

Genetic response to  
pollution in sticklebacks;  
natural selection in the wild.

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To the sticklebacks and thanks for all the fins!

“But man is a part of nature, and his war against nature is inevitably a war against himself.”

— Rachel Carson



## ABSTRACT

The last century, humans have been altering almost all natural environments at an accelerating rate, including the Baltic Sea that has highly eutrophicated areas and many coastal industries such as Pulp-mills. For animals living in a habitat that changes there are basically two alternatives, either to cope with the change or become locally extinct. This thesis aims to investigate if recent anthropogenic disturbance in the Baltic Sea can affect natural populations on a genetic level through natural selection.

First, we found a fine-scale genetic structure in three-spine sticklebacks (*Gasterosteus aculeatus*) populations along the Swedish coast (paper I), indicating limited gene-flow between populations in geographic proximity. Different genetic markers, specifically Amplified Fragment Length Polymorphism (AFLP, and microsatellites, gave different results, highlighting the heterogeneous character of genomes which demonstrates that it is important to choose a genetic marker that is relevant for the question at hand. With a population genomic approach, and a multilocus genetic marker (AFLP), we detected convergent evolution in genotype composition in stickleback populations living in environments affected by pulp-mill effluent (paper II) and in highly eutrophicated environments (paper III), compared to adjacent reference populations. We found loci, in both studies (paper II, III), that were different from a neutral distribution and thus probably under divergent selection for the habitat differences investigated. The selective effect from pulp-mill effluents were more pronounced, but the two different habitats had mutual characters AFLP loci). In paper IV, we converted five anonymous AFLP loci to sequenced markers and aligned them to the stickleback genome. Four out of five loci aligned within, or close to, coding regions on chromosome I, chromosome VIII, chromosome XIX and chromosome XX. One of the loci, located on chromosome VIII and identified as under divergent selection in both paper II and III, has been identified in other studies as to be under selection for fresh water adaptation, including Baltic Sea stickleback populations.

In conclusion, anthropogenic alterations of natural environments can have evolutionary consequences, probably adaptive, for the animals living there and the evolutionary response exhibited by natural populations can be very fast.

**Keywords** Population genomics, genome scan, divergent selection, *Gasterosteus aculeatus*, Baltic Sea, pollution



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## LIST OF PAPERS

This thesis is based on the following four papers, referred to in the text by their Roman numerals

- I. **Lind E, Ohlin H, Grahn M.** Fine scale genetic structure in Threespine stickleback (*Gasterosteus aculeatus*) along Sweden's coast. *Manuscript*
- II. **Lind E, Mats G.** Directional genetic selection by pulp mill effluent on multiple natural populations of three-spined stickleback (*Gasterosteus aculeatus*). *Ecotoxicology* 20(3):503-12.\*
- III. **Lind E, Larsson J, Tuomainen U, Borg M, Candolin U, Grahn M.** Genetic response to eutrophication in three-spined sticklebacks (*Gasterosteus aculeatus*). *Manuscript*
- IV. **Larsson J, Lind E, Hallgren S, Grahn M.** From AFLP to sequence specific markers. *Manuscript*

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## INTRODUCTION

Understanding selection and adaptive genetic processes in natural populations is a central objective in evolutionary biology and ecology (for reviews see Stinchcombe and Hoekstra 2007, Nosil et al. 2009). This may be especially relevant now, when humans are altering most natural environments at an accelerating rate as we have entered “the Anthropocene” (Millennium Ecosystem Assessment 2005, Crutzen 2006, Steffen et al. 2007). Marine environments are no exceptions, for example Halpers et al. (2008) estimate that all marine areas to some degree are negatively affected by human influence, and a daunting 41% is strongly affected by multiple drivers of ecological change. For populations living in an environment that undergoes rapid changes there are essentially two alternatives; they can either cope with the change and survive by adapting to the new conditions or become locally extinct (Potvin and Tousignant 1996, Van Straalen 2003, Johannesson et al. 2011).

Environmental changes can cause altered selection pressures and initially, individuals often respond with behavioral changes that depend on phenotypic plasticity (the capacity of a single genotype to exhibit variable phenotypes in different environments) and therefore genetically determined reaction norms (Hendry et al. 2008, Tuomainen and Candolin 2011, Candolin and Wong 2012). Such phenotypic changes, for instance behavioral or physiological, can allow an animal to survive and reproduce in the new environment and thus pass on heritable traits to its offspring. This can potentially cause genetic changes on a population level. The ability of a population to adapt to a change in the environment depends on the genetic variation available within the population. If the population has a lot of standing genetic variation, the likelihood increases that a trait (an allele or a combination of alleles), beneficial in the new environment, is present. Thus, knowledge about a population’s genetic variation is important if one wants to understand a population's potential to cope with environmental changes.

### **Genetic variation; how and what**

The genetic variation in a population is affected by four factors; gene-flow, genetic drift, mutations and selection.

Gene-flow is when migrants from populations with different genetic compositions, with other genotypes and alleles, contribute to the next generation. The genetic variation in the receiving population consequently increases and the genetic differentiation between the two populations is reduced as they become more similar. If the gene-flow decreases with geographic distance in a spatial system a continuous distribution will appear and the genetic similarity among populations decreases with distance in accordance with an Isolation by distance model (Wright 1938, 1943).

Since gene-flow erases the genetic differentiation between populations it can inhibit local genetic adaptation (Slatkin 1987). But, theoretically, it can also have contrasting effects by promoting adaptations in an “adaptive landscape”. Under this model, Wrights shifting balance theory, adaptive genes or gene combinations are spread to other populations by gene-flow (e.g. Wright 1982, Coyne et al. 1997). Variation *among* populations first arises through genetic drift and past selection, and then current local selection pressure can drive a population towards another adaptive peak (e.g. gene combination) in the adaptive landscape. Consequently, that gene combination can then spread to other populations through gene flow. For an adaptive shift to occur the population must first be forced down to the adaptive valley with genetic drift to be able to reach another adaptive peak. Moreover, a build-up of adaptive differentiation in a population through gene-flow is possible with hitch-hiking effects. That is when genetic regions are under linkage disequilibrium, and therefore selectively neutral loci can hitch-hike with non-neutral loci because they are in fact physically linked. Hitch-hiking can be defined as “the indirect effects of selection at one or more loci on the rest of the genome” (Barton 2000, Flaxman 2013). Flaxman et al. (2013) use a simulation approach to explore “divergence hitchhiking”, that is when selection on one loci reduces gene-flow in linked loci, and “genome hitchhiking”, that is when divergent selection cause reduction in average genome-wide and effective recombination rates, and finds that hitchhiking facilitates speciation with gene-flow and that especially genome hitchhiking promotes the differentiation.

Genetic drift is when purely stochastic events affect the genotypic composition of the next generation resulting in non-adaptive evolution. Evolution by genetic drift proceeds faster in small populations and affects allele frequencies at all loci simultaneously, in the absence of selection and/or in selectively neutral alleles. Genetic drift has severe effects in small populations, either due to founder effects or

genetic bottlenecks and genetic variation will always be reduced (Futuyma 1998). The Neutral theory (Kimura 1977) holds that most genetic variation is selectively neutral and thus suggests that molecular evolution is mostly due to drift, making mutations negligible (and mostly neutral) in the long run.

Mutations are alterations in the DNA sequence from damage to the DNA (often caused by radiation or chemical mutagens), replication errors, or insertions or deletions of DNA. Mutations increase the amount of genetic variation. Even though most mutations will be either selectively neutral or slightly deleterious, and then erased by selection, there is abundant evidence that some mutations are advantageous (Futuyma 1998). In sexually reproducing organisms, genetic recombination, when new allele combinations are generated in the meiosis, is another factor generating genetic variation.

So heritable variation is the raw material for evolutionary change, but the engine for adaptive evolution is natural selection. Natural selection is the process whereby the frequency of heritable traits changes in a population due to the effect traits have on individual fitness. Traits that are beneficial in the current environment will increase in frequency as individuals carrying the heritable trait will to a greater extent affect the genotypic composition of the next generation. Selection is non-random, decreases variation, and will vary in both direction and intensity over time (Endler 1986, Hendry et al. 2008). There are different modes of selection where the outcome depends on fitness of the trait; directional selection is when one phenotype has the highest fitness, disruptive selection when two (or more) genotypes have higher fitness than intermediate genotypes, and stabilizing (normalizing) selection is when the intermediate genotype is the most fit (see Fig 1).

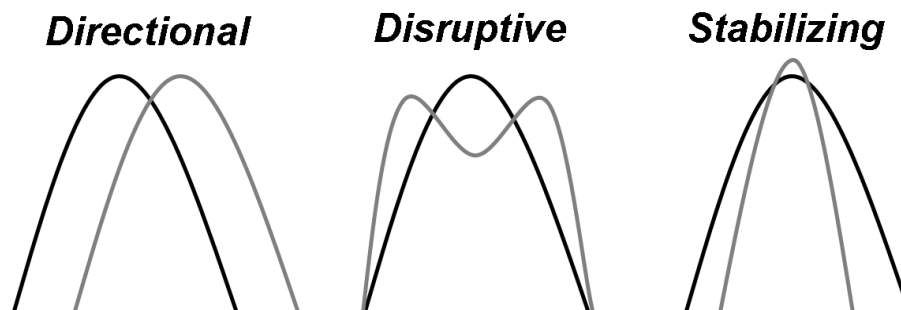


Fig1. The three different modes of selection, black lines represent the frequencies of traits before selection and grey lines after selection. Directional selection changes the heritable traits in one direction and one genotype has the highest fitness, disruptive selection is when two genotypes have higher fitness than intermediates, and stabilizing selection happens when the intermediate is fittest.

## Genomic heterogeneity and Genome scans

Not only is there genetic variation within and between populations, the genome itself is highly heterogeneous (e.g. Nosil et al. 2008, 2009), where some regions are selectively neutral and mainly affected by gene-flow and random genetic drift whereas other regions are influenced by directional selection, either directly or by linkage disequilibrium (non-random association of alleles at two or more loci). When assessing spatial genetic structuring this heterogeneity can have a huge impact. For example De Faveri et al. (2013) found that population divergence in Baltic sticklebacks increased substantially when using genic markers compared to non-genic markers, and with genic markers there was a pattern in the differentiation with several genetic clusters.

When studying selection and adaptation on the molecular level, a common goal is to identify loci that are under selection and understand the underlying mechanisms (Lewontin and Krakauer 1973). Typically this has been done through investigations of candidate genes, with known biological functions thought to be important in the selective regime investigated. However, it can be challenging, or even impossible, to know which genes to survey due to a lack of genomic information. The last decade, population genomics and the use of genomic scans to find genetic regions potentially under selection have become popular (Luikart et al. 2003, Bonin et al. 2006, Excoffier et al. 2009, Shimada et al. 2011, Radwan and Babik 2012). This approach can also be fruitful in non-model organisms to identify links between environmental factors and loci under selection. (e.g. Wilding et al. 2001, Storz 2005, Hoffman and Willi 2008, Nosil et al. 2008, 2009, Schluter et al. 2010).

The underlying principle is that loci that are influenced by directional selection, either directly or by linkage disequilibrium will show a larger genetic differentiation than selective neutral loci. Loci that have been subject to balancing selection will show a lower genetic differentiation. Generally this is done by identifying outlier loci with  $F_{ST}$  coefficients significantly different from those expected under the neutral theory (e.g. Lewontin and Krakauer 1973, Wilding et al. 2001). The main difficulty with this is to obtain a proper expected  $F_{ST}$  distribution (Beumont 2005). One approach is to use an island model (Wright 1931) in which every population's allele frequencies are correlated through a common gene pool, from which they differ to varying degrees. The differences in allele frequency between the gene pool and each subpopulation is measured by a population specific  $F_{ST}$ . Therefore, this model

considers both differences in effective population size and differences in gene-flow (Foll and Gaggiotti 2006). Still, there are problems with this model if some populations share recent ancestry, populations contribute to different migrant pools or if there is a hierarchical structure (Excoffier et al. 2009). By using a hierarchical island model, where populations are assigned to pre-defined groups and that allows for more migration within the pre-defined groups, the occurrence of false-positives is reduced (Excoffier et al. 2009).

Still, underlying genetic structure with complex past demographic events such as bottlenecks, demographic expansions, colonization patterns and clines across geographical gradients can still lead to an excess of false positives (Excoffier et al. 2009, Strand et al. 2012)

### **Rapid evolution**

There are several well known examples of rapid genetic adaptation caused by a strong selection pressures. This is most often documented in species that are targets for eradication. For example, almost all disease bacteria have evolved some level of resistance to antibiotics, not uncommonly within only a few years after introduction of the antibiotics. Penicillin, for instance, was introduced 1943 and resistance had evolved by 1946 (Palumbi 2001). In animals, insecticides are a good example of strong selective agents (Roush and McKenzie 1987). In the nineties over 500 insects species were resistant to at least one insecticide and many were resistant to several different insecticides (Palumbi 2001). DDT-resistance was noted within a year after the first treatment of lice (Herbert et al. 1952). It is now known that a quite simple mutation is associated with resistance to both DDT and other insecticides in *Drosophila* (Daborn et al. 2002), whereas resistance in other insects also can depend on up-regulation of another gene. The same phenomenon has been observed in weeds that rapidly have evolved resistance to different herbicides (Jasieniuk et al. 1996).

There are much fewer examples from natural populations that have not been targets for eradication, but instead suffer from unintended anthropogenic stressors acting as selection pressure. One example comes from Massachusetts, North America, where some well investigated populations of Mummichogs (*Fundulus heteroclitus*) inhabit highly polluted marine superfund sites. The sites have been contaminated the last 60 years with PCBs (polychlorinated biphenyls), PAHs (Polycyclic aromatic

hydrocarbons), dioxins and other contaminants, that represent a cocktail of toxins. Both embryos and larvae from contaminated sites are less responsive to dioxin-like compounds compared to individuals from reference sites, and this responsiveness is inherited, which suggests a genetic adaptation (Nacci et al. 1999). The genetic diversity measured with Amplified Fragment Length Polymorphism(AFLP) within and between populations do not differ between polluted and reference sites (McMillan et al. 2006) but genome scans can identify AFLP outlier loci assumed to be under divergent selection and when excluded, an otherwise strong Isolation by distance pattern disappears (Williams and Oleksiak 2008). When metabolic genes were investigated, two genes have a common response in all three populations demonstrating that high selective pressures may favor specific convergent responses (Fisher and Oleksiak 2007). With SNPs (Single Nucleotide Polymorphism) the CYP1A gene (xenobiotic metabolizing enzyme) is identified in all polluted sites as under selection, and the same study suggests that hundreds of loci are involved in the rapid evolutionary response to pollution (Williams and Oleksiak 2011). Ancestral populations have probably harbored genotypes that enabled this fast response; in common-garden experiments it has been shown that tolerant populations independently evolved a transcriptional response and hence tolerance to dioxin-like compounds (Whitehead et al. 2012). This system serves as an excellent example of a natural population under strong selection from unintended toxins.



## STUDY SYSTEM

### Baltic Sea

The Baltic Sea is a young brackish sea with a history as a freshwater lake that became connected to the North Sea about 8 500 years ago (Johannesson and André 2006). It has an inflow of saltwater from the south and freshwater from the north that create a salinity gradient from about 2 psu in North to 25 psu in the South-West (HELCOM 2007). This can create a rather stressful environment for the organisms living there, as they either have marine or aquatic origins (Johannesson and André 2006). The Baltic Sea is also heavily affected by anthropogenic stressors in terms of nutrients, heavy metals and toxins (Ducrotoy and Elliott 2008, Jansson and Dahlberg 1999). It has a very large catchment area, now populated by approximately 85 million people. What in the 1940s was a nutrient poor sea with clear water has now changed. The nitrogen load has increased threefold (mostly from agriculture and air pollution) and the phosphorous load has increased fivefold (mostly from sewage) creating highly, and in some spots acute, eutrophicated areas (HELCOM 2009, Jansson and Dahlberg 1999). The eutrophication is manifested as an increase in phytoplankton primary production and increased growth of short-lived macroalgae. This causes turbidity in the water and thus a decrease in light penetration. In addition, eutrophication increases the occurrence of oxygen depletion, caused by the oxygen consumption of microbial processes that degrades organic matter which accumulates in the sediment. Thus, oxygen depletion (both hypoxia and even anoxia) is a common effect of eutrophication, and in 2002 the Baltic Sea suffered from the worst hypoxia ever in terms of area affected (HELCOM 2009).

A major source of pollution and nutrients in the Baltic Sea is the pulp- and paper mill industry. Today, around 50 mills produce more than 12 million tons of pulp annually in Sweden. Pulp mill effluents contain toxins (Pokhrel and Viraraghavan 2004, Ali and Sreekrishnan 2001), nutrients (Hansson 1987) and fresh water (Thompson et al. 2001) and thus affect run-off areas with both eutrophication and a cocktail of toxins. It is well documented that the effluents affect fish reproduction resulting in decreased gonad size, altered expression of secondary sex characteristics and reductions in fecundity (for review see Hewitt et al. 2008). The masculinization occurring, for instance in sticklebacks (Katsiadaki et al. 2002), probably originates

from androgenic steroids such as androstenedione, androstadienedione and progesterone, all byproducts derived from the microbial degradation of phytosterols leaking from the wood (Denton et al. 1985). So even if the industries has vastly improved since the 1980s, when malformed fishes with curved spines were a common sight outside the industries, there are still documented effects of the effluents.

## **Stickleback**

The three-spined stickleback (*Gasterosteus aculeatus*) is a small bony fish found in marine, brackish and freshwater environments around the northern hemisphere and is also common in the Baltic Sea. During breeding season, sticklebacks enter their breeding grounds, shallow coastal areas, and the male sticklebacks then develop blue eyes, blue dorsal and red ventral color and establish territories where they build nests. After courtship, as soon as there are eggs in the nest, the male use his pectoral fins to fan the eggs (Wootton 1976).

It is a well studied model organism in behavioral and evolutionary biology (e.g. Bell & Foster 1994). During a comparatively short history (10 000 - 15 000 years) marine sticklebacks are thought to have re-colonized freshwater habitats after the last glaciation, presumably from adjacent estuarine or marine refugia (reviewed in McPhail 1994) and the marine genotypes rapidly diverged into several sympatric and parapatric ecotypes. The populations have diverged as a response to the different conditions in the new habitats resulting in a large number of distinct morphological forms. Stickleback populations now exhibit variation in for example the number of body armour and gill rakers, coloration, behavior, number and shape of dorsal spines, number, shape and pattern of lateral plates and pelvic fin development, and most of these traits are thought to be heritable. As the ancestral populations of marine sticklebacks have independently and repeatedly invaded fresh water habitats across the northern hemisphere (Bell and Foster 1994), a fair amount of research has been conducted in the context of genetic adaptation to fresh water habitats (Jones et al. 2012a, b, DeFaveri et al. 2011, Shimada et al. 2011, Hohenlohe et al. 2010, Mäkinen et al. 2008a, b). Jones et al. (2012) did an extensive study where they used whole genome sequencing and found that probably only about 20% of freshwater adaptations are situated in coding DNA and the remaining 80 % situated in

regulatory DNA (Jones et al. 2012b). Hence, molecular adaptation to freshwater largely happens through genetic regulation in the non-coding region of the genome.

It is known that the Ectodysplasin (EDA) signaling pathway is responsible for armor loss in freshwater sticklebacks. But, the EDA allele responsible for lateral plate reduction is rare in marine stickleback populations and occurs only in about 1%. However, due to the strong selection pressure favoring this allele in freshwater environment, its frequency increases dramatically in freshwater (to 100%) (Colosimo et al. 2005, Barrett et al. 2008, Mäkinen et al. 2008, Hohenlohe et al. 2010).

Consequently, rare alleles that are part of populations standing genetic variation can be vital for a population's potential to adapt to a novel environment, despite the possibility that they are neutral or slightly deleterious under some conditions, (Barrett and Schluter 2008).

This thesis had the aim to investigate if recent anthropogenic disturbances in marine environments can affect natural populations on a genetic level by natural selection.

## METHODS

### AFLP

AFLP, Amplified Fragment Length Polymorphism, was first developed by Vos et al. (1995) and is basically a combination of RFLP and RAPD. Genomic DNA of high quality, and molecular weight, is digested with two different restriction enzymes, one four- cutter and one six-cutter, resulting in thousands of DNA fragments. Double stranded DNA-adaptors, fitting into cut sites of respective restriction enzymes, are then ligated to the DNA fragments enabling replication of fragments with Polymerase Chain Reaction (PCR) with primers complementary to the adaptors. Two different PCR-reactions are then done, first with one arbitrarily chosen nucleotide extending into the restriction fragment, pre-amplification, to narrow down the number of fragments, and secondly a selective amplification with three arbitrarily chosen nucleotides to even further reduce the complexity (Vos et al. 1995, Bonin et al. 2007). The remaining fragments are separated based on length, using polyacrylamid gel electrophoresis or with sequencing robots, and the presence of an allele of a certain length is scored as 1 whereas the absence is scored as 0 resulting in matrices with 1/0 for every individual and for all scored alleles. Hence, the genotypic information obtained is not complete since individuals scored as 1 can be either homozygote (1/1) or heterozygote (1/0) for the presence of an allele making every locus less informative than a multi-allelic marker such as microsatellites. On the contrary the large number of markers that easily are obtained and their random distribution in the genome may compensate the dominant character of AFLP (Mueller and Wolfenbarger 1999, Bensch and Åkesson 2005, Bonin et al. 2007). It is worth noticing that there are studies indicating that fragments do not appear randomly in the genome but rather cluster together near centromeres (Lindner et al. 2000) which could increase patterns of differentiation and linkage disequilibria (Carneiro 2009). As with microsatellites there can be problems with size homoplasy and co-migration fragments can represent different loci in the genome, a problem that increases for very short fragments (Vekeman et al. 2002).

That AFLP yields fragments that are randomly distributed in the genome, easily obtained, reproducible and from both neutral and non-neutral genetic regions, make it very suitable for population genomic approaches and genome scans to detect

genetic regions under selection. It is also feasible to convert AFLP fragments to codominant SNPs (Bensch et al. 2002) and to use the SNP to find candidate genes.

## **Statistical methods**

The dominant character of AFLPs and the difficulty to distinguish whether an individual is heterozygote or homozygote at the presence loci (i.e. have a band) makes the traditional way to measure genetic diversity with average level of heterozygosity impossible. Instead there have been several statistical models developed for dominant markers (reviewed in Bensch and Åkesson 2005, Bonin et al 2007) most are allele-frequency based where allele frequencies at every loci are estimated with preliminary assumptions, as for example Hardy-Weinberg equilibrium. Here, some of the statistical methods used in this thesis are briefly described.

### *AFLP-surv*

AFLP-surv was developed for dominant markers by Vekemans et al.(2000), in order to estimate for example genetic diversity ( $H_j$ ), differentiation between populations (e.g.  $F_{ST}$ ) and between sites (fixed geographic locations). It assumes a non-uniform prior distribution of allele frequencies and Hardy-Weinberg equilibrium (it is optional to apply FIS-values) and uses the Bayesian method by Zhivotovsky (1999). *Significance values of  $F_{ST}$  are obtained with a permutation test where  $F_{ST}$ -values are computed after each permutation consisting of a random rearrangement of individuals among existing populations. The set of  $F_{ST}$  values obtained by permutation, gives an ad-hoc distribution of the statistic under the null hypothesis (no genetic differentiation) and observed  $F_{ST}$  are then tested against the distribution (Vekeman 2002).*

### *ARLEQUIN*

The ARLEQUIN 3.5 package has several applications suited for dominant markers. The  $F_{ST}$ -outlier analysis (a genome scan approach) simulates evolution in a hierarchical set of populations according to the Wright island model. Coalescent simulations are used to get a null distribution of locus-specific  $F_{ST}$  values and confidence intervals around the observed values to test if observed  $F_{ST}$  values can be considered as outliers in relation to the overall observed  $F_{ST}$  value for each locus (Excoffier et al. 2009). In addition, the AMOVA (Analysis of molecular variance)

partitions how much of the genetic variation in predefined groups that can be explained by differences among pre-defined groups, among populations within groups and within populations, under the assumption that loci are unlinked.

### *STRUCTURE*

The 2.2 release of STRUCTURE allows the use of dominant markers. STRUCTURE implements a model-based clustering method for inferring population structure in which there are K populations (genetic clusters), each of which is characterized by a set of allele frequencies at each locus. Individuals are probabilistically assigned to a cluster (K) and it is assumed that within a cluster (K) loci are at Hardy-Weinberg equilibrium, and that loci are unlinked (linkage equilibrium) (Pritchard et al. 2000).

### *R; Vegan package and Random Forest*

The vegan package (Oksanen et al. 2006) implemented in R 2.5.1 (R Development Core Team 2007) provides an implementation of constrained principal coordinate analysis (cPCoA) using the capscale procedure. This is similar to a redundancy analysis but allows non-Euclidian dissimilarity indices; we use Jaccard distance. We also use constraining variables in our analyses (population and habitat), the constraints are tested for significance using a permutation test. We also use constraints to partial out variation explained by one variable before testing the next (i.e. conditioning).

Random Forest is classification algorithm, developed by Breiman (2001) and implemented in R (R Development Core Team 2007) that builds classification trees using a bootstrap sample of the data. But instead of using the best split among all variables, the program randomly chooses a subset of variables and calculates the best split for each node making it very robust against over-fitting (Breiman 2001). Here, data can be divided between different habitats and Random forest will be used to identify loci that contributed the most to the difference in the genotypes between the habitats.

## SUMMARY OF THE PAPERS

### **I. Fine scale genetic structure in Threespine stickleback (*Gasterosteus aculeatus*) along Sweden's coast.**

In this study we aimed to assess the genetic structure of marine threespine sticklebacks along Sweden's coastline. We used two different marker systems, AFLP and microsatellites to enable both comparison of markers and to identify the spatial genetic structure. Eight sites (252 individuals) were sampled and genotyped with five microsatellites and 173 AFLPs. We found that sticklebacks in the Baltic Sea have a fine scale spatial genetic structure that follows Isolation by distance, even on a smaller scale, and that populations sampled as close as 2 km from each other are genetically separated with limited gene-flow. Since there are no distinguishable geographical barriers, we interpret this result as an effect of the sticklebacks' behavior that limits the gene-flow. The two different molecular markers used gave to some extent different results and to summarize, AFLPs had higher resolution and showed a pattern in the genetic variation whereas the microsatellites did not reveal a population structure.

### **II. Directional genetic selection by pulp mill effluent on multiple natural populations of three-spined stickleback (*Gasterosteus aculeatus*)**

In paper II we aimed to investigate whether pollution from point sources could act as a selective force and drive local adaptive change in sticklebacks inhabiting polluted areas. In a hierarchical approach we sampled sticklebacks from four pulp-mill effluent polluted habitats and four adjacent reference sites (in total eight sites) in the Baltic Sea. We did AFLP genotyping (248 loci, 244 individuals) and found that genotype composition had changed in response to habitat. There were convergent genetic changes in the polluted sites. With a genome scan approach we also found several loci statistically different from a neutral distribution that indicated that sticklebacks were subject to divergent selection.

### **III. Genetic response to eutrophication in the three-spined stickleback (*Gasterosteus aculeatus*) - A study of multiple Baltic Sea populations**

Here we sampled five stickleback populations in habitats identified as in poor ecological states, assessed as highly affected by eutrophication by the Finnish Environmental Administration, and five reference sites classified as in good ecological states. Candolin and colleagues have shown that increased eutrophication has complex effects on reproductive behavior and sexual selection in the stickleback (e.g. Candolin et al. 2007, 2008). Furthermore, one major impact of pulp-mill effluent is eutrophication (Hansson 1987). We did AFLP genotyping with the same primer combinations as in paper II (292 individuals, 204 loci) and found that eutrophication caused a genetic response manifested in eutrophicated areas having higher frequencies of certain genotypes compared to reference sites. Genome scans found AFLP-loci that were likely to be affected by divergent selection by eutrophication. Several of these loci were also found in paper II suggesting similarities in selective factors between eutrophication and pulp-mill effluent affected habitats. Still, the response found here was much weaker than the response found to pulp-mill effluents.

### **IV. From AFLP to sequence specific markers - Identifying genomic regions under selection in the three-spined stickleback caused by pulp mill effluents**

In this study we further investigated the loci identified in Paper II as being under divergent selection for sticklebacks living in habitats with pulp-mill effluents. The anonymous AFLP loci were converted into sequenced markers and aligned to the stickleback genome. Four out of five loci aligned within or close to coding regions, on chromosome I (7kb from the *TIAM1* gene), chromosome VIII (within the coding region for the enzyme Dipeptidyl-peptidase9), chromosome XIX (within the coding region for myosinIXA (*MYO9A*), 195kb from the microsatellite locus Gaest 31) and chromosome XX (31kb from the homeobox gene *POU3F1*). The fifth loci had multiple locations within the genome (< 40 hits). The locus located on chromosome VIII has been identified to be under selection for fresh water adaption in other studies, including Baltic Sea stickleback populations (Mäkinen et al. 2008a,b), and was also found to be under divergent selection in paper III (eutrophication).



## RESULTS & DISCUSSION

This thesis had the aim to investigate if recent anthropogenic disturbances in marine environments can affect natural populations on a genetic level by natural selection. In order to do that we first wanted to find out if there was any spatial genetic structure in three-spined sticklebacks in the Baltic Sea and at the same time compare different genetic markers to ensure that we choose a proper marker (Paper I). We found a fine-scale genetic structure in the Baltic Sea where populations only separated by 2 km had a significant amount of genetic variation between them ( $F_{ST}$ ). Genetic differentiation followed the Isolation by distance model, where the amount of genetic variation between populations increased with increasing geographic distance. But this pattern was only found using AFLPs, with microsatellites there were no structuring in the genetic variation. Even if we only genotyped five microsatellites there are other studies that have addressed genetic structuring in sticklebacks in the Baltic Sea, and for example Mäkinen et al. (2006) found  $F_{ST} = 0,003$  using 18 microsatellites among five coastal Baltic Sea sites and DeFaveri et al. (2012) found low levels of population structure in Baltic Sea sticklebacks using 15 microsatellites. Another study by Rafiński et al. (1988) analyzed 13 enzymatic loci in six Baltic Sea populations of sticklebacks and found very low genetic distances ( $D_n$ ) between sites. Our other studies using AFLP on sticklebacks from the Baltic Sea (paper II and paper III) corroborate the results from paper I, as they identified a similar pattern with significant genetic differences between sites and Isolation by distance. This clearly indicates that there is a limited gene-flow between the populations we have sampled so that gene-flow is counteracted by genetic drift and/or natural selection.

Restricted gene-flow can either be due to geographic barriers, where the animals cannot physically migrate, or due to behavior where the animals either choose not to migrate to other sites, or do migrate without reproducing in the new population. Therefore, ecological studies of the movement of organisms may overestimate gene-flow if they do not consider reproductive success. On the other hand, one important assumption when estimating gene-flow is that the genetic markers are selectively neutral, since loci under divergent selection would underestimate gene-flow and loci under stabilizing selection overestimate gene-flow. One of the characteristics of AFLPs is that the whole genome is sampled, both neutral loci and loci under

selection or loci linked to selected loci. In paper I we tried to overcome that by removing loci identified by the  $F_{ST}$  outlier analyses as under divergent selection by grouping populations according to salinity at sampled site since there is a strong salinity gradient in the Baltic Sea. When re-analyzing without the outlier loci pairwise  $F_{ST}$  between sites were lowered by about 50-70% but still significant between all populations and the isolation by distance pattern remained ( $R^2= 0.44$ ,  $p=0.005$ ). The remaining patterns of genetic structuring could be an effect of non-random mating and there are studies that have found indices of homing in sticklebacks (e.g. Kynard 1978, Saimoto 1993, Bolnick et al. 2009, Cano et al. 2008). The genetic pattern we observed could be caused by homing behavior, but our study is not sufficient to confirm that (e.g. de Campos Telles et al. 2011) and the results could also support alternative explanations such as resident populations, selection, legacy of post-glacial colonization etc. Thus, it suggests that it would be interesting to look further into the system. Paper I does, however, establish that AFLP is an adequate marker system to study genetic signs of natural selection in the study system (paper II, III and IV) and by using the same primer combinations we tried to avoid ascertainment bias, which potentially can be a problem in AFLP studies (Guillot and Foll 2009). Using the same primer combinations also enable direct comparisons of loci between paper II, III and IV.

One implication of the spatial genetic structure in paper I is that it can be expected to find a significant amount of variation between sites despite habitat differences. Therefore, using for example  $F_{ST}$  between populations cannot be regarded as a good method to detect genetic differences caused by habitat (pulp-mill and eutrophication). We used a hierarchical sampling regime in both paper II and III to try to minimize the geographical effects on genetic variation in the study area and the effects of other environmental factors, for example salinity.

We could see that both pulp-mill effluents and eutrophication caused a convergent change in the exposed populations' genetic compositions. The effect was much more pronounced in populations living outside pulp-mills, possibly best visualized in the cPCoA plots where pulp mill vs. reference (unconditioned analyze) separated well on the second principal coordinate axes (Fig 2). In analyses with habitat as condition, both datasets separate the samples according to habitat. In both paper II and III we did genome scans to detect loci that have a non-neutral distribution and thus might be under divergent selection for the habitat differences investigated. Here, the

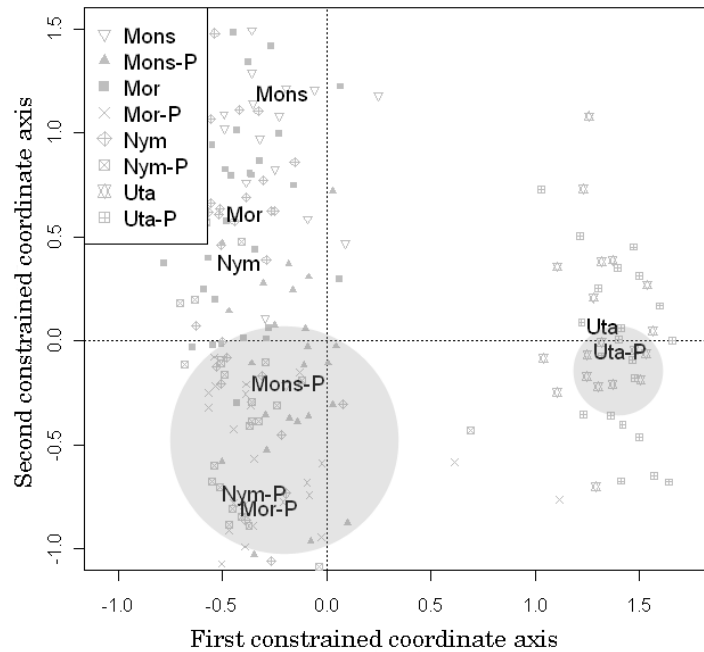


Figure 2. A constrained principal coordinate analysis (cPCoA) with population as constrain, based on 248 AFLP loci. A total of eight sites, four recipients sampled outside pulp-mills (noted with pop-P) marked out with grey circles and four close-by reference sites, the first principal coordinate axis explains 59.6% of the variation and the second principal coordinate axis 12% of the variation. The populations separate on habitat in the second principal coordinate axis.

hierarchical sampling regime enabled the construction of dendrograms based on the identified loci, one based on selective neutral and one based on outliers presumably under directional selection. The dendrograms mirror the genomes heterogeneity. With selectively neutral loci that are affected by gene flow and demography the dendrograms separate populations according to geography. While with outlier loci the populations group according to habitat, which further support that both pulp-mill effluent and human-induced eutrophication can act as selection pressures. We could also identify joint genetic characters between the two habitat types; nine out of 13 outlier loci found in paper III (eutrophication) were also found in Paper II (pulp-mill, where we found more than 21 outlier loci).

There is a possibility that other differences exist between the populations that we have not accounted for, which could give rise to outlier loci (Strand et al. 2012). Attempting to overcome that, we conducted two very different outlier-methods (for further details on the methods see paper II and III) and in Paper III we further tried to assess the occurrence of false-positives by employing the non-hierarchical  $F_{ST}$ -outlier approach in ARLEQUIN (Excoffier 2009) on each pair of eutrophic-control

populations, on solely eutrophic populations and solely control populations. We found a few loci only reappearing in eutrophic and/or control populations that might be false positives.

In paper IV we converted five AFLP loci from paper II (of which two also appeared in paper III) to sequenced markers and aligned them to the stickleback genome. Four out of five fragments were located within or close (< 30 kb) to coding regions and two were found within protein-coding regions, coding for Dipeptidyl-peptidase9 (fragment  $E_{TAG-M_{CAC33}}$ ) and Myosin9A (fragment  $E_{TAG-M_{CAC88}}$ ). The biological functions of these proteins are not fully known, but Dipeptidyl-peptidase9 seems to be important in peptide turnover and antigen presentation (Geiss-Friedlander et al. 2009, Tang et al. 2009). In mammals, Myosin9A is important for the function and maturation of epithelial cells in the brain and involved in cell to cell contacts (Bähler et al. 2011), in the collective migration of cells (Omelchenko and Hall 2012) and also in selective estrogen receptor modulation in human breast cancer (Cirillo et al. 2013). The other two fragments are located close, < 32kb, to coding regions.  $E_{TCT-M_{CAC119}}$  31kb from the homeobox gene POU3F1 that is involved in differentiation of Myelinating Schwann cells (Birmingham et al. 2002). In mice, misexpression of POU3F1 may lead to improper myelination and axonal loss (Ryu et al. 2007) and in developing zebra-fish embryos the class III POU genes coordinate expressions that contribute to pattern formation or cell fate determination in the developing CNS and other structures (Hauptmann and Gerster 2000). The fragment  $E_{TCT-M_{CAC76}}$  is located 7kb from TIAM1 gene that is involved in the regulation of various cell functions depending on cell type and substrate. TIAM1 seems to be essential for hippocampus neural development (Zhang and Macara 2006) and it also seems to increase invasion of T-lymphoma cells and is involved in cell polarity and cell adhesion among other suggested functions. The fifth loci had multiple locations in the genome (< 40 hits). Even though four loci were within, or close to, coding regions they might as well be linked to other loci that could be the actual target for selection. One of the loci that were both found in paper II and paper III ( $E_{TAG-M_{CAC33}}$ ) is located within a region that were under directional selection when Mäkinen et al. (2008a,b) compared marine, brackish and freshwater populations from Norway, Sweden, Finland and Barents Sea. This was the only joint character to fresh-water adaptation we could find when comparing to studies regarding fresh water adaptation (Jones et al. 2012a,b, DeFaveri et al. 2011, Shimada et al. 2011, Hohenlohe et al. 2010, Mäkinen et al. 2008a,b).

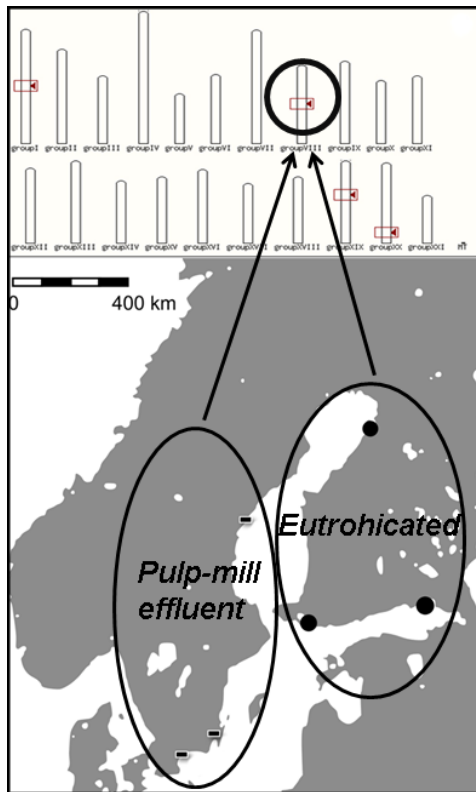


Figure 3. Map over the Baltic Sea there sites sampled in pulp mill effluent are marked with - and sites sampled in eutrophicated areas are marked with •. One AFLP-fragment indicated to be under selection in Paper II (pulp mill effluence), converted into a SNP and mapped to chromosome VIII was also identified in paper III (eutrophicated) as under divergent selection.

## Conclusions

With a population genomic approach we have found that stickleback populations living in habitats with recent anthropogenic disturbances, pulp-mill effluents and human-induced eutrophication, have responded evolutionarily to the change in their environment. There are convergent changes in genotype composition in populations from polluted vs. adjacent reference populations. We have also converted anonymous AFLP-markers, statistically identified to be under divergent selection from pulp-mill effluent, into sequenced markers and aligned them to the stickleback genome. Four out of five sequenced markers were within, or close to, protein coding genes. However, we do not understand the cause and effect of the selection and the genetic effects we have found. In conclusion, the alterations in natural environments, caused by humans, can have evolutionary consequences for the animals living there and the evolutionary response from natural populations can be very fast.

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## POPULÄRVETENSKAPLIG SAMMANFATTNING

Evolution är den process genom vilken levande organismers egenskaper förändras över tiden, och evolutionsteorin kan förklara både hur dinosaurier förändrades till fåglar och hur bakterier kan bli resistenta mot penicillin.

Det som driver evolutionen är naturligt urval tillsammans med slumpen. Begreppet naturligt urval, eller naturlig selektion, innebär att individer med egenskaper som främjar överlevande och fortplantning i den *aktuella miljön* tenderar att sprida sig till kommande generationer, under förutsättning att egenskaperna är ärftliga.

Egenskaper som helt enkelt gör att djur överlever bättre och får fler ungar blir vanligare och vanligare.

Om man lämnar individer och istället talar om populationer, en grupp av individer, ser man att en populations förmåga att anpassa sig till en förändrad miljö beror på hur mycket ärftlig variation det finns i de egenskaper som påverkas av selektionen. Skulle till exempel vår miljö förändras på ett sätt så att vi skulle överleva bättre, och få fler barn, om vi bara hade ett öga mitt i pannan skulle ingen förändring över generationer ske. Det är inte möjligt för det existerar ingen variation i egenskapen "antal ögon och deras placering" hos människor. Alla har två ögon, ett på varje sida av näsan. Nästan all variation vi kan se har en genetisk grund. Men många egenskaper påverkas också av den miljö man växer upp i, som till exempel i viss utsträckning hur långa vi blir. I fallet kroppslängd bestämmer genetiken hur lång det är möjligt att bli, men det är miljön individen växer upp i (näring, solljus etc.) som kommer avgöra hur lång man verkligen blir. Men här fokuserar jag på genetisk variation, variation i arvsmassan, och då på populationsnivå. Har en population mycket genetisk variation så är det större chans att evolutionära förändringar kan ske när selektionstrycket förändras, eftersom det är större chans att det finns gynnsamma egenskaper i populationen.

Den genetiska variationen i en population påverkas av fyra faktorer; genflöde eller migration, genetisk drift, mutationer och naturlig selektion. Kortfattat är genflöde när individer flyttar till en annan population, parar sig i den nya populationen och därmed påverkar hur genetiska sammansättningen kommer se ut i nästa generation. Genetiska variationen i den nya populationen kommer då att öka. Genetisk drift är rena slumpändelser och minskar alltid variationen. Ett exempel på genetisk drift, och när den har stor påverkan är vargpopulationen i Sverige, där från början bara tre individer var förfäder till hela vargpopulationen. Då kan det inte bli så mycket variation (men nu gäller inte detta längre eftersom det har kommit in mer variation

genom genflöde). Ett annat exempel är om en population helt plötsligt drabbas av en katastrof, till exempel en jordbävning, och av rena slumpen överlever bara några få individer och därmed fortlever endast de gener som just dessa hade. Mutationer i sin tur är när den genetiska koden, arvsmassan, förändras genom till exempel slumpmässiga kopieringsfel under celldelningen, strålning eller av vissa kemiska ämnen. Mutationer ökar variationen men dessa förändringar är sällan gynnsamma. Och slutligen naturlig selektion som inte alls är innebär en slumpmässig förändring, utan då minskar den genetiska variationen för att de som är bäst anpassade till miljön de lever i har större framgång. Det är alltså en missuppfattning att det alltid är de starkaste som överlever, utan det är det gör de individer som överlever och fortplantar sig i störst utsträckning i just den miljön de lever i.

Inte nog med att det finns genetisk variation i populationer av organismer, våra genom (vårt DNA, arvsmassan) är också väldigt variabla. Vissa delar av genomet består av gener som gör olika proteiner, de kallar man för kodande gener. Medan andra delar bestämmer över, reglerar, dessa gener och bestämmer hur mycket protein det ska bli. Men till största del består våra genom av icke-kodande DNA, alltså DNA som inte leder till någon konkret produkt. Så olika delar av genomet kommer att påverkas olika av naturlig selektion. Vissa delar påverkas till största del av slumpen, genetisk drift, och inte alls av naturlig selektion. Dessa delar kallas därför för neutrala delar. Andra delar kan påverkas mycket av naturlig selektion och de kallas således för icke-neutrala.

Om man vill undersöka genomet och försöka hitta delar som påverkas av selektion brukar man använda metoder för att få fram många, helst jättemånga, genetiska fragment som är utspridda i genomet och genom att använda statistiska modeller kan man uppskatta vilka fragment som är neutrala och vilka fragment som förmodligen är påverkade av selektion. Oftast har man emellertid ingen aning om vilken del av organismens genom fragmenten kommer ifrån, mer om det senare.

Vi påverkas dagligen av hur snabb och kraftfull naturlig selektion kan vara. Vi får antibiotika mot en infektion, men har vi otur hjälper inte medicinen för de bakterier som orsakat infektionen har blivit resistenta, de har genom generationer utvecklat en motståndskraftighet. Det är livsviktigt för bakterien, men förödande för människan. Vi måste därför hela tiden utveckla nya strategier för att förhindra att insekter äter upp våra grödor, i en konstant pågående kapprustning där skadeinsekter och ogräs genom generationer utvecklar resistens mot de gifter vi använder för att kontrollera dem. Ett välkänt exempel är DDT. Det upptäcktes 1939 och användes mycket flitigt, men redan 1945 hade flera insekter utvecklat resistens mot DDT och mitten av

sjuttioalet fanns det ungefär 200 insektsarter som genom resistens alltså inte påverkas nämnvärt av DDT, ett tidigare mycket potent gift.

Så när miljön förändras kommer med största sannolikhet även det selektiva trycket att förändras, eftersom levnadsförhållandena ändras. De sista hundra åren har påverkan från människor kraftigt förändrat olika levnadsmiljöer på olika sätt till exempel genom föroreningar. Östersjön är inget undantag, det är ett av världens mest förorenade havsområden. Östersjön är ett bräckt inlandshav med smala passager till världshaven och ett stort inflöde av sötvatten från floder främst i norra delen. Brackvattnet gör det till en ganska tuff miljö för de arter som lever där då de från början var anpassade till antingen söt- eller saltvatten. Dessutom är havet som sagt hårt utsatt för miljöpåverkan såsom övergödning orsakat av till exempel avrinning från jordbruk och alla de pappersmassabruk som finns längst kusten och som har avrinningsrör från industrin ut i havet.

I den här avhandlingen har vi undersökt storspigg. Det är en vanligt förekommande fisk i Östersjön som dessutom är känd för att snabbt anpassa sig till olika förhållanden. Därför är det en populär fisk hos evolutionsbiologer som tidigare undersökt bland annat hur spigg anpassat sig när de upprepade gånger flyttat från salt- till sötvatten, till exempel vilka delar av genomet som har påverkats. Vi har gjort fyra olika studier där vår främsta målsättning har varit att undersöka om spiggpopulationer som bor i förorenade miljöer har en annorlunda genetisk sammansättning än spiggpopulationer som bor geografiskt nära men i mycket renare miljö. Nedan kommer jag kort redogöra för de fyra olika studierna utgör stommen i den här avhandlingen.

- I. I den här studien samlade vi in spigg från åtta olika lokaler längst Sveriges kust och undersökte deras genetiska sammansättning. Vi såg att spiggpopulationer från olika lokaler signifikant skiljer sig åt i genetisk sammansättning. Till och med spigg insamlade så nära varandra som på olika sidor av ön Öja (endast 2 kilometer ifrån varandra) var genetiskt åtskilda. Vi kunde också se att populationerna blir mer genetiskt olika ju längre ifrån varandra de är insamlade, vilket tyder på att migrationen, genflödet, blir mindre med ökat avstånd. Vi tolkar våra resultat som att de här spiggarna möjligen är filopatriska dvs. att de återvänder till en speciell geografisk plats för att föröka sig, något som är vanligt hos djur. Vi använde två olika genetiska markörer, där man tittar på olika ställen i genomet och såg att det var stor skillnad mellan markörerna med avseende på hur tydliga resultaten var.

- II. Här undersökte vi spiggpopulationer som lever utanför fyra olika pappersmassabruk och jämförde dem med spiggpopulationer från fyra närliggande, men renare, områden. Andra forskare har sett att spigg som lever utanför massabruk kan påverkas fysiologiskt, bland annat genom maskulinisering av honor. Det beror förmodligen på ämnen som finns naturligt i veden man använder i pappersmassa-tillverkningen. Vi använde samma genetiska markörer som i studie I och med hjälp av statistiska modeller kunde vi se att spigg som lever utanför massabruk har en förändrad genetisk sammansättning jämfört med sina grannar från renare vatten. Dessutom var förändringen konvergent. Det betyder att det har skett liknande förändring i den genetiska sammansättningen utanför flera olika massabruk. Vi kunde identifiera små anonyma genetiska fragment, som sannolikt är under selektion för just den här miljöskillnaden. I miljön utanför massabruken är det alltså ett annat selektionstryck än i de närliggande, betydligt renare vattnen.
- III. Massabruk har flera effekter på miljön, varav en är övergödning, så kallad eutrofiering. Övergödning sker när det finns för mycket näring i vattnet (såsom kväve och fosfor) vilket kan leda till algbloomningar och syrebrist i havet. I den här studien undersökte vi spigg från Finska kustområden som är starkt påverkade av eutrofiering (men utan massabruk i närheten). Andra forskare har sett att spigg som lever i eutrofa vatten förändrar sitt parningsbeteende, kanske främst för att vattnet blir grumligt. Vi använde återigen genetiska markörer och undersökte spigg från fem olika eutrofa lokaler och fem närliggande lokaler med (ganska) klart vatten och såg att det även nu fanns en förändring av spiggarnas genetiska sammansättning. Men den här förändringen var inte lika tydlig som den vi fann utanför massabruk. Vi kunde även här identifiera genetiska fragment som är under selektion, och vissa av dem kom sannolikt från samma delar av DNA:t som de fragmenten vi upptäckte hade förändrats utanför massabruken.
- IV. I det sista kapitlet ville vi ta reda på mer om de anonyma genetiska fragment som vi hittade hos spigg utanför massabruken och som troligen var kopplade till delar av genomet som påverkats av naturlig selektion. Vi tog fram DNA-sekvenser från fem olika fragment och eftersom man sedan tidigare har kartlagt hela spiggens DNA (man känner till DNA sekvensen på spiggens alla 21

kromosomer) möjliggjorde det en direkt jämförelse mellan de fragment vi funnit och var i spiggens DNA dessa fragment kommer ifrån. Vi fick helt enkelt reda på var i genomet våra DNA-fragment passade in. Vi såg att två fragment kan komma från gener som är proteinkodande och två andra låg väldigt nära andra proteinkodande gener (så pass nära att de rent fysiskt förmodligen kommer nedärvas tillsammans). Ett av fragmenten återfanns även hos spiggar som bor i eutrofa miljöer och dessutom ligger det väldigt nära en del av genomet andra forskare menar är under selektion för saltvattenanpassning hos spigg. Tyvärr är det svårt att säga vad det betyder att just de här generna troligen verkar ha varit under selektion, det kräver vidare studier. Men det är ganska anmärkningsvärt att vi träffade på kodande gener eftersom det bara är ungefär 1.5 % av spiggens genom som består av kodande gener.

Slutsatsen i den här avhandlingen bli följaktligen att organismer som bor i miljöer starkt påverkade av miljöförstöring kan vara utsatta för ett annat selektionstryck och att en evolutionär detekterbar förändring kan ske.



## Tack-

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