Nordic Council of Ministers

Transcriptomic Tool to determine European Eel marine residency

for use in Monitoring and Management (2TEM)

Contents

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Preface

This report presents the outcome for the Nordic Council of Ministers' funded project "Transcriptomic Tool to determine European Eel marine residency for use in Monitoring and Management (2TEM).". This Nordic project has been led by Caroline Durif (IMR) in close collaboration with Francesca Bertolini (DTU Aqua), Jonna Tomkiewicz (DTU Aqua), and Mehis Rohtla (IMR/University of Tartu). Everyone has contributed with either collecting samples, analyzing data and statistics. We thank the fishers who helped collect the eel involved in this project Atle Nilsen, Per Henning Gjuvsland and Kjell Henning Sjo.

More details about the methods used in this study can be found in:

Bertolini, F., Rohtla, M., Parzanini, C., Tomkiewicz, J., & Durif, C. M. F. (provisionally accepted). Blood-based gene expression as non-lethal tool for inferring salinity-habitat history of European eel. Scientific Reports.

Rohtla, M., Daverat, F., Arts, M. T., Browman, H. I., Parzanini, C., Skiftesvik, A. B., Thorstad, E. B., van der Meeren, T., Vøllestad, L. A., & Durif, C. M. F. (in press). Marine and freshwater habitat use of the European eel along a latitudinal gradient in Norway. Canadian Journal of Fisheries and Aquatic Sciences.

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Caroline Durif, PhD, Project manager
Institute of Marine Research
Marine Ecosystem Acoustics Group
Austevoll Research Station

Introduction
Background and objective

Like all anguillid eels, the European eel (*Anguilla anguilla*) is catadromous, meaning it spawns in the sea, but grows in freshwater habitats. It is the most widespread single fish stock in Europe (Dekker 2003). Yet, today, the population is at a historical minimum and the species is Critically Endangered (International Union for Conservation of Nature (IUCN), ICES 2021). The causes are linked to the cumulative effects of habitat loss, overfishing, hydropower, pollution, parasite introduction and changes in ocean-atmospheric conditions (Miller et al. 2016; Castonguay and Durif 2016; Drouineau et al. 2018).

Eels occur in many different habitats and can adapt to a wide range of salinities. They are present in lakes, rivers, and marshes, but also in estuaries and coastal marine areas. Eels in the northern part of the geographic distribution – countries around the Baltic and the North Sea – tend to grow in more saline habitats than eels in the central part of Europe (Daverat et al. 2006, Rohtla et al 2022). Eels in northern regions (but not exclusively) may also shift between habitats throughout their growth phase complicating their assessment (Tsukamoto et al. 1998; Jessop et al. 2002; Lamson et al. 2006; Daverat and Tomas, 2006). For example, individuals collected in estuaries or coastal habitats might be transient (migratory) rather than resident in the habitat where they were sampled. Very little is known about these behaviors or about the proportion of habitat shifter eels. Yet, this information is needed to manage the species and provide appropriate measure to improve migratory conditions (safe passage) and habitat availability.

It is possible to determine the salinity habitat of eel by analyzing certain chemicals accumulated in their ear stones (also called otoliths) which are involved in sensing gravity and movement in fish. However, these analyses are time-consuming and require sacrifice of the eel to dissect the otoliths. The aim of the project 2TEM was to determine whether it is possible to determine the salinity habitat of an eel from a blood sample (liquid biopsy) and whether this can be done in field conditions. Such sampling could, for example, be implemented during eel monitoring surveys on many individuals without sacrificing eels.

In this study, eels were caught in different salinity environments. Individual blood samples were taken on site, then eels were sacrificed to collect the otoliths. The elemental composition of the otoliths was later analyzed by microchemistry to obtain information on individual eel salinity history, which was compared to the transcriptomic information, obtained by RNA sequencing, in the blood.

The European eel

The European eel is distributed across most coastal countries in Europe and North Africa, spanning the entire Mediterranean basin. Its northern limit is situated in the Barents Sea at 72°N and its southern limit is in Mauritania 30°N. This widely dispersed species represents a single genetic population (panmixia), which spawns in the Sargasso Sea, in the Atlantic Ocean (Als et al. 2011). Newly hatched eel larvae (named *leptocephalus*) drift with the ocean currents to the continental shelf of Europe and North Africa, where they metamorphose into glass eels which swim actively toward the continent (Cresci, 2020). Most eels will colonize freshwater habitats and live their growth phase as 'yellow eels'. Some individuals never colonize freshwater but instead inhabit and grow in coastal marine areas during all their lifecycle. Others shift between habitats several times during the yellow stage. This stage can last up to 20 years on average, but sometimes 30–40 years depending on the growth conditions (Vøllestad and Jonsson, 1986; Poole and Reynolds, 1996; Durif et al. 2020). Eels then become 'silver', undergoing morphological and physiological modifications that announce their reproductive migration back to the Sargasso Sea. This phase marks the beginning of sexual maturation, although eels will never be mature until they reach the Sargasso Sea. Silver eels display counter-shading similar to pelagic fishes and marine mammals and their eyes enlarge and their pectoral fin elongate (Figure 1). Their stage can be evaluated based on these external modifications (Durif et al. 2005).

Figure 1: Eels at the yellow (upper picture) and silver stage (lower picture). During silvering, the eel undergoes color changes, their eyes become enlarged, and their pectoral fins elongated. Photos Caroline Durif

Otolith: the eel's black box

Otoliths are calcium carbonate structures that allow fish to perceive horizontal and vertical movements, gravity and balance. They are the first calcified structures to appear during the early development (Radtke and Dean, 1982; Lecomte-Finiger 1999). Otoliths grow with the fish incorporating trace elements from the water that they occupy. Otoliths are useful for deriving age of fish (Figure 2), but their elemental composition may also provide information on the environment they lived in — for example, they can be used to reconstruct the salinity history of fish (Tzeng and Tsai, 1992). This is how in the late 1990s, it was discovered that some eels spend all their growth phase in coastal marine habitats, thus skipping the freshwater phase (Tzeng et al., 1997; Tsukamoto et al., 1998). It was further revealed that some eels shift habitats several times during their growth phase (Daverat and Tomas, 2006). The time recording property of otoliths (*i.e.* the presence of incremental growth rings) can be used to extract a retrospective chemical habitat use profile from otoliths. This chemical history can, in turn, be interpreted in the context of movement and habitat use patterns through time. The prevalent chemical marker used in eel otolith microchemistry studies is the strontium to calcium ratio (Sr/Ca) (e.g. Tsukamoto et al. 1998; Arai et al. 2019). The salinity history of an eel can be inferred by plotting the Sr/Ca values against the radius of the otolith, i.e. the distance between the core and the outer edge (Figure 3). Identification of inter-habitat movement is based on the changes of Sr/Ca relative to defined life-history changes, such as the larval stage and the metamorphose into glass eel (Jessop et al. 2008).

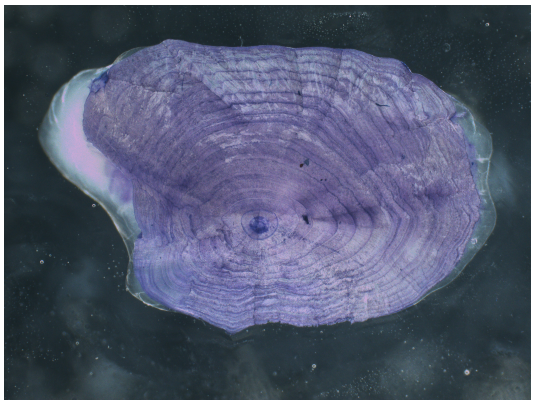
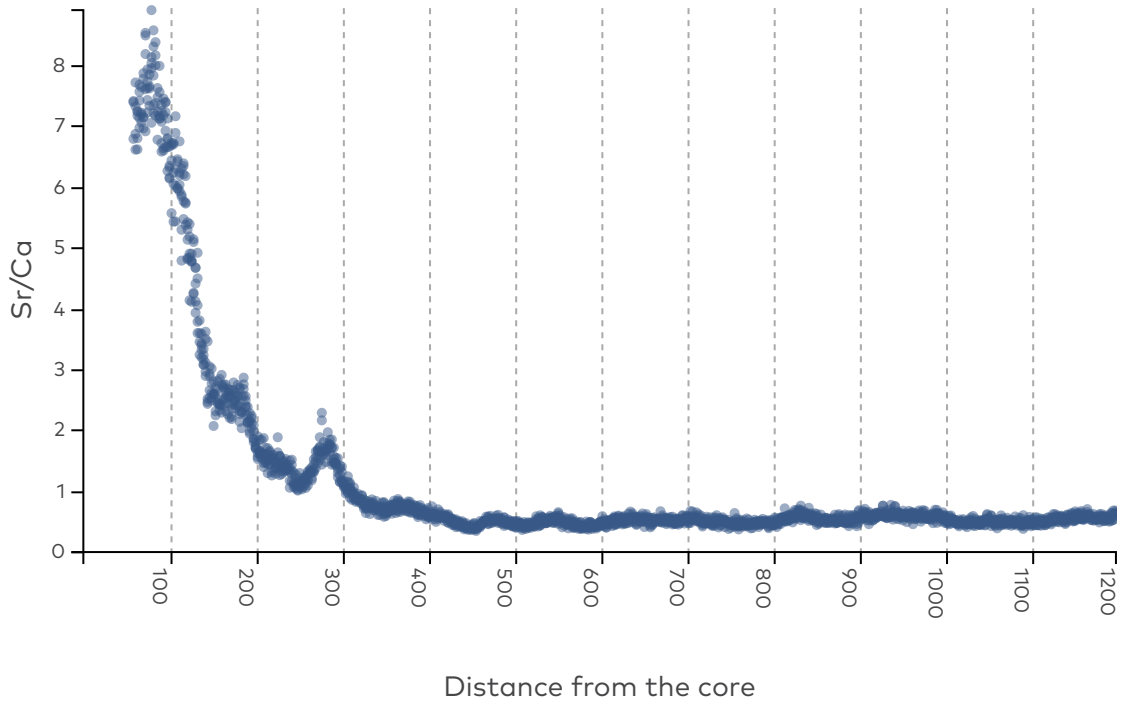


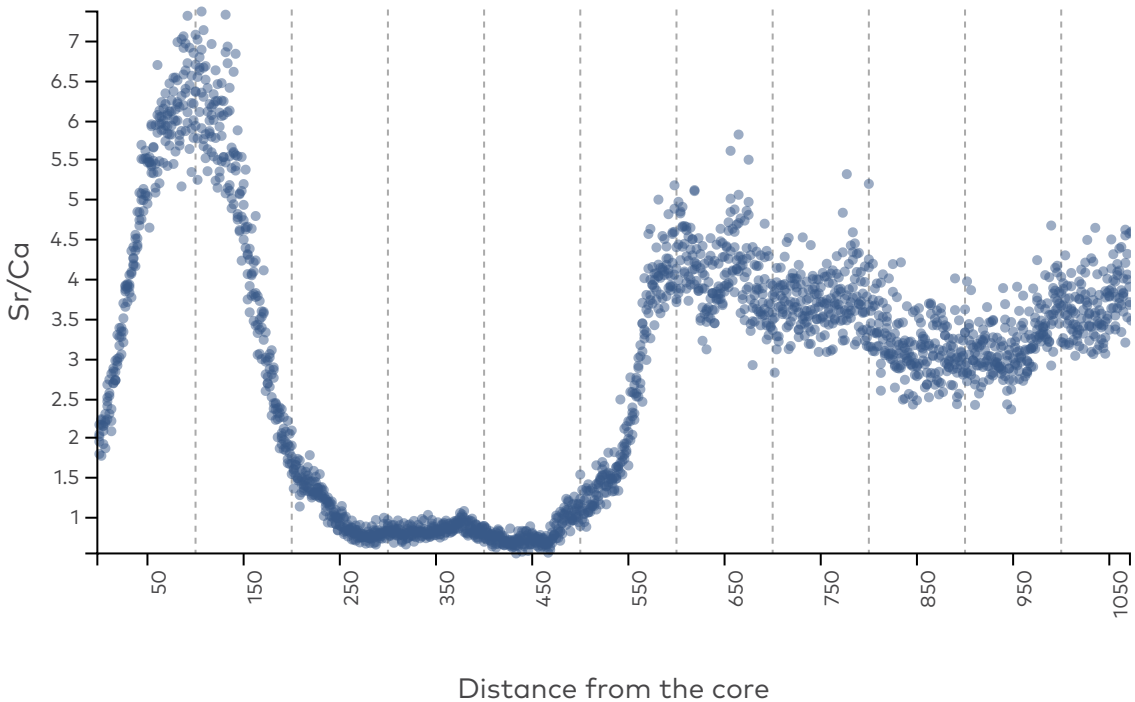
Figure 2.a. European eel otolith, which has been prepared for age analysis. Once the otolith is grinded, decalcified and stained (here with Toluidine blue), zones of slow and fast growth are revealed. The number of rings corresponds to the age of the fish. In the case of eel, the first ring corresponds to the arrival of the juvenile eel in continental habitats. Photo: Marina Mihaljevic.

Figure 2.b. European eel otolith prepared for microchemistry analysis. Chemical elements such as strontium and calcium are measured along a transect (white line between the core and the outer ring) and can give information about the salinity habitat of the eel. Photo: Mehis Rohtla.

Freshwater resident



Habitat shifter



Seawater resident

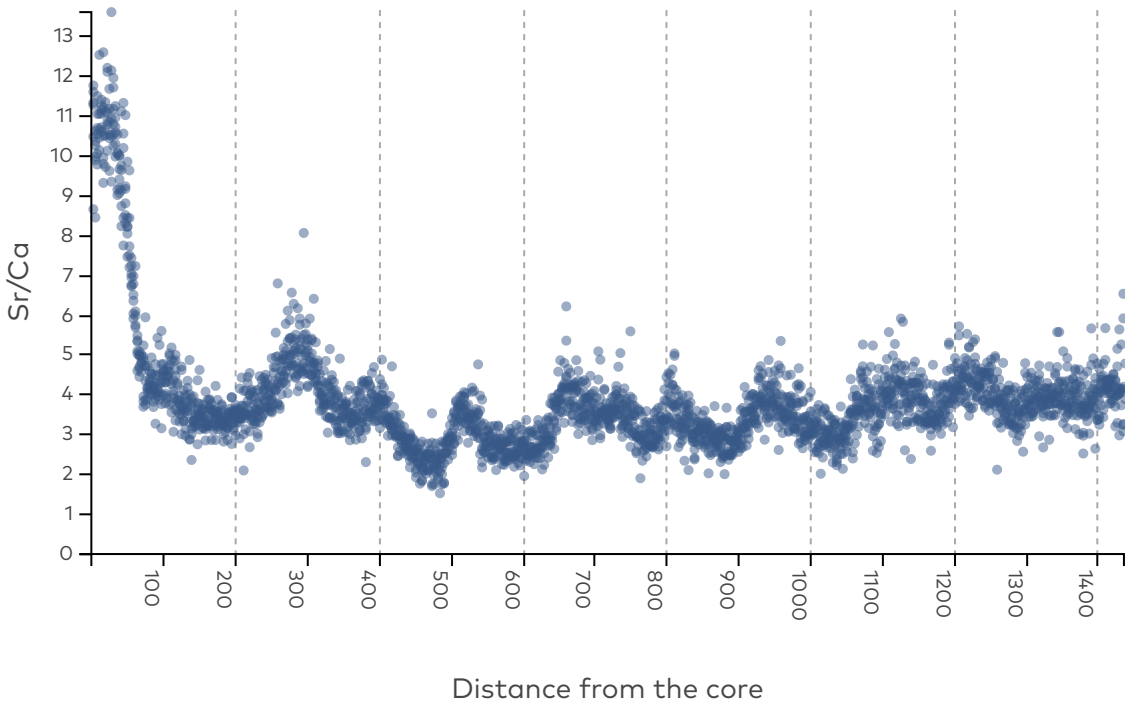


Figure 3. Examples of typical strontium/calcium profiles of eel otoliths showing three different life patterns with regards to salinity habitat. The initial peak corresponds to the larval stage. The blue dashed lines indicate habitat changes between saltwater and freshwater.

Methods

Study sites and eel sampling

Eels were collected in seven sites in Norway which represented three different salinity habitats (Figure 4): freshwater (FW; Arendal and Bergen), brackish water (BW; Arendal), and seawater (SW; Bergen and Haugesund). Individuals were caught using fyke nets and anesthetized on site. Eels were measured to determine their stage using body length, body mass, eye diameters and pectoral fin length (Durif et al. 2009). Blood samples were drawn on site either on the boat or on land close to the collections site (see Blood sampling protocol). Samples were kept on ice before they were transferred at -20°C in the laboratory. Eels were transported to the laboratory of the Institute of Marine Research (IMR, Norway) where they were euthanized by an overdose of anesthetic (MS-222), then immediately dissected for sex determination and otoliths. Otoliths were prepared at IMR, and microchemistry analyses of the otoliths were carried out by the University of Tartu (Estonia) (for details see Rohtla et al. 2022). Sampling and handling of eels in this study were approved by the Norwegian Animal Research Authority and all procedures followed local animal welfare regulations (FOTS id 15952).

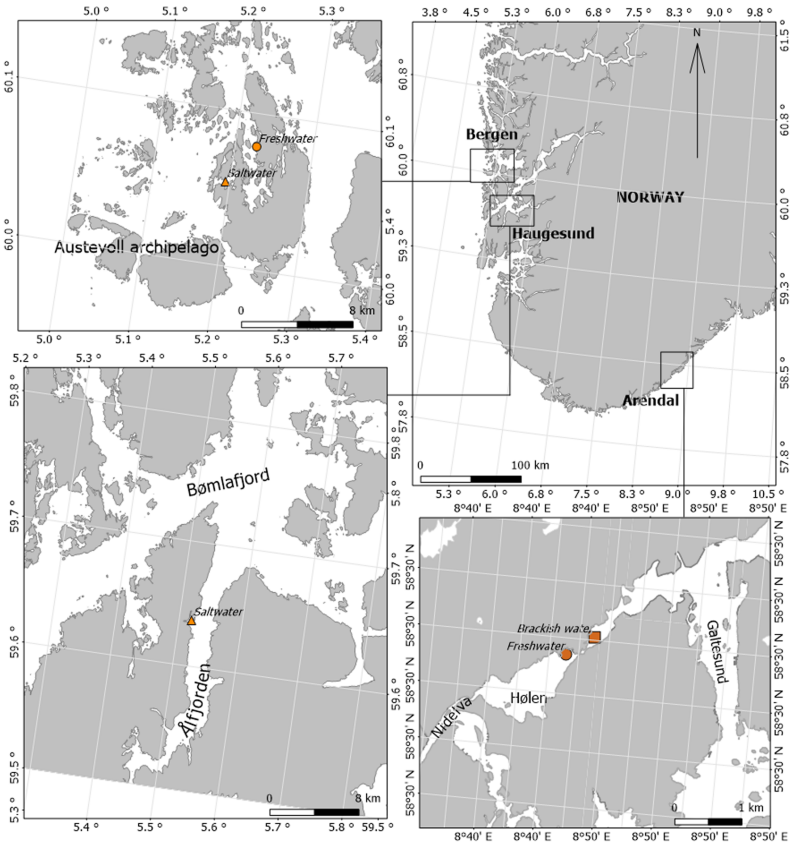


Figure 4: Locations where eels were caught and sampled in different salinity habitats.

Transcriptomic analyses

Blood samples were sent to partners at DTU aqua (Denmark) for RNA extraction (see RNA extraction). The mRNA sequencing (from 60 eels) was done from approximately 800 ng of extracted total RNA by the company Novogene. Briefly, messenger RNA (mRNA) was isolated using specific poliT probes, then the isolated RNA was converted in more stable complementary DNA, fragmented and at the end sequenced in parallel.

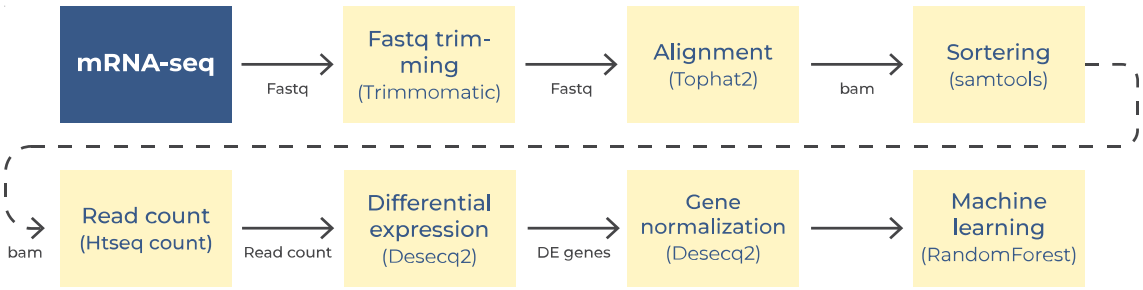


Figure 5. Overall transcriptomic analysis pipeline.

Read quality was assessed by Fastqc (Figure 5, Andrews, 2015). Reads were trimmed with Trimmomatic v0.38 (Bolger et al., 2014), removing the first 9 bases at the beginning of the reads, as well as part of reads that had lower quality and reads that were shorter than 36bp after trimming (HEADCROP:9, SLIDINGWINDOW: 4:15, MINLEN:36). Mapping of trimmed reads was performed with Tophat2 v0.13 (Kim et al., 2013) with default options using the latest version of the European eel reference genome and annotation fAngAng1.pri (NCBI; GCF_013347855.1) to guide the read mapping. A further filter was performed with Samtools v1.10 (Li & Durbin, 2009), removing reads that mapped in multiple places and sorting the reads by read name, as condition to run the reads count. For every sample, reads count at each annotated gene was performed with htseq-count (Anders et al., 2015).

Differential expression was done with the Deseq2 R package (Love et al., 2014) considering only freshwater residents and seawater resident eels derived from the otolith analyses. Here, genes with adj P values <0.05 were considered as significantly differentially expressed (DE) and used for machine learning Random Forest (RF) analyses. RF on the DE gene set was performed with the R package Randomforest (www.stat.berkeley.edu/~breiman/RandomForests/) over normalized gene expression data, obtained through a log-transformation of the whole gene set with Deseq2 (Love et al., 2014). Then, the log-transformed values of the DE genes were used for the RF analysis considering the whole sample set. Samples were classified according to the otolith microchemistry analyses into three groups: freshwater, seawater, and habitat shifters (Figure 3). Among the DE genes used for classification, sorting was done to their Mean Decrease in the Gini Index, retaining smaller number of genes.

Blood sampling protocol

Material:

- Anesthetic
- Syringes (3 mL) with 21-gauge needles
- Blood collection tubes (2 mL) with gel and lithium heparin anticoagulant, vacuum
- 2 mL Eppendorf tubes pre-filled with 1.2 mL RNAlater

Blood sampling:

1. Anesthetize the eel, until it loses balance and becomes unresponsive. Common anesthetics that can be used for eel are, for example, tricaine methane sulfonate (MS-222) and eugenol.
2. Place the eel on its side, and insert the syringe in the ventral midline, along the anal fin. Push the needle toward the vertebral column until it reaches the base (do not pierce the vertebral column). Gently pull it back less than 1 mm so it is not against the base and pull the plunger of the syringe to draw the blood.
3. Empty the blood into a vacuum tube (lithium heparin anticoagulant) and mix through inversion
4. Transfer 0.6 mL of the collected blood in a microcentrifuge tube (sterile, 2 ml) and shake well
5. Place the samples on dry ice or similar (to avoid any sedimentation) and then at -20C. This can be done at the end of the sampling (at room temperature), only ensure there is no sedimentation when they are placed on dry ice.
6. Transfer 0.6 mL of blood in a 2 mL tube that is prefilled with 1.2 mL of RNA-later.

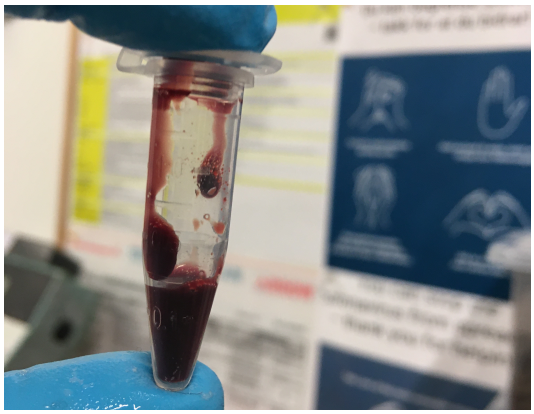


Figure 6. Blood sampling of eel.

RNA extraction

1. Place 200 µL blood in RNA-later in a clean 1.5 ml tube
2. Centrifuge 1,5 min 10.000 rpm at 4 °C and discard the supernatant
3. Add 600 µL of TRIzol™ Reagent, mix and incubate for 5 min at room temperature
4. Add 120 µL of Chloroform, mix and incubate for 2–3 min
5. Centrifuge 15 min 12.000 rpm at 4 °C and retain the aqueous upper phase and transfer it in a new 1.5ml tube
6. Add 300 µL of Isopropanol to re retained aqueous phase turn the tube few times and incubate for 10 min
7. Centrifuge 10 min 12.000 rpm, 4 °C and discard the supernatant
8. Add 600 µL of 75% Ethanol and resuspend
9. Centrifuge 5 min 7500rpm, 4 °C
10. Repeat point 8 and centrifuge 10 min 16.000 rpm, 4 °C
11. Air dry the RNA pellet for 10–15 min
12. Resuspend in 20 µL of RNAse free water
13. Incubate at 57°C for 10 min

Results

Among the 60 eels included in this study, 27 individuals were captured in saltwater, 7 in brackish water and 26 in freshwater. Otolith analysis classified 23 eels as seawater resident (SWR), 25 as habitat shifters (HS), and 11 of these eels as freshwater resident (FWR), 1 sample was unclear (SWR/HS).

The DE analyses retrieved 3,451 genes, that correctly classified 79% of the eel into their salinity habitat. The subset of genes further selected considering Mean Decrease in the Gini Index improved the overall results, with a maximum value of rate of 93%. Only four eels in the 50 and 30 genes panels were misclassified (CCP HS= 0.84). These four eels were classified as habitat shifters by otolith analyses, but as seawater resident by their transcriptome, reflecting the recent salinity habitat, (i.e., capture location in seawater). Among the top 30 genes, 10 genes (mctp2a, inppl1b, asap1b, itk, adra1ba, rerea, tead1b, nelfb, acin1a and rev1) are included in one or more biological processes mainly related to general metabolic and regulation processes, or developmental processes, hematopoiesis and immune system.

Table 1. OOB error rate (%) and correct prediction proportion (CPP) of the different gene sets: all differentially expressed (DE), and top 150, 100, 50 and 30 bases on Mean Decrease in the Gini index. CCP =(1-classification error). FWR: Freshwater resident, SWR: Seawater resident, HS: Habitat shifter.

		Mean Decrease in the Gini			
	All DE	**150**	**100**	**50**	**30**
OOB error rate (%)	18.97	8.62	8.62	6.9	6.9
CCP FWR	0.80	1.00	1.00	1.00	1.00
CCP SWR	0.96	1.00	1.00	1.00	1.00
CCP HS	0.67	0.80	0.80	0.84	0.84

Conclusion and perspectives

Blood transcriptional profile is influenced by salinity habitat history. The combination of machine learning and transcriptomic profiling allowed the assessment of salinity-habitat history with high accuracy (up to 93%), including habitat shifting behavior. The ability of transcriptomic-based biomarkers to distinguish with a relatively high level of accuracy (75%) eels with habitat-shifting history from residents (fresh- or seawater) regardless of the salinity at the sampling site suggests that salinity habitat history leaves a fingerprint in blood transcriptomics. This could hypothetically be through e.g., epigenetic mechanisms, however further studies would be needed to decipher this aspect.

Only four eel samples (out of 60) were misclassified. The misclassification of these four samples was detected in all gene panels. These eels corresponded to habitat shifters as determined by otolith microchemistry. Specifically, otolith analysis detected that those misclassified samples had had early FW experience. This indicates that transcriptomics analysis fails to show very early FW experience. For complete reconstructions of habitat shifter chronological salinity history, otolith microchemistry seems inevitable. Otolith analysis can further characterize habitat shifting, by estimating when and how many times, shifts between FW and SW habitats have occurred (Rohtla et al., 2022). However, in a management context, only the recent habitat history (1–2 years) is relevant. Collecting blood and determining salinity-habitat history during annual monitoring surveys would provide important information improving management of this species. Knowledge on the proportion of habitat shifters is needed to improve seasonal migratory conditions and habitat availability.

Collecting blood and preserving the blood samples was possible under field conditions – in a fishing boat or on land near the capture site. Once in RNA later, samples can be kept at room temperature for a few hours. Such sampling could therefore be carried out during eel monitoring surveys to determine the proportions of the different life patterns with regards to salinity. Overall knowledge on eel habitat use, such as migratory season and proportion of individuals that shift habitat, is needed for eel population assessment.

Practically, an adequate increase and maintenance of the training population (for the random forest analysis), using otolith microchemistry analysis as known phenotype, would be needed to greatly reduce, and in the future substitute, otolith microchemistry analysis for general estimation of fish salinity history. This approach is promising for the replacement or reduction of other lethal analyses in fish research, especially for critically endangered species, such as the European eel. In this study, we only tested whether blood transcriptomics could detect salinity habitat, but they may also detect other physiological parameters related to the health status and overall quality of the fish. This is an important aspect of eel monitoring to consider for the recovery of the species (ICES 2012).

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Caroline Durif, Francesca Bertolini, Amaya Zaratiegui Pedrosa, Mehis Rohtla, Jonna Tomkiewicz

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Nordic Council of Ministers/Publication Unit
Ved Stranden 18
DK-1061 Copenhagen
Denmark
pub@norden.org

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