revised 18 June 2022
Accepted 29 June 2022