Response to oncogenic pollution in two fish species: are there differences in adaptive potential?

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Abstract

Environmental factors cause cancer in both wild animals and humans. In ecological settings, genetic variation and natural selection can 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 amongst 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 28 flatfishes. By comparing cancerous and healthy fishes, and non-cancerous fishes from clean and polluted sites, we suggest 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 that in flounders, either hormetic upregulation of tumour suppression or a stronger natural selection pressure for higher cancer resistance has evolved in a polluted environment. For pollution-induced liver cancer to develop in flounders, genetic defence mechanisms need to be suppressed, while in dabs, analogous process is weak or absent in our samples. We conclude that wild species could offer novel insights and ideas for understanding the nature and evolution of natural cancer defence mechanisms.

Keywords

Cancer defence mechanisms, wildlife cancer genetics, pollution-induced cancer, pollution adaptation, fish cancer, liver cancer

Introduction

While medical and evolutionary sciences have traditionally developed in separation from one another, applying evolutionary principles and approaches to understand cancer could offer new insights for understanding the ecological and evolutionary aspects affecting this disease. A key

consideration in the global effort against cancer is the influence of the anthropogenic environmental change, from exposures to ultraviolet radiation, air pollution, disruptions in the food and water supply, environmental toxicants, and infectious agents (Lewandowska et al. 2019; Hiatt & Beyeler, 2020, Baines et al. 2021). While we know that anthropogenic environmental change strongly affects 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 natural cancer defence mechanisms and 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 (Enriquez-Navas et al. 2015; Nesse 2017; Gatenby & Brown 2020, Sepp & Giraudeau 2022). Cancer is a disease that developed with the transition from unicellular to multicellular life, simultaneously with mechanisms that control unregulated divisions of "selfish" cells (Smith & Szathmáry 1995). These control mechanisms provided a strong selective advantage (Nesse 2017), and include tumour suppressor genes, immune surveillance, and systems that prevent and repair DNA damage (Nunney & Muir 2015). Knowledge about defence mechanisms against cancer development is limited and is mostly based on the study of model organisms with low genetic diversity in laboratory environments (Ducasse et al. 2015). The costs and benefits of implementing cancer defences should also be studied in natural settings, where resources are limited, environmental stressors are multiple, predators and competitors are present, and fitness effects of adaptations are ruthlessly revealed.

Among animal species, a lack of correlation between body size and cancer incidence, called Peto's paradox, has improved our understanding of cancer defences (Caulin & Maley 2011). Depending on their body size, but also longevity, life history strategy, and environmental (oncogenic) pressures (Ducasse et al. 2015), species should exhibit different vulnerabilities to cancer, and deploy different tumour suppression strategies. Recent comparative genomic

studies have described the differences between genomic cancer defences in mammals (Tollis et al. 2020, Tejada-Martinez et al. 2021, Vazquez & Lynch 2021, Yu et al. 2021), but also in a wider range of vertebrates (Nair et al. 2022), including fishes (Baines et al. 2022). A focus on a wider range of model species, and on ecological and environmental factors, should contribute to the discovery of naturally selected mechanisms of cancer resistance. 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 & Newton 2009; Vittecoq et al. 2018; Giraudeau et al. 2018; Sepp et al. 2019). However, if we could identify some species and/or populations that have been exposed to oncogenic pollution for extended periods, but do not show increased cancer prevalence, we have a situation that is similar to Peto's paradox – cancer should be there, but it is not. This suggests stronger defence mechanisms in these species/populations against pollution-induced cancer (Vittecoq et al. 2018), indicating that natural selection can sometimes produce solutions to avoid and control oncogenic processes in pollutant-exposed populations.

In aquatic habitats, animals live in close contact with oncogenic pollutants, and have been exposed to pollution for extended time periods. Over the last two centuries, anthropogenic contamination has resulted in the rapid increase in aquatic habitats of both evolutionarily old, familiar oncogenic substances (e.g. polycyclic aromatic hydrocarbons, PAHs), that can also be produced by natural processes, albeit in much smaller quantities than by anthropogenic activities, and those that are novel (such as polybrominated biphenyls, PBBs, or trinitrotoluene, TNT) (Häder et al. 2020). Due to the interconnectedness of aquatic ecosystems through highly effective marine and atmospheric transport routes, and the long persistence of pollutants in sediments, aquatic species are exposed to markedly higher levels of oncogenic pollutants compared to terrestrial species. This can lead to pollution-induced cancer epidemics in aquatic species, for example, in beluga whales (*Delphinapterus leucas*, Martineau et al. 2002),

California sea lions (*Zalophus californianus*, Randhawa et al. 2015), and the numerous discoveries of different types of fish tumours in polluted habitats (Brown et al. 1973, Black & Baumann 1991, Murchelano & Wolke, 1991). In particular, "risk factors" for fish to develop cancers are residing in polluted areas near urban centres, bottom-dwelling life style, and advanced age (Hinton et al. 1989). The environmental crisis of habitat contamination has unintentionally created opportunities to use polluted aquatic environments as "ecological laboratories" for studying the evolution, nature and costs of cancer defence mechanisms, as suggested by Vittecoq et al. (2018). Whilst 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 (2015) presents, in his review, the notorious case of the Elisabeth River (USA), where there is evidence of adaptation against the effects of PAHs, including liver neoplasms, possibly through changes in several different pathways and mechanisms.

The current study tests the hypotheses that (1) the vulnerability and defence mechanisms to pollution-induced cancer differ between two bottom-dwelling flatfish species, and (2) that environments contaminated with oncogenic pollutants select for stronger cancer defence mechanisms (Figure 1). 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 (HELCOM 2018; Lehtonen et al. 2006). Both regions contain high levels of common contaminants with known oncogenic effects (Mathew et al. 2017; 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*) and dab (*Limanda limanda*) 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 (mean age we derived from the original data files of the following five studies [Betancur-R.et al. 2015, Bryne et al. 2018, Rabosky et al. 2018, Ribeiro et al. 2018, Sanciangco et al. 2016], range is 7.3 to 15.2 million years ago). 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. Potential for local adaptation was shown in Baltic Sea flounders, indicating that populations here are separated enough to develop local adaptations. For example, Nissling et al. (2017) described local adaptation of flounders, increasing the survival of pelagic eggs at specific salinities in different spawning areas. Similarly, Larsen et al. (2006) have indicated local adaptation in flounder populations for osmoregulatory and stress genes. There are several local populations of both species that inhabit a gradient of environmental contamination, forming a "natural laboratory" with replicates of polluted and clean sites. 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 with varying selective pressures. It is noteworthy that in the ecotoxicological literature, tumours in flatfishes are generally considered to result in low mortality (Koehler et al. 2004), suggesting that selection has already eliminated the most vulnerable genotypes, or that flatfishes have mechanisms that prevent these tumours from developing into mortal malignancy. Flounders living in highly polluted sites do not seem to have higher cancer prevalence compared to their conspecifics in cleaner sites (Vethaak & Wester, 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 flounders are less susceptible to cancer development compared to dabs, or are more likely to develop higher levels of cancer defences in populations living in polluted environments by natural selection. In the latter case, it is not known if this apparent tolerance to oncogenic pollution evolved recently in direct response to pollution, or is a feature that has a deeper evolutionary history in the flounder which may always be considered active, or may become activated through some direct physiological or biochemical pathways in response to environmental conditions.

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*	31	Lyons et al. 2006
Dab	8.56	50	Stentiford et al. 2009
Dab	~10	600	CEFAS report 2004

*Depending on location

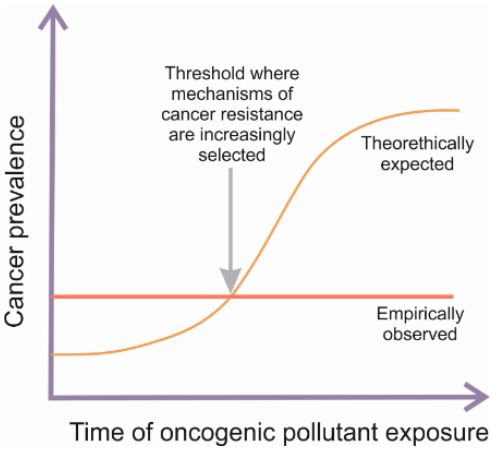


Figure 1. Pollution as a selection pressure shaping cancer defences. Based on the conceptual figure presented in Roche et al. 2012

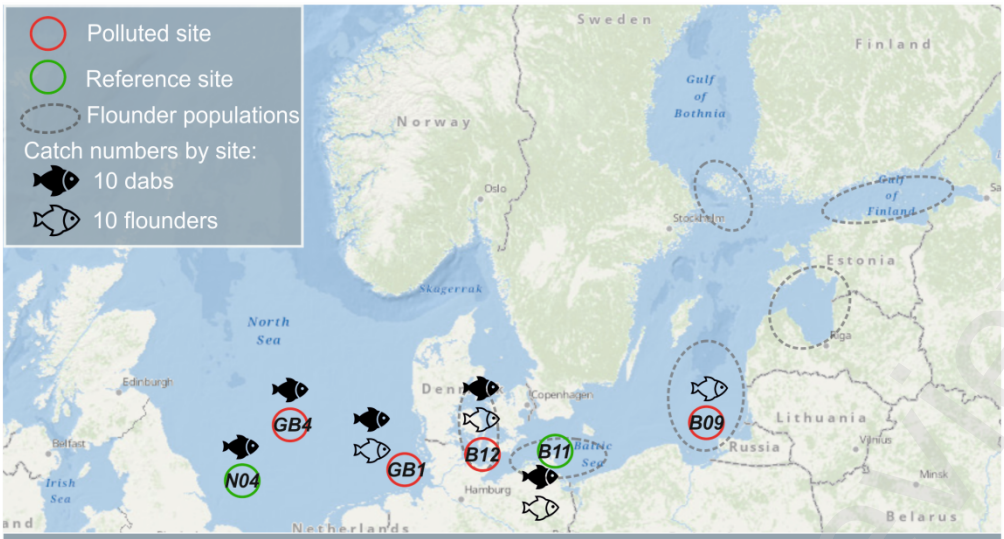
Gene expression analyses provide a new avenue to study the molecular basis of adaptation. Variation in gene expression has large functional consequences and is considered a key component of environmental adaptation in natural populations (Oleksiak et al. 2002, Babu & Aravind, 2006). In flounders, for example, intraspecific variation has been shown between populations in gene expression related to osmoregulation, heme biosynthesis, and stress resistance (Larsen et al. 2008). The possibility of local adaptation to pollutant levels has rarely been considered in flatfishes. For cancer-related studies in flounders, previous research has mostly focused on the mutations that cause cancer, as opposed to the genetic defences preventing it from developing (Williams et al. 2011).

Here, we present the results of a study with samples from 30 flatfishes: 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 were conducted to identify cancer-related genes that were (1) differently expressed between flounders and dabs with and without cancer, and (2) differently expressed between flounders and dabs without cancer, originating from

reference and polluted sites. We hypothesized that cancer affects the two species differently in their levels of gene expression, possibly explaining the differences in vulnerability. We also suggest that as a result of local adaptation, cancer-related gene expression patterns differ in non-cancerous flounders and dabs living in polluted and reference sites, possibly showing higher expression of defence mechanism related genes in flounders.

Table 2. Description of the whole sample, and the subsample of fish chosen for transcriptome

Site	Site status	Total flounders/ with cancer	N N	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

Figure 2. Map of sites, catch size, and subsamples chosen for transcriptome analysis. Sites are further characterized in Table 5 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.

Materials and Methods

Sample collection and processing

Two fish species were included in the current study: dab (*Limanda limanda; Linnaeus, 1758*) and flounder (*Platichthys flesus; Linnaeus, 1758*). Both species are benthic flatfishes that are exposed to the environmental contaminants accumulated in sediments through feeding and direct contact. These species are widely used in ecotoxicological studies and are also part of several monitoring programmes (Hylland et al., 2017). Cancerous lesions (liver, skin) are common in both species (Vethaak et al. 2009). Specimen of dab and flounder were collected by means of bottom trawling during a research cruise with RV Walther Herwig III (WHIII-

429) in August-September 2019. Sampling comprised three areas in the North Sea and three areas in the Baltic Sea (Table 5). 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 2009). Such distribution was based on public data of monitoring programmes (HELCOM 2018, HELCOM 2010; OSPAR 2009, OSPAR 2010) and previous studies of the areas (e.g. Zaborska et al., 2019, Hylland et al., 2017; Table 5). 128 fishes in total were sampled including 40 flounders and 88 dabs, (Table 2). Average trawling speed was 3,5 km for 60 minutes, after which fish were quickly transferred to a large flow-through seawater tanks and sampled within 1 hour.

Fishes were killed by a blow on the head, and liver and otholites were collected. Livers were assessed for external lesions, and then an approximately 3 mm slice of middle section of liver was cut and stored in 4% formalin for 24 hours 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 24h, 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, mean no cancer 3.9, t-test p=0.20), and flounders (mean cancer 6.5, mean no cancer 5.2, t-test p=0.24). Samples were collected from bile and liver to analyse pollutant content from fish tissues. Bile was collected directly from the gall bladder using a disposable syringe, and liver tissue by hand with a scalpel. Both samples were snap frozen immediately. Results are indicated in Table S3 (Supplementary materials) and Figure 4.

Pollutant analyses from tissues

In order to measure PAH metabolites from fish bile, fixed wavelength fluorescence (FF) analyses were performed. A 96-well plate-reader with fluorescence spectrophotometer (BMG Omega Fluostar) was used. Bile samples were diluted in 48% ethanol (1:1600)(Aas et al. 2000) and measured using fixed wavelength pairs (excitation and emission, respectively) to detect PAH metabolites: 290/380 nm representing naphthalene (2-ring PAH); 256/380 nm representing phenanthrene (3-ring PAH); 341/383 nm representing pyrene (4-ring PAH); and 380/430 nm representing benzo(a)pyrene (5-ring PAH) (Lee and Anderson 2005). The results are presented as fluorescence units (FU), which are proportional to the concentration of PAH metabolite in the dilution (Beyer et al. 2010).

Liver samples were freeze-dried for 48 hours and ground to powder for homogenization prior to trace element analyses. Total Hg concentrations were measured in subsamples of ~1mg of homogenized liver using an advanced Hg analyzer spectrophotometer (Altec AMA 254) as described in Bustamante et al. (2006). Hg analyses were repeated two times for each sample to ensure the relative standard deviation for the aliquots was <10%. Remaining samples were analysed for trace elements with known toxic effects (arsenic (As), cadmium (Cd), lead (Pb). Briefly, samples were digested with a mixture of 3 mL HNO3 and 5 mL HCl Suprapur quality, and then heated in a microwave oven. Samples were then diluted to 50 ml with deionized water. Concentrations of trace elements were analysed by Inductively Coupled Plasma Atomic Emission Spectrometry (Varian Vista-Pro ICP-AES) and Mass Spectrometry (ICP-MS II Series Thermo Fisher Scientific). All metallic trace element analyses were performed at the laboratory Littoral, Environnement et Société (LIENSs, La Rochelle).

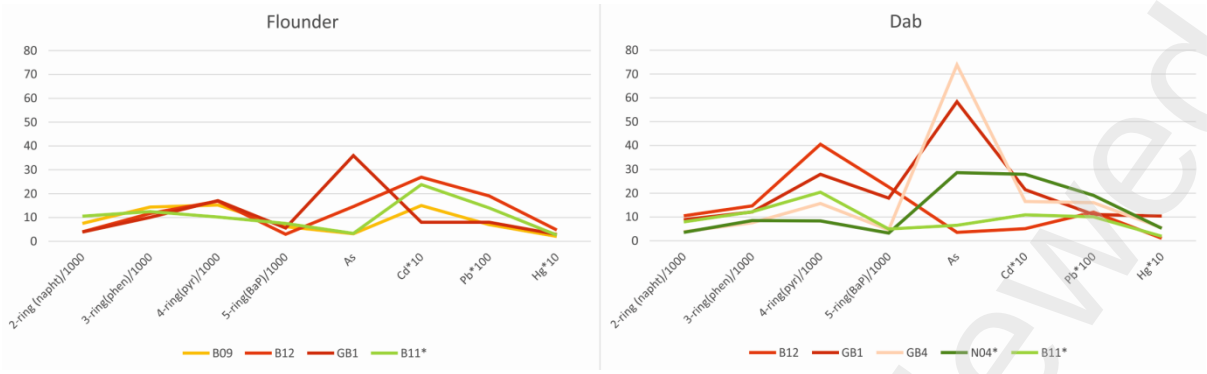


*Figure 4. Site-specific levels of selected pollutants in fish liver (heavy metals; µg g^{-1} dry weight) and bile (polycyclic aromatic hydrocarbon metabolites; fluorescence units (FU)). Napht – naphthalene, phen – phenanthrene, pyr – pyrene, BaP - benzo(a)pyrene, As – arsenic, Cd – cadmium, PG – lead, Hg – mercury. The results have been brought to the same scale with the transformations shown on x-axis for each pollutant. Green hues (and * by site code) indicate reference sites and red hues indicate polluted sites. Site (B09, B12, GB1, GB4, NO4, B11) codes are further explained in Table 5.*

For histopathology, liver samples were processed according to Feist et al (2004). Tissue was fixed with buffered formalin and following the transfer of samples to ethanol they were further dehydrated and then embedded in paraffin wax. Samples were then sliced 4µm 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. Samples were diagnosed as either: cancerous (eg. adenoma or carcinoma according to diagnostic methods described by Feist et al (2004)) or not cancerous (including any preneoplastic lesions such as foci of cellular alteration).

Table 3. Sampling locations and description in six areas of the North and the Baltic Seas.

Code	Site name	Coordinates	Category	Description

B09	Gulf of Gdańsk	55°06,93N 018°10,90E	polluted	Inflow from the Vistula estuary. Effect of industry (Zaborska et al., 2019).
B11	Arcona Sea	54°45,39N 013°11,91E	reference	Wind parks and marine protected areas.
B12	Kiel Area	54°14,87N 011°44,34E	polluted	Heavy marine traffic. TBT-specific effects are still found in maritime areas even after global 2008 ban (OSPAR 2010). Therefore harbours can have a noticeable impact, highlighting the importance of local sources and historic contamination of harbour sediments.
GB1	German Bight South	54°04,54N 007°53,71E	polluted	Area of extensive maritime activities. Inflow from the rivers Elbe and Weser (Hylland et al., 2017). Heavy metal concentrations in sediments are at levels that pose a risk of pollution effects for marine life in the southern North Sea (OSPAR 2010).
GB4	German Bight North	55°23,29N 004°32,44E	polluted	Area of extensive maritime activities.
N04	Dogger Bank	54°46,26N 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 (OSPAR 2010).

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275 *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 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 20-30mg. The tissue was disrupted using a pestle and mortar and homogenised by adding both 600µL RLT buffer with β-mercaptoethanol (10µL β-mercaptoethanol added to 1ml RLT buffer) and 0.5mm glass beads to the sample tube and homogenised in Bullet Blender 24 (Next Advance Inc, USA) on speed 4 for 2 minutes. 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 a 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 80bp 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 separately for both species as in Meitern et al. (2020). Briefly, the sequencing resulted in 1013M PE raw reads that were cleaned and trimmed using Trimmomatic 0.38 (Bolger et al. 2014). After quality control *de novo* transcriptome assembly was performed with Trinity 2.8.4 (Haas et al. 2013). Downstream analyses for aligning reads for assembly were performed with scripts within Trinity using Salmon (Patro et al. 2017). For flounder transcriptome assembly we added liver transcriptome data from 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 (McCarthy et al. 2012) and DESeq2 (Love et al., 2014). To annotate the obtained transcriptome, we used Dammit (Scott 2016) using orthologous genes database (OrthoDB) version 10.1 (Kriventseva et al. 2019). Human orthologues for each transcript were retrieved through OrthoDB using the cluster ID. The final tables and graphs were prepared in R version 4.1.3 (R Core Team, 2022). KEGG plots were produced using pathview (Luo et al. 20213). Other R packages used included several packages from the tidyverse (Wickham et al., 2019) 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 used pathways defined in the KEGG database (Kyoto Encyclopedia of Genes and Genomes, http://www.kegg.jp/). We 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 figures S1-S3 display the human genes for which we could identify an ortholog as coloured and those without a human ortholog match as white.

Results

Cancer prevalence and pollution levels within tissues

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 categorised 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 2015-2022) 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 from protected marine areas, while the polluted habitats experience heavy marine traffic or inflow from industrial areas. We also measured levels of polycyclic aromatic hydrocarbons (PAH's) and heavy metals from fish livers. However, these pollutant measurements were not included to validate the definition of reference and polluted sites, as many other pollutants than those we measured could vary in space and between the two groups, and site characteristics + long term health data give a better view of the site category than one timepoint pollutant analysis. 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 to protect themselves against environmentally induced liver cancer.

Histopathology analysis confirmed cancer in 3 out of 40 flounders (1 from polluted 2 from reference sites, total prevalence 7.5%) and in 8 out of 50 dabs (3 from polluted, 5 from reference sites, total prevalence 16%, Table 2). Cancer in this case is defined as neoplastic changes and were 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. 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. We suggest that this decrease is due to successful international regulation of marine contamination. 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.

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 had cancer, and 6 out of 16 dabs. We used two methods, the EdgeR method and DESeq2 method, which perform the differential expression analysis slightly differently. In both approaches, p-values were adjusted and presented: for the EdgeR method, as the false discovery rate (FDR), and for the DESeq2 method, the p-value (adjusted for multiple testing with the Benjamini-Hochberg procedure). Of the 25378 gene ID's for flounder, the DESeq2 method recovered 449 gene ID's that were significantly differently expressed between fish with and without cancer (p-value adjusted <0.05), whilst the EdgeR method recovered 123 significantly differently expressed genes (FDR<0.05). For dabs, of the 23311 gene ID's, 86 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 (Figure 3, Ashburner et al. 2000, Bryant et al. 2017, The Gene Ontology Consortium, 2021). We ran similar analysis with only females included to control for the potential confounding effect of sex (Figure 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.

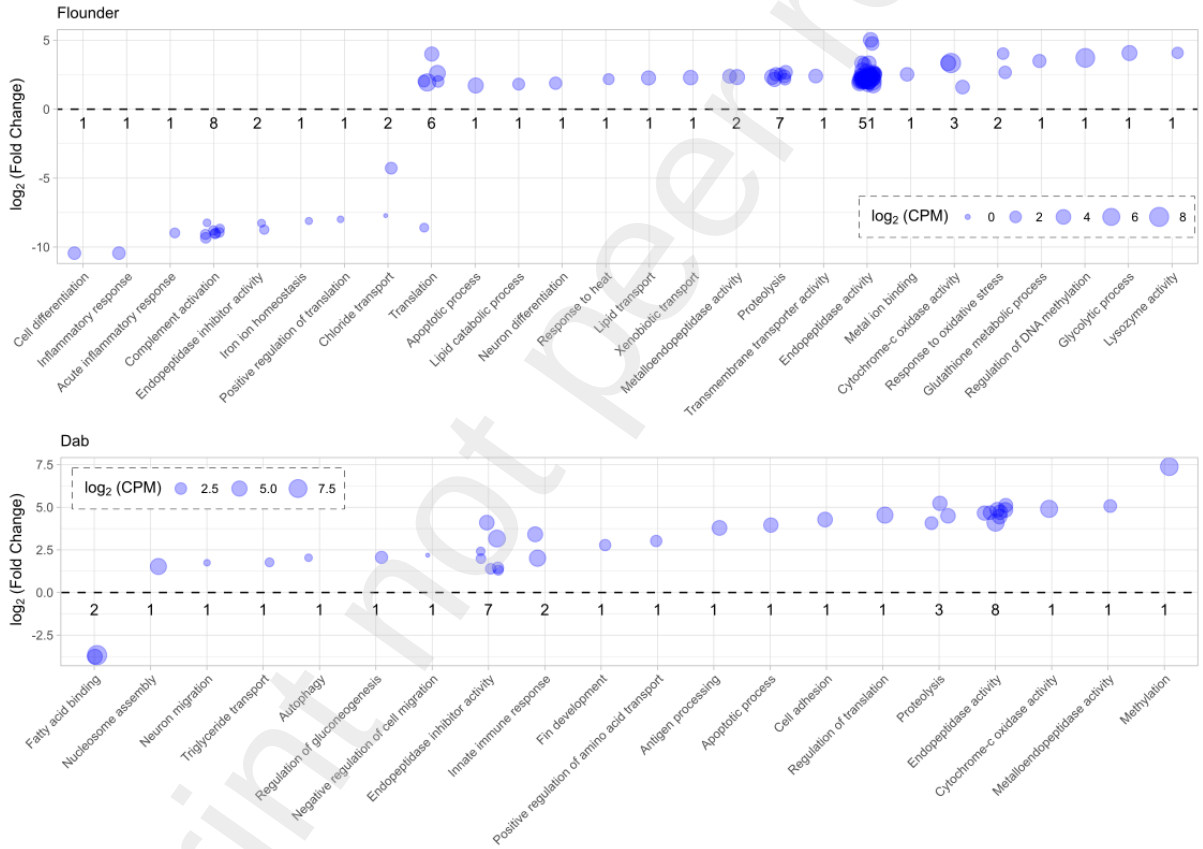

Figure 3. Comparison of transcripts from flounders (upper part) and dabs (lower part) with and without cancer. Y-axis indicates log(2) transformed ratio between gene expression levels in cancerous fish compared to healthy fish. Only annotated transcripts with false discovery rate under 0.05 are shown. 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).

Gene expression of healthy fishes from polluted and reference sites

Looking further into the possibility of local adaptation, we also compared non-cancerous fishes from polluted and reference sites to find possible signs of local adaptations in defence mechanisms against oncogenic pollution. Using the DESeq2 approach, 43 of transcripts were significantly differently expressed between polluted and reference sites in flounders (adjusted p-value <0.05), whilst 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), whilst 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, 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, whilst 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 amongst 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, Figure S1), and chemical carcinogenesis pathways for DNA adducts, reactive oxygen species and receptor activation (Supplementary materials, Figures 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 figures S1-S3).

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 ($p<0.001$). Compared to flounders, dab living in polluted vs reference sites showed fewer differences in gene expression (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

Table 3. 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.

Gene ID	Mean Reference Site	Mean Polluted Site	EdgeR FDR	Seq2 p - value (adjusted)	GO Category Molecular Function	Best Protein Match Actinopterygii
TRINITY_DN25209_c0_g1	48.35	9305.55	0.001	<0.001	fructose-1-phosphate aldolase activity	Fructose-bisphosphate aldolase
TRINITY_DN5857_c0_g1	0	199.00	0.004	<0.001	N/A	ubiquitin carboxyl-terminal hydrolase CYLD-like
TRINITY_DN25228_c0_g1	0	27.66	0.001	<0.001	N/A	uncharacterized protein LOC108412876

TRINITY_DN26272_c0_g1	0	25.59	0.001	<0.001	Apoptotic process	DNA damage-regulated autophagy modulator protein 2
TRINITY_DN15305_c0_g2	34.58	0	<0.001	<0.001	N/A	G protein-regulated inducer of neurite outgrowth 1
TRINITY_DN93825_c0_g1	21.15	0	0.001	<0.001	N/A	Salt-inducible kinase 1
TRINITY_DN1340_c1_g1	944.29	231.92	0.006	0.002	Carbohydrate binding	C-type lectin domain family 10 member A-like
TRINITY_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

TRINITY_DN5751_c0_g1	5771.08	485.34	0.029	0.0190	N/A	complement factor H-like
TRINITY_DN2799_c0_g1	5920.67	771.66	0.030	0.023	Antigen processing and presentation	major histocompatibility complex class I-related gene protein-like
TRINITY_DN3684_c1_g1	199.77	6.90	0.041	0.038	N/A	N/A
TRINITY_DN4442_c0_g1	497.79	56.84	0.041	0.043	Meiotic cell cycle	inactive peptidyl-prolyl cis-trans isomerase FKBP6

441

442 Table 4. Significant gene matches using both EdgeR and Seq2 methods comparing non-cancerous dab (*Limanda limanda*) from reference and
443 polluted sites. The best protein match is from OrthoDB at *Actinopterygii* level. Transcripts are categorized according to Gene Ontology (GO)
444 knowledgebase molecular functions. FDR – false discovery rate.

Gene ID	Mean Reference Site	Mean Polluted Site	EdgeR FDR	Seq2 p - value (adjusted)	GO Category Molecular Function	Best Protein Match Actinopterygii
TRINITY_DN31277_c1_g1	23.87	0	<0.001	<0.001	Endopeptidase inhibitor	uncharacterized protein LOC106633487
TRINITY_DN6114_c0_g2	856.52	114.27	<0.001	<0.001	N/A	hemopexin-like
TRINITY_DN41167_c0_g1	1670.71	40.46	0.004	<0.001	N/A	complement C1s subcomponent-like
TRINITY_DN20872_c0_g1	410.42	81.40	0.004	<0.001	Metal iron binding	fibrinogen beta chain

TRINITY_DN6706_c0_g1	1130.39	74.07	0.006	<0.001	Calcium ion binding	complement C1s subcomponent-like
TRINITY_DN10063_c0_g1	1497.18	109.39	0.006	<0.001	Calcium iron binding	complement C1s subcomponent-like
TRINITY_DN855_c0_g2	0	19.47	0.008	<0.001	N/A	guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase MESH1
TRINITY_DN3541_c0_g1	9259.83	402.54	0.019	<0.001	Calcium ion binding	complement C1s subcomponent-like
TRINITY_DN22589_c0_g1	1245.74	68.84	0.023	<0.001	N/A	Immunoglobulin-like domain
TRINITY_DN4021_c0_g1	193.27	0	0.027	<0.001	Metal ion binding	N/A
TRINITY_DN21243_c0_g3	33.23	0	0.047	0.013	Peptidase activity	transmembrane protease serine 6-like

Discussion

We found that gene expression patterns in the livers of dabs differ from flounders, with dabs showing mainly upregulation of genes caused by cancer, while in flounders we see downregulation of potential defence mechanisms. 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 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.

In dabs, we mostly observed the upregulation of genes in cancerous fish. 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 & 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. As a possible active defence mechanism against cancer in both species, we see upregulation of apoptosis-related transcripts in cancerous individuals. In flounders with cancer, transcripts related to antioxidant defences are also upregulated. We also see the upregulation of cytochrome C oxidase activity in cancerous individuals from both species, as well as an upregulation of xenobiotic transport in cancerous flounders, supporting 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 byproducts (metabolites of lipophilic chemicals with increased polarity for better excretion) of active pollutant metabolism leads to carcinogenesis via DNA adduct formation (Stegeman & 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). Similar correlations to DNA adducts and damage are also well-described in fish exposure studies (Peters et al. 1997; Santos et al. 2018). 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 & Mattson, 2017). Notably, hormetic effects have been found to be very useful in describing organisms' responses to toxicological challenges (Calabrese & Baldwin, 2003). 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 procarcinogenes (Oziolor et al., 2019; Celander et al. 2021). Interestingly, there is no evidence of similar adaptations through Phase II enzymatic activity in fishes published so far. Upregulation of the conjugation and transport systems would streamline the elimination of toxic metabolites from the body.

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 fished (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 (Bu et al. 2018, 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 (Peters et al. 1998). It remains controversial whether this gene is a tumour promoter or suppressor (Fang & Shen, 2017, Yu et al. 2008). 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 tumor 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 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 tumor 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, Zhuang et al. 2020, Zhou et al. 2021). Note that GPRIN1 expression level in fish from polluted sites was 0. 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 tumor suppressors or oncogenes, depending on the tissue of expression, but are more often seen as proto-oncogenes (Sun et al. 2020). Indeed, inhibition of SIK2 has been suggested to be a potential target in cancer therapy (Chen et al. 2019), and 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 (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 hemolysis 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

595 was annotated as transmembrane protease serine 6-like, which is involved in iron absorption
596 (uniprot.org) and inflammatory immune responses (Patel, 2017). Taken together, most of the
597 differences in gene expression between polluted and reference site dabs suggest lowered
598 (innate) immune responses, and there is, compared to flounders, little support to potential
599 adaptation with oncogenic pollution. This finding supports the KEGG pathway analysis, and
600 could also explain the higher prevalence of liver cancer observed in dabs compared to
601 flounders.
602 In conclusion, using whole genome transcriptome analysis, our study highlighted potential
603 mechanisms and pathways that protect flounders against the oncogenic effects of pollution,
604 lowering their pollution-induced cancer vulnerability compared to dabs. Specifically, more
605 active pollutant metabolism is observable in the liver tissue of flounders, potentially leading to
606 either hormetic upregulation of tumour suppression mechanisms, or to a stronger natural
607 selection pressure for higher cancer resistance. In flounders, we were able to link the
608 differences in gene expression levels to known tumour suppression mechanisms, while in dabs,
609 only indication of immune suppression was found in polluted sites. This study indicates that
610 study of wild species could offer novel insights for understanding the nature and evolution of
611 natural cancer defence mechanisms, and the differing potential of wild species to adapt to
612 anthropogenic environmental change.
613

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Competing interests statement

Authors declare no competing interests.

Figure legends

Figure 1. Pollution as a selection pressure shaping cancer defences. Based on the conceptual figure presented in Roche et al. 2012

Figure 2. 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.

Figure 3. 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).

Figure 4. Site-specific levels of selected pollutants in fish liver (heavy metals; μg g^{-1} dry weight) and bile (polycyclic aromatic hydrocarbon metabolites; fluorescence units (FU)). Napht – naphthalene, phen – phenanthrene, pyr – pyrene, BaP - benzo(a)pyrene, As – arsenic,

Cd – cadmium, PG – lead, Hg – mercury. The results have been brought to the same scale with the transformations shown on x-axis for each pollutant. Green hues (and * by site code) indicate reference sites and red hues indicate polluted sites. Site (B09, B12, GB1, GB4, NO4, B11) codes are further explained in Table 3.

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