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CIARA BAINES

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Department of Zoology, Institute of Ecology and Earth Sciences, Faculty of Science and Technology, University of Tartu, Estonia

Department of Fish Biology and Fisheries, Estonian Marine Institute, Faculty of Science and Technology, University of Tartu, Estonia

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Supervisors: Dr.Tuul Sepp, University of Tartu, Estonia
Dr. Lauri Saks, University of Tartu, Estonia
Dr. Mathieu Giraudeau, University of La Rochelle, France

Opponent: Prof. Joachim Sturve, University of Gothenburg, Sweden

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1. Baines, C., Lerebours, A., Thomas, F., Fort, J., Kreitsberg, R., Gentes, S., Meitern, R., Saks, L., Ujvari, B., Giraudeau, M. and Sepp, T., 2021. Linking pollution and cancer in aquatic environments: A review. *Environment International*, *149*, p.106391.

2. Baines, C., Meitern, R., Kreitsberg, R. and Sepp, T., 2022. Comparative study of the evolution of cancer gene duplications across fish. *Evolutionary Applications, 15*(11), pp.1834–1845.

3. Sepp, T., Baines, C., Kreitsberg, R., Scharsack, J. P., Nogueira, P., Lang, T., Fort, J., Sild, E., Clarke, J. T., Tuvikene, A., Meitern, R. 2023. Response to oncogenic pollution in two fish species: are there differences in adaptive potential? Under review

4. Baines, C., Meitern, R., Kreitsberg, R., Fort, J., Scharsack, J. P., Nogueira, P., Giraudeau, M., Sepp, T. 2023. Correlations between oxidative DNA damage and formation of hepatic neoplasms in two flatfish species from contaminated environments. Accepted in Biology Letters

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Authors contribution to the papers (* denotes a moderate contribution, ** denotes a high contribution, *** denotes a leading role).

	I	II	III	IV
Original Idea	**	**		***
Study Design	**	*	*	**
Data Collection	***	***	***	***
Data Analysis	***	*		***
Manuscript Preparation	***	**	**	***

1. INTRODUCTION

1.1 Cancer and cancer defences in wild animals

It was estimated by the world health organisation that worldwide, nearly 1 in 6 deaths in humans were caused by cancer in 2020 (WHO, 2022). Cancer is a malignant growth of cells that is capable of invading other tissues (WHO, 2022). Cancer came into existence with the evolution from unicellular to multicellular animals and it is widely understood that cancer can affect any multicellular animal from invertebrates to mammals (Aktipis *et al*., 2015). The evolution from unicellular organisms to multicellular organisms involved developing processes to ensure cell cooperation which reduced the risk of cells mutating and developing into cancer (Trigos *et al.*, 2018). Processes such as metabolism and proliferation are generally considered unicellular processes, as single celled organisms did not need to evolve processes that would encourage cell cooperation (Trigos *et al.*, 2018). However, with the evolution of multicellularity came the need to control for potential 'anti-social' mutations and, as a result, cancer. This led to the promotion of genes involved in tissue maintenance, differentiation, and communication with the environment (Trigos *et al.,* 2018). Connections between these unicellular and multicellular genes regulate homeostasis in normal tissue, however, when disrupted they can contribute to malignant growth (Trigos et al, 2018). Cancer can occur through mutations that are either inherited through the germline or occur somatically, and it is estimated that approximately 90% of cancers occur from somatic mutations (Sondka *et al.,* 2018). Somatic mutations can accumulate across an organism's lifetime. There are numerus causes for mutations that can alter DNA and they can occur from either endogenous or exogenous damage (Caulin & Maley, 2011). Some examples of processes that can lead to endogenous damage are oxidation, hydrolysis and alkylation whereas exogenous sources occur for example from radiation or different chemicals (Hakem, 2008). To reduce the risk of cancer from cell mutations, all species have evolved defences that protect against cancer, for example, through the evolution of genes that either promote or control mutations.

Proto-oncogenes and tumour suppressor genes (TSGs) are amongst the oldest gene classes found in multicellular organisms. These genes limit cell mutation rates, that could lead to neoplastic growth (Makashov *et al.,* 2019). Oncogenes (OGs) are mostly derived from proto-oncogenes. This group of genes are highly conserved because of their important roles in encoding proteins to inhibit cell differentiation, halt cell death and stimulate cell division (Chial, 2008), which ensure growth of an organism through the maintenance of tissues and organs (Lodish, 2000). As a result of their important role in tissue maintenance, proto-oncogenes have been highly conserved (Lodish, 2000). However, when proto-oncogenes are converted into oncogenes through gain-of function mutations, they start to encode proteins that induce cancer (Croce, 2009). To counteract the increased risk of cancer from oncogenes, species have evolved genetic controls for potential carcinogenic mutations, called tumour suppressor genes (Kumari *et al.,*

2014). These are divided into two main categories; caretakers and gatekeepers based on their main functions. Caretaker genes are involved in the protection of genome stability by maintaining the integrity of genetic information within each cell through, for example, DNA repair. Gatekeepers, however, directly regulate tumour growth by encoding for proteins that either stimulate or inhibit proliferation, differentiation, or apoptosis (Weitzman, 2001).

Multicellular animals have evolved cancer defences such as TSGs, however, not all species have the same rate of cancer. According to Peto's paradox, body size and lifespan are not related to cancer rate between species (Peto, 1975; Caulin & Maley, 2011; Tollis *et al.,* 2017). As a result, an animal that is 1000 times larger than another would be expected to develop much higher rates of cancer (due to an increased number of cells) than actually occurs (Caulin & Maley, 2011) and equally, small animals such as rodents would likely not develop cancer (Litchenstein, 2005). Cancer mortality ranges from 0% to 57.14% across mammals (Vincze *et al.,* 2022) with body size or lifespan appearing to have little influence on this variation. For example, cancer is responsible for approximately 17% of human deaths (WHO, 2022), 18.7% of deaths in equids (Miller *et al.,* 2016), and 4.81% in elephants (Abegglen *et al.,* 2015). However, on the intraspecific level, cancer rate is related to body size which suggests different cancer defences occur between species compared to between individuals within a species. In humans, for example, a leg length of 3–4 mm above average increases non-smoking cancer risk by as much as 80% (Albanes, 1998).

One important mechanism for creating genetic novelty is gene duplication (Magadum *et al.,* 2013; Ohno, 1970), and the duplication of TSGs is one way of reducing a species cancer risk (Vazquez & Lynch, 2021). Species that are at higher risk of cancer due to bigger size and longer lifespans are likely to have evolved increased copies of genes that, in turn, reduce their cancer risk. For example, elephants (*Proboscidean* lineage) have evolved 20 copies of the TSG TP53, whereas humans have only one copy (Sulak *et al.,* 2016, Abegglen *et al.,* 2015). Additionally, in blind mole rats (*Spalax* sp.), the duplication of genes related to the interferon pathway, leads to interferon-mediated concerted cell death, a strategy that has been proposed to counteract the weakened pro-apoptotic function of the p53 protein in this species (Gorbunova *et al.,* 2012).

The use of comparative analyses to determine differences in the number of copies of TSGs, between species, has the potential to increase our understanding of how different species have evolved mechanisms to reduce their cancer risk. However, most comparative analyses have so far, been limited to mammals (e.g. Abegglen *et al.,* 2015; Seluanov *et al.,* 2018; Tejada-Martinez *et al.,* 2021; Tollis *et al.,* 2020; Vazquez & Lynch, 2021; Yu *et al.,* 2021). Widening the scope for comparative analysis to other vertebrate groups and beyond could provide novel understandings into the evolution of cancer defences across the tree of life (Nair *et al.,* 2022; Sepp & Giraudeau, 2022). Fishes for example, and more specifically bony fishes, are evolutionarily older and genetically more diverse than mammals (Buchmann, 2014). They are a paraphyletic group, whose last common ancestor is also an ancestor of the tetrapod's and, consequently, all mammals. Furthermore,

fishes may provide insight into novel and potentially phylogenetically older tumour suppression mechanisms that are not present in mammals. There is evidence that fish lineages have evolved increased rates of duplicated genes compared to mammals (Robinson-Rechavi & Laudet, 2001), suggesting a possibility that tumour suppression and gene duplications could be related to life-history more closely in fish compared to mammals. For example, all teleost fish have gone through three rounds of whole genome duplication (WGD), and a fourth round of duplication has taken place in salmonids (the salmonid-specific auto-tetraploidization event), which occurred in the common ancestor of salmonids ~100 Mya (Lien *et al.,* 2016). Genetic cancer defences have evolved to reduce cancer risk from natural sources. However, anthropogenic change has the potential to disrupt the effectiveness of these defences, potentially leading to increased rates of cancer in our rapidly changing environments.

1.2 Aquatic environment and cancer

Whilst we know that anthropogenic environmental change can affect cancer occurrence in humans (Boffetta, 2006) and wild animals (Giraudeau *et al.*, 2018), environmental factors are often difficult to study in the laboratory ("The global challenge of cancer" 2020). For example, many pollutants have been linked to cancer, but the interactions between various cocktails of contaminants can increase cancer risk at much lower concentrations than seen in studies of individual pollutants (Lagunas-Rangel *et al.*, 2022). Due to the interconnectedness of aquatic ecosystems through highly effective marine, and atmospheric transport routes, and the persistence of many pollutants in the sediments, aquatic species face increasing oncogenic pressures from anthropogenic contamination. Although there are some pollutants, produced by humans that also have natural sources, such as polycyclic aromatic hydrocarbons (PAHs), many pollutants, that have oncogenic potential are not naturally found in our aquatic systems and increase pressure on species inhabiting contaminated environments. It is estimated by the European Union (EU) that approximately 100,0000 new substances will become emerging pollutants in aquatic systems in the near future (Brack *et al.,* 2018) and as such, organisms living in these environments will face an ever-increasing pressure from anthropogenic contamination. There is increasing evidence that anthropogenic contamination can cause cancer in aquatic species (e.g. Martineau *et al.,* 2002; Randhawa *et al.,* 2015; Brown *et al.,* 1973; Black & Baumann, 1991). The majority of the pollutants that have been linked to cancer in aquatic animals are heavy metals, PAHs and pesticides but it is likely that there are other pollutants that could potentially increase cancer rates in aquatic systems that have yet to be studied.

Anthropogenic pollution has put increasing pressure on aquatic systems for around 200 years, since the start of the industrial revolution (Quadra *et al*., 2019). This length of time therefore could provide the possibility for rapid adaptation, or acclimation to these new pressures in the environment and there is evidence to suggest that this can occur in some species. For example, brown bullheads

(*Ameiurus nebulosus*) are adapted to life in habitats with high levels of industrial pollution and get less cancer than fish living in cleaner environments (Baumann & Harshbarger, 1998) and Atlantic killifish (*Fundulus heteroclitus*) show evidence of adaptation to the exposure to PAHs through various pathways and mechanisms (reviewed in Di Giulio, 2015).

The North and Baltic Seas are considered some of the most polluted marine areas in the world (HELCOM 2018; Lehtonen *et al*., 2006), because of the release of high levels of known oncogenic contaminants (Mathew *et al*., 2017; HELCOM, 2018) since the beginning of the industrial revolution (HELCOM, 2018). As a result, it is possible to use these polluted environments as 'natural laboratories' to better understand the interactions between the evolution of cancer defences and pollution. Both European flounder (*Platichthys flesus* L.) and dab (*Limanda limanda* L.), which have been regularly used in biomonitoring studies in the North Sea and the Baltic Sea, inhabit a gradient of relatively clean to severely polluted habitats in the Baltic and North Seas. These species diverged from each other approximately 10.9 million years ago (mean age derived from the following 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). With a generation time of only 2–3 years, these species have been subjected to anthropogenic contamination for a minimum of 50 generations. Both benthic species are considered marine sentinel species due to their close contact with marine sediments, where they are exposed to higher levels of accumulated contaminants.

European flounders and dabs both show susceptibility to cancerous skin and liver lesions (Vethaak *et al*., 2009) and evidence suggests that dabs have almost 10 times higher prevalence of liver neoplasms than European flounders (Cachot *et al.,* 2013; Lang *et al.,* 2006; Stentiford *et al.,* 2003; Vethaak *et al.,* 1996; Lyons *et al.,* 2006; Stentiford *et al.,* 2009; CEFAS report, 2004). Furthermore, flounders living in highly polluted sites do not seem to have higher cancer prevalence compared to their conspecifics in cleaner sites (Vethaak & Jol, 1996; De Boer *et al*., 2001). Whereas in dabs, cancer prevalence does vary relative to local pollution levels (Lerebours *et al*., 2014). This suggests that there are differences in cancer defences both when comparing flounders and dabs but also within the species, dependant on whether a population is living in a heavily polluted area or not. Exploring these potential differences in cancer defences in wild organisms could help to predict cancer dynamic in our changing environments, and whether a species has adapted to cope with pollution-induced cancer.

The number of copies of tumour suppressor genes are an important part of natural cancer defences however, neither flounders nor dabs have had their genomes sequenced to date. Therefore, exploring other variables such as the expression of genes in cancerous and normal cells is a possible avenue for understanding how species reduce cancer risk (e.g. Zhang *et al.,* 1997). Knowledge about how cancer defence mechanisms vary between species is limited and is mostly based on the study of model organisms with low genetic diversity in laboratory environments (Ducasse *et al*., 2015). 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 the expression of genes related to osmoregulation, heme biosynthesis, and stress resistance (Larsen *et al*., 2008). 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). Exploring the differences in gene expression between individuals with and without cancer could offer a better understanding of how individuals prevent neoplastic development.

Additionally, focusing on specific physiological pathways is another way to unravel the mechanisms behind cancer defences in natural populations. One of these mechanisms that has been extensively linked to both cancer and anthropogenic contamination in humans is oxidative DNA damage (Valavanidis *et al.,* 2009; Singh *et al.,* 2007; Hengstler *et al.,* 2003). However, relatively little is known about how changes in oxidative stress impact the risk of cancer in wild organisms, particularly in pollution-exposed populations. The majority of studies have so far focused on the changes in antioxidant levels following exposure to a specific contaminant (e.g. in goldish [*Carassius auratus*, Liu *et al.,* 2015], medaka [*Oryzias latipes* Tu *et al.,* 2016], and reviewed in Isaksson, 2010). However, due to time-lagged and hormetic upregulation of protective mechanisms, there are limitations in relying on antioxidants as an accurate indication of oxidative stress (Meitern *et al.*, 2013). The biomarker, 8-hydroxy-2′ -deoxyguanosine (8-OHdG), quantifies oxidative lesions, formed in nuclear or mitochondrial DNA, induced by free-radicals. The levels of 8-OHdG have been used as a biomarker for measuring pollution-induced oxidative damage for both organic pollutants (Singh *et al.*, 2007) and heavy metals (Hengstler *et al.*, 2003)

1.3 Aims and Hypotheses

1. To undertake a literature review to determine whether there is evidence that anthropogenic pollution can increase cancer rates in aquatic animals in wild environments (review paper **I**).
2. To analyse and compare the number of copies of cancer-related genes across fish genomes to test whether there is a relationship between the number of copies of cancer related genes and the lifespan of fishes (comparative analysis **II**).
3. To test whether there are differences between tumour rates in wild populations of flounders and dabs and determine if these differences could, at least in part, be explained by the differences in gene expression and levels of oxidative damage seen between the two species (field studies **III**, **IV**).
4. To use a field study of wild populations of flounders and dabs caught in the North and Baltic Sea to test whether there is a difference between cancer rates, oxidative DNA damage and gene expression in populations living in more heavily polluted environments compared to reference sites of both species (field studies **III**, **IV**).

2. METHODS

2.1 Literature review (I)

In order to collate the literature on both cancer in aquatic animals and research linking cancer with pollution in aquatic environments (paper I), various google scholar searches were undertaken. Searches included a variety of terms including: 'cancer', 'histopathology', 'neoplasia', 'histology', 'aquatic animals', 'marine', freshwater', 'pollution', 'contamination', 'PAH', 'pesticides', 'heavy metals'. Data was collected to determine the species affected, whether the study was done in the wild/laboratory, the cancer prevalence (if available), the organ affected, the type of cancer, the habitat of the species and whether pollution was linked to increased cancer prevalence. Additionally, other information such as the type of cancer (including transmissible and viral-induced cancers), the species migratory status and mechanisms (e.g. metabolic pathways and oxidative damage) that could influence cancer progression in aquatic systems were also reviewed.

2.2 Comparative analysis (II)

For the comparative analysis of cancer-related gene copy numbers in fish (paper II), the list of cancer-related genes used in our study was extracted from the COSMIC database. This database lists human cancer-related genes, which are categorised as either oncogenes (OGs) or tumour suppressor genes (TSGs). The Ensemble Biomart orthology database and Ensemble CAFE were used to calculate gene copy numbers across species. The Ensemble CAFE species trees were downloaded for all COSMIC genes to provide an estimation of gene gain and loss for each species while accounting for lineage (De Bie *et al.,* 2006; Herrero *et al.,* 2016). Next, the unique confident orthologs in each fish species were counted and represented in the Ensembl database, using BioMart, for each gene.

Trait data, specifically maximum length and lifespan data was mostly collected from FishBase (Froese & Pauly, 2021) and AnAge databases (Tacutu *et al.,* 2017) but if unavailable then it was collected from other reliable sources (e.g. articles or websites). Species with no maximum lifespan data were excluded from the dataset.

Normalisation of the copy number counts (by dividing the sum of all gene copies, for all genes, with the total number of orthologous genes found for that species (Tollis *et al.,* 2020)) accounted for any potentially missing orthologs from incomplete genome sequencing or assembly. The normalized copy number counts were standardized (i.e., converted to z-scores) prior to all analyses. Genes classified as both TSGs and OGs in the COSMIC database were excluded from the calculation of copy numbers. Additionally, the TSG/OG ratio was calculated by dividing the normalized TSG count with the normalized OG count.

The longevity quotient (LQ) was calculated according to Tollis *et al*. (2020), to give an indication of how lifespan compares to other species of a similar size

(LQ = observed longevity/expected longevity). For each species, expected longevity was calculated by fitting a linear regression to log10(maximum longevity) and log10(body mass).

Phylogenetic trees for each species, with branch lengths, were obtained from timetree.org (Kumar *et al.,* 2017). Maximum body length and lifespan were log transformed prior to analysis. All statistical analysis was performed in R (version 4.0.5, R Core Team, 2021) using the caper package (Orme *et al.,* 2013) for phylogenetically informed regressions. Branch lengths were optimised using maximum likelihood which are provided as λ, κ and δ values which correspond to Pagel's branch-length modifications (Pagel, 1997; 1999). Branch optimisation was undertaken to confirm that the results were not heavily dependent on default λ, κ and δ values. For more details on λ, κ and δ, see the caper package manual (Orme *et al.,* 2013). Other used packages included base, utils, stats, (R Core Team, 2021) ggplot2 (Wickham *et al.,* 2016), ggtree (Yu, 2020), tidytree (Yu, 2021), biomaRt (Durinck *et al.,* 2009), ape (Paradis & Schliep, 2019), AnnotationDbi (Pagès *et al.,* 2019), dagitty (Textor *et al.,* 2016) and numerous dependencies within those.

2.3 Study System and Fieldwork (III, IV)

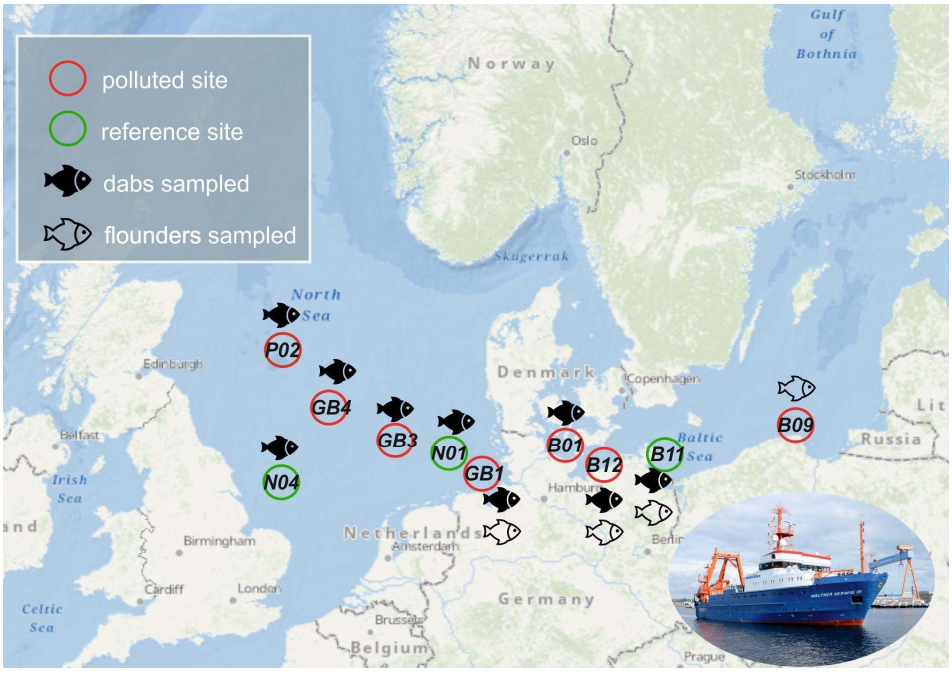

Figure 1. *Map of the 10 sampling locations in the North Sea and the Baltic Sea. The coloured circles represent whether a site was categorised as reference (green) or polluted (red) based on the local anthropogenic pressures (see Table 1 for more details). Fish symbols represent which species were caught and therefore sampled at each location. Transparent fish represent locations with flounders and black fish represent locations with dabs. Photo shows research vessel Walter Herwig III (modified from paper* ***III****).*

Two species, dab and flounder, were chosen to study local adaptations to oncogenic contamination. Both species are benthic flatfish that are widely used in ecotoxicological studies and monitoring studies (e.g. Hylland *et al.,* 2017; Vethaak *et al*., 2009; CEFAS report, 2004; Cachot *et al*., 2013). The liver was focused on for both cancer and contaminant measurements as the liver is heavily involved in contaminant removal (Feist *et al.,* 2004) and liver cancer is common in both species (Vethaak *et al*., 2009), although, it appears to be less prevalent amongst flounders (Cachot *et al*., 2013; Lang *et al.*, 2006; Stentiford *et al*., 2003; Lyons *et al*., 2006; Stentiford *et al*., 2009; CEFAS report, 2004).

The fieldwork was conducted in collaboration with the Thünen Institute, Germany. A research cruise was undertaken in the North Sea and Baltic Sea in August 2019 onboard fishing vessel Walter Herwig III. Fish were caught using a trawl at 10 sites (Figure 1). 1-hour trawls were undertaken, and fish were immediately sorted and placed into fresh sea water at ambient temperature and sampled within 1 hour. A maximum of 10 dab and 10 flounder were sampled at each site, where present, and a total of 128 fish were sampled consisting of 88 dab and 40 flounder. They were weighed (g) and length measured (mm) and then euthanized by a percussive blow to the head followed by the destruction of the brain using a surgical knife (following FELASA guidelines (EU directive 2010/63)). Otoliths were collected to determine the age of the fish. Livers were assessed for external lesions and a 3 mm slice was cut for histopathology analysis and stored in 4% formalin for 24 hours then transferred to 70% ethanol for storage until analysis (Feist *et al.,* 2004). If external lesions were present, then healthy and tumorous tissue was included in the sample. Additional liver samples were collected for trace metal, transcriptome and oxidative DNA damage analysis. Bile samples were collected for PAH analysis. All samples were flash frozen in liquid nitrogen immediately and then stored at –80 °C until analysis.

All study areas were categorised based on local anthropogenic pressure and divided into more polluted/affected (marked as ‘polluted’) and less affected areas (marked as ‘reference’). Site categories were 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/, see Table 1). Pollutant measurements (PAH’s and trace metals) were not included to validate the definition of reference and polluted sites, as many other pollutants could be present.

Table 1. *Sampling locations and description of all sites sampled in the North and the Baltic Seas. Asterix (*) next to site names represents the locations used in the transcriptomics analysis.*

Code	Site name	Coordinates	Number sampled	Category	Description
B01	Kiel Bay	54°32.743'N 010°47.946'E	10 dabs	polluted	Heavy marine traffic. TBT-specific effects are still found in maritime areas even after global 2008 ban (OSPAR, 2010).
B09*	Gulf of Gdańsk	55°06.93'N 018°10.90'E	10 flounders	polluted	Inflow from the Vistula estuary. Effect of industry (Zaborska *et al.,* 2019).
B11*	Arcona Sea	54°45.39'N 013°11.91'E	10 dabs and 10 flounders	reference	Wind parks and marine protected areas.
B12*	Kiel Area	54°14.87'N 011°44.34'E	10 dabs and 10 flounders	polluted	Heavy marine traffic. Historical TBT effects.
GB1*	German Bight South	54°04.54'N 007°53.71'E	10 dabs and 10 flounders	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).
GB3	German Bight Central	54°58.706'N 006°22.933'E	10 dabs	polluted	Area of extensive maritime activities.
GB4*	German Bight North	55°23.29'N 004°32.44'E	10 dabs	polluted	Area of extensive maritime activities.
N01	German Bight South	54°11.886'N 007°38.978'E	10 dabs	polluted	Area of extensive maritime activities. Inflow from the rivers Elbe and Weser (Hylland *et al.,* 2017).
N04*	Dogger Bank	54°46.26'N 002°02.23'E	10 dabs	reference	Shallow 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).
P02	Ecofisk oil field	56°40.979'N 003°11.859'E	10 dabs	polluted	Anthropogenic impacts from offshore oil drilling facilities.

A new species of flounder was recently described in the Baltic Sea (Momigliano *et al.,* 2018) and we ran genetic analyses, to confirm that all flounders caught during this study were *Platichthys flesus* and not the newly described *Platichthys solemdali.* Therefore, the Purelink Genomic DNA Mini Kit (Invitrogen) was used for DNA extraction from liver samples. Distinguishing flounder species was performed by analysing species-specific single nucleotide polymorphisms (SNPs) according to Momigliano *et al*. (2018), except that five SNP loci were analysed instead of the original six (886, 1882, 3556, 3599, 4474), as recommended in Momigliano *et al.* (2019). Analyses were performed at the University of Tartu, department of Zoology by Urmas Saarma and Egle Tammeleht.

2.4 Sample analysis (III, IV)

Histopathology analysis was undertaken according to Feist *et al.* (2004). Samples were dehydrated before being embedded in paraffin wax. Samples were sliced using a microtome to a width of 4–5 μm, floated in a water bath and embedded onto clean glass slides and dried. Samples were then stained with haematoxylin and eosin (H&E), dehydrated, cleared and mounted for analysis. Slides were analysed using a Nikon Eclipse 80i microscope to determine the prevalence of neoplastic changes. These neoplastic changes were diagnosed using the criteria set out by Feist *et al.* (2004). Both neoplastic lesions (adenomas and carcinomas) and pre-neoplastic lesions (foci of cellular alteration, FCA) were diagnosed.

Pollutants were measured from bile and liver tissue samples. Firstly, polycyclic aromatic hydrocarbons were measured in bile samples. Samples were diluted with 48% ethanol to a ratio of 1:1600 (Aas *et al.,* 2000) and pipetted into a 96-well plate. Samples were analysed using a fluorescence spectrophotometer (BMG Omega Fluostar). Excitation and emission fixed wavelengths were measured, respectively for the detection of 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 & Anderson, 2005). Results were normalised against samples only containing 48% ethanol and presented as fluorescence units (FU), which were proportional to the concentration of PAH metabolites (Beyer *et al.,* 2010).

Heavy metal concentrations were measured in liver tissue. Samples that had been frozen and stored at –80. Liver samples were freeze-dried for 48 hours and ground to powder for homogenization. Total Hg concentrations were measured, in duplicate (ensuring relative standard deviation for aliquots was <10%), in subsamples of ~1 mg of homogenized liver using an advanced Hg analyser spectrophotometer (Altec AMA 254, Bustamante *et al*., 2006). Remaining samples were analysed for trace elements arsenic (As), cadmium (Cd), and lead (Pb). Briefly, samples were digested with a mixture of 3 mL HNO3 and 5 mL HCl Suprapur quality, heated in a microwave oven and diluted to 50 ml with deionized water. Trace element concentrations 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).

Oxidative damage to DNA was analysed from liver samples. The Purelink Genomic DNA Mini Kit (Invitrogen) was used for DNA extraction from liver samples stored at –80 °C. Extracted DNA was quantified using a Qubit 4 fluorometer. Standards were read on the dsDNA HS setting to calibrate the machine. 1 µL of sample was added to 199 µL of the working solution in triplicates, analysed, and the means calculated in ng/µL. The ELISA kits, for measuring oxidative DNA damage (8-OHdG quantification), were purchased from Cell Biolabs Inc (cat number STA-320). Samples were prepared following the protocol for cell or tissue DNA samples by converting to single strand DNA, digesting DNA using nuclease P1 (M0660S, New England Biolabs), adding alkaline phosphatase (CIP, M0371S New England Biolabs), centrifuging at 6000g for 5 minutes and collecting supernatants. The assay protocol was followed as described. Plates were washed using BioTek ELx50 microplate washer and absorbance (wavelength 450 nm) measured using a spectrophotometer (Biotek Synergy 2). Results were normalised against the DNA concentrations, converted from 1 to 4 strand DNA, and presented as 'ng oxidised DNA bases per mg DNA'.

A total of 30 fishes were chosen for transcriptome analysis, split as 14 flounders and 16 dabs which represented a subsample of fish with neoplasms (2 flounders, 6 dabs) and without neoplasms (12 flounders, 10 dabs). They were caught from two reference (N04, B11) and 4 polluted sites (GB1, GB4, B09, B12). Whole transcriptome sequencing analysis (RNA-Seq) was performed to acquire gene expression data. Total RNA was extracted from liver samples that were stored in RNAlater using the RNeasy mini kit from Qiagen (cat. 47104). The tissue was disrupted using a pestle and mortar and then homogenised by adding 600 µL 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. Samples were analysed using the mini kit protocol part 1 and the DNase digestion in part 2 of the mini kit protocol with one final elution step. 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.

2.5 Statistical analysis (III, IV)

Transcriptome sequencing and analysis was 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). 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 the latest 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). Other used R packages included several packages from 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 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).

Statistical analyses for oxidative DNA damage were run using R 4.0.5(R Core Team, 2021). Principle component analysis (PCA), using packages stats (Revelle, 2022) and psych (Harrell, 2022), grouped 3 components splitting the variables into 'size + age' (age, weight, length), metals (As, Cd, Pb, Hg) and hydrocarbons (2, 3, 4, 5 ring). Generalised linear models tested whether oxidative DNA damage or tumours could be predicted by the PCA components, location (Baltic Sea or North Sea), or whether sites were polluted/reference (Table 1). Chi-squared tests tested for a difference between the number of fishes with or without neoplasms/ FCAs. Finally, ANOVA and post-hoc Tukey tests tested for differences between oxidative DNA damage and tumours. Other packages used for plotting and data reorganisation were ggplot2 (Wickham *et al.,* 2016), tidyverse (Wickham *et al.,* 2019), car (Fox & Weisberg, 2019) and various dependencies within them.

3. RESULTS

3.1 Cancer in aquatic animals (I)

Throughout the literature, cancer has been diagnosed in approximately 300 aquatic species ranging from invertebrates to mammals. However, only around 30 of these species have been included in studies that link pollution to cancer prevalence (Figure 2). Most of these studies have been focused on fish and molluscs.

Studies on cancer in invertebrates were relatively common and mostly focused on molluscs. Molluscs develop two main types of cancer, disseminated neoplasia and gonadal neoplasia. A few studies have found links between pollution exposure and cancer prevalence. For example, polychlorinated bisphenols (PCBs) led to cancer rates of 90% in a population of soft-shell clams (*Mya arenaria*, Reinisch *et al.,* 1984). Three species of planaria have been studied regarding pollution-induced cancer due to their regeneration capabilities (*Girardia tagrina*, Voura *et al.,* 2017; *Girardia dorotocephala*, Hall *et al.,* 1986; *Bdellocephala brunnea*, Hoshina &Teshirogi, 1991). In cnidaria and crustaceans, studies have found neoplastic changes, but no studies have linked pollution to them.

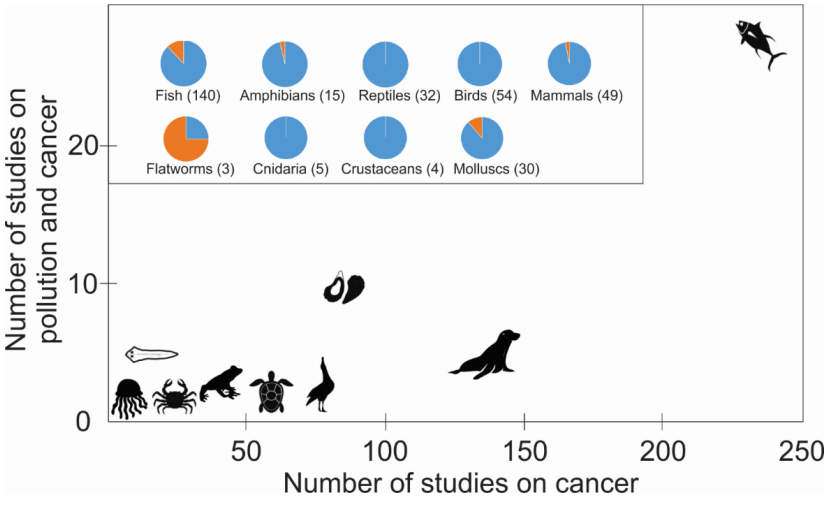

***Figure 2**. Number of studies that indicate cancer in aquatic and semi-aquatic species, and the number of these studies that link cancer occurrence to pollution. Groups of animals depicted in this figure include (from left) cnidarian, flatworms, crustaceans, amphibians, reptiles, birds, molluscs, mammals, and fish. Pie charts illustrate proportions of pollution-associated studies per group, numbers in brackets indicate the number of species studied so far (from paper **I**).*

Fish studies linking cancer and pollution have been approached with three main methods. Firstly, laboratory studies, exposing animals to specific types and concentrations of carcinogens to determine their susceptibility to neoplasia, for example in the zebrafish (*Danio rerio*, Beckwith *et al.,* 2000; Spitsbergen *et al.,* 2000a; Spitsbergen *et al.,* 2000b). Secondly, studies exploring the relationship between cancer in wild fish and the level of contaminants within the tissues of the animal (usually liver or bile) have been undertaken in a range of species including the dab (Lerebours *et al.,* 2014), winter flounder (*Pseudopleuronectes americanus,* Chang *et al.,* 1998) and European eel (*Anguilla Anguilla*, Ribeiro *et al.,* 2005). Thirdly, there have been studies testing for relationships between the concentrations of contaminants in the sediments and cancer prevalence in wild animals. One example is a study of brown bullheads following the closure of a coking plant which found reductions in the levels of PAHs in the sediments, leading to a significantly decreased prevalence of neoplasia in the local population of fish (Baumann & Harshbarger, 1998).

In aquatic mammals, there were two species that have been targeted for pollution and cancer studies: the beluga whale (*Delphinapterus leucas*) and the Californian sea lion (*Zalophus californianus*). In an isolated population of beluga whales in the St. Lawrence estuary, Canada, higher rates of cancer resulting from benzopyrenes in the sediment have been found compared to Arctic populations (Martineau *et al.,* 2002). In Californian sea lions, the carcinomas have a viral etiology, specifically the Otariine herpesvirus 1, but studies have also found a positive relationship between the concentrations of PCBs present in the sea lion blubber and the rate of carcinomas (Ylitalo *et al.,* 2005).

In other vertebrates, cancer and pollution studies are limited to date. The main reptile studied is the green turtle (*Chelonia mydas*) which develops fibropapillomas. For example, dos Santos *et al.* (2010) used an ecological index to determine water quality and showed an increase in fibropapillomas prevalence from 0% in clean waters to 58.8% at polluted sites. In amphibians, one laboratory study explored how exposure to the carcinogenic PAH, 3-methylcholanthren induces sarcomas in leopard frogs (*Rana pipiens*) (Outzen *et al.,* 1976). Additionally, a study in marsh frog (*Rana ridibunda*) found histopathological alterations, diagnosed as the early stages of holangiofibroma, from a polluted river in northern Greece. No histopathological alterations were observed in frogs from a clean river (Loumbourdis, 2007). Currently, aquatic birds are known to accumulate contaminants but there are no studies linking increased cancer prevalence to pollution exposure in this taxon.

Of the 30 species studied in publications linking pollutants and aquatic wildlife cancer, the choice of contaminants investigated is limited. Heavy metals and PAHs dominate, with few studies focusing on PCB's, pesticides, chlorine or general industrial or sewage pollution. Additionally, the main organs studied in wildlife cancer studies are the skin and liver, with other organs being largely ignored or unstudied.

Understanding the mechanistic causes of pollution induced cancer is another area that is understudied in aquatic species. The studies that have investigated the physiological mechanisms linking cancer and pollution have suggested mechanisms such as ethoxyresorufin-O-deethylase (EROD) activity and mitochondrial DNA damage (Wills *et al.,* 2010), the activation of K-ras oncogene (Wirgin *et al.,* 1989) or the inactivation of Rb genes (Lerebours *et al.,* 2014), immunosuppression and DNA adducts (Martineu *et al.,* 1994) and genotoxicity (Baumann, 1998).

3.2 Comparative analysis of cancer gene copy numbers in fishes (II)

The duplication of cancer-related genes is known to be associated with varying levels of cancer prevalence's in different species (e.g. Seluanov *et al.,* 2018; Tejada-Martinez *et al.,* 2021; Tollis *et al.,* 2020). In order to explore this topic on a taxon not studied before, a comparative analysis was run to determine differences in the number of copies of cancer-related genes in fish species.

A total of 69 fish genome assemblies were available from a total of 3 clades (65 species from *Actinopterygii* (ray-finned fish), 3 species from *Cyclostomata* (jawless fish) and 1 species from *Sarcopterygii* (fringe-finned fish, Figure 3). Of these species, lifespan data was available for 54 *Actinopterygii*, 2 *Cyclostomata* and 1 *Sarcopterygii*. Most lifespan data was from reliable sources (23 from Anage, 11 from Fishbase, 10 from articles). Analysis was completed on all species with lifespan data. Maximum lifespan was related to maximum body size when branch lengths were optimised using maximum likelihood ($R^2 = 0.34$, $p = 0.00001$). However, at fixed branch lengths this relationship only holds for reliably sourced maximum lifespan data.

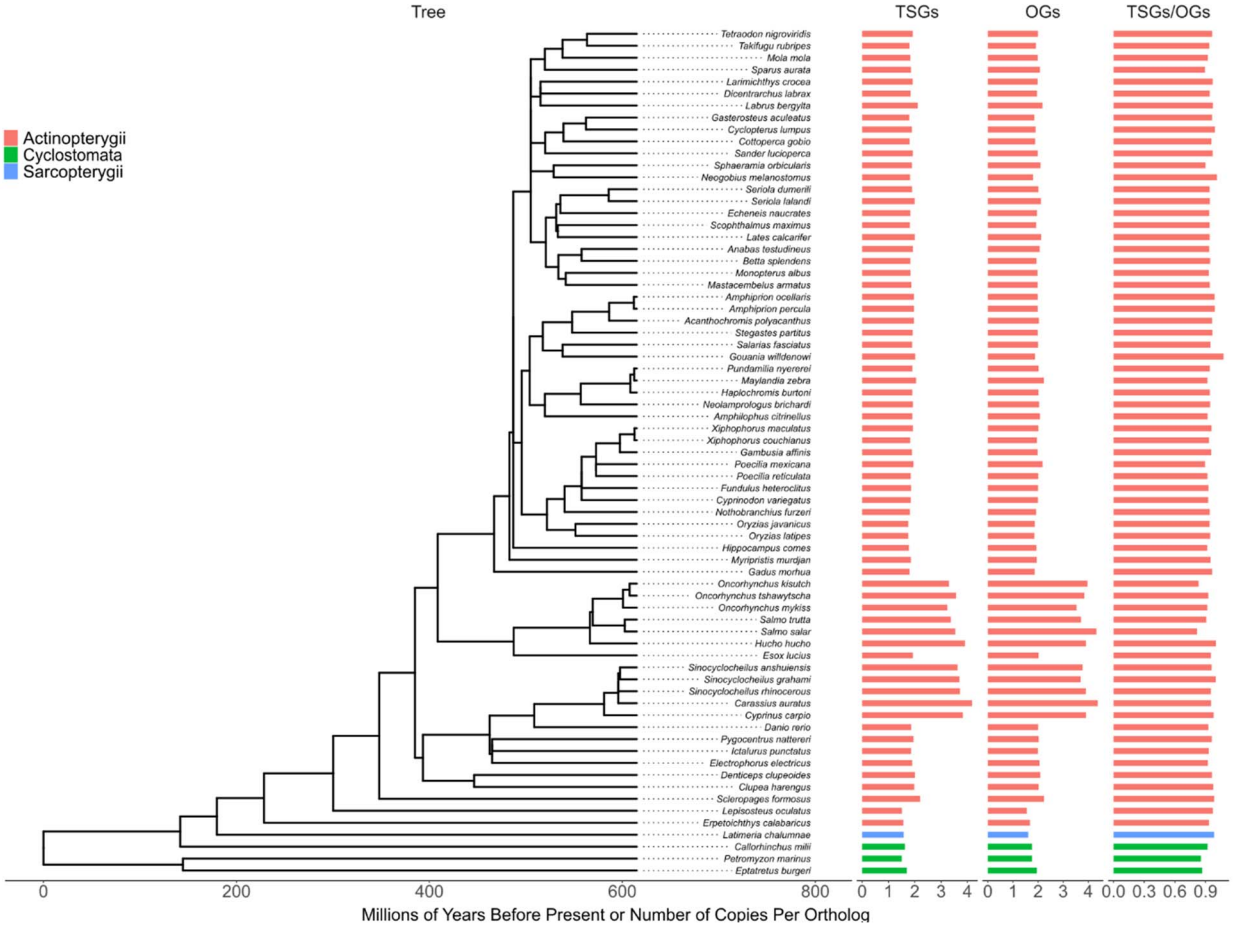

***Figure 3**. The species tree with branch lengths and counts for each species for normalized TSGs and OGs. TSGs/OGs is the ratio between normalized TSG counts and normalized OG counts (from paper **II**).*

From all queried human cancer genes, an average of 218 (±11 SD) TSG and 192 (±12 SD) OG orthologs were identified using the CAFE approach and 170 (±31 SD) TSG and 152 (±27 SD) OG orthologs for the homolog approach. Using phylogenetically adjusted regressions, there was a strong positive correlation between the number of copies of OGs and TSGs (all TSGs: $R^2 = 0.93$, $p < 0.00001$, gatekeeper genes: $R^2 = 0.93$, $p < 0.00001$ and caretaker genes: $R^2 = 0.43$, $p < 0.00001$). Removing the two fish families that have undergone an extra round of whole genome duplication (Salmonidae and Cyprinidae) from the analysis did not remove the significant correlation between OGs and TSGs.

Lifespan is positively related to the total number of TSGs and negatively to the total number of OGs, irrespective of branch length optimization (Figure 5, for optimised branch lengths $p < 0.00001$ $R^2 = 0.37$, at fixed $p < 0.00001$ $R^2 = 0.36$), the inclusion or exclusion of body size, or low-quality data points (maximum lifespan data from less reliable sources) in the model. However, the relationship was significant only when both OG and TSG counts, or their ratio were included in the model.

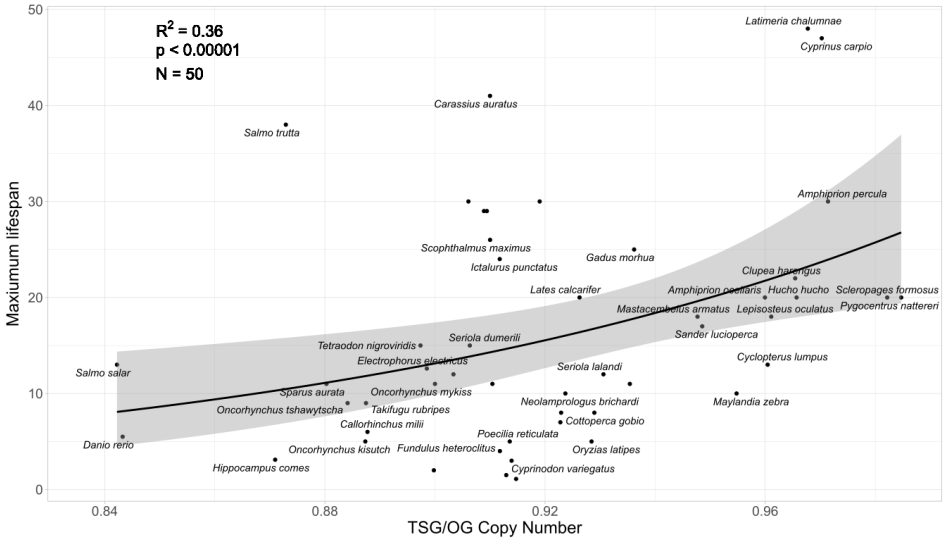

Figure 4*. Linear regression between maximum lifespan and the normalized count of tumour suppressor genes (TSG), divided by the normalized count of oncogenes (OG) obtained from the CAFE approach and including only COSMIC Tier 1 genes. Each point in the plot represents a species in the dataset. The line and the confidence intervals depicted in the plot come from a log linked general linear model (i.e. not adjusted phylogenetically), the values R^2, p and N are from phylogenetically adjusted linear regression where the maximum lifespan is log transformed. The λ, κ and δ values are fixed at 1 (from paper* ***II****).*

We also found that many human cancer genes were duplicated in fish genomes. The number of copies of genes that were duplicated varied between species, as did the ratio TSGs/OGs. As expected, the species from the fish families that had undergone an extra round of whole genome duplication (*Salmonidae* and

Cyprinidae) stand out as species with the highest copy numbers of TSGs and OGs. However, even within the fish species with smaller genomes, the number of copies of TSGs and OGs ranged from 1.5 to 2.2. When looking separately at fish species outside the salmonid and cyprinid families, species with highest number of copies of TSGs are two tropical fish, Asian arowana (*Scleropages formosus*) and mormyrid electric fish (*Paramormyrops kingsleyae*), and one temperate fish, the ballan wrasse (*Labrus bergylta*; based on COSMIC tier 1 gene list, which is more reliable in regards of links of genes with cancer compared to the full list). As the number of copies of TSGs and OGs are correlated ($R^2 = 0.93$, $p < 0.00001$), we also calculated the TSG/OG ratio for all studied species (Figure 3), with the suggestion that species with the highest ratio invest more into cancer defences compared to species with the lowest ratio. According to this calculation, the three species with the highest TSG/OG copy number ratios were blind cave tetra (*Astyanax mexicanus*, TSG/OG copy number ratio 1.017), Asian arowana (0.985), and the red-bellied piranha (*Pygocentrus nattereri*, 0.982). The three species with the lowest TSG/OG copy number ratio were zebrafish (0.843), Atlantic salmon (*Salmo salar*, 0.842), and reedfish (known also as ropefish, *Erpetoichthys calabaricus*, 0.837).

3.3 Cancer and oncogenic pollution in two flatfish (III, IV)

3.3.1 Differences between species

Many anthropogenic pollutants are persistent in the environment, therefore understanding how pollution affects different biological mechanisms that can lead to increased cancer prevalence's, such as changes in gene expression and levels of oxidative DNA damage, could provide insights into how pollution affects cancer at a mechanistic level. From 10 sites across the Baltic Sea and North Sea (Figure 1), flounders were present at 4 sites (GB1, B09, B11, B12) and dabs were present at 9 (all except B09). We collected 40 flounders and 88 dabs (only 8 dabs were caught at site N01). Genetic analysis of the flounder samples showed that all 40 fishes were indeed *Platichthys flesus* and not *Platichthys solemdali*. Histopathology analysis of the livers of these 128 fishes found that 28 had pre-neoplastic lesions (foci of cellular alteration (FCAs)) and 14 fishes had liver neoplasms (Table 2). Neoplasms were diagnosed as either hepatocellular adenoma or hepatocellular carcinoma (Figure 5) and there was no significant difference in the prevalence of neoplasms or pre-neoplastic lesions between flounders and dabs (Chi-squared =2.81, df=2, p=0.2). A subsample of these fishes (14 flounders and 16 dabs, from both polluted and reference sites) was selected for gene expression analysis; of these, 2 flounders and 6 dabs had neoplasms. Pre-neoplastic (FCAs) and other histopathological changes were not included in the transcriptome analysis as the focus was to explore differences in gene expression between fish with and without neoplasms and between fish living in polluted and reference sites.

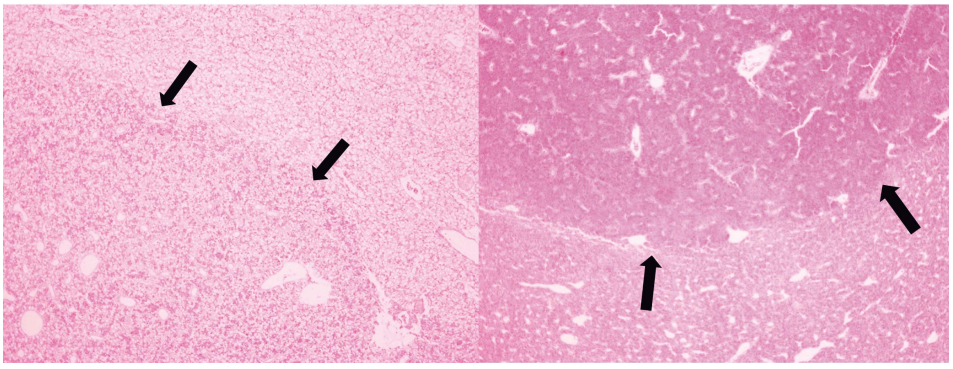

Figure 5. *Histology images of two hepatic adenomas found in dab* (*Limanda limanda*). *More intensely coloured areas are tumorous tissue and black arrows indicate the edge of the tumour.*

Table 2. *Histopathology results by species where total number of flounders sampled was 40 and total dabs was 88. Brackets indicate the percentage of each species that had neoplastic or foci of cellular alterations (FCA) of the liver.*

Species	Histopathology diagnosis	Number of Individuals (% of fish)
Flounder	FCA	6(15)
	Neoplasm	3(7.5)
Dab	FCA	22(25)
	Neoplasm	11(12.5)

For the transcriptome study, analysis was run to explore the differences in gene expression between fishes with and without neoplasms, using two methods, the EdgeR and DESeq2. These methods perform the differential expression analysis slightly differently. Of the 25378 genes for flounder, the DESeq2 method recovered 449 genes that were significantly differently expressed between fish with and without neoplasms (p-value adjusted <0.05), whilst the EdgeR method recovered 123 significantly differently expressed genes (FDR<0.05). For dabs, of the 23311 genes, 86 were significantly differently expressed between fish with neoplasms and those with no neoplasms detected using the DESeq2 method (p-value adjusted <0.05), whereas using the EdgeR approach, there were 42 significant differences (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 6, Ashburner *et al*., 2000; Bryant *et al*., 2017; The Gene Ontology Consortium, 2021). In dabs, we mostly observed the upregulation of genes in fishes with neoplasms. The only transcripts that were downregulated in dabs with neoplasms were related to fatty acid binding proteins. In flounders, we could also see downregulation of different categories of genes in fishes with neoplasms, although upregulation was still more common.

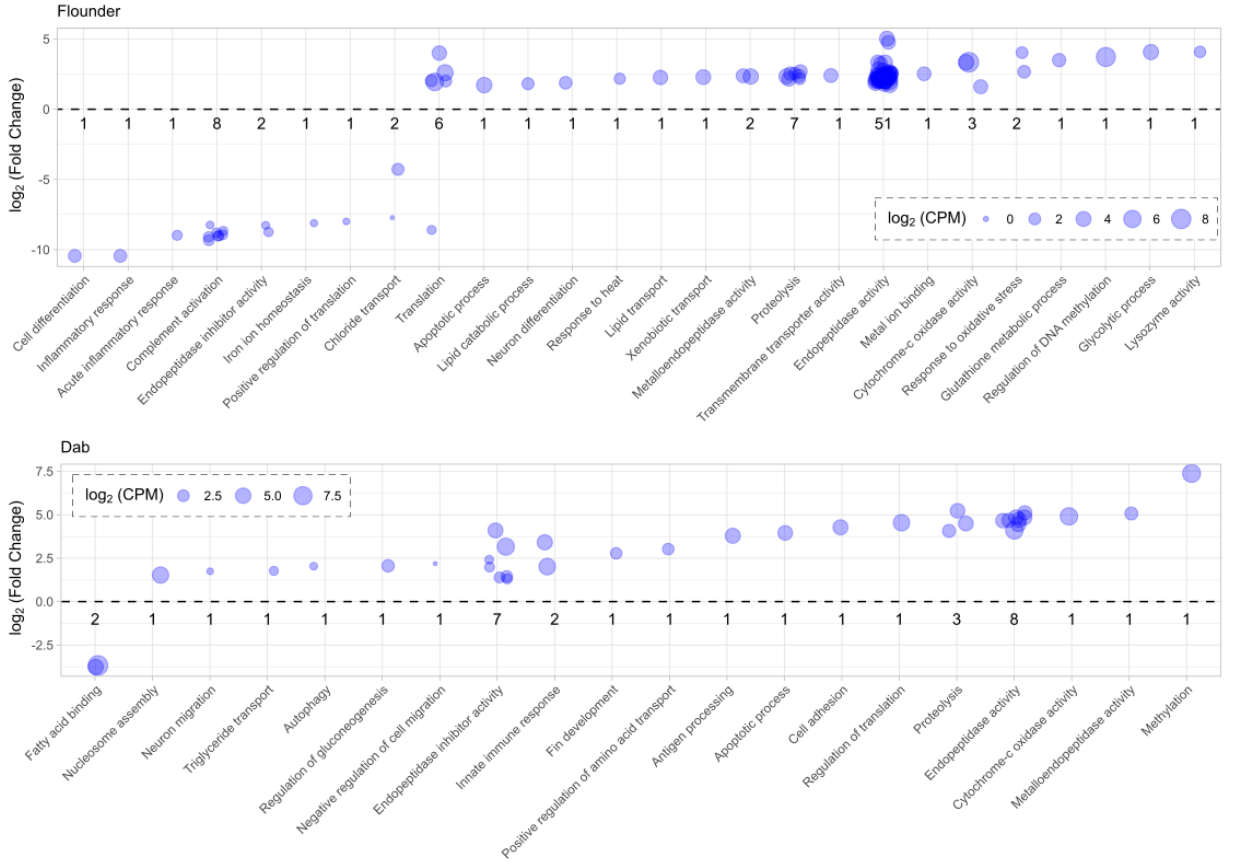

***Figure 6**. Comparison of transcripts from flounders (upper part) and dabs (lower part) with and without neoplasms. Y-axis indicates log(2) transformed ratio between gene expression levels in fishes with neoplasms compared to healthy fishes. Only annotated transcripts with false discovery rate under 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, from paper **III**).*

In addition to sites being described as either reference or polluted based on environmental data (Table 1), pollutant levels (PAH metabolites and trace metals) were measured in the bile and liver of the 128 fishes sampled. A principal component analysis was run to group different variables within the data set. These components were hydrocarbons (2-, 3-, 4-, 5- ring hydrocarbons), trace metals (Cd, Pb, As, Hg) and 'size + age' (age, length, weight). According to the logistic regression model, flounders were bigger/older and had higher levels of oxidative DNA damage than dabs. In regard to pollutant levels, dabs had significantly higher levels of organic pollutants (PAHs) in the bile than flounders but there was no significant difference between species in the levels of trace metals in the liver. There was significantly greater variation (F-test) in the levels of contaminants in dabs than flounders (metals: $F=6.2909$, num df= 72, $p<0.001$ and hydrocarbons: $F=2.1857$, num df=72, $p=0.0115$). There was no significant variation in the size (length, age, weight, $F= 0.899$, num df= 72, $p=0.689$) or levels of oxidative DNA damage ($F=0.560$, num df=87, $p=0.555$) between flounders and dabs.

3.3.2 Differences between populations

A generalised linear model was run to test whether the effects of the PCA components (metals, hydrocarbons or 'size + age'), the levels of oxidative DNA damage, the location (Baltic Sea or North Sea) and whether a site was categorised as polluted or reference, influenced the likelihood of the fish developing neoplasms. The model tested both species combined and then flounder and dabs individually. The generalised linear model suggested that neither size/age, contaminant levels, oxidative DNA damage, or location (North Sea vs Baltic Sea) influenced the likelihood of a fishes developing neoplastic/pre-neoplastic tumours (both species and dabs, $p>0.05$). However, larger/older flounders had more tumours than smaller/younger ones ($p=0.0284$). Additionally, there was significantly higher proportion of tumours in fishes living in reference sites compared to more polluted sites (both species $p=0.007$; flounders $p=0.0419$; dabs, $p=0.0322$).

A second generalised linear model was run to determine which variables affected the levels of oxidative DNA damage. The models tested both species combined and then flounder and dabs individually. There was a significantly higher level of oxidative damage in the fishes from the Baltic Sea than the North Sea ($p<0.001$) but 'size + age', contaminant levels or whether a site was categorised as polluted/reference did not affect oxidative damage levels ($p>0.05$). In dabs, there was significantly higher oxidative damage in the Baltic than the North Sea ($p<0.001$). In flounders, oxidative damage was significantly higher in the Baltic Sea ($p<0.001$) and in flounders with lower trace metal concentrations ($p=0.03$).

An ANOVA model indicated that oxidative damage was significantly higher in dabs with no alterations detected compared to those with an FCA ($p=0.027$) but not different between fishes with no alterations detected and those with neoplasms, or between individuals with neoplasms and those with an FCA (Figure 7). There were no significant differences for flounders.

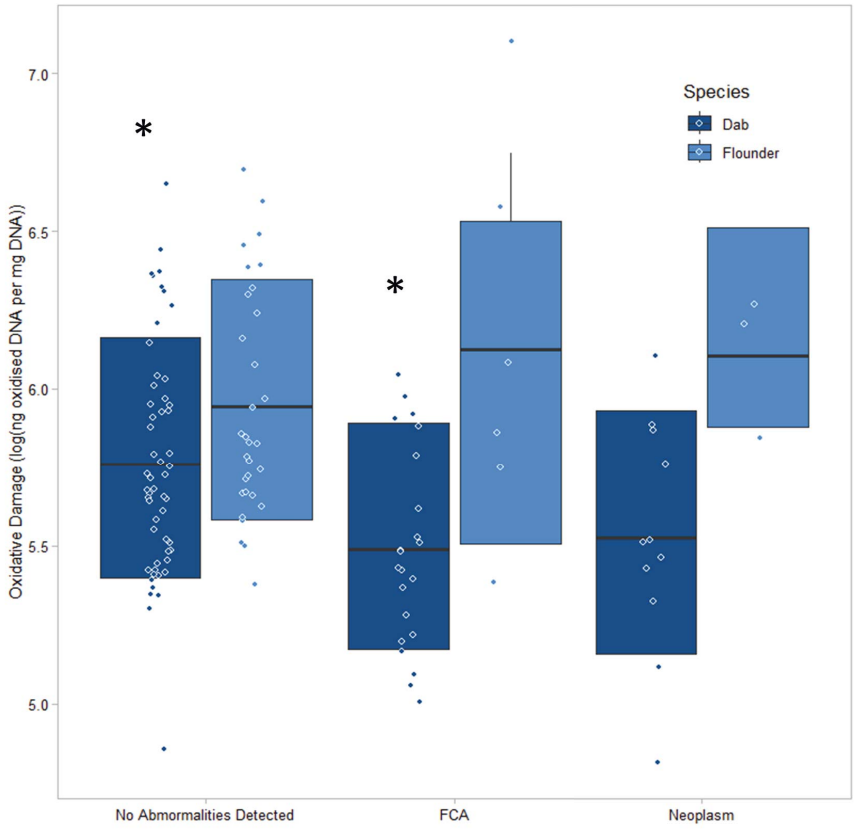

***Figure 7**. Mean (log-transformed) oxidative DNA damage between fish with neoplasms, foci of cellular alterations (FCA) or no abnormalities detected. Asterix (*) represents p<0.05. Bars represent mean +/– standard deviation. Dots are individual data points (from paper **IV**).*

Looking further into the possibility of local adaptation, fishes without liver neoplasms from polluted and reference sites were compared, using gene expression analysis, to find possible signs of local adaptations in defence mechanisms against pollution exposure. 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 differently expressed between reference and polluted sites (FDR<0.05). In dabs, the DESeq2 approach found 67 genes that were significantly different between polluted and reference sites (p-value adjusted <0.05), whereas the EdgeR approach recovered only 12 genes that were significantly different between polluted and reference sites (FDR<0.05).

Using the OrthoDB to search for the best protein match for each significant gene ID, for both species, GO categories for molecular processes were added, if available. There was greater diversity in the best protein matches for flounders than dabs, and different molecular functions for transcripts were indicated. In flounders,

the available GO categories suggested changes in processes linked with immune response, apoptosis, and cell cycle regulation, whilst in dabs, metal ion binding processes and peptidase activity regulation were indicated. In flounders, most of the transcripts could be linked with potential oncogenes or tumour suppressor genes, however, in dabs only links with immune suppression could be made based on best protein match analysis.

4. DISCUSSION

As cancer is a disease that can impact all multicellular organisms, studies focused on a wider range of wildlife species could develop our understand of the ecology and evolution of the disease, especially when considered alongside the increasing anthropogenic pressures that are likely to be increasing cancer rates across the tree of life. It could therefore be surprising that only 300 aquatic species have had some form of cancer diagnosed in them, and only around 30 species have been included in studies linking pollution to increased cancer rate, either in a laboratory setting or in the wild (**I**). Most of these studies have been on fish. This could well be because of the ease of sampling fish in comparison to other vertebrate groups such as mammals, birds and reptiles which are often protected by law. Another possibility is there is less interest or disease monitoring in species that are not commonly used as a source of food for humans.

The next best studied group of aquatic animals, regarding cancer studies, are molluscs (**I**). Again, this focus could be due to their use as a source of human food, but additionally, bivalves develop a form of transmissible cancer, known as disseminated neoplasia (DN), that has attracted the attention of evolutionary biologists. Of the nine known transmissible cancers, the majority are found in the marine environment in species such as soft-shell clams (*M. arenaria*), mussels (*Mytilus trossulus*, *M. edulis*, *M. chilensis*), cockles (*Cerastoderma edule,* reviewed in Carballal *et al*., 2015), with cross-species contagion observed in golden carpet shell clams (*Polititapes aureus*), derived from the pullet shell clam (*Venerupis corrugate,* Metzger *et al.,* 2016). However, the effect of pollution on DN outbreaks, that can cause high mortality in mollusc populations is not well studied and understood. The impact of several chemical pollutants on DN in several host species has been assessed, mostly through correlative studies where sample sizes and/or chemical measurements are not sufficient to draw firm conclusions (reviewed in Carballal *et al.,* 2015).

Despite the high number of potential emerging pollutants (Brack *et al.,* 2018), only a handful of them have been studied with regards to their effects on wildlife cancer. Heavy metals and PAHs have been the main focus of studies to date (**I**). The lack of literature, focusing on a wider range of contaminants, and their effect on cancer needs to be addressed. However, the wide spectrum and low concentrations of these substances in the environment presents challenges for understanding their effects on the health of aquatic organisms. Especially, when such linkages between tumour prevalence and presence of potentially mutagenic toxicants are not always causal. The additive, synergistic and/or antagonistic effects of pollutant 'cocktails' (Laetz *et al.,* 2009; Ansari *et al.,* 2004) presents challenges in studies on wild animals and their effects on cancer. Particularly when these interactions between different contaminants can be impacted by other environmental stressors, such as climate change and the bioavailability of contaminants (Crain *et al.,* 2008; Parmesan & Yohe, 2003). Additionally, marine debris such as microplastics which can form biofilms have the potential to act as vectors for

oncogenic viral transmission. As much as 15–20% of human cancers are estimated to have a viral aetiology and it is therefore unsurprising that viruses can cause cancer in wild animals too. The papilloma virus is responsible for cancers in sperm whales (*Physeter macrocephalus*, Lambertsen *et al.,* 1987), Burmeister's porpoise (*Phocoena spinipinnis*, Van Bressem *et al.,* 2007) and beluga whale (De Guise *et al.,* 1994), herpes virus in California sea lions (King *et al.,* 2002) and green turtles (Lu *et al.,* 2000) and retrovirus in walleye fish, (*Sander vitreus*, Martineau *et al.,* 1992) and Atlantic salmon (*Salmo salar*, Paul *et al.,* 2006). Pollution has the potential to exacerbate the effects of oncogenic viruses, for example in Californian sea lions where a relationship between PCB's and cancer prevalence was found (Ylitalo *et al.,* 2005). Other pollutants have been shown to have effects such as altering biological pathways, for example, endocrine disruptors (Schug *et al.,* 2016; Soho & Sonnenschein, 2010), changes in the expression of aryl hydrocarbon receptors (Zhou *et al.,* 2010) or the induction of heat shock proteins (Rajeshkumar & Munuswamy, 2011). Some of these changes to biological pathways could potentially increase the risk of diseases, such as cancer, in aquatic animals.

Studying pollution and cancer in wild organisms presents a range of issues. For example, many species migrate long distances, therefore it is difficult to determine the exposure of an organism to pollutants (**I**). For example, European flounders can undertake migrations up to 80–95 km with the longest recorded migration reaching approximately 700 km (Ojaveer & Drevs, 2003). However, there are also benefits to using wild animals as they provide a more realistic understanding of natural cancer rates in a population that is subjected to all its natural and introduced stressors. Dependant on the location a species migrates to, it could increase or decrease their risk of accumulating contaminants that are carcinogenic. For example, great skuas (*Stercorarius skua*), breeding in the pristine Arctic, overwinter along industrialized coasts of Europe, North Africa and North America where accumulated concentrations of persistent organic pollutants (POPs) might affect some populations (Leat *et al.,* 2013).

A focus on natural habitats that have been subjected to human contamination, and particularly remediation projects, could provide insight into the effect of contamination on cancer prevalence's in natural populations. For example, a study on brown bullheads found reduced neoplasia following the closure of a coking plant (Baumann & Harshbarger, 1995). These restoration projects have the potential to act as natural laboratories to better explore the physiological and genetic mechanisms that increase both a species and an individual animal's risk of cancer. Understanding these processes could improve our understanding of the evolution of cancer defences and the adaptation or acclimation processes of species exposed to anthropogenic oncogenic pressures.

The small and biased selection of species studied in the context of cancer and pollution so far hints that species could be differently vulnerable to pollution-induced cancer. This could be due to varying pollutant vulnerability, but also due to variation in cancer defences. As comparative studies (e.g. Tejada-Martinez *et al.,* 2021; Tollis *et al.,* 2020; Vazquez & Lynch, 2021) have shown before, cancer

defences vary between species, depending on their life-history. Studying a wider range of species in the context of cancer and pollution could also provide valuable information about the differing vulnerabilities to environmental change between species, and to the evolution of cancer defences in general.

Different species have evolved different cancer defences, in the form of tumour suppressor genes depending on their evolutionary pressures and trade-offs. This has mostly been studied using mammalian genomes to date (Tejada-Martinez *et al.,* 2021; Tollis *et al.,* 2020; Vazquez & Lynch, 2021). However, in my thesis, I focused on a phylogenetically older, and genetically more diverse group, fishes, to determine whether there is a link between body size or lifespan and cancer related genes (**II**). I aimed to determine how the number of copies of 715 genes, that have been linked to cancer in humans, varied across the spectrum of fishes. Since there is no current database on cancer genes in wildlife, and OGs and TSGs are amongst the oldest gene classes, it is reasonable to use human cancer genes as a proxy for wildlife cancer genes. Additionally, as the evolutionary distance between fishes and humans is much larger than that of mammals with humans, it should not influence the results as much as a similar comparative analysis on mammals. However, it should be noted that until there is experimental verification that human cancer genes do have the same function in fishes as in humans, these results should be taken with caution.

Interestingly, the results (**II**) suggest that there is a masked relationship between the number of copies of OGs and TSGs with lifespan that is only visible when using the OG/TSG ratio in the model. When we include these variables separately, this relationship is not significant. The evolutionary pressure of this increased risk of oncogenic mutations, because of a higher number of copies of OGs, appears therefore to be counteracted by the evolution of higher numbers of copies of TSGs (Figure 4). However, this relationship does not hold for mammals (Tollis *et al.,* 2020), unless only using genomes sequenced in Ensembl (**II**). One possible explanation is that the genomes sequenced in Ensembl are considered of better quality (Kinsella *et al.,* 2011). Additionally, it is possible that the relatively larger phylogenetic distances between different fish species genomes compared to that of mammals contributes to this relationship holding in fishes but not mammals. It might be that such a relationship emerges only on a larger phylogenetic scale. Additionally, it could be speculated that this masked relationship holds for fishes and not mammals because only the most important human cancer genes, in terms of lifespan, are conserved in fishes. It is possible that other less relevant cancer-related genes are present in mammals that weaken this relationship, but this remains to be studied.

Focusing on the ratio of OGs/TSGs is potentially more informative when considering the susceptibility or resistance of a species to cancer, compared to focusing solely on which species have the highest numbers of TSGs (**II**). One reason for this could be that by compensating for increased copies of (proto)oncogenes by increasing the copies of TSGs, lifespan could increase, without increasing the susceptibility of a species to cancer. The three species with the highest ratio of OGs to TSGs are the blind cave tetra, Asian arowana and red-bellied piranha. The blind

cave tetra has undergone recent rapid evolutionary change, dividing into two subspecies. One of these subspecies has undergone extreme morphological evolution through cave colonisation, losing eyes and pigmentation, and living in permanent darkness whilst the other is an 'ancestral' multi-coloured tropical freshwater fish (Torres-Paz *et al.,* 2018). This species could be used to better understand the evolution of specific traits and genetic mechanisms that support rapid habitat-based evolutionary change (Torres-Paz *et al.,* 2018) and the trade-offs that could lead to increased tumour resistance. The Asian arowana and red-bellied piranha are two fish species for which parental care is described (Queiroz *et al.,* 2010, Scott & Fuller, 1976). Additionally, the Asian arowana is a highly valuable ornamental species with a late sexual maturation. It is understood that having a slow life history is a trait that leads to increased tumour resistance (Boddy *et al.,* 2020).

The species with the lowest OG/TSG ratio were zebrafish, Atlantic salmon and reedfish (**II**). Zebrafish is a well-established model organism for cancer studies due to the similarities in tumorigenesis with the human species (Stoletov & Klemke, 2008). However, as this species has a fast life history, it may have more similarities to mice than humans particularly regarding lifespan (Hu & Brunet, 2018). Many other salmon species, in addition to the Atlantic salmon, had low ratio of OG/TSG. These semelparous species, which only reproduce once, may prioritise growth/reproduction over the evolution of increased tumour suppression mechanisms. Reproduction in semelparous species can lead to severe, rapid pathology known as reproductive death due to lower investment in self-maintenance to compensate for an increase in reproductive effort (Gems *et al.,* 2021). The species with the lowest ratio was the reedfish, a facultative airbreather that can move between aquatic and terrestrial environments (Sacca & Burggren, 1982). Changes in oxygen pressure could be one reason for the lower investment in TSGs in this species. It has been shown in humans that hyperbaric oxygenation has the potential to inhibit the proliferation of tumour cells (Granowitz *et al.,* 2005). However, whether this lower investment in genome level tumour suppression mechanisms is a result of reduced cancer risk from changes to oxygen pressures in the environment has yet to be studied. Gathering more information on natural cancer prevenances from a wider range of wild organisms would be one way to better understand how cancer defences vary between species dependant on their environment.

The study of wildlife cancer genetics is still in its infancy and as cancer is a complex disease, impacted by both genetic and environmental factors, developing our understanding of the evolution of cancer defences in a wider range of species could be valuable. Relatively few fish genomes, compared to mammals, have been sequenced, and often the ones that have, have been done so because they are of specific interest. For example, the mormyrid electric fish has been sequenced to explore the evolution of electric organs. Unfortunately to date, although both species have been used as ecotoxicological models for decades, neither flounders nor dabs have had their genomes sequenced. Therefore, we could not explore the differences in genetic cancer defences between these two species. However, understanding how different species have evolved to reduce their cancer risk is only one step in the process of understanding the evolution of cancer

defences across the tree of life. In addition, understanding whether there are differences in gene expression or mechanisms such as oxidative DNA damage between species, and how environmental pressures, particularly novel anthropogenic change, affects this could provide insight into differences in cancer prevalence between different populations.

I conducted field studies (**III**, **IV**) to explore the differences in cancer defence mechanisms between two flatfish species and how oncogenic contamination influences the expression of these defences and the initiation of oncogenic processes. We explored the differences in neoplasm prevalence, oxidative DNA damage and gene expression between these two species exposed to different levels of oncogenic contamination. When the genomes of these species will be sequenced, it would be interesting to see if the evolution of the numbers of copies of cancer related genes differs between the two species, which would suggest that there is a genetic difference in cancer susceptibility or resistance between flounders and dabs.

We did not find any significant difference in the prevalence of neoplasms between dabs and flounders (**IV**). This result contradicted that seen throughout the literature, which has generally found liver neoplasm prevalence's of around 8–10% in dab (Lyons *et al*., 2006; Stentiford *et al*., 2009; CEFAS report, 2004) compared to between 0.7–1.5% in flounder (Cachot *et al*., 2013; Lang *et al*., 2006; Stentiford *et al*., 2003; Vethaak *et al*., 1996). This decreasing trend in liver neoplasms in dab could be due to the successful international regulation on marine pollution. However, information from sediments (OSPAR Data and Information Management System, https://odims.ospar.org/) and historic tumour prevalence data still support the possibility of locally differing selection pressures by the oncogenic effects of pollutants. It should also be noted that these results did have the same downward trend of less neoplasms in flounders than dabs seen throughout the literature, even though it was not significant. It is possible that as these results only covered 1 sampling year there could have been biases that could not be accounted for, either from sample size or environmental conditions that were present in that year.

We detected differences in gene expression patterns between the two species. Dabs with neoplasms showed a general upregulation of different cancer related genes. The only transcripts which were downregulated were related to fatty-acid binding proteins. In flounders with neoplasms, we found a downregulation of various other genes linked to immune responses that could potentially act as cancer defence mechanisms, such as inflammatory responses and complement activation. Other downregulated processes in flounders included cell differentiation, chloride transport, iron homeostasis, regulation of translation, and endopeptidase inhibition. However, in flounders with neoplasms, there were more genes upregulated than downregulated. This was expected considering that, in liver cancer transcriptome studies in humans, around 90% of differently expressed genes were upregulated (Jin *et al*., 2019). In both flounders and dabs, endopeptidase activity was the process with the highest number of linked transcripts and the highest transcript abundance. These proteases were involved in breaking

down proteins that promote angiogenesis, invasion and metastasis in cancerous tissues (Lopez-Otin & Overall, 2002). Interestingly, endopeptidase inhibiting processes were downregulated in flounders with neoplasms, suggesting that these processes need to be actively suppressed in healthy individuals for neoplasia to occur. This is not actively observable in dabs which suggests the possibility that less efficient cancer defence mechanisms occur in dabs.

The link between cancer and oxidative stress is well studied in humans. Oxidative stress is defined as imbalance of oxidants to antioxidants leading to oxidative damage to tissues. Measuring antioxidant/oxidant levels in an organism as an accurate representative of oxidative stress presents challenges due to time-lagged and hormetic upregulation of protective mechanisms (Meitern *et al.,* 2013). Focusing on a biomarker that measures damage to DNA, reduces the risk of misinterpreting high antioxidant levels that could have been upregulated to prevent oxidative stress. In my study (**IV**), the levels of oxidative DNA damage in an individual could not predict neoplasm occurrence. However, flounders had higher levels of oxidative damage than dabs. This suggests that there are biological processes or mechanisms, other than oxidative DNA damage, that are more important or more reliable for determining whether these fishes develop neoplasms or not. Interestingly though, dabs with no detected abnormalities appear to have higher levels of oxidative DNA damage compared to individuals showing signs of preneoplastic changes (FCA). This relationship did not hold for dabs with neoplasms suggesting the possibility that DNA repair mechanisms are upregulated in dabs with neoplasms, following pre-neoplastic liver changes.

The links between increased oxidative DNA damage and exposure to environmental contamination (e.g. PAH's and metals) are well studied (e.g. Machella *et al.,* 2004; El-Agri *et al.,* 2022), hence it could be assumed that there would be a link between pollution and oxidative damage to DNA in flounder and dab (**IV**). However, this was only the case for flounders, not dabs, where individuals with lower metal concentrations in the liver had higher levels of oxidative damage. This suggests that flounders upregulate DNA repair processes when exposed to higher contaminant burdens. The lack of relationship between contaminants and oxidative DNA damage in dabs could potentially be because dabs are able to regulate their antioxidant processes to reduce the effect on DNA following exposure to contamination. Additionally, it suggests both species have either acclimated and/or adapted to cope with the ongoing pressure from anthropogenic contamination in marine ecosystems. The most significant factor affecting oxidative DNA damage levels was which sea the fishes were caught in, either the Baltic Sea or the North Sea. Oxidative damage was significantly higher in the Baltic Sea for both flounders and dabs. Whilst hyposaline conditions have been found to increase oxidative stress in olive flounders (*Paralichthys olivaceus*, Lee *et al.,* 2022) it is worth also noting that a larger proportion of the Baltic (96%) was considered polluted compared to the North Sea area (75%, European Environment Agency, 2019). It is possible that this difference in other present contaminants, that were not measured in this study, could be influencing the levels of oxidative DNA damage in flounders and dabs.

The North Sea and the Baltic Sea have been subjected to ongoing anthropogenic pollution since the industrial revolution. In my thesis (**IV**), the focus on PAH's and oncogenic trace metals, specifically As, Pb, Hg and Cd, was chosen because these are the two types of contaminants focused on in cancer and pollution studies (**I**). It is interesting to note however, that the levels of these contaminants, in the fish's tissue, did not vary significantly between sites. However, there were differences between the two species in the levels of contaminants present in the tissues. The variation in the levels of contaminants was higher in dabs than flounders and PAH levels were significantly lower in flounders which suggests potentially better pollutant metabolism in flounders than dabs. In other words, physiological mechanisms that either remove pollutants or metabolise them can vary between species, populations, and individuals, resulting in similar levels of tissue pollutants despite differing exposure levels. Organic pollutant metabolism can have implications for increased cancer development (Stegeman & Lech, 1991; Willett *et al*., 2006). For example, cytochrome p450 (CYP) enzymes have been linked to oncogenic pollutant metabolism in a range of species from humans to fish (Kwon *et al.,* 2021; Uno *et al.,* 2012). One example is the benzo(a)pyrene metabolite, benzo(a)pyrene-diol-epoxide that becomes carcinogenic following CYP metabolism rather than in its original form (Newbold & Brookes, 1976; Kim *et al*., 1998). The transcriptomic analysis (**III**) indicates increased CYP activity in flounders with neoplasms compared to dabs with neoplasms. However, this increased pollutant metabolism does not appear to increase neoplasm rates in flounders. It is possible that in flounders this increased pollutant metabolism triggers cancer defence mechanisms to be activated in the liver. 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).

When testing to see if there was a difference in the proportions of fish with neoplasms (**IV**), based on whether they were living in sites categorised as polluted or reference, there was a significantly higher proportion of fish with neoplasms in reference sites compared to polluted. The number of fish sampled from reference sites was lower than that of polluted sites. However, assuming that the sample size used in the study gave a representative screen of the population, fish living in areas considered polluted could have acclimated or adapted to cope with oncogenic contamination pressures. This hypothesis is supported also by the gene expression analysis, at least in flounders (**III**). Our transcriptome results, comparing flounders without neoplasms living in polluted vs reference sites, found 12 differently expressed transcripts. The transcripts that were upregulated in flounders living in polluted sites include aldolase genes, ubiquitin/proteasome system and DNA damage-regulated autophagy modulator protein 2 (DRAM2). All of these three can be linked to tumour formation or suppression mechanisms. Aldolase genes can regulate proliferation, apoptosis and metastasis in human liver cancer (Bu *et al*., 2018; Li *et al*., 2019). Ubiquitin/proteasome systems lead

to the 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). DRAM2 can be activated by the TP53 gene, a well-known tumour suppressor with links to apoptosis, autophagy, and programmed cell death (Crighton *et al.,* 2006). Additionally, some of the genes that were upregulated in flounders from reference sites have been linked to mechanisms promoting tumour development. Dabs however, showed fewer differences in gene expression between reference and polluted sites, suggesting that adaptation to polluted habitats is more likely to occur in flounders compared to dabs, potentially due to stronger selection pressures caused by more active pollutant metabolism (**III**).

The study of these two species in this thesis (**III, IV**) suggests potential differences in natural cancer defences both between the species and between areas considered polluted when compared to 'reference' sites. Wildlife cancer studies are limited, and studying a wider range of species could provide novel insights into both the ecology and evolution of cancer defences but also how species are able to adapt or acclimate to survive in environments with long-term anthropogenic pressures, such as marine contamination.

5. CONCLUSIONS

My thesis explores cancer ecology and evolution in aquatic wild animals, with a focus on fish and oncogenic pollution. The main conclusions of my work are as follows:

i. Whilst cancer in wild aquatic ecosystems is unquestionably under-researched within the literature, studies in 30 species have linked increased cancer prevalence with pollution exposure. Links between cancer and pollution are mainly studied in fish, in addition to a few studies in marine mammals, and several transmissible cancer studies in molluscs.

ii. Using restoration projects could be a useful tool for better understanding the effects of contaminants on cancer prevalence in exposed populations, but there is a need for developing less invasive methods to detect cancer in wild species than necropsies, so that a wider range of species could be studied.

iii. In this thesis, I undertook the first comparative study on the duplication of cancer-related genes in fishes. Whilst all studies have to date been done on mammals, fishes are phylogenetically older, more diverse, and have the potential to have evolved previously unknown cancer defences. There was a masked relationship between cancer related genes and lifespan which suggests that higher ratio of copies of tumour suppressor genes to oncogenes could be associated with the evolution of longer lifespans in some species.

iv. Oxidative DNA damage was not related to pollutant levels and cancer occurrence in either flounders or dabs. However, DNA damage differed between large and small fishes, and was higher in the Baltic Sea fish compared to North Sea fishes. Pollutant levels did vary less in flounders compared to dabs, suggesting that flounders are better able to control their pollutant metabolism.

v. Gene expression analysis suggests potential mechanisms that protect flounders from developing pollution-induced cancer. More active pollutant metabolism shown in transcriptome results for flounders compared to dabs could protect flounders from high levels of pollution. Additionally, it could contribute to stronger selection pressure for locally evolved cancer defences, as pollutant metabolites are known to be oncogenic within tissues. This suggests variation in tumour suppression mechanisms both between the two species but also within a species. This variation indicates that some species can be more resistant to pollution-induced cancer, but also that there could be potential for local adaptation for stronger cancer defences.

vi. Understanding both the genetic differences between species and the biological processes that increase/decrease cancer risk is important in understanding the effect of anthropogenic contamination on the long-term health of wild populations.

6. SUMMARY

Multicellular species have evolved natural cancer defences and understanding how cancer defences in wild animals differs between species can expand our understanding of cancer evolution across the tree of life. Additionally, wild animals now face ever-increasing threats from anthropogenic change and one of biggest threats is the ever-increasing types and quantities of contaminants released into natural environments. It is well known that some of the contaminants in aquatic systems are associated with an increased cancer risk in humans and studying the effect of contamination on cancer rates in wild organisms can, not only improve our understanding of physiological mechanisms that cause cancer in wild animals, but also improve our understanding of the long-term impacts of anthropogenic pollution on our aquatic systems.

In my thesis, I explored different aspects of cancer evolution and adaptation in aquatic species. Firstly, I explored the extent of current knowledge on the pollution induced cancer in aquatic organisms. Cancer studies in aquatic animals have linked pollution exposure to cancer prevalence in around 30 species to date, incorporating the influence of various additional factors such as viral aetiology in green turtles and Californian sea lions and transmissible cancers in bivalves. However, most studies only looked at a limited number of contaminants: heavy metals and polycyclic aromatic hydrocarbons. There are numerous other contaminants that affect biological mechanisms that may directly or indirectly increase the risk of cancer in a population.

I also investigated the difference in the number of copies of both oncogenes (OGs) and tumour suppressor genes (TSGs) in fish genomes. We discovered a masked relationship with the ratio of OGs to TSGs with lifespan. This suggests that a higher number of copies of OGs leads to the selection of more copies of TSGs to potentially compensate for the increased cancer risk that comes with longer life.

In addition to the evolution of different copy numbers of cancer related genes, other biological mechanisms can influence cancer risk within a population. Therefore, the next stages of my thesis involved a field study of two flatfish species, European flounder, and dab. I explored whether gene expression and oxidative DNA damage levels could explain differences in cancer prevalence's in fish from polluted or reference sites, to investigate whether fish living in polluted areas could adapt or acclimate to reduce their cancer risk. This seems to be the case as tumours were significantly more prevalent in populations living at sites categorised as reference rather than polluted. We found that oxidative DNA damage did not appear to act as a mediator between tumours and contamination for either species. This suggests that mechanisms other than oxidative damage play a more significant role in determining the likelihood of cancer in these two species. I hypothesized that there would be differences between flounders and dabs living in polluted areas and gene expression. I found this to be the case and that flounders appear to have stronger pollutant metabolism. This is evident both

by the smaller variation in pollutants within the tissues of flounders compared to dabs, but also in the upregulated expression of cytochrome p450 genes that play a role in pollutant metabolism. However, in dabs there were fewer differences in gene expression between populations living in polluted or reference sites. It is possible that these differences in gene expression seen between flounders and dabs living in polluted/reference sites is a sign of adaptation of flounders due to stronger selection pressures caused by more active pollutant metabolism.

7. SUMMARY IN ESTONIAN

Kohastumine onkogeense reostusega ja looduslikud vähikaitsemehhanismid veekeskkonnas

Vähk on hulkraksete organismide evolutsiooniline pärand ajast, mil toimus üleminek üherakulisuselt hulkraksusele. Vähirakkudes toimuvad protsessid, muuhulgas kontrollimatu paljunemine ja energiatootmine, sarnanevad ainuraksete organismide omaga. Hulkraksuse evolutsiooni käigus tuli ehitada ainuraksetele omaseid protsesse kontrollivate mehhanismide süsteem, mis surub alla rakkude iseka, kontrollimatu jagunemise ning suunab rakud omavahel koostööd tegema. See süsteem pole aga veatu, ja nii ongi leitud, et vähihaigus võib ohustada kõiki hulkrakseid organisme, alates selgrootutest ja lõpetades imetajatega. Ka inimestel on Maailma Tervishoiuorganisatsiooni andmetel pea iga kuuenda surmajuhtumi taga vähk.

Eelnevast lähtub, et kõik hulkraksed organismid peavad pidevalt tegelema rakkude „isekate“ huvide kontrollimise ehk vähikaitsega. Selleks on evolutsiooni käigus kujunenud välja kindlate ülesannetega geenid. Osa neist kaitseb genoomi stabiilsust, näiteks parandades DNA-d. Teised aga takistavad vähirakkude teket, tootes valke, mis takistavad rakkude jagunemist või suunavad vigaseid rakke programmeeritud rakusurma. Kui sellistes vähikaitsegeenides tekivad mutatsioonid, mis takistavad nende toimimist, suureneb organismi vähirisk. Vähki soodustavad ka mutatsioonid rakkude kasvu ja kudede uuenemist soodustavates geenides, mida nimetatakse proto-onkogeenideks. Need geenid on organismile vajalikud, sest aitavad organismil kasvada ja paraneda, kuid oma töö iseloomu tõttu võivad need kontrolli alt väljudes kutsuda esile vähitekke.

Kuigi kõik hulkraksed organismid peavad vähitekke ohuga arvestama, pole liikide võime vähki alla suruda sarnane. Kuna suuremaks kasvamine ja kauem elamine on suurema hulga rakkude ja rakujagunemiste tõttu ohutegurid, mis suurendavad organismide vähiriski, on just suurekasvulistes ja pikaealistel liikidel kõige tugevamad kaitsemehhanismid vähi vastu. Imetajatel tehtud uuringud on näidanud, et vähki suremus varieerub liigiti 0–60% ulatuses. Näiteks kui inimeste vähkisuremus on umbes 17%, siis hobustel on see umbes 19% ja elevantidel alla 5%. Vähirisk võib varieeruda ka liigisiseselt. Inimeste puhul on leitud näiteks, et pikem kasv on vähi riskiteguriks.

Vähikaitse (ja vähirisk) on seega seotud liikide elukäigustrateegiatega. Pikemaealised liigid peavad vähikaitsesse rohkem investeerima. Looduslikul valikul on mitmeid võimalusi tugevamat vähikaitset tekitada, kuid ehk üks kiiremaid ja lihtsamaid võimalusi on duplitseerida olemasolevaid vähikaitsegeene. Nii on näiteks leitud, et elevantidel on ühte tuntuimat vähikaitsegeeni nimega TSG genoomis tervelt 20 koopiat.

Organismide risk haigestuda vähki ei sõltu mitte ainult nende elukäigustrateegiast, vaid ka keskkonnast, milles nad elavad. Vähitekkeni viivaid mutatsioone võivad põhjustada näiteks erinevad keskkonnas leiduvad kemikaalid või kiirgus. Inimtekkelised keskkonnamuutused on seega nii inimeste kui ka teiste loomade

vähki haigestumise riski suurendanud. Selliseid seoseid on aga raske uurida laboritingimustes ja klassikaliste, madala geneetilise mitmekesisusega mudelorganismidega. Looduses esinevad vähki tekitavad tegurid, näiteks reoained, erinevates kombinatsioonides, mille täpse koostise kopeerimine laboris ei pruugi olla võimalik. Ka on loomad looduslikes tingimustes mõjutatud korraga paljudest stressitegurtitest, sh piiratud ressursid või haigustekitajad (nt viirused), mis võivad keskkonnamuutustega koosmõjus vähiriski suurendada.

Veekeskkonnas elavad loomad on reostuse vähki tekitava mõju osas eriti haavatavad, kuna reostus levib veekeskkonnas kiiresti ning akumuleerub veekogude setetes pika aja jooksul. Veekogudes leidub seega nii vanu, nn pärandreostusega seotud aineid kui ka uusi, veel tundmatu keskkonna- ja tervisemõjudega reoaineid. Mitmed uuringud on kinnitanud, et reoained põhjustavad veeloomadel vähi esinemissageduse tõusu. Peamisteks ohuteguriteks on seniste andmete põhjal raskmetallide ning orgaaniliste reoainete esinemine.

Põhjameri ja Läänemeri on ühed maailma reostunuimad merealad, kuna nendesse veekogudesse on reoained kuhjunud juba tööstusrevolutsiooni algusest saadik. Selle aja jooksul on nendes meredes elanud palju mereloomade põlvkondi. Seega on olemas eeldused, et mõnede liikidel/populatsioonidel on toiminud looduslik valik, mis on soosinud kas tugevamate vähikaitsemehhanismide kujunemist või siis paremate vähikaitsemehhanismidega isendite eelistatud ellujäämist. Nii võib Põhja- ja Läänemerd vaadelda kui „looduslikke laboreid“, kus vähikaitsemehhanisme ja nende evolutsiooni on võimalik uurida.

Reoainete akumulatsiooni tõttu setetes on nende mõjule kõige haavatavamad veekogu põhjas elavad liigid. Kuigi Põhja- ja Läänemeres elab mitmeid põhjaeluviisiga liike, on reoainete mõju uurimisel peamisteks mudelliikideks kujunenud jõelest (Platichthys flesus L.) ja soomuslest (Limanda limanda L.). Nende kahe liigi põhjal on juba eelmise sajandi keskpaigast saadik läbi viidud ökotoksikoloogilisi monitoorimisprogramme, kus vähi esinemine on üheks reostuse mõju hindamise markeriks. Seega on nende liikide puhul olemas nii metoodika vähi diagnoosimiseks kui ka kui pikaajalised ja geograafiliselt laiaulatuslikud andmed vähi esinemissageduse kohta. Mõlemal liigil on leitud, et reostuse toimel kujuneb neil välja naha- ja maksavähk. Varasemate uuringute põhjal on soomuslestade vähi esinemissagedus kümme korda kõrgem kui jõelestadel, kuid selle erinevuse põhjus pole teada.

Oma doktoritöös uurisingi reostuse ja vähitekke seoseid veekeskkonnas elavatel liikidel, seades eesmärgiks ka vähikaitsega seotud kohastumuste uurimise. Alustasin oma tööd laiaulatusliku ülevaate tegemisest varasematest uuringutest, et mõista, kui palju üldse veeloomadel reostuse ja vähi vahelisi seoseid on uuritud. Leidsin, et praeguseks on umbes 30 liigi puhul seda seost näidatud. Reostuse ja vähi vahelist seost võivad mõjutada ja vahendada ka muud tegurid, näiteks vähki esile kutsuvad viirused, mille mõju on uuritud veekilpkonnadel ja California merilõvidel, aga ka nakkavate vähivormide esinemine, nagu on leitud mitmel karbiliigil. Oma ülevaates leidsin, et kõige paremini on reostuse ja vähi vaheline seos tõestatud aga kaladel.

Kuna siiani on võrdlevaid uuringuid vähikaitsemehhanismidest tehtud peamiselt vaid imetajatel, seadsingi endale järgmiseks ülesandeks uurida vähikaitsegeene kaladel. Sarnaselt varasemalt avaldatud uuringutega keskendusin vähiga seotud geenide koopiate arvule. Minu artikkel, mis on esimene võrdlev uuring vähigeenidest kaladel, näitas, et kaladel on tasakaalustatud vähki tekitavate geenide (onkogeenide) ja vähki alla suruvate geenide koopiate arv genoomis. Kalad, kes kasvavad suureks ja elavad kaua, peavad suureks kasvamiseks vajalike proto-onkogeenide koopiate suuremat arvu kompenseerima ka vähikaitsegeenide koopiate arvu suurendamisega. Võrdlev uuring võimaldas ka ennustada, millised kalaliigid on kõige tugevama vähikaitsega (suhteliselt rohkem koopiaid vähikaitsegeenidest võrreldes onkogeenidega) ja millised, vastupidi, kõige nõrgema vähikaitsega. Seda teadmist saab edaspidi kasutada, hindamaks erinevate liikide ja populatsioonide ohustatust näiteks reostuse või muude vähki esile kutsuvate keskkonnategurite tõttu.

Jõe- ja soomuslestade mudelsüsteemis sain küsida aga uurimisküsimusi teiste bioloogiliste mehhanismide kohta, mis lisaks geenikoopiate arvule võiksid vähi allasurumisega looduslikus keskkonnas seotud olla. Kuna kummagi liigi genoom pole sekveneeritud, saab nende geene uurida avaldumise kaudu, ehk siis geeniekspresiooni tasemeid võrreldes. Uurisin, kas geenikspressioon erineb jõelestade ja soomuslestade vahel, lähtudes teadmisest, et soomuslestadel esineb vähki rohkem kui jõelestadel. Samuti uurisin võimalust, et üsna lokaalse iseloomuga lestapopulatsioonides võivad sõltuvalt kohalikest reostusoludest tekkida kohastumused, mis aitavad neil populatsioonidel reostuse vähki tekitava mõjuga toime tulla.

Geenikspressiooni analüüsid viitasid, et vähi tekkeks peab jõelestadel olema alla surutud mitmete geenide avaldumise tase, mis võib viidata sellele, et need geenid on seotud vähikaitsega. Sama ei ole näha soomuslestadel. See viitab võimalusele, et jõelestadel on tõepoolest olemas vähikaitsemehhanismid, mis erinevad soomuslestade omadest ja aitavad neil reostunud keskkonnas elades hoida madalamat vähitaset kui soomuslestade populatsioonides täheldada võib. Samal ajal leidsin, et jõelestadel avalduvad kõrgel tasemel mitmed reoainete ainevahetusega seotud geenid. Varasemates uuringutes on aktiivset reoainete ainevahetust peetud pigem vähki soodustavaks teguriks, sest reoainete metabolismi vaheproduktid on veelgi tugevamad onkogeenid kui reoained ise. Seega võib spekuleerida, et just aktiivne reoainete metabolism on jõelestade puhul valikusurveks, mille tulemusena on populatsioonidest kadunud vähile vastuvõtlikumad isendid ning alles jäänud vaid need, kellel on tugevam vähikaitse.

Kohaliku kohastumise mustrid olid vähem selged. Leidsin küll rea geene, mis avaldusid reostunud keskkonnas, aga mitte puhtas keskkonnas, kuid ei ole selge, kas need on seotud erinevustega vähivastases kaitses. Lisaks ei olnud neid geene kuigi palju. Need tulemused viitavad, et liikide vahelisel võrdlusel on tõenäoliselt rohkem perspektiivi kui liigisisesel lähenemisel, asurkondade võrdlemisel.

Kuna ei ole täpselt teada, mis mehhanismide kaudu reostus rakkudes vähitekkeni viib, testisin jõe- ja soomuslesta mudelsüsteemi peal ka võimalust, et

selleks vahendajaks on oksüdatiivsed kahjustused lestade DNA-s. Reoained põhjustavad teadaolevalt vabade hapniku radikaalide teket organismis ning need omakorda lõhuvad DNA-d. Vead DNA-s võivad rivist välja viia näiteks geenid, mis vastutavad vähikaitse eest. Selle hüpoteesi testimiseks mõõtsin lisaks reostustasemele ja vähi esinemise hindamisele maksarakkudest oksüdatiivseid kahjustusi. Minu andmetel aga oksüdatiivsed kahjustused DNA-s see rakusisene mehhanism ei ole – DNA kahjustused ei olnud seotud vähi esinemise või puudumisega. Mõned huvitavad mustrid siiski leidsin. Suurematel ja vanematel kaladel oli rohkem DNA kahjustusi ning Läänemeres oli kaladel kõrgem DNA kahjustuste tase võrreldes Põhjamerega. Läänemerd peetakse võrreldes Põhjamerega veelgi kehvemas seisus olevaks veekoguks ning tundub, et jälgi sellest võib näha ka kalade DNA-s.

Hulkraksetel liikidel on välja kujunenud looduslikud vähikaitsemehhanismid, mis liikide vahel erinevad. Nende erinevuste uurimine aitab mõista vähi evolutsioonilist rolli ning vähikaitse arenemist erinevates fülogeneesipuu harudes. Tänapäevases maailmas on looduslike vähikaitsemehhanismide ja vähikaitsega seotud kohastumuste mõistmine muutunud eriti oluliseks, kuna inimtekkelised keskkonnamuutused on suurendanud vähi esinemissagedust nii inimestel, inimesega koos elavatel loomadel kui ka looduslikel liikidel. Looduslike vähikaitsemehhanismide ja liikide erineva haavatavuse mõistmine vähitekke kontekstis võib tulevikus aidata paremini mõista reostuse mõju looduslikele liikidele. See võib panustada ka parematesse regulatsioonimehhanismidesse ning võimalustesse kaitsta looduslikke liike ja populatsioone väljasuremise eest.

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PUBLICATIONS

CURRICULUM VITAE

Name:	Ciara Baines
D.O.B:	26/12/1993
Email:	baines.ciara@gmail.com

Education

2019–present	PhD at University of Tartu, Estonia Adaptation to oncogenic pollution and natural cancer defences in the aquatic environment.
2018–2019	MSc Tropical Marine Biology, University of Essex – Merit
2013–2016	BSc Honours, Environmental Science, University of Hull – 2.1
2005–2012	St Mary Catholic Comprehensive Sixth form, Menston, Leeds
A level:	Maths: C, Physics: C, Geography: C
GCSE:	11 passes ranging from A* to B including Maths, English, Science

Work Experience

04/2019	Marine Conservation Society Seychelles
07/2017–09/2018	Hallmark Cards plc
03/2017–06/2017	Athena Innovations
06/2016–09/2016	Tigerprint, a division of Hallmark Cards
10/2015–07/2016	Sea Watch
08/2015	UK Environment Agency
07/2014–08/2014	Operation Wallacea
09/2010–06/2016	Argos Ltd
01/2013–03/2013	Voluntary Services Overseas (Kenya)

Publications

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Poster presentation at PRIMO21 conference in Gothenburg Sweden (May 2022)

Europaeum Winter School on Planetary Wellbeing (February 2022)

Oral presentation at WildCan Network meeting (May 2023)

ELULOOKIRJELDUS

Nimi:	Ciara Baines
Sündinud:	26/12/1993
E-mail:	baines.ciara@gmail.com

Haridustee

2019–…	Doktorantuur, Tartu Ülikool Kohastumine onkogeense reostusega ja looduslikud vähikaitsemehhanismid veekeskkonnas
2018–2019	MSc troopiline merebioloogia, University of Essex,
2013–2016	BSc *cum laude*, University of Hull – 2.1

Töökogemus

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07/2017–09/2018	Hallmark Cards plc
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