Evolution and Ecology of AhR genes in Atlantic salmon (Salmo salar L.)

Hansson, Maria

2004

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"Først i den nyeste tid begynder der att falde lys over mange hidtil gaadefulde sider af laksens liv men endnu er langtfra alt opklaret"

C. V. Otterstrøm, 1914, *Danmarks Fauna*

Evolution and ecology of AhR genes in Atlantic salmon (*Salmo salar* L.)

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Abstract

In vertebrates, studies of the aryl hydrocarbon receptor (AhR) have been of particular interest due to its direct involvement in the toxic effects exerted by several environmental pollutants, such as PCB and dioxin. After binding to the toxic molecule, this receptor initiates transcription of several genes that are part of the biotransformation battery but most notably the cytochrome P450 (CYP) genes, which results in an excessive release of oxidative enzymes and free radicals. The AhR is represented by a single gene in mammals (AhR1) but two distinct genes reside in other vertebrates like birds and fish (AhR1 and AhR2). The Atlantic salmon (Salmo salar L.) is a common top-predator to the Baltic Sea, a region that is one of the most contaminated bodies of water within Europe after a long history of high pollution exposures. In this thesis, I describe the identification and characterization of six AhR genes in the Atlantic salmon, two AhR1 and four AhR2 genes. I have investigated if AhR genotypes interact differently with individual load of PCBs so mediating differences in AhR/CYP1A1 transcription levels and thereby also affecting tissue levels of antioxidants in foraging individuals from natural populations of Atlantic salmon in the Baltic Sea. I have identified allelic variation in the AhR2 5´-flanking region and measured the levels of PCB and astaxanthin in muscle tissue. The transcription levels of the AhR2 genes and the CYP1A1 gene were measured by real-time quantitative PCR. The analyses revealed that the relative transcription levels of the AhR2a, AhR2g and AhR2d genes were significantly correlated with the relative transcription levels of the CYP1A1 gene and that the transcription of CYP1A1 was negatively correlated with antioxidant levels (i.e. astaxanthin) in muscle. The transcription level of one of the salmon AhR genes, AhRa, was significantly associated with the AhR2a 5´-flanking region genotype and with the interaction of the genotype and individual PCB level. There was no correlation between the levels of PCB in muscle and the transcription levels of CYP1A1. These results suggest that biotransformation activity in wild Atlantic salmon individuals from the Baltic Sea may be affected by genetic polymorphisms at the AhR loci. Finally, I have investigated the evolution of AhR genes in Atlantic salmon and vertebrates by searching for AhR homologs in the database containing the complete genome of the pufferfish (Fugu rubripes). I have described the evolution of vertebrates and fish in an attempt to explain the incidence of multiple AhR genes in the salmon genome, concluding that these genes probably arose from several whole-genome duplication events in the ancestors of salmonid fish.

Key words: Atlantic salmon, gene evolution, gene characterization, environmental pollutants, ecotoxicology, biotransformation, antioxidants, astaxanthin, phylogenetics, population genetics, Baltic Sea

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Evolution and ecology of AhR genes in Atlantic salmon (*Salmo salar* L.)

Introduction

The exposure to environmental pollutants like PCB and dioxin (TCDD) has had a great impact on many marine vertebrates living in the Baltic Sea for a long period of time. The uptake of many pollutants from the water, sediments and food is facilitated by the lipophilic ("fat-friendly") properties of many toxic chemicals, which enable the molecules to readily cross the cellular membranes and potentially bioaccumulate inside organisms. Consequently, the elimination of environmental pollutants from biological tissues by a process known as biotransformation in which the toxic molecules are enzymatically converted to easily excreted, water-soluble chemicals is of outmost importance to vertebrates. Fish, and especially salmonid fish, are extremely sensitive to the toxic effects induced by environmental pollutants (Horning et al., 1999; Elonen et al., 1998; Walker et al., 1991).

The Biotransformation system

All organisms are constantly exposed to foreign chemicals (xenobiotics), which include both natural (e.g. toxins produced by plants, animals and molds) and synthetic (drugs, pesticides, pollutants) chemicals. The mission of the enzymes in the biotransformation system is to transform toxic molecules that cannot be directly removed by the liver and kidney into water-soluble and easily excreted chemicals by modifying the chemical composition of the foreign compounds (by changing or removing functional groups such as -OH, -NH_2, -SH or -COOH; Parkinson, 1996). Several different enzymes participate in the biotransformation processes, which include hydrolysis, reduction, oxidation, sulfation, acetylation, methylation and conjugation reactions with glutathione or amino acids (Alexander, 1994). In the oxidation reactions, for example cytochrome P450 (CYP) enzymes, alcohol dehydrogenases (ADH) and aldehyde dehydrogenases (ALDH) are important participants. Occasionally, the ultimate toxicant is not the original toxicant to which the organism was exposed but a metabolite of the parent compound or a reactive oxygen species generated during the enzymatic biotransformation of the toxicant (Gregus and Klaassen, 1996). Many of the man-made environmental pollutants become highly toxic specifically due to the activation of the biotransformation enzymes (Alsharif et al., 1994; Nebert et al., 1996). After exposure to these toxicants, an excessive transcription of oxidative enzymes from the biotransformation genes is initiated. The extreme levels of enzymes result in the formation of high levels of free radicals which cause damage to DNA, proteins and lipids (Lackner, 1998; Marnett, 2000). Consequently, the levels of antioxidants are quickly depleted within exposed organisms (Spear and Moon, 1986; Hakansson et al., 1994; Rolland, 2000; Simms and Ross, 2001; Hallgren et al., 2001), which in turn affects important biological processes causing immune-function decline (Anderson, 1996) and ultimately contributes to cancer and degenerative diseases (Ames et al., 1993; Nishino, 1998; Chew et al., 1999). In humans, high numbers of xenobiotic-metabolizing enzymes have been identified, which in addition display differences in gene expression and genetic polymorphisms between individuals (Daly et al., 1993). In Fig. 1, the biotrans-

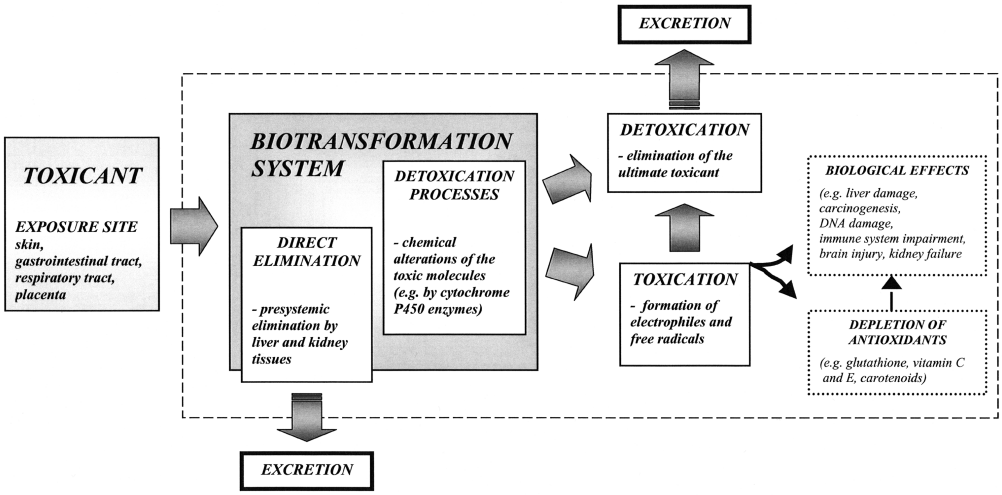

Fig. 1. The basic principles of the biotransformation system. The enzymatic processes that chemically alter the composition of xenobiotics results in the formation of reactive products, which affect antioxidant levels and ultimately cause a decline in the function of the immune system and the internal organs.

formation pathway and its consequences, are displayed schematically.

The Aryl hydrocarbon Receptor (AhR)

In vertebrates, the aryl hydrocarbon receptor (AhR) has been the most extensively studied inducer of biotransformation enzymes since it mediates the responses induced by several environmental pollutants like PCBs and dioxin (Hankinson, 1995; Schmidt and Bradfield, 1996; Denison and Heath-Pagliuso, 2000). The AhR resides in the cytosol of cells (Fig. 2) and is a ligand-activated nuclear transcription factor which is part of the basic-helix-loop-helix (bHLH) and Per-ARNT-Sim (PAS) protein superfamily (Schmidt and Bradfield, 1996; Hahn, 1998; Crews and Fan, 1999). After binding to the ligand the AhR-complex associates with specific DNA elements, which initiates transcription of several genes that are part of the biotransformation battery (Dong et al., 1996; Nebert et al., 2000) but most notably the CYP genes (Gonzalez, 1989; Whitlock, 1999). This initiates the release of an excessive amount of oxidative enzymes which ultimately results in a state of oxidative stress which has physiological (Alsharif et al., 1994; Shertzer et al., 1998) and evolutionary (von Schantz et al., 1999) consequences.

From studies on different strains of mice it has been demonstrated that allelic variation at the AhR locus could result in differences in dioxin binding affinity (Poland et al., 1994). Furthermore, in populations of Atlantic killifish (*Fundulus heteroclitus*) from highly contaminated environments, heritable resistance to halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons has been ascribed to a down-regulation of the AhR pathway (Nacci et al., 1999). Specific alleles in the coding regions of the killifish AhR1 gene were found to be under-represented in a population of TCDD- and PCB-resistant fish as compared to fish from a less contaminated reference site in a study by Hahn et al. (2004). However, functional analyses found no differences in binding capacities and affinities for TCDD between these different AhR proteins (Hahn et al., 2004). A study by Roy and Wirgin (1997) showed that polymorphisms in the coding region at the Atlantic tomcod (*Microgadus tomcod*) AhR locus (AhR2) differed between individuals sampled from a population in the contaminated Hudson River compared with fish from cleaner rivers. In addition, the incidence of tumors in the Hudson River population had decreased substantially during recent years as compared with previous measurements even though the levels of pollutants in the waters remained

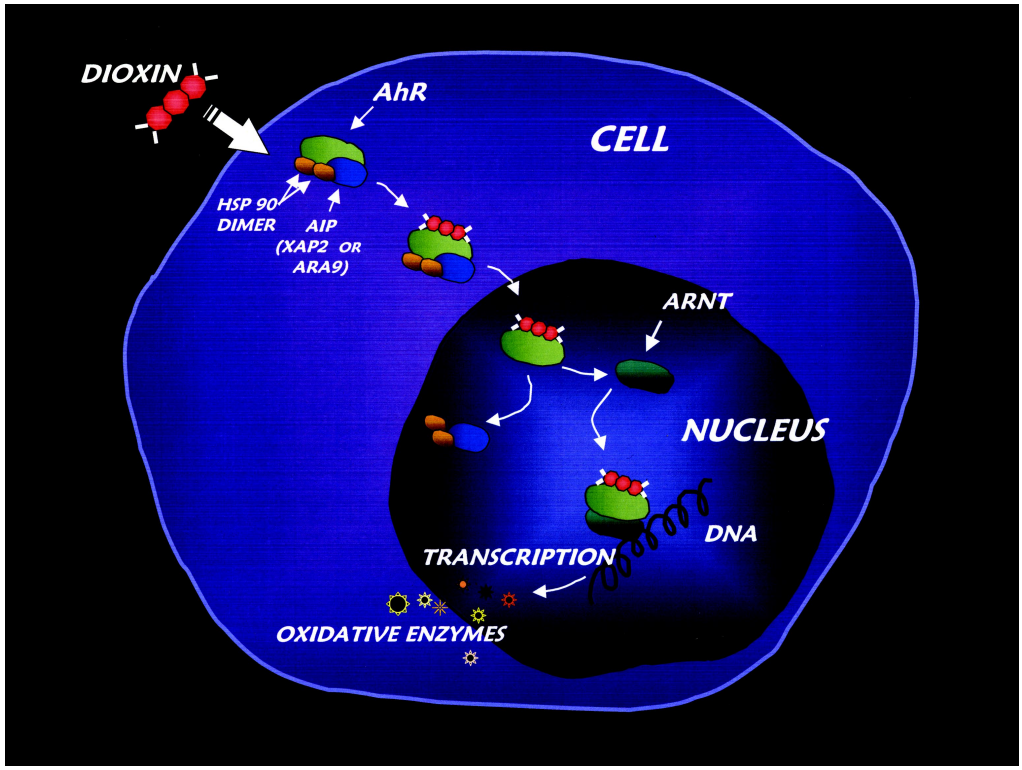

Fig. 2. The AhR pathway. After entering the cell, the dioxin molecule attaches to the AhR protein complex which initiates a translocation to the cell nucleus where the AhR-dioxin complex binds to the ARNT protein and subsequently triggers the expression of genes which form part of the biotransformation system.

high. The conclusion from the latter study was that tomcods from the Hudson River population had adapted to their contaminated environments by specifically down-regulating the AhR pathway and thereby transcription of CYP1A1. However, the significance of these AhR polymorphisms was not investigated further.

AhR diversity

AhR genes are generally divided into two distinct evolutionary lineages, AhR1 and AhR2 (Fig. 3). In mammals, only the AhR1 gene is present in contrast to birds and fish where genes from both lineages have been identified within organisms (Hahn, 2002). All vertebrate AhRs examined to date share the dioxin-binding properties first demonstrated for mammals (Hahn, 2003). AhR genes have been characterized in several species of fish since fish are particularly sensitive to the toxic effects induced by AhR-mediated xenobiotics, especially during their early life stages (Walker et al., 1991; Elonen et al., 1998; Hornung et al., 1999). AhR gene homologs have been identified and found expressed in several invertebrates including a nematode *Caenorhabditis elegans* (Powell-Coffman et al., 1998), the fruit fly *Drosophila melanogaster* (Duncan et al., 1998) and the soft-shell clam *Mya arenaria* (Butler et al., 2001) which would indicate that the AhR gene emerged very early in animal evolution. A common feature of the invertebrate AhRs, however, is the relatively low sensitivity to dioxin-like compounds which is in strong contrast to the vertebrate AhRs (Hahn, 1998; Butler et al., 2001).

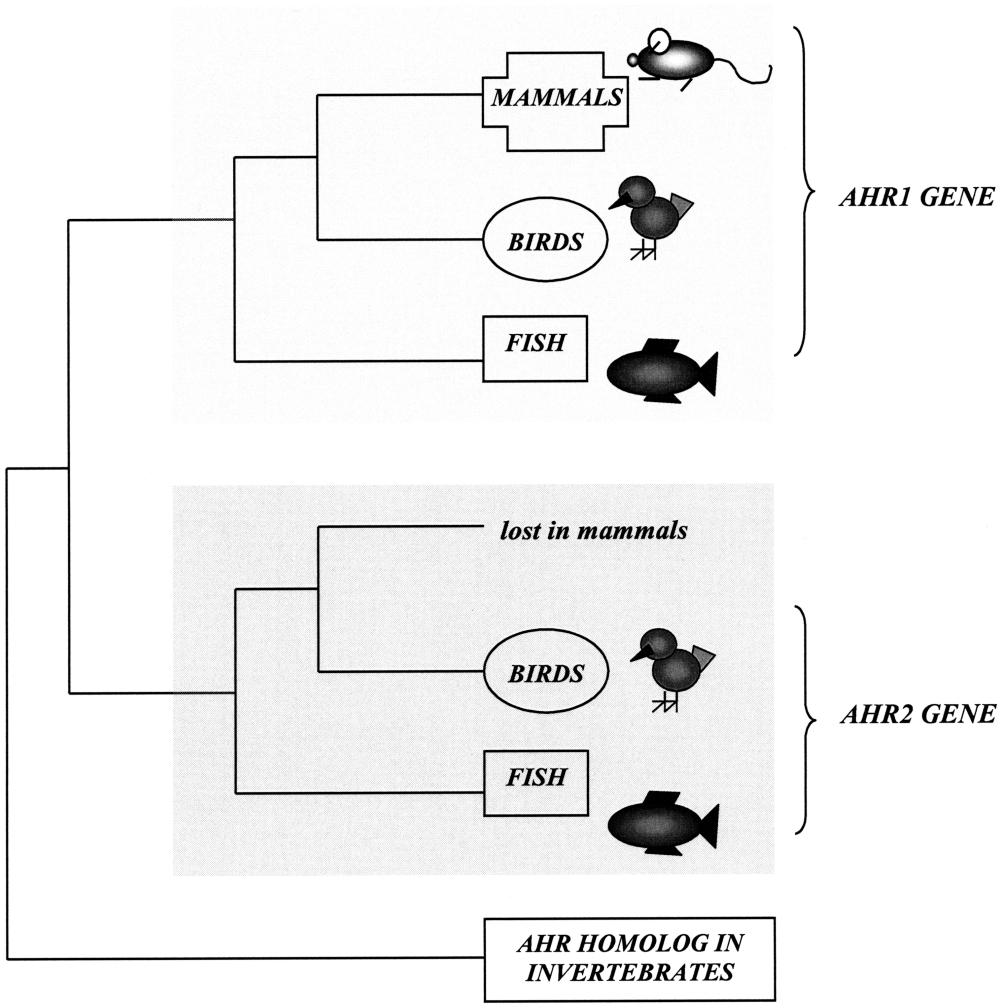

Fig. 3. In vertebrates, there are two distinct AhR gene lineages, AhR1 and AhR2. In avian and fish species, both genes are retained in contrast to mammals where the AhR2 gene is missing and most likely was lost in a early mammalian ancestor. In invertebrates a single AhR gene homolog has been identified. However, the invertebrate AhR lacks the dioxin-binding properties characteristic of the vertebrate AhRs.

The Atlantic salmon

The Atlantic salmon (*Salmo salar* Linnaeus 1758, with the Latin name meaning "the leaper") belongs to the Salmonidae family which is divided into three subfamilies; Salmoninae (charr, salmon and trout), Coregoninae (whitefish and cisco) and Thymallinae (grayling) all together comprising approximately 66 species (Nelson, 1994). The species is distributed around the North Atlantic basin (northeastern America and Europe), in the European Arctic and surrounding streams. The salmon population in the Baltic Sea, a region that was colonized stepwise by immigrating species during the retreat of the continental ice which ended approximately 10,000 years ago, is considered evolutionary distinct from the two populations (West and East) in the Atlantic Ocean (Ståhl, 1987). The Atlantic salmon is mainly an

anadromous species (i.e. spawn in fresh water but spend the majority of its life in marine environments) but resident freshwater populations occur (Klemetsen et al., 2003). Salmon spawn in shallow tributaries during fall where the eggs hatch and the fry migrate out to sea after one to six years where they typically spend one or several years at feeding grounds before migrating back to the native birthplace for spawning. As a consequence of their migratory behavior, salmon have a high content of fat deposited in their muscles (10–20%) (Larsson et al., 1996), which is stored for growth of reproductive organs as well as for energy to be utilized during the migratory swimming phase.

The Atlantic salmon has a high commercial value. However, numerous populations are in continued decline and numbers have dropped greatly in the past half-century. To compensate for the losses directly caused by the damming of rivers for hydropower production, several populations are subject to artificial rearing in hatcheries. Salmon decline has, in addition to the damming of rivers, been ascribed to the high sensitivity of salmonids to environmental pollutants which occur in high levels in many marine and fluvial habitats. The Baltic Sea (Fig. 4) is one of the most contaminated bodies of water within Europe, with high levels of pollutants such as PCBs in both water and sediments (Olsson and Reutergårdh, 1986; Andersson et al., 1988; Larsson et al., 1996) – a direct consequence of the high pollution exposure that has been mediated by the numerous rivers which transport contaminants from large areas in the surrounding countries out to open sea (Jansson and Dahlberg, 1999). The Atlantic salmon is a common top-predator to the Baltic Sea. Accordingly, the levels of pollutants in fat tissue of Baltic salmon are high (Larsson et al., 1996).

Evolution and Ecology of AhR genes in Atlantic salmon

The scope of my thesis has been (1) to identify and characterize the AhR genes in Atlantic salmon, (2) conduct phylogenetic analyses of the genes, (3) study their variation and identify differ-

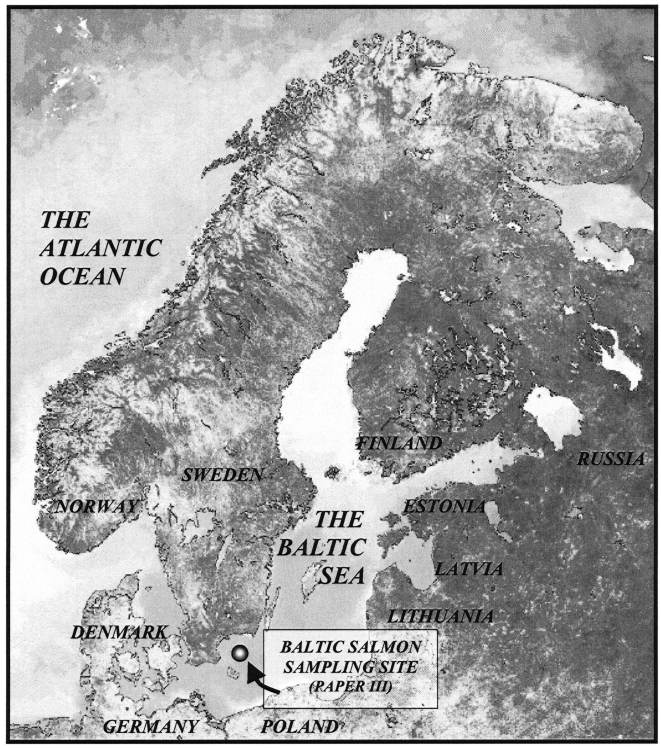

Fig. 4. The Baltic Sea and its surrounding countries. Numerous rivers drain the adjacent land areas, transporting high levels of sediments, nutrients and hazardous substances out to open sea.

ent AhR genotypes and (4) to investigate to what extent tissue loads of PCB congeners and AhR genotypes contribute to biotransformation activity in foraging individuals from natural populations of Atlantic salmon in the highly polluted Baltic Sea.

Identification of AhR genes

To identify AhR genes in Atlantic salmon we initially constructed a cDNA library, which was screened with a 636 bp long sequence of the killifish AhR2 gene (paper I). This revealed a single AhR clone that contained an AhR gene sequence, which was incomplete at least at the 3´end. To obtain complete 5´and 3´ends of this AhR gene, I used the RACE (rapid amplification of cDNA ends) method which gave me the complete AhR cDNA sequence However, amplifications and subsequent sequencing revealed several polymorphic sites and two distinct 5´and 3´ends. After an additional cloning step, two complete and distinct AhR cDNA gene sequences were isolated. Since both AhR sequences could be amplified from 30 different salmons (using PCR amplifications and direct sequencing) we concluded that the two AhRs were two distinct genes and not alleles. To verify their transcription activity in salmon tissues, we performed reverse-transcriptase (RT) PCR, using mRNA isolated from muscle, spleen and liver as template, which demonstrated that these AhRs were transcribed in all three tissues. Subsequent phylogenetic analyses demonstrated that they belonged to the AhR2 gene family but also that they were more similar to each other than to any other AhR gene identified in other organisms, including the two AhR2 genes (α and β) identified in the closely related rainbow trout (*Oncorhynchus mykiss*). Thus, it was concluded that the two salmon genes were not the orthologs[1] of the rainbow trout genes but represented a separate and distinct AhR2 lineage in salmonid fish. The two paralogous[1] Atlantic salmon AhR genes were designated as AhR2γ (gamma) and AhR2δ (delta) to distinguish them from the trout genes. However, in the same study (paper I), we successfully amplified a partial AhR sequence from rainbow trout DNA that was more similar to the Atlantic salmon AhR2 genes than to the two previously known α and β genes in the trout. This fragment likely represented an AhR2 gene (or genes) in trout that was orthologous to the novel salmon genes. This demonstrated that multiple AhR genes and lineages reside in salmonid fish and that the Atlantic salmon genome also may contain additional AhR genes other than the AhR2γ and AhR2δ.

To investigate this possibility more thoroughly (paper II) we constructed a cosmid library where large sequences of genomic DNA (30,000–42,000 bp) were inserted into cosmid vectors and subsequently screened for AhR gene homologs using the AhR2γ sequence as a probe. The screening revealed several partial AhR sequences in isolated cosmids (cosmid sequences were subcloned before sequencing, see Fig. 5). Coding regions were identified by alignments with the two Atlantic salmon AhR2γ and AhR2δ cDNA sequences as well as the Atlantic tomcod AhR2 gene where exon-intron borders are determined. The partial genomic sequences obtained from cosmids were used to construct gene specific primers, which were used in RACE analyses resulting in the complete characterization of two additional AhR2 genes. These genes were more similar to the trout AhR2α and β genes than the salmon AhR2γ and δ. Accordingly, these two novel Atlantic salmon AhR genes were determined to be the orthologs of the trout genes and were designated the salmon AhR2α and AhR2β genes.

In paper II, two additional, highly similar but not identical genomic AhR sequences were identified from the cosmid screenings that were more similar to other fish AhR1 genes than any of the four salmon AhR2 genes thus far identified. However, RACE analyses did not readily amplify these sequences from the synthesized cDNA (from liver and spleen mRNA). Eventually, repeated amplification attempts revealed two sequences that were most likely complete in the coding regions but the 5´and 3´end sequences remain uncertain. It is possible that these genes are not transcribed in liver and spleen but can be isolated from other tissues in the future. These genes were designated as the salmon AhR1α and AhR1β genes.

[1] Two genes are said to be orthologous if they are derived from a speciation event and paralogous if they are derived from a duplication event.

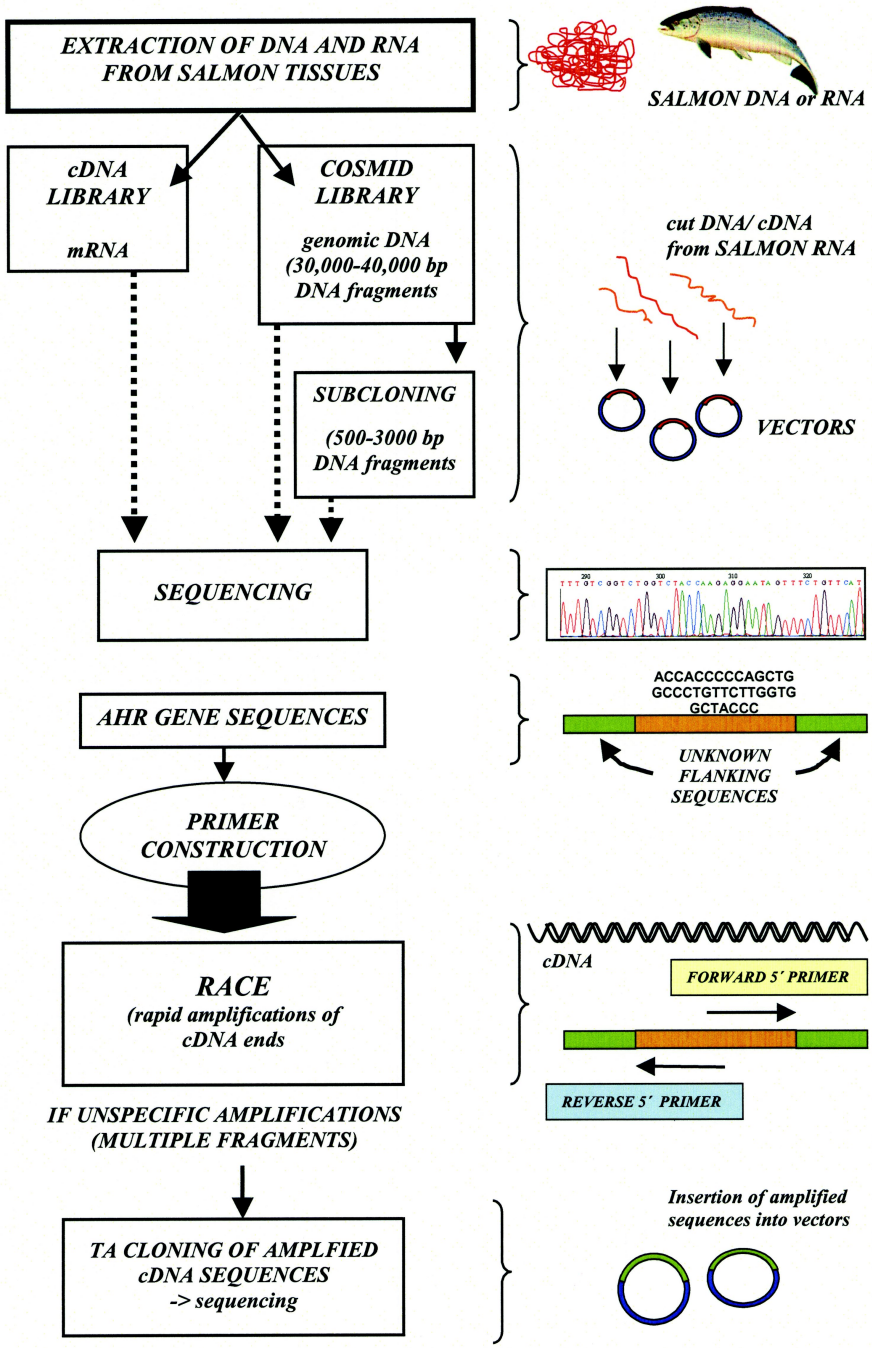

Fig. 5. The principles of gene characterization. Initially, DNA and/or RNA is extracted from tissues and can be utilized for construction of cosmid and cDNA libraries, respectively. From the obtained sequences, gene specific primers are designed and used in Rapid Amplification of cDNA Ends (RACE) analyses with cDNA modified in the 5´and 3´ ends as template (5´and 3´oligo sequences are added during the cDNA synthesis).

Identification of AhR alleles

There were no indications of allelic variation residing in the coding regions of any of the six AhR genes from the RACE analyses. Previous studies on Atlantic tomcod (Roy and Wirgin, 1997) and killifish (Hahn et al., 2004) have demonstrated allelic variation in both the conserved ligand-binding region (exon 5 to 9) as well as in the non-conserved (exon 10–11) region. However, repeated amplifications of both regions using DNA from 10 to 20 salmon (sampled in rivers in both northern and southern Sweden) revealed no polymorphic sites in any of the genes, even when including the interspaced intron sequences (unpublished).

Instead, I decided to search for genetic polymorphisms in the 5′-flanking regions of each AhR gene (paper III) where transcription and gene expression is regulated (Garrison and Denison, 2000; Racky et al., 2004). To obtain previously uncharacterized sequences of the 5′-flanking regions of the genes, we utilized the inverse PCR (IPCR) amplification method (Ochman et al., 1988). Restriction digests were carried out using genomic DNA from one salmon. Since the 5′-flanking regions of each of the four salmon AhR2 genes were the objects of the amplifications, primers were placed in the close vicinity of the start codons of the AhR2α, AhR2β, AhR2γ and AhR2δ genes. The AhR1α and AhR1β genes were not included in these analyses since the sequences for these genes were not complete in the 5′end, thus preventing IPCR primer design.

From the IPCR characterizations I found evidence of allelic variation in the 5′-flanking regions of all four investigated AhR2 genes (paper III). The polymorphic sites were confined to distinct stretches in all four sequences in a region 250–530 bp before the start codon. I identified a total of two alleles in the AhR2α 5′-flanking region, which differed by a single nucleotide at position –530. Seven distinct sequences were identified from the AhR2β 5′-flanking region in a stretch between positions –175 to –191. I found two polymorphic sites in the AhR2γ 5′-flanking region at position –253 and before position –482 and at total of three alleles were identified. Two polymorphic sites were identified in the AhR2δ 5′-flanking region at positions –322 and –345 and three alleles were identified.

Baltic salmon analyses

Having identified several different allelic variants in the 5′-flanking regions of the AhR genes, we wanted to investigate to what extent tissue loads of PCB congeners and AhR genotypes contribute to biotransformation activity in foraging individuals from natural populations of Atlantic salmon residing in the Baltic Sea (paper III). From 1999 to 2003, 96 adult (1–4 years in the sea) wild Atlantic salmon in their foraging phase were captured in the open part of the southern Baltic Sea (Fig. 3). Liver samples for the genetic transcription analyses were immediately frozen on board in liquid nitrogen. Individual fish was weighed and the length and sex were determined. Muscle tissues were sampled for PCB and astaxanthin level analyses (a measure of antioxidant status) by removing a muscle section behind the neck region of each salmon. Subsequently in the lab, individuals were screened for their 5′-flanking region alleles and the genotype of each salmon was determined. In addition, the PCB and astaxanthin levels of 53 and 51 salmon, respectively, were determined. To measure the relative transcription levels of each of the AhR2 genes, I performed real-time quantitative PCR on 88 salmon using cDNA from liver RNA as template. The values obtained for the AhR2β gene were not included in the statistical analyses due to unreliable amplifications. To get a measure of biotransformation activity, I also measured the transcription levels of the CYP1A1 gene.

In paper III, we found that the total PCB ($\sum$PCB) values (i.e. the sum of 21 PCB congeners) measured in muscle tissue (wet weight) averaged 69.3 ng/g and varied from 5.02 to 314.7 ng/g (n=53). The lipid content in the same muscle tissue averaged 12.6% and ranged from 2.2 to 31.2% (n=53). $\sum$PCB was highly correlated with lipid content in muscle tissue ($r=0.533$, $p<0.001$, n=53; Fig. 6). An ANCOVA analysis showed that the lipid content, the gender as well as the interaction between lipid content and gender, were significantly associated with $\sum$PCB. The standardized residual value (RESPCB; concentration of $\sum$PCBs, i.e. sum of 21 congeners identified in muscle controlled for the effects of lipid content and gender) of this ANCOVA analysis was used as the measure of $\sum$PCB controlled for the effects

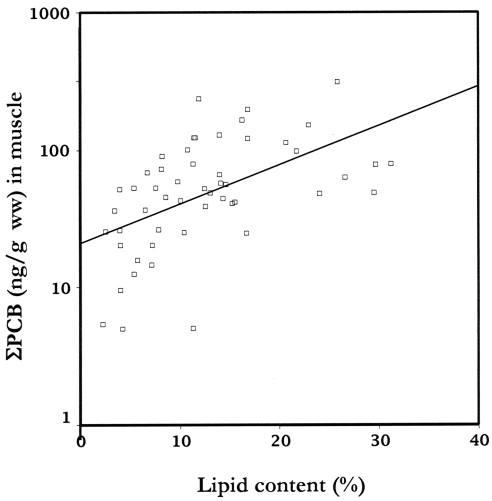

Fig. 6. The correlation between the log transformed values of ∑PCB (ng/g wet weight) in muscle and muscle lipid content (%), (r=0.533, p<0.001, n=53).

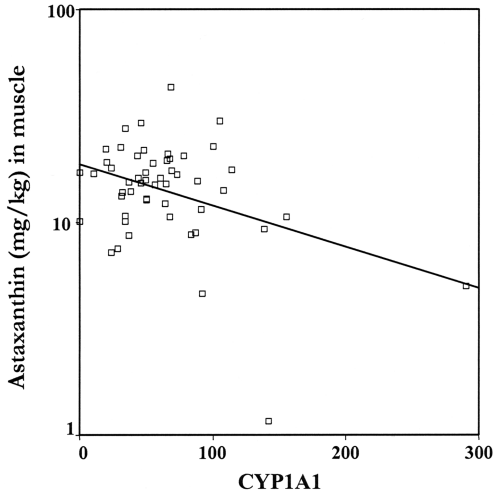

Fig. 7. The correlation between the transcription levels (normalized values) of CYP1A1 in liver and the log transformed astaxanthin levels (mg/kg) in muscle (r=–0.380, p<0.01, n=51).

of lipid content and gender. Astaxanthin was the dominant carotenoid in muscle tissue and constituted between 25–80% of the total amounts of carotenoids in the samples. The sum-astaxanthin levels in muscle averaged 0.489 mg/kg and ranged from 0.037 to 1.361 mg/kg. The RESPCB did not correlate (p>0.05) with any of the transcription levels of AhR2α, AhR2γ, AhR2δ or CYP1A1, respectively. Nor did RESPCB correlate with muscle concentration of astaxanthin.

The transcription levels of each of the three AhR2 genes were significant predictors (p<0.01, n=88 in all cases) of the transcription levels of CYP1A1 (AhR2α, r=0.349; AhR2γ, r=0.406; AhR2δ, r=0.321). Multiple regression analysis revealed that AhR2α and AhR2γ were more influential than AhR2δ as predictors of the transcription of CYP1A1.

Muscle concentration of astaxanthin was negatively correlated with the transcription levels of CYP1A1 (r=–0.380, p<0.01, n=51, Fig. 7). The transcription levels of AhR2α, AhR2γ or AhR2δ were not directly associated with astaxanthin level.

An ANOVA analysis revealed that the transcription level of the AhR2α gene was significantly associated with AhR2α 5′-flanking region genotype. The transcription of the other two measured AhR genes (AhR2γ and AhR2δ) was not associated with their 5′-flanking region genotype. As mentioned previously, RESPCB *per se* was not associated with the transcription of the AhR2α gene. However, by including RESPCB as covariate in an ANCOVA with the transcription levels of the AhR2α gene as dependent variable and AhR2α 5′-flanking region genotype as category variable, we found a significant effect of the interaction between genotype and RESPCB (paper III).

Thus, the results of our study supported, at least in part, our initial idea that biotransformation activity in wild Atlantic salmon individuals from the Baltic Sea may be affected by genetic polymorphisms in the AhR genes. However, the RESPCB in muscle was not directly associated with any of our measured criterions of induced biotransformation activities. Other studies have previously found significant relationships between the levels of PCB in muscle and CYP1A1 transcription in Atlantic salmon (Arukwe et al., 2000) and that levels of PCB can be directly correlated with levels of antioxidants in lake trout (Palace et al., 1996). Those results were, however, obtained under controlled laboratory conditions

by specific injections of PCBs directly into sampled fish. In contrast, the results presented in this study originate from analyses on a free-ranging Baltic Sea salmon population that has not been subjected to any prior modulations other than natural selection.

Since Atlantic salmon is an anadromous species, lipids to be utilized during migration to spawning rivers are mainly deposited in muscle tissue during the life stage that is spent foraging out at open sea. Throughout their lives pollutants from prey accumulate in tissues and may reach high levels. When the lipophilic pollutants are deposited together with lipids in the muscle tissues, the pollutants do not necessarily have effects on the biotransformation system and the major part of the pollutants can be "deactivated" in the accumulating lipids. Accordingly, migratory species like salmon and eel (*Anguilla anguilla*) are mostly affected by the toxic effects induced by bioaccumulated pollutants during the energy-demanding migration phase (Larsson et al., 1991) when fat content decrease by up to 69% (Henderson and Tocher, 1987). This is a direct result of energy being utilized for swimming, sexual maturation, gonad development and spawning. Considering that our study has focused on individuals from a foraging salmon population, it is possible that the ∑PCB deposited in the muscle lipids do not affect the activity of genes involved in the processes of the biotransformation system (AhR and CYP1A1). It is also possible that the levels of PCB in blood and/or in the liver of foraging salmon would better reflect the pollutant exposure and, thus, the induced biotransformation activities of AhR and CYP1A1. Hence, the exposure on foraging fish ought to be far less compared to fish in their migration phase and most especially to when the lipid deposits of salmon are exhausted at spawning.

Evolution of the AhR genes

The identification of six distinct AhR genes in Atlantic salmon in this thesis is evidence of an unprecedented genomic diversity of AhR genes within one single organism. In paper IV, I investigate how many AhR gene homologs can be detected within the genome of another teleost, namely the pufferfish *Fugu rubripes* whose complete genome was recently made publicly available (Aparicio et al. 2002). From these searches, five separate *Fugu* AhR sequences were identified which were determined to be two AhR1 and three AhR2 genes by phylogenetic analyses. One AhR1 and one AhR2 gene were found in a tandem repeat suggesting that the two gene lineages may originate from a tandem duplication that occurred in an early teleost ancestor. Two of the AhR2 *Fugu* homologs and one of the AhR1 homologs were orthologous to the three paralogous Atlantic salmon AhR gene pairs. An ortholog to the fifth AhR *Fugu* gene has however, not been identified in salmon and I, accordingly, suggest the possibility that additional uncharacterized AhR genes may still reside in the salmonid genome.

The alleles that were identified in the 5´flanking regions of the Atlantic salmon AhR2 genes revealed that none of the four AhR2 genes appear to be linked. Thus, all four Atlantic salmon AhR2 genes may lie on separate chromosomes and linkage may instead reside between the salmon AhR1 and AhR2 genes – a scenario that is supported by the tandem repeat of AhR1 and AhR2 genes in *Fugu*. The lack of linkage between the four AhR2 genes in Atlantic salmon would suggest that these genes are likely the result of two separate genome duplication events – most likely from the fish specific as well as salmon specific whole-genome duplications and not from separate tandem duplications. Thus, the analysis in paper IV indicates that at least two rounds of genome duplications have created the surplus of salmonid AhR genes (Fig. 8).

One major explanation as to why fish, and most notably salmonid fish, retain multiple copies of several genes comes from their evolutionary history (paper IV). Several whole-genome duplication events have resulted in a high incidence of duplicated genes in fish. In addition, the ancestor to salmonid fish is believed to have undergone an additional genome duplication 25–100 million years ago (Allendorf and Thorgaard, 1984). It was suggested early that the complexity and success of the vertebrates was facilitated by a series of gene duplication events, which allowed new gene functions to evolve (Ohno, 1970). The surplus of genes that resulted allowed new functions to be assigned to the duplicated genes. Having a tetra-

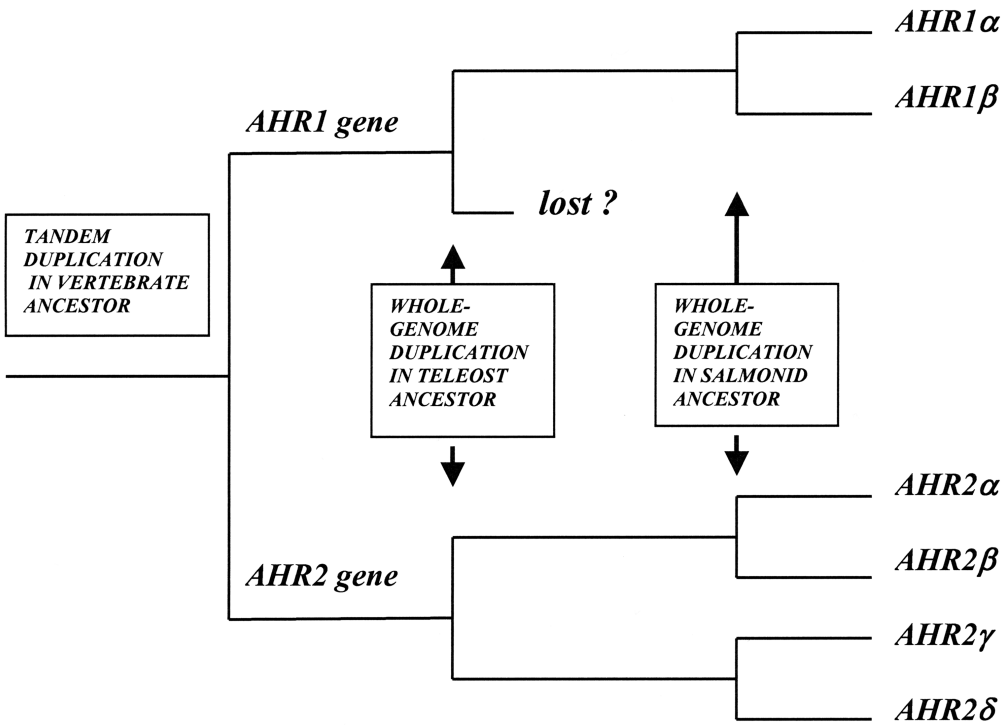

Fig. 8. Evolution of AhR genes in Atlantic salmon. Two genome duplications (one in the teleost fish lineage after the split between the fish, mammals, birds and reptilian lineages had occurred, followed by a an additional whole-genome duplication in a salmonid ancestor) most likely duplicated the AhR genes twice resulting in the four AhR2 genes seen in Atlantic salmon today. Presently, it is not known if additional AhR1 or AhR2 genes reside in salmon.

ploid genome might have been beneficial and may have facilitated the quick and successful local adaptations of salmonids to habitats in the northern hemisphere since the end of the latest glaciation some 10,000 years ago (Allendorf and Thorgaard, 1984). In salmonids, the observed rate of gene silencing has been slower than predicted by theoretical models, which has been suggested to be due to retained tetrasomic segregation (Ferguson and Allendorf, 1991). Allendorf and Thorgaard (1984) suggested that the tetraploidization event facilitated the evolution and adaptation of salmonid anadromous life-history by producing different genes which could be expressed during either the fresh water or the marine part of their lives. It is clear that the AhR did not evolve to respond to man-made chemicals but what the endogenous functions of the vertebrate AhRs are, remains largely unknown even today. I suggest in paper IV that a possible explanation as to why salmonids have the highest sensitivity among vertebrate species to the detrimental effect of environmental pollutants (Hahn, 2001; Tanguay et al., 2003), may be the high incidence of AhR genes retained specifically in these species.

Conclusions and future directions

In this thesis, I show that multiple AhR genes reside in the Atlantic salmon genome; AhR1α, AhR1β, AhR2α, AhR2β, AhR2γ and AhR2δ. I demonstrate that there is allelic variation in the 5´-flanking region of the AhR2 genes and that, in the case of the AhR2α gene, the level of transcription is significantly associated with AhR2α genotype and that the genotypes interact differently

with individual level of PCB. These results suggest that biotransformation activity in wild Atlantic salmon individuals from the Baltic Sea may be affected by genetic polymorphisms in the AhR genes. Finally, I discuss the evolutionary history of the AhR genes and vertebrates in an attempt to explain the multiplicity of AhR genes specifically in salmonid species.

There are still many unanswered questions about the evolution and ecology of AhR genes, especially in salmonid species. For the future, characterization of the dioxin-binding abilities of the Atlantic salmon AhR genes will provide a more complete picture of the effects exerted by the numerous toxins prevailing in our ecosystems today. This could help resolve why salmonid species are more sensitive to the toxic effects of environmental pollutants than other vertebrates. It is today essential to study the evolution of allele frequencies of the biotransformation genes and the receptors transcribing them, especially in species that are highly sensitive to the toxic effects induced by environmental pollutants.

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Svensk sammanfattning

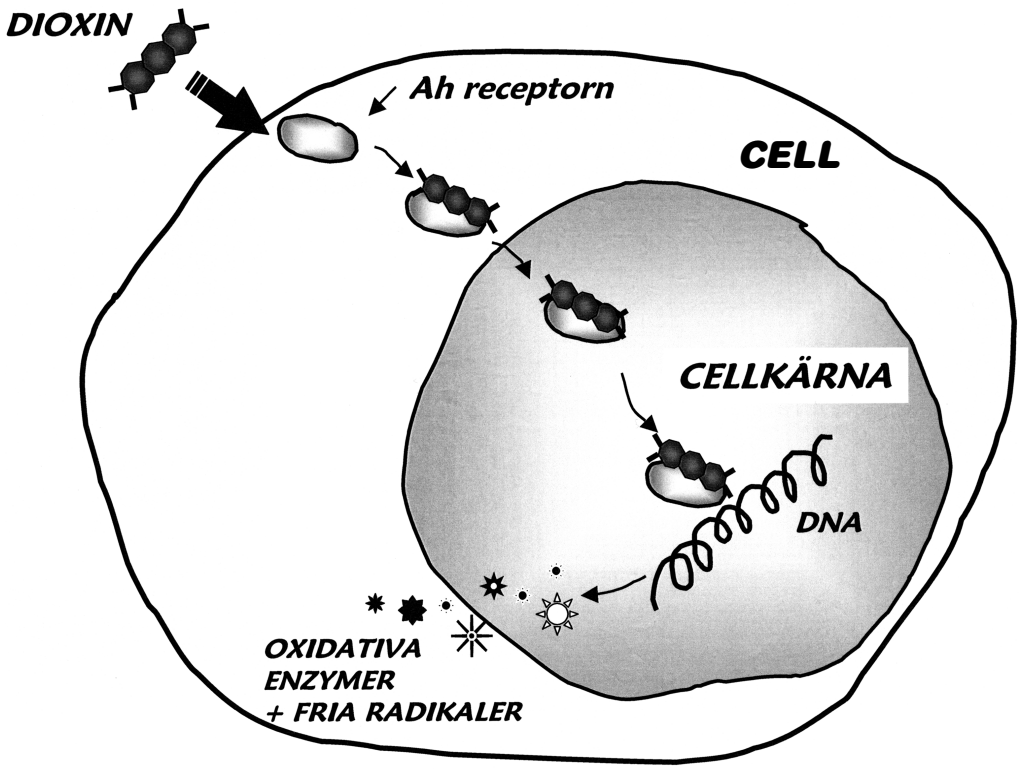
När främmande fettlösliga ämnen som miljögifter (ex. PCB och dioxin) hamnar inuti kroppen bearbetas de ofta av det så kallade biotransformations-systemet. När inte levern och njurarna direkt kan utsöndra de främmande molekylerna, får enzymer ändra de oönskade ämnenas kemiska sammansättning. Detta görs genom att ändra molekylernas kemiska egenskaper så att de blir vattenlösliga och lättare att få ut ur kroppen via galla eller urin. I processen där de främmande molekylerna görs vattenlösliga bildas ofta även reaktiva sidoprodukter, s.k. fria radikaler som inverkar skadligt på omgivande celler. För att skydda sig mot dessa är det därför viktigt för organismer att ha antioxidanter som fångar upp de fria radikalerna innan de hinner göra för mycket skada.

Hos alla ryggradsdjur är det en speciell receptor som specifikt binder till PCB – och dioxinmolekylerna – den s.k. aryl hydrocarbon receptorn (Ah-receptorn). Receptorn återfinns inuti cellerna och när exempelvis dioxin binder till receptorn sätts en kedjeprocess igång som leder till att mängder av olika enzymer från biotransformationssystemet börjar produceras (se figur). Den stora produktion av fria radikaler som denna biotransformation ofta genererar kan i slutändan ge upphov till de symptom som är klassiska vid miljögiftsexponering, dvs. cellförändringar som kan bilda tumörer, nedsatt immunförsvar, dålig leverfunktion, mm. Dioxin – ett av världens mest farliga kemikalier – är så farligt just på grund av att Ah-receptorn sätter igång biotransformationssystemet i så hög grad.

Ah-receptorn upptäcktes först hos laboratoriemöss där det visade sig att individer som hade olika varianter av den gen som kodade för Ah-receptorn även hade olika förmåga att binda dioxin. När man sen kunde helt ta bort den gen som kodar för Ah-receptorn insåg man vilken stark betydelse denna receptor har för biotransformationen av dioxin. Dessa möss kunde nämligen inte bara överleva utan genen utan var dessutom helt resistenta mot dioxinets normala skadeeffekter. Vad som är Ah-receptorn egentliga, endogena funktion i kroppen är idag oklart. Att den har utvecklats för att binda just miljögifter är dock inte troligt eftersom dessa kemikalier bara funnits i större mängder i ekosystemet under det senaste århundradet. Dessa nya kemikalier bara ”råkar” binda till Ah-receptorn.

Fiskar, och framförallt laxfiskar, har i jämförelse med däggdjur påvisats vara extra känsliga för påverkan av miljögifter. I svårt förorenade amerikanska floder där antalet friska fiskar varit på neråtgående under flera decennier observerades det under 1990-talet plötsligt att fiskarna blev allt friskare trots att mängden gifter i flodernas vatten var i stort sätt oförändrad. Detta tillskrevs förändringar i den gen som kodar för Ah-receptorn och att dessa förändringar hade minskat receptorns kapacitet att starta produktionen av reaktiva enzymer. Anledningarna till varför laxfiskar är de allra mest känsliga för miljögifter är ännu inte klarlagda. I Östersjön har miljögiftshalterna varit mycket höga under lång tid. Det var därför intressant att undersöka om Östersjölaxen har utvecklat olika varianter av Ah-receptorer som gör laxarna mindre känsliga för miljögiftspåverkan.

I min avhandling visar jag att det finns fler Ah-receptor gener hos laxen än vad som hittills påvisats hos någon annan organism – hela sex stycken – vilket troligtvis är en direkt konsekvens av att hela arvsmassan hos laxens förfader fördubblades flera gånger för miljontals år sedan. Jag visar också



i min avhandling att det hos laxar från Östersjön finns genetiska skillnader i den del av Ah-receptor genen som bestämmer hur mycket som ska uttryckas i cellerna (dvs. hur många receptorer som genen ska producera) och hur många Ah-receptorer som det faktiskt finns i levern. Min avhandling visar alltså att aktiviteten av biotransformationen hos Östersjölaxar kan påverkas av genetiska varianter av Ah-receptorn. Dessutom visar jag att mängden antioxidanter (i form av det röda pigmentet astaxanthin) i laxarnas muskler minskar i takt med att halten enzymer ökar och att ju mer fett en lax från Östersjön har, desto mer PCB finns lagrat i musklerna. Eftersom laxarna i Östersjön lagrar stora mängder fett i musklerna till deras energikrävande vandring tillbaka till lekplatserna där de själva kläcktes, är det troligt att miljögifterna kommer att påverka laxarna den dag när de påbörjar förbränningen av fettreserverna.

Tacksamhet…

(*This is written in Swedish. For those of you who do not understand the following, look upon it as an educational lesson in a culturally advanced and sophisticated language*.)

Sedan jag började som doktorand på Ekologihuset i oktober 1999 har mitt liv i princip kretsat runt dess innevånare så det känns som om jag nu ska säga tack för så mycket av vad ni låtit mig ta del av under dessa år. Ni har format mig till den jag är idag, begrunda detta… detta är ett axplock av vad ni har gett och eventuellt lärt mig…

Torbjörn; min handledare som gav mig möjligheten att genomföra det jag drömt om sedan jag gick i första klass i skolan.. Jag tackar dig för att du gav mig chansen till detta, din passion för laxar har smittat av sig, tack vare dig kan jag aldrig mer gå förbi fiskdisken i affären utan att klappa på mina silverglänsande vänner… (låter det lite sjukt kanske…).
Håkan; min andre handledare, trots att du arbetade i Paris och sedan hamnade på Botaniska museét så har du alltid haft tid för mig närhelst jag behövt din hjälp och läst mina manuskript mellan besöken på alla franska krogar och tunnelbanefärder runt Paris… jag är så väldigt tacksam för att du lärt mig att bli den noggranna och högst motiverade molekylärbiologiska forskare som jag är idag. Varhelst i världen jag befinner mig så kommer jag alltid ha tid att plocka en maskros och tänka på dig (antar att du förstås vill att jag istället skickar den till dig va!).
Kerstin; du har inte bara varit min arbetspartner under fyra år, du har framförallt varit en kär vän som jag alltid kunnat diskutera allt från molekylära metoder till kreativa eskapader med. Tack för allt din hjälp på labbet, uppskattar det så väldigt mycket!
Jakob; jag är tacksam för att du har du gett mig smak för de finare sakerna i livet och lärt mig att man inte ska nöja sig med vad som helst här i livet – utan alltid satsa på att nå det allra bästa bara för att man helt enkelt inte förtjänar mindre än just det! Det ni…
Maria P.; ta väl hand om våra små laxar, jag ger dig vårdnaden nu – men jag reserverar mig rätten till besöksrätt hos mina små sötnosar när som…
Staffan; att jag har haft ditt stöd och uppmuntran har betytt mycket för den här avhandlingen. Du är så förbaskat intelligent att jag blir helt matt (och lite avundsjuk). Jag har kommit en bit sedan jag gjorde mitt exjobb, eller vad tycker du…
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Niklas och Patrik; hoppas att ni inser vad ni gjort – ert laxfiske blev min avhandling, tänk vad man kan åstakomma genom att förena (vissas) nytta med (vissas) nöje… sänder även ett stort tack till **professor Larsson** för hans grandiosa laxinsats i denna avhandling
Helena; Du har lärt mig att se mer kritiskt på världen och dess företeelser – och det är bra att ha lite skeptisism med i framtiden, stort tack för detta!
Erik; det är så skönt med uppskattning här i livet – tack för att du är så frikostig med att dela ut den till oss utvalda, det värmer.

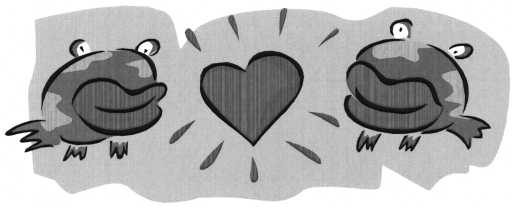
Henrik; du var faktiskt den som fick mig att inse att ekologi var det som gällde inom biologin när jag var student en gång i tiden (jag var helt bestämd vid att bli zoolog…), se där, det blev t.o.m. en avhandling. Hoppas du inser vad dina ord kan åsamka din omgivning…
Mats Grahn; strävar alltid efter att bli en lika slipad och brilliant forskare som du.
Sam; Den skarpa hjärnan måste slipas med motion för hela kroppen, d.v.s. utan en tränad kropp förtvinar hjärnan. Du är en stor förebild för mig!
Anna och Maj; Vi tillsammans stärker den kvinnliga självkänslan! Kan behövas ibland, eller hur..
Micke Åkesson; Konsert, du och jag, när som, var som, hur som…
David & Jean; tack för att ni drog med mig ut så att jag inte fick tillbringa all min fritid på labbet! Ni har varit mig två väldigt kära vänner – jag är framförallt väldigt tacksam för att jag fått lyssna på era inbördes diskussioner som belyst skillnaderna mellan England och Frankrike… jag ler fortfarande bara vid tanken…
Olof; Min femte och siste rumskompis, alltid trevligt att ha en inredningsfrände bredvid (bakom) sig. Det har varit så trevligt att höra dig spela spel bakom min rygg (fick jag inte nämna det…?)
Helene; tack för att du är mig en så god vän, jag hoppas på framtida trädgårdsupplevelser tillsammans och att du håller min danska uppe även i framtiden. Som skåning är det alltid skönt att få höra sitt evolutionära modersmål talas, kan annars bli lite frustrerande att alltid umgås med ockuperande svenskar hela dagarna… (nu får jag väl spö för det också..). Passar på att säga hej till **Richard** nu när jag har chansen! (minns du när vi fångade strömstarar tillsammans…).

Till sist vill jag säga några ord till…

mina kära vänninor **Camilla, Maj** och **Liv** som alltid finns där när det gäller.

… och de som är mig mer nära besläktade…

Min familj som har fått utstå mig under de här åren, mitt humör har gått upp och ner i rungande fart beroende på hur resultaten på labbet gått just för tillfället och ni har inte kunnat göra annat än att lyssna på mitt DNA-blabbel… men eftersom jag inte blivit utesluten ännu så betyder det väl att ni helt enkelt lärt er att jag behöver lite extra förståelse…
Tack för att ni finns där för mig när jag behöver det i form av kaffe och kaka-pauser, bilskjutsar, utflykter, luncher, öringmiddagar och allt annat! Stor kram till er alla!



Har jag nu glömt någon som borde vara med
så är det bara att ta för sig av ovanstående hjärta,
för det är till alla som gjort min omgivning ljusare

Maria