# Differences on the level of hepatic transcriptome between two flatfish species in response to liver cancer and environmental pollution levels

Tuul Sepp, Ciara Baines, Randel Kreitsberg, Jörn Peter Scharsack, Pedro Nogueira, Thomas Lang, Jérôme Fort, Elin Sild, John Clarke, Arvo Tuvikene, et al.

Comparative Biochemistry and Physiology, Part C 275 (2024) 109781

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

# Differences on the level of hepatic transcriptome between two flatfish species in response to liver cancer and environmental pollution levels

Tuul Sepp [a,*], Ciara Baines [a,b], Randel Kreitsberg [a], Jörn Peter Scharsack [c], Pedro Nogueira [c], Thomas Lang [c], Jérôme Fort [d], Elin Sild [a], John T. Clarke [a,e,f,g], Arvo Tuvikene [h], Richard Meitern [a]

[a] Institute of Ecology and Earth Sciences, University of Tartu, Liivi 2, 50409 Tartu, Estonia
[b] Estonian Marine Institute, University of Tartu, Mäealuse 14, 12618 Tallinn, Harju County, Estonia
[c] Thünen Institute of Fisheries Ecology, Herwigstraße 31, 27572 Bremerhaven, Germany
[d] Littoral, Environnement et Sociétés (LIENSs), UMR7266 CNRS - La Rochelle University, 2 rue Olympe de Gouges, 17000 La Rochelle, France
[e] GeoBio-Center, Ludwig-Maximilians-Universität München, Richard-Wagner-Str. 10, 80333 Munich, Germany
[f] Department of Earth and Environmental Sciences, Paleontology & Geobiology, Ludwig Maximilians-Universität München, Richard-Wagner-Str. 10, 80333 Munich, Germany
[g] Department of Ecology and Biogeography, Nicolaus Copernicus University in Toruń, Lwowska 1, 87-100 Toruń, Poland
[h] Estonian University of Life Sciences, Friedrich Reinhold Kreutzwaldi 1a, 51014 Tartu, Estonia

ARTICLE INFO

Edited by Martin Grosell

*Keywords:*
Cancer defence mechanisms
cancer evolution
Fish cancer
Liver cancer
Pollution-induced cancer
Transcriptome
Wildlife cancer genetics

ABSTRACT

Environmental factors can cause cancer in both wild animals and humans. In ecological settings, genetic variation and natural selection can sometimes produce resilience to the negative impacts of environmental change. An increase in oncogenic substances in natural habitats has therefore, unintentionally, created opportunities for using polluted habitats to study cancer defence mechanisms. The Baltic and North Sea are among the most contaminated marine areas, with a long history of pollution. Two flatfish species (flounder, *Platichthys flesus* and dab, *Limanda limanda*) are used as ecotoxicological indicator species due to pollution-induced liver cancer. Cancer is more prevalent in dab, suggesting species-specific differences in vulnerability and/or defence mechanisms. We conducted gene expression analyses for 30 flatfishes. We characterize between- and within-species patterns in potential cancer-related mechanisms. By comparing cancerous and healthy fishes, and non-cancerous fishes from clean and polluted sites, we suggest also genes and related physiological mechanisms that could contribute to a higher resistance to pollution-induced cancer in flounders. We discovered changes in transcriptome related to elevated pollutant metabolism, alongside greater tumour suppression mechanisms in the liver tissue of flounders compared to dabs. This suggests either hormetic upregulation of tumour suppression or a stronger natural selection pressure for higher cancer resistance for flounders in polluted environment. Based on gene expression patterns seen in cancerous and healthy fish, for liver cancer to develop in flounders, genetic defence mechanisms need to be suppressed, while in dabs, analogous process is weak or absent. We conclude that wild species could offer novel insights and ideas for understanding the nature and evolution of natural cancer defence mechanisms.

## 1. Introduction

A key consideration in the global effort against cancer is the influence of anthropogenic environmental change, including that of environmental contamination. While we know that contaminants can strongly affect cancer occurrence in humans, environmental factors are often difficult to study in the laboratory ("The global challenge of cancer", 2020). Using wild model organisms to understand the link between increased habitat pollution levels and cancer is, currently, an underexplored avenue of research that shows great promise due to the similarity of oncogenic processes across species (Nesse, 2017; Sepp and Giraudeau, 2022). Knowledge about defence mechanisms against cancer

* Corresponding author.
*E-mail address:* tuul.sepp@ut.ee (T. Sepp).
@TuulSeppLab (T. Sepp)

https://doi.org/10.1016/j.cbpc.2023.109781
Received 2 August 2023; Received in revised form 20 September 2023; Accepted 23 October 2023
Available online 3 November 2023

development is limited and is mostly based on the study of model organisms with low genetic diversity in laboratory environments. Recent comparative genomic studies have described the differences between genomic cancer defences in mammals (e.g. Tollis et al., 2020; Yu et al., 2021), but also in a wider range of vertebrates (Nair et al., 2022), including fishes (Baines et al., 2022).

In the context of the evolution of cancer defences, oncogenic pollution is currently an underexplored factor. Ecotoxicological studies have connected oncogenic pollutants with increased prevalence of wildlife cancer (McAloose and Newton, 2009; Vittecoq et al., 2018). While numerous ecotoxicological studies analyse the link between pollutants and cancer incidence in aquatic species (reviewed in Baines et al., 2021), studies that focus on the possibility of differing vulnerabilities to (or adaptations against) oncogenic processes arising in wild populations living in polluted environments are scarce. So far, only Di Giulio and Clark (2015) present, in their review, the notorious case of the Elisabeth River (USA), where there is evidence of adaptation against the effects of polycyclic aromatic hydrocarbons (PAHs), including liver neoplasms, possibly through changes in several different pathways and mechanisms.

Two of the marine areas with the longest history of high levels of pollution are the Baltic and North Seas, which are among the most polluted marine areas worldwide (e.g. heavy metals, PAHs, polychlorinated biphenyls (PCBs), tributyltin (TBT), HELCOM, 2018). The long time period of high selective pressure by contamination has created opportunities for natural selection to act, potentially selecting for genotypes that are better protected against oncogenic pollution, or producing novel defence mechanisms through evolutionary processes. Two flatfish species, flounder (*Platichthys flesus, Linnaeus, 1758*) and dab (*Limanda limanda, Linnaeus, 1758*) inhabit a gradient of relatively clean to severely polluted habitats in the Baltic and North Seas. These two species diverged from one another approximately 10.9 million years ago (details of this calculation are given in Supplementary materials). As benthic organisms, they have been exposed to high levels of anthropogenic contamination (that accumulates in the sediments) since the beginning of industrialization in the 19th century (HELCOM, 2018). With a generation time of approximately 2–3 years, flatfishes have lived in close contact with oncogenic pollution here for 50 generations or more, and are considered marine sentinel species. European flounder as well as dab have separately spawning sub-populations in the Baltic Sea (Berkström et al., 2022; Nissling et al., 2017), such as the Bornholm Basin, the Gdansk Deep, the Arcona Sea and Belt Sea. Analysis of tagging experiments has indicated several local populations with limited migration patterns. For example, based on active migrations the maximum home range of flounder is described to be in between 50 and 200 km (Berkström et al., 2022) and a similar scale for dab during described within-basin migrations (Rijnsdorp et al., 1992). Reduced migration may also be the reason for the existence of genetic structure at a regional level in dab as discussed by Tysklind et al. (2013). Accordingly, flatfish populations here are separated enough to develop local adaptations (Nissling et al., 2017; Larsen et al., 2008). There are several local populations of both species that inhabit a gradient of environmental contamination. Cancerous lesions (liver, skin) are common in both species (Vethaak et al., 2009). The two species seem to differ in their vulnerability to pollution-induced cancer, with dab generally showing higher overall cancer prevalence than flounder (Table 1). This provides us with a model that allows investigation into the evolution of defence mechanisms. Additionally, flounders living in highly polluted sites do not seem to have higher cancer prevalence compared to their conspecifics in cleaner sites (Vethaak et al., 1996; de Boer et al., 2001), while in dabs, cancer prevalence does vary relative to local pollution levels (Lerebours et al., 2014). It would seem that either the flounder is less susceptible to cancer development compared to the dab, or is more likely to develop higher levels of cancer defences in populations living in polluted environments by natural selection.

As a first study to look into the possibility of evolved cancer defences in flatfish, we have conducted a study of gene expression levels in 14 flounders and 16 dabs. These fishes represent a subsample of flounders and dabs caught from two reference sites and four polluted sites (90 fishes in total, 40 flounders, 50 dabs, Table 2). Histopathological analysis was conducted from all fishes to confirm if individuals had liver cancer or not, and subsequently, representatives from both species with or without cancer were chosen for transcriptome analysis. Gene expression analyses provide a new avenue to study the molecular basis of adaptation, as variation in gene expression has large functional consequences and is considered a key component of environmental adaptation in natural populations (Babu and Aravind, 2006). This approach allowed us to start characterizing cancer defences in the two species, describing within- and between population patterns. Based on these patterns, we set out to test the following hypothesis, being however aware of the limitations for this raising from our small sample size: (1) gene expression related to cancer vulnerability and defences differs between two bottom-dwelling flatfish species with known differences in liver cancer risk, and (2) environments contaminated with oncogenic pollutants select for stronger cancer defence mechanisms, observable on the level of gene expression.

**Table 1**
Studies reporting liver cancer prevalence (benign and malignant neoplasms) in flounders and dabs. In addition, large-scale (about 60.000 individuals) monitoring data presented by Vethaak et al. (2009) indicated cancer prevalence in flounders to be 0.5–1.5 %, and in dabs, 0.8 %, but in this study, histopathological analyses were only conducted from fish with visible liver nodules, likely therefore underestimating cancer prevalence.

| Species | Liver neoplasm (%) | Sample size | Source |
|---|---|---|---|
| Flounder | 1.5 | 338 | Cachot et al., 2013 |
| Flounder | 0.7 | 436 | Lang et al., 2006 |
| Flounder | 1.49 | 201 | Stentiford et al., 2003 |
| Flounder | 0.9 | 443 | Vethaak et al., 1996 |
| Dab | 2 or 10[a] | 31 | Lyons et al., 2006 |
| Dab | 8.56 | 50 | Stentiford et al., 2009 |
| Dab | ~10 | 600 | CEFAS report, 2004 |

[a] Depending on location.

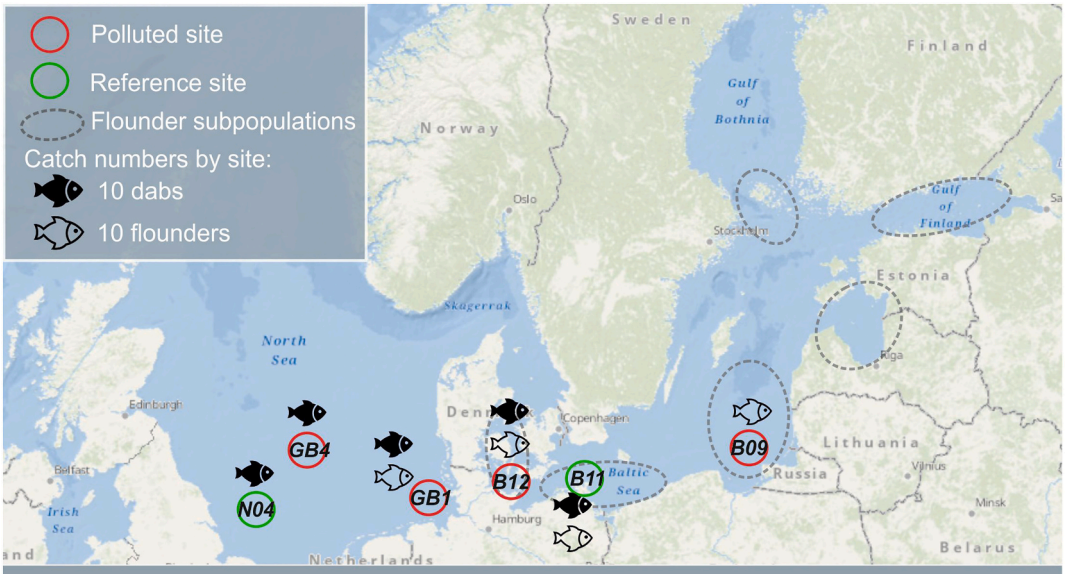
## 2. Materials and methods

### 2.1. Sample collection and processing

Fish were collected using bottom trawling during a research cruise with RV Walther Herwig III (WHIII-429) in August–September 2019. Sampling comprised of three areas in the North Sea and three areas in the Baltic Sea (Fig. 1, Table 3). Study areas were chosen on the basis of differences in anthropogenic pressure and divided into more polluted/affected (marked as 'polluted') and less affected areas (marked as 'reference'). Key factors and pressures described include land-based inputs, long-range air transport, and sea-based activities (OSPAR,

**Table 2**
Description of the whole sample, and the subsample of fish chosen for transcriptome. Site categorization data is presented in Table S1.

| Site | Site status | Total N flounders/N with cancer | Total N dabs/N with cancer | Chosen for transcriptome |
|---|---|---|---|---|
| B09 | Polluted | 10/0 | – | 1 healthy flounder |
| B11 | Reference | 10/2 | 10/2 | 5 flounders (1 cancer, 4 healthy) 7 dabs (2 cancer, 5 healthy) |
| B12 | Polluted | 10/0 | 10/1 | 2 flounders (healthy), 6 dab (1 cancer, 5 healthy) |
| GB1 | Polluted | 10/1 | 10/0 | 6 flounder (5 healthy, 1 cancer) |
| GB4 | Polluted | – | 10/2 | 1 dab with cancer |
| N04 | Reference | – | 10/3 | 1 dab with cancer |




| Chosen for transcriptome analysis: | | | | | | | |
|---|---|---|---|---|---|---|---|
| Flounder (*Platichthys flesus*) | | | | Dab (*Limanda limanda*) | | | |
| *Polluted site* | | *Reference site* | | *Polluted site* | | *Reference site* | |
| Healthy 8 | Cancer 1 | Healthy 4 | Cancer 1 | Healthy 5 | Cancer 2 | Healthy 5 | Cancer 4 |

**Fig. 1.** Map of sites, catch size, and subsamples chosen for transcriptome analysis. Sites are further characterized in Table 3 in the Methods section. Dashed ovals indicate spawning areas of flounders in the Baltic Sea, representing separate populations (Nissling et al., 2017), no comparable data was available for dab. Base map from Esri.

2010). Such distribution was based on public data of monitoring programs and previous studies of the areas (Table S1). A total of 90 fishes in total were sampled including 40 flounders and 50 dabs, (Table 2). Average trawling speed was 3,5 km for 60 min, after which fish were quickly transferred to a large flow-through seawater tanks and sampled within 1 h. Fish sampling and handling was performed by experienced and licensed researchers, and all procedures were in accordance with institutional, national, and European legislation.

Fishes were killed by a blow on the head, and liver and otoliths were collected. Livers were assessed for external lesions, and then an approximately 3 mm slice of liver was cut and stored in 4 % formalin for 24 h before being transferred to 70 % ethanol. Another piece of liver was stored in RNA later in 1 to 5 ratio (sample/buffer), snap frozen in liquid nitrogen and stored at −80 °C until further analysis. Otoliths were collected, air-dried for 24 h, and preserved at −20 °C for age determination. An overview of the age and size of the specimen chosen for transcriptomics (14 flounders, 16 dabs) is given in Table S2 (Supplementary materials). Groups (cancer vs no cancer) did not differ in average age for dabs (mean cancer 3.2 years, mean no cancer 3.9 years, *t*-test $p = 0.20$), and flounders (mean cancer 6.5, mean no cancer 5.2, t-test $p = 0.24$).

For histopathology, liver samples were processed according to Feist et al. (2004). Samples stored in ethanol were further dehydrated and then embedded in paraffin wax, sliced to 4 μm and mounted onto glass slides. These sections were stained with haematoxylin and eosin (H&E), dehydrated, cleared and mounted for analysis of microscopic lesions using a microscope. Tumours were diagnosed as either cancerous (e.g. adenoma or carcinoma according to diagnostic methods described by Feist et al. (2004)).

### 2.2. Transcriptomics

14 flounders and 16 dabs were chosen for transcriptome analysis. These fishes represent a subsample of flounders and dabs with and without cancer and were caught from two reference sites and four polluted sites. We performed a whole transcriptome sequencing (RNA-Seq) to acquire gene expression data. Total RNA was extracted from liver samples stored in RNAlater using the RNeasy mini kit from Quiagen (cat. 47104). Briefly, samples were cut into sections between 20 and 30 mg. The tissue was disrupted using a pestle and mortar and homogenised by adding both 600 μL RLT buffer with β-mercaptoethanol (10 μL β-mercaptoethanol added to 1 mL RLT buffer) and 0.5 mm glass beads to the sample tube and homogenised in Bullet Blender 24 (Next Advance Inc., USA) on speed 4 for 2 min. The samples were then processed according to the RNeasy Mini Kit protocol including optional steps for DNase digestion. Determination of the quality and quantity of RNA was undertaken using TapeStation (Agilent). Samples with an RNA integrity number (RIN) value of 7.3 and above were chosen for transcriptomic analysis. Extraction of mRNA and generation of cDNA was undertaken using IlluminaTruSeq Stranded mRNA Library Prep Kit. Paired end 80 bp sequencing was performed on an Illumina NextSeq500 sequencer (Sequencing kit: NextSeq HIGH150, Flowcell version: NextSeq HIGH) at the Institute of Genomics at the University of Tartu. The initial quality of the reads was then assessed using FastQC.

Transcriptome sequencing and analysis were performed as in Meitern et al. (2020). Briefly, the sequencing resulted in 1013M PE raw reads that were cleaned and trimmed using Trimmomatic 0.38. After quality control de novo transcriptome assembly was performed with Trinity 2.8.4. Downstream analyses for aligning reads for assembly were performed with scripts within Trinity using Salmon. For flounder transcriptome assembly we added liver transcriptome data from

**Table 3**
Sampling locations and description in six areas of the North and the Baltic Seas. Detailed data and references added in supplementary materials, Table S1.

| Code | Site name | Coordinates | Category | Description |
|---|---|---|---|---|
| B09 | Gulf of Gdańsk | 55°06,93 N 018°10,90E | Polluted | Inflow from the Vistula estuary. Effect of industry. |
| B11 | Arcona Sea | 54°45,39 N 013°11,91E | Reference | Wind parks and marine protected areas. |
| B12 | Kiel Area | 54°14,87 N 011°44,34E | Polluted | Heavy marine traffic. TBT-specific effects are still found in maritime areas even after global 2008 ban. Therefore harbors can have a noticeable impact, highlighting the importance of local sources and historic contamination of harbor sediments. |
| GB1 | German Bight South | 54°04,54 N 007°53,71E | Polluted | Area of extensive maritime activities. Inflow from the rivers Elbe and Weser. Heavy metal concentrations in sediments are at levels that pose a risk of pollution effects for marine life in the southern North Sea. |
| GB4 | German Bight North | 55°23,29 N 004°32,44E | Polluted | Area of extensive maritime activities. |
| N04 | Dogger Bank | 54°46,26 N 002°02,23E | Reference | Swallow sandbank. Feeding and spawning area for fish. Partly protected area. Although cadmium and mercury concentrations in fish and shellfish were rising in early 2000s. |

Pomianowski et al. (2021) to increase assembly quality. We ran the analyses for flounder also without this additional data to control if the differences with dab might have arisen from assembly quality. Both flounder assembly versions (only our data vs our data + data from Pomianowski et al., 2021) gave quantitatively (but not qualitatively) similar results (described in Supplementary Materials, part 2). Differential expression analysis between groups was conducted using both edgeR and DESeq2. EdgeR method and DESeq2 method perform the differential expression analysis slightly differently, with edgeR giving more conservative results. In both approaches, *p*-values were adjusted for multiple testing with the Benjamini-Hochberg procedure. To annotate the obtained transcriptome, we used Dammit using orthologous genes database (OrthoDB) version 10.1. Human orthologues for each transcript were retrieved through OrthoDB using the best match cluster ID. We matched against human gene database, as curated cancer gene lists exist for humans but not for other species. However, as cancer genes are among the oldest gene classes in vertebrates (Makashov et al., 2019), using human gene databases for cancer gene annotation is a reasonable proxy. In addition to OrthoDB we utilized Trinotate for the assignment of Gene Ontology (GO) terms and best SwissProt gene matches. Trinotate is a comprehensive functional annotation suite specifically designed for automatic functional annotation of transcriptomes, particularly de novo assembled transcriptomes, from model or non-model organisms (Bryant et al., 2017). Trinotate leverages a number of different well-known bioinformatics tools and databases to assign various functional annotations to transcripts. These include homology search to known sequence data (BLAST+/SwissProt), protein domain identification (HMMER/PFAM), protein signal peptide and transmembrane domain prediction (signalP/tmHMM), and leveraging various annotation databases (eggNOG/GO/Kegg databases) to provide functional annotation information. We followed the standard Trinotate protocol. After obtaining a set of assembled transcripts, we first identified the likely coding regions within these transcripts using TransDecoder. The resulting protein sequences were then subjected to homology search against the SwissProt database. The top matching entries were retrieved. The GO terms were assigned based on the homology search results. Trinotate uses both Pfam and BLAST databases for this purpose, which allows for a more comprehensive functional assignment compared to other tools that use only one database (Das and Mykles, 2016). For the GO category analysis, we used the differentially expressed genes obtained from DeSeq2 instead of EdgeR. This allowed compiling larger number of genes into a reasonable number of categories for interpretation. For most genes a set of GO categories was retrieved from annotation so we manually selected for the categories that allowed maximal grouping of the transcripts for simplifying interpretation. This approach makes also sense biologically, as it is expected that biological processes active in sampled fish involve the products of several genes. However, we acknowledge also the subjective nature of such approach. In addition, this approach does not take into account the possible bias of having more GO categories on shorter transcripts. The full list of GO categories linked to the transcripts is shown in Supplementary data. The transcripts were categorized either according to GO biological processes (if available) or GO molecular functions (Fig. 4, The Gene Ontology Consortium, 2021). The final tables and graphs were prepared in R version 4.1.3 (R Core Team, 2022). KEGG plots were produced using Pathview. Heatmaps were generated from log transformed individual expression levels. For heatmaps, we used the edgeR differential expression analysis, which is more conservative compared to the DeSeq2 method. Having less transcripts simplifies the visualization. The gene symbols for heatmaps are the SwisProt best matches (if available) or shortened transcript names corresponding to the respective field is supplementary tables. Other R packages used included several packages from the tidyverse and their dependencies. The raw sequencing data along with the assembled transcriptome is openly available in EMBL-EBI European Nucleotide Archive under the primary study accession number PRJEB53201.

In order to generate specific hypotheses on the molecular pathways linked with cancer and modulated in cancerous liver tissue, or as compensatory adaptation to oncogenic pollution, we additionally used pathways defined in the KEGG database (Kyoto Encyclopedia of Genes and Genomes, http://www.kegg.jp/) (note that the analyses mentioned in previous paragraphs were all conducted on the whole transcriptome). Since no such pathway mapping yet exists for fish, but the main genes represented in these pathways belong to a phylogenetically old gene family (CYP genes, Nelson et al., 2013), we used best human gene match from OrthoDB. Selected pathways related to hepatocellular carcinoma (hsa05225), chemical carcinogenesis (receptor activation, hsa05207, DNA adducts, hsa05204, and reactive oxygen species, hsa05208), and pancreatic cancer (hsa05212) in humans. To perform pathway enrichment, we checked if our annotation of the assembled transcriptome enabled us to identify a human orthologue for each gene in the pathway. The pathway maps presented in Supplementary Figs. S1–S3 display the human genes for which we could identify an ortholog as coloured and those without a human ortholog match as white.

## 3. Results

### 3.1. Cancer prevalence

We compared the gene expression of cancerous and non-cancerous flounders and dabs (based on liver histopathology), and the gene expression of non-cancerous flounders and dabs living in polluted vs reference areas. Sites were categorized based on long-term flatfish health monitoring data by the Thünen Institut (unpublished data), habitat disturbance levels, and environmental pollutant data from OSPAR (OSPAR Data and Information Management System, https://odims.ospar.org/, Tables 5 and S1, see methods and Supplementary materials for more details). For reference site B11, OSPAR sediment pollution data was not available, but longitudinal health monitoring data from Thünen Institut (spanning from 2015 to 2022) indicates lower

pollutant content and better health of fish from this site (Rügen island wind park area) compared to nearby (more anthropogenically disturbed) areas. Our reference areas comprised mainly protected marine areas, while the polluted habitats experienced heavy marine traffic or inflow from industrial areas. This study design allowed us to suggest mechanisms that drive the higher vulnerability to pollution-induced cancer in dabs compared to flounders, and describe the genes and physiological pathways that help flatfishes protect themselves against environmentally induced liver cancer.

Histopathology analysis confirmed cancer in 3 out of 40 flounders (1 from polluted and 2 from reference sites, total prevalence 7.5 %) and in 8 out of 50 dabs (3 from polluted and 5 from reference sites, total prevalence 16 %, Table 2). Cancer in this case is defined as neoplastic changes and was diagnosed as either hepatocellular adenoma and/or hepatocellular carcinoma. Preneoplastic (e.g. foci of cellular alteration) and other histopathological changes were not included in the analysis as they were beyond the scope of this study.

### 3.2. *Characterizing patterns of cancer-related gene expression*

We created transcriptome heat maps to visualize the expression values for the returned genes of interest, specifically, transcripts that were differently expressed between groups (cancerous vs healthy or polluted vs reference). We present here the heat maps characterizing individual gene expression patterns in cancerous and healthy flounders and dabs (Fig. 2) and flounders and dabs from polluted and reference

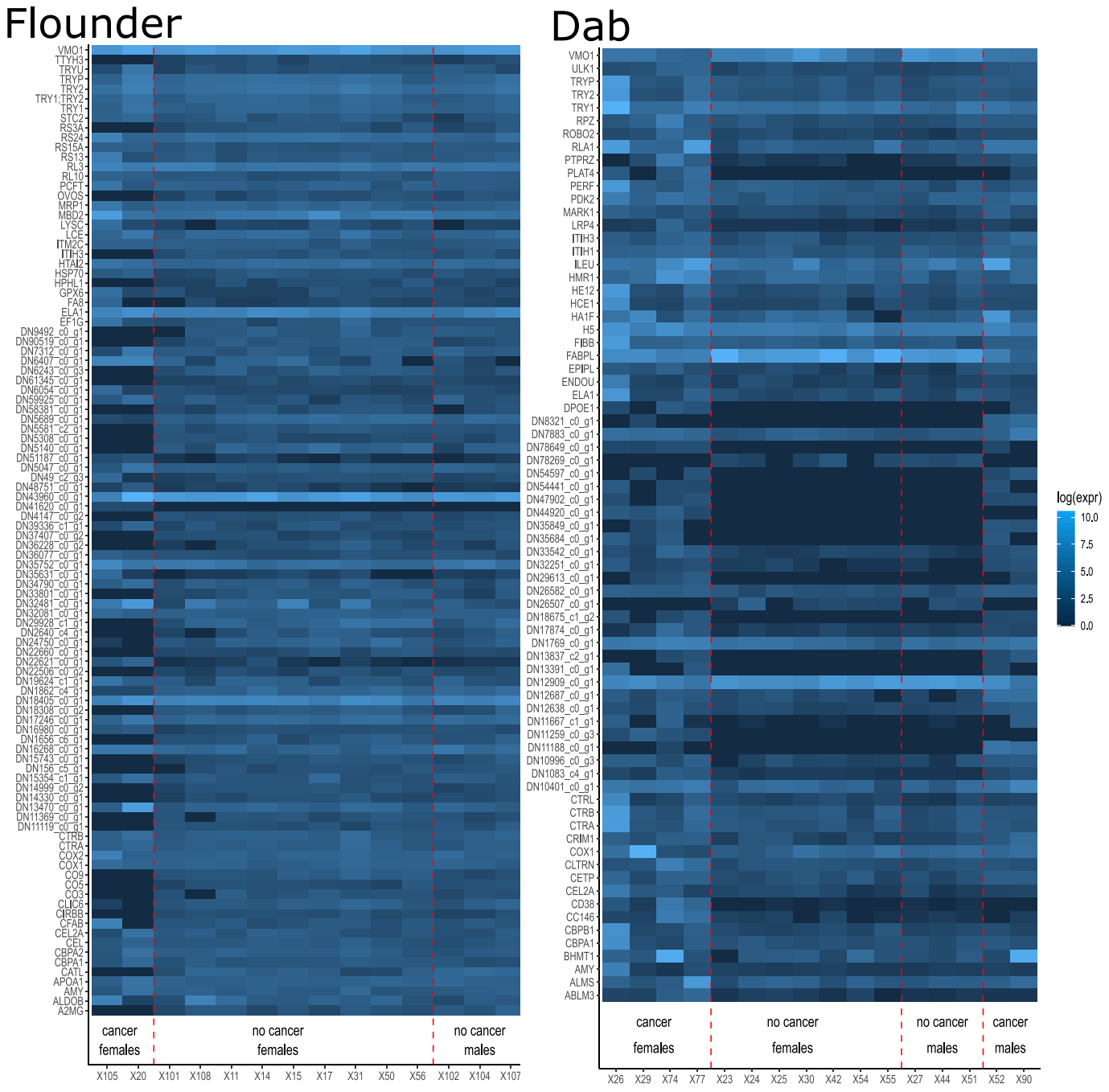



**Fig. 2.** Heat maps describing the $\log_e$ transformed expression levels of transcripts with significantly different expression levels between cancerous and healthy individuals. Fish IDs are shown on x-axis. On Y axis, transcripts for which a protein homologue was available in Swissprot are marked with corresponding protein name abbreviation, the rest of the transcripts are marked with transcript ID (see also Supplementary data table).

sites (Fig. 3), indicating also the sex of the individuals. When available, transcripts on heatmaps were linked with corresponding protein names (based on Swissprot homology search). If no match was available, transcript ID was used. Heatmaps suggest a downregulation of a number of transcripts (both with available protein homologue and unknown) in cancerous flounders. This pattern is less clear in dabs. Rather, a list of unknown transcripts seems to be upregulated in cancerous dabs, both males and females. From the annotated transcripts, fatty acid binding protein 1 (FABP1), responsible for lipoprotein-mediated cholesterol uptake in hepatocytes, shows highest expression levels in dabs, possibly with a, lower expression level in cancerous individuals. In flounders, the two transcripts showing highest expression levels are not annotated, however, the expression levels are similar between cancerous and healthy individuals. The comparison of polluted and reference sites indicates lower expression levels of four unknown transcripts in reference sites, and six unknown transcripts with higher expression levels in reference sites for flounders. For transcripts that could be associated with a protein homologue, aldolase B (ALDOB, active in carbohydrate metabolism in the liver) and DNA damage-regulated autophagy modulator (DRAM2) show increased expression levels in polluted sites. In dabs, three unknown transcripts indicate lower expression levels in reference sites. From transcripts with protein link, the clearest pattern seems to be higher expression of ITIH3 (inter-alpha-trypsin inhibitor heavy chain 3), a protein possibly linked with carrying hyaluronan in extracellular matrix.

### 3.3. Gene expression in cancerous vs non-cancerous individuals

We compared gene expression between fishes with and without cancer. In our analysis, we did not target the cancer tissue for RNA extraction, but we cannot exclude that some of the cancerous tissue was included in the liver samples used for transcriptome analysis. In our subsample for transcriptomics, 2 out of 14 flounders and 6 out of 16 dabs had cancer. Of the 25,378 gene ID's for flounder, the less conservative DESeq2 method recovered 445 gene ID's that were significantly differently expressed between fish with and without cancer ($p$-value adjusted $<0.05$), while the more conservative EdgeR method recovered 123 significantly differently expressed genes (FDR $<0.05$). For dabs, of the 23,311 gene IDs, 68 were significantly differently expressed between cancerous and non-cancerous fish using the DESeq2 method (p-value adjusted $<0.05$), whereas using the EdgeR approach, there were 42 significant results (FDR $<0.05$) (differentially expressed transcripts listed in Supplementary data file). We categorized the annotated transcripts according to GO biological processes (if available) or GO molecular functions (Fig. 4, The Gene Ontology Consortium, 2021). We ran similar analysis with only females included to control for the potential confounding effect of sex (Fig. S4), ending up with qualitatively similar results. In dabs, we mostly observed the upregulation of genes in cancerous fish, but in flounders, we could also see downregulation of different categories of genes in cancerous fish, although upregulation was still more common.

### 3.4. Gene expression of healthy fishes from polluted and reference sites

We compared non-cancerous fishes from polluted and reference sites to find possible signs of local adaptations in defence mechanisms against oncogenic pollution. For these results, it is important to keep in mind that the sites categorized as “polluted” or “reference” might also differ in some other environmental factors besides anthropogenic pollutant pressure, however, in a study in ecological settings, these potential factors cannot be controlled for. Using the DESeq2 approach, 43 of transcripts were significantly differently expressed between polluted and reference sites in flounders (adjusted $p$-value $<0.05$), while using the EdgeR method, only 13 of these transcripts were significantly different between reference and polluted sites (FDR $<0.05$). In order to get the best possible matches for proteins, Table 3 shows the 12 gene ID's that were significantly different with both methods.

In dabs, the DESeq2 approach found 67 gene ID's that were significantly different between polluted and reference sites (p-value adjusted $<0.05$), while the EdgeR approach recovered only 12 gene ID's that were significantly different between polluted and reference sites (FDR $<0.05$). Table 4 shows the 11 gene ID's that were significantly different in both methods in dabs.

Using the OrthoDB we searched for the best protein match for each significant gene ID (See Tables 3 and 4) for both species, and also added GO categories for molecular processes from Trinotate, if available. There is greater diversity in the best protein matches for flounder than dab, and different molecular functions for transcripts are indicated. In flounders, the available GO categories suggest changes in processes linked with immune response, apoptosis, and cell cycle regulation, while in dab, metal ion binding processes and peptidase activity regulation are indicated. In flounders, most of the transcripts described in Table 3 can be linked with potential oncogenes or tumour suppressor genes, while in dabs (Table 4), only links with immune suppression can be made based on best protein match analysis (see the discussion below for further analysis).

Oncogenes, tumour-suppressor genes, and differentiation genes are among the oldest gene classes in humans and are shared by most animal species (Makashov et al., 2019). As a result, it is possible to compare the functional pathways related to pollution-induced carcinogenesis between species. The assembled transcriptomes for non-cancerous flounders and dabs from polluted and reference sites were mapped against genes in the human KEGG pathways relevant to pollution induced cancer. These included a pathway for hepatocellular carcinoma (Supplementary materials, Fig. S1), and chemical carcinogenesis pathways for DNA adducts, reactive oxygen species and receptor activation (Supplementary materials, Figs. S2-S3). Note that the genes from KEGG pathways are not shown in Tables 3 and 4, as the selection criteria were stricter for the transcripts included in the tables as compared to KEGG pathway mapping (using adjusted $p$-values gave no responses in pathway mapping). For both species most of the genes in the examined human KEGG pathways were represented in the assembled transcriptomes (shown in grey in Figs. S1-S3).

## 4. Discussion

Our first aim was to describe inter- and intraspecific variation in the gene expression between healthy and cancerous flatfish, and between fish caught from polluted and reference sites. We showed that many transcripts that vary between these groups in expression levels are currently unknown. Especially in flounders with known lower prevalence of pollution-induced cancer compared to dabs, we could see the downregulation of many unknown transcripts in cancerous individuals, suggesting that these could be related to natural cancer defence mechanisms. For the transcripts that could be linked with known protein homologues, the most noteworthy is probably higher expression levels of transcript linked to DRAM2, an effector molecule that is critical for p53-mediated apoptosis, in flounders living in polluted environments.

In addition to characterizing variation, we also set out to test the hypotheses about the potential inter-specific variation in gene expression related to cancer vulnerability and defences, and the possibility that environments contaminated with oncogenic pollutants select for stronger cancer defence mechanisms. When we compared fish with and without liver cancer, we found that gene expression patterns in the livers of dabs differ from flounders. Cancerous dabs show mainly upregulation of genes when compared with healthy dabs. In cancerous flounders, however, we also see downregulation of some genes when comparing gene expression with healthy flounders. This could suggest that these downregulated genes may act as potential defence mechanisms that need to be suppressed for cancer to develop. Our results also suggest that the gene expression patterns in non-cancerous flounders living in polluted vs reference sites are linked to different mechanisms of

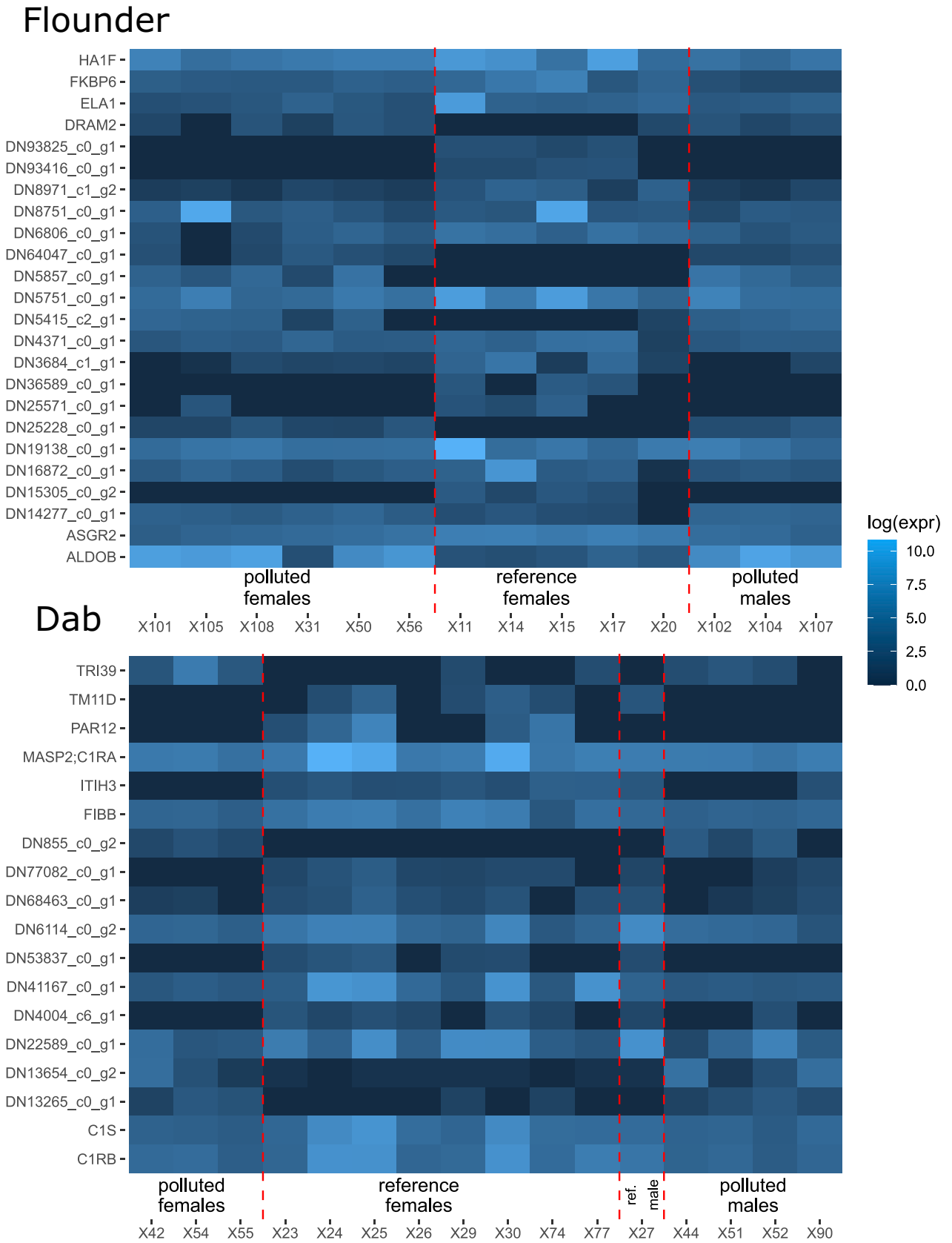



**Fig. 3.** Heat maps describing the $\log_e$ transformed expression levels of transcripts with significantly different expression levels between fish caught from polluted and reference sites. Fish IDs are shown on x-axis, an Asterix by the ID indicates cancerous individuals. On Y axis, transcripts for which a protein homologue was available in Swissprot are marked with corresponding protein name abbreviation, the rest of the transcripts are marked with transcript ID (see also Supplementary data table).

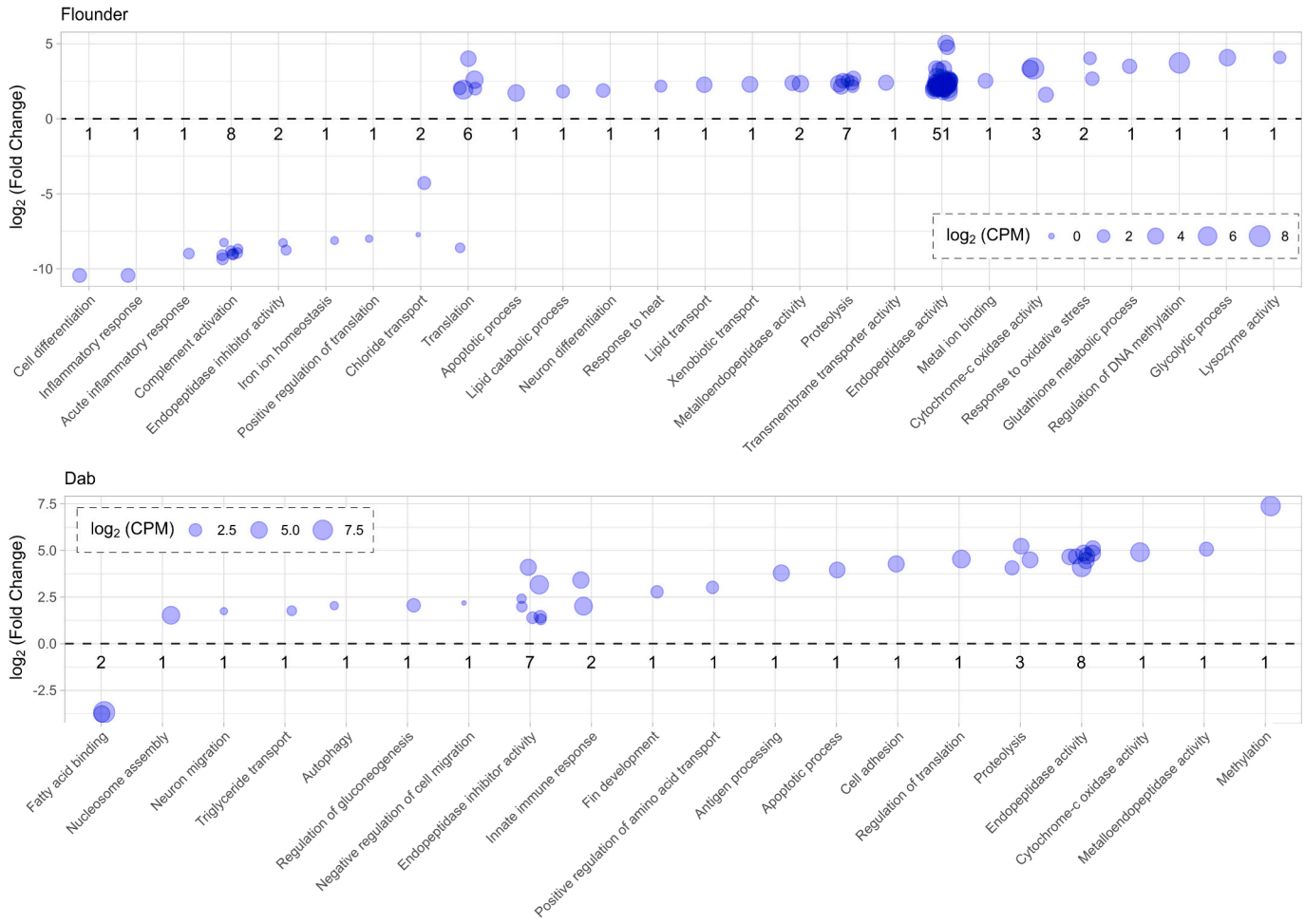



**Fig. 4.** Comparison of transcripts from flounders (upper part) and dabs (lower part) with and without cancer. Y-axis indicates $\log_2$ transformed ratio between gene expression levels in cancerous fish compared to healthy fish. Only annotated transcripts with false discovery rate under 0.05 are shown. Transcripts are categorized according to GO biological processes (if available) or GO molecular functions. If several processes were linked to one transcript, the most informative GO category in terms of cancer-related processes was chosen. The numbers below the X-axis show the number of transcripts categorized under named GO category. Size of the dot indicates $\log_2$ transformed relative transcript abundance (CPM – counts per million).

**Table 4**
Significant gene matches using both EdgeR and Seq2 methods comparing non-cancerous flounder (*Platichthys flesus*) from reference and polluted sites. The best protein match is from OrthoDB at *Actinopterygii* level. Transcripts are categorized according to Gene Ontology (GO) knowledgebase molecular functions. FDR – false discovery rate. Adj.p – p-value adjusted using Benjamini-Hochberg procedure.

| Gene ID | Mean reference site | Mean polluted site | EdgeR FDR | DeSeq2 Adj. p | GO category molecular function | Best protein match actinopterygii |
|---|---|---|---|---|---|---|
| DN25209_c0_g1 | 48.35 | 9305.55 | 0.001 | <0.001 | Fructose-1-phosphate aldolase activity | Fructose-bisphosphate aldolase |
| DN5857_c0_g1 | 0 | 199.00 | 0.004 | <0.001 | N/A | ubiquitin carboxyl-terminal hydrolase CYLD-like |
| DN25228_c0_g1 | 0 | 27.66 | 0.001 | <0.001 | N/A | uncharacterized protein LOC108412876 |
| DN26272_c0_g1 | 0 | 25.59 | 0.001 | <0.001 | Apoptotic process | DNA damage-regulated autophagy modulator protein 2 |
| DN15305_c0_g2 | 34.58 | 0 | <0.001 | <0.001 | N/A | G protein-regulated inducer of neurite outgrowth 1 |
| DN93825_c0_g1 | 21.15 | 0 | 0.001 | <0.001 | N/A | Salt-inducible kinase 1 |
| DN1340_c1_g1 | 944.29 | 231.92 | 0.006 | 0.002 | Carbohydrate binding | C-type lectin domain family 10 member A-like |
| DN8971_c1_g2 | 56.29 | 5.31 | 0.008 | 0.005 | N/A | LOW QUALITY PROTEIN: general transcription factor II-I repeat domain-containing protein 2-like |
| DN5751_c0_g1 | 5771.08 | 485.34 | 0.029 | 0.0190 | N/A | complement factor H-like |
| DN2799_c0_g1 | 5920.67 | 771.66 | 0.030 | 0.023 | Antigen processing and presentation | major histocompatibility complex class I-related gene protein-like |
| DN3684_c1_g1 | 199.77 | 6.90 | 0.041 | 0.038 | N/A | N/A |
| DN4442_c0_g1 | 497.79 | 56.84 | 0.041 | 0.043 | Meiotic cell cycle | inactive peptidyl-prolyl cis-trans isomerase FKBP6 |

pollutant metabolism or tumour suppression, while these connections are less clear in dabs. These results suggest that flounders may have stronger genomic cancer defence mechanisms compared to dabs, and that, as a result, flounder populations may acclimatize or develop adaptations against pollution-induced cancer more efficiently than dabs. This indicates that flatfishes, and especially flounders, can be studied as a natural model system for understanding the evolution of cancer defence mechanisms in polluted environments. However, as the sample

**Table 5**
Significant gene matches using both EdgeR and Seq2 methods comparing non-cancerous dab (*Limanda limanda*) from reference and polluted sites. The best protein match is from OrthoDB at *Actinopterygii* level. Transcripts are categorized according to Gene Ontology (GO) knowledgebase molecular functions. FDR – false discovery rate. Adj. p – p-value adjusted using Benjamini-Hochberg procedure.

| Gene ID | Mean reference site | Mean polluted site | EdgeR FDR | DeSeq2 Adj. p | GO category molecular function | Best protein match actinopterygii |
|---|---|---|---|---|---|---|
| DN31277_c1_g1 | 23.87 | 0 | <0.001 | <0.001 | Endopeptidase inhibitor | Uncharacterized protein LOC106633487 |
| DN6114_c0_g2 | 856.52 | 114.27 | <0.001 | <0.001 | N/A | Hemopexin-like |
| DN41167_c0_g1 | 1670.71 | 40.46 | 0.004 | <0.001 | N/A | Complement C1s subcomponent-like |
| DN20872_c0_g1 | 410.42 | 81.40 | 0.004 | <0.001 | Metal iron binding | Fibrinogen beta chain |
| DN6706_c0_g1 | 1130.39 | 74.07 | 0.006 | <0.001 | Calcium ion binding | Complement C1s subcomponent-like |
| DN10063_c0_g1 | 1497.18 | 109.39 | 0.006 | <0.001 | Calcium iron binding | Complement C1s subcomponent-like |
| DN855_c0_g2 | 0 | 19.47 | 0.008 | <0.001 | N/A | Guanosine-3′,5′-bis(diphosphate) 3′-pyrophosphohydrolase MESH1 |
| DN3541_c0_g1 | 9259.83 | 402.54 | 0.019 | <0.001 | Calcium ion binding | Complement C1s subcomponent-like |
| DN22589_c0_g1 | 1245.74 | 68.84 | 0.023 | <0.001 | N/A | Immunoglobulin-like domain |
| DN4021_c0_g1 | 193.27 | 0 | 0.027 | <0.001 | Metal ion binding | N/A |
| DN21243_c0_g3 | 33.23 | 0 | 0.047 | 0.013 | Peptidase activity | Transmembrane protease serine 6-like |

sizes for the current study were rather low, these results can be viewed as an exploratory pilot study encouraging more comparative studies with different fish species exposed to oncogenic pollutants.

There were 445 differently expressed genes between cancerous and healthy flounders, with 281 upregulated and 164 downregulated transcripts in cancerous flounders. For dabs, less differentially expressed genes between healthy and cancerous individuals was found. From 42 differentially expressed transcripts, four were downregulated and 38 upregulated in cancerous dabs. Many of these transcripts could not be linked with a human homologue neither on the levels of genes nor proteins. This suggests the possibility that novel cancer-related genetic mechanisms, including natural cancer defence mechanisms, could be discovered from studying wild fish. For a better discussion of such a large number of transcripts, we have divided the transcripts with a known homologue from human genes into categories based on their biological functions. The following section therefore describes the differentially expressed transcripts based on their biological function category.

The only transcripts that were downregulated in cancerous dabs were related to fatty acid binding proteins. In flounders, we could also see downregulation of different categories of genes in cancerous fish, although upregulation was still more common. This is in accordance with studies of the human liver cancer transcriptome, where about 90 % of differently expressed genes were upregulated in cancerous individuals (Jin et al., 2019). Most of the transcripts downregulated in flounders with cancer could be linked to immune responses (inflammatory responses, complement activation). In addition, processes downregulated in cancerous flounders included cell differentiation, chloride transport, iron homeostasis, regulation of translation, and endopeptidase inhibition. In both species, the process that was most convincingly upregulated in cancerous fishes (highest number of linked transcripts and highest transcript abundance) was endopeptidase activity. Endopeptidases are proteases, and it is known that in cancerous tissues, proteases are implemented to break down proteins to promote angiogenesis, invasion and metastasis (Lopez-Otin and Overall, 2002). We suggest that in flounders, defence mechanisms that inhibit excessive endopeptidase activity occur in healthy individuals and need to be suppressed for cancer to occur. This aligns with immune suppression seen in cancerous flounders but not in dabs, suggesting that for flounders to develop cancer, defence mechanisms need to be actively suppressed, while little evidence for this activity is observable in dabs, suggesting that less efficient defence mechanisms occur in dabs to begin with.

Some of the transcripts upregulated in flatfishes could also be linked to organismal defence mechanisms against pollution or pollution-induced cancer. For example, in both species there was upregulation of apoptosis-related transcripts in cancerous individuals. In flounders with cancer, transcripts related to antioxidant defences are also upregulated. Additionally, the upregulation of cytochrome C oxidase activity in cancerous individuals from both species, as well as an upregulation of xenobiotic transport in cancerous flounders, supports the link with pollutant metabolism in the development of liver cancer in flatfishes (Stegeman and Lech, 1991). Cytochrome p450 (CYP) enzymes have been shown to play an important role in organic pollutant metabolism, specifically oncogenic contaminant metabolism in a range of species, from humans to fishes (Kwon et al., 2021; Uno et al., 2012). However, upregulation of CYP enzymes have also been linked to higher cancer incidence, as the by-products (metabolites of lipophilic chemicals with increased polarity for better excretion) of active pollutant metabolism leads to carcinogenesis via DNA adduct formation (Stegeman and Lech, 1991; Willett et al., 2006). Mammalian studies have also suggested that some CYP enzymes are upregulated in cancer cells compared to adjacent non-diseased cells (Willett et al., 2006). CYP activity is higher in cancerous flounders compared to dabs (higher number of linked transcripts and higher transcript abundance), suggesting that compared to dabs, flounders show more active pollutant metabolism. This is usually linked to higher cancer risk, but we did not see this in flounders compared to dabs. We suggest two possible explanations for this. First, it is possible that the more active pollutant metabolism in flounders produces some signals in the liver that trigger cancer defence mechanisms. This could be described as a potential hormesis effect, which is defined as an adaptive response of biological systems to moderate environmental challenges through which the system improves its functionality and/or tolerance to more severe challenges (Calabrese and Mattson, 2017). Notably, hormetic effects have been found to be very useful in describing responses to toxicological challenges in a wide range of organisms, including fish (Rix et al., 2022). While a hormesis effect could also be described as an acclimation as opposed to adaptation, we suggest an adaptive process here for flounders. More active pollutant metabolism in flounder would increase their risk of pollution-induced cancer (resulting in a higher selection pressure than for dabs), and therefore, it is possible that only flounders with the strongest defence mechanisms have survived to reproduce in polluted habitats. Whether this hypothesis is valid remains to be tested with an experimental approach using either common garden or multi-generational set-ups. When comparing pathway mapping results (KEGG pathways relevant to pollution induced cancer) for non-cancerous flounders and dabs, an interesting pattern emerges. We see that in flounders, each pathway (except the pancreatic cancer pathway) contains genes that are differently expressed between polluted and reference sites, while no such genes can be found in dabs. In the pathways related to chemical carcinogenesis, upregulation of cytochrome genes (e.g. CYP1A1, CYP2A, CYP1B1 and CYP2Bs) in flounders living in polluted environments is observed. Such site-specific difference of CYP upregulation is not observable in KEGG pathway mapping for dab living in polluted environments, although expression of CYP-genes occurs – which may be the result of widespread pollution that also affects chosen reference areas. The higher expression of CYP enzymes in

non-cancerous flounders in polluted compared to reference sites, but not in dabs, supports the previous suggestion of more active pollutant metabolism in flounders. When considering the lower prevalence of cancer in flounders compared to dabs, this suggests that some mechanisms other than adaptive AhR or CYP-inhibition are occurring, a method which has been previously described in fish populations living in extremely polluted environments to tackle the constant activation of pro-carcinogenes (Oziolor et al., 2019; Celander et al., 2021). Interestingly, there is little evidence of similar adaptations through Phase II enzymatic activity in fishes published so far, but see Collier et al. (1992) for a discussion of the potential role of Phase II enzymes in differential susceptibility to cancer in flatfish.

In our sample, fish from polluted sites did not show higher cancer prevalence compared to fish from reference sites. We see three possible explanations for the seen pattern. (1.) It can possibly stem from the low sample size. (2) It is possible that sites do not differ that much in their actual quality, as pollutants spread quickly in aquatic environment, and no area in the North and Baltic Sea can be considered really "clean". Alternatively, the international regulation of marine contamination may have resulted with less pollution in "polluted sites", also reducing the site differences. As has been shown by long-term monitoring programs from the Thünen Institut, dab from the North Sea historically displayed a much higher prevalence of liver tumours than flounder from the Baltic Sea, but this regional difference has somewhat disappeared in recent years due to a decreasing trend in the prevalence of liver tumours in dab from the North Sea. However, information from sediments (OSPAR Data and Information Management System, https://odims.ospar.org/) and historic cancer prevalence data still support the possibility of locally differing selection pressures against the oncogenic effects of pollutants. (3) It is therefore possible that what we see today in terms of cancer prevalence result from adaptation. In more polluted sites, fish may have been selected to demonstrate more active tumour suppressor mechanisms, resulting in lower prevalence.

When comparing non-cancerous flounders from polluted and reference sites, we found 12 transcripts that were differently expressed on the adjusted *p*-value level of significance ($p < 0.001$). From these 12, one could not be linked to any known protein, and one gave the response of an uncharacterized protein (Table 3). From the rest of the genes, six gave the same best protein match on both the vertebrate and the ray-finned fish (*Actinopterygii*) levels. Looking closer at the function of the three proteins that were linked to transcripts having higher expression in fish from polluted sites, we could suggest links with tumour formation and tumour suppression. First, in polluted sites, we see higher expression of the transcript related aldolase genes, which regulate tumour cell proliferation, apoptosis, and metastasis in human liver cancers (Li et al., 2019). Second, we see the higher expression of the transcript related to ubiquitin/proteasome system, with the function of degradation of abnormal proteins generated under normal and stress conditions. It remains controversial whether this gene is a tumour promoter or suppressor (Fang and Shen, 2017). The third protein that was linked to a transcript having higher expression in polluted site fishes was the DNA damage-regulated autophagy modulator protein 2 (DRAM2), which could be a part of tumour suppression pathway. Transcriptional activation of DRAM2 by the well-known tumour suppressor gene, TP53, has important links to autophagy, apoptosis and programmed cell death (Crighton et al., 2006).

The rest of the transcripts had higher expression in reference sites compared to polluted sites. The lower expression of the following three genes in polluted sites can be suggested to be mechanisms for suppressing tumour development. First, the flounders from reference sites showed markedly higher expression of asialoglycoprotein receptor gene (note that for this gene, the best match on the ray-finned fish levels was different than in the vertebrate level, namely, C-type lectin domain family 10 member A-like), which is upregulated in several human cancer cell lines and, notably, liver cancers (proteinatlas.org). Second, the downregulation of GPRIN1 is a potential tumour suppression mechanism, as it has shown to significantly decreased cell viability, colony formation, and the number of invasive and migrating cells (for human cancers of the lung and the kidneys, Zhou et al., 2021). Note that fish from polluted sites did not show expression of GPRIN1. The same was true for the third transcript, which was linked to serine/threonine protein kinase (salt-inducible kinases, SIK). SIKs can act both as tumour suppressors or oncogenes, depending on the tissue of expression, but are more often seen as proto-oncogenes, as the inhibition of SIK2 has been suggested to be a potential target in cancer therapy (Chen et al., 2019). We speculate that this mechanism might already be used by flounders living in polluted sites. Two of the transcripts that showed markedly lower expression in polluted sites can be linked with immune genes. These include complement factor H-like, and immunoglobulin-like domain (major histocompatibility complex class I-related gene protein-like in ray-finned fish level). In previous work, increases in the expression of mRNAs coding for proteins of innate immunity and inflammation have been observed in response to experimental long-term chronic exposure to a polluted sediment (Leaver et al., 2010). A similar study using liver tissues from individual fish (as opposed to pooled hepatocytes) indicated both upregulation and downregulation of different genes related to immune response resulting from experimental pollution exposure (Williams et al., 2014). Downregulation of some aspects of the immune response could be a direct effect of pollution exposure, but also a potential cost of adapting to high levels of pollution, investing resources into defence mechanisms against pollution as opposed to immune responses.

Compared to flounders, dab living in polluted vs reference sites showed fewer differences in gene expression when we look at the differently expressed transcripts on the functional level (Table 4). We found 11 transcripts that were differently expressed on the adjusted *p*-value level ($p < 0.001$), and three of these could not be linked to any known proteins. Four transcripts from the rest all gave the response of mannan-binding lectin serine peptidase on the vertebrate level, which were complement C1s subcomponent-like at the ray-finned fish level. Both annotations can be linked to innate immune system (Thiel et al., 2012). All of these transcripts showed higher expression in reference sites, indicating immune suppression in polluted sites, which is similar to what was observed in flounders. Only one transcript showed higher expression in polluted site, and this was linked to guanosine-3′,5′-bis (diphosphate) 3′-pyrophosphohydrolase MESH1, which is a little studied hydrolyzing enzyme involved in starvation response (24niprot.org). Three additional transcripts showed lower expression in polluted sites. The first was linked to hemopexin, which is an acute phase reactant protein that binds heme that is released into the blood as the result of haemolysis and transports it to the liver, and is therefore a mechanism against deleterious inflammation and oxidative stress induced by the presence of free heme (Mauk et al., 2011). Previous studies in fishes have linked a protein with high resemblance to hemopexin, teleostean WAP65, with pollution exposure, but usually the response is upregulation of WAP65 to protect cells from oxidative damage (Olsvik et al., 2011). The second transcript that corresponded to fibrinogen beta chain, can also be linked to immune system and inflammatory response, and has been shown to be affected by pollution exposure in fish livers in previous studies (i.e. Leaver et al., 2010). The third transcript was annotated as transmembrane protease serine 6-like, which is involved in iron absorption (uniprot.org) and inflammatory immune responses (Patel, 2017). Taken together, most of the differences in gene expression between polluted and reference site dabs suggest lowered (innate) immune responses, and there is, compared to flounders, little support to potential adaptation with oncogenic pollution. This finding supports the KEGG pathway analysis, and could also explain the higher prevalence of liver cancer observed in dabs compared to flounders.

In conclusion, using whole genome transcriptome analysis, our study highlighted potential mechanisms and pathways that could explain the differing cancer risk described previously for flounders and dabs in polluted habitats. Specifically, more active pollutant metabolism is

observable in the liver tissue of flounders, potentially leading to either hormetic upregulation of tumour suppression mechanisms, or to a stronger natural selection pressure for higher cancer resistance. In flounders, we were able to link the differences in gene expression levels to known tumour suppression mechanisms, while in dabs, only indication of immune suppression was found in polluted sites. This study indicates that study of wild species could offer novel insights for understanding the nature and evolution of natural cancer defence mechanisms, and the differing potential of wild species to adapt to anthropogenic environmental change.

## Funding sources

This work was supported by the Estonian Research council grant (PSG653) to TS. RM was supported by the Estonian Research council grant (PRG1137). JC's work was supported by a postdoctoral fellowship of the Alexander von Humboldt Foundation, the Polish National Agency for Academic Exchange (NAWA; PPN/ULM/2019/1/00248/U/00001), and the Estonian Research Council (grant PRG741).

## CRediT authorship contribution statement

**Tuul Sepp:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Ciara Baines:** Investigation, Methodology, Writing – original draft, Writing – review & editing. **Randel Kreitsberg:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Jörn Peter Scharsack:** Resources, Supervision, Writing – review & editing. **Pedro Nogueira:** Investigation, Methodology, Writing – review & editing. **Thomas Lang:** Methodology, Writing – review & editing. **Jérôme Fort:** Investigation, Methodology, Writing – review & editing. **Elin Sild:** Resources, Writing – review & editing. **John T. Clarke:** Methodology, Writing – review & editing. **Arvo Tuvikene:** Conceptualization, Resources, Writing – review & editing. **Richard Meitern:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Visualization, Writing – review & editing.

## Declaration of competing interest

Authors declare no competing interests.

## Data availability

I have shared my data as attached file.

## Acknowledgements

We are grateful to the crew of RW Walther Herwig III for all-round help during the fieldwork. This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 951963.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2023.109781.

## References

Babu, M.M., Aravind, L., 2006. Adaptive evolution by optimizing expression levels in different environments. Trends Microbiol. 14, 11–14.
Baines, C., Lerebours, A., Thomas, F., Fort, J., Kreitsberg, R., Gentes, S., Meitern, R., Saks, L., Ujvari, B., Giraudeau, M., Sepp, T., 2021. Linking pollution and cancer in aquatic environments: a review. Environ. Int. 149, 106391.
Baines, C., Meitern, R., Kreitsberg, R., Sepp, T., 2022. Comparative study of the evolution of cancer gene duplications across fish. Evol. Appl. 15, 1834–1845.
Berkström, C., Wennerström, L., Bergström, U., 2022. Ecological connectivity of the marine protected area network in the Baltic Sea, Kattegat and Skagerrak: current knowledge and management needs. Ambio 51, 1485–1503.
de Boer, J., van der Zande, T.E., Pieters, H., Ariese, F., Schipper, C.A., van Brummelen, T., Vethaak, A.D., 2001. Organic contaminants and trace metals in flounder liver and sediment from the Amsterdam and Rotterdam harbours and off the Dutch coast. J. Environ. Monitor. 3, 386–393.
Bryant, D.M., Johnson, K., DiTommaso, T., Tickle, T., Couger, M.B., Payzin-Dogru, D., Lee, T.J., Leigh, N.D., Kuo, T.H., Davis, F.G., Bateman, J., Bryant, S., Guzikowski, A. R., Tsai, S.L., Coyne, S., Ye, W.W., Freeman Jr., R.M., Peshkin, L., Tabin, C.J., Regev, A., Haas, B.J., Whited, J.L., 2017. A tissue-mapped axolotl de novo transcriptome enables identification of limb regeneration factors. Cell Rep. 17 (18), 762–776.
Cachot, J., Cherel, Y., Larcher, T., Pfohl-Leszkowicz, A., Laroche, J., Quiniou, L., Morin, J., Schmitz, J., Burgeot, T., Pottier, D., 2013. Histopathological lesions and DNA adducts in the liver of European flounder (*Platichthys flesus*) collected in the Seine estuary versus two reference estuarine systems on the French Atlantic coast. Environ. Sci. Pollut. Res. 20, 723–737.
Calabrese, E.J., Mattson, M.P., 2017. How does hormesis impact biology, toxicology, and medicine? Aging Mech. Dis. 3, 13.
CEFAS, Marine Environment Monitoring Group, 2004. UK National Marine Monitoring Programme - Second Report (1999–2001). ISBN 0 907545 20 3.
Celander, M.C., Goldstone, J.V., Brun, N.R., Clark, B., Jayaraman, S., Nacci, D., Stegeman, J.J., 2021. Resistance to Cyp3a induction by polychlorinated biphenyls, including non-dioxin-like PCB153, in gills of killifish (*Fundulus heteroclitus*) from New Bedford Harbor. Environ. Toxicol. Pharmacol. 83, 103580.
Chen, F., Chen, L., Qin, Q., Sun, X., 2019. Salt-inducible kinase 2: an oncogenic signal transmitter and potential target for cancer therapy. Front. Oncol. 9, 18.
Collier, T.K., Singh, S.V., Awasthi, Y.C., Varanasi, U., 1992. Hepatic xenobiotic metabolizing enzymes in two species of benthic fish showing different prevalences of contaminant-associated liver neoplasms. Toxicol. Appl. Pharmacol. 113, 319–324.
Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P.R., Gasco, M., Garrone, O., Crook, T., Ryan, K.M., 2006. DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell 126, 121–134.
Das, S., Mykles, D.L., 2016. A comparison of resources for the annotation of a de novo assembled transcriptome in the molting gland (Y-organ) of the blackback land crab, *Gecarcinus lateralis*. Int. Comp. Biol. 56, 1103–1112.
Di Giulio, R.T., Clark, B.W., 2015. The Elizabeth river story: a case study in evolutionary toxicology. J. Toxicol. Environ. Health B Crit. Rev. 18, 259–298.
Fang, Y., Shen, X., 2017. Ubiquitin carboxyl-terminal hydrolases: involvement in cancer progression and clinical implications. Cancer Metastasis Rev. 36, 669–682.
Feist, S.W., Lang, T., Stentiford, G.D., Köhler, A.I.C.E.S., 2004. Biological effects of contaminants: use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.) for monitoring. ICES TIMES. 38, 42.
HELCOM, 2018. State of the Baltic Sea – second HELCOM holistic assessment 2011-2016. Balt. Sea Environ. Proc. 155.
Jin, Y., Lee, W.Y., Toh, S.T., Tennakoon, C., Toh, H.C., Chow, P.K., Chung, A.Y., Chong, S.S., Ooi, L.L., Sung, W.K., Lee, C.G., 2019. Comprehensive analysis of transcriptome profiles in hepatocellular carcinoma. J. Transl. Med. 17, 273.
Kwon, Y.J., Shin, S., Chun, Y.J., 2021. Biological roles of cytochrome P450 1A1, 1A2, and 1B1 enzymes. Arch. Pharm. Res. 44, 63–83.
Lang, T., Wosniok, W., Baršienė, J., Broeg, K., Kopecka, J., Parkkonen, J., 2006. Liver histopathology in Baltic flounder (*Platichthys flesus*) as indicator of biological effects of contaminants. Mar. Poll. Bulletin. 53, 488–496.
Larsen, P.F., Nielsen, E.E., Williams, T.D., Loeschcke, V., 2008. Intraspecific variation in expression of candidate genes for osmoregulation, heme biosynthesis and stress resistance suggests local adaptation in European flounder (*Platichthys flesus*). Heredity 101, 247–259.
Leaver, M.J., Diab, A., Boukouvala, E., Williams, T.D., Chipman, J.K., Moffat, C.F., Robinson, C.D., George, S.G., 2010. Hepatic gene expression in flounder chronically exposed to multiply polluted estuarine sediment: absence of classical exposure 'biomarker' signals and induction of inflammatory, innate immune and apoptotic pathways. Aquat. Toxicol. 96, 234–245.
Lerebours, A., Stentiford, G.D., Lyons, B.P., Bignell, J.P., Derocles, S.A., Rotchell, J.M., 2014. Genetic alterations and cancer formation in a European flatfish at sites of different contaminant burdens. Environ. Sci. Technol. 48, 10448–10455.
Li, X., Jiang, F., Ge, Z., Chen, B., Yu, J., Xin, M., Wang, J., An, L., Wei, J., Wu, L., 2019. Fructose-bisphosphate aldolase a regulates hypoxic adaptation in hepatocellular carcinoma and involved with tumor malignancy. Dig. Dis. Sci. 64, 3215–3227.
Lopez-Otin, C., Overall, C.M., 2002. Protease degradomics: a new challenge for proteomics. Nat. Rev. Mol. Cell Biol. 3, 509–519.
Lyons, B.P., Stentiford, G.D., Bignell, J., Goodsir, F., Sivyer, D.B., Devlin, M.J., Lowe, D., Beesley, A., Pascoe, C.K., Moore, M.N., Garnacho, E., 2006. A biological effects monitoring survey of Cardigan Bay using flatfish histopathology, cellular biomarkers and sediment bioassays: findings of the Prince Madog prize 2003. Mar. Environ. Res. 62, 342–346.
Makashov, A.A., Malov, S.V., Kozlov, A.P., 2019. Oncogenes, tumor suppressor and differentiation genes represent the oldest human gene classes and evolve concurrently. Sci. Rep. 9, 1–12.
Mauk, M.R., Smith, A., Mauk, A.G., 2011. An alternative view of the proposed alternative activities of hemopexin. Protein Sci. 20, 791–805.
McAloose, D., Newton, A.L., 2009. Wildlife cancer: a conservation perspective. Nat. Rev. Cancer 9, 517–526.
Meitern, R., Fort, J., Giraudeau, M., Rattiste, K., Sild, E., Sepp, T., 2020. Age-dependent expression of cancer-related genes in a long-lived seabird. Evol. Appl. 13, 1708–1718.

Nair, N.U., Cheng, K., Naddaf, L., Sharon, E., Pal, L.R., Rajagopal, P.S., Unterman, I., Aldape, K., Hannenhalli, S., Day, C.P., Tabach, Y., 2022. Cross-species identification of cancer resistance-associated genes that may mediate human cancer risk. Sci. Adv. 8, eabj7176.

Nelson, D.R., Goldstone, J.V., Stegemen, J.J., 2013. The cytochrome P450 genesis locus: the origin and evolution of animal cytochrome P450s. Philos. Trans. R. Soc. Lond. B Biol. Sci. 368, 20120474.

Nesse, R., 2017. Introduction: five evolutionary principles for understanding cancer. In: Ecology and Evolution of Cancer. Elsevier Inc., pp. 15–21

Nissling, A., Nyberg, S., Petereit, C., 2017. Egg buoyancy of flounder, *Platichthys flesus*, in the Baltic Sea—adaptation to salinity and implications for egg survival. Fish. Res. 191, 179–189.

Olsvik, P.A., Brattås, M., Lie, K.K., Goksøyr, A., 2011. Transcriptional responses in juvenile Atlantic cod (*Gadus morhua*) after exposure to mercury-contaminated sediments obtained near the wreck of the German WW2 submarine U-864, and from Bergen Harbor, Western Norway. Chemosphere 83, 552–563.

OSPAR Commission, 2010. OSPAR Quality Status Report 2010. https://qsr2010.ospar.org/en/index.html.

Oziolor, E.M., Reid, N.M., Yair, S., Lee, K.M., VerPloeg, S.G., Bruns, P.C., Shaw, J.R., Whitehead, A., Matson, C.W., 2019. Adaptive introgression enables evolutionary rescue from extreme environmental pollution. Science 364, 455–457.

Patel, S., 2017. A critical review on serine protease: key immune manipulator and pathology mediator. Allergol. Immunopathol. 45, 579–591.

Pomianowski, K., Burzyński, A., Kulczykowska, E., 2021. A de novo transcriptome assembly of the european flounder (*Platichthys flesus*): the preselection of transcripts encoding active forms of enzymes. Front. Mar. Sci. 8, 618779.

R Core Team, 2022. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL. https://www.R-project.org/.

Rijnsdorp, A.D., Vethaak, A.D., van Leeuwen, P.I., 1992. Population biology of dab *Limanda limanda* in the southeastern North Sea. Mar. Ecol. Progr. Series. 91, 19–35.

Rix, R.R., Guedes, R.N.C., Cutler, G.C., 2022. Hormesis dose-response contaminant-induced hormesis in animals. Curr. Opin. Toxicol. 30, 100336.

Sepp, T., Giraudeau, M., 2022. Wild animals as an underused treasure trove for studying the genetics of cancer. BioEssays 45, 2200188.

Stegeman, J.J., Lech, J.J., 1991. Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. Environ. Health Perspect. 90, 101–109.

Stentiford, G.D., Longshaw, M., Lyons, B.P., Jones, G., Green, M., Feist, S.W., 2003. Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. Mar. Envir. Res. 55, 137–159.

Stentiford, G.D., Bignell, J.P., Lyons, B.P., Feist, S.W., 2009. Site-specific disease profiles in fish and their use in environmental monitoring. Mar. Ecol. Progr. Series. 381, 1–15.

The Gene Ontology resource: enriching a GOld mine. Nucleic Acids Res. 49, 2021, D325–D334.

The global challenge of cancer. Nat. Cancer 1, 2020, 1–2.

Thiel, S., Jensen, L., Degn, S.E., Nielsen, H.J., Gál, P., Dobó, J., Jensenius, J.C., 2012. Mannan-binding lectin (MBL)-associated serine protease-1 (MASP-1), a serine protease associated with humoral pattern-recognition molecules: normal and acute-phase levels in serum and stoichiometry of lectin pathway components. Clin. Exp. Immunol. 169, 38–48.

Tollis, M., Schneider-Utaka, A.K., Maley, C.C., 2020. The evolution of human cancer gene duplications across mammals. Mol. Biol. Evol. 37, 2875–2886.

Tysklind, N., Taylor, M.I., Lyons, B.P., Goodsir, F., McCarthy, I.D., Carvalho, G.R., 2013. Population genetics provides new insights into biomarker prevalence in dab (*Limanda limanda* L.): a key marine biomonitoring species. Evol. Appl. 6, 891–909.

Uno, T., Ishizuka, M., Itakura, T., 2012. Cytochrome P450 (CYP) in fish. Environ. Toxicol. Pharmacol. 34, 1–13.

Vethaak, A.D., Jol, J.G., Meijboom, A., Eggens .L., M, Rheinallt, T.A., Wester, P.W., Van De Zande, T., Bergman, A., Dankers, N., Ariese, F., Baan, R.A., 1996. Skin and liver diseases induced in flounder (*Platichthys flesus*) after long-term exposure to contaminated sediments in large-scale mesocosms. Environ. Health. Persp. 104, 1218–1229.

Vethaak, A.D., Jol, J.G., Pieters, J.P., 2009. Long-term trends in the prevalence of cancer and other major diseases among flatfish in the southeastern North Sea as indicators of changing ecosystem health. Environ. Sci. Technol. 43, 2151–2158.

Vittecoq, M., Giraudeau, M., Sepp, T., Marcogliese, D.J., Klaassen, M., Renaud, F., Ujvari, B., Thomas, F., 2018. Turning natural adaptations to oncogenic factors into an ally in the war against cancer. Evol. Appl. 11, 836–844.

Willett, K.L., Ganesan, S., Patel, M., Metzger, C., Quiniou, S., Waldbieser, G., Scheffler, B., 2006. In vivo and in vitro CYP1B mRNA expression in channel catfish. Mar. Environ. Res. 62, S332–S336.

Williams, T.D., Davies, I.M., Wu, H., Diab, A.M., Webster, L., Viant, M.R., Chipman, J.K., Leaver, M.J., George, S.G., Moffat, C.F., Robinson, C.D., 2014. Molecular responses of European flounder (*Platichthys flesus*) chronically exposed to contaminated estuarine sediments. Chemosphere 108, 152–158.

Yu, Z., Seim, I., Yin, M., Tian, R., Sun, D., Ren, W., Yang, G., Xu, S., 2021. Comparative analyses of aging-related genes in long-lived mammals provide insights into natural longevity. The Innov. 2, 100108.

Zhou, Q., Li, D., Zheng, H., He, Z., Qian, F., Wu, X., Yin, Z., Bao, P.T., Jin, M., 2021. A novel lncRNA-miRNA-mRNA competing endogenous RNA regulatory network in lung adenocarcinoma and kidney renal papillary cell carcinoma. Thorac. Cancer. 12, 2526–2536.