2013

ADAPTIVE RESPONSES TO AQUATIC POLLUTION: DISPERSAL, PHYSIOLOGICAL ACCLIMATION, GENETIC ADAPTATION IN THE BROWN BULLHEAD (AMEIURUS NEBULOSUS)

Linda Söderberg
University of Windsor

ADAPTIVE RESPONSES TO AQUATIC POLLUTION: DISPERSAL, PHYSIOLOGICAL ACCLIMATION, GENETIC ADAPTATION IN THE BROWN BULLHEAD (*AMEIURUS NEBULOSUS*)

By

Linda I. Söderberg

A Dissertation
Submitted to the Faculty of Graduate Studies
through the **Department of Biological Sciences**
in Partial Fulfillment of the Requirements for
the Degree of **Doctor of Philosophy**
at the University of Windsor

Windsor, Ontario, Canada

2013

ADAPTIVE RESPONSES TO AQUATIC POLLUTION: DISPERSAL, PHYSIOLOGICAL ACCLIMATION, GENETIC ADAPTAION IN THE BROWN BULLHEAD (*AMEIURUS NEBULOSUS*)

by

Linda I. Söderberg

APPROVED BY:

_____

Isaac Wirgin, External Examiner
New York University, School of Medicine

_____

Melania Cristescu, GLIER

_____

Ken Drouillard
Biological Sciences

_____

Doug Haffner
Biological Sciences

_____

Andrew Hubberstey, Advisor
Biological Sciences

_____

Daniel Heath, Advisor
Biological Sciences

June 13th 2013

DECLARATION OF CO-AUTHORSHIP/ PREVIOUS PUBLICATIONS

I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

This thesis also incorporates the outcome of a joint research undertaken in collaboration with and under supervision of Professor Daniel Heath. The contribution of co-authors was primarily in an advisory capacity. The key ideas, primary contribution, experimental design, data analysis and interpretation were performed by the author. This thesis also incorporates the outcome of a joint research undertaken in collaboration with Dr Juan Galarza and Dr Ryan Walter. The contribution of co-authors was primarily technical and analytical advice and Mr Matthew Ouellette for technical assistance. The key ideas, primary contribution, experimental design, data analysis and interpretation were performed by the author.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

II. Declaration of Previous Publication

This thesis includes two original papers that have been previously published/submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status*
Chapter 3	L.I Söderberg, R.P. Walter, M.J. Ouellette, D.D. Heath. Dispersal and genetic structure among brown bullhead populations: The role of distance and degraded habitats	*In review at CJFAS*
Appendices	L.I Söderberg, J.A Galarza, D.D. Heath. 2010. Novel and optimized polymorphic microsatellite loci for brown bullhead (*Ameiurus nebulosus*). Conservation Genetics Resources 2:211-213	*Published in Conservation Genetics Resources*

I certify that I have obtained a written permission from the copyright owner(s) to include the above published material(s) in my thesis. I certify that the above material describes work completed during my registration as graduate student at the University of Windsor.

I declare that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.

ABSTRACT

Organisms are likely to display adaptive responses to their local environment, it may be local adaptation or physiological acclimation, and both improve performance (increase fitness) in stressful habitats. In this dissertation, I explore adaptive responses to pollution stress in the brown bullhead (*Ameiurus nebulosus*) from the Detroit River, as a model for integration of evolutionary and ecotoxicologial analyses.

I develop a systematic hierarchical scheme to investigate the role of adaptive processes in response to stressful environments. My literature-based review suggests initial investigation of dispersal as confounding adaptive response to degraded local environment. If there is low dispersal I suggest variation in gene transcription as a biomarker for accurate and repeatable measures of the response to pollution stress, as gene transcription is a very early response to contaminant stress. Following my proposed approach, I examined dispersal and molecular adaptive responses in brown bullhead and developed tools for the analyses: population genetic markers, a custom microarray and transcriptome libraries. The population genetic study demonstrates high population structure F_{ST} = 0.095 indicating limited long-term gene flow but contemporary dispersal associated with high contaminant levels (37% dispersals within each region). My initial transcriptome characterisation was done with next generation sequencing (NGS) on challenged and control individuals from two sites (degraded and clean). The NGS transcriptome characterisation was resulted in 3.4 million assembled reads and identified 5515 transcribed genes across clean and polluted background populations. Many gene transcription patterns were as expected as part of an adaptive response; however, some

expected transcription induction was not observed. Thus I used a 128 gene custom ecotoxicology response microarray to quantify dose and temporal response of selected genes in brown bullhead exposed to B[a]P. This identified 5 up-regulated and 5 down-regulated gene responses: up-regulation included a variety of response profiles, while down-regulation was simple gene repression.

All forms of adaptive responses in contaminant indicator species have the potential to confound our interpretation of toxicity in natural and lab environments. This may have important management and legislative implications. Of equal interest, my thesis research highlights some behavioural and molecular mechanisms for adaptive responses in Detroit River brown bullhead.

DEDICATION

All this work and this dissertation is for my most beloved and fantastic daughter Ebba who spent her first years with mamma always working and being stressed. Ebba I am so proud of you and I am proud to be your mother. We have had good times between my long hours of work and I promise you we will have many more, I can't wait to have more time to spend with you and to be able to make you laugh. You have been here all along for me and it makes me so happy that you are proud over me too. It you in 20 years want to become a doctor like mamma as you said this morning I promise you I will do everything in my power to help you.

I always loved you and I always will love you and be proud of you.

Alla timmar of arbete så väl som själva avhandlingen tillägnar jag min älskade fantastiska dotter Ebba som spenderade sina första år med en mamma som alltid jobbade och var stresad. Ebba jag är så stolt att vara din mamma och jag är stolt över dig. Vi har haft det bra och roligt mella min långa timmar av jobb och jag lovar att vi ska ha många fler nu. Jag ser sån fram emot att kunna spendera mer tid med dig och ha roligt och skratta med dig. Du har funnits här for mig hela tiden och att veta att du ä stolt över vad jag har åstakommit gör mig sa glad. Om du om 20 år fortfarande vill doktorera som mamma så lovar jag dig att göra allt i min makt för att hjälpa dig.

Jag älskar dig av hela mitt hjärta och komme alltid vara stolt över dig

ACKNOWLEDGEMENTS

I want to thank my supervisor Daniel Heath for giving me this opportunity to come to Canada and to do this cool project. Furthermore I want to thank him for always being there and supporting me through these years even when I have struggled and I think we both doubted that I would make it through. I also want to thank my co-supervisor Andrew Hubberstey and my committee for their advice and support to make my thesis better.

I want to thank Todd Leadley for all his help and support in field, during challenges and with fish care. Todd's support has been very important for this study and for me. Further I want to acknowledge everyone that has helped me in the field especially Michelle Farwell, Rebecca Williams, Kevin Robinson, and Steve Cho. Rebecca needs a further thank you for sharing her samples with me. I also recognise the people that helped me in the lab: Matt Ouellette, Betty Helou, and during challenges Rob Ginson and Jessica O'Neil, Joe Robinet, Gord Patterson and Ken Drouillard.

I cannot thank Dr. Ryan Walter, Dr. Tutku Aykanat, Dr Subbarao Chaganti and Kyle Wellband enough for always having time for discussions and great ideas on how to proceed or improve things, their contribution has been invaluable to me and I don't know how I can show my appreciation. There have also always been people around that help me with English or just help me to find a word. These people have helped me tremendously.

I also want to thank my family that has been so supportive during all this time, especially my mom and my sister Ellinor, who came to Canada to help me take care of Ebba in stressful times. My family's support has helped me through a lot of hard times

during the last five years despite coming all the way from Europe. It is great to know that I always have your love and support.

There have been so many people that has helped take care of Ebba and helped me keep my sanity while trying to juggle PhD, caring for my daughter, and at times just surviving the day. There are too many people that should have credit for helping me succeed but persons that need special mentioning is Mirela Metea, Seda Karsioglu, Anna Constantin, Rachel Franzblau, and Katerina Stojanovic for babysitting when I have been desperate. Anna Constantin, Rachel Franzblau Nadine Loick, Jill Olin, Melissa Farmer, Jenn Bronnenhubber and people in my office that have heard about my struggles and been supportive over the years. I would not have been able to get where I am today without my amazing support and I would not have been the person I am today without your love and support.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 3

CHAPTER 4

CHAPTER 5

APPENDICES TABLES

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF APPENDICES

CHAPTER 1

GENERAL INTRODUCTION

Adaptive responses: Adaptive responses to changed or stressful environments will provide an organism superior performance in their environment compared to individuals that do not mount such a response. There are different types of adaptive responses, they can be evolutionary (genetic adaptation which is non-plastic selection on polymorphic loci), or plastic (phenotypic plasticity or physiological acclimation which can have a genetic background). Either genetic or plastic adaptive responses will result in the organism having a higher fitness in their environment.

Evolution – Local adaptation: Evolutionary responses to novel or changing environments form the basis for generating biological diversity over time. It was previously believed that evolutionary processes generally act over thousands - or even millions - of years, which is often the case for major evolutionary events. However, more recently it has been reported that evolution can occur rapidly (Grant and Grant 2002; Reznick *et al.* 1997; Hendry *et al.* 2000; Hendry 2001; Kinnison and Hendry 2001; Heath *et al.* 2003). This type of rapid evolution is often in response to novel environments (Hendry *et al.* 2000; Hendry 2001), changing environments (Grant and Grant 2002), or degraded environments (Wirgin *et al.* 2011). Independent of the cause of the evolutionary response, the population's allele frequency changes to be better suited to the current environment.

Evolutionary processes drive major events such as speciation, or for maladapted species, extinction. However, the same evolutionary principles can contribute to smaller population-level changes, with some individuals performing better than others, and thus they have higher survival and reproductive success (i.e., have a higher fitness) in their local environment. That is, fitness is determines by interactions between their genotype and their environment. In the absence of other forces, such as gene flow and drift, selection will lead to local populations evolving traits that provide an advantage in their local environment, independent of their effect in other environments; this is referred to as "local adaptation", a form of genetic adaptation (Kawecki and Ebert 2004). High levels of gene flow are believed to inhibit local adaptation, as immigrant alleles will dilute the local gene pool, unless the selective force is very strong (Hoffman and Hercus 2000; Kawecki and Ebert 2004). Thus for adaptive responses to a stressor (for example, pollution) to evolve, some level of philopatry is advantageous. Simple dispersal without reproduction will not affect genetic diversity and effective population size, and hence it will not affect the evolution of local adaptation. However if immigrants reproduce, gene flow will result increasing genetic variation and effective population size – the net result will be to slow the effect of selection.

Plastic responses: Plastic responses are individual-level phenomena and can occur in two ways. Phenotypic plasticity is where the phenotype of the organism will depend on the natal and developmental environment, rather than genotype. This can be adaptive under some conditions, but can also be maladaptive if the environment is highly stochastic. The other type of plastic response is acclimation, which is a physiological response that returns an organism closer to its homeostatic condition in a stressful

environment. Acclimation can thus increase short-term survival and maintain the highest possible fitness under stressful conditions. However, acclimation is energetically costly to the organism, and thus if the environment changes to reduce or eliminate the stressor, the acclimation will disappear (Klerks *et al.* 1997; Wirgin and Waldman 2004). Depending on the nature of the stress that elicited acclimation, a physiological trade off might occur, such as a reduced life span or reduced reproductive success (Wilson and Franklin 2002; Wood and Harrison 2002; Farwell *et al.* 2012). It has even been argued that the benefit of physiological acclimation may be lost due to the cost of the physiological response (Hoffman 1995). An organism (or a group of organisms) can acclimate for an extended period of time to maintain survival rates; however, acclimation is not an optimal long-term stress response. Another aspect of acclimation is that previous exposure and acclimation can result in a more rapid acclimatory response upon re-exposure. However, acclimation cannot be inherited across generations, and it is an individual response (Wirgin and Waldman 2004).

Transcription and gene expression: Every organismal trait and response originates in gene transcription or variation in gene transcription. Gene transcription is the process whereby information stored in the DNA (genes) is transcribed to RNA and ultimately messenger RNA (mRNA). The mRNA may be subsequently translated into amino acid chains which then form functional proteins. We can measure mRNA copy number, thus estimating transcription levels for individual genes, or for the entire transcriptome. Many studies assume a close to 1:1 ratio between mRNA and functional protein, though this is generally not true (Maier *et al.* 2009), as there are several post-transcriptional regulation processes taking place that will affect the relationship between mRNA levels and final

protein function. However, the post-transcriptional regulation processes are generally poorly understood (at least for non-model organisms) and often difficult to quantify. Nevertheless, the primary and initial response of a cell to external stimuli is gene transcription, even if the final protein is not achieved.

When adaptive responses occur it will first affect the transcription patterns as all proteins, enzymes and physiological responses depend on gene transcription. For example, genes that would normally respond to a stressful stimulus may not be transcribed at all, due to adaptive responses: the most commonly studied example is the reduced induction of cytochrome P450 1A (CYP1A) in chronically stressed fish (Wirgin and Waldman 1998; Elskus *et al.*1999; Meyer *et al.*2002; Grey *et al.* 2003; Wirgin and Waldman 2004; Wirgin *et al.*2011; Brammell *et al.*2013). However, if an acclimation process inhibits the response at one gene locus, there might be other genes that are regulated differently to compensate and maintain the individual at or near homeostasis.

Adaptive responses that mediated altered transcription can be measured and compared to control populations or individuals that are from a similar habitat but without the environmental stressor. There are different methods for measuring transcription, one of the earlier methods (that is still in use) is Northern Blotting, which measures mRNA semi-quantitatively. More quantitative methods such as quantitative real-time PCR (qRT-PCR) have been developed; however both allow targeted study of a few genes. There are two different approaches to measuring transcription in many genes at once. Microarray analysis will quantify a few hundred gene transcripts to the entire transcriptome. Although microarrays are commonly used, they have some limitations, for example, they do not allow the detection of novel transcripts (David *et al.* 2010). The new upcoming

method to quantify transcription across the entire transcriptome is next generation sequencing (NGS; Brenner *et al.* 2000), which can detect both known and unknown mRNA sequences and is generally more sensitive than microarrays. I use both methods for different purposes to investigate the potential for adaptive responses in the brown bullhead (*Ameiurus nebulosus*).

Pollution and its effect on transcription: Pollution is becoming an increasingly important issue today as global pollution levels are increasing in the environment. Aquatic environments have especially high levels of pollution, as they have been used as disposal sites for decades with justification such as the "solution to pollution is dilution". Another historic reason to dispose of waste in aquatic environments is that it is less visible when mixed or submerged in water. The level of pollution in natural aquatic ecosystems is high enough to cause measurable harm to organisms. Observed effects include increased cancer rates, endocrine disruption, and reduced reproductive ability in exposed organisms (Johnson *et al.* 2003; Ketata *et al.* 2007; Ruzi *et al.* 2011). Initially, there is an instantaneous acute response to the stressor which is energetically very costly for the organism, but is an often necessary defence against the stress.

When organisms are exposed to any stressor, including pollutants, a change in transcription profile and gene expression will occur. This change in transcription is the first response that occurs at the sub-cellular level. This change in transcription level can be used as an early indicator of possible biohazards. Using this information will provide an opportunity to remediate the site to reduce further effects on the ecological community (Medeiros *et al.* 2008). For individuals experiencing chronic exposure, we can still compare transcription profiles from clean and polluted environments to identify genes

and signalling pathways that are up- or down-regulated in the polluted environment. However, such an analysis will not provide information on the adaptive nature of the response they are presenting.

Different stressors and pollutants will trigger different transcriptional responses. Organic pollutants, such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), activate the aryl hydrocarbon receptor (AhR) and the metabolites may be toxic (genotoxic). When an organism is exposed to PAHs or PCBs, their CYP1A expression is generally increased in a predictable fashion (Wirgin and Waldman 1998; Meyer *et al.* 2003; Kilemade *et al.* 2009), making CYP1A the most studied biomarker for this pathway. However, other pollutants will activate different gene responses, for example, metals will induce metallothionein (Bervoets *et al.* 2013) and estradiols and xenoestrogen will induce vitellogenin expression (Mortensen and Arukwe 2007). Those are examples of gene induction we know and understand in the fish detoxification processes. There are likely many other gene transcription responses that have neither been described nor characterised, while fish signalling pathways that are induced / inhibited in response to contaminant stress are even more poorly understood.

Adaptive responses to pollution: When organisms are first exposed to pollution, they will mount their initial stress response, the nature of this response will depend on the stressor. If the organisms remain exposed to prolonged stress (chronic stress) they may display a wide diversity of adaptive responses.

Several studies have shown that organisms under chronic pollution exposure do not show a change or a limited induction in CYP1A expression when challenged with a pollutant they are normally exposed to (Gray *et al.* 2003; Meyer *et al.* 2003). Although, if

they are exposed to a novel organic pollutant, they will still display a characteristic CYP1A response (Meyer *et al.* 2003; Wirgin and Waldman 2004). Thus the nature and strength of transcriptional and gene expression response to contaminant stress can vary unpredictably depending on previous exposure and the adaptive effects associated with the previous exposure. To reduce the chance of adaptive responses interfering with the interpretation of an organisms' response to acute contaminant exposure, we can use a broader approach with a wide selection of genes included for transcriptional analysis. This is becoming a more common approach to study pollution effects (Williams *et al.*2003; Holth *et al.*2008; Oleksiak 2008; Carlson *et al.*2009; Lie *et al.*2009; Bozinovic and Oleksiak 2011; Whitehead *et al.*2011), but is still not common practice. Microarray studies have been used to measure the response of multiple genes to pollution in several aquatic species (reviewed in Bozinovic and Oleksiak 2011), but no general pattern has been found. Indeed, there are even different responses among populations within a species (Fisher and Oleksiak 2007; Oleksiak 2008; Bozinovic and Oleksiak 2011; Whitehead *et al.* 2011). With both microarrays and next generation sequencing (NGS) approaches one is likely to find changes that occur in the transcriptome due to the pollutant, even if single candidate gene responses may be masked by adaptive responses.

Brown bullhead: The brown bullhead (*Ameiurs nebulosus*) is a benthic catfish native to the Great Lakes and has long been used as an indicator species for aquatic pollution studies. They are reported to be highly tolerant to contaminants in the sediment (Scott and Crossman 1979), but also display high levels of neoplasia and tumours in highly contaminated habitats (Baumann *et al.* 1987; Baumann 1992; Leadley *et al.*1998). It has been shown that the frequency of tumours in brown bullhead correlate with PAH

concentration in the sediment (Baumann *et al.* 1996). On the other hand, it has also been shown that there is reduced CYP1A induction in brown bullhead from highly polluted areas in Presque Isle Bay (Grey *et al.* 2003). One interpretation of this is that there is a potential for adaptive responses in this species. I chose to use the brown bullhead as a model species for this study due to their high tumour rate, their contact with the sediment and as their possibly adaptive responses to contaminant stress.

Thesis Overview: This thesis begins with a literature-based review (Chapter 2) discussing evolutionary responses and biomarker use, in which I make suggestions to use ecotoxicogenomic (transcription) methods as an additional biomarker approach. I also highlight the potential confounding effects of adaptive responses in bioindicator species. I further develop a systematic and hierarchical approach to the quantification and characterisation of possible adaptive responses to aquatic contaminants. The remainder of my dissertation follows that suggested approach. Initially I developed microsatellite markers for brown bullhead (appendices) and used these markers in a population genetic study investigating population structure and dispersal among brown bullhead populations across the lower Great Lakes. I found high population structure and that populations are genetically differentiated even in close proximity (Chapter 3). Consequently, I explored the possibility of adaptive responses between brown bullhead from a clean and a polluted site using next generation sequencing of genome-wide transcription comparing the transcriptome profile between challenged and control individuals from a clean and a polluted site (Chapter 4). Finally, I developed a targeted microarray which was used to determine the timing of induction of gene transcription after an injection of benzo[a]pyrene (B[a]P) using a dose response design (Chapter 5). I end by discussing my

findings and conclude that brown bullhead are likely displaying adaptive responses; however, my work is unable to separate physiological acclimation from genetic adaptation (Chapter 6). I close with suggestions on how to practically approach the issue of determining the nature of adaptive transcriptional responses to aquatic contaminant stress (Chapter 6).

REFERENCES

Baumann, P. C., W. D. Smith, and W. K. Parland. 1987. Tumor frequencies and contaminant concentrations in brown bullheads from an industrialized river and a recreational lake. Transactions of the American Fisheries Society 116:79-86

Baumann, P. 1992. The use of tumors in wild populations of fish to assess ecosystem health. Journal of Aquatic Ecosystem Health 1:135-146

Baumann, P. C., I. R. Smith, and C. D. Metcalfe. 1996. Linkage between chemical contaminants and tumour in benthic Great Lakes fish. Journal of Great Lakes Research 22:131-152

Bervoets, L., D. Knapen, M. De Jonge, K. Van Campenhout, and R. Blust. 2013. Differential hepatic metal and metallothionein levels in three feral fish species along a metal pollution gradient. Public Library of Science One 8:1-11

Bozinovic, G., and M. F. Oleksiak. 2011. Genomic approaches with natural fish populations from polluted environments. Environmental Toxicology and Chemistry 30:283-28

Brammell, B. F., D. J. Price, W. J. Brige, and A. A. Elskus. 2013. Lack of CYP1A responses in species inhabiting chronically contaminated habitats: two varieties of resistance? Comparative Biochemistry and Physiology Part C 157:212-219

Brenner, B., M. Johnson, J. Bridgham, G. Golda, D. H Lloyd, D. Johnson, S. Luo, S. McCurdy, M. Foy, M. Ewan, R. Roth, D. George, S. Eletr, G Albrecht, E. Vermaas, S. R. Williams, K. Moon, T. Burcham, M. Pallas, R. B. DuBridge, J. Kirchner, K. Fearon, J. Mao, and K. Corcoran. 2000. Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. Nature Biotechnology 18:630-634

Carlson, E. A., N. K. Roy, and I. I. Wirgin. 2009. Microarray analysis of polychlorinated biphenyl mixture-induced changes in gene expression among Atlantic tomcod populations displaying differential sensitivity to halogenated aromatic hydrocarbons. Environmental Toxicology and Chemistry 28:759-771

David, J. P., E. Coissac, C. Melodelima, R. Poupardin, M. A Riaz, A. Chandor-Proust and S. Reynaud. 2010. Transcriptome response to pollutants and insecticides in the dengue vector *Aedes aegypti* using next-generation sequencing technology. BMC Genomics 11:216

Elksus, A. A., E. Monosson, A. E. McElroy, J. J. Stegeman, D. S. Woltering. 1999. Altered CYP1A expression in *Fundulus heteroclitus* adults and larvae: a sign of pollutant resistance? Aquatic Toxicology 45:99-113

Farwell M., K. G. Drouillard, D. D. Heath, T. E Pitcher. 2012. Acclimation of life-history traits to experimental changes in environmental contaminant concentration in brown bullhead (*Ameiurs nebulous*). Environmental Toxicology and Chemistry 31:863-869

Fisher, M. A. and M. F. Oleksiak. 2007. Convergence and divergence in gene expression among natural populations exposed to pollution. BMC Genomics 8:108

Grant, P.R., and B. R. Grant. 2002. Unpredictable evolution in 30-year study of Darwin's finches. Science 296:707-711

Grey, J. P., T. L. Leas, E. Obert, D. Brown, G. C. Clark, and J. P. Vanden Heuvel. 2003. Evidence of aryl hydrocarbon receptor ligands in Presque Isle Bay of Lake Erie. Aquatic Toxicology 64:343-358

Heath, D. D., J. W. Heath, C. A. Bryden, R. M. Johnson, and C. W. Fox. 2003. Rapid evolution of egg size in captive salmon. Science 299:1738-1740

Hendry, A. P., J. K. Wenburg, P Bentzen, E. C. Volk, and T. P. Quinn. 2000. Rapid evolution of reproductive isolation in the wild: evidence from introduced salmon. Science 290:516-518

Hendry, A. P. 2001. Adaptive divergence and the evolution of reproductive isolation in the wild: an empirical demonstration using introduced sockeye salmon. Genetica 112-113:515-534

Hoffman, A. A. 1995. Acclimation: increased survival at a cost. Trends in Ecology and Evolution 10:1-2

Hoffman, A. A., and M. J. Hercus. 2000. Environmental Stress as an Evolutionary Force. BioScience 50:217-226

Holth, T. F., R. Nourizadeh-Lillabadi, M. Blaesbjerg, M. Grung, H. Holbech, G. I. Petersen, P. Aleström, and K. Hylland. 2008. Differential gene expression and biomarker in zebrafish (*Danio rerio*) following exposure to produced water components. Aquatic Toxicology 90:277-291

Johnson, L. L., T. K. Collier, and J. E. Stein. 2002. An analysis in support of sediment quality thresholds for polycyclic aromatic hydrocarbons (PAHs) to protect estuarine fish. Aquatic conservation: Marine and Freshwater Ecosystems 10:517-538

Kawecki, T. J., and D. Ebert. 2004. Conceptual issues in local adaptation. Ecology Letters 7:1225-1241

Ketata, I., W Smaoui-Damak, F. Guermazi, T. Rebai, and A. Hamza-Chaffai. 2007. *In situ* endocrine disrupting effects of cadmium on the reproduction of *Ruditapes decussates*. Comparative Biochemistry and Physiology, Part C 146: 415-430

Kilemade, M., M. G. J. Hartel, J. O'Halloran, N. M. O'Brien, D. Sheehan, C. Mothersill, and F. N. A. M. van Pelt. 2009. Effects of contaminated sediment from Cork Harbour, Ireland on the cytochrome P450 system of turbot. Ecotoxicology and Environmental Safety 72:747-755

Kinnison, M. T., A. P. Hendry. 2001. The pace of modern life II: from rates of contemporary microevolution to pattern and process. Genetica 112-113:145-164

Klerks P. L., P. L. Leberg, R. F. Lance, D. J. McMillin, and J. C. Means. 1997. Lack of development of pollutant-resistance or genetic differentiation in darter gobies (*Gobionellus boleosoma*) inhabiting a produced water discharge site. Marine Environment Research 44:377-395

Leadley, T. A., G. Balch, C. D. Metcalfe, R. Lazar, E. Mazak, J. Habowsky, and G. D. Haffner. 1998. Chemical accumulation and toxicological stress in three brown bullhead (*Ameiurus nebulosus*) populations of the Detroit River, Michigan, USA. Environmental Toxicology and Chemistry 17:1756-1766

Lie, K. K., A. Lanzen, H. Berelid, and P. A. Olsvik. 2009. Gene expression profiling in Atlantic cod (*Gadus Morhua L.*) from two contaminated sites using a custom-made cDNA microarray. Environmental Toxicology and Chemistry 28:1711-1721

Maier, T., M. Güell, and L. Serrano. 2009. Correlation of mRNA and protein in complex biological samples. FEBS Letters 583:3966-3973

Medeiros, I.D., M. N. Siebert, G. Toledo-Silva, T. B. Rodrigues, M. R. F. Marques, and A. C. D Bainy. 2008. Induced gene expression in oyster *Crassostrea gigas* exposed to sewage. Environmental Toxicology and Pharmacology 26:362–365

Meyer, J. N., D. E. Nacci, R.T. Di Giulio. 2002. Cytochrome P4501A (CYP1A) in killifish (*Fundulus heteroclitus*): heritability of altered expression and relationship to survival in contaminated sediments. Toxicological Science 68:69-81

Meyer, J. N., D. M. Wassenberg, S. I. Krachner, M. E. Hahn, and R. T. Di Giulio. 2003. Expression difference and inducibility of aryl hydrocarbon receptor pathway genes in wild-caught killifish (*Fundulus heteroclitus*) with different contaminant-exposure histories. Environmental Toxicology and Chemistry 22:2337-2343

Mortensen, A. S., and A. Arukwe. 2007. Interactions between estrogen- and Ah-Receptor signalling pathways in primary culture of salmon hepatocytes exposed to nonylphenol and 3,3',4,4'-tetrachlorobiphenyl (congener 77). Comparative Hepatology 6:2

Oleksiak, M. F. 2008. Change in gene expression due to chronic exposure to environmental pollutants. Aquatic Toxicology 90:161-171

Reznick, D. N., F. H. Shaw, F. H. Rodd, and R. G. Shaw. 1997. Evaluation of the rate of evolution in natural populations of guppies (*Poecilia reticulata*). Science 275:1934-1936

Ruiz, Y., P. Suarez, A. Alonso, E. Longo, A. Villaverde and F. S. Juan. 2011. Environmental quality of mussel farms in the Vigo Estuary: Pollution by PAHs, origin and effects on reproduction. Environmental Pollution 159:250-265

Scott, W. B., and E. J. Crossman. 1973. Freshwater fishes of Canada. Bulletin – Fisheries Research Board of Canada No. 184

Whitehead, A., F. Galvez, S. Zhang, L. M. Williams and M. F. Oleksiak. 2011. Functional genomics of Physiological plasticity and local adaptation in killifish. Journal of Heredity 102:499-511

Williams, T. D., K. Gensberg, S. D. Minchin, and J. K. Chipman. 2003. A DNA expression array to detect toxic stress response in European flounder (*Platichthys flesus*). Aquatic Toxicology 65:141-157

Wilson, R. S., and C. E. Franklin. 2002. Testing the beneficial acclimation hypothesis. Trends in Ecology & Evolution 17:66-70

Wirgin, I., J. R. Waldman. 1998. Altered gene expression and genetic damage in North American fish populations. Mutation Research 399:193-219

Wirgin, I., J. R. Waldman. 2004. Resistance to contaminant in North American fish populations. Mutation Research 552:73-100

Wirgin, I., N. K. Roy, M. Loftus, R. C. Chambers, D. G. Franks, and M. E Hahn. 2011. Mechanistic basis of resistance to PCBs in Atlantic tomcod from the Hudson River. Science 331:1322-1325

Wood, H. A., and J. F. Harrison. 2002. Interpreting rejections of the beneficial acclimation hypothesis: when is physiological plasticity adaptive? Evolution 56:1863-1866

CHAPTER 2

CONTAMINANT EFFECTS IN AQUATIC ECOSYSTEMS: GENETIC ADAPTATION, ACCLIMATION AND DISPERSAL CONFOUND BIOINDICATOR RESPONSE

INTRODUCTION

Pollution in aquatic environments has become an increasingly important issue as our rivers, lakes, seas and oceans have long been used by humans as a solution for unwanted waste and pollutants (Costello and Read 1994; Takada *et al.* 1994; Wölz *et al.* 2009). Many substances disposed of in our waters ultimately end up in the sediments, which can absorb and retain chemicals in higher concentrations than the water itself (DeValls *et al.* 2002; Aldarondo-Torres *et al.* 2010; De Domenico *et al.* 2011). The average level of pollution in many ecosystems is still increasing (Percy and Ferretty 2004; Law and Stohl 2007; Ramos *et al.* 2009; Wölz *et al.* 2009) and many studies indicate that pollutants are reaching critical concentration levels, such that organisms are facing survival challenges (e.g., carcinomas, mutagenic-related birth defects, neurological damage, endocrine disruption and loss of reproductive capacity; Johnson *et al.* 2003; Ketata *et al.* 2007; Aldarondo-Torres *et al.* 2010; Chopra *et al.* 2011; Ruiz *et al.* 2011). The impact of the pollutants on aquatic ecosystems may be even greater due to variable and complex mixtures of pollutants. Conservation biologists and environmental agencies need an objective way to measure the potential impact of pollution, especially since

immigrants (dispersals) and naïve organisms (including exposed humans) are likely to be highly susceptible to novel exposure or combinations of pollutants making impact predictions problematic.

We are capable of detecting and quantifying aquatic and sediment contaminations at very low concentrations. However, the limitation with analytic approaches is that we only detect chemicals that we are looking for. Standard contaminant analyses are limited in the number and classes of compounds routinely measured, meaning that the agent responsible for toxicity may often avoid detection. To address this potential limitation, environmental monitoring often includes biological measurements of the effect of pollution on the function and health of specific organisms (biomonitor species). However, while this approach does allow quantification of toxic effects of unknown contaminants, it can suffer from potentially confounding evolutionary responses. Organisms may exhibit "adaptive" responses to pollution, where "adaptive" is defined as any response (molecular, cellular, physiological or behavioural) that will increase their fitness relative to organisms that do not mount that response. If such adaptive responses are not considered, the pollutant effects on immigrant or naïve species or ecosystems in general, are likely to be misinterpreted. Physiological acclimation (an adaptive physiological response that is energetically costly), or genetic adaptation (an adaptive change in population allele frequencies) in response to pollutants which will affect their tolerance to exposure, are possible adaptive responses. Furthermore organisms are neither static nor stationary; they can disperse under contaminant stress (either into or out of the area) even if normally philopatric. Such a behavioural response may be adaptive or not, depending on the fitness outcome; however, in either case contaminant-related dispersal will affect

the interpretation of resident response to the local habitat. To be able to predict the effect of local habitat degradation on naïve organisms, we need to consider the potential for adaptive responses in native organisms to confound our assessment of the hazards of the polluted habitat. The use of non-native indicator species can overcome this problem; however, it will depend critically on the source and genetic background of the animals (or plants). If the indicator species population has undergone acclimation or genetic adaptation to any of the pollutants, we may misinterpret a lack of response as a lack of hazard to naïve species. The potential for adaptive responses to confound biomonitoring is compounded by the common use of late-stage organismal responses, or the "end of the pipe" types of measurement (Eason and O'Halloran 2002). Such late-stage effects (e.g., morbidity, mortality and reproductive failure) are also perhaps the most likely to exhibit acclimation or genetic adaptation.

In this review we propose a novel approach to biomonitoring; the use of native organisms or communities of organisms to predict possible effects on naïve biota (or humans) resulting from exposure to an area of contaminant concern. We suggest that biomonitoring should reflect evolutionary principles and explicitly incorporate adaptive responses (see Figure 2.1). We further recommend that biomonitoring incorporate genomic methods for screening gene transcription, a sensitive and innate marker of an organism's response to their environment (Busch *et al.* 2004; Bozinovic and Oleksiak 2011). Gene transcription fulfils the need for early stage biological response, as transcription is the first response of an organism to exposure to stress.

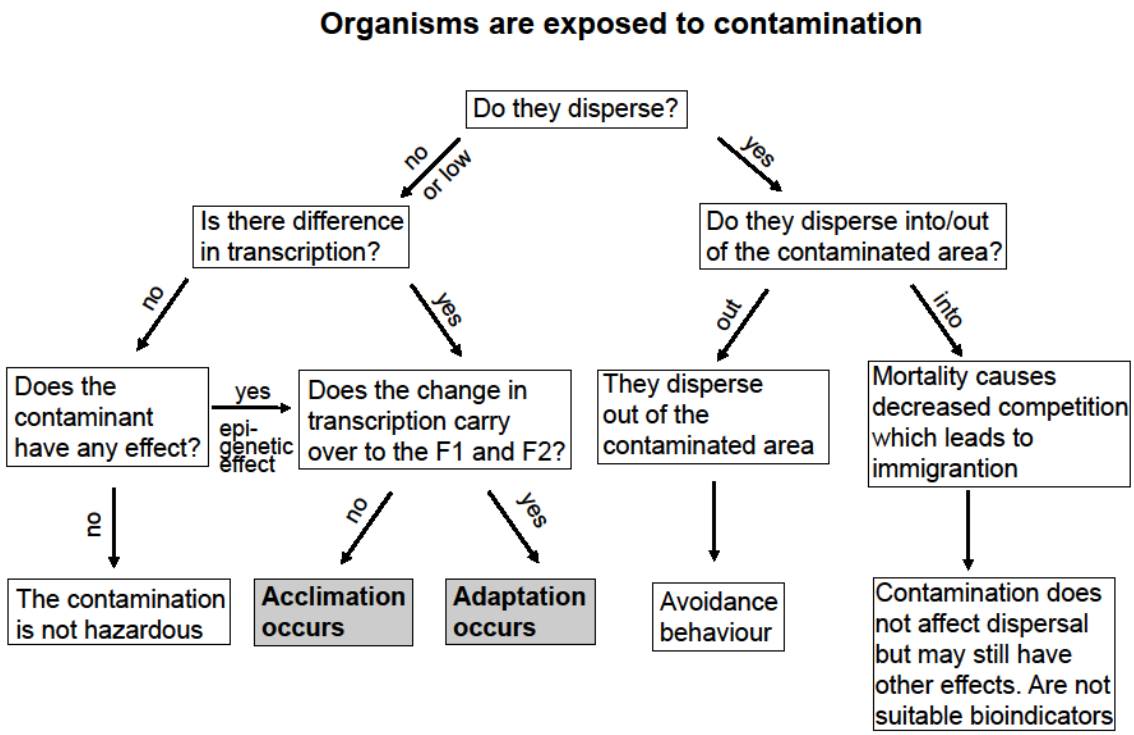

Figure 2.1 Systematic hierarchical approach to interpret contamination's effect on biomarkers such as transcription when adaptive responses may be present.

ADAPTIVE RESPONSES TO POLLUTION

When organisms are exposed to either acute or chronic contamination or pollution they can display one of several adaptive responses to survive and maintain highest possible fitness. For example, the logical first response to aquatic pollution is avoidance away from localised sources (avoidance). Although aquatic organisms technically have a three-dimensional space to utilise, their ability to disperse is often functionally quite limited (Wong *et al.* 2004; Evans *et al.* 2009; Millard *et al.* 2009); in fact, in some species no dispersal is observed, despite a lack of physical barriers (Rico and Turner 2002). In other species, dispersal occurs only at a specific life stage; for instance reef fishes disperse at early life stages only, while the adults are sedentary (Cowen and Sponaugle 2009; Salas *et al.* 2010), thus limiting their ability to avoid point-source pollution. High levels of aquatic contamination have been shown to affect dispersal frequency and pattern (reviewed in Bickham *et al.* 2000; Theodarkis 2003; Bickham 2011; Söderberg *et al.* 2013). Clearly, before any reliable estimate of the impact of aquatic pollutants on specific organismal, or sub-organismal, response can be made, we must determine the potential for and magnitude of dispersal. There are many methods used to quantify dispersal; direct methods involving mark and recapture or tagging and tracking are common in the literature. Tagging and tracking are highly effective, especially for larger species that can carry a satellite tag that provides detailed information on the path of dispersal (Millard *et al.* 2009; Block *et al.* 2012). However, all forms of tagging and tracking are expensive and time consuming, and for smaller aquatic organisms, tagging is not suitable. Indirect methods of quantifying dispersal include genetic and microchemical analysis of endogenous tags (Bradbury *et al.* 2008; Selkoe *et al.* 2008). Genetic methods can measure

dispersal indirectly by estimating gene flow among sampling sites, this has been done for several aquatic taxa, including invertebrates, fish and sea birds (Pogson *et al.*2001; Luttikhuizen *et al.* 2003; Manel *et al.*2003; Samadi *et al.* 2006; Friesen *et al.* 2007; Demarchi *et al.* 2008; Luttikhuizen *et al.* 2008; White *et al.* 2009; Zakas and Wares 2012). Another common indirect method of dispersal tracking in aquatic organisms is the use of microchemical characterization of hard structures (e.g., otoliths; Bradbury *et al.* 2008; Selkoe *et al.* 2008; Humston *et al.* 2009). Trace elements in the water column can characterise rearing location, identify natal origin, and potentially, migration pathways without the assumption of gene flow (Humston *et al.* 2009); however, otholiths may also pick up heavy metal pollution from their environment (Ranaldi and Gangnon 2010; Li *et al.* 2011). Polluted habitat may affect dispersal in one of two ways; 1) induce dispersal away from the source of the stress (avoidance), this behaviour was observed in the Detroit River where a higher number of individuals were found to leave contaminated areas than observed in uncontaminated reference sites (Söderberg *et al.* 2013); or 2) result in dispersal into the polluted area in response to available habitat due to increased mortality or reproductive failure in the native population (sink population dynamics – Theodorakis *et al.* 2001; Bickham 2011). While avoidance is an adaptive response (assuming the migrant moves to a less stressful habitat), immigration into a degraded habitat is not likely to be an adaptive response. Independent of the direction of the dispersal, pollution driven dispersal will confound measurements of pollution effects, as exposed individuals will end up in clean sites or naïve organisms will be included in the polluted site sample. Clearly, the first step in quantifying the effect of pollution on a bioindicator species is measuring dispersal into or out of the study area (Figure 2.1).

If an indicator species can be shown to have limited dispersal (even under pollution stress) and the individual and the population survives the pollutant stress, the population will genetically adapt and/or the individuals will physiologically acclimate in response to the polluted environment (Klerks *et al.* 1997). Generally, genetic adaptation and physiological acclimation in natural systems are hard to differentiate without additional experiments. When first exposed to a novel pollutant, an organism will mount an acute response to cope and possibly to survive: if it can tolerate the stress (i.e., survive) it will have the potential to acclimate or genetically adapt in the longer term. Physiological acclimation to a stressor is reversible and thus disappears with environmental remediation (Wirgin and Waldman 2004). Acclimation is often energetically costly; it has even been argued that it is almost to the point when the benefit of the acclimation is less than the energy cost (Hoffamn 1995; Wood and Harrison 2002). Physiological acclimation will increase tolerance, and the organism will gain an advantage in a stressful environment, as a result of earlier or prolonged exposure (Leroi *et al.* 1994; Klerks *et al.* 1997; Sabban and Kvetňanský 2001; Wood and Harrison 2002). Acclimation is a mechanism that facilitates the return towards the homeostatic state that may involve changes in transcription and/or gene expression at specific gene loci (López-Maury *et al.* 2008). The ultimate effect of acclimation is to reduce the stress response; hence the organism will appear to be at, or near, homeostasis, masking its initial (acute) response to pollutants. This masking of the acute response may lead to incorrect conclusions about the pollution status (hazard) of the environment to other organisms. However, it is important to remember that acclimation does have costs, and acclimated individuals may not respond well to changing conditions (even towards "normal"

conditions). Indeed, they may experience reduced reproductive success (Farwell *et al.* 2012) and early mortality due to the trade-offs associated with the energetic costs of acclimation (Wilson and Franklin 2002; Wood and Harrison 2002). Physiological acclimation generally acts more rapidly in individuals with previous exposure (Klerks *et al.* 1997; Sabban and Kvetňanský 2001; Wood and Harrison 2002). Although physiological acclimation is not genetically heritable, it can carry over to offspring in the F1 generation (Meyer *et al.* 2002) by either maternal or epigenetic effects (Wirgin and Waldman 2004). However, acclimation does not generally persist across multiple generations. Physiological acclimation is a plastic response but should not be confused with; (i) phenotypic plasticity (Wilson and Franklin 2002), including behavioural phenotypic plasticity (Wood and Harrison 2002), (ii) developmental phenotypic plasticity (Bradshaw 1965), or (iii) genotype x environment (GxE) interactions (Fry 1992). The subtle, but important, distinctions among the various forms of plasticity are summarized in Box 1.

Genetic adaptation is an evolutionary population-level response and will generally take longer to emerge than physiological acclimation. It is essentially genetic change in the population over time; genotypes and alleles that move the organism closer to homeostasis or alter homeostatic thresholds will be favoured by natural selection, ultimately resulting in better adapted genotypes and phenotypes. Genetic adaptation occurring at the population level is substantially different from the plastic adaptive responses discussed above (see Box 1). Genetic adaptation usually evolves over an extended period of time, although genetic adaptation to local environments ("local adaptation") may be "rapid" (over a few generations; Hendry and Kinnison 1999). Rapid

local adaptation occurs in new or changing environments, and has been documented across diverse taxa in nature (e.g., Trinidad guppies (*Poecilia reticulata*), sockeye salmon (*Oncorhynchus nerka*), Galapagos finches (*Geospiza spp*), Reznick *et al.* 1997; Hendry and Kinnison 1999; Hendry *et al.* 2000; Grant and Grant 2002). Rapid genetic adaptation to contaminated and/or polluted environments has also been documented in midges (*Chironomus riparius*), Atlantic tomcod (*Microgadus tomcod*), and Atlantic killifish (Elskus *et al.* 1999; Groenendijk *et al.* 1999; Groenendijk *et al.* 2002; Nacci *et al.* 2002; Wirgin and Waldman 2004; Nacci *et al.* 2010; Wirgin *et al.* 2011). However, despite strong theoretical expectation and some empirical evidence for evolutionary responses (i.e., genetic adaptation) in populations inhabiting polluted aquatic environments, the potential for such adaptation to confound biomonitoring efforts has not been systematically explored.

Physiological acclimation, genetic adaptation and phenotypic plasticity may alter the response of biomarkers and other traits used for biomonitoring of polluted aquatic ecosystems in unpredictable ways. Although important for field studies of native populations and communities, adaptive responses generally do not apply to tests for the impacts of pollution or contamination under lab conditions using naïve organisms. However, dispersal, acclimation and genetic adaptation should be taken into consideration for all studies designed to quantify contaminant exposure effects in the wild. The best approach would be a systematic series of validation experiments prior to interpreting biomarker or trait data in natural contaminated systems (Figure 2.1); however, the choice of biomarker or response trait is also critical for minimizing the potential to have adaptive responses confounding measures of contaminant effects.

RESPONSE MEASUREMENTS

Traditional approaches to measuring contaminant effects on organisms or ecosystems in nature use endpoints such as median lethal concentration or dose (LC_{50}, LD_{50}), dose or concentration giving specific response in 50% of the test animals (ED_{50} EC_{50}), lowest observable effect level or concentration (LOEL, LOEC), and no observable effect level or concentration (NOEL, NOEC). Such measurements work well for controlled experiments in the lab on naïve organisms and give good indication of toxicity, but when applied to natural systems, they suffer limitations because the dose in nature is not controlled, and often difficult to quantify. Furthermore, endpoints such as LC_{50} or even lethal concentration of five per cent (LC_5) of the animals are likely only seen in populations or ecosystems that are highly stressed. It is logistically difficult to determine the limit between NOEL and the LOEL in wild populations, and even harder when considering an entire ecosystem consequently they are rarely used as such. However, traditional endpoints are conceptually straightforward and have been well characterized in a large number of toxicological studies in a variety of organisms (Kroes *et al.* 2000; Stark and Banks 2003; Niyogi and Wood 2004) and provide integrated measures of cumulative impacts. Thus, given a known contamination load, traditional endpoints provide a relative quantification of substance toxicity, and are widely used in spite of their known and acknowledged limitations (Eason and O'Halloran 2002).

Another approach commonly used to quantify the biological effects of contamination in nature involves biomarker measurement. A biomarker is defined as a biological response to pollution at sub-organismal levels (such as at the cellular, tissue, physiological or biochemical levels) that provides an early measure of departure from

homeostasis (Newman and Unger 2003). The use of bioindicator species for biomonitoring in the field provides highly sensitive measures of the effects of contaminants in a natural habitat, but it also has limitations. Because individual organisms, populations, and species differ in tolerance, exposure history (which may affect adaptive response) and dispersal ability, biomarker/bioindicator response may vary unpredictably. However, the major advantage of using biomonitoring is that it can be used to evaluate cumulative effects of multiple pollutants (Kopecka-Pilarczyk and Correia 2009), while bioindicators are preferably well calibrated for the contaminant response (Gewurtz *et al.* 2002; Wannaz and Pignata 2006). Although biomarker and bioindicator approaches provide powerful measurements of toxicity, Eason and O'Halloran (2002) refer to them as "end of pipe" analyses, where the biological effect has progressed to the point of severe consequences (exceptions exists such as EROD, an early stage biomarker). As late stage biomarkers are often associated with mortality, they can be the basis for selective forces that ultimately drive adaptive responses, which in turn will act to mask the acute toxicity responses, as described above. Ideally then, we need biomarkers that provide measurement very early in the organismal response to contaminant stress (such as EROD), that is, they show predictable response to a wide range of pollutants at non-lethal concentrations across a breadth of taxa.

There have been a number of studies that have used genetic-based approaches to characterising the effect of pollutants on aquatic organisms and ecosystems. Studies using population genetics have mainly tested for mutation and genetic diversity differences between clean and contaminated sites (Belfiore and Anderson 2001; Theodorakis 2003; Johnston and Roberst 2009; Bickham 2011). Population genetic studies generally address

pollution-mediated changes in population characteristics, but they still focus on "end of the pipe" responses. Elevated mutation levels result from mutagenic effects of the pollutant, coupled with a breakdown of the repair processes, while a loss of genetic diversity is predicated on genetic drift associated with high levels of mortality resulting from the pollution stress.

Recent studies have explored gene expression response to pollutants in an effort to develop more immediate and fundamental sub-organismal biomarkers (Busch *et al.* 2004). A Scopus search for articles with the most common pollutants and the different methods to detect transcription resulted in 453 publications, with a steady increase in publication rate from the first publication in 1996 to the end of 2012 (Figure 2.2). From the first microarray study in 2002 the use of that approach has increased, and overall 14% of all the studies in the Scopus search results were in fact on microarrays. Though the main focus of the 453 articles was on the aryl hydrocarbon receptor (AhR) pathways, particularly the Cytochrome P450 (CYP1A 36% with 19% transcriptional studies; Figure 2.2). Despite the well characterised function of AhR and CYP1A in pollutant detoxification, and the attention they have received in the literature, there is growing evidence that the AhR gene expression pathway may have serious technical limitations. Several studies have reported a gender difference in both CYP1A mRNA concentrations and/or EROD (Elksus *et al.* 1992; Williams *et al.* 1998; Meyer *et al.* 2002; Nacci *et al.* 2002b; Brammell *et al.* 2010; Diniz *et al.* 2010; Huang *et al.* 2012). This likely reflects crosstalk (either positive or negative) between AhR and estrogen receptors, indeed

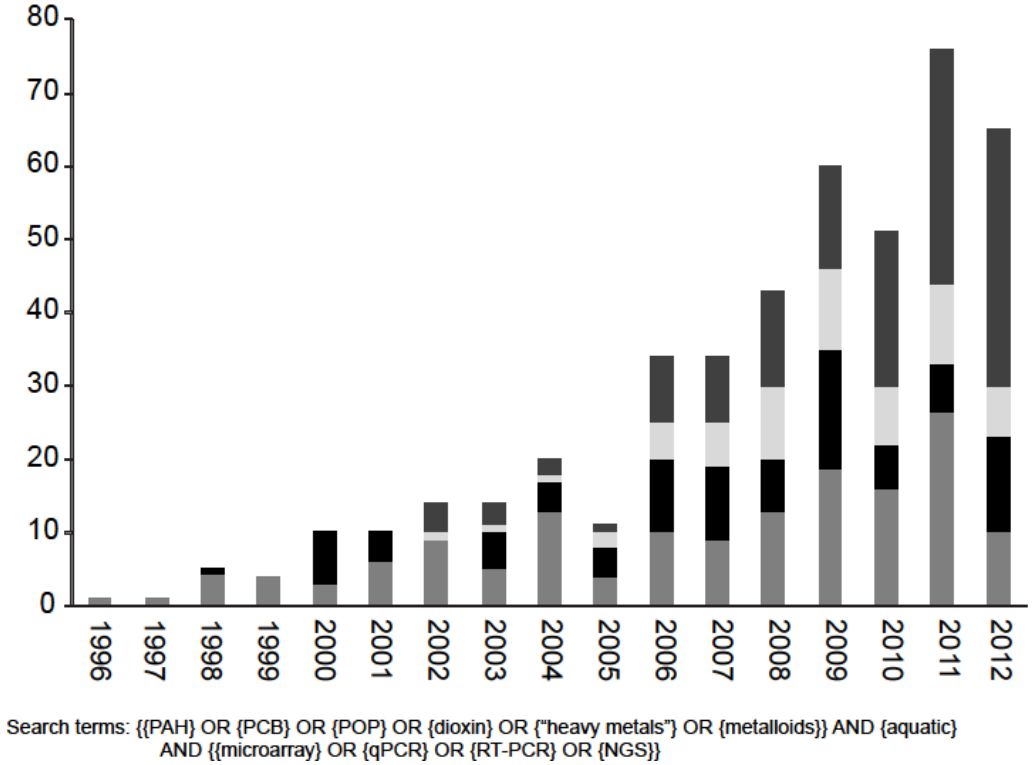

Figure 2.2 The frequency (number) of journal publications (total = 453) concerning CYP1A, MT and microarrays for transcriptional response to contaminants from the first one 1996 until the end of 2012 when doing a Scopus search. The search terms used were the most widespread contaminants and common ways of detecting transcription: {{PAH} OR {PCB} OR {POP} OR {dioxin} OR {"heavy metals"} OR {metalloids}} AND {aquatic} AND {{microarray} OR { qPCR} OR {RT-PCR} OR {NGS}}. Grey is the articles for CYP1A and black is MT, light grey are microarrays on response to pollution and dark grey are the remaining articles.

hepatic AhR function can be directly affected by female sex hormones (Navas and Segner 2001; Mortensen *et al.* 2006; Mortensen and Arukwe 2007; Gräns *et al.* 2010). Another problem with CYP1A is that fish have been shown to mount adaptive responses to chronic exposure, but will still respond to novel substances (Wirgin *et al.* 1992; Meyer *et al.* 2002; Nacci *et al.* 2010; Whitehead *et al.* 2011; Wirgin *et al.* 2011; Clark and Di Giulio 2012). The other well-studied area of toxicology that has made a systematic use of gene expression assays is response to heavy metals in aquatic systems (21% of 453 studies with 11.5 % being transcriptional studies). An increase in the expression of metallothionein (MT) decreases the sensitivity of the organism to metals by binding to, and thus limiting, toxic metal availability (Monserrat *et al.* 2007; Bell and Vallee 2009). Again, other factors unrelated to toxicological exposure play significant roles in affecting MT gene expression (e.g., osmoregulatory and oxidative stress will also affect the MT expression; Monserrat *et al.* 2007; Spearow *et al.* 2011). Recent gene expression/transcription research has focussed on CYP1A and MT (likely due to their well-characterised function). Such a focus is a logical starting point; however, the potential confounding effects of complex regulation pathway redundancy and specific adaptive responses on individual genes make the use of single (or few) gene analyses suspect. Instead, we suggest that a multi-gene approach would be preferable, and would reduce the likelihood of strong adaptive responses and single gene biases, allowing the best possible biomarker resolution.

Ecotoxicogenomics is a field that is growing rapidly, defined by Snape *et al.* (2004) as the incorporation of gene and protein expression (transcriptomics,

metabolomics and proteomics) into ecotoxicology. The shift towards ecotoxicogenomic approaches is driven mainly by the high sensitivity and early response of transcription and gene expression biomarkers coupled with the need for multi-gene assays. The choice of biomarker will affect the point in the response timeline one is exploring (Figure 2.3), and hence the likelihood of adaptive response confounding actual impacts. Once exposed to pollution (or any toxin), the organism will attempt to regain homeostasis through the generalised stress response, cellular protection, toxin transportation, and toxin metabolism; all of which require specific proteins/enzymes and hence changes in gene expression. Genes whose expression is mediated by exposure to a toxicant often exhibit rapid induction; for example, within a few hours to a day in response to PAHs and heavy metals (Courtenay *et al.* 1999; Nuwaysir *et al.* 1999; Bugiak and Weber 2009; Durieux *et al.* 2012; Liu *et al.* 2012) or a few days for polychlorinated biphenyls (PCBs; Courtenay *et al.* 1999; Nuwaysir *et al.* 1999; Doyen *et al.* 2012). Thus gene expression biomarkers could function as the "beginning of the pipe" indicators identified by Eason and O'Halloran (2002). An advantage of early biomarkers is that they are likely to be under less selection (i.e. they have less effect on reproductive success), and consequently adaptive responses are less likely to act upon it. Therefore if a change in transcription is observed after the initial stress, especially in naïve organisms, we know that the pollution is eliciting an effect and we can then look for other effects.

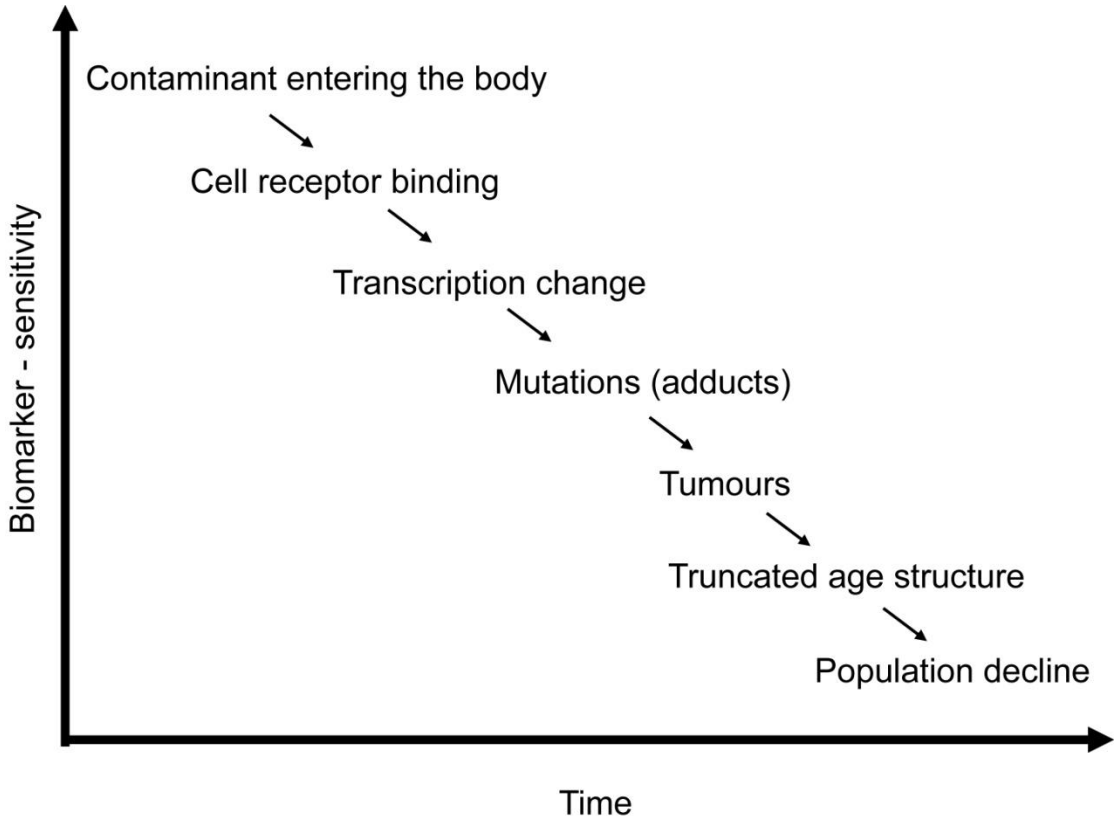

Figure 2.3. The graph illustrate that with increasing time after pollutant exposure there are diverse set of biomarkers with different sensitivity and ecological effect that can be measured. Early biomarkers can be detected at lower contaminant exposures and has less ecologic effect. Less sensitive biomarkers generally take longer before it can be reliably observed and quantified and the higher chance that it will have a significant effect on the individual, population or ecosystem.

Although a variety of molecular genetic techniques have been developed to allow the quantification of transcriptional responses to environmental stressors (e.g., Northern blots, quantitative real-time PCR (qRT-PCR), DNA microarrays), few of them provide broad, multi-gene transcription data. Genome-wide transcriptomic methods can be divided into open and closed technologies. Microarray analysis, which is a commonly used genomic approach, is a closed technology in that it does not allow the detection of novel or unexpected transcriptional responses, i.e. genes not spotted on the array (David *et al.* 2010). Microarrays also suffer from low sensitivity and non-specific binding (David *et al.* 2010), but are still good for studies with a large number of individuals, and have been used successfully for response to pollution analyses (reviewed in Bozinovic and Oleksiak 2011). Massively parallel-, or Next Generation-, sequencing (NGS; Brenner *et al.* 2000) can be applied to the transcriptome (RNA-seq), and is an open technology in that it can detect both known and unexpected or novel mRNA transcripts as well as smRNA, miRNA and alternative splicing. RNA-seq also generally has higher resolution than microarrays (Wilhelm and Landry 2009). Subtle changes in transcription as a result of genetic adaptation or acclimation can be detected with RNA-seq (Margurerat and Bähler 2010). Furthermore, the expression of alternative alleles or signalling pathways are also detectable using NGS transcriptomic approaches. In general, NGS provides excellent potential for use in non-model species ecotoxicogenomics (Mehinto *et al.* 2012) and the falling costs of NGS will make it suitable for environmental monitoring in the near future. The rapidly advancing transcriptomic technology is outpacing calibrated applications in ecotoxicogenomics, but given the potential for transcriptomics to address many of the shortcomings of existing biomarkers, we predict widespread applications

using microarray and NGS approaches to characterising bioindicator species response to contaminated aquatic ecosystems.

TRANSCRIPTIONAL ADAPTATION AND ACCLIMATION

Gene transcription is the first step in gene expression, and thus represents one of the earliest biomarkers possible (i.e., "beginning of the pipe"). There are mechanisms by which genetic adaptation and physiological acclimation can alter transcriptional response to pollution in the wild, likely driven by transcription regulation modifications and epigenetic effects on transcription. For example, the non-heritable lack of induction of CYP1A to PAH exposure in Elizabeth River killifish is carried over to the F1 generation but not further (Meyer *et al.* 2002). Wirgin and Waldman (2004) suggested that DNA methylation could explain such a pattern of apparent resistance "inheritance"; however, no difference in methylation of the cytochrome P450 1A (CYP1A) promoter region was found between the clean and polluted sites (Timme-Laragy *et al.* 2005). Thus the mechanism for the killifish resistance is still to be resolved, though some form of epigenetic mechanism seems likely. The potential for genetic adaptation and physiological acclimation to alter or mask transcriptional responses to environmental stress is intriguing and has been reported in CYP1A studies (Elskus *et al.* 1999; Wirgin *et al.* 1992; Meyer *et al.* 2002; Nacci *et al.* 2010; Whitehead *et al.* 2011; Wirgin *et al.* 2011; Clark and Di Giulio 2012), however, not seen on a broader genome-wide scale. However, if acclimation and genetic adaptation is acting, it is likely very gene specific, and we argue that a multi-gene ecotoxicogenomic approach should minimize the potential for single gene biases resulting from adaptive transcriptional responses. Such an approach would have the additional advantage of providing data necessary to identify the

individual genes showing adaptive effects. Such information would help our understanding of the mechanisms behind acclimation, adaptation and phenotypic plasticity in degraded habitats. Responding to a stressor such as a pollutant can be an important adaptive response; however it is important to recognise that some sub-organismal responses are not adaptive, but are simply part of the stress response syndrome.

Much of the observed phenotypic variation in natural populations is likely due to differences in the level of transcription and/or gene expression, rather than gene coding polymorphisms (Esteller 2008; Aykanat *et al.* 2011). Indeed, evolution by transcriptional modification has been proposed as a mechanism driving rapid local adaptation (Jeukens *et al.* 2008; St-Cyr *et al.* 2008; Aykanat *et al.* 2011), possibly through non-additive genetic and epigenetic mechanisms. Epigenetic effects may be particularly relevant to ecotoxicological studies using gene transcription as a biomarker. Environmental effects and short term exposure to chemicals can result in epigenetic effects (Reamon-Buettner *et al.* 2008; Kotubash *et al.* 2011) rather than the more classically expected DNA mutations, and unlike mutations, epigenetic changes are reversible (Esteller 2008; Reamon-Buettner *et al.* 2008). Although transcriptional modification provides a unified mechanistic explanation for both acclimation and genetic adaptation in response to environmental stressors, it is unique in that the basis for epigenetic effects in the regulation of transcription under pollution stress is well known. Finally, as epigenetic effect are semi-heritable (may be passed down over a few generations) it could contribute to the observed phenomenon of single generation inheritance of physiological acclimation to contaminant stress.

CONCLUSION

Acclimation and genetic adaptation can mask or skew the response of organisms exposed to pollution in controlled challenges or exposure in their native environment. This could lead to an underestimation of the level of risk posed to naïve organisms (such as ourselves) resulting from the pollutant stress present. Furthermore, the potential for dispersal to bias interpretation of *in situ* measures of pollution stress is always present for bioindicator species capable of dispersal. Clearly we must first rule out dispersal, then test for acclimation and genetic adaptation before we can interpret *in situ* pollutant response in any aquatic ecosystem (Figure 2.1). We also call for broader use of multiple early response biomarkers, such as transcription, that can provide early warning, prior to permanent deleterious effects in the organisms, population or ecosystem. We propose a logical and hierarchical approach to addressing pollution effects on aquatic animals in nature that addresses cryptic adaptive responses such as dispersal, physiological acclimation and genetic adaptation. Such an approach uses comparisons between organisms from exposed and naïve populations and breeding experiments with offspring observed into the F1 or even later generations (Figure 2.1). This approach will allow partitioning pollution response into that mediated by pollution stress, and that mediated by adaptive processes. Although our proposed approach may not identify the adaptive mechanism behind the unexpected transcriptional and/or gene expression response, it will address the possible role that physiological acclimation and genetic adaptation may (or may not) play in biasing biomonitoring efforts by regulatory agencies.

We probably only know a fraction of the effects and responses that take place at the cellular and organism levels in response to contaminant stress. Given the variety of pathways and mechanisms available to animals to physiologically acclimate or genetically adapt to pollution, a broad survey-style approach to biomarker measurement should be taken. For ecotoxicogenomic approaches, we suggest using NGS to characterise the transcriptome of exposed or challenged organisms as well as naïve organisms to identify differentially expressed genes – this information can be used independently or to guide microarray design or qRT-PCR applications. The use of a variety of gene transcription quantification technologies will provide sensitive transcriptional biomarkers of contaminant effects. Focussed approaches (such as skewed responses at known-function genes such as CYP1A; Wirgin *et al.* 1992; Meyer *et al.* 2002; Nacci *et al.* 2010; Whitehead *et al.* 2011; Wirgin *et al.* 2011; Clark and Di Giulio 2012) are perhaps better suited to secondary applications, after broad survey analyses have been completed.

Independent of the nature of the stimulus or stressor and past acclimation or genetic adaptation, there should always be a measureable change in the transcriptional profile in response to environmental stress, if enough genes are assayed. Therefore the use of transcription as a biomarker provides not only early detection of organismal, population or ecosystem effects, it will also be relatively robust to acclimation and genetic adaptation masking effects. However, we caution that despite using transcriptional biomarkers at multiple genes, adaptive responses must be taken into consideration, as they may affect gene transcription, and ultimately confound the outcome, interpretation and potentially the regulatory response.

Box 1. **Definitions of terminology used in this paper.**

Tolerance – tolerance is an acute response to a contaminant that allows the organism to survive short-term in the presence of the stressor.

Physiological acclimation – the physiological change in an individual in response to environmental change or stress to return towards homeostasis. The organism becomes more resistant due to earlier or prolonged exposure. It is reversible, costly and not always beneficial. This can be seen as a plastic response but then it has to be separated from phenotypic plasticity.

Phenotypic plasticity – an irreversible change of the phenotype (Wilson and Franklin 2002). Can be an irreversible behavioural (Wood and Harrison 2002), morphological, or physiological change that increases fitness in a different environment.

Gene x Environment (GxE) interaction – Identical genotypes exhibit different phenotypes in different environments. GxE is not reversible nor is it adaptive (Figure I; Fry 1992).

Developmental phenotypic plasticity – the traditional view of phenotypic plasticity (Bradshaw 1965). Individuals with identical genotypes will display different phenotypes when experiencing different environments during development (Figure II). The parallel reaction norms define the plasticity.

Genetic adaptation – a population-level response involving a change in the allele frequencies over time in response to natural selection. It is not reversible and is inherited across generations

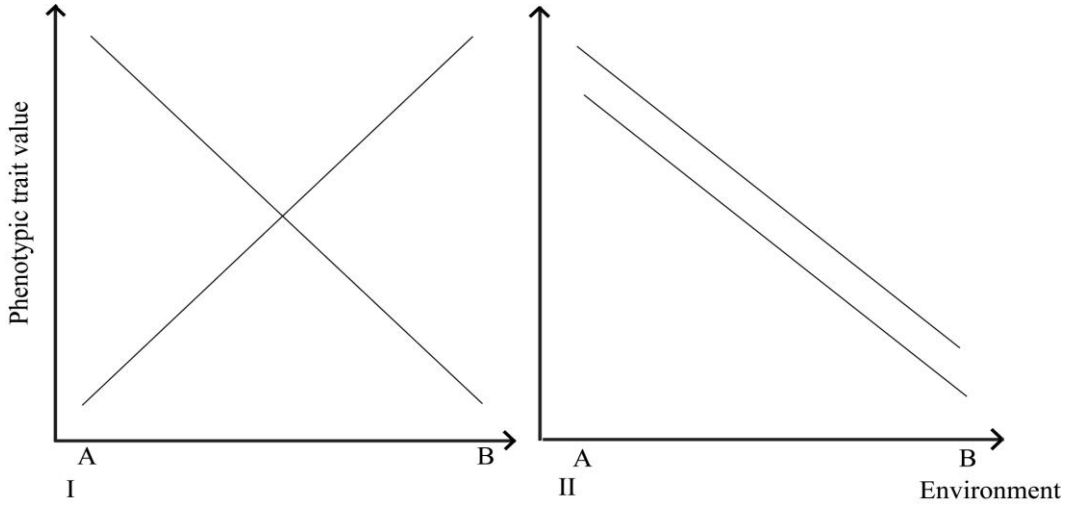

Figure I: Schematic reaction norm diagram showing gene x environment (GxE) interaction. Each line represents a unique genotype in two environments (A and B), while the y-axis is phenotypic trait value. Note that there is no difference in mean phenotype in the two environments (indicating no phenotypic plasticity) but high levels of GxE since the phenotype rank differs in the two environments.

Figure II: Schematic reaction norm diagram showing phenotypic plasticity. Each line represents a unique genotype in two environments (A and B), while the y-axis is phenotypic trait value. Note that there is a net difference in mean phenotype between the two environments indicating phenotypic plasticity, but there is no evidence for GxE effects.

REFERENCES

Aldarondo-Torres, J. X., F. Samara, I. Mansilla-Rivera, D. S. Aga, and C. J. Rodríguez-Sierra. 2010. Trace metals, PAHs, and PCBs in sediments from the Jobos Bay area in Puerto Rico. Marine Pollution Bulletin 60:1350-1358.

Aykanat, T., F. P. Thrower, and D. D Heath. 2011. Rapid evolution of osmoregulatory function by modification of gene transcription in steelhead trout. Genetica 139:233-242.

Belfiore, N. M., and S. L. Anderson. 2001. Effects of contaminants on genetic patterns in aquatic organisms: a review. Mutation Research 489:97-122.

Bell, S. G., and B. L. Vallee. 2009. The metallothionein / thionine system: an oxidoreductive metabolic zinc link. ChemBioChem 10:55-62.

Bickham, J. W., S. S. Sandhu, P. D. N. Hebert, L. Chikhi, and R. Anthwal. 2000. Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. Mutation Research 463:33-51.

Bickham, J. W. 2011. The four cornerstones in evolutionary toxicology. Ecotoxicology 20:497-502.

Block, B. A., I. D. Jonsen, S. J. Jorgensen, A. J.Winship, S. A. Shaffer, S. J. Bograd, E. L. Hazen, D. G. Foley, G. A. Breed, A.-L. Harrison, J. E. Ganong, A. Swithenbank, M. Castleton, H. Dewar, B. R. Mate, G. L. Shillinger, K. M. Schaefer, S. R. Benson, M. J. Weise, R. W. Henry, and D. P. Costa. 2011. Tracking apex marine predator movements in a dynamic ocean. Nature 479:86-90.

Bozinovic, G., and M. F. Oleksiak. 2011. Genomic approaches with natural fish populations from polluted environments. Environmental Toxicology and Chemistry 30:283-289.

Bradbury, I. R., S. E. Campana, and P. Bentzen. 2008. Estimating contemporary early life-history dispersal in an estuarine fish: integrating molecular and otolith elemental approaches. Molecular Ecology 17, 1438-1450.

Bradshaw, A. D. 1965. Evolutionary significance of phenotypic plasticity in plants. Advances in Genetics 13:115-155.

Brammell, B. F., D. J. Price, W. J. Birge, E. M. Harmel-Laws, J. A Hitron, and A. A, Elskus. 2010. Differential sensitivity of CYP1A to 3,3′,4′,4-tetrachlorobiphenyl and

benzo(a)pyrene in two Lepomis species. Comparative Biochemistry and Physiology, Part C 152:42-50.

Brenner, B., M. Johnson, J. Bridgham, G. Golda, D. H Lloyd, D. Johnson, S. Luo, S. McCurdy, M. Foy, M. Ewan, R. Roth, D. George, S. Eletr, G Albrecht, E. Vermaas, S. R. Williams, K. Moon, T. Burcham, M. Pallas, R. B. DuBridge, J. Kirchner, K. Fearon, J. Mao, and K. Corcoran. 2000. Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. Nature Biotechnology 18:630-634.

Bugiak, B., and L. P Weber. 2009. Hepatic and vascular mRNA expression in adult zebrafish (*Danio rerio*) following exposure to benzo-a-pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Aquatic Toxicology 95:299-306.

Busch, C. R., D. D. Heath, and A. Hubberstey. 2004. Sensitive genetic biomarkers for determining apoptosis in the brown bullhead (*Ameiurus nebulosus*) Gene 329:1-10.

Chopra, A. K., M. K. Sharma, and S. Chamoli. 2011. Bioaccumulation of organochlorine pesticides in aquatic system – an overview. Environmental Monitoring and Assessment 173:905-916.

Clark, B. W., and R. T. Di Giulio. 2012. *Fundulus heteroclitus* adapted to PAHs are cross-resistant to multiple insecticides. Ecotoxicology 21:465-474.

Costello, M. J., and P. Read. 1994. Toxicity of sewage sludge to marine organisms: a review. Marine Environmental Research 37:23-46.

Courtenay, S. C., C. M. Grunwald, G. Kreamer, W. L. Fairchild, J. T. Arsenault, M. Ikonomou, and I. I. Wirgin. 1999. A comparison of the dose and time response of CYP1A1 mRNA induction in chemically treated Atlantic tomcod from two populations. Aquatic Toxicology 47:43-69.

Cowen, R. K., and S. Sponaugle. 2008. Larva dispersal and marine population connectivity. Annual Review of Marine Science 1:443-466

David, J. P., E. Coissac, C. Melodelima, R. Poupardin, M. A Riaz, A. Chandor-Proust and S. Reynaud. 2010. Transcriptome response to pollutants and insecticides in the dengue vector *Aedes aegypti* using next-generation sequencing technology. BMC Genomics 11:216.

De Domenico, E., A. Mauceri, D. Giordano, M. Maisano, G. Gioffrè, A. Natalotto, A. D'Agata, M. Ferrante, M. V. Brundo, and S. Fasulo. 2011. Effects of "in vivo" exposure to toxic sediments on juveniles of sea bass (*Dicentrarchus labrax*). Aquatic Toxicology 105:688-697.

Demarchi, M., M Chiappero, J. Laudien, and R Sahade. 2008. Population genetic structure of the ascidian *Styela rustica* at Kongsfjorden, Svalbard, Arctic. Journal of Experimental Marine Biology and Ecology 364:29-34.

DeValls, T. A, J. M. Forja, and A. Gómez-Parra. 2002. Seasonality of contamination, toxicity, and quality values in sediments from littoral ecosystems in the Gulf of Cádiz (SW Spain). Chemosphere 46:1033-1043.

Diniz, M. S., I. Peres, L. Castro, A. C. Freitas, T. A. P. Rocha-Santos, R. Pereira, and A. C. Duarte. 2010. Impact of a secondary treated bleached kraft pulp mill effluent in both sexes of goldfish (*Carassius auratus* L.). Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering 45:1858-1865.

Doyen, P., F. Rodius, L. L'Hoste, L. Hoffmann, and H. Cauchie. 2012. Identification and mRNA expression of pi-class glutathione S-transferase and selenium-dependent glutathione peroxidase in the gudgeon *Gobio gobio* exposed to PCB 77. Comparative Biochemistry and Physiology, Part C 155:300-306.

Durieux, E. D. H, R. E. Connon, I. Werner, L. S. D'Abronzo, P. S. Fitzgerald, J. L. Spearow, and D. J. Ostrach. 2012. Cytochrome P4501A mRNA and protein induction in striped bass (*Morone saxatilis*). Fish Physiology and Biochemistry 38:1107-1116.

Eason, C., and K. O'Halloran. 2002. Biomarkers in toxicology versus ecological risk assessment. Toxicology 181-182:517-521.

Elksus, A. A., R. J. Pruell, J. J. Stegeman. 1992. Endogenously-mediated, pretranslational suppression of cytochrome P450IA expression in PCB-contaminated flounder. Marine Environment Research 34:97-101.

Elksus, A. A., E. Monosson, A. E. McElroy, J. j. Stegeman, D. S. Woltering. 1999. Altered CYP1A expression in *Fundulus heteroclitus* adults and larvae: a sign of pollutant resistance? Aquatic Toxicology 45:99-113.

Esteller, M. 2008. Epigenetics in cancer. The New England Journal of Medicine 358:1148-1159.

Evans, K. M., V. A. Chepurnov, H. J. Sluiman, S. J. Thomas, B. M. Spears, and D. G. Mann. 2009. Highly differentiated populations of the freshwater diatom *Sellaphora capitata* suggest limited dispersal and opportunities for allopatric speciation. Protist 160:386-396.

Farwell M., K. G. Drouillard, D. D. Heath, T. E Pitcher. 2012. Acclimation of life-history traits to experimental changes in environmental contaminant concentration in brown bullhead (*Ameiurs nebulous*) Environmental Toxicology and Chemistry 31:863-869.

Friesen, V. L., M. Burg, and K. D. McCoy. 2007. Mechanisms of population differentiation in seabirds. Molecular Ecology 16: 1765-1785.

Fry, J. D. 1992. The mixed-model analysis of variance applied to quantitative genetics: biological meaning of the parameters. Evolution 46:540-550.

Gewurtz, S. B., K. G. Drouillard, R. Lazar, and G. D. Haffner. 2002. Quantitative biomonitoring of PAHs using the barnes mussel (*Elliptio complanata*). Archives of Environmental Contamination and Toxicology 43:497-504.

Gräns, J., B. Wassmur, and M. C. Celander. 2010. One-way inhibiting cross-talk between aryl hydrocarbon receptor (AhR) and estrogen receptor (ER) signalling in primary cultures of rainbow trout hepatocytes. Aquatic Toxicology 100:263–270.

Grant, P.R., and B. R. Grant. 2002. Unpredictable evolution in 30-year study of Darwin's finches. Science 296:707-711.

Groenendijk, D., B. van Opzeeland, L. M. Dionisio Pires, and J. F. Postma. 1999. Fluctuating life-history parameters indicating temporal variability in metal adaptation in riverine chironomids. Archives of Environmental Contamination and Toxicology 37:175-181

Groenendijk, D., S. M. G Lücker, M. Plans, M. H. S Kraak, and W. Admiraal. 2002. Dynamics of metal adaptation in riverine chironomids. Environmental Pollution 117:101-109.

Hendry, A. P., and M. T. Kinnison. 1999. Perspective: The pace of modern life: measuring rates of contemporary microevolution. Evolution 53:1637-1653

Hendry, A. P., J. K. Wenburg, P Bentzen, E. C. Volk, and T. P. Quinn. 2000. Rapid evolution of reproductive isolation in the wild: evidence from introduced salmon. Science 290:516-518

Hoffman, A. A. 1995. Acclimation: increased survival at a cost. Trends in Ecology and Evolution 10:1-2

Huang, G. Y. G. G. Ying, S. Liu, and Y. X. Fang. 2012. Regulation of reproduction- and biomarker-related gene expression by sex steroids in the livers and ovaries of adult female western mosquitofish (*Gambusia affinis*) Comparative Biochemistry and Physiology, Part A 162:36-43

Humston, R., B. M. Priest, W. C. Hamilton, and P. E. Bugas Jr. 2010. Dispersal between tributary and main-stem rivers by juvenile smallmouth bass evaluated using otolith microchemistry. Transactions of the American Fisheries Society 139:171-184

Jeukens, J., D. Bittner, R. Knudsen, and L. Bernatchez. 2009. Candidate genes and adaptive radiation: insights from transcriptional adaptation to the limnetic niche among coregonine fishes (*Coregonus* spp., Salmonidae). Molecular Biology and Evolution 26:155-166

Johnson, L. L., T. K. Collier, and J. E. Stein. 2002. An analysis in support of sediment quality thresholds for polycyclic aromatic hydrocarbons (PAHs) to protect estuarine fish. Aquatic conservation: Marine and Freshwater Ecosystems 10:517-538

Johnston, E. L., and D. A. Roberst. 2009. Contaminants reduce the richness and evenness of marine communities: A review and meta-analysis. Environmental Pollution 157 (2009) 1745-1752.

Ketata, I., W Smaoui-Damak, F. Guermazi, T. Rebai, and A. Hamza-Chaffai. 2007. *In situ* endocrine disrupting effects of cadmium on the reproduction of *Ruditapes decussates*. Comparative Biochemistry and Physiology, Part C 146: 415-430

Klerks, P. L., P. L. Leberg,. R. F. Lance, D. J. McMillin, and J. C. Mean. 1997. Lack of development of pollutant-resistance or genetic differentiation in darter gobies *(Gobionellus boleosoma)* inhabiting a produced-water discharge site. Marine Environmental Research 44:317-395

Kopecka-Pilarczyk, J., and A. D Correia. 2009. Biochemical response in gilthead seabream (*Sparus aurata*) to *in vivo* exposure to a mix of selected PAHs. Ecotoxicology and Environmental Safety 72: 1296-1302.

Kotubash, I., A. Scherhag, J. Sorrentino, K. Sexton, W. Bodnar, J. A. Swenberg, F. A. Beland, F. Pardo-Manuel de Villena, I. Rusyn, and I. P. Pogribny. 2011. Epigenetic mechanisms of mouse interstrain variability in genotoxicity of the environmental toxicant 1,3-Butadiene. Toxicological Science 122:448–456

Kroes, R., C. Galli, I. Munro, B. Schilter, L. A. Tran, R. Walker, and G. Würtzen. 2000. Threshold of toxicological concern for chemical substances present in the diet: A practical tool for assessing the need for toxicity testing. Food and Chemical Toxicology 38: 255-312

Law, K. S., and A. Stohl. 2007. Arctic air pollution: origins and impacts. Science 315, 1537-1540

Leroi, A. M., A. F. Bennett, and R. E. Lenski. 1994. Temperature acclimation and competitive fitness: An experimental test of the beneficial acclimation assumption. Proceedings of the National Academy of Science of the United States of America 91:1917-1921

Li, S., Y. Gao, J. Luo, Y. Cao, Y. Gao, L. Yang, X. Zhang, L. Yan, and F Du. 2011. The thermoluminescence of carp otoliths: A fingerprint in identification of lake pollution. African Journal of Biotechnology 10: 18440-18449.

Liu, Y., S. Yu, Y. Chai, and Q. Zhu. 2012. Transferrin gene expression in response to LPS challenge and heavy metal exposure in roughskin sculpin (*Trachidermus fasciatus*). Fish and Shellfish Immunology 32:223-229

López-Maury, L., S. Marguerat, and J. Bähler. 2008. Tuning gene expression to changing environments: from rapid responses to evolutionary adaptation. Nature Reviews Genetics 9:583-593.

Luttikhuizen, P. C., J. Drent, W. Van Delden, and T Piersma. 2003. Spatially structured genetic variation in a broadcast spawning bivalve: quantitative vs. molecular traits. Journal of Evolutionary Biology 16:260-272.

Luttikhuizen, P. C., J. Campos, J. van Bleijswijk, K. T. C. A Peijnenburg, and H. W. van der Veer. 2008. Phylogeography of the common shrimp, Crangon crangon (L.) across its distribution range. Molecular Phylogenetics and Evolution 46: 1015-1030.

Manel, S., M. K Schwartz, G. Luikart, and P Taberlet. 2003. Landscape genetics: combining landscape ecology and population genetics. TRENDS in Ecology and Evolution 18:189-197.

Marguerat, S. and J. Bähler. 2010. RNA-seq: from technology to biology. Cellular and Molecular Life Science 67:569-579

Mehinto, A. C., C. J. Martyniuk, D. J. Spade, N. D. Denslow. 2012. Applications of next-generation sequencing in fish ecotoxicogenomics. Frontiers in Genetics 3:62.

Meyer, J. N., D. E. Nacci, R.T. Di Giulio. 2002. Cytochrome P4501A (CYP1A) in killifish (*Fundulus heteroclitus*): heritability of altered expression and relationship to survival in contaminated sediments. Toxicological Science 68:69-81

Millard M. J., D. R. Smith, E. Obert, J. Grazio, M. L. Bartron, C. Wellington, S. Grisè, S. Rafferty, R. Wellington, and S. Julian. 2009. Movements of brown bullheads in Presque Isle Bay, Lake Erie, Pennsylvania. Journal of Great Lakes Research 35:613-619

Monserrat, J. M., P. E. Martínez, L. A. Geracitano, L. L. Amado, C. M. H. Martins, G. L. L Pinho, I.S. Chaves, M. Ferreira-Cravo, J. Ventura-Lima, and A. Bianchini. 2007. Pollution biomarkers in estuarine animals: Critical review and new perspectives. Comparative Biochemistry and Physiology, Part C 146:221-234

Mortensen A. S., and A. Arukwe. 2007. Interactions between estrogen- and Ah-receptor signalling pathways in primary culture of salmon hepatocytes exposed to nonylphenol and 3,3',4,4'-tetrachlorobiphenyl (congener 77). Comparative Hepatology 6:2

Mortensen A. S., C. C Tolfsen, and A. Arukwe. 2006. Gene expression patterns in estrogen (nonylphenol) and aryl hydrocarbon receptor agonists (PCB-77) interaction using rainbow trout (*Oncorhynchus mykiss*) primary hepatocyte culture. Journal of Toxicology and Environmental Health, Part A, 69:1-19

Nacci, D. E., D. Champlin, L. Coiro, R. McKinney, and S. Jayaraman. 2002. Predicting the occurrence of genetic adaptation to dioxin like compounds in populations of the estuarine fish *Fundulus heteroclitus*. Environmental Toxicology and Chemistry 21:1525-1532

Nacci[b], D. E., M. Kohan, M. Pelletier, and E. George. 2002. Effects of benzo[a]pyrene on a fish population resistant to the toxic effects of dioxin-like compounds. Aquatic Toxicology 57:203-215

Nacci, D. E., D. Champlin, and S. Jayaraman. 2010. Adaptation of the estuarine fish *Fundulus heteroclitus* (Atlantic Killifish) to polychlorinated biphenyls (PCBs). Estuaries and Coasts 33: 853-864

Navas, J. M., and H. Segner. 2001. Estrogen-mediated suppression of cytochrome P4501A (CYP1A) expression in rainbow trout hepatocytes: role of estrogen receptor. Chemico-Biological Interactions 138:285–298

Newman, M. C., and M. A. Unger. 2003. Fundamentals of ecotoxicology 2ed Lewis Publishers of CRC Press LLC. CRC press LLC, 2000 N.W Corporated Blvd., Boca Raton, Florida 33431. USA. ISBN 1-56670-598-3

Niyogi, S., and C. M. Wood. 2004. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. Environmental Science and Technology 38:6177-6192.

Nuwaysir, E. F., M. Bittner, J. Trent, J. C. Barrett, and C.A. Afshari. 1999. Microarrays and toxicology: the advent of toxicogenomics. Molecular Carcinogenesis 24:153-159

Percy, K. E., and M. Ferretty. 2004. Air pollution and forest health: toward new monitoring concepts. Environmental Pollution 130:113-126

Pogson, G. H., C. T. Taggart, K. A. Mesa, and R. G. Boutilier. 2001. Isolation by distance in the Atlantic cod, *Gadus morhua*, at large and small geographic scale. Evolution 55:131-146.

Ramos, R., J. González-Solís, M. G. Forero, R. Moreno., E Gómez-Díaz, X Ruiz and K. A. Hobson. 2009. The influence of breeding colony and sex on mercury, selenium and lead levels and carbon and nitrogen stable isotope signatures in summer and winter feathers of *Calonectris* shearwaters. Oecologia 159:345-354

Ranaldi, M. M., and M. M. Gangnon. 2010. Trace metal incorporation in otoliths of pink snapper (*Pagrus auratus*) as an environmental monitor. Comparative Biochemistry and Physiology, Part C 152:248-255.

Reamon-Buettner, S. M., V. Mutschler, and J. Borlak. 2008. The next innovation cycle in toxicogenomics: Environmental epigenetics. Mutation Research 659:158-165

Reznick, D. N., F. H. Shaw, F. H. Rodd, and R. G. Shaw. 1997. Evaluation of the rate of evolution in natural populations of guppies (*Poecilia reticulata*). Science 275, 1934-1937

Rico, C., and G.F Turner. 2002. Extreme microallopatric divergence in a cichlid species from Lake Malawi. Molecular Ecology 11:1585-1590

Ruiz, Y., P. Suarez, A. Alonso, E. Longo, A. Villaverde and F. S. Juan. 2011. Environmental quality of mussel farms in the Vigo Estuary: Pollution by PAHs, origin and effects on reproduction. Environmental Pollution 159:250-265

Sabban, E. L., and R. Kvetňanský. 2001. Stress-triggered activation of gene expression in catecholaminergic systems: dynamics of transcriptional events. TRENDS in Neurosciences 24:91-98

Salas, E., H. Molina-Ureña, R. P. Walter, and D. D. Heath. 2010. Local and regional genetic connectivity in a Caribbean coral reef fish. Marine Biology 157:437-445

Samadi, S., L. Bottan, E. Macpherson, B. R. De Foges, and M.C Boisselier. 2006. Seamount endemism questioned by the geographic distribution and population genetic structure of marine invertebrates. Marine Biology 149:1463-1475.

Selkoe, K. A., C. M. Henzler, and S. D. Gaines. 2008. Seascape genetics and the spatial ecology of marine populations. Fish and Fisheries 9:363-377

Snape, J. R., S. J. Maund, D. B. Pickford, T. H. Hutchinson. 2004. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. Aquatic Toxicology 67:143-154

Söderberg, L. I, R. P. Walter, M. J. Ouellette, and D. D. Heath. 2013. Dispersal and genetic structure among brown bullhead populations: The role of distance and degraded habitats. Journal of Canadian Fisheries and Aquatic Science (in review)

Spearow, J. L., R. S. Kota, and D. J Ostrach. 2011. Environmental contaminant effect on juvenile striped bass in the San Francisco estuary, California, USA. Environmental Toxicology and Chemistry 30:393-402

Stark, J. D., and J. E. Banks. 2003. Population-level effects of pesticides and other toxicants on arthropods. Annual Review of Entomology 48:505-519.

St-Cyr, J., N. Derome, and L Bernatchez. 2008. The transcriptomics of life-history trade-offs in whitefish species pairs (*Coregonus* sp.). Molecular Ecology 17:1850-1870

Takada, H., J.W. Farringtion, M.H. Bothner, C.G., Johnson, and B.W. Tripp. 1994. Transport of sludge-derived organic pollutants to deep-sea sediments at deep water dump site 106. Environmental Science and Technology 28:1062-1072

Theodorakis, C. W. 2001. Integration of genotoxic and population genetic endpoints in biomonitoring and risk assessment. Ecotoxicology, 10:245-256

Theodorakis, C. W. 2003. Establishing causality between population genetic alterations and environmental contamination in aquatic organisms. Human and Ecological Risk Assessment 9:37-58

Timme-Laragy, A. R., J. N. Meyer, R. A Waterland, and R. T. Di Giulio. 2005. Analysis of CpG methylation in the killifish CYP1A promoter. Comparative Biochemistry and Physiology, Part C 141:406-411

Wannaz, E. D., and M. L. Pignata. 2006. Calibration of four of *Tillandsia* as pollution biomonitors. Journal of Atmospheric Chemistry 53:185-209

White, T. A., S. Stefanni, J. Stamford, and A. R. Hoelzel. 2009. Unexpected panmixia in a long-lived, deep-sea fish with well-defined spawning habitat and relatively low fecundity. Molecular Ecology 18: 2563-2573.

Whitehead, A., F. Galvez, S. Zhang, L. M. Williams, and M. F. Oleksiak.2011. Functional genomics of physiological plasticity and local adaptation in killifish. Journal of Heredity 102:499-511

Wilheim, B. T., and J-R. Landry. 2009. RNA-Seq – quantitative measurement of expression through massively parallel RNA-sequencing. Methods 48:249-257.

Williams, P. J., S. C Coutenay, and C. E. Wilson. 1998. Annual sex steroid and effect of gender and season on cytochrome P450 mRNA induction in Atlantic Tomcod (*Microgadus tomcod*). Environmental Toxicology and Chemistry 17:1582-1588

Wilson, R. S., and C. E. Franklin. 2002. Testing the beneficial acclimation hypothesis. Trends in Ecology & Evolution 17:66-70

Wirgin, I., G. L. Kreamer, C. Grunwald, K. Squibb, S. J. Garte, and S Courtenay. 1992. Effects of prior exposure history on cytochrome P4501A mRNA induction by PCB congener 77 in Atlantic tomcod. Marine Environmental Research 34:103-108.

Wirgin, I., and J. R.Waldman. 2004. Resistance to contaminants in North American fish populations. Mutation Research 552:73-100

Wirgin, I., N. K. Roy, M. Loftus, R. C. Chambers, D. G. Franks, and M. E Hahn. 2011.Mechanistic basis of resistance to PCBs in Atlantic tomcod from the Hudson River. Science: 331-1322-1325

Wölz, J., D. Borck, G. Witt, and H. Hollert. 2009. Ecotoxicological characterization of sediment cores from the western Baltic Sea (Mecklenburg Bight) using GC–MS and in vitro biotests. Journal of Soils Sediments 9:400-410

Wong, B. B. M., J. S. Keogh, and D. J. M. Glashan. 2004. Current and historical patterns of drainage connectivity in eastern Australia inferred from population genetic structuring in a widespread freshwater fish *Pseudomugil signifier* (Pseudomugilidae). Molecular Ecology 13:391-401

Wood, H. A., and J. F. Harrison. 2002. Interpreting rejections of the beneficial acclimation hypothesis: when is physiological plasticity adaptive? Evolution 56:1863-1866

Zakas, C., and J. P. Wares. 2012. Consequences of a poecilogonous life history for genetic structure in coastal populations of the polychaete *Streblospio benedicti*. Molecular Ecology 21: 5447-5460.

CHAPTER 3

DISPERSAL AND GENETIC STRUCTURE AMONG BROWN BULLHEAD POPULATIONS: THE ROLE OF DISTANCE AND DEGRADED HABITATS*

INTRODUCTION

Organisms faced with a degrading environment have two options to survive: 1) they can physically relocate to better environmental conditions; or 2) they remain *in situ* and physiologically acclimate and/or genetically adapt to the stressful conditions. Assessing the level of population isolation is important for evaluating whether stress tolerance has evolved *in situ* or has been acquired through gene flow. While isolation is generally challenging to directly demonstrate (Nosil *et al.* 2005), patterns of dispersal and gene flow can be assessed indirectly using molecular genetic data. Quantifying patterns of dispersal and gene flow among populations experiencing anthropogenic habitat degradation is critical not only for effective conservation, but for the prediction of short- and long-term impacts. Dispersal and gene flow may be constrained by physical barriers (Steeve *et al.* 2005), long distances (Milá *et al.* 2009; Taylor *et al.* 2001), unsuitable habitat (i.e., habitat fragmentation, Rico and Turner 2002) and behavioural isolation (i.e., sexual selection, Seehausen and van Alphen 1998). When challenged by a degraded

*L. I. Söderberg, R. P. Walter, M. J. Ouellette, D. D. Heath

environment, emigration would be an obvious response, and even philopatric species may disperse to avoid toxic levels of pollution. This response would result in elevated dispersal rates out of polluted areas; curiously, dispersal in response to degraded environments is rarely reported and the studies considering it showed the opposite from what we would expect (Bickham 2011).

Aquatic sediments act as a sink for organic pollutants (Johnston and Roberts 2009; Dachs and Méjanelle 2010), resulting in high levels of impact on benthic organisms. Brown bullhead *(Ameiurus nebulosus*) is a benthic fish native to fresh waters in eastern and central North America, and they are generally tolerant of contaminated environments (Scott and Crossman 1979; Schofield and Driscoll 1987). This tolerance to high contaminant loads has resulted in the use of brown bullhead being as a bioindicator of habitat degradation by pollution – especially sediment contaminants (Baumann *et al.* 1996; Leadley *et al.* 1998; Pyron *et al.* 2001). Brown bullhead are believed to be philopatric based on observations of breeding system behaviour (Blumer 1985); however, no systematic analysis of gene flow has yet been performed on this species. Telemetry studies show that brown bullhead generally remain within 500 - 800 meters of their release site, but occasionally they were found up to six kilometres away, over the span of 2.5 months to a year (Dedual 2002; Sakaris *et al.* 2005; Millard *et al.* 2009). Studies using indirect genetic methods also support philopatry with evidence for genetic structure and limited gene flow: Murdoch and Herbert (1994) showed high levels of genetic structure among bullhead from sites in Lake Erie and Lake Ontario using mitochondrial DNA restriction fragment length polymorphisms (RFLPs). Using RAPD polymorphisms, Silbiger *et al.* (2001) also showed restricted gene flow among four brown bullhead

populations from two clean and two contaminated river sites, 35 km to 190 km apart on the southern shores of Lake Erie. Interestingly, both studies reported reduced genetic diversity in populations inhabiting heavily contaminated sites, and the authors speculated that it may have been due to population bottlenecks resulting from mortality resulting from selection against sensitive individuals. Another study, at a smaller spatial scale (e.g., lake embayment), used microsatellite markers and showed no detectable bullhead population structure within Presque Isle Bay, Lake Erie (Millard *et al.* 2009). Though there are published records of brown bullhead population genetic structure, a systematic survey of dispersal and genetic structure at small and large geographic scales coupled with a focussed analysis of the effects of contaminant loads is needed. Such an analysis would quantify the relative roles of short and long range dispersal versus anthropogenic impacts on relocation, and hence on acclimation and adaptation potential in the brown bullhead.

In this paper, we used 11 polymorphic microsatellite loci to assess population genetic structure, dispersal and genetic diversity in brown bullhead at multiple spatial scales: 1) small (5 - 60 km); 2) intermediate (5 - 450 km); and 3) large (5 - 900 km). We also explicitly test the hypothesis that elevated chemical pollutants will correlate with lower genetic diversity (due to population bottlenecks) and higher dispersal rates (avoidance response). Our study provides the first comprehensive genetic analysis of a benthic fish species in contaminated and relatively pristine habitats in the Laurentian Great Lakes.

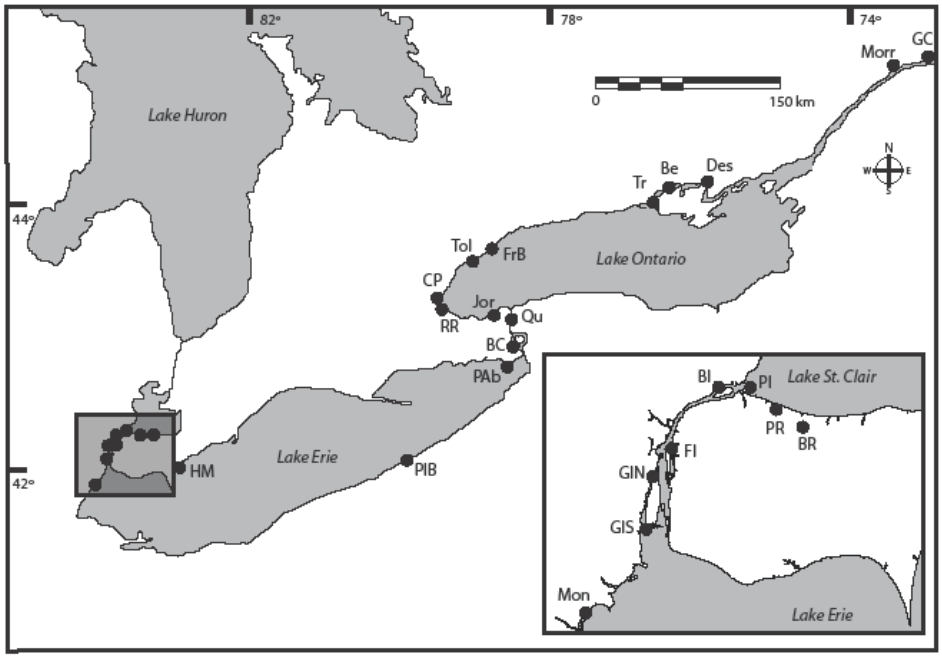

Figure 3.1. Brown bullhead sampling locations in the Great Lakes (N = 23), insertion shows an enlargement of the shaded box (Detroit River region). Study site abbreviations are given in Table 1.

MATERIAL AND METHODS

Sampling

Brown bullhead were sampled from 2003 – 2008 by electro-shocking at 23 sites within the Great Lakes drainage basin, and the St. Lawrence River (Figure 3.1). Fish from each site were collected in an area less than 0.5 km^2. Two sites, Deserento and Trenton, were each sampled in two consecutive years, allowing temporal genetic comparisons. Fin clips were collected directly from fish at 13 sites, and from whole frozen fish sampled by Environmental Canada, and stored in high-salt preservative or 95% ethanol.

Microsatellite genotyping

Genomic DNA was extracted from tissue samples using the Elphinstone *et al.* (2003) plate-based extraction method. Individual samples were PCR amplified at 11 microsatellite loci: Amn-3, Amn-16, Amn-34, Amn-42, Amn-44, Amn-43, An-12, Ip-365, Ip-372 and Ip-607 following Söderberg *et al.* (2010), and Ane-359 (Millard *et al.* 2009) using the following protocol: ~50ng DNA, 2.5mM $MgCl_2$, 0.25 μM of each primer, 200 μM dNTP, 1x reaction buffer [75 mM Tris-HCl, 20mM $(NH_4)2SO_4$], 0.5 units of Taq polymerase (Applied Biosystems) and water to a final volume of 12.5 μL. PCR conditions for Ane-359 were an initial denaturation at 94°C for 2 min, followed by 32 cycles of 94°C denaturation for 15 s, 56°C annealing for 15 s, 72°C extension for 30s, and a final extension at 72°C for 1 min, while PCR conditions for the remaining loci are described in Söderberg *et al.* (2010). PCR fragments were separated and visualised on a Li-Cor 4300 DNA Analyser (Biosciences, New Life Science products Inc. for Li-Cor Inc.) and allele sizes were scored using GENEIMAGIR 4.05 software (Scanalytics).

Population genetic characterisation

The average number of alleles, allele richness and F_{IS} for each site was calculated in FSTAT 2.9.3.2 (Goudet 2002). The number of private alleles and observed and expected heterozygosity were calculated in GENALEX 6.2 (Peakall and Smouse 2006). We tested for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium in ARLEQUIN 3.1 (Excoffier *et al.* 2005). Cavalli-Sforza and Edwards' chord distance for all site pairs was calculated in PHYLIP 3.69 (Phylogeny Inference Package; Felsenstein 2009).

Population structure analysis

Population structure was assessed at multiple spatial scales within the Great Lakes: 1) all sites combined, 2) within Lake Erie (including Detroit River and Southern Lake St Clair) and Lake Ontario (including Niagara River and St Lawrence River) separately, and among 3) selected population clusters (within a 60 km radius). The 60 km population cluster radius was chosen because brown bullhead have been shown to swim up to six km (Millard *et al.* 2009), thus we defined population clusters at ten times the known dispersal distance to ensure the inclusion of all normally dispersing fish. The population clusters also coincided with spatial clusters in our sampled sites (Figure 3.1). Thus, the population cluster analyses were performed on a subset of eight sites in Detroit River and southern Lake St Clair (Detroit River region) and eight sites in the Niagara River and western Lake Ontario region (Niagara-L.Ont)

Pair-wise F_{ST} was calculated in ARLEQUIN for all sites, while global F_{ST} was calculated and jacked-knifed over loci in FSTAT. Two sites (Deserento and Trenton) that were sampled in two consecutive years were used to test for temporal variation using

Exact Tests of Sample Differentiation (Raymond and Rousset 1995) between years with a Markov chain length of 100 000 steps and 10 000 dememorisation steps in ARLEQUIN. A Mantel test was used to test for isolation by distance at all spatial scales using pair-wise F_{ST} and geographical distance in GENALEX.

We also performed a cluster-based assignment analysis to assess population genetic structure in STRUCTURE 2.1 (Pritchard *et al.* 2000). Runs were performed on all 23 sites in the Great Lakes with K ranging between 1 and 13, then within lakes with K between 1 and 12 and finally within region with K between 1 and 8 (three replicates, with a burn in of 300 000 and 300 000 MCMC repetitions). The number of genetic clusters (K) was chosen according to the ΔK method (Evanno *et al.* 2005) using STRUCTURE HARVESTER (Earl and vonHoldt 2012). Individual assignments from STRUCTURE HARVESTER were compiled in CLUMPP 1.1.2 (Jakobssen and Rosenberg 2007) and plotted with DISTRUCT 1.1 (Rosenberg 2004). A neighbour-joining tree was created using the Cavalli-Sforza and Edwards' chord distance in PHYLIP with all 23 sampled sites.

Genotype Assignment

Due to previous reports of limited dispersal, we only performed genotype assignment for first generation migrants within the Detroit River and Niagara–L.Ont regions. The assignment was carried out using the Bayesian method of Rannala and Mountain (1997) in GENECLASS 2 (Piry *et al.* 2004) excluding fish ($P<0.05$) with no likely source population among the sampled sites using 10^4 Monte Carlo resampling simulations (Paetkau *et al.* 2004). For the fish that were not excluded, we identified the most likely source population with a rank-based assignment (Paetkau *et al.* 1995). We assigned a fish

to a source population when the likelihood of assignment to that population was three times (or higher) the likelihood of assignment to the next most likely source population. Assignment likelihood ratios lower than three resulted in a failed assignment. Our choice of a likelihood ratio of three was based on a sensitivity analyses where we preformed the rank-based assignment across a range of likelihood ratios and plotted the proportion (per cent) of individuals that assigned to a source population against the different likelihood ratios (Figure 3.2). The sensitivity analysis showed that our choice of likelihood ratio value does not bias our outcome (Figure 3.2). To test whether the pattern of dispersal differed from random expectation, we calculated the pattern of dispersal assuming random migration and placed the simulated migrants into distance travelled bins. We repeated the randomised dispersal calculation 20 times and took the average number of migrants for each distance travelled bin. We then compared the random migrant numbers within each distance bin to the observed number of migrants using a chi square test.

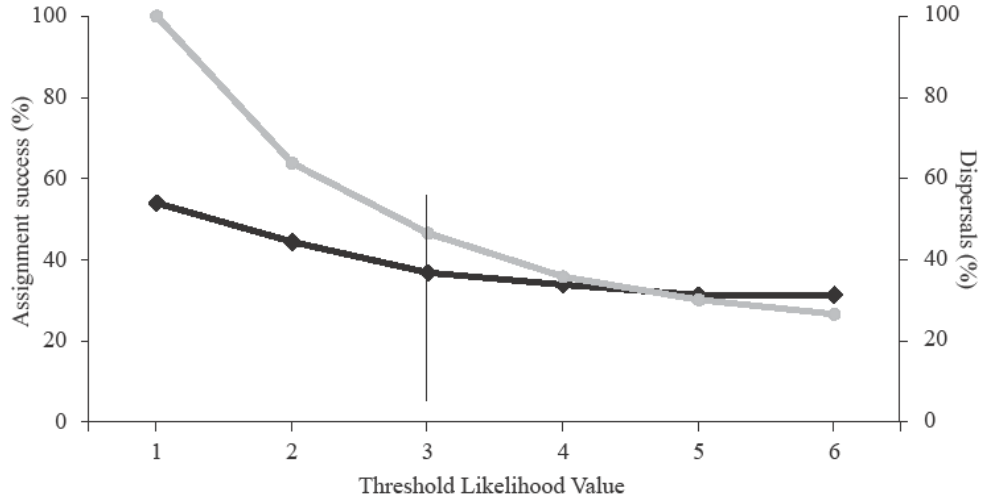

Figure 3.2. Results of a sensitivity analysis of the genotype assignment protocol to changes in the choice of threshold likelihood ratio for brown bullhead sampled in the lower Great Lakes. Threshold likelihood value refers to the ratio of the likelihoods of the most likely to the second most likely source population. The black line (◆) shows the proportion of first generation migrants out of all of the successfully assigned. The Grey line (●) shows the proportion of fish successfully assigned, and decreases as the assignment threshold increases.

Contaminant effects

To test for the effect of elevated sediment contaminant load on genetic diversity, genetic structure and dispersal patterns in the brown bullhead, I classified sites based on their contamination status. I used three sources of information to classify capture sites: 1) environmental agency evaluations, 2) sediment analyses, and 3) body burden analyses. Sites in Lake Ontario, Niagara River as well as PIB were sampled by Environment Canada and the US Environmental Protection Agency for habitat degradation, as a part of their monitoring program. A habitat can be identified as degraded for several reasons, but generally it is where environmental quality is low compared to other areas in the Great Lakes, assessed by high chemical levels and loss of fish or wildlife habitat due to pollution (http://www.ec.gc.ca/raps-pas/default.asp?lang=En&n=A0270A32-1). Sites classified as "degraded" by environmental agencies were considered polluted in thus study, and all such sites have an associated "clean" reference population sampled simultaneously. Contaminant status for sites sampled within the Detroit River area (an Area of Concern) was confirmed with sediment and body burden contaminant data (Farwell *et al*. 2012, Drouillard *et al.* 2013). Hillman Marsh has been delisted and is considered clean by Environment Canada. My Monroe site is considered polluted based on body burden data from unpublished contaminant analysis data (K. Drouillard, GLIER, University of Windsor, pers. comm.). The relationship between contaminant status and genetic diversity was assessed using a one way ANOVA for allele richness, total number of alleles and F_{IS} in SPSS 16 at small local, intermediate and large spatial scales. We tested for the effect of contaminant status on genetic structure using a hierarchical AMOVA in ARLEQUIN where genetic variance was partitioned into between-

contaminant groups (contaminated and clean), among sites within contaminant group, and among individuals within sites. We used the migrants identified by GENECLASS genotype assignment to test for contaminant effects on dispersal using a contingency table with the expected pattern to be equal dispersal away from contaminated and clean sites. We excluded the Belle River site due to anomalous genetic structure and first generation migrant assignment results. We also performed this analysis on a subset of sites within the Detroit River to explore the role of the deep channel separation resulting from with high water flow.

RESULTS

Population genetic characterisation

The number of alleles observed across all sites ranged from 11 to 37 among loci. Frenchman's Bay had two loci (Amn-3 and Amn-42) fixed for alleles while Carols Point, Jordan, Toronto Island and Trenton had one locus each that was fixed for a single allele (Amn-42, Amn-34, Amn-3 and Amn-34). The average number of alleles per site, per locus, ranged between 5.3 and 10.2 with an average of 7.1 over all sites (Table 1). Allele richness ranged between 4.81 and 7.54, with a value of 6.1 over all sites (Table 1). The total number of private alleles was 38 across all loci and all sites, ranging from zero at several sites to nine at Monroe (Table 1). F_{IS} values ranged from negative at Peche Isle, Belle Isle and Point Abino to 0.18 in Presque Isle Bay, with a median of 0.037 (Table 1). Observed heterozygosity ranged between 0.33 (Morrisburg) and 0.48 (Peche Isle), and expected heterozygosity ranged between 0.35 (Morrisburg) and 0.52 (Monroe; Table 1). The number of alleles per locus per site ranged between being fixed for six loci/site combinations to 26 alleles for two sites for locus Ane-359 with the average over sites

ranging between 2.74 for Ip-607 to 20.6 for Ane-359 (Supplementary Material Table S1), the average observed number of alleles was 7.12. Within the Detroit River region, the mean number of alleles per locus was 7.94, higher than that in the Niagara–L.Ont region at 6.51. Departure from HWE was observed at 13 out of 275 locus-by-population comparisons (Supplementary Material Table S1) after Bonferroni correction. The Belle River population had the highest number of loci (4) out of HWE, and the locus out of HWE in highest number of populations (5 of 23 populations) was Amn-44. No significant linkage disequilibrium was found after Bonferroni correction. Cavalli-Sforza and Edwards' chord distance ranged between 0.007 and 0.053 with average of 0.028 (Supplementary Material Table S2).

Population structure analysis

Study-wide global F_{ST} was 0.095 (SE = 0.023), compared to 0.046 (SE = 0.015) and 0.033 (SE = 0.008) in Lake Erie and Lake Ontario, respectively. Regional F_{ST} for the Detroit River region was 0.031 (SE = 0.012) and 0.022 (SE = 0.006) in the Niagara–L.Ont region. The majority of the pair-wise F_{ST} values were significantly different from zero after Bonferroni correction (Supplementary Material Table S2), with the exception of the Deserento – Belleville, Gross Isle South – Gross Isle North, Gross Isle South – Fighting Island and Gross Isle South – Puce River comparisons. Gross Isle North and Fighting Island are spatially close to Gross Isle South but Puce River is not (Figure 3.1). Non-significant pair-wise F_{ST} were also found for Randalph Reef – Carols Point, Randalph Reef – Jordan and Randalph Reef – Frenchman’s Bay (Supplementary Material Table S2). There was no significant allele frequency differences found between 2004 and 2005

replicated temporal samples at either the Deserento or Trenton sites, indicating minimal temporal variation from year to year.

The brown bullhead populations sampled by us followed an isolation by distance (IBD) pattern of genetic divergence across all sampled sites (R^2 = 0.79, Mantel P = 0.010; Figure 3.3A). Also, within each lake, we found significant IBD (Lake Ontario R^2 = 0.53, P=0.01 Figure 3.3B; Lake Erie R^2 = 0.67, P= 0.01 Figure 3.3C). We found significant IBD at the regional level as well, with the sampled sites in the Niagara–L.Ont region following IBD (R^2 =0.19, Mantel P = 0.020 Figure 3.3D). However, the Detroit River

Table 3.1. Summary of sample size (n), average number of alleles (A), allele richness (A_R), the number of private alleles (A_P), observed heterozygosity (Ho), expected heterozygosity (He), and Fixation index (Fis) for each brown bullhead sample location, based on 11 microsatellite loci.

Sampling location	**Abbr**	**n**	**A**	**A_R**	**A_P**	**Ho**	**He**	**F_{IS}**
Belle River	BR	79	9.8	7.2	4	0.40	0.47	0.14
Puce River	PR	35	7.0	6.3	4	0.40	0.40	0.02
Peche Isle	PI	49	6.8	5.9	0	0.48	0.47	-0.01
Belle Isle	BI	63	8.1	6.6	0	0.46	0.46	-0.01
Fighting Island	FI	31	6.7	6.5	1	0.43	0.44	0.04
Gross Isle North	GIN	53	7.2	6.1	1	0.41	0.42	0.04
Grosse Isle South	GIS	38	7.7	7.0	1	0.44	0.44	0.01
Monroe	Mon	93	10.2	7.5	9	0.48	0.52	0.09
Hillman Marsh	HM	38	7.4	6.7	0	0.37	0.41	0.12
Presque Isle Bay	PIB	35	8.1	7.5	2	0.40	0.48	0.18
Point Abino	PAb	59	7.5	6.3	1	0.42	0.41	-0.02
Black Creek[a]	BC	65	7.1	6.0	0	0.38	0.39	0.02
Queenston	Qu	59	6.5	5.8	0	0.38	0.41	0.09
Jordan	Jor	39	6.4	5.8	1	0.40	0.41	0.05
Randandalph Reef	RR	40	6.1	5.6	1	0.40	0.41	0.03
Carols Point	CP	29	5.3	5.2	1	0.40	0.41	0.03
Toronto Island	ToI	40	7.3	6.5	1	0.40	0.43	0.08
Frenchman's Bay	FrB	39	6.0	5.4	0	0.34	0.35	0.05
Belleville	Be	49	6.2	5.4	2	0.36	0.38	0.06
Deserento	Des	65	6.9	5.6	0	0.37	0.38	0.03
Trenton	Tr	65	7.5	6.0	1	0.39	0.40	0.03
Gray's Creek	GC	57	6.4	5.4	1	0.41	0.41	0.01
Morrisburg	Morr	54	5.6	4.8	2	0.33	0.35	0.05

[a]Actual sample site was in the Niagara River near Black Creek site; it has been named Black Creek to differentiate it from the Niagara River mainstream site.

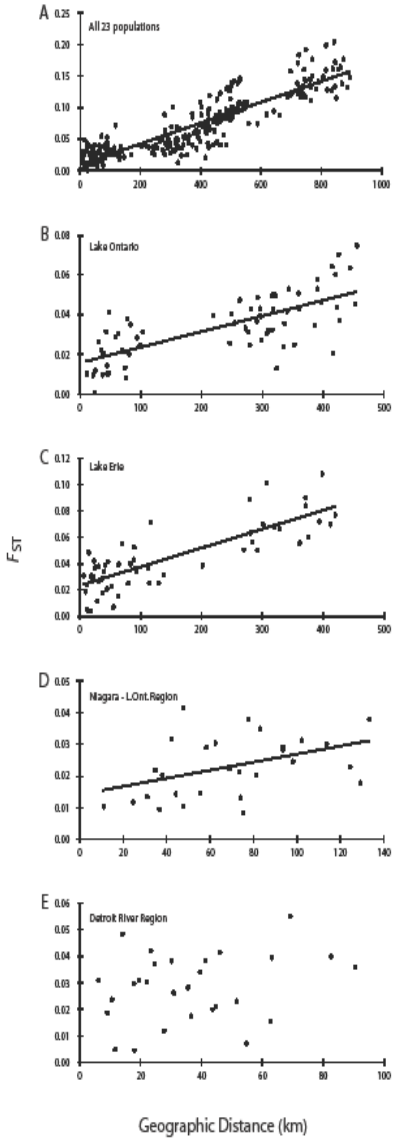

Figure 3.3. Scatterplots of pair-wise F_{ST} versus pair-wise shortest water distance (km) between sampled brown bullhead sites in the lower Great Lakes. Panel A shows the relationship for all 23 populations within the Great Lakes ($R^2 = 0.79$, P = 0.010); Panel B shows the relationship for Lake Ontario ($R^2 = 0.53$, P = 0.010); Panel C shows the relationship for Lake Erie ($R^2 = 0.67$, P = 0.010); Panel D shows the relationship for Niagara–L.Ont region ($R^2 = 0.19$, P = 0.020); and Panel E shows no significant relationship for the Detroit River region ($R^2 = 0.065$, P = ns)

region did not follow an IBD pattern of divergence (R^2 =0.065, Mantel P = 0.20 Figure 3.3E).

Study-wide (23 sample sites), STRUCTURE divided the two lakes into two separate genetic groups. Individuals collected across the 23 sampled sites formed 9 genetic groups (K = 9). Lake Ontario, including Queenston and Black Creek in Niagara River (12 sites) consisted of two genetic groups a lake and a river cluster; however, when Niagara River sites were excluded, Lake Ontario (10 sites) showed three groups (K=3). Lake Erie (11 sites) had ten genetic clusters (K = 10). The Detroit River region (8 sites) consisted of four genetic clusters (K = 4), with Fighting Island, North and South Gross Isle make up one cluster within (Figure 3.4). Niagara–L.Ont (8 sites), a subset of the Lake Ontario sites, showed a K = 2, suggesting separate river and lake populations (Figure 3.4).

The neighbour joining tree based on Cavalli-Sforza and Edwards' chord distance (23 sampling sites; Figure 3.5) show a cluster pattern mainly consistent with the STRUCTURE analysis. Lake Ontario has three clusters with membership matching the STRUCTURE results. Lake Erie sampling sites form a single branch, while the Detroit River region sites show a mixed pattern with some sites not clustering with their spatial neighbours (Figure 3.5).

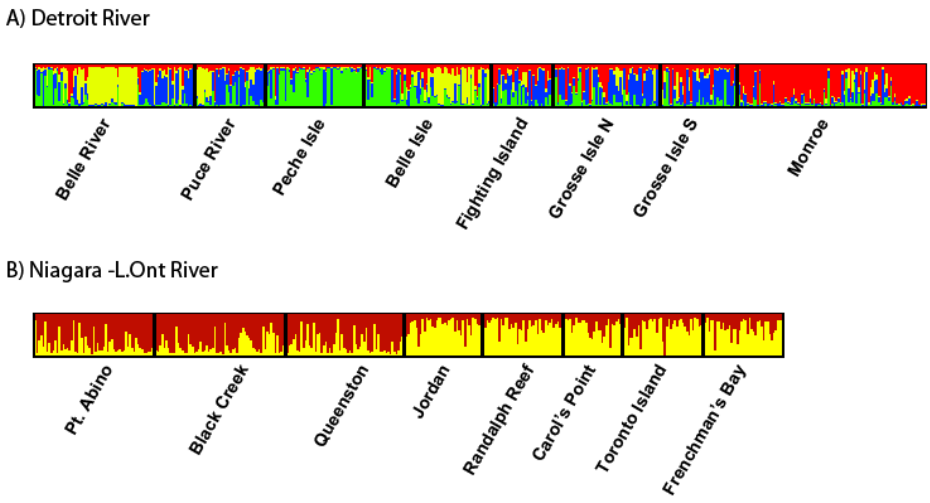

Figure 3.4. Microsatellite-based genotype assignments in STRUCTURE for each site in the Detroit River region sites (panel A: K = 6) and the Niagara–L.Ont region sites (panel B: K = 2).

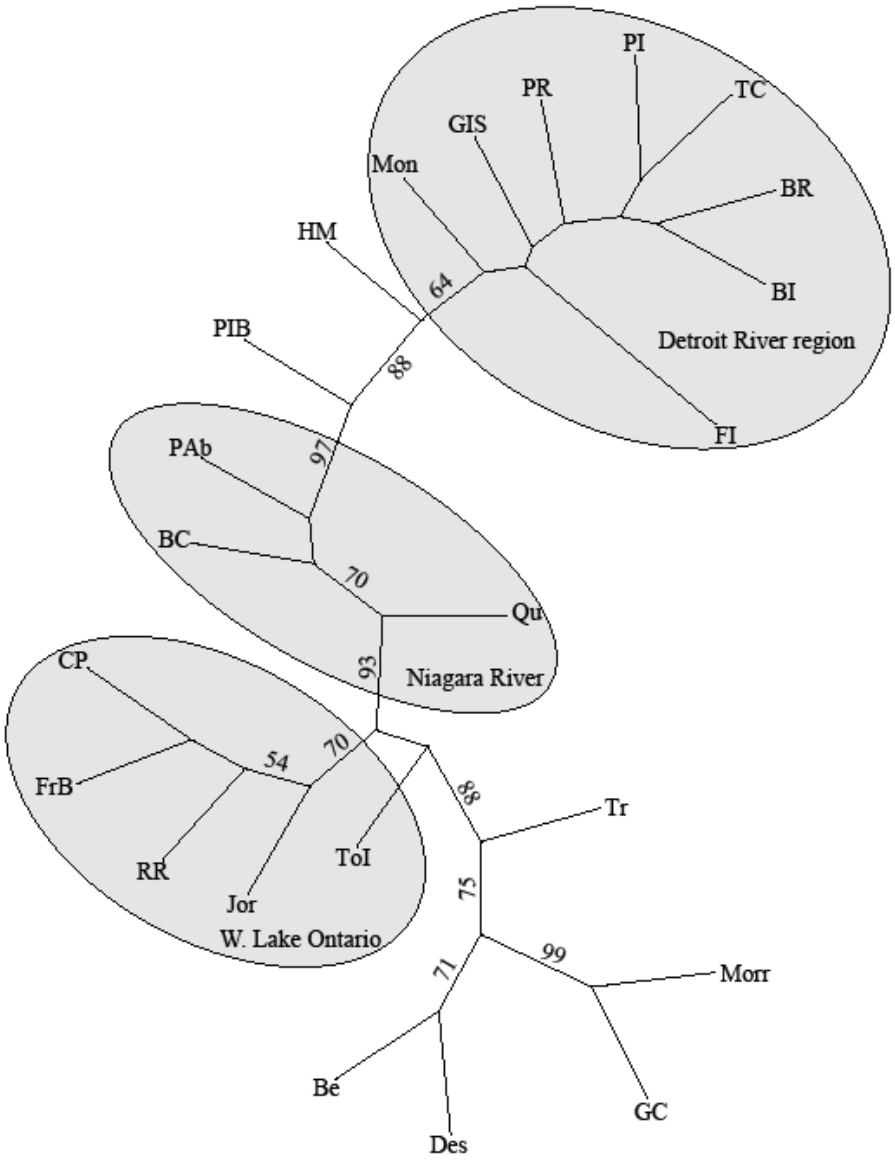

Figure 3.5. Un-rooted Neighbour joining tree based on pair-wise Cavalli-Sforza and Edwards' chord distances in PHYLIP 3.69. Numbers indicate bootstrap support following 1000 replicates (bootstrap values below 50 per cent are not shown).

Genotype assignment

There were only a few fish that were excluded as coming from all sampled source populations in the genotype assignment; 18 out of 441 fish (4.1%) in the Detroit River region and 13 out of 370 fish (3.5%) in the Niagara–L.Ont region were excluded from all sampled putative source populations. In general, we appear to have sampled most of the potential source populations, aided by the relatively strong IBD which would allow assignment to geographically close (and genetically related) source populations. In the Detroit River region, 48.2 % (204 of 423) of the fish were assigned to a specific source population using the likelihood ratio threshold of 3. Of the successfully assigned fish in the Detroit River region, 75 (36.8%) were first generation migrants (Table 2). Most dispersal events were between spatially adjacent sites (Table 2), and the number of first generation migrants decreased with geographic distance, with the exception that Monroe had more first generation migrants from Belle River than expected (Figure 3.6A). We cannot explain this anomaly as there are few or no first generation migrants at intermediate distances. The observed dispersal distance distribution was significantly different from the random generated null distribution ($\chi^2 = 6.9$; $p < 0.01$) in the Detroit River region. In the Niagara–L.Ont region 44.8 % (160 of 357) were successfully assigned with 3 as the likelihood ratio threshold for assignment, and 59 (36.9%) of the assigned fish were first generation migrants. Again, most dispersal was between neighbouring sites (Table 2, Figure 3.6B), and the dispersal distance distribution was also significantly different that the null distribution for the Niagara-L.Ont region populations ($\chi^2 = 17.9$; $p < 0.001$).

Table 3.2. Result of genotype assignment analyses (GENECLASS) for brown bullhead sampled in two regions – the Detroit River region (Panel A) and the Niagara – L.Ont region (Panel B). Self-assigned fish (to sampled site) are highlighted in bold along the diagonal while the first generation migrants (dispersed fish) are off the diagonal. The population where the fish were sampled is listed in the left column while the assigned source populations are listed in the top row.

A	BR	PR	PI	BI	FI	GIN	GIS	Mon
BR	**20**	3	3	1	3	1	1	2
PR		**7**						1
PI	1	1	**32**	2	3		1	
BI	3	2	4	**21**	1	1		1
FI		1	1		**7**	1	2	
GIN		2	2		2	**11**	1	4
GIS		1			5	2	**3**	3
Mon	4				3	3	3	**28**

B	PAb	BC	Qu	Jor	RR	CP	ToI	FrB
PAb	**19**	3	2		1	1	1	1
BC	1	**22**	1			2		2
Qu	1	3	**17**	2				2
Jor			2	**9**	2	1		
RR				2	**7**	4	4	1
CP				1		**12**		3
ToI	1		1	2	2	1	**14**	
FrB		1		2	1	3	2	**1**

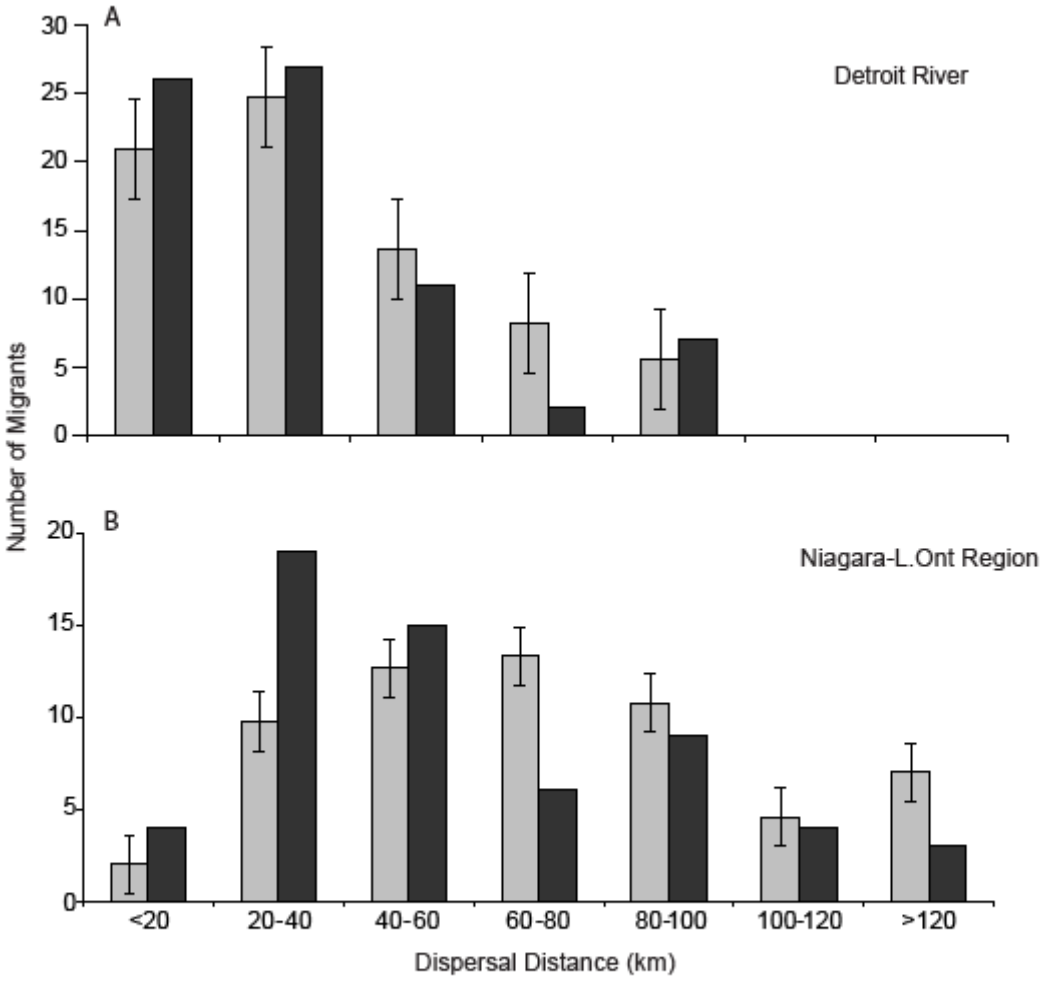

Figure 3.6. Frequency distribution of the dispersal distances for first generation migrants identified by GENECLASS genotype assignment for brown bullhead from two selected regions in the lower Great Lakes. The random-generated null distribution is shown in light grey bars (± 1.0 standard error), and the observed distribution is shown in black bars. Panel A shows the distributions for the Detroit River region, Panel B shows the distribution for the Niagara–L.Ont region populations.

Contaminant effects

No differences in allele richness, number of alleles or F_{IS} were found between contaminated and clean sites at any spatial scale ($P > 0.05$). AMOVA revealed no significant partitioning of genetic variance among clean and contaminated sites. Genetic variation was highest among individuals within sampling sites (Table 3) and also significant between sampling sites within clean or contaminated sites (Table 3). In the Detroit River 0.08% ($P > 0.05$) of the genetic variance between clean and contaminated sites (Table 3).

We did find a significant difference in dispersal away from contaminated versus clean sites in the Detroit River region (excluding Belle River) with more fish leaving contaminated sites than are leave clean sites ($\chi^2 = 5.53$, $P = 0.019$; Table 4). This results held for analyses that included only the five sites in Detroit River itself ($\chi^2 = 6.37$; $P=0.012$; Table 4). We found no significant differences in the pattern of dispersal from contaminated and clean sites in the Niagara–L.Ont region ($\chi^2 = 1.69$, $P =0.194$; Table 4).

Table 3.3. AMOVA genetic variance partitioning at different spatial levels between clean versus contaminated sites, among sites within clean/contaminated sites and among individuals within sites.

Spatial level	Per cent variance	P-value
23 populations		
Among individuals within sites	92.5	0.000
Among sites within clean or contaminated areas	8.0	0.000
Between clean and contaminated sites	-0.5	0.901
Lake Erie		
Among individuals within sites	95.4	0.000
Among sites within clean or contaminated areas	4.6	0.000
Between clean and contaminated sites	-0.1	0.637
Lake Ontario		
Among individuals within sites	96.6	0.000
Among sites within clean or contaminated areas	3.5	0.000
Between clean and contaminated sites	-0.1	0.537
Detroit River		
Among individuals within sites	96.8	0.000
Among sites within clean or contaminated areas	3.1	0.000
Between clean and contaminated sites	0.1	0.408
Niagara–L.Ont		
Among individuals within sites	97.8	0.000
Among sites within clean or contaminated areas	2.4	0.000
Between clean and contaminated sites	-0.2	0.766

Table 3.4. The number of fish staying (self-assigned) versus leaving (first generation migrants) the clean and contaminated sites within each study area.

Study area	**Detroit River**[a]		**Five sites within the Detroit River**		**Niagara–L.Ont**	
Behaviour	**stay**	**leave**	**stay**	**leave**	**stay**	**leave**
Contaminated	63	47	35	34	29	24
Clean	46	14	39	13	72	36
Chi-square	5.53		6.37		1.69	
P-value	0.019		0.012		0.194	

[a]The Detroit River without Belle River

DISCUSSION

For large water bodies (such as the Great Lakes) with few physical barriers the primary limitation to fish dispersal is geographic distance. Distance-based limits to dispersal should result in a pattern of isolation by distance (IBD), which has been reported for a number of philopatric freshwater fishes (Bernatchez 2001; Koblmüller *et al.* 2008; Barson *et al.* 2009; VanDeHey *et al.* 2009; Wagner *et al.* 2009). Our study shows that population differentiation in brown bullhead is primarily distance driven, with 79% of the variation in genetic differentiation (i.e. $r^2 = 0.79$) among sampling sites attributable to geographic distance among those sites. Similarly distance contributes substantially to genetic divergence among sites at smaller spatial scales; 67% and 53% of the variation in genetic divergence explained by distance in Lake Erie and Lake Ontario, respectively.

These values are higher than those previously reported in the Great Lakes: for example, Lake Michigan lake white fish (*Coregonus clupeaformis*) exhibit significant IBD (r^2 of 18%; VanDeHey *et al.* 2009), which is comparable to our findings among the Niagara-L.Ont region sites ($r^2 = 0.19$). We found significant IBD at almost all spatial scales, including quite small spatial scales (<90 km). This, combined with high levels of genetic divergence among sites (e.g., pairwise Fst), indicates that brown bullhead are limited in their dispersal, supporting previous claims of philopatry. However, despite the high levels of genetic structure indicative of low long-term gene flow (supporting previous genetic studies; Murdoch and Herbert 1994; Silbiger *et al.* 2001; Dedual 2002; Sakaris *et al.* 2005), we identified high levels of dispersal, with genotype assignment identifying approximately 30% first generation migrants within most sampled sites. Thus our dispersal estimates contradict our population genetic divergence results. However, the distribution of the dispersal distances tend to cluster at short distances (< 60 km), and since we do not know the spatial extent of brown bullhead populations, it is possible that we inadvertently sampled individual brown bullhead populations more than once. This would also explain the relatively high failed genotype assignment rate in our study – GENECLASS would identify two source "populations" with similar assignment probabilities resulting in a failed assignment to a single population. We cannot rule out the possibility that brown bullhead commonly disperse, perhaps seasonally, but return to their natal site to reproduce, thus maintaining high genetic structure while generating high numbers of "stray" bullhead.

In highly contaminated areas there are two possible demographic outcomes in the absence of acclimation or adaptation; increased dispersal and/or increased mortality.

Increased mortality will lead to reduced population density in the contaminated area which might attract other fish to the area – the contaminated site would thus act as a sink (Bickham 2011). On the other hand, the contaminant stress may elicit an avoidance behaviour resulting in individuals moving out of the area. As brown bullhead live in the sediment in continuous contact with the contaminants, we would predict the high stress would drive a net emigration away from affected sites. A higher number of fish leaving polluted sites (relative to clean sites) was observed in the Detroit River region. However, we did not find the same effect in the Niagara–L.Ont region, despite similar or higher levels of PAHs in those contaminated sites (Drouillard *et al.* 2006; Sofowote *et al.* 2008). This discrepancy likely reflects the fact that dispersal is affected by more than stress avoidance, with factors such as distance, unsuitable habitat or competition contributing to the dispersal patterns among first generation migrants. The lack of an IBD pattern of genetic divergence among the Detroit River region sites suggests that avoidance of the polluted areas over time may be breaking down the migration - drift equilibrium that contributes to IBD.

There is a strong theoretical expectation for aquatic pollutants to drive substantial change in genetic structure and diversity of affected populations resulting from elevated mortality and reduced reproductive success associated with high contaminant load (Bickham 2011). We found no evidence that sediment pollution has a measureable effect on microsatellite genetic diversity or structure at any spatial level in Great Lakes brown bullhead. This is despite very high levels of pollution at some sites (Drouillard *et al.* 2006) and reports of widespread neoplasia and tumours in Detroit River bullhead collected from contaminated sites (Leadley *et al.* 1998). Previous analyses of genetic

variation in brown bullhead showed reduced genetic diversity at sites associated with polluted habitat (Murdoch and Herbert 1994; Silbiger *et al.* 2001). Such a pattern has also been reported in other species (Roark *et al.* 2005; Johnston and Roberts 2009). However, a loss of genetic diversity is not universally observed: studies have shown that even when selection (and associated mortality) has resulted in measurable adaptation to contaminants, no change in neutral genetic diversity was detectable (McMillan *et al.* 2006; Lind and Grahn 2011). This was true even when very rare alleles had been selected to substantially higher frequencies (Wirgin *et al.* 2011). In our sample sites most heavily polluted (e.g., Carols Point, Gross Isle North) brown bullhead were the only fish observed and captured, and in other contaminated sites they were clearly the dominant species. If the brown bullhead are particularly tolerant of contaminant stress, and have large population sizes, our measures of genetic variation and structure may simply be not sensitive enough to detect subtle changes.

Overall, our results show that brown bullhead in highly contaminated habitats neither abandon their sites, nor are extirpated, thus it is likely that they are coping with pollution via acclimation and/or genetic adaptation. Although pollution tolerant, bullhead do display dramatic phenotypic effects in response to sediment contaminants, including high levels of tumours and neoplasia (Baumann *et al.* 1996). We propose that sophisticated physiological acclimation or genetic adaptations are plausible and likely responses to the stress caused by the contamination in the lower Great Lakes. Previous research using Detroit River brown bullhead demonstrated both physiological acclimation (Robinson 2011; Farwell *et al.* 2012) and genetic adaptation (Breckels and Neff 2010) in response to contaminant stress. Future studies focussing on identifying which mechanism

(acclimation versus adaptation) is more prevalent will need to incorporate multiple generations of controlled breeding of brown bullhead. If brown bullhead are indeed responding to contaminated sediment stress through acclimation and adaptation, they will no longer provide accurate biomonitoring information for the assessment of the biological impacts of the sediment pollution, and action plans based on native bullhead response should be reassessed.

REFERENCES

Barson, N. J., T. O Haugen, L. A. Vøllestad, and C. R Primmer. 2009. Contemporary isolation-by-distance, but not isolation-by-time, among demes of European grayling (*Thymallus thymallus*, Linnaeus) with recent common ancestors. Evolution 63:549-556.

Baumann, P. C., I. R. Smith, and C. D. Metcalfe. 1996. Linkages between chemical contaminants and tumors in benthic Great Lake Fish. Journal of Great Lakes Research 22:131-152.

Bickham, J. W. 2011. The four cornerstones of evolutionary toxicology. Ecotoxicology 20:497–502.

Blumer, L. S. 1985. Reproductive natural history of the brown bullhead *Ictalurus nebulosus* in Michigan. American Midland Naturalist 114:318-330.

Breckels, R. D., and B. D. Neff. 2010. Pollution-induced behavioural effects in the brown bullhead (*Ameiurus nebulosus*). Ecotoxicology 19:1337-1346.

Bernatchez, L. 2001. The evolutionary history of brown trout (*Salmo trutts* L.) inferred from phylogeographic, nested clade, and mismatch analysis of mitochondrial DNA variation. Evolution 55:351-379.

Dachs, J., and L. Méjanelle. 2010. Organic pollutants in coastal waters, sediment and biota: A relevant driver for ecosystem during the anthropocene? Estuaries and Coasts 33:1-14.

Dedual, M. 2002. Vertical distribution and movements of brown bullhead (*Ameiurus nebulosus* Lesueur 1819) in Motuoapa Bay, southern Lake Taupo, New Zealand. Hydrobiologia 483:129-135.

Drouillard, K. G., M. Tomczak, S. Reitsma, and G. D. Haffner. 2006. A River-wide survey of polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and selected organochlorine pesticide residues in sediments of the Detroit River—1999. Journal of Great Lakes Research 32:209-226.

Earl, D., and B. vonHoldt. 2011. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetic Research 4:359-361.

Elphinstone, M. S., G. N. Hinten, M. J. Anderson, and C. J. Nock. 2003. An inexpensive and high-throughput procedure to extract and purify total genomic DNA for population studies. Molecular Ecology Notes 3:317-320.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14:2611-2620.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1:47-50.

Farwell, M., K. G. Drouillard, D. D. Heath, and T. E. Pitcher. 2012. Acclimation of life-history traits to experimental changes in environmental contaminant concentrations in brown bullhead (*Ameiurus nebulosus*). Environmental Toxicology and Chemistry 31:863-869.

Goudet, J. 1995. FSTAT version 1.2: a computer program to calculate Fstatistics. Journal of Heredity 86:485-486.

Jakobsson, M., and N. A. Rosenberg. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23:1801-1806.

Johnston, E. L., and D. A. Roberts. 2009. Contaminants reduce the richness and evenness of marine communities: A review and meta-analysis. Environmental Pollution 157:1745-1752.

Kasumyan, A. O. 2002. Sturgeon food searching behaviour evoked by chemical stimuli: a reliable sensory mechanism. Journal of Applied Ichthyology 18:685-690.

Kobmüller, S., N. Duftner, K. M. Sefc, U. Aigner, M. Rogetzer, and C. Sturmbauer, 2008. Phylogeographic structure and gene flow in the scale-eating cichlid *Perissodus microlepis* (Teleostei, Perciformes, Cichlidae) in southern Lake Tanganyika. Zoologica Scripta 38:257-268.

Leadley, T. A., G. Balch, C. D. Metcalfe, R. Lazar, E. Mazak, J. Habowsky, and G. D. Haffner. 1998. Chemical accumulation and toxicological stress in three brown bullhead (*Ameiurus nebulosus*) populations of the Detroit River, Michigan, USA. Environmental Toxicology and Chemistry 17:1756-1766.

Lind, E. E., and M. Grahn. 2011. Directional genetic selection by pulp mill effluent on multiple natural populations of three-spined stickleback (*Gasterosteus aculeatus*). Ecotoxicology 20:503-512.

McMillan, A. M., M. J. Bagley, S. A. Jackson, and D. E. Nacci. 2006. Genetic diversity and structure of an estuarine fish (*Fundulus heteroclitus*) indigenous to sites associated with a highly contaminated urban harbor. Ecotoxicology 15:539-548.

Milá, B., R. K. Wayne, P. Fitze, and T. B. Smith. 2009. Divergence with gene flow and fine-scale phylogeographical structure in the wedge-billed woodcreeper, *Glyphorynchus spirurus*, a Neotropical rainforest bird. Molecular Ecology 18:2979-2995.

Millard, M. J., D. R. Smith, E. Obert, J. Grazio, M. L. Bartron, C. Wellington, S. Grisè, S. Rafferty, R. Wellington, and S. Julian. 2009. Movements of brown bullheads in Presque Isle Bay, Lake Erie, Pennsylvania. Journal of Great Lakes Research 35:613-619.

Murdoch, M. H., and P. N. D. Herbert. 1994. Mitochondrial DNA diversity of brown bullhead from contaminated and relatively pristine sites in the Great Lakes. Environmental Toxicology and Chemistry 13:1281-1289.

Nosil, P., T. H. Vines, and D. J. Funk. 2005. Respective: reproductive isolation caused by natural selection against immigrants from divergent habitats. Evolution 59:705-719.

Paetkau, D., W. Calvert, I. Stirling, and C. Storbeck. 1995. Microsatellite analysis of population structure in Canadian polar bears. Molecular Ecology 4:347-354.

Paetkau, D., R. Slade, M. Burden, and A. Estoup. 2004. Genetic assignment method for direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. Molecular Ecology 13:55-65.

Peakall, R., and P. E. Smouse. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6:288-295.

Piry, S., A. Alapetite, J. M. Cornuet, D. Paetkau, L. Baudouin, and A. Estoup. 2004. GeneClass2: A Software for Genetic Assignment and First-Generation Migrant Detection. Journal of Hereditary 95:536-539.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945-959

Pyron, M., E. C. Obert, and R. Wellington. 2001. Tumor rates and population estimates of brown bullhead (*Ameiurus nebulosus*) in Presque Isle Bay, Lake Eire. Journal of Great Lakes Research 27:185-190.

Rannala, B., and J. L. Mountain. 1997. Detecting immigrants by using multilocus genotypes. Proceedings of the National Academy of Science of the United States of America 94:9197-9201.

Raymond, M., and F. Rosset. 1995. An exact test for population differentiation. Evolution 46:1280-1283.

Rico, C., and G. F. Turner. 2002. Extreme microallopatric divergence in a cichlid species from Lake Malawi. Molecular Ecology 11:1585-1590.

Roark, S. A., D. Nacci, L. Coiro, D. Champlin, and S. I. Guttman. 2005. Population genetic structure of a nonmigratory estuarine fish (*Fundulus heteroclitus*) across a strong gradient of polychlorinated biphenyl contamination. Environmental Toxicology and Chemistry 24:717-225.

Robinson, K. 2011. Characterizing the immune function of the brown bullhead (*Ameiurus nebulosus*) from less contaminated and highly contaminated locations along the Detroit River. M.Sc. thesis, Department of Biology, University of Waterloo, Waterloo, ON.

Rosenberg, N. A. 2004. DISTRUCT: a program for the graphical display of population structure. Molecular Ecology Notes 4:137-138.

Sakaris, P. C., R. V. Jesien, and A. E. Pinkney. 2005. Brown bullhead as an indicator species: seasonal movement patterns and home ranges within the Anacostia River, Washington, D.C. Transaction of American Fisheries Society 134:1262-1270.

Schofield, C. L., and C. T. Driscoll. 1987. Fish species distribution in relation to water quality gradients in the North Branch of the Moose River Basin. Biogeochemistry 3:63-85.

Scott, W. B., and E. J. Crossman. 1973. Freshwater fishes of Canada. Bull. Fish. Res. Board Can. No. 184.

Scott, G. R., and K. A. Sloman. 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquatic Toxicology 68:369-392.

Seehausen, O., and J. J. M. van Alphen. 1998. The effect of male coloration on female mate choice in closely related Lake Victoria cichlids (*Haplochromis nyererei* complex). Behavioural Ecology and Sociobiology 42:1-8

Silbiger, R. N., A. C. Leonard, P. Dimsoski, S. Fore, S. I. Guttman, A. C. Roth, D. A. Gordon, T. Wessendarp, G. P. Toth, and M. K. Smith. 2001. Use of molecular markers to study the effects of environmental impacts on genetic diversity in brown bullhead (*Ameiurus nebulosus*) populations. Biogeochemistry 20:2580-2587.

Söderberg, L. I., J. A. Galarza, and D. D. Heath. 2010. Novel and optimized polymorphic microsatellite loci for brown bullhead (*Ameiurus nebulosus*). Conservation Genetic Resource 2:211-213.

Sofowote, U., B. E. McCarry, and C. H. Marvin. 2008. Source apportionment of PAH in Hamilton Harbour suspended sediments: comparison of two factor analysis methods. Environmental Science and Technology 42: 6007-6014.

Steeve, T. E., D. J Anderson, and V. L. Friesen. 2005. The Isthmus of Panama: a major physical barrier to gene flow in a highly mobile pantropical seabird. Journal of Evolutionary Biology 18:1000-1008.

Taylor, M. I., L. Rüber, and E. Verheyen. 2001. Microsatellites reveal high levels of population substructuring in the species–poor Eretmodine cichlid lineage from Lake Tanganyika. Proceedings of the Royal Society B. 268:803-808.

VanDeHey, J. A., B. L Sloss, P. J. Peeters, and T. M Sutton. 2009. Genetic structure of lake whitefish (*Coregonus clupeaformis*) in Lake Michigan. Canadian Journal of Fisheries and Aquatic Science 66:382-393.

Wagner, C. E., and A. R. McCune. 2009. Contrasting patterns of spatial genetic structure in sympatric rock-dwelling cichlid fishes. Evolution 63:1312-1326.

Wirgin, I., N. K Roy, M. Loftus, R. C. Chambers, D. Franks, and M. E. Hahn. 2011. Mechanistic basis of resistance to PCBs in Atlantic tomcod from the Hudson River. Science 331:1322-1325.

Supplementary Table S3.1. Summary of the number of alleles (A) observed (Ho) and expected (He) heterozygosity for each microsatellite locus within each sample site, summarised across all populations at the bottom. The 13 locus-by-population combinations that depart from HWE after Bonferroni correction are underlined and highlighted in bold

Site		Ip-372	Ip-365	Amn-34	Amn-3	Amn-42	Amn-16	An-12	Amn-44	Amn-43	Ip-607	Ane-359
	A	13	4	7	5	7	12	10	9	12	3	26
Belle River	Ho	0.71	**0.06**	**0.17**	0.19	0.56	**0.70**	0.40	0.33	0.42	**0.06**	0.86
	He	0.82	0.15	0.23	0.18	0.52	0.79	0.46	0.44	0.44	0.18	0.94
	A	10	3	3	5	4	6	7	3	13	5	18
Puce River	Ho	0.77	0.14	0.09	0.14	0.37	0.60	0.38	0.26	0.69	0.06	0.85
	He	0.77	0.13	0.08	0.14	0.36	0.56	0.39	0.27	0.60	0.20	0.89
	A	8	3	4	3	3	11	7	4	10	2	20
Peche Isle	Ho	0.71	0.10	0.29	0.33	0.53	**0.71**	0.63	0.39	0.53	0.10	0.98
	He	0.69	0.10	0.28	0.28	0.41	0.83	0.54	0.51	0.55	0.10	0.92
	A	11	3	2	2	7	11	9	4	14	3	23
Belle Isle	Ho	0.92	0.11	0.02	0.22	0.53	**0.68**	0.52	0.46	0.57	0.15	0.91
	He	0.87	0.11	0.02	0.22	0.46	0.79	0.51	0.46	0.54	0.14	0.90
	A	9	2	8	4	5	5	5	4	9	2	21
Fighting Island	Ho	0.84	0.06	0.52	0.10	0.42	0.48	0.57	0.35	0.52	0.03	0.87
	He	0.83	0.06	0.47	0.12	0.41	0.59	0.50	0.35	0.56	0.03	0.93
	A	12	2	5	2	6	10	7	4	8	3	20
Gross Isle N.	Ho	0.83	0.13	0.11	0.11	0.38	0.66	0.45	0.28	0.47	0.14	0.89
	He	0.76	0.12	0.18	0.11	0.37	0.64	0.43	0.30	0.48	0.28	0.93
	A	12	3	5	3	3	7	8	5	12	3	24
Gross Isle S.	Ho	0.71	0.11	0.29	0.16	0.45	0.66	0.65	0.24	0.42	0.24	0.95
	He	0.77	0.10	0.26	0.15	0.39	0.62	0.56	0.30	0.50	0.25	0.94
	A	14	4	7	4	9	7	12	4	20	6	25
Monroe	Ho	0.82	0.15	0.29	0.25	0.51	0.58	0.52	**0.43**	0.57	**0.24**	0.92
	He	0.77	0.17	0.31	0.24	0.46	0.70	0.51	0.51	0.63	0.53	0.94
	A	9	4	5	2	5	6	11	5	8	3	23
Hillman marsh	Ho	0.74	0.39	0.18	0.11	0.24	0.24	0.50	0.32	0.45	0.06	0.84
	He	0.76	0.34	0.17	0.10	0.30	0.38	0.52	0.38	0.44	0.20	0.94

	A	14	4	3	3	4	7	11	7	7	3	26
Presqe Isle Bay	Ho	0.69	**0.20**	0.03	0.20	0.23	0.31	0.97	0.26	0.23	0.40	0.89
	He	0.84	0.37	0.21	0.18	0.45	0.39	0.86	0.35	0.21	0.45	0.94
	A	13	7	3	3	3	6	10	4	11	2	21
Point Abino	Ho	0.92	0.25	0.08	0.12	0.15	0.51	0.90	0.10	0.46	0.15	0.95
	He	0.85	0.26	0.08	0.11	0.14	0.53	0.84	0.16	0.43	0.14	0.93
	A	12	3	3	3	3	7	11	4	9	2	21
Black Creek	Ho	0.77	0.08	0.13	0.18	0.05	0.38	0.88	0.17	0.38	0.26	0.92
	He	0.76	0.07	0.15	0.17	0.05	0.38	0.88	0.24	0.39	0.25	0.93
	A	11	4	2	2	3	6	12	4	6	2	19
Queenston	Ho	0.90	0.14	0.05	0.02	0.14	0.36	0.81	**0.12**	0.39	0.27	0.98
	He	0.83	0.13	0.05	0.02	0.14	0.48	0.87	0.40	0.42	0.28	0.93
	A	9	4	1	2	3	7	12	3	10	2	17
Jordan	Ho	0.74	0.08	0.00	0.00	0.15	0.38	0.85	0.18	0.79	0.23	0.97
	He	0.82	0.12	0.00	0.05	0.14	0.39	0.89	0.29	0.65	0.28	0.91
	A	9	6	4	3	2	2	12	2	7	3	17
Randalph Reef	Ho	0.88	0.20	0.21	0.10	0.08	0.30	0.90	0.30	0.25	0.28	0.93
	He	0.85	0.19	0.19	0.10	0.07	0.32	0.86	0.26	0.40	0.32	0.93
	A	9	3	3	4	1	2	12	3	4	3	14
Carols Point	Ho	0.86	0.10	0.25	0.55	0.00	0.21	0.79	0.21	0.34	0.34	0.78
	He	0.83	0.10	0.23	0.42	0.00	0.19	0.88	0.29	0.30	0.38	0.87
	A	13	4	3	1	6	5	11	4	10	3	20
Toronto Island	Ho	0.73	0.30	0.13	0.00	0.15	0.30	0.88	0.03	0.68	0.33	0.93
	He	0.87	0.26	0.12	0.00	0.17	0.29	0.86	0.16	0.73	0.34	0.93
	A	9	5	5	1	1	4	13	2	7	2	17
Frenchman Bay	Ho	0.74	0.15	0.15	0.00	0.00	0.26	0.72	0.08	0.44	0.18	0.97
	He	0.83	0.15	0.15	0.00	0.00	0.31	0.87	0.07	0.41	0.16	0.89
	A	10	4	2	2	4	2	13	3	8	2	18
Belleville	Ho	0.84	0.31	0.06	0.04	0.10	0.06	0.90	0.04	0.29	0.38	0.91
	He	0.80	0.36	0.06	0.04	0.14	0.06	0.84	0.15	0.32	0.45	0.92
	A	10	3	3	2	4	5	12	4	8	2	23
Deserento	Ho	0.83	0.28	0.11	0.05	0.06	0.12	0.89	**0.05**	0.37	0.41	0.92
	He	0.80	0.27	0.10	0.05	0.09	0.13	0.84	0.17	0.41	0.41	0.94

	A	11	6	1	3	4	5	12	3	11	2	25
Trenton	Ho	0.86	0.35	0.00	0.03	0.12	0.11	0.89	**0.02**	0.60	0.38	0.92
	He	0.79	0.37	0.00	0.03	0.15	0.16	0.85	0.13	0.54	0.39	0.94
	A	10	3	4	4	3	5	8	3	9	2	19
Gray's Creek	Ho	0.81	0.23	0.09	0.18	0.04	0.23	0.82	0.12	0.75	0.26	0.95
	He	0.81	0.21	0.12	0.16	0.04	0.21	0.80	0.15	0.79	0.28	0.90
	A	7	2	4	5	2	3	9	2	7	3	18
Morrisburg	Ho	0.62	0.15	0.21	0.09	0.00	0.06	0.85	**0.00**	0.63	0.20	0.84
	He	0.68	0.14	0.22	0.12	0.04	0.05	0.79	0.14	0.54	0.18	0.91
	A	23	12	15	10	13	20	17	11	26	11	37
Over all populations	A - mean	10.7	3.74	3.78	2.96	4.00	6.13	10.2	3.91	9.57	2.74	20.6
	Ho	0.79	0.18	0.15	0.14	0.23	0.39	0.73	0.21	0.49	0.23	0.91
	He	0.81	0.19	0.16	0.13	0.23	0.43	0.72	0.29	0.50	0.27	0.93

Supplementary Table S3.2. Cavalli-Sforza and Edwards' cord distance above the diagonal and pair wise F_{ST} below diagonal, non-significant F_{ST} values after Bonferroni correction highlighted in bold and underlined.

	Belle Isle	Belle River	Peche Isle	Gross Isle N	Belle-ville	Black Creek	Dese-rento	Fightin Island	French Bay	Gray's Creek	Gross Isle S	Carol Point
Belle Isle	*	0.017	0.020	0.018	0.037	0.029	0.038	0.023	0.038	0.042	0.020	0.041
Belle River	0.012	*	0.025	0.017	0.045	0.031	0.044	0.023	0.042	0.048	0.019	0.043
Peche Isle	0.031	0.031	*	0.019	0.047	0.037	0.048	0.027	0.044	0.051	0.024	0.051
Gross Isle N	0.037	0.023	0.038	*	0.044	0.026	0.040	0.020	0.042	0.048	0.011	0.046
Belleville	0.144	0.150	0.192	0.171	*	0.021	0.007	0.039	0.018	0.017	0.035	0.023
Black Creek	0.090	0.088	0.119	0.102	0.050	*	0.021	0.026	0.020	0.027	0.022	0.021
Deserento	0.139	0.143	0.184	0.158	**0.001**	0.039	*	0.039	0.020	0.017	0.033	0.026
Fighting Island	0.030	0.021	0.042	0.024	0.133	0.070	0.123	*	0.035	0.041	0.016	0.038
Frenchmans Bay	0.101	0.106	0.143	0.133	0.043	0.025	0.036	0.084	*	0.028	0.032	0.012
Gray's Creek	0.139	0.149	0.178	0.172	0.049	0.064	0.041	0.116	0.053	*	0.042	0.033
Gross Isle S	0.028	0.016	0.039	**0.005**	0.127	0.061	0.118	**0.005**	0.088	0.128	*	0.034
Carols Point	0.107	0.108	0.146	0.140	0.053	0.031	0.050	0.092	0.020	0.075	0.092	*
Hillmans Marsh	0.037	0.032	0.071	0.053	0.095	0.049	0.089	0.034	0.062	0.111	0.025	0.067
Jordan	0.092	0.097	0.131	0.123	0.043	0.031	0.037	0.080	0.013	0.037	0.078	0.032
Monroe	0.040	0.036	0.055	0.034	0.122	0.089	0.121	0.042	0.114	0.133	0.026	0.101
Morrisburg	0.162	0.165	0.205	0.199	0.047	0.064	0.040	0.129	0.043	0.027	0.149	0.071
Point Abino	0.072	0.077	0.108	0.084	0.052	0.014	0.037	0.060	0.018	0.057	0.056	0.038
Presqe Isle Bay	0.070	0.067	0.101	0.089	0.041	0.022	0.041	0.057	0.039	0.074	0.051	0.035
Puce River	0.031	0.019	0.049	0.020	0.145	0.078	0.136	0.018	0.101	0.132	**0.007**	0.109
Queenstown	0.082	0.084	0.113	0.098	0.043	0.012	0.034	0.066	0.021	0.060	0.062	0.038
Randalph Reef	0.096	0.099	0.139	0.127	0.036	0.028	0.032	0.080	**0.008**	0.045	0.083	**0.010**
Toronto Island	0.100	0.107	0.143	0.131	0.031	0.035	0.025	0.083	0.022	0.021	0.087	0.042
Trenton	0.132	0.141	0.178	0.161	0.010	0.042	0.010	0.117	0.027	0.031	0.117	0.051

	Hillm Marsh	Jordan	Monroe	Morris -burg	Point Abino	Presque Isle Bay	Puce River	Queens town	Randa- lph Reef	Toronto Island	Trenton
Belle Isle	0.017	0.037	0.020	0.047	0.025	0.029	0.020	0.033	0.039	0.033	0.036
Belle River	0.019	0.041	0.022	0.050	0.030	0.026	0.018	0.035	0.041	0.035	0.044
Peche Isle	0.024	0.045	0.026	0.053	0.037	0.035	0.024	0.039	0.050	0.046	0.044
Gross Isle N	0.015	0.041	0.018	0.051	0.024	0.029	0.016	0.029	0.043	0.039	0.041
Belleville	0.027	0.018	0.035	0.016	0.020	0.027	0.039	0.020	0.019	0.016	0.010
Black Creek	0.019	0.020	0.027	0.027	0.011	0.021	0.026	0.012	0.022	0.021	0.020
Deserento	0.027	0.019	0.035	0.016	0.018	0.027	0.040	0.018	0.019	0.017	0.011
Fighting Island	0.018	0.037	0.021	0.041	0.025	0.028	0.020	0.030	0.038	0.035	0.038
Frenchmans Bay	0.025	0.013	0.039	0.024	0.017	0.030	0.038	0.016	0.013	0.019	0.015
Gray's Creek	0.036	0.027	0.042	0.013	0.026	0.035	0.042	0.028	0.025	0.023	0.020
Gross Isle S	0.015	0.032	0.016	0.043	0.022	0.026	0.015	0.024	0.035	0.031	0.032
Carols Point	0.030	0.018	0.042	0.029	0.021	0.031	0.040	0.023	0.013	0.023	0.024
Hillmans Marsh	*	0.027	0.017	0.036	0.019	0.024	0.017	0.022	0.028	0.028	0.027
Jordan	0.063	*	0.035	0.023	0.019	0.031	0.039	0.017	0.013	0.016	0.013
Monroe	0.043	0.092	*	0.044	0.025	0.025	0.024	0.028	0.039	0.032	0.035
Morrisburg	0.124	0.046	0.159	*	0.025	0.035	0.045	0.025	0.024	0.024	0.016
Point Abino	0.050	0.029	0.090	0.062	*	0.022	0.025	0.013	0.018	0.020	0.018
Presqe Isle Bay	0.039	0.042	0.064	0.075	0.026	*	0.030	0.024	0.030	0.024	0.029
Puce River	0.026	0.091	0.040	0.162	0.070	0.068	*	0.030	0.038	0.035	0.037
Queenstown	0.055	0.021	0.082	0.058	0.015	0.025	0.080	*	0.019	0.019	0.018
Randalph Reef	0.062	**0.010**	0.095	0.044	0.023	0.029	0.096	0.022	*	0.014	0.017
Toronto Island	0.070	0.011	0.097	0.035	0.030	0.037	0.094	0.029	0.014	*	0.015
Trenton	0.088	0.024	0.120	0.026	0.040	0.041	0.129	0.033	0.025	0.013	*

CHAPTER 4

ADAPTIVE TRANSCRIPTIONAL RESPONSES TO POLLUTION IN THE BROWN BULLHEAD (*AMEIRUS NEBULOSUS*).

INTRODUCTION

Urbanisation and industrialisation have caused an increase in point-source pollution, especially in the aquatic environment. Many pollutants reach aquatic ecosystems through rain and runoff as well as through intentional human disposal of waste into water-bodies. This has driven pollution levels in aquatic ecosystems to extremely high levels with particularly high sediment concentrations, as sediment retains contaminants within the organic matter. Concentrations of pollutants have been reported to reach critical levels, high enough to cause animals damage such as endocrine disruption, reproductive failure and death (Ketata *et al.* 2007; Chopra *et al.* 2011; Ruiz *et al.* 2011). Organic pollutants have also been correlated to tumour rates in fish (Baumann *et al.*1996; Myers *et al.*2008).

Any environmental perturbation can cause a change in transcription levels and gene expression. The magnitude of gene transcription change will depend on the nature of the stressor (here defined as organisms' response to environmental perturbation) as well as on the initial transcription profile that the organism had prior to the stress. Resting and challenge response transcriptional profiles have been shown to differ among habitats and populations, likely reflecting both the organism's environment and local adaptation

(Fisher and Oleksiak 2007; Falciani *et al.*2008; Oleksiak 2008; Carlson *et al.* 2009; Lie *et al.*2009; Whitehead *et al.*2011). Organisms that remain in degraded habitats can either locally adapt or physiologically acclimate to maximize their fitness. Local adaptation is an evolutionary process that occurs at the population level and provides a long-term adaptive response to a given environment. Organisms can be locally adapted to both natural and degraded habitats, for example tomcod and killifish have been shown to locally adapt to polluted environments (Elskus *et al.* 1999; Meyer and Di Giulio 2002; Nacci *et al.*2010; Whitehead *et al.*2011; Wirgin *et al.*2011). However, if local adaptation does not occur, or before local adaptation has evolved, organisms may display other adaptive responses such as physiological acclimation, which is an individual physiological response to return the individual towards homeostasis. Both local adaptation and physiological acclimation will lead to a change in gene transcription at specific gene loci as a response to pollution (López-Maury *et al.* 2008). Such changes in transcription can be investigated either by examining individual genes or through technologies that have a broader, genome-wide, coverage. DNA microarrays are one such technology which have been used in several studies to characterise pollution effects on the transcriptome (Fisher and Oleksiak 2007; Carlson *et al.*2009; Oleksiak 2008; Carlson *et al.* 2009; Lei *et al.*2009; Bozinovic and Oleksiak 2011; Whitehead *et al.*2011; Vidal-Dorsch *et al.*2012). Microarrays have also been used to show that different genes can exhibit very different transcriptional profiles among sites (or populations) within the same species (Fisher and Oleksiak 2007; Whitehead *et al.*2011). This highlights the need for a broad approach across many functional groups of genes to investigate organismal responses rather than an individual gene level analysis. The majority of gene transcription

studies on pollution response have focused on cytochrome P450 1A (CYP1A) and the Aryl hydrocarbon receptor (AHR) pathway, which provides an indication of specific detoxification processes. However, if the transcriptional response or gene expression of one (or a few) gene(s) is used as a biomarker, there is a chance that the response will be biased by some form of adaptive response (see Chapter 2; Grey *et al.*2003; Meyer *et al.*2003; Wirgin *et al.*2011). Alternatively, it is possible that in the study species, the selected gene does not respond to that particular stressor in the expected fashion.

Adaptive responses may bias biomarker response, and thus, to reduce the potential for adaptive response bias, it is important to study early responses to pollutants such as transcription (Chapter 2). Later biomarkers, such as tumours, endocrine disruption or reduced reproductive success, reflect past ecological effects, and possibly evolutionary effects as well. If there are no adaptive effects (either genetic adaptation or acclimation) occurring, organisms from clean and polluted sites should respond in the same manner to an acute stress. If adaptive effects have occurred, it is unlikely that they affect all genes equally, and there may be genes and signalling pathways that may not be affected (although the function of those may not be known). To avoid the effects of adaptive responses and to detect transcriptional responses to unexpected loci, it is important that we use whole transcription investigation technologies such as microarrays or next generation sequencing (NGS) of the transcriptome. Microarrays are useful for large studies; however, they do have limitations, with high background and non-specific hybridisation substantially reducing sensitivity (Hurd and Nelson 2009) and gene coverage restricted to those that have been characterized and spotted. Next generation sequencing (NGS) provides a solution to many of the limitations associated with

microarrays; prior genome information is not needed as NGS will detect all transcribed genes. NGS can also be quantitative, qPCR as well as direct comparison to microarray data indicate that NGS may be more specific in its quantification than microarrays (Hurd and Nelson 2009; Wilhem and Landry 2009; Meyer *et al.* 2011; Garcia et al. 2012). Assuming high transcriptome sequencing coverage, NGS is highly specific and sensitive; however, coverage may become an issue of technical limitations affecting the success of any specific step leading up to a sequencing run. Coverage considerations require limiting the number of individuals per NGS run to ensure sufficient coverage. Low coverage can result in the failure to detect genes that are transcribed at low levels. The cost of NGS is still relatively high, making High sample sizes and experimental replication is unreasonably expensive.

Brown bullhead (*Ameiurus nebulosus*) have long been used as an indicator species for sediment pollution in the Great Lakes. For example, they have been shown to have increased tumour rates in the presence of polycyclic aromatic hydrocarbons (PAH; Baumann *et al.*1987, Baumann *et al.*1996). Given their history as an indicator species, and the volume of literature on their response to contaminants, surprisingly little research has been published on their molecular response to pollution. EROD has been investigated in hatchery reared brown bullhead (Watson and Di Giulio 1997; Ploch *et al.* 1998) and in wild caught fish from the Niagara River area (Eufemina *et al.* 1997). Transcriptional response was investigated in apoptosis related genes in brown bullhead cell lines (Busch *et al.*2004). CYP1A1 in Presque Isle Bay fish was also investigated using quantitative real time-PCR (qRT-PCR), with different induction between Presque Isle Bay and a reference site (Grey *et al.* 2003). As brown bullhead live in sediment, and in constant

contact with pollutants, their high tumour load is not surprising. However, brown bullhead populations also appear to be thriving in even highly polluted areas, with evidence for successful breeding in some extremely polluted areas. A pattern of viable brown bullhead populations, even under severe pollution stress, indicates possible adaptive responses in action. An investigation of brown bullhead gene expression response to acute contaminant stress using fish from both polluted and clean sites would allow a test of the potential for adaptive responses (including both genetic adaptation and physiological acclimation) to be occurring in brown bullhead that allows them to survive and persist in highly degraded habitat.

Here I describe a study that uses NGS of the whole brown bullhead transcriptome to investigate changes in transcription profile of brown bullheads from two sites within the Detroit River, one highly polluted and one less polluted, when challenged with polluted sediment. I tested for transcription differences between challenged and control brown bullhead: I predict that the fish from the contaminated site would show reduced transcriptional responses to the challenge relative to the naive fish due to a combination of adaptive effects in the population experiencing chronic pollination stress. Furthermore, I expected to see the transcriptional differences in specific functional groups of genes, while other groups would show little variation either between the two populations or in response to the challenge. It is important to investigate early adaptive responses by transcription to be able to detect and remediate degraded sites before ecological effects are too severe. This study also highlights the potential central role that evolutionary forces may have in our interpretation of ecotoxicological biomarkers.

MATERIAL AND METHODS

Sampling and Challenges: Eight brown bullhead were sampled by electro-shocking in mid-October 2009 at two different sites: Trenton Channel (TC – polluted – four fish) and Peche Isle (PI – less contaminated – four fish) in the Detroit River (Figure 4.1). In the TC, brown bullhead was the dominant species and few other species were observed, while on the other hand, the fish community at PI was highly diverse. The TC fish commonly displayed skin lesions which were absent in PI fish, this is consistent with previous reports of skin lesions (Leadley et al. 1998). Upon dissection, TC fish had dramatically dark red liver tissue, while PI fish had more normal light-brown coloured liver tissue; however, liver pathology was not investigated further. During the sampling efforts sediment was also collected with a petite ponar for contaminant analysis and for challenges. Fish were selected to minimize size differences. The fish were held separately in two aerated 4x4 meter pools filled with well water for 72 h prior to the challenge, to recover from capture stress. After the 72 h recovery, two fish from each site were randomly selected and placed in 1 meter diameter pools filled with water and sediment from the polluted TC site for 24 h ("challenge"). Two fish from each site were placed in identical pools but with clean water (PI site) and sediment for the same period of time ("control"). After 24 hours, the fish were humanely euthanized (overdose of MS222), weight was measured, and liver tissue was flash frozen and stored at -80°C. Difference in weight between groups were tested using a contingency table analysis (SPSS 16.0). Previous studies have shown that adaptive responses often occur for specific compounds to which the organism has been chronically exposed, while sensitivity remains for other compounds (Meyer *et al.* 2002; Wirgin and Waldman 2004; Brammel *et al.* 2010). As the

goal of this study was to elicit possible adaptive responses, I chose to challenge the fish with the mixed contaminant sediment from TC, which should contain compounds that those fish may have developed adaptive responses to. Had I chosen a single challenge compound, it may or may not have resulted in an adaptive response.

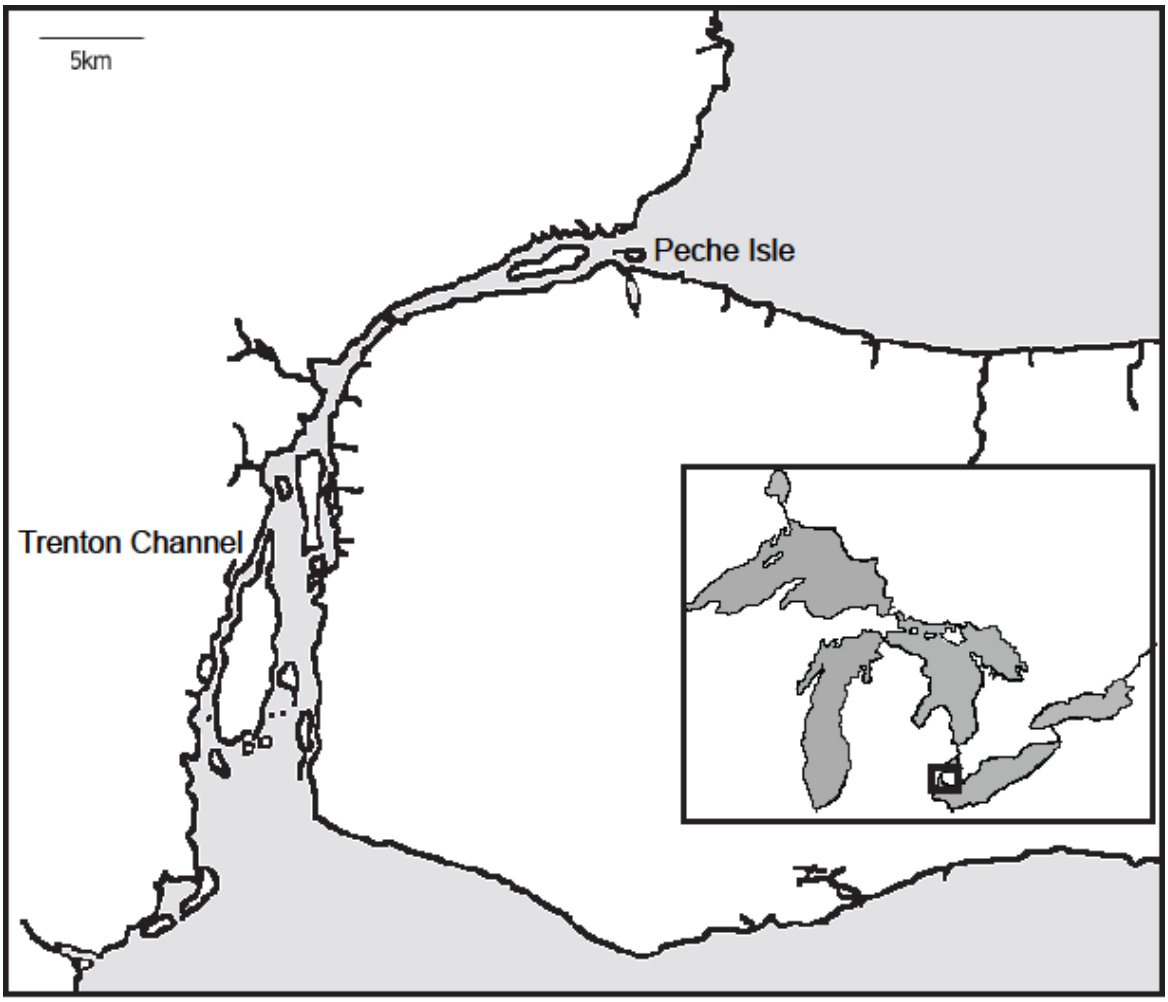

Figure 4.1. Location of sampling sites, Trenton Channel (TC) in US waters and Peche Isle (PI) in Canada, inserted is a Great Lakes map with small square indicating location of large map.

RNA extraction, Library preparation and NGS: From each fish, a 10 mg sample of liver was mechanically homogenised with glass beads in 0.75 ml TriZol (Ambion) and total RNA extraction was carried out following Chomczynski and Sacchi (1987). The total RNA was diluted in 22 μL of MilliQ H_2O. RNA quality was initially determined on a 1.8% agarose gel to ensure the RNA was not degraded and that ribosomal RNA was detected. Concentrations and quality were determined by UV-spectrophotometry (Victor 3V plate reader, Perkin Elmer), A260/A280 values between 1.9 and 2.1 were considered good quality and clean. An oligo(dT) selection was performed with GenElute mRNA miniprep kit (Sigma-Aldrich) to increase the relative concentration of mRNA to rRNA.

Preparation of the eight cDNA libraries was done with the Ion Total RNA-seq Core kit v2, the Ion RNA-seq Primer Set v2 kit and the Magnetic Bead clean-up Module (Life Technologies) following the manufacturer's protocols. Assessment of yield, fragment size distribution, and quality was performed on a Qubit 2.0 flourometer (Invitrogen Life Technologies) and Bioanalyser 2100 (Agilent Technologies, USA) using a Qubit RNA Assay kit (Invitrogen Life Technologies), Qubit dsDNA HS Assay kit (Invitrogen Life Technologies), and bioanalyser kit High Sensitivity DNA kit and RNA 6000 Pico kit (Agilent). The separate libraries were bar-coded and pooled. An emulsion PCR was run on an Ion OneTouch System (Life Technologies) using an Ion OneTouch template kit 200 bp (Life Technologies). The NGS was performed on an Ion Torrent Personalised Genome Machine (PGM) using two 318 chips, with 500 flows, with an expected yield of ~6 million reads per chip. On each chip there was one challenged and one control fish library from each site for a total of 4 libraries per chip.

Analysis: All sequences from the PGM were technical quality filtered (with ION Torrent software to remove machine-generated artefacts), and data were exported as FastQ files. The data were imported into DNASTAR, SeqMan NGen version 4.1.2(25) and assembled using the *Danio renio* package as the closest species with a reference genome. The individually bar-coded samples were pooled per treatment per site for better general coverage during assembly and analysis. The number of successfully assembled sequences was noted in DNASTAR SeqManPro version 10.1.2(20) and compared to the original number of sequences to estimate the total coverage and the level of transcription in each treatment group after rRNA and other unassembled and unassigned sequences were removed.

Transcriptome analysis: To compare gene transcription differences at both the functional group level as well as at the individual gene level, Q-seq/ArrayStar version 5.1.2 was used to initially process the sequence data, this includes quantification and normalisation of individual mRNA sequence reads per kilobase per million ("RPKM" = number of mapped reads per length of transcript (kb) per total number of reads in a million) in ArrayStar. All further analysis described below was performed using ArrayStar and data that was RPKM normalised. The total number of transcribed genes over all sites and treatments was recorded. To compare and characterise patterns of gene transcription across the transcriptome of the four treatment groups, the total number of transcripts as well as overlapping number of genes transcripts within site were recorded, so was the overlap between the two challenges.

Site comparison of transcription level: If there are adaptive responses occurring to affect the contaminant challenge response, there will be a difference between sites in the

number and/or function of genes that are transcribed when the fish are challenged. To test for the difference in transcribed genes between sites (contaminated TC vs. clean PI), the genes that showed differences in transcription in response to the challenge within each site were examined. The number of genes that had a four-fold (or higher) difference in transcription level was recorded and compared. The number of differentially transcribed genes in each treatment per site was compared in a contingency table analysis in SPSS 16.0.

Gene function and assignment comparison: To further test for adaptive responses, I determined the putative functions of the transcribed genes and organised them into functional groups. I compared membership among functional groups to see if the same functional groups were activated in both sites or if there were differences in the types of genes transcribed. Genes that were four-fold (or higher) differently transcribed within a site were used to determine which functional groups were up- vs. down-regulated. Gene ontology (GO) annotation within ArrayStar was used to assign gene function. GO terms were assigned to each unique gene based on the GO terms annotated to the corresponding homologs in the UniProt database, defining the functional groups was done following the gene ontology web site (http://amigo.geneontology.org/cgi-bin/amigo/browse.cgi?session_id=498amigo1371665781).

Candidate gene comparisons: There are several genes that are known to be involved in detoxification, and as my main interest is adaptive response to pollution, I investigated the transcription level for those specific genes. Genes that were included were CYP1A1, aryl hydrocarbon receptor 1 and 2 (AHR1 and 2), aryl hydrocarbon receptor nuclear translocator (ARNT), heat shock protein 90 (hsp90), and aryl

hydrocarbon receptor integrating protein (AIP), for organic pollutants, glutathione S-Transferase (GST) for genotoxic or carcinogenic compounds and metallothionein (MT or MTa) and superoxide dismutase 1 (SOD1) and superoxide dismutase 2 (SOD 2) for heavy metals.

There are other genes that are not traditionally identified as "detoxifying", but rather characterized as "responding to xenobiont stimulus", I also investigate their transcription level. The GO annotation was used to assign transcribed genes to the response to xenobiont stimulus category, and I explored their transcription among treatments and sites.

Outlier transcription response analysis: To account for the possibility of adaptive responses at unknown or unexpected genes, the functions of genes that had very high up- or down-regulation (8-fold or more) between treatments within a site were also explored.

RESULTS

Fish weight varied between 181 g and 278 g and the average for TC was 260 ± 19g and the PI average was 199 ± 15 g, though there was no significant difference between sites or treatments (P=0.24; Table 4.1). TC sediment is three times higher in polycyclic aromatic hydrocarbon (PAH) and almost twice the polychlorinated biphenyls (PCB) than PI sediments (Figure 4.2; Drouillard pers. communication). The total body burden of total PCB in eggs from TC brown bullhead females is significantly higher (536.2 ±11.7 μg/kg) than for females from PI (70.1 ±0.1 μg/kg; Farwell *et al.* 2012).

Table 4.1. Average length and weight for each brown bullhead from each site (TC = Trenton channel; PI = Peche Isle) used in the two treatments (challenged = the polluted sediment challenge; control = control sediment)

Origin	Treatment	Length (cm)	Weight (g)
TC	challenged	28	272
TC	challenged	28	278
TC	**average**	**28**	**275**
TC	control	28	253
TC	control	28	236
TC	**average**	**28**	**244.5**
PI	challenged	30	204
PI	challenged	26	195
PI	**average**	**28**	**199.5**
PI	control	27	217
PI	control	27	181
PI	**average**	**27**	**199**

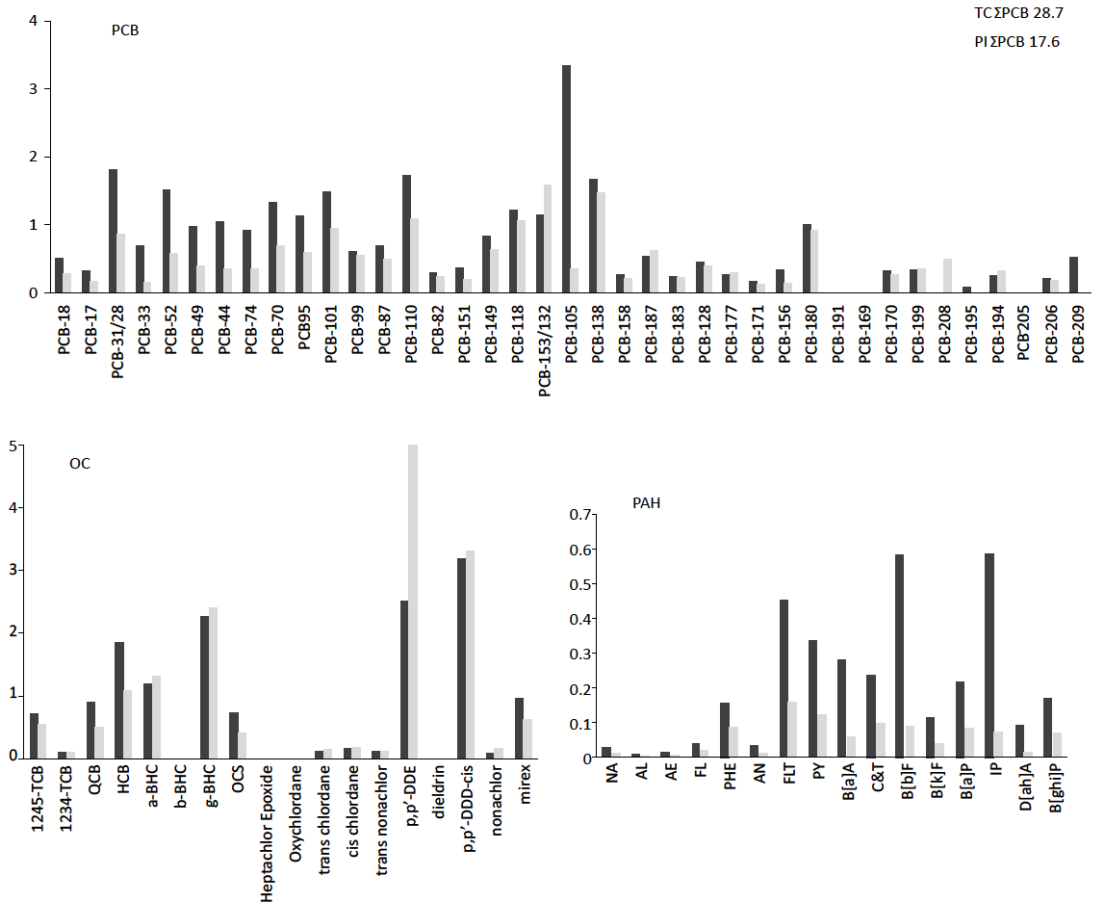

Figure 4.2. Sediment concentrations of PCBs, OCs and PAHs for polluted Trenton Channel (TC - dark bars) and the less polluted Peche Isle (PI - light bars). Data from Ken Drouillard (unpublished data).

Next generation sequencing data: There were initially 8.7 million reads from the two NGS runs; however, ~1.3 million sequences were excluded due to quality control or short read length. The final usable number of sequences was 7.4 million (7 386 421), with the number of reads per site and per treatment ranging from 1.6 to 2 million (Table 4.2). After the assembly analysis had excluded non-assembled and rRNA sequences, the number of reads in both of the PI treatments was lower than the TC treatments (Table 4.2). Assembled sequences had an average length of 97 base pairs, ranging from 95 to 99 base pairs (Table 4.2).

Table 4.2 Sequence distribution from each site (TC = Trenton channel; PI = Peche Isle) and for each treatment (challenged = the polluted sediment challenge; control = control sediment). The number of reads that were from the PGM (# reads PGM), number of sequences that were assembled (#assembled), and the percentage of sequences that were assembled (% assembled) are shown. The average length of assembled reads for each site per treatment (aver. read length)

Site and treatment	# read PGM	# assembled	% assembled	Aver. read length
TC challenged	1 809 715	931 289	51 %	99 base pairs
TC control	1 638 610	1 062 739	65 %	95 base pairs
PI challenged	1 937 510	693 961	36 %	99 base pairs
PI control	2 000 586	680 454	34 %	96 base pairs
Total	**7 386 421**	**3 368 443**	**45.6 %**	**97 base pairs**

Transcriptome analysis: After assembly, the total number of transcribed genes identified was 5 515 (for complete list: http://www.uwindsor.ca/glier/reference-material) across all the data; however, there was substantial variation in their representation among sites and treatments: many genes were only transcribed in one group (Figure 4.3). There was relative high overlap between the replicate fish from each site-by-treatment group, with exception of TC challenged fish which display little gene transcription overlap indicating that sampling more individuals for this group would be an ideal (Figure 4.4). PI had a higher number of transcribed genes in total (N=4418), with over 3000 transcribed genes in each treatment (challenged N = 3183, control N = 3467), and 1933 genes in shared in both treatments (Figure 4.3). TC had a lower number of total transcribed genes (N= 3015), but with more transcribed genes in the challenge than the

control treatment (challenged N = 2522, control N = 1378) with 885 genes overlapping (Figure 4.3). There were 2217 genes that were transcribed in both PI and TC, 798 that were only transcribed in TC and 2500 only in PI (Figure 4.5a). The two challenge treatments had 1572 transcribed genes in common (Figure 4.5b) which is 49% of the transcripts for the challenged fish in PI and 62% for same treatment in TC. The PI control fish had the highest number of uniquely transcribed genes (1096), while the TC control had the lowest number of uniquely transcribed genes (193; Figure 4.3). There were only 704 genes that overlap in both populations in both treatments (Figure 4.3).

Site comparison of transcription level: Within each population there were a different number of genes transcribed both in the challenged fish and the control fish. PI had twice as many genes than TC that were transcribed in both treatments (1933 vs. 885 in TC; Figure 4.5; Figure 4.6). At the four-fold difference in transcription level, the number of genes that were differently transcribed was relative equal between the sites. TC had 108 differently transcribed while PI had 99. However in the challenged TC the up- vs. down-regulated gene transcription were highly divergent with 99 genes up-regulated and 9 genes down-regulated ($P<0.001$), while the PI transcription pattern was more balanced with 42 genes down-regulated and 54 up-regulated ($P\geq0.05$).

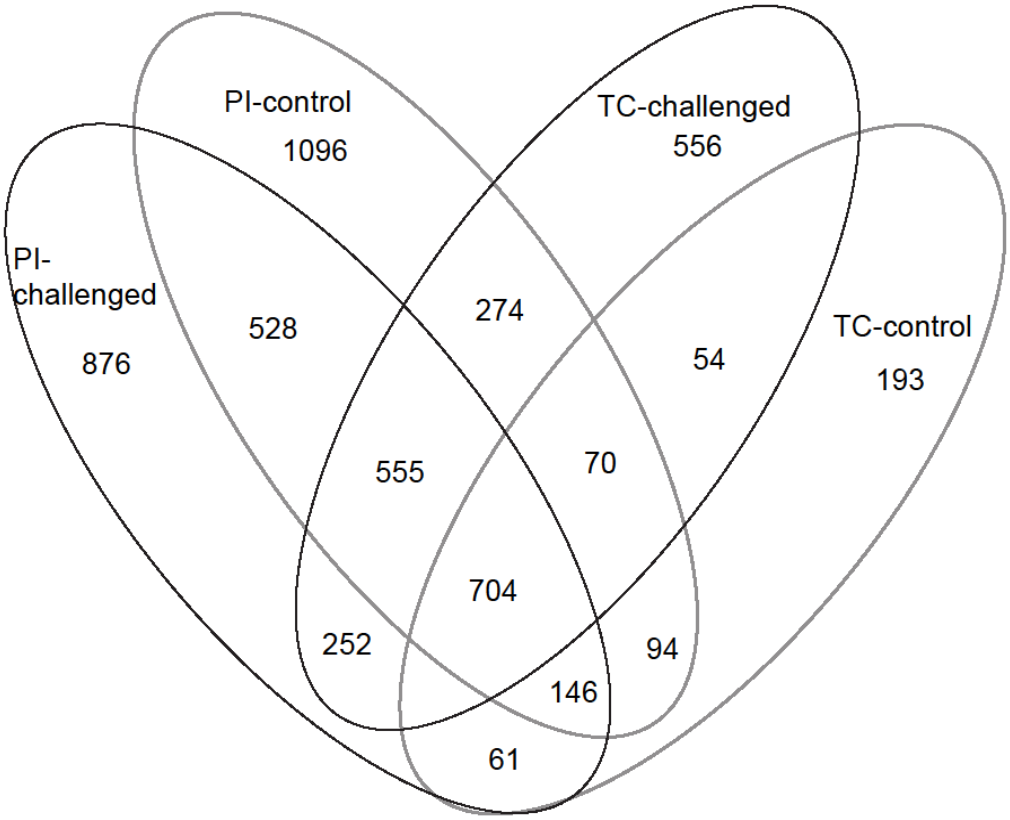

Figure 4.3 A Venn diagram showing the pattern of transcribed gene sharing among the 4 site-by-treatment brown bullhead groups**.** The number of transcribed genes is displayed for all intersecting and unique groups based on the different sites and treatments.

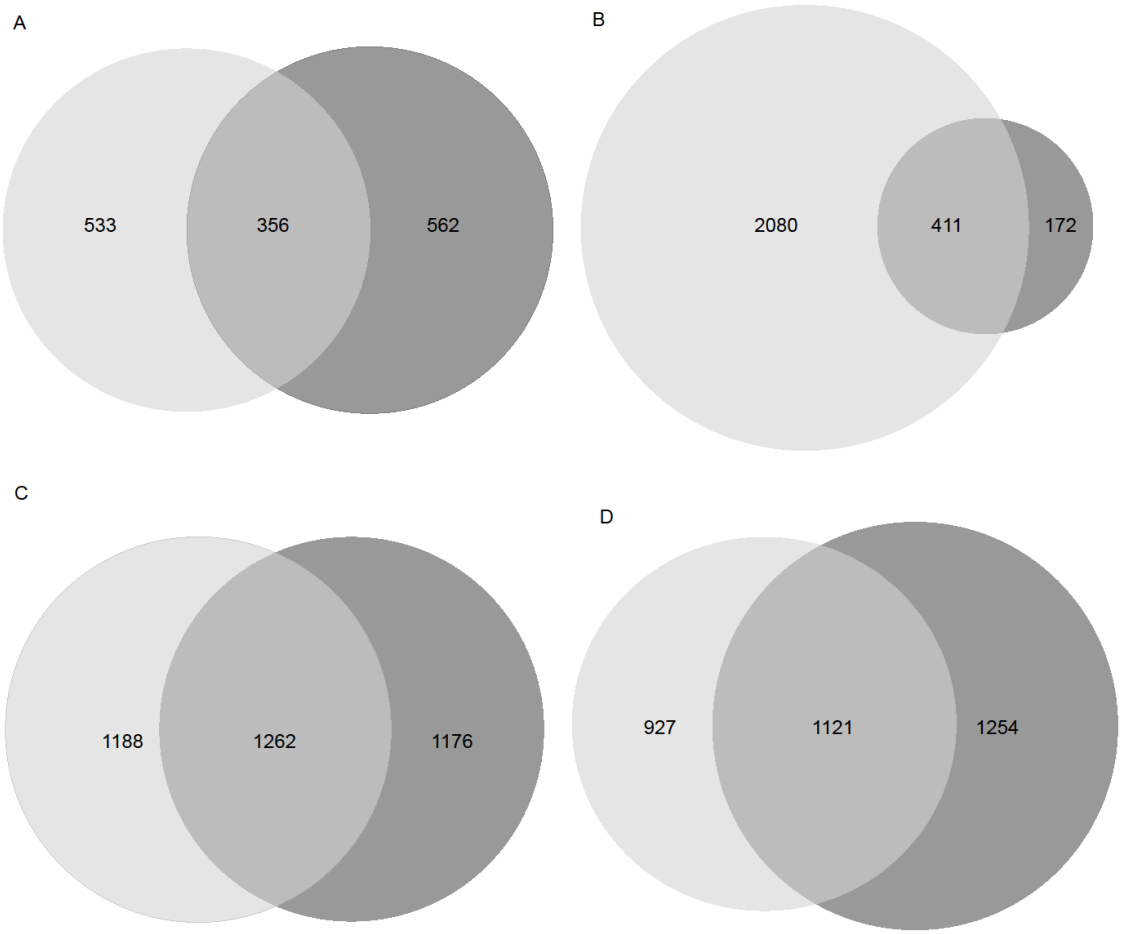

Figure 4.4. The level of overlapping genes transcribed for replicate individuals within a treatment for each site-by-treatment group. Panel A is TC control, panel B is TC challenged, panel C is PI control and D is PI challenged. Numbers on the circles refer to total number of genes transcribed.

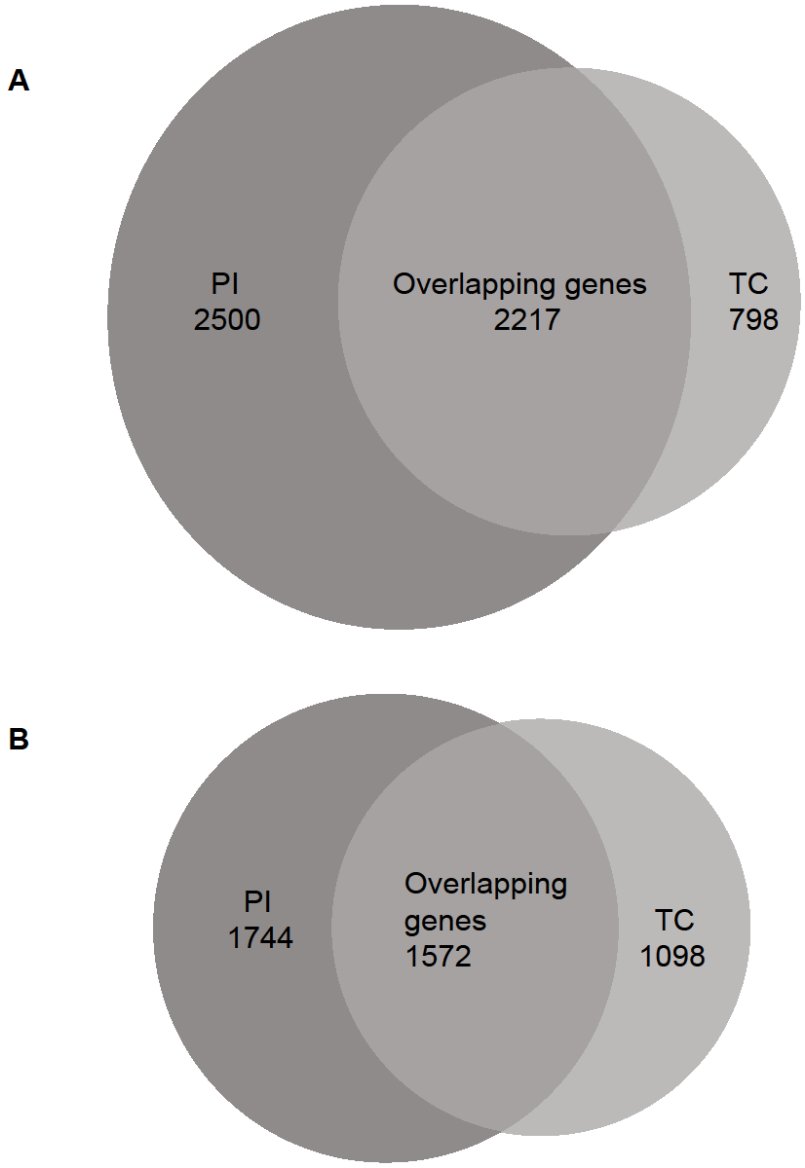

Figure 4.5 Venn diagrams showing the distribution of the 5515 transcribed genes between the populations. Panel A: There are 2217 genes overlapping between the populations, PI has 2500 genes that are only expressed within PI (dark grey) while TC has 798 genes that are unique (light grey). Panel B Overlap between the two sites when they were challenged is shown, 1573 transcribed genes overlap between PI (49%) in dark grey and TC (62%) in light grey.

Gene function and assignment comparison: I classified the function of differently transcribed genes into broad functional groups (pathways) and 21 functional groups were identified (Figure 4.7 for definitions see supplementary information). As there were multiple genes transcribed in each functional group, both up- and down-regulated transcriptional responses to the polluted sediment challenge are identified (Figure 4.7). Also, a single gene may be included in multiple functional groups, thus the total number of genes across all functional groups may be more than the total number of genes that were differentially transcribed. Genes with unknown function are not assigned to any functional groups. The individual functional groups were further categorised under the broad areas of; Biological Processes, Cellular Components, and Molecular Functions (Figure 4.7). The 99 genes that were up-regulated (at 4X) under the challenge in TC fish occurred in 20 functional groups, and the 9 down-regulated transcribed genes were in 8 functional groups (Figure 4.7). The PI fish exhibited 54 up-regulated genes in 13 functional groups, and 42 down-regulated genes in 14 functional groups (Figure 4.7). Among the functional groups, metabolic processes and multicellular organismal processes have only up-regulated transcription levels in the challenged fish. There are several of the functional groups that are up-regulated in the TC challenged fish (i.e., cellular processes, metabolic processes, single organism processes, cell part, and binding) - these groups also were up-regulated in PI fish. There are four groups that showed up-regulation in the TC challenge, but that did not appear in PI the fish (i.e., growth, cell junction, extracellular region part, and organelle part). Response to stimulus, membrane part, structural molecular activity, and transporter activity show variable regulation patterns: up-regulated in TC but down-regulated in PI.

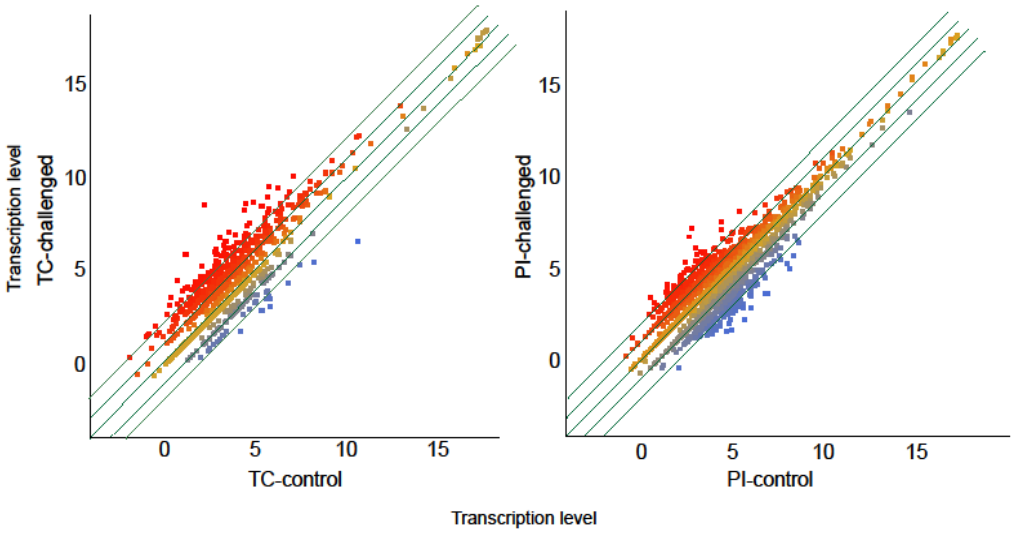

Figure 4.6. Scatterplot showing estimated (log2 transformed) transcription levels for fish held on clean (control) versus contaminated (challenged) sediment for two populations of brown bullhead. In TC there are 885 genes that are transcribed in both challenge and control, at a 4-fold transcription level difference there are 108. In PI there are 1933 genes that are transcribed in both the challenge and control, at a 4-fold transcription level difference there are 99. The middle line is the 1:1 ratio – no difference in transcription, the dots are yellow. Outside of the 1:1 line are the 2-fold and the 4-fold changes in transcription, up-regulated genes have in red dots while down-regulated have blue.

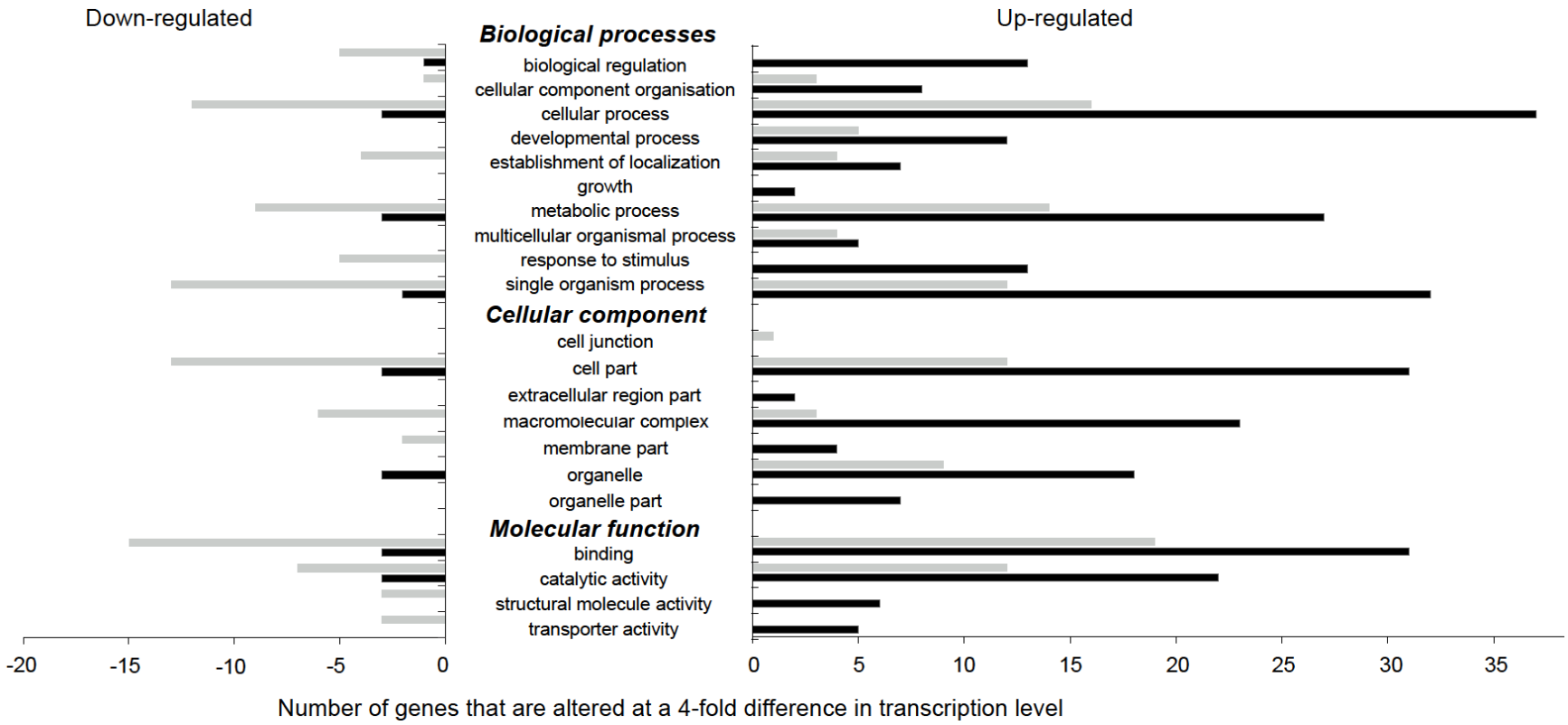

Figure 4.7. Gene transcription in challenged relative to control treatment brown bullhead organized by gene function groups (based on gene ontology). Only genes showing 4-fold or more difference in transcription level are included. The down-regulated are to the left and up-regulated are to the right. The two populations are shown as black bars (TC) and grey bars (PI) Definitions for functional groups can be found in the supplementary information.

Candidate gene comparison: None of the detoxification genes were significantly up- or down-regulated, in fact most were only expressed in the challenged fish (in TC or PI), so fold differences could not be calculated. CYP1A1 was only expressed in the challenged PI fish, while AHR2 and ARNT were only transcribed in challenged TC fish. Hsp90 was transcribed at high levels in fish from both sites and groups, but was up-regulated in challenged TC fish. AIP and GST were not detected at all. MTa was transcribed in both the challenged groups, but in neither control group. SOD2 was transcribed in the PI challenged and control fish as well as the TC challenged fish but not in the TC control fish; SOD1 transcripts were not detected at all.

Using the GO annotation to explore the "responding to xenobiont stimulus" group, there were five genes that were identified as such and were present in the transcriptome of the brown bullhead in this experiment: AHR2, estrogen receptor (esr1), CYP1A, cytochrome P450 3a65 (CYP3a65), and vitellogenin (vtg1). All but vtg1 only occurred in challenged treatments. Vtg1 was transcribed in both control and challenged treatments in TC fish, but was highly (26 fold) up-regulated in the challenge treatment. The AHR2 and esr1 genes were transcribed in only the TC challenged fish, at low levels. CYP1A and CYP3a65 were transcribed only in the PI challenged fish at low levels. The genes that have been described as responding to xenobiont stimuli detected in the brown bullhead transcriptome all show evidence for up-regulation under contaminated sediment challenge.

Outlier transcription response analysis: There were 24 genes that were highly differently transcribed (eight or higher fold difference) in TC fish, only one of them was down-regulated, the remaining 23 were up-regulated in response to the challenge (Table

4.3). In the PI fish there were ten genes that were highly differently transcribed, with four up- and six down-regulated in response to the challenge (Table 4.3). These genes had a range of functions with no consistent functional patterns, this is perhaps not surprising as the only common factor among them is an arbitrary level of differential gene transcription. Most of the identified genes were part of the Biological Processes category, with metabolism and cellular processes as the main contributors, although also binding and catalytic activity (Molecular Functions) were present. In the TC fish, there were two genes that were of interest; vitellogenin (vtg) and heme oxygenase (decycling) 1 (hmox1), both were highly up-regulated (Table 4.3). In the PI fish, growth arrest and DNA damage inducible protein (gadd45) were highly up-regulated in response to the contaminant stress. Another gene of interest in the PI fish response was signal transduction and the activator of transcription (stat5.1) gene, which was highly down-regulated. Stat genes are involved in development and function of the immune system, and but also in tumour control (Meinke *et al.*1996).

Table 4.3. Highly differently transcribed genes within sites (A) Trenton channel and (B; on the following page) Peche Isle. The gene symbol is the identification that was given by DNAStar, the fold change reflects the change in the challenge vs. the control. The function of the transcribed genes was gathered with the gene symbol from UniProt for each transcript.

A

Gene symbol	Fold change	Function
usp5	8.0	regulation of transcription, metal ion binding
hmgb1	8.2	--------
eno2	8.5	glycolysis
LOC100330675	8.5	--------
COX2	8.8	electron transport
hspa5	9.0	nucleotide binding, ATP binding
ncor1	9.4	DNA binding
sox5	9.4	transcription factor
nucks1a	9.4	phosphorylation
si,ch73-131e21.5	9.4	protein transport
zgc,123327	10.1	--------
eno1	10.4	glycolytic enzyme
plcl1	10.5	phospholipid binding, lipid metabolism
hmox1	12.3	redox response to chemical stimulus and hypoxia
kif13ba	13.2	ATPbinding, microtubili associated
sox11b	13.2	response to wounding
rpl36	14.1	ribosomal
rpl30	14.1	ribosomal
zgc,162608	15.1	--------
si,xx-by187g17.1	18.9	binding, transport
vtg1	26.4	lipid transport, response to estradiol, xenobiont, chemical
vtg7	85.3	lipid transport
try	-16.3	catalytic activity

B **Gene symbol**	**Fold change**	**Function**
bzw1a	9.0	regulation of transcription
gadd45aa	9.0	regulation of cell cycle, response to stress
ik	17.9	heart contraction
dkc1	23.5	RNA processing and binding
LOC555748	-8.9	--------
ppa1	-8.9	Mg ion binding
ppdpfb	-8.9	cell differentiation
tmem169	-9.8	--------
ube4b	-9.8	protein degradation
stat5.1	-10.7	regulate transcription, immune function

DISCUSSION

Site comparison of transcription level: There were substantial differences between transcriptomes between the Trenton Channel (TC) and Peche Isle (PI) fish reflecting fundamental differences in the way the fish from these populations respond to challenges. The PI fish had overall higher numbers of transcribed genes then TC fish. This is despite TC having a higher number of assembled sequences (sequence depth); which would indicate that the picture is not an artefact of sequence depth but true transcriptome differences between TC and PI fish. PI fish had about the same number of genes in the control and the challenge transcriptomes. There can be different reasons for this pattern, perhaps the PI fish were not responding specifically to the contaminant challenge but rather having a more transcriptome-wide response. The PI fish do have a large proportion of transcripts that are differentially expressed between treatments, so there is a possibility the fish in the challenged treatment are initiating detoxification, but perhaps for them to mount the full response takes time, as it is a novel challenge. The TC fish had twice the number of genes transcribed under challenge relative to control conditions. These genes were biased towards up-regulation (as opposed to PI where it was about 50:50; Figure 4.6). TC fish may be genetically adapted to deal with the contaminants, or physiologically acclimated with a fast response due to previous exposure. Either of the adaptive responses may explain the difference in overall transcription.

Gene function and assignment comparison: When considering the different gene functional groups that have transcription levels that are up-/down-regulated (four fold or higher), it is clear that TC fish generally have much more up-regulation. The most up-regulated groups include; cell processes, metabolic processes, single organism processes,

cell part, binding and catalytic activity. Those functional groups are also up-regulated in PI fish; however, they also have substantial numbers of genes that are down-regulated.

Certain genes are expected to respond to both stressors and contaminant challenges, such genes come under the "response to stimuli" category. Interestingly, the genes characterised as "response to stimulus" that show a 4-fold difference in transcription are only up-regulated in TC fish and only down-regulated in PI fish. The response to stimuli functional group of genes include a wide variety of responses, such as response to chemicals, immune response, redox state, and many more. The pattern of up- and down-regulation between the PI and TC fish in this class of genes indicates that the fish from TC respond to the challenge with a consistent induction of gene transcription, while the PI fish do not show such a pattern,. Other functional groups of genes that are up-regulated in TC fish and down-regulated in PI fish include "membrane part" with diverse functions such as proton transport and respiratory chain. The other two groups are transporter activity (transport of a variety of cellular components from vitamins to xenobionts), and structural molecular activity.

The emerging pattern of transcriptional response to the pollution challenge is that fish from TC and PI respond differently to the challenge. More specifically, the TC fish exhibit a more consistent pattern of up-regulation for genes known or suspected to be important in an adaptive response to contaminants stress, while the PI fish appear to mount a less focussed response that may reflect either different timing of transcription, or a lack of previous exposure. Additionally, the transcriptome approach used here provides an overview of the differences (and similarities) in transcriptional regulation between the two populations, highlighting the need to use broad, multi-gene transcriptional assays.

Candidate gene comparison: Previous work has identified genes and pathways that are active in detoxification processes in the contamination response. These genes were expected to respond to the present challenge, and although some of them did, I expected them to have a higher and more consistent response. The PI fish that are normally not exposed to the high pollution levels they faced in the challenged were expected to have a more acute response. CYP1A is only expressed in the challenged PI fish (though at low levels) indicating that they are responding appropriately to the challenge; however at lower levels than expected. It is possible that the PI fish required more than a 24 h challenge to mount a full CYP1A response. Indeed previous work in other species indicates that CYP1A mRNA synthesis may peak only after a much longer exposure time (up to 3 – 6 days after initiating exposure; Courtney *et al.*1999; Durieux 2012; Ruiz *et al.*2012). A response delay may also be why I observed no noticeable induction of ARH, ARNT, GST and AIP in the PI fish. The fish from TC that are normally under chronic pollutant exposure were also expected to express detoxifying genes in response to the challenge. The TC fish did not display any CYP1A mRNA induction, and this could again be due to response delay. Interestingly, there have been several studies showing a lack of CYP1A induction (or very low induction) in fish experiencing chronic exposure (Wirgin *et al.*1996; Wirgin and Waldman 1998; Meyer *et al.*2002; Nacci *et al.*2002; Grey *et al.*2003; Meyer *et al.*2003; Wirgin and Waldman 2004; Kilemade *et al.*2009; Brammell *et al.*2010; Brammell *et al.*2013). That anomalous low induction of CYP1A in response to chemicals that the fish are chronically exposed to has been interpreted as physiological acclimation or genetic adaptation effects (Meyer *et al.*2002; Grey *et al.*2003; Meyer *et al.*2003; Wirgin and Waldman 2004; Fisher and

Oleksiak 2007; Carlson *et al.*2009; Kilemade *et al.*2009; Nacci *et al.*2010; Whitehead *et al.*2011; Wirgin *et al.*2011; Brammell *et al.*2013).

Both AHR2 and ARNT transcripts were found at low levels in TC fish (indicating use of the AHR pathway) though they were absent from PI fish. This is consistent with TC fish having an altered AHR response relative to the PI fish. TC fish may increase their AHR transcription faster as part of a previously primed response to rapidly changing heavy pollutant loads. A longer exposure time may have allowed the difference between populations to reach significance. The AHR pathway is important to an organism's ability to survive contaminant stress, and the observed pattern may result from previous exposure driving an adaptive response.

Both TC and PI fish are exposed to relative high metal levels in their native environments (Szalinska *et al.* 2006), though the levels in TC are higher. This implies that all the fish are chronically exposed, and hence it is not surprising that fish from the two sites mount similar responses with induced MT and SOD. MT and SOD respond with up-regulation to heavy metals in bacteria, marine invertebrates and fish (Roesijadi 1994; Monserrat *et al.*2007; Kim *et al.* 2011; Navarro *et al.* 2011; Bervoets *et al.* 2013; Fang *et al.* 2013)

Outlier transcription response analysis: There are a number of genes that exhibited extreme transcriptional responses to the challenge (either up- or down-regulation). Of particular interest is the heme oxygenase (decycling) 1 (Hmox1) gene in the TC fish. Hmox1 is involved in redox reactions and responses to hypoxia or chemical stimuli, and has been shown to respond to PAH as a part of the phase II enzyme response (Bekki *et al.*2012). The heat shock 70kDa protein 5 (hspa5) was highly over expressed in

the TC challenged fish and is involved in stress response, it has been shown to have a regulatory function in stress response to environmental damage (Falahatpisheh *et al.*2007). Enolase 1 (eno1) was also up-regulated in TC fish, and is a glycolytic enzyme in mammals with a short isoform functional as a tumour suppressant (Feo *et al.*2000), while it is unknown what function these genes have in fish, it is likely that eno1 has similar functions in fish. None of those genes are up-regulated in the PI fish, although the PI fish showed highly up-regulated growth arrest and DNA-damage-inducible alpha a gene (gadd45aa) transcription. Gadd45aa is another stress-related gene that is among those active in demethylation and DNA repair (Dengke *et al.*2009; Niehrs and Schäfer2012). Bugiak and Weber (2009) found that Benzo[a]Pyrene did not induce CYP1A in liver of *Danio renio*, but instead found an increase of cyclooxygenase 1 (COX1) and cyclooxygenase (COX2), indicating the possibility of alternative pathways for detoxification or contaminant responses. In my experiment, there is no CYP1A induction in the liver of the challenged TC fish, but COX2 is among the genes that stand out as being highly up-regulated (8.8 times). There is a possibility that TC fish have adopted an alternative pathway to handle extreme contamination in a similar way as *D. renio* displayed. Other genes that are highly differentially transcribed are vitellogen (vtg) 1 and vtg7, in fact vt7 in TC fish exhibited the highest fold change of all gene detected in this study (85-fold). Vitellogens are lipid transporters and constitute the main egg-yolk protein, but they are also biomarkers for environmental estrogen. Vtg1 and esr were found to be present in significantly higher levels in the challenged TC than in the control. There are several other genes that exhibit very high transcriptional responses to contaminant exposure in the TC and PI fish, but their known or suspected function in

other animals do not make them obvious candidates for contaminant stress response. Perhaps they have an unsuspected detoxification or stress response roles, clearly they are interesting candidate genes for future ecotoxicological study.

Conclusion: The brown bullhead from the polluted Trenton Channel and the fish from the cleaner Peche Isle habitats are responding to the pollution in the sediment in the challenge, and the nature of their responses is fundamentally different. Although this experiment cannot conclusively show that such differences are adaptive (either acclimation or genetic adaptations), the skew towards up-regulated transcription in the challenged TC fish is certainly consistent with an adaptive response. When one considers the known and suspected function of the differentially transcribed genes, I would argue that there are adaptive responses occurring in the TC fish exposed to contaminated sediment. To determine whether these differences are due to physiological acclimation or genetic adaptation, a similar analysis of F1 offspring (or preferably second generation offspring fish – see Chapter 2) would be necessary. Other research focusing on this question in the Detroit River brown bullhead has shown both acclimation (Robinson 2011; Farwell *et al.*2012) and genetic adaptation (Breckels and Neff 2010) in molecular and whole organism traits. The fish from TC do not respond to pollution in the same manner as the naïve fish from PI, and this could affect our interpretation of hazard levels if brown bullhead from TC are used as bioindicators.

REFERENCES

Baumann, P. C., W.D. Smith, and W. K Parland. 1987. Tumor frequencies and contaminant concentrations in brown bullhead from an industrialized river and a recreational lake. Transaction of the American Fisheries Society 166:79-86

Baumann, P. C., I. R. Smith, and C. D. Metcalfe. 1996. Linkage between chemical contaminates and tumor in benthic Great Lakes fish. Journal of Great Lakes Research 22:131-152

Bekki, K., H. Takigami, G. Suzuki, A. Toriba, N. Tang, T. Kameda, and K. Hayakawa. 2013. Gene expression changes of phase I and II metabolizing enzymes induced by PAH derivates. Polycyclic Aromatic Compounds 32:141-153

Bervoets, L., D. Knapen, M. De Jonge, K. Van Campenhout, and R. Blust. 2013. Differential hepatic metal and metallothionein levels in three feral fish species along a metal pollution gradient. Public Library of Science One 8:1-11

Bozinovic, G. and M. F. Oleksiak. 2011. Genomic approaches with natural fish populations from polluted environments Environmental Toxicology and Chemistry 30:283-289

Brammell, B. F., D. J. Price, W. J. Brige, E. M. Harmel-Leaws, and A. A. Elskus.2010. Differential sensitivity of CYP1A to 3,3',4',4-tetrachlorobiphenyl and benzo(a)pyrene in two *Lepomis* species. Comparative Biochemistry and Physiology, Part C 152:42-50

Brammell, B. F., D. J. Price, W. J. Brige, and A. A. Elskus. 2013. Lack of CYP1A responses in species inhabiting chronically contaminated habitats: two varieties of resistance? Comparative Biochemistry and Physiology Part C 157:212-219

Breckels, R.D., and B.D. Neff. 2010. Pollution-induced behavioural effects in the brown bullhead (*Ameiurus nebulosus*). Ecotoxicology 19:1337-1346.

Bugiak, B., and L. P Weber. 2009. Hepatic and vascular mRNA expression in adult zebrafish (*Danio rerio*) following exposure to benzo-a-pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Aquatic Toxicology 95:299-306

Busch, C. R., D. D. Heath, and A. Hubberstey. 2004. Sensitive genetic biomarkers for determining apoptosis in the brown bullhead (*Ameiurus nebulosus*) Gene 329:1-10

Carlson, E. A., N. K. Roy, and I. I. Wirgin. 2009. Microarray analysis of polychlorinated biphenyl mixture-induced changes in gene expression among Atlantic tomcod populations displaying differential sensitivity to halogenated aromatic hydrocarbons. Environmental Toxicology and Chemistry 28:759-771

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical biochemistry 162:156-159

Chopra, A. K., M. K. Sharma, and S. Chamoli. 2011. Bioaccumulation of organochlorine pesticides in aquatic system – an overview. Environmental Monitoring and Assessment 173:905-916

Courtenay, S. C., C. M. Grunwald, G. Kreamer, W. L. Fairchild, J. T. Arsenault, M. Ikonomou, and I. I. Wirgin. 1999. A comparison of the dose and time response of CYP1A1 mRNA induction in chemically treated Atlantic tomcod from two populations. Aquatic Toxicology 47:43-69

Dengke, K. M., J. U. Guo, G-I. Ming, and H. Song. 2009. DNA excision and repair proteins and Gadd45 as molecular players for active DNA demethylation. Celle Cycle 8:1526-1531.

Durieux, E. D. H, R. E. Connon, I. Werner, L. S. D'Abronzo, P. S. Fitzgerald, J. L. Spearow, and D. J. Ostrach. 2012. Cytochrome P4501A mRNA and protein induction in striped bass (*Morone saxatilis*). Fish Physiology and Biochemistry 38:1107-1116

Elksus, A. A., E. Monosson, A. E. McElroy, J. j. Stegeman, D. S. Woltering. 1999. Altered CYP1A expression in *Fundulus heteroclitus* adults and larvae: a sign of pollutant resistance? Aquatic Toxicology 45:99-113

Eufemia, N. A., T. K. Collier, J. E. Stein, D. E. Watson, and R. T. Di Giulio. 1997. Biochemical responses to sediment-associated contaminates in brown bullhead (*Ameiurus nebulosus*) from the Niagara River ecosystem. Ecotoxicology 6:13-34

Falahatpisheh, H., A. Nanzez, D. Montoya-Durango, Y. Qian, E. Tiffany-Castiglioni, and K. S. Ramos. 2007. Activation profiles of HSPA5during the glomerular mesanglial cell stress response to chemical injury. Cell Stress and Chaperones 12:209-218

Falciani, F., A. M. Diab, V. Sabine, T. D. Williams, F. Ortega, S. G George, and J. K. Chipman. 2008. Hepatic transcriptomic profiles of European flounder (*Platichthys flesus*) from field sites and computational approaches to predict site from stress gene responses following exposure to model toxicants. Aquatic Toxicology 90:92-101

Fang, Y., H. Yang, B. Liu, and L Zhang. 2013. Transcriptional response of lysozyme, metallothionein, and superoxide dismutase to combined exposure to heavy metals and

bacteria in *Mactra veneriformis*. Comparative Biochemistry and Physiology, Part C 157:54-62

Farwell M., K. G. Drouillard, D. D. Heath, T. E Pitcher. 2012. Acclimation of life-history traits to experimental changes in environmental contaminant concentration in brown bullhead (*Ameiurs nebulous*) Environmental Toxicology and Chemistry 31:863-869

Feo, S., D. Arcuri, E. Piddini, R. Passantino, and A. Giallongo. 2000. ENO1 gene product binds to the c-myc promoter and acts as a transcriptional repressor: relationship with Myc promoter-binding protein 1 (MBP-1). Federation of European Biochemical Societies Letters 473:47-52

Fisher, M. A. and M. F. Oleksiak. 2007. Convergence and divergence in gene expression among natural populations exposed to pollution. BMC Genomics 8:108

Garcia, T. I., Y. Shen, D. Crawford, M. F. Oleksiak, A. Whitehead, and R. B. Walter. 2012. RNA-Seq reveals complex genetic response to deepwater horizon oil release in *Fundulus grandis*. BMC Genomics 13:474

Grey, J. P., T. L. Leas, E. Obert, D. Brown, G. C. Clark, and J. P. Vanden Heuvel. 2003. Evidence of aryl hydrocarbon receptor ligands in Presque Isle Bay of Lake Erie. Aquatic Toxicology 64:343-358

Hurd, P. J., and C. J. Nelson. 2009. Advantage of next generation sequencing versus the microarray in epigenetics. Briefings in Functional genomics and proteomics 8:174-183

Ketata, I., W Smaoui-Damak, F. Guermazi, T. Rebai, and A. Hamza-Chaffai. 2007. *In situ* endocrine disrupting effects of cadmium on the reproduction of *Ruditapes decussates*. Comparative Biochemistry and Physiology, Part C 146: 415-430

Kilemade, M., M. G. J. Hartel, J. O'Halloran, N. M. O'Brien, D. Sheehan, C. Mothersill, and F. N. A. M. van Pelt. 2009. Effects of contaminated sediment from Cork Harbour, Ireland on the cytochrome P450 system of turbot. Ecotoxicology and Environmental Safety 72:747-755

Kim, B-M., J-S. Rhee, G. S. Park, J. Lee, Y-M Lee, J-S. Lee. 2011. Cu/Zn- and Mn-superoxide dismutase (SOD) from the copepod *Tigriopus japonicus*: Molecular cloning and expression in response to environmental pollutants. Chemosphere 84: 1467-1475

Lie, K. K., A. Lanzen, H. Berelid, and P. A. Olsvik. 2009. Gene expression profiling in Atlantic cod (*Gadus Morhua L.*) from two contaminated sits using a custom-made cDNA microarray. Environmental Toxicology and Chemistry 28:1711-1721

López-Maury, L., S. Marguerat, and J. Bähler. 2008. Tuning gene expression to changing environments: from rapid responses to evolutionary adaptation. Nature Reviews Genetics 9:583-593

Meinke, A., F. Barahamand-Pour, S. Wöhrl, D. Stoiber, and T Decker. 1996. Activation of different Stat5 isoforms contributes to cell-type-restricted signalling in response to interferon's. Molecular and Cellular Biology 16:6937-6944

Meyer, J. N., and Di Giulio. 2002. Patterns of heritability of decrease EROD activity and resistance to PCB 126-induced tertatogenesis in laboratory-reared offspring of killifish (*Fundulus heteroclitus*) from a creosote-contaminated site in the Elisabeth River, VA, USA. Marine Environment Research 54:621-626

Meyer, J. N., D. E. Nacci, R.T. Di Giulio. 2002. Cytochrome P4501A (CYP1A) in killifish (*Fundulus heteroclitus*): heritability of altered expression and relationship to survival in contaminated sediments. Toxicological Science 68:69-81

Meyer, J. N., D. M. Wassenberg, S. I. Krachner, M. E. Hahn, and R. T. Di Giulio. 2003. Expression difference and indelibility of aryl hydrocarbon receptor pathway genes in wild-caught killifish (*Fundulus heteroclitus*) with different contaminant-exposure histories. Environmental Toxicology and Chemistry 22:2337-2343

Meyer, E., G. V. Aglymova and M. V. Matz. 2011. Profiling gene expression responses of coral larvae (*Acropora millepora*) to elevated temperature and settlement inducers using a novel RNA-Seq procedure. Molecular Ecology 20:3599-3616

Monserrat, J. M., P. E. Martínez, L. A. Geracitano, L. L. Amado, C. M. H. Martins, G. L. L Pinho, I.S. Chaves, M. Ferreira-Cravo, J. Ventura-Lima, and A. Bianchini. 2007. Pollution biomarkers in estuarine animals: Critical review and new perspectives. Comparative Biochemistry and Physiology, Part C 146:221-23

Myers, M., B. F. Anulacion, B. L. French, W. L. Reichert, C. A. Laetz, J. Buzitis, O. P. Olson, S. sol and T. K. Collier. 2008. Improved flatfish health following remediation of a PAH-contaminated site in Eagle Harbor, Washington. Aquatic Toxicology 88:277-288

Nacci, D. E., M. Kohan, M. Pelletier, and E. George. 2002. Effects of benzo[a]pyrene on a fish population resistant to the toxic effects of dioxin-like compounds. Aquatic Toxicology 57:203-215

Nacci, D. E., D. Champlin, and S. Jayaraman. 2010. Adaptation of the estuarine fish *Fundulus heteroclitus* (Atlantic Killifish) to polychlorinated biphenyls (PCBs). Estuaries and Coasts 33: 853-864

Navarro, A., M. Faria, C. Barata, and B. Piña. 2011. Transcriptional response of stress genes to metal exposure in zebra mussel larvae and adults. Environmental Pollution 159:100-107

Niehrs, C., and A. Schäfer. 2012. Active DNA demethylation by Gadd45 and DNA repair. 2012. Trends in Cell Biology 22:220-227

Oleksiak, M. F. 2008. Change in gene expression due to chronic exposure to environmental pollutants. Aquatic Toxicology 90:161-171

Ploch, S. A., L. C. King, M. J., Kohan and R. T. Di Guiulio. 1998. Comparative *in vitro* and *in vivo* Benzo[a]pyrene–DNA adduct formation and its relationship to CYP1A activity in two species of Ictalurid catfish. Toxicology and Applied Pharmacology 149:90-98

Robinson, K. 2011. Characterizing the immune function of the brown bullhead (*Ameiurus nebulosus*) from less contaminated and highly contaminated locations along the Detroit River. M.Sc. thesis, Department of Biology, University of Waterloo, Waterloo, ON. Available at: http://hdl.handle.net/10012/6032

Roesijadi, G. 1994. Metallothionein induction as a measure of response to metal exposure in aquatic animals. Environmental Health Perspective 102:91-96

Ruiz, Y., P. Suarez, A. Alonso, E. Longo, A. Villaverde and F. S. Juan. 2011. Environmental quality of mussel farms in the Vigo Estuary: Pollution by PAHs, origin and effects on reproduction. Environmental Pollution 159:250-265

Ruiz, P., M. Ortiz-Zarragoitia, A. Orbea, M. Theron, S. Le Floch, and M. P. Cajaraville. 2012. Responses of conventional and molecular biomarkers in turbot *Scophthalmus maximus* exposed to heavy fuel oil no. 6 and styrene. Aquatic Toxicology 116-117: 116-128

Szalinska, E., K. G. Drouillard, B, Fryer, and G. D. Haffner. 2006. Distribution of heavy metals in sediment of the Detroit River. Journal of Great Lakes Research 32:442-454

Vidal-Dorsch, D. E., S. M. Bay, M. A. Mays, D. J. Greenstein, D. Young, J. C. Wolf, D. Pham, A. V. Loguinov, and C. Vulpe. 2011. Using Gene Expression to Assess the Status of Fish from Anthropogenically Influenced Estuarine Wetlands. Environmental Science and Technology 46: 69-77

Watson, D. E., and R. T. Di Giulio. 1996. Hepatic CYPlA in brown bullhead catalyzes the binding of 2-aminoanthracene to DNA *in vivo* and *in vitro*. Aquatic Toxicology 37:21-36

Whitehead, A., F. Galvez, S. Zhang, L. M. Williams and M. F. Oleksiak. 2011. Functional genomics of Physiological plasticity and local adaptation in killifish. Journal of Heredity 102:499-511

Wilhem, B. T., and J. R. Landry. 2009. RNA-Seq—quantitative measurement of expression through massively parallel RNA-sequencing. Methods 48: 249-257

Wirgin, I., B. Konkle, M. Pedersen, C. Grundwald, J. Williams, S. C. Courtenay. 1996. A comparison of cytochrome P4501A (CYP1 A) mRNA inducibility in four species of Atlantic Coast anadromous fishes. Estuaries 19:913-922

Wirgin, I., J. R. Waldman. 1998. Altered gene expression and genetic damage in North American fish populations. Mutation Research 399:193-219

Wirgin, I., J. R. Waldman. 2004. Resistance to contaminant in North American fish populations. Mutation research 552:73-100

Wirgin, I., N. K. Roy, M. Loftus, R. C. Chambers, D. G. Franks, and M. E Hahn. 2011. Mechanistic basis of resistance to PCBs in Atlantic tomcod from the Hudson River. Science 331:1322-1325

Supplementary information Definitions according to AmiGO at the gene ontology (GO) web page (with *Danio rerio* selected as species; http://amigo.geneontology.org/cgi-bin/amigo/browse.cgi?action=minus_node&target=GO:0008150&open_1=GO:0044699,GO:0008150,all,GO:0043025&closed=GO:0005575,GO:0003674,GO:0044297,GO:0050789,GO:0016265,GO:0044464,GO:0065007&session_id=498amigo1371665781)

***Biological Process*:** *process or sets of molecular events with defined initiation and ending, relevant to the function of cells, tissues, organs, and organisms.*

Biological Regulation: A process that alter measurable characteristics of any function or process.

Cellular Component Organisation or Biogenesis: A process that causes biosynthesis of constituent macromolecules, or disassembling of cellular components.

Cellular Process: A process on the cellular level (can be several cells such as cell communication occurring among multiple cells, but at cellular level).

Developmental Processes: A process resulting in is the development of; a living unit, an anatomical structure (sub-cellular, cell, tissue, organ) or an organism developing through sequential stages.

Establishment of Localization: A directed movement of a cell or substance, such as protein complexes or organelles moving to an active location.

Growth: Increase in mass in an organism (or part of an organism), or cell.

Metabolic Processes: Chemical reactions and pathways (catabolism and anabolism) with which an organism converts chemical substances, such as protein synthesis, degradation and DNA repair.

Multicellular organismal process: Any process occurring at the level of multicellular organism.

Response to Stimuli: A process starting with the detection of a stimulus causing a change in activity or state of a cell or organism due to the stimulus.

Single Organism Process: Any biological process involving a single organism.

Cellular Component*: parts of a cell or its extracellular environment*

Cell Junction: A cellular component which forms a connection between two cells or to the extra-cellular matrix.

Cell Part: Any component (part) of a cell, such as basic structure.

Extracellular Region Part: Any constituent part of the external structure of a cell.

Macromolecular Complex: A stable cluster of more than one macromolecule (i.e., protein, lipids, nucleic acid, and carbohydrates) where the components act together.

Membrane Part: Any component (part) of the membrane (lipid bilayer), also including proteins coupled to it.

Organelle: An organized structure which has a specific function and morphology (include nucleus, mitochondria, plastids, vacuoles, vesicles, ribosomes and cytoskeleton), excluding the plasma membrane.

Organelle Part: any component (part) of an organelle.

Molecular function: *the essential activities of a gene product at molecular level*

Binding: Selective interaction between molecules, or between a molecule and one or more specific sites.

Catalytic Activity: Catalysis of biochemical reactions, enzymes and RNA with catalytic activity (ribozymes).

Structural Molecular Activity: Molecular action affecting structural integrity of complex assemblies.

Transporter Activity: Mechanisms that drive directed movements of molecules (macromolecules and small molecules) and ions in or out of a cell, or between cells.

CHAPTER 5

MULTIPLE GENE RESPONSE TO A B[a]P CHALLENGE IN BROWN BULLHEAD, EFFECT OF DOSE AND RECOVERY TIME ON GENE TRANSCRIPTION

INTRODUCTION

The aquatic environment is constantly changing, both due to natural processes, but also, and more importantly, from anthropogenic impacts. When the environment changes, or organisms disperse to a new environment, they will respond to the changing stimuli to maximize their likelihood for survival and reproduction. Pollution is a major form of anthropogenic disturbance and it has reached critical levels in many aquatic ecosystems (Ketata *et al.* 2007; Aldarondo-Torres *et al.* 2010; Chopra *et al.* 2011; Ruiz *et al.* 2011). Fish have been shown to be capable of rapid responses to new or degraded environments (Reznick *et al.* 1997; Hendry *et al.*2000; Oleksiak 2008; Wirgin *et al.*2011), and rapid adaptive responses to polluted environments act to increase tolerance by mechanisms such as physiological acclimation (Meyer *et al.* 2002; Meyer *et al.*2003; Farwell *et al.*2012) or genetic adaptation (Wirgin *et al.*2011). All forms of adaptive response originate as changes in gene transcription and gene expression. Alterations in gene transcription can be an early sign of stress (depending on the response and the gene), and transcription markers are increasingly used as biomarkers (Wirgin and Waldman 1998; Nacci *et al.*1999; Meyer *et al.*2002; Meyer and Di Giulio 2002; Nacci *et al.*2002; Grey *et*

*al.*2003; Fisher and Oleksiak 2007; Oleksiak 2008; Carlson *et al.* 2009; Lei *et al.*2009; Nacci *et al.*2010; Bozinovic and Oleksiak 2011; Whitehead *et al.*2011; Wirgin *et al.*2011; Brammell *et al.* 2013). Cytochrome P450 1A (CYP1A) and metallothionein (MT) are commonly used gene loci to test for environmental effects (see Chapter 2); however, adaptation and physiological acclimation can bias the interpretation of such single gene assays (Wirgin and Waldman 1998; Meyer *et al.*2002; Meyer *et al.*2003; Grey *et al.*2003; Wirgin and Waldman 2004; Monserrat *et al.*2007; Kilemade *et al.*2009; Brammell *et al.*2013). Therefore broader, multi-gene screening studies are needed for detecting and quantifying pollution effects; however those approaches needs be properly calibrated and characterised before widespread application in ecotoxicological studies.

Although aquatic ecosystems experience complex combinations of contaminants under polluted conditions, single contaminant studies provide an excellent starting point for biomarker development. For example, many fish species have been shown to respond to aromatic hydrocarbons (AH) toxicity (i.e. killifish (*Fundulus heteroclitus*), brown bullhead (*Ameirus nebulosus*), European flounder (*Platichthys flesus*), tomcod (*Microgadus tomcod*), darter goby (*Ctenogobus boleosoma*), rainbow trout (*Oncorhynchus mykiss*) and turbot (*Scophthalmus maximus*; Meyer *et al.*2002; Meyer and Di Giulio 2002; Nacci *et al.*2002; Grey *et al.*2003; Williams *et al.*2003; Wirgin and Waldman 2004; Hook *et al.*2006; Kilemade *et al.*2009). However, as adaptive responses can obscure this response, we need sensitive biomarkers that will capture the response to the contamination among all species, despite possible adaptive responses. Ecotoxicogenomics has been proposed as a powerful alternative biomarker approach to examine responses to pollution. There are currently good technologies that have broader

gene coverage, and can be used to compare transcripts from many genes at the same time. For example, DNA microarrays have already been used for biomarker measurement (Fisher and Oleksiak 2007; Oleksiak 2008; Carlson *et al.* 2009; Lei *et al.*2009; Bozinovic and Oleksiak 2011; Whitehead *et al.*2011). Although microarrays have been used previously and are becoming more common in ecotoxicology, they have not yet become as wide spread as CYP1A gene analyses. Microarrays can be designed for either cDNA from the entire genome, or for expressed sequence tags (ESTs) representing the entire genome. Such approaches should identify all the genes that are altered in response to the challenge. However, microarrays do not have to include all of the genes in the genome; a well-planned, targeted microarray may be a good choice as a biomarker tool. Targeted microarray data should be interpreted with caution, as they reflect a small part of the genome (less than 1%), and important transcriptional changes may be overlooked. Nevertheless, a targeted microarray with genes that are important for detoxification, stress, and possibly others that have been shown to exhibit altered transcription under similar conditions may be close to ideal to quantify environmental effects of contaminants, and to determine the stress level of the challenged organisms. Such a custom microarray could be applied to both model and/or indicator-species, and if properly calibrated, would serve as a powerful biomarker for early detection of the effects of contamination.

Brown bullhead (*Ameiurus nebulosus*) are a benthic catfish native to North America and are tolerant to a wide range of environmental conditions (Scott and Crossman 1979). Due to their high tumour prevalence and the correlation of the incidence of tumours with sediment contamination (Baumann 1987; Baumann *et al.*1996), bullhead

have been used as an indicator species of contamination for some time (Baumann *et al.*1987; Leadley *et al.* 1998). Despite their long history as an indicator species for contamination and tumour development, brown bullhead have been surprisingly under-studied for the molecular genetic basis of their response to pollution (but see Grey *et al.*2003; Busch *et al.*2004).

In this study I describe the design and development of a targeted microarray for brown bullhead, using genes known to be involved in detoxification, or that have been found to exhibit transcriptional responses to contaminants in other studies. Using the custom microarray, I measure gene transcription as a dose response in brown bullheads after exposure to various doses of benzo[a] pyrene (B[a]P) at two time points after exposure. The development and calibration of a targeted microarray to investigate brown bullhead response to carcinogenic toxicants will not only generate a valuable brown bullhead tool, but also provide a template for future custom microarray development for use as biomarkers. Microarray technology provides a rapid and inexpensive tool that capitalises on the potential for multi-gene transcriptional biomarkers that are insensitive to biases introduced by possible adaptation and acclimation. This study also serves to explore some anomalies in expected gene transcriptional responses identified in my Next Generation sequencing of the transcriptome of the brown bullhead exposed to contaminated sediment (Chapter 4).

MATERIAL AND METHODS

Fish collection: Brown bullhead were collected by electroshocking from Belle River and held in an aerated semi-natural pond. They were fed trout chow (Martin Mills

3.-6.PT) every second day. In October 2011, 40 F1 offspring fish were transferred to indoor flow-through tanks and held for 3 months, fed trout chow (Martin Mills 3.-6.PT) twice a week

Challenges: To investigate response timing and the effect of dose on gene transcription, I challenged the brown bullhead with a single dose of B[a]P. Bullhead were divided into individual tanks (N=8 per tank) in March 2012. The fish were held for one week to allow them to adjust to the environment, and were not fed during the week leading up to the challenge. The bullhead were interperitoneally (ip) injected with a single dose of B[a]P. Fish in each group tank were injected with one of following doses: 0 mg/kg, 5 mg/kg, 10 mg/kg, 25 mg/kg, or 50 mg/kg. Four bullheads from each group were sacrificed after 24 h and an additional four fish were sampled after 96 h. Liver tissue was collected and placed in RNAlater within 3 minutes of being humanely euthanized and stored, first at room temperature overnight, and then at -80° C until further analysis.

The mixtures for the ip injections were prepared by dissolving 1g B[a]P in 10ml dichloromethane (DMC), then 10ml safflower oil was slowly added on low heat while the suspension was stirred continuously. The flask was left in the fume hood overnight and stirred slowly to evaporate the DMC. The various diluted B[a]P solutions were made by serial dilution with safflower oil.

Microarray design: 128 genes relevant to ecotoxicological response and were PCR amplified and sequenced (see below) in brown bullhead cDNA, those sequences with 4 plant gene sequences as negative control were used to design oligonucleotide probes in OligoArray 2.1 (Rouillard *et al.* 2003). Oligo probes (49-51 bases) were purchased from Sigma and spotted on poly-L-lysine coated glass slide using a SpotArray

24 microarray printer (Perkin Elmer). Probes (all 132 genes) were printed in triplicate in each block and the blocks were replicated three times per slide. The replication of each oligonucleotide spot nine times (3 replicate spots x 3 replicate blocks) allows the partitioning of experimental variation to provide greater power to detect small transcription signal variation. After printing, the slides were blocked with UV-light and succinate anhydride, as described in Massimi *et al.* (2002).

The genes selected for inclusion on the microarray was based on a literature search performed to identify genes that have been found to exhibit responses to contaminant stress in prior studies in fish (Williams *et al.*2003; Holth *et al.*2008; Oleksiak 2008; Carlson *et al.*2009; Lie *et al.*2009; Whitehead *et al.*2011). Identified genes were searched in the NCBI GenBank, I recorded the number of species with the target gene sequences – this varied among genes; however, 2 to 17 sequences were downloaded per gene. Sequence alignments were used to design degenerate 10-16 base primers with PriFi (Fredslund *et al.*2005). Those primers sets were designed to PCR amplify a 300-500 base pair (bp) sequence from bullhead cDNA. If the NCBI sequences for a single gene varied too much, it was removed – unless one of the NCBI sequences was from the *Ameiurus* or *Ictalurus* genera (i.e., catfishes).

Degenerate primers were used in two 25 μL PCRs with brown bullhead single stranded cDNA to create a fragment of brown bullhead sequence. Several of the degenerate primers produced more than one band. Bands in the expected size range were excised from agarose gel, and extracted with a gel extraction kit (Epoch Life Science) following the manufacturer's protocol. 160 extracted fragments were sequenced (Applied Biosystem's 3730xl DNA analyser) and confirmed with NCBI's blastx. Sequences that

were not in the forward reading frame were reversed and complemented using The Bio-Web.

RNA extraction, reverse transcription, labelling, and hybridisation: A small piece of tissue (~10 mg) from the challenged and control bullhead was mechanically homogenised using 400 mL glass bead solution and 0.75 ml TriZol (Ambion). Total RNA was extracted following Chomczynski and Sacchi (1987) and dissolved in 30µL of MilliQ water. RNA quality was determined by gel-electrophoresis on 1% agarose gels, and the presence of 18S and 28S rRNA was confirmed. RNA concentrations were determined in a Victor 3V plate reader (Perkin Elmer) using UV spectrophotometry; only total RNA samples with values between 1.6 and 2.2 (A260/A280) were used for the subsequent analyses.

Reverse transcription of 30µg RNA was done using the Genisphere 3DNA Array 50 kit (details can be found at http://genisphere.com/sites/default/files/pdf/Array50_Jan2011.pdf) with SuperScript II Reverse Transcriptase (Life Technologies). In short, reverse transcription was carried out using oligo d(T) primers with a 5' sequence tag (dye specific). RNA and primers were heated to 80 °C for 10 minutes and put on ice, 10U of RNase inhibitor was added. Then the reverse transcription reaction (including; 5X superscript buffer (Life Technologies), dNPT mix (Genisphere), 5mM DTT (Life Technologies) and 400 U Superscript II (Life Technologies)) was incubated at 42°C for 2.5 hours. The reaction was stopped by adding EDTA/NaOH, followed by heating to 65°C for 15 minutes. The reaction was neutralised with Tris-HCl, and two samples were pooled, with different sequence tags. Synthesised cDNA was precipitated with acrylamide, NaOAc and 95% EtOH at room temperature

overnight, then centrifuged at 13000g for 1 hour, washed with 70% EtOH, and the pellet was dissolved in 25µL MilliQ water.

Hybridisation was carried out in a two-step process. In the first step microarrays and cover-slips were pre-heated in the hybridisation chamber prior to hybridisation. The cDNA (with the 5' sequence tag) was hybridised to the microarrays by mixing 2X formamide hybridisation buffer (25% formamide, 4xSSC, 0.5%SDS, 2X Denhardt's solution) with the cDNA. The mixture was heated to 80°C for 10 min, then pipetted directly onto the microarray and covered with the pre-heated coverslip. The hybridisation reaction was carried out at 42°C for 17.5h. The arrays were then washed in 2xSSC, 0.1% SDS at 42°C for 5 minutes and then at room temperature in 2xSSC, 0.1% SDS for 3 minutes, followed by two 1xSSC washes for 3 minutes and two 0.1xSSC washes for 3 minutes and dried by centrifuging at 1000 rpm for 5 minutes.

In the second hybridisation step, to hybridise the dyes (Cy3 and Cy5) to the cDNA attached the array, the microarrays and cover slips were heated in the hybridisation chamber. 3DNA hybridisation mixtures containing 3DNA capture reagents, Cy3 and Cy5, 2 X formamide hybridisation buffers, and locked nucleic acid dT blocker (LNA dT blocker) were mixed and incubated with the microarrays for three hours at 42°C. Previous washes were repeated: 2xSSC, 0.1% SDS at 42°C for 5 minutes and then at room temperature in 2xSSC, 0.1% SDS for 3 minutes, followed by two 1xSSC washes for 3 minutes and two 0.1xSSC washes for 3 minutes and dried by centrifuging at 1000 rpm for 5 minutes.

Scanning and Data preparation: Slides were scanned within 24h of hybridisation on a ScanArray 4000 XL Microarray analysis System (Perkin Elmer) using ScanArray

Express software version 4.0 (Perkin Elmer). Spots were detected and quantified with spotfinder 3.2.1 (Saeed *et al.* 2003*).* Spotfinder searched each grid and measured each spots' signal and the background signal around the spot and performed a background correction by subtracting the background intensity from the spot intensity. This analysis gave an intensity measurement and a quality code for each spot. The data was filtered using the quality scores, retaining codes that according to the program were high quality spots (A, B, and C) and deleting codes S, U, X, Y and Z (low quality). Of the 132 spotted oligos (including four negative controls and 128 target genes) 45 had sufficient positive signal data to be analysed further (Supplementary Table S5.1.) These 45 spots had either complete fluorescence data or incomplete data, but patterns that made biological sense, such as presence at one sample time or that followed the dose pattern.

*Data analysis***:** Analyses were performed using R 2.15.1 (R Development Core Team, 2009). The analyses were performed as a one-channel microarray experiment in a mixed-effects model in the R package lme4 (Bates *et al.*2011) using the following model:

$$x_{alkj} = \mu + F_a + I_j + B_{k(j)} + e_{alkj}$$

where x_{alkj} is the normalised average intensity value (one gene) for the l^{th} replicate spot in the k^{th} block, nested in the j^{th} individual as random effects. The fixed effect (F_a) was the parameter being tested (i.e., weight, sex, and dose). A likelihood ratio test (ANOVA) between the model with the fixed effect included and the model without the fixed effect was used to determine significance of the fixed effect on gene transcription for a particular gene. This analysis was performed on each gene as independent markers of gene expression response.

Tests for an effect of sex and weight on transcription followed this approach, and sex was found to have a significant effect for a two genes, and I therefore modified the basic model to include sex as a random effect (S_i):

$$x_{alkij} = \mu + F_a + S_i + I_{j(i)} + B_{k(j(i))} + e_{alkij}$$

To test for the effect of B[a]P dosage on transcription, the data was analysed separately for each of the two sampling times, with dose as a fixed effect (F_a). For genes with significant dose effects on transcription, Tukey post-hoc tests were performed among all pairwise doses in the R package multcomp (Hotorn *et al.* 2008) to identify specific dose-related transcriptional differences.

RESULT AND DISCUSSION

The genes spotted on the custom bullhead microarray for the contaminant challenge were selected based on known detoxification genes and genes that have been reported to show altered transcription in other species after exposure to pollution stress. Surprisingly, the bullhead microarray exhibited a limited number of genes with altered transcription in response to the challenge. Furthermore, only 45 of 128 (35%) spotted genes had detectable transcription. There are a number of possible explanations for this pattern of transcription: 1) the selected genes do not respond to contaminants in the expected fashion in brown bullhead, 2) the bullhead oligo-microarray is not sensitive enough to detect low levels of transcripts, or 3) there is high variability in the transcription control among species, or perhaps even populations. Species-level variation in gene response to a challenge has been shown in other microarray studies (Williams *et*

*al.*2003; Oleksiak 2008; Carlson *et al.*2009; Bozinovic and Oleksiak 2011). Killifish have even been shown to have transcriptional differences among populations, depending on exposure (Fisher and Oleksiak 2007; Oleksiak 2008; Whitehead *et al.*2011). One of the limitations of microarrays is their low sensitivity and low signal-to-noise ratios, which affects their detection capabilities, and hence my ability to analyse and interpret data from some of the selected genes (David *et al.*2010). However, a microarray such as the custom brown bullhead array described here is likely to show transcription for different subsets of the spotted genes, depending on the nature of the challenge.

There was no significant effect of body size (weight) in any of the models for any of the genes, so body size was excluded from all further analyses. All fish were one or two years old, and most were entering sexual maturity (sampled in March-April). Sex was found to have a significant effect ($p<0.01$) for two genes: elongation factor 1 alpha (EF1α) and phosphoenolpyruvate carboxykinase (PEP carboxykinas). Difference in transcription between sexes has been previously reported for some genes associated with stress response (Williams *et al.*2003; Derks *et al.*2008; Lie *et al.*2009), and thus this result is not surprising. The lack of effect of body mass on gene transcription at any gene may be due to the fish being of similar age and size.

Induction time effects: There is a difference in transcription between the two sampling times (24h and 96h), overall there were fewer gene transcripts detected at 24h than at 96 h (40 vs. 45). Of the genes that are transcribed at both sample times, many have a higher transcription value at 96h relative to 24 h (Figure 5.1). If transcription is used as a biomarker, variation in induction timing must be taken into consideration. For example, some genes may be transcriptionally induced at 24 h, but their transcription levels will

keep increasing, as has been shown for both phase I and phase II enzymes in the aryl hydrocarbon receptor pathway (AHR i.e. CYP1A1 and glutathione S-transferase (GST); Beyer *et al.*1997; Courtenay *et al.* 1999; Wang *et al.*2006; Le Goff *et al.*2006).

The five genes that are not transcribed at 24 h but do show a detectable signal at 96 h are; elongation factor 1 gamma (EF1γ), glutathione S-transferase (GST), phospholipase –B (P76), nesprin-1, and β-actin. Curiously, β-actin is often used as a "housekeeping" or endogenous control gene, thus the lack of detectable transcription at 24h, and the weak up-regulation at 96h is not expected. Perhaps they are expressed at 24h, but below the detection limit of the assay, and as the level of transcription at 96h is low, the apparent variation in transcription over time may be an artefact. Of the other 4 genes not detected at 24h, but measureable at 96h, nesprin-1 has been associated with cell death, GST is a metabolic protein involved in xenobiotic metabolism (it metabolises glutathione and xenobiotic chemicals) and is a phase II enzyme in the aryl hydrocarbon receptor (AHR) pathway which breaks down contaminants, while P76 is a metabolic rate related gene and EF1γ is involved in the elongation process (translation). That some genes are not transcribed (or transcribed below the detection limit) at the 24 h time point suggests that other genes spotted might show detectable signal at other times or under different stress challenges.

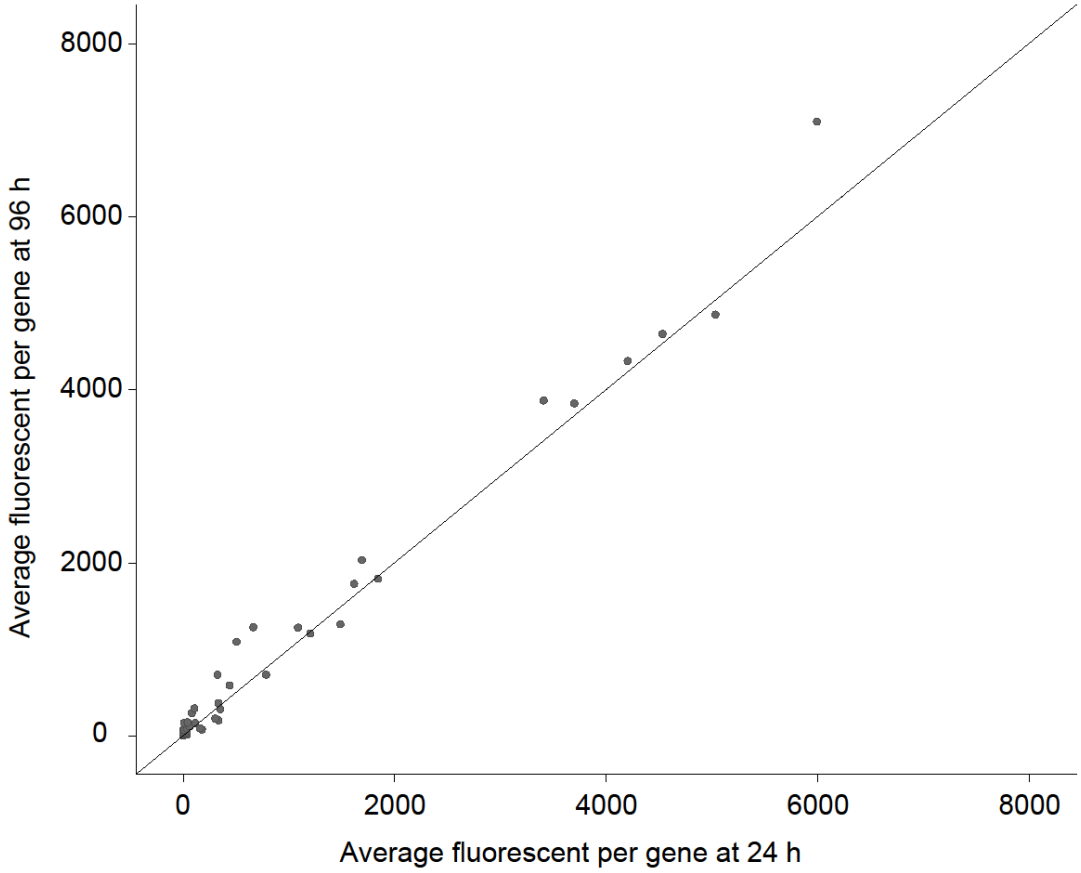

Figure 5.1. Average fluorescent intensity representing transcription level of the 45 analysed genes at 24 h vs. 96 h. Above the 1:1 line are genes that are transcribed at higher levels at 96 h, while below the line are genes transcribed at higher levels at 24h. Genes with values on the 1:1 line are those that show no transcription induction timing effects.

Dose response effect: Gene transcription can be either up- or down-regulated in response to a challenge. This can occur in three different ways 1) threshold response, where at a specific does the gene is activated or repressed with an abrupt change in transcription levels, 2) linear response, where the increase (decrease) in transcription changes in proportion to dose increments, and 3) asymptotic response, where there is initially an increase / decrease in transcription, however eventually the transcription level will asymptote with no further change with changes in dose. Of the 40 transcribed genes that were transcribed at 24 h there was an overall effect of dose for two genes: Catechol-O-methyltransferase (COMT) and NADH dehydrogenase 1β (NADH). The Tukey's post-hoc tests resulted in no significant pairwise differences among doses for NADH; however, this is likely due to a loss of statistical power due to multiple simultaneous tests and reduced sample sizes when two doses were compared. Transcriptional variation among doses at COMT show significant up- and down-regulation among doses; however, there does not appear to be any pattern. There were no other significant overall dose effects after the 24 h challenge. This may mean that although transcription induction occurs within a few hours for some genes (Beyer *et al.*1997; Courtenay *et al.* 1999), a longer (or shorter) challenge may have detected a dose-response that would make the genes effective as biomarkers. This lack of effect at 24h post-challenge would also explain our low transcription detection of several of the detoxification genes described in Chapter 4 (Next Generation Sequencing of the transcriptome).

In the 96h sampling after the challenge, there was a dose effect on transcription for ten genes: CYP1A1, superoxide dismutase [Cu-Zn] (SOD1), and compliment factor 9 (C9), are all up-regulated with an asymptotic response (Figure 5. 2) while β-actin was up-

regulated with less of a particular response pattern and little difference between doses. NADH shows an initial down-regulation followed by up-regulation (Figure 5. 2), not following any specific pattern. The remaining genes were down-regulated relative to the control with the lowest dose of B[a]P being enough to pass the threshold and repress transcription for these genes are: hypoxia inducible factor 2 (HIF2; was not specific for either HIF2α or HIFβ) and hypoxia inducible factor 2 alpha (HIF2α; there was no difference between the two HIF homologues), isocitrate dehydrogenase (IDH), kinase D-interacting (unknown function), and c-fos oncogene (c-fos; Figure 5.3). All responses were found to follow either a threshold or asymptotic response pattern, if a change in response to dose was observed.

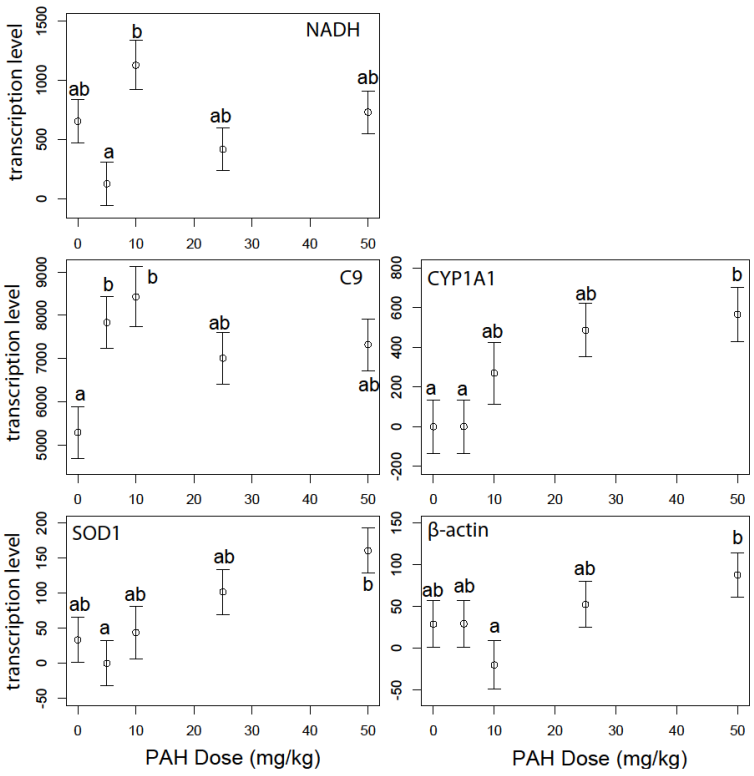

Figure 5.2 Average transcription (fluorescence) levels with standard error for up-regulated genes. Shared lower-case letters indicate no significant differences between doses, a different letter shows significantly different transcription.

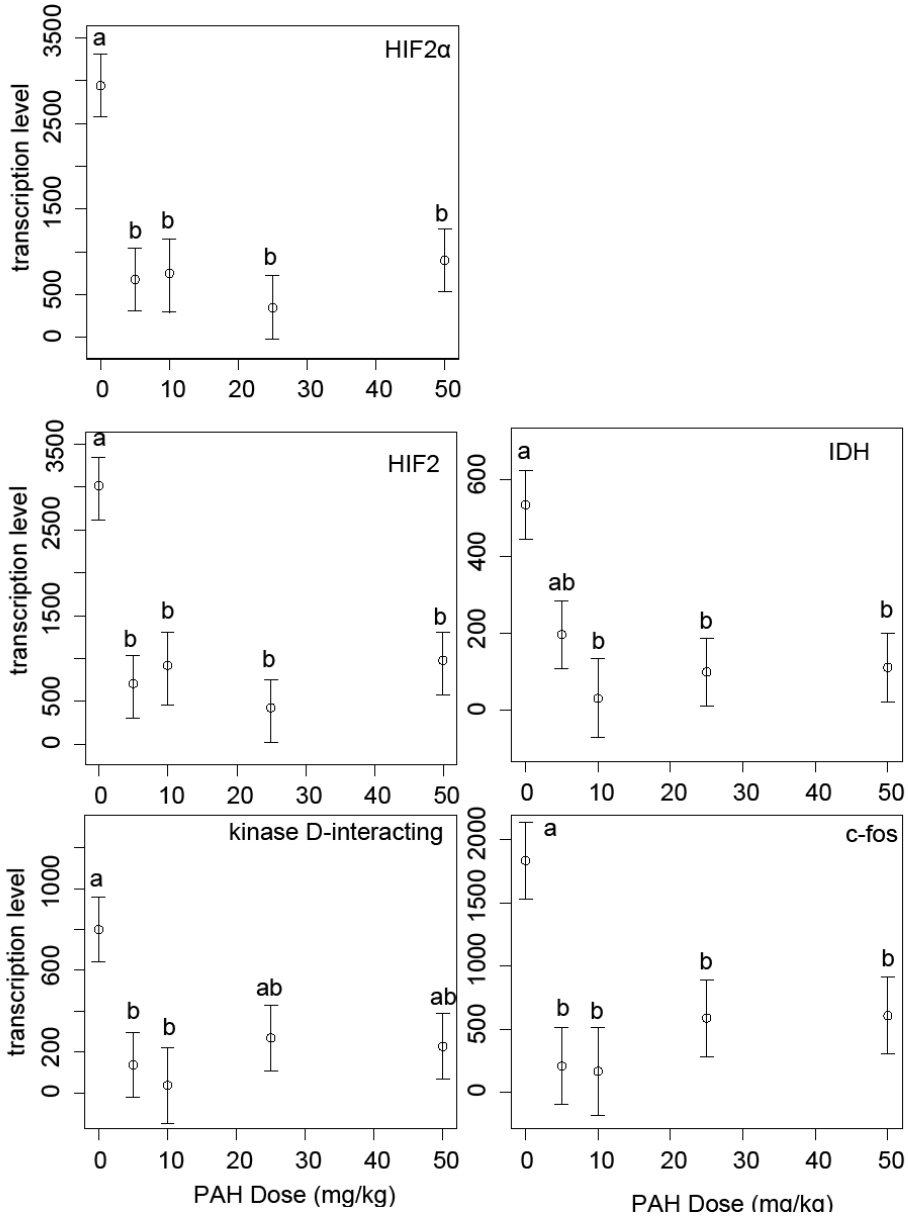

Figure 5.3. Average transcription (fluorescence) levels with standard error for down-regulated genes. Shared lower-case letters indicate no significant differences between doses, a different letter shows significantly different transcription.

HIF2 and HIF2α are a transcription factors and were down regulated at all doses of B[a]P. HIF2α, HIF2β, HIF1α, HIF1β all have very similar sequences but produce different proteins, the sequence similarity makes it likely that the oligo probes on the array hybridized with cDNA from all four genes, thus no differences can be seen between HIF2α and HIF2. HIF1β (aryl-hydrocarbon receptor nuclear translocator; ARNT) is also important in the induction of the AHR pathway, and studies have shown that there is cross talk among the genes (Stregman *et al.*2010; Garcia-Travera *et al.*2013).

Isocitrate dehydrogenase (IDH) was down-regulated in all doses relative to the sham-injected fish – very similar to what was reported for killifish in polluted sites (Oleksiak 2008). IDH metabolises isocitrate in the carbohydrate pathway, and the energy generated is used for catabolising intermediate compounds. The c-fos gene was down-regulated in response to my B[a]P 96h challenge, c-fos is known to respond as a part of an immune and stress response in mammals when exposed to PAH, where it was also reported to increase with CYP1A (White *et al.*2011; Nobles *et al.*2012), which does not agree with my results for brown bullhead. C9 was up-regulated at lower challenge doses of B[a]P at 96h post-challenge. C9 is involved in the cytolysis process in the immune system (Wang *et al.*2013), in previous studies, immune genes were generally down-regulated under PAH challenge (Reynaud and Deschaux 2006; Hur *et al.*2013).

CYP1A1 induction is a commonly used biomarker for contaminant exposure, and this study confirms the consistent up-regulation in response to the B[a]P challenge 96h post-challenge. Previous studies have shown that induction of CYP1A1 may not be instantaneous and the lack of a dose effect at 24h post challenge in this study, coupled

with the CYP1A1 results in Chapter 4 supports the 3-6 day induction delay in CYP1A1 (Beyer *et al.*1997; Courtenay *et al.* 1999).

SOD1 together with GST are two of the phase II enzymes in the AHR pathway that respond after phase I enzymes (such as CYP1's; Sharma *et al.*2013) are induced. SOD1 has been shown to be induced by PAHs as a part of the antioxidant response (Timme-Laragy *et al.*2009) and it has also been shown to be active in double stand DNA repair and regulation of the apoptotic processes.

β-actin was significantly up-regulated at 50 mg/kg relative to 10 mg/kg. Given that I found a significant sex effect on β-actin transcription, coupled with a lack of signal detection at 24 h, I recommend that it not be used as either a biomarker or housekeeping gene until further investigation of β-actin gene function in brown bullheads is complete.

Brown bullhead clearly responded transcriptionally to the B[a]P challenge. It is curious that there was relatively limited evidence for consistent dose effects. None of the 128 genes showed a significant dose response curve, although CYP1A and SOD1 show a consistent increase with dose. Microarrays have been used in ecotoxicogenomics, and is considered quantitative; however my data do not support a functional dose response to B[a]P challenge for any gene. It is know that transcription regulation for some genes is highly sensitive to the environment, but other genes may be regulated as a simple on or off fashion – this would not result in the expected dose response curve. Even if a gene has an on/off transcription regulation, there may be post-transcriptional regulation that adjusts the final protein levels. The six genes that showed a consistent response at 96 h (i.e. CYP1A1, SOD1, HIF2α, HIF2, IDH, c-fos, D-interacting) show promise as biomarkers,

though my data suggest that microarrays may not be sensitive enough to detect adaptive responses.

REFERENCES

Aldarondo-Torres, J. X., F. Samara, I. Mansilla-Rivera, D. S. Aga, and C. J. Rodríguez-Sierra. 2010. Trace metals, PAHs, and PCBs in sediments from the Jobos Bay area in Puerto Rico. Marine Pollution Bulletin 60:1350-1358.

Bates, D., M. Maechler, B. Bolker. 2011. lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-42. http://CRAN.R-project.org/package=lme4

Baumann, P. C., W.D. Smith, and W. K Parland. 1987. Tumour frequencies and contaminant concentrations in brown bullhead from an industrialized river and a recreational lake. Transaction of the American Fisheries Society 166:79-86

Baumann, P. C., I. R. Smith, and C. D. Metcalfe. 1996. Linkage between chemical contaminates and tumour in benthic Great Lakes fish. Journal of Great Lakes Research 22:131-152

Beyer, J., M. Sandvik, J. U. Skåre, E. Egaas, K. Hylland, R. Waggø, and A. Goksøyr. 1997. Time- and dose- dependent biomarker response in flounder (*Platichthys flesus L*) exposed to benzo[a]pyrene, 2,3,3',4,4',5-hexachlorobiphenyl (PCB-156) and cadmium. Biomarkers 2:35-44

Bozinovic, G. and M. F. Oleksiak. 2011. Genomic approaches with natural fish populations from polluted environments Environmental Toxicology and Chemistry 30:283-289

Brammell, B. F., D. J. Price, W. J. Brige, and A. A. Elskus. 2013. Lack of CYP1A responses in species inhabiting chronically contaminated habitats: two varieties of resistance? Comparative Biochemistry and Physiology Part C 157:212-219

Busch, C. R., D. D. Heath, and A. Hubberstey. 2004. Sensitive genetic biomarkers for determining apoptosis in the brown bullhead (*Ameiurus nebulosus*) Gene 329:1-10

Carlson, E. A., N. K. Roy, and I. I. Wirgin. 2009. Microarray analysis of polychlorinated biphenyl mixture-induced changes in gene expression among Atlantic tomcod

populations displaying differential sensitivity to halogenated aromatic hydrocarbons. Environmental Toxicology and Chemistry 28:759-771

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical biochemistry 162:156-159

Chopra, A. K., M. K. Sharma, and S. Chamoli. 2011. Bioaccumulation of organochlorine pesticides in aquatic system – an overview. Environmental Monitoring and Assessment 173:905-916

Courtenay, S. C., C. M. Grunwald, G. Kreamer, W. L. Fairchild, J. T. Arsenault, M. Ikonomou, and I. I. Wirgin. 1999. A comparison of the dose and time response of CYP1A1 mRNA induction in chemically treated Atlantic tomcod from two populations. Aquatic Toxicology 47:43-69

David, J. P., E. Coissac, C. Melodelima, R. Poupardin, M. A Riaz, A. Chandor-Proust and S. Reynaud. 2010. Transcriptome response to pollutants and insecticides in the dengue vector *Aedes aegypti* using next-generation sequencing technology. BMC Genomics 11:216

Derks, N. M., M. Müller, B. Gaszner, D. T. W. M. Tilburg-Ouwens, E.W. Roubos and L.T Kozicz. 2008. Housekeeping genes revisited: different expressions depending on gender, brain area and stressor. Neuroscience 156:305-309

Farwell M., K. G. Drouillard, D. D. Heath, T. E Pitcher. 2012. Acclimation of life-history traits to experimental changes in environmental contaminant concentration in brown bullhead (*Ameiurs nebulous*). Environmental Toxicology and Chemistry 31:863-869.

Fisher, M. A. and M. F. Oleksiak. 2007. Convergence and divergence in gene expression among natural populations exposed to pollution. BMC Genomics 8:108

Fredslunds, J., L. Schauser, L. H. Madsen, N. Sandal, and J. Stougaard. 2005. *PriFi: using a multiple alignment of related sequences to find primers for amplification of homologs. Nucleic Acid Research 33:W516-W520*

Garcia-Travera, J. L., D. Valdés-Lozanos, I. Poblete-Naredo, A. Albores-Medina, and O. Zapate-Pérez. 2013. Bile benzo[a]pyrene concentration and hepatic CYP1A induction in hypoxic adult tilapia (*Oreochromis niloticus*) Chemosphere 92:16-23

Grey, J. P., T. L. Leas, E. Obert, D. Brown, G. C. Clark, and J. P. Vanden Heuvel. 2003. Evidence of aryl hydrocarbon receptor ligands in Presque Isle Bay of Lake Erie. Aquatic Toxicology 64:343-358

Hendry, A. P., J. K. Wenburg, P Bentzen, E. C. Volk, and T. P. Quinn. 2000. Rapid evolution of reproductive isolation in the wild: evidence from introduced salmon. Science 290:516-518

Holth, T. F., R. Nourizadeh-Lillabadi, M. Blaesbjerg, M. Grung, H. Holbech, G. I. Petersen, P. Aleström, and K. Hylland. Differential gene expression and biomarker in zebrafish (*Danio rerio*) following exposure to produced water components. Aquatic Toxicology 90:277-291

Hook, S. E., A. D.Skillman, J. A. Small and I. R. Schultz. 2006. Gene expression pattern in rainbow trout, *Oncorhynchus mykiss*, exposed to a suit of model toxicants. Aquatic Toxicology 77:372-385

Hotorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous inference in general parametric models. Biometrical Journal 50:346-363

Hur, D., J.-K. Jeon, and S. Hong. 2013. Analysis of immune gene expression modulated by benzo[a]pyrene in head kidney of olive flounder (*Paralichthys olivaceus) Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 165:49-57*

Ketata, I., W Smaoui-Damak, F. Guermazi, T. Rebai, and A. Hamza-Chaffai. 2007. *In situ* endocrine disrupting effects of cadmium on the reproduction of *Ruditapes decussates*. Comparative Biochemistry and Physiology, Part C 146: 415-430

Kilemade, M., M. G. J. Hartel, J. O'Halloran, N. M. O'Brien, D. Sheehan, C. Mothersill, and F. N. A. M. van Pelt. 2009. Effects of contaminated sediment from Cork Harbour, Ireland on the cytochrome P450 system of turbot. Ecotoxicology and Environmental Safety 72:747-755

Le Goff, G., F. Hilliou, B. D. Siegfried, S. Boundy, E. Wajnberg, L. Sofer, P. Audant, R. H. ffrecnch-Constant, and R. Feyereisen. 2006. Xenobiotic response in Drosophila melanogaster: Sex dependence of P450 and GST gene induction. Insect biochemistry and Molecular Biology 36:674-682

Leadley, T. A., G. Balch, C. D. Metcalfe, R. Lazar, E. Mazak, J. Habowsky, and G. D. Haffner. 1998. Chemical accumulation and toxicological stress in three brown bullhead (*Ameiurus nebulosus*) populations of the Detroit River, Michigan, USA. Environmental Toxicology and Chemistry 17:1756-1766.

Lie, K. K., A. Lanzen, H. Berelid, and P. A. Olsvik. 2009. Gene expression profiling in Atlantic cod (*Gadus Morhua L.*) from two contaminated sits using a custom-made cDNA microarray. Environmental Toxicology and Chemistry 28:1711-1721

Massimi, A., t. Harris, G. Childs, and S. Somerville. 2002. Printing on glas slides. In a Molecular Cloning Manuel: DNA Microarrays. Bowtell D, Sambrook J, eds. Cold Spring Harbor Laboratory Press Cold Spring Harbor, NY. 71-78

Meyer, J. N., and Di Giulio. 2002. Patterns of heritability of decrease EROD activity and resistance to PCB 126-induced tertatogenesis in laboratory-reared offspring of killifish (*Fundulus heteroclitus*) from a creosote-contaminated site in the Elisabeth River, VA, USA. Marine Environment Research 54:621-626

Meyer, J. N., D. E. Nacci, R.T. Di Giulio. 2002. Cytochrome P4501A (CYP1A) in killifish (*Fundulus heteroclitus*): heritability of altered expression and relationship to survival in contaminated sediments. Toxicological Science 68:69-81

Meyer, J. N., D. M. Wassenberg, S. I. Krachner, M. E. Hahn, and R. T. Di Giulio. 2003. Expression difference and inducibility of aryl hydrocarbon receptor pathway genes in wild-caught killifish (*Fundulus heteroclitus*) with different contaminant-exposure histories. Environmental Toxicology and Chemistry 22:2337-2343

Monserrat, J. M., P. E. Martínez, L. A. Geracitano, L. L. Amado, C. M. H. Martins, G. L. L Pinho, I.S. Chaves, M. Ferreira-Cravo, J. Ventura-Lima, and A. Bianchini. 2007. Pollution biomarkers in estuarine animals: Critical review and new perspectives. Comparative Biochemistry and Physiology, Part C 146:221-234

Nacci, D. E., L. Coiro, D. Champlin, S. Jayaraman, R. McKinney, T. R. Gleason, W. R. Munns Jr, J. L. Specker and K. R. Cooper. 1999. Adaptations of wild populations of the estuarine fish *Fundulus heteroclitus* to persistent environmental contaminants. Marine Biology 134:9-17

Nacci, D. E., M. Kohan, M. Pelletier, and E. George. 2002. Effects of benzo[a]pyrene on a fish population resistant to the toxic effects of dioxin-like compounds. Aquatic Toxicology 57:203-215

Nacci, D. E., D. Champlin, and S. Jayaraman. 2010. Adaptation of the estuarine fish *Fundulus heteroclitus* (Atlantic Killifish) to polychlorinated biphenyls (PCBs). Estuaries and Coasts 33: 853-864

Nobles, I., C. Vanparys, R. Van den Heuvel, J Vercauteren, and R. Blust. 2012. Added value of stress related gene inductions in HepG2 cells as effect measurement in monitoring of air pollution. Atmospheric Environment 55:154-163

Oleksiak, M. F. 2008. Change in gene expression due to chronic exposure to environmental pollutants. Aquatic Toxicology 90:161-171

Reynaud, S., P. Deschaux. 2006. The effects of polycyclic aromatic hydrocarbons on the immune system of fish: a review. Aquatic Toxicology 77:229-238

Reznick, D. N., F. H. Shaw, F. H. Rodd, and R. G. Shaw. 1997. Evaluation of the rate of evolution in natural populations of guppies (*Poecilia reticulata*). Science 275, 1934-1937

Rouillard, J-M., M. Zuker, and E. Gulari. 2003. OligoArray 2.0: Design of oligonucleotide probes for DNA microarrays using a thermodynamic approach. Nucleic Acids Research 31:3057-3062

Ruiz, Y., P. Suarez, A. Alonso, E. Longo, A. Villaverde and F. S. Juan. 2011. Environmental quality of mussel farms in the Vigo Estuary: Pollution by PAHs, origin and effects on reproduction. Environmental Pollution 159:250-265

Saeed, A.I., V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa, T. Currier, M Thiagarajan, A. Sturn, M. Snuffin, A. Rezantsev, D. Popov, A. Ryltsov, E. Kostukovich, I. Borisovsky, Z. Liu, A. Vinsavich, V. Trush, and J. Quackenbush. 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34:374-378

Scott, W. B., and E. J. Crossman. 1973. Freshwater fishes of Canada. Bulletin – Fisheries Research Board of Canada No. 184

Sharma, A., K. Saurabh, S. Yadav, S. K. Jain, and D. Parmar. 2013. Expression profiling of selected genes of toxication and detoxication pathways in peripheral blood lymphocytes as a biomarker for predicting toxicity of environmental chemicals. International Journal of Hygiene and Environmental Health in press

Stegeman, J. J., J. V. Goldstone, and M. E. Hahn. 2010. 10 – Perspective on zebrafish as a model in environmental toxicology. Fish Physiology 29:367-439

Timme-Laragy, A., L. A. Van Tiem, E. A. Linney, and R. T. Di Giulio. 2009. Antioxidant response and NRF2 in synergic developmental toxicity of PAH in zebrafish. Toxicological Science 109:217-227

Wang, C., Y. Zhao, R. Zheng, X. Ding, W. Wei, Z. Zuo, and Y. Chen. 2006. Effects of tributyltin, benzo[a]pyrene, and their mixture on antioxidant defense systems in (*Sebastiscus marmoratus*) Ecotoxicology and Environmental Safety 65:381-387

Wang, Y., S. Xu, Y. Su, B. Ye, and Z. Hua. 2013. Molecular characterization and expression analysis of complement component C9 gene in the whitespotted bambooshark, *Chiloscyllium plagiosum*. Fish and Shellfish Immunology In Press

White, L. Loughlin, Z. Maqbool, M. Nilsen, J. McClure, Y. Dempsie, A. H. Baker, and M. R. MacLean. 2011. Serotonin transporter, sex, and hypoxia: microarray analysis in the pulmonary arteries of mice identifies genes with relevance to human PAH. Physiological Genomics 43:417-437

Whitehead, A., F. Galvez, S. Zhang, L. M. Williams and M. F. Oleksiak. 2011. Functional genomics of Physiological plasticity and local adaptation in killifish. Journal of Heredity 102:499-511

Williams, T. D., K. Gensberg, S. D. Michin, and J. K. Chipman. 2003. A DNA expression array to detect toxic stress response in European flounder (*Platichthys flesus*). Aquatic toxicology 65:141-157

Wirgin, I., J. R. Waldman. 1998. Altered gene expression and genetic damage in North American fish populations. Mutation Research 399:193-219

Wirgin, I., J. R. Waldman. 2004. Resistance to contaminant in North American fish populations. Mutation research 552:73-100

Wirgin, I., N. K. Roy, M. Loftus, R. C. Chambers, D. G. Franks, and M. E Hahn. 2011. Mechanistic basis of resistance to PCBs in Atlantic tomcod from the Hudson River. Science 331:1322-1325

Supplementary Table S5.1. Names, symbols and transcription value of the genes whose transcription was measurable in the two sacrificing times.

Gene name	symbol	transcribed	
		24 h	96 h
ADP/ATP traslocase		8	53
adrenergic receptor		334	181
Aryl hydrocarbon receptor nuclear tranlocator	ARNT	4202	4333
ATP binding cassette B-MDR/TAP		160	90
ATP synthase α		113	150
ATP synthase β		7	151
ATP synthase δ		39	86
cAMP-dependent, regulatory		178	75
Catechol-O-methyltransferase	COMT	Dose effect	1253
CD63 antigen		9	28
Compliment factor 4	C4	5032	4869
Compliment factor 9	C9	5991	Dose effect
Compliment factor B	CFB	504	1089
Cytochrome P450 1A1	CYP1A1	81	Dose effect
Elongation factor 1 alpha	EF1α	3697	3842
Elongation factor 1 beta	EF1β	1841	1817
Elongation factor 1 gamma	EFIγ	0	44
Ferreterin		1690	2033
Fibrinogen β		3408	3876

Glutathione S-transferase	GST	0	68
Glutathione S-transferase -3	GST-3	333	381
Heat shock protein 70	Hsc 70	325	707
Heat shock protein 70 71 kDa	Hsc 70 cong 71 kDa	42	159
4- hydroxyphenylpyruvate dioxygenase	HPPD/HPD	106	320
Hypoxia inducible factor 2	HIF 2	1486	Dose effect
Hypoxia inducible factor 2 alpha	HIF 2α	1300	Dose effect
Inhibition of apoptosis protein	IAP	34	15
Interlectin 2		21	18
Isocitrate dehydrogenase	IDH	304	Dose effect
Kinase D-interacting		351	Dose effect
Major histocompatibility complex IIβ	MHC IIβ	18	35
Methylcystosine dioxygenase	TET3	61	113
Myogenin		4	8
NADH dehydrogenase 9	NADH -9	18	35
NADH dehydrogenase 1β	NADH1β	Dose effect	Dose effect
nesprin-1		0	6
Nexin precursor		31	31
c-fos oncogene	c-Fos	784	Dose effect
phosphenolpyruvate carboxylkinase	PEP carboxykinase	10	76
phospholipase -B	P76	0	24
ribosomal protein	L13	4531	4645
superoxidase dimutase [Cu/Zn]	SOD1	17	Dose effect

Transferin precursor		1617	1759
Translation tumour protein	TPT1	663	1257
β-actin		0	Dose effect

CHAPTER 6

CONCLUSION

Evolution is continuously acting on all populations: it can contribute to biodiversity in nature, or it can reduce diversity through extinction or through the loss of alleles under negative selection. The process of evolution, while historically thought of as a long-term gradual process, can act rapidly (< 20 generations), and rapid evolution is most often associated with novel, fast-changing and/or stressful environments. Throughout my thesis I have referred to several examples of rapid evolution in aquatic systems in response to both novel and stressful environments (Reznick *et al.*1997; Elskus *et al.* 1999; Hendry *et al.* 2000; Wirgin and Waldman 2004; Nacci *et al.*2010; Wirgin *et al.*2011). While the paradigm of slow gradual evolutionary change has itself evolved, the potential for rapid evolution in response to anthropogenic habitat destruction it still relatively new.

Part of the difficulty with studying rapid evolution in response to polluted or degraded habitats is that fitness advantage may be, but does not have to be, a result of genetically based adaptation. Any response which provides advantages (increased fitness) in the local environment is identified as an "adaptive response". Within that definition of adaptive responses, there can be a number of specific processes acting, such as, behavioural responses, population genetics, plastic responses, cellular responses and true genetic adaptation. What unites the various processes within the concept of adaptive

responses is that they will alter the organism's response to the environment relative to a true naïve organism.

It is important to consider adaptive responses in degraded environments. An adaptive response is beneficial for the organism/population that exhibits the response, but the environment will still be stressful for all other (less adapted) organisms in the habitat. The contaminated environment may also be very harmful for naïve organisms. Naïve organism (including humans) may suffer severe stress when exposed, which is important to consider when developing biomarkers or using bioindicators. Despite the fact that humans may only be occasional "visitors" to an aquatic habitat, they and other naïve and visiting organisms may be able to experience enough exposure to be harmed – indeed that is the goal of biomarker and bioindicator species study.

Another reason to consider adaptive responses in ecotoxicological work is for informing environmental monitoring and restoration effort. If bioindicator species are used to assess the level of pollution hazard for a number of areas there may be some areas where the indicators show little effect due to adaptive responses. Those areas may then be wrongly ranked and assumed to be "clean" relative to other equally or less polluted sites where the indicator species do not display adaptive responses. When environmental and government agencies then determine priorities for remediation, these highly polluted areas will not be correctly ranked or prioritised for remediation. I have, in my dissertation, developed a systematic hierarchical approach for testing for adaptive responses to stressful environments (degraded environments; Figure A.1) which I think is important to consider for regulatory bodies working with organisms under any kind of stress.

Any organism (including naïve ones) have the potential to develop adaptive responses to any stressor, although genetic constraints and trade-offs may limit such responses. For short term impacts, acclimation is probably the adaptive response that occurs more often than genetic adaptation. For genetic adaptation to be able to evolve, a more chronic state of stress and hence selection pressure is needed. Despite the potential for rapid evolution, it still needs a few generations to come about. The scope for acclimation is likely genetically based (hence a genetic adaptation), even if the physiological acclimation in itself may not be. I believe acclimated individuals in a population may affect the evolutionary process towards genetic adaptations. How acclimation will affect the evolutionary process to adaptation will vary the stressor, and the species / population. However, variation in acclimation or phenotypic plasticity can slow down or speed up evolution, for example moderate plasticity will promote evolution (Price *et al.* 2003). Thus plastic responses in general may be important for not only short-term survival, but also for promoting genetic adaptation to stressful environments.

With the creation and discharge of new compounds driven by human activities, there are an increasing number of possible stressors occurring in nature. Novel compounds such as polychlorinated biphenyls (PCBs) have promoted genetic adaptation in fish (Elksus *et al.* 1999; Wirgin *et al.* 2011), or possibly acclimation in cases where the type of adaptive response has not been determined. On the other hand, naturally occurring contaminants, such as polycyclic hydrocarbons (PAHs) can result in plastic responses (Meyer *et al.* 2002; Nacci *et al.* 2002). Maybe there has been an advantage through time to be able to acclimate, when the benefits outweighed the costs and genetic adaptation did not occur, that is, when acclimation was efficient enough to block the evolution of genetic

adaptation. Now the chronic presence of PCBs is such a strong selection agent that it has driven rapid evolution. Thus an evolutionary response to novel stressors is possible, while acclamatory responses to natural and long-term stressors are also a possible outcome.

With the widespread study of local adaptation to new environments, coupled with physiological acclimation to degraded environments, fish have been shown to both genetically adapt and acclimate (for example Reznick *et al.*1997; Hendry *et al.* 2000; Meyer *et al.* 2002; Grey at al. 2003; Heath *et al.* 2003; Meyer *et al.* 2003; Williams *et al.* 2003; Fisher and Oleksiak 2007; Jeukens *et al.* 2008; Carlson *et al.* 2009; Clark *et al.* 2010; Aykanat *et al.* 2011; Whitehead *et al.* 2011; Clark and Di Giulio2012; Brammell *et al.* 2013). However, there will be high costs, both in terms of mortality and bioenergetics, until adaptive responses are fully developed (Wilson and Franklin 2002; Wood and Harrison 2002; Johnson *et al.* 2002; Ketata *et al.* 2007; Aldarondo-Torres *et al.* 2010; Chopra *et al.* 2011; Farwell *et al.* 2011; Ruiz *et al.* 2011). Many fish and aquatic invertebrates have short generation times, and they will thus evolve adaptive responses apparently rapidly, or at least rapidly relative to longer generation time species (such as large mammals). For humans and other long-lived animals, we now see increasing reproductive failure and cancer rates resulting from chronic contaminant exposure (Jemal *et al.* 2010; Soto and Sonnenschein 2010; Silber and Barbey 2012); however, the main effects of pollution may yet to be seen in longer-lived animals. This implies that it is critically important for both our future and for the future of our ecosystem to understand the fundamental processes that underlie acclimation and genetic adaptation in response to environmental stress and change. It is apparent that we need to consider adaptive responses in general for all changes occurring, both natural and anthropogenic, especially

as it is likely that various environmental stressors will act synergistically to impact individuals and populations.

Contribution to science

1) I challenged the paradigm of assuming organisms have a naïve response to stress challenges, this highlights the need to quantitatively partition the roles of cell and whole organism physiological responses

2) I combine and ecotoxicogenomic approaches with evolutionary principles to provide a systematic hierarchical approach for addressing the complexities of potential adaptive responses to environmental stress.

3) I show, for the first time, a behavioural adaptive response to leave polluted areas (avoidance) at a higher frequency that random dispersal can explain.

4) I show that brown bullhead (*Ameiurus nebulosus*) from polluted Trenton Channel display an alternative transcriptional response profile relative to fish from the cleaner Peche Isle site, and interpret this as an indication of adaptive response.

5) I am among the first to use next generation sequencing of the transcriptome in ecotoxicogenomics

6) I developed novel tools (microsatellite markers, custom microarray) for future researchers interested in brown bullhead as a model species.

7) I characterise the transcriptional response of the brown bullhead to an acute B[a]P challenge at over 40 toxicologically relevant genes.

Future directions: I would increase the number of species studied within the Detroit River to see the impact of pollution more generally, not just in brown bullhead. I would investigate how other fish species respond, whether more fish species display adaptive responses and how invertebrates respond. There are many other fish species living in the Detroit River, but the brown bullhead has been singled out for its high tumorigenesis rate; is that because of a difference in their behavioural, physiological cellular or molecular responses or is it their habitat exposure levels? I have shown that brown bullhead (a common indicator species) display adaptive responses and I would investigate if there are more species (fish and other) that display adaptive responses in the Detroit River. Further, I would examine correlations of gene expression co-occurring contaminants, different taxa may be responding to different pollutants.

Detroit River is not the only polluted aquatic ecosystem, and an extensive investigation of organisms from different polluted ecosystem in a similar manner would be interesting to see which species respond with adaptive responses. Assuming that the most highly responsive species to pollution will have been already identified as indicator species (that has been determined to be sensitive), perhaps they respond to pollution in unique ways and thus may exhibit different adaptive responses. Sensitive species may be more likely to develop adaptive responses that will skew our estimation of impacted sites.

Pollution is not the only possible stressor for organisms, other anthropogenic changes such as increased temperature and acidity as well as eutrophication impact aquatic organisms. Gene transcription would still be a good biomarker for all of those stressors, but we need to learn more about gene interactions, signalling pathways and specific gene functions to make true progress. When we get a better understanding of

individual stressors, their transcriptional effects and adaptive responses, then we can look into multiple stressor effects. There are already studies reporting on multiples stressors effects (Stone *et al.*2001; Eder *et al.*2009; Vanhoudt *et al.*2012), but we need to understand what will happen with adaptive responses when there are multiple stressors. Acclimation is costly and multiple stressors may be too demanding for some organisms and may ultimately cause mortality rather than acclimation.

I focused on genetic adaptation and physiologic acclimation but there are other adaptive responses and plastic responses. Most studies are conducted to look for genetic adaptation or physiological acclimation; however, Bozinovic and Oleksiak (2010) report phenotypic plasticity in pollution response among killifish (*Fundulus heteroclitus*). Phenotypic plasticity is considered relatively common in nature (Price *et al.* 2003). However, although my work adds to our understanding of the nature of adaptive responses that are commonly occurring, we need to address the question of other adaptive effects I did not consider (such as phenotypic plasticity). There are so many reposes that may occur but very little have been done to study most of them.

As is the case for all research studies, a number of methodological improvements could be made to increase the scope and impact of my work. These include: expand the next generation sequencing experiment. Though I report clear response differences, I would recommend doing the experiment on individuals challenged for 96 h and with greater sequencing depth per individual. I also believe that the deeper transcription coverage would pick up more genetic mechanisms that are occurring, such as alternative spicing and alternative allele expression. A sample with less rRNA (a better rRNA depletion) and a *de novo* assembly should be possible with deeper coverage. Next

generation sequencing could also be combined with epigenetic analyses, which would be an interesting investigation approach (Hurd and Nelson 2009) when adaptive responses have been shown. Despite some shortcomings of my methods, I was still able to address a number of important issues in my dissertation

REFERENCES

Aldarondo-Torres, J. X., F. Samara, I. Mansilla-Rivera, D. S. Aga, and C. J. Rodríguez-Sierra. 2010. Trace metals, PAHs, and PCBs in sediments from the Jobos Bay area in Puerto Rico. Marine Pollution Bulletin 60:1350-1358.

Aykanat, T., F. P. Thrower, and D. D Heath. 2011. Rapid evolution of osmoregulatory function by modification of gene transcription in steelhead trout. Genetica 139:233-242.

Bozinovic, G., and M. F. Oleksiak. 2010. Embryonic gene expression among pollutant resistant and sensitive *Fundulus heteroclitus* populations. Aquatic Toxicology98:221-229.

Brammell, B. F., D. J. Price, W. J. Brige, and A. A. Elskus. 2013. Lack of CYP1A responses in species inhabiting chronically contaminated habitats: two varieties of resistance? Comparative Biochemistry and Physiology Part C 157:212-219

Carlson, E. A., N. K. Roy, and I. I. Wirgin. 2009. Microarray analysis of polychlorinated biphenyl mixture-induced changes in gene expression among Atlantic tomcod populations displaying differential sensitivity to halogenated aromatic hydrocarbons. Environmental Toxicology and Chemistry 28:759-771

Chopra, A. K., M. K. Sharma, and S. Chamoli. 2011. Bioaccumulation of organochlorine pesticides in aquatic system – an overview. Environmental Monitoring and Assessment 173:905-916

Clark, B. W., C. W. Matson, D. Jung, and R. T. Di Giulio. 2010. AHR2 mediates cardiac teratogenesis of polycyclic aromatic hydrocarbons and PCB-126 in Atlantic killifish (*Fundulus heteroclitus*). Aquatic Toxicology 99:232-240

Clark, B. W., and R. T. Di Giulio. 2012. *Fundulus heteroclitus* adapted to PAHs are cross-resistant to multiple insecticides. Ecotoxicology 21:465

Eder, K. J., H. R. Köhler, and I. Werner. 2009 Pesticide and pathogen: Heat shock protein expression and acetylcholinesterase inhibition in juvenile Chinook salmon in response to multiple stressors. Environmental Toxicology and Chemistry 26:1233-1242

Elksus, A. A., E. Monosson, A. E. McElroy, J. j. Stegeman, D. S. Woltering. 1999. Altered CYP1A expression in *Fundulus heteroclitus* adults and larvae: a sign of pollutant resistance? Aquatic Toxicology 45:99-113

Farwell M., K. G. Drouillard, D. D. Heath, T. E Pitcher. 2012. Acclimation of life-history traits to experimental changes in environmental contaminant concentration in brown bullhead (*Ameiurs nebulous*) Environmental Toxicology and Chemistry 31:863-869.

Fisher, M. A. and M. F. Oleksiak. 2007. Convergence and divergence in gene expression among natural populations exposed to pollution. BMC Genomics 8:108

Grey, J. P., T. L. Leas, E. Obert, D. Brown, G. C. Clark, and J. P. Vanden Heuvel. 2003. Evidence of aryl hydrocarbon receptor ligands in Presque Isle Bay of Lake Erie. Aquatic Toxicology 64:343-358

Heath, D. d., J. W. Heath, C. A. Bryden, R. M. Johnson, and C. W. Fox. Rapid evolution of egg size in captive salmon. Science 299:1738-1740

Hendry, A. P., J. K. Wenburg, P Bentzen, E. C. Volk, and T. P. Quinn. 2000. Rapid evolution of reproductive isolation in the wild: evidence from introduced salmon. Science 290:516-518

Hurd, P. J., and C. J. Nelson. 2009. Advantage of next generation sequencing versus the microarray in epigenetics. Briefings in Functional Genomics and Proteomics 8:174-183

Jemal, A., M. M Center, C. DeSantis, and E. M. Ward. 2010. Global patterns of cancer incidence and mortality rates. Cancer Epidemiology, Biomarkers and Prevention 19:1893-1907

Jeukens, J., D. Bittner, R. Knudsen, and L. Bernatchez. 2009. Candidate genes and adaptive radiation: insights from transcriptional adaptation to the limnetic niche among coregonine fishes (*Coregonus* spp., Salmonidae). Molecular Biology and Evolution 26:155-166

Johnson, L. L., T. K. Collier, and J. E. Stein. 2002. An analysis in support of sediment quality thresholds for polycyclic aromatic hydrocarbons (PAHs) to protect estuarine fish. Aquatic conservation: Marine and Freshwater Ecosystems 10:517-538

Ketata, I., W Smaoui-Damak, F. Guermazi, T. Rebai, and A. Hamza-Chaffai. 2007. *In situ* endocrine disrupting effects of cadmium on the reproduction of *Ruditapes decussates*. Comparative Biochemistry and Physiology, Part C 146: 415-430

Meyer, J. N., D. E. Nacci, R.T. Di Giulio. 2002. Cytochrome P4501A (CYP1A) in killifish (*Fundulus heteroclitus*): heritability of altered expression and relationship to survival in contaminated sediments. Toxicological Science 68:69-81

Meyer, J. N., D. M. Wassenberg, S. I. Krachner, M. E. Hahn, and R. T. Di Giulio. 2003. Expression difference and inducibility of aryl hydrocarbon receptor pathway genes in wild-caught killifish (*Fundulus heteroclitus*) with different contaminant-exposure histories. Environmental Toxicology and Chemistry 22:2337-2343

Nacci, D. E., M. Kohan, M. Pelletier, and E. George. 2002. Effects of benzo[a]pyrene on a fish population resistant to the toxic effects of dioxin-like compounds. Aquatic Toxicology 57:203-215

Nacci, D. E., D. Champlin, and S. Jayaraman. 2010. Adaptation of the estuarine fish *Fundulus heteroclitus* (Atlantic Killifish) to polychlorinated biphenyls (PCBs). Estuaries and Coasts 33: 853-864

Price, D., A. Qvarnström, and D. E. Irwin. 2003. The role of phenotypic plasticity in driving genetic evolution. Proceedings of the Royal Society B 270:1433-1440

Reznick, D. N., F. H. Shaw, F. H. Rodd, and R. G. Shaw. 1997. Evaluation of the rate of evolution in natural populations of guppies (*Poecilia reticulata*). Science 275, 1934-1937

Ruiz, Y., P. Suarez, A. Alonso, E. Longo, A. Villaverde and F. S. Juan. 2011. Environmental quality of mussel farms in the Vigo Estuary: Pollution by PAHs, origin and effects on reproduction. Environmental Pollution 159:250-265

Silber, S. J., and N. Barbey. 2012. Scientific molecular basis for treatments of reproductive failure in the human: An insight into the future. Biochimica and Biophysica Acta 1822:1981-1996

Soto, A. M., and C. Sonnenschein. 2010. Environmental causes of cancer: endocrine disruptors as carcinogens. Nature Reviews Endocrinology 6:363-370

Stone, D., P. Jepson, P. Kramarz, and R. Laskowski. 2001. Time to death response in carabid beetles exposed to multiple stressors along a gradient of heavy metal pollution. Environmental Pollution 113:239-244

Vanhoudt, N., H. Vandenhove, A. Real, C. Bradshaw, and K. Stark. 2012. A review of multiple stressor studies that include ionising radiation. Environmental Pollution 168:177-192

Whitehead, A., F. Galvez, S. Zhang, L. M. Williams, and M. F. Oleksiak.2011. Functional genomics of physiological plasticity and local adaptation in killifish. Journal of Heredity 102:499-511

Williams, T. D., K. Gensberg, S. D. Michin, and J. K. Chipman. 2003. A DNA expression array to detect toxic stress response in European flounder (*Platichthys flesus*). Aquatic Toxicology 65:141-157

Wilson, R. S., and C. E. Franklin. 2002. Testing the beneficial acclimation hypothesis. Trends in Ecology & Evolution 17:66-70

Wirgin, I., and J. R.Waldman. 2004. Resistance to contaminants in North American fish populations. Mutation Research 552:73-100

Wirgin, I., N. K. Roy, M. Loftus, R. C. Chambers, D. G. Franks, and M. E Hahn. 2011. Mechanistic basis of resistance to PCBs in Atlantic tomcod from the Hudson River. Science 331:1322-1325

Wood, H. A., and J. F. Harrison. 2002. Interpreting rejections of the beneficial acclimation hypothesis: when is physiological plasticity adaptive? Evolution 56:1863-1866

APPENDICES

NOVEL AND OPTIMIZED POLYMORPHIC MICROSATELLITE LOCI FOR BROWN BULLHEAD (*AMEIURUS NEBULOSUS*)*

Brown bullhead (*Ameiurus nebulosus*) are native to freshwaters of eastern and central North America (Scott & Crossman 1998), and are thought to be particularly tolerant to stressful environment conditions (e.g., contaminates and low oxygen; Scott & Crossman 1998). Brown bullhead have been used as a contaminant sentinel species due to their benthic habitat (Baumann *et al.* 1996). However, an understanding of their dispersal patterns is needed to investigate possible local adaptation in response to aquatic contaminants. Although mitochondrial and RAPD based studies showed brown bullhead are philopartric (Murdoch & Herbert 1994; Silbiger *et al.* 2001), more precise gene flow estimates are needed to confirm their suitability as a contaminant sentinel species.

We developed microsatellite markers using an enriched genomic library following the protocol of Galarza *et al.* (2007). Briefly, approximately 10 μg genomic DNA was extracted from five individuals using phenol-chloroform extraction (Sambrook *et al.* 1989). Genomic DNA was simultaneously digested using *Dra*I and ligated with double-stranded Super SNX linkers (Hamilton *et al.* 1999). Ligated fragments were enriched with a biotin-labelled probe mixture of $(AC)_7$ and $(GCTG)_5$ at 10 μM each and selectively detained by streptavidin-coated Dynabeads (Roche). Enriched DNA was

*L.I. Söderberg, J.A. Galarza and D.D Heath

eluted in 100 µl ddH_2O, PCR amplified, and the products were ligated into a pGEM-T Easy Vector following the manufactures' protocol (Promega). Sequences from 48 positive clones were used to design primer pairs for 18 potential microsatellite DNA markers using Primer3 (Rozen & Skaletsky 2000).

Primer sets were tested by PCR amplification preformed in 25 µl reaction volumes: 50 ng DNA, 2.0mM $MgCl_2$, 0.25 µM of each primer (forward primers dye-labelled), 200 µM of dNTP, 1x reaction buffer [75mM Tris-HCl, 20mM $(NH_4)_2SO_4$] and 0.5 units of Taq polymerase (Applied Biosystems). Reaction conditions were: initial denaturation of 2 min at 94°, 33 cycles consisting of 30 s at 94°, 45 s at various annealing temperatures (Table 1) and 1 min at 72°; ending with a 2 minute final extension at 72°. Primer Amn-42 worked best with a "touch-down" PCR protocol with an initial 10 cycles decreasing one degree per cycle (from 56° to 46°). Genotypes (fragment sizes) were determined using a Li-Cor 4300 DNA analyser and alleles scored using GENEIMAGER 4.05 software (Scanalytics). Of the 18 primers pairs, eight were variable and showed consistent amplification (Table 1). An additional five primers were optimised for use in brown bullhead (Table 1) from channel catfish (*Ictalurus punctatus*; Liu *et al.* 1999; Tan *et al.* 1999) and yellow bullhead (*A. natalis*; Creer & Trexler 2006).

Table A.1 Summary of the characteristics of polymorphic microsatellite loci in the brown bullhead (*Ameiurus nebulosus*). Locus name, GenBank Accession number, repeat motif, primer sequence (5'to 3'), annealing temperature (°C), final $MgCl_2$ concentration (Mg), number of alleles, range of allele sizes, observed and expected heterozygosity (H_0 and H_E) and inbreeding coefficient (F_{IS}) are presented.

Locus	Repeat motif	GenBank Accession no.	Primer sequences (5'-3')	°C	Mg (mM)	No of alleles	Allele size (bp)	H_O	H_E	F_{IS}
Amn-3	$(AC)_{10}$	GQ869778	F-ACAACCTGGAACCTCAATCG R- TAACAGCAAAAGGGGGAACA	58	1.5	3	175-179	0.327	0.276	-0.182
Amn-16	$(CA)_{14}$	GQ869779	F-ACAACCGAAAGGATCTGGTG R-ACGACCACTTCAACGATGC	60	2.2	8	111-133	0.735	0.698	-0.053
Amn-34	$(GT)_7AT(GT)_6CT(CA)_3$	GQ869780	F-TTGTGTTCAGTCCGATAAATGT R-CCCCTGGCTTTCCAATTACT	60	1.5	4	187-227	0.286	0.282	-0.014
Amn-41	$(AC)_{14}$	GQ869781	F- ACGTCAATCAGGTTTGAGCA R- GGCCGCAACTTACAAGACAC	60	1	9	106-134	0.776	0.738	-0.051
Amn-42	$(GT)_{11}$	GQ869782	F-CGCTTGATTATGCACACCTG R-TAAGGCAAGCCAAGATGAGC	TD58	1.5	3[a]	135-149	0.531	0.412	-0.289
Amn-43	$(CA)_{15}$	GQ869783	F-TGATTGAGACAAATTCAAGGAAG R-GATGGTCAGGTGTCCACAAA	65	1.5	11	148-198	0.531	0.597	0.112
Amn-44	$(AC)_{10}$	GQ869784	F- CGGAAACGAGACACTACATGG R- AGTGGAACCCTTTGCCTTTT	60	2	4[a]	125-141	0.388	0.508	0.237
Amn-46	$(CA)_{16}$	GQ869785	F-CCGGTGTCGTGCTAATACCT R-CAGCCACGTCATGTACCACT	58	2.2	10	125-159	0.306[b]	0.449	0.318
An 12[c]	$(TATC)_{11}$		F-ACCATCTCAGTGGGAGCCAA R-AAGAAAACAGACTGCAACAT	60	1.5	7	126-170	0.673	0.561	-0.201
Ip 30[d]	$(CA)_{11}$		F-CTAAAGTTGGAGAAGAGTTCAGC R-AAGACAAGGACATCTCAATGC	50	2.2	5	196-234	0.469	0.454	-0.034
Ip 365[d]	$(CA)_{13}$		F-TAAAGGATCTGATTCACCGTATC R-AAACCGCTAACCTACCCTCT	55	2	4	110-134	0.122	0.117	-0.048
Ip 372[d]	$(CA)_8$		F-GGCACTGAGGTTTGGGCTGCAC R-TGGCATCGCTCCTCATCATCCTG	60	1.5	8	161-193	0.714	0.692	-0.033
Ip 607[e]	$(GA)_{24}$		F-TCAGGCACAAATCTTGTGATGG R-TTGTAGTTCTGCCTCTAACCGC	50	2.2	3	143-147	0.125	0.119	-0.051

[a] For both Amn-42 and Amn-44 two additional alleles were found in samples from other locations.

[b] Significant departure from Hardy Weinberg equilibrium, homozygote excess was indicated by MICROCHECKER 2.2.3 (Van Oosterhout *et al.*2004)

[c] Previously described for *A. natalis* in Creer & Trexler (2006)

[d] Previously described for *I. punctatus* in Liu *et al.* (1999)

[e] Previously described for *I. punctatus* in Tan *et al.* 1999

We assessed microsatellite variability in 96 individuals from several populations, 49 of the 96 individual were from a single Detroit River population which was used for all further analyses. All loci were polymorphic, with allele number ranging from three to eleven, and observed heterozygosity ranging from 0.286 to 0.776 (Table 1). Deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium was tested using GENEPOP 1.2 (Raymond & Rousset 1995; Table 1) with 500 batches. One locus (Amn-46) showed significant deviation from HWE after Bonferoni correction: the homozygote excess was likely due to null alleles based on MICROCHECKER 2.2.3 (Van Oosterhout *et al.*2004). There was no evidence of linkage disequilibrium. The primer pairs were tested in four closely related species: the yellow bullhead, black bullhead (*A. maleas*) channel catfish and tadpole madtom (*Noturus gyrinus*), and four or more primer pairs proved useful in all four species (Table 2). These 12 microsatellites will be useful in assessment of gene flow and dispersal, as well as help monitor ecosystems for the effects of contaminant loads.

Table A.2 Cross-species amplification of *Ameiurus nebulosus* microsatellite markers. Where PCR amplification was successful, size range and numbers of alleles are included; amplification failure is indicated by a dash.

	Yellow bullhead *Ameiurus natalis* (N=7)		*Black bullhead* *Ameiurus maleas* (N=6)		Channel catfish *Ictalurus punctatus* (N=6)		Tadpole madtom *Noturus gyrinus (N=5)*	
	No of alleles	Size range (pb)	No of alleles	Size range (pb)	No of alleles	Size range (pb)	No of alleles	Size range (pb)
Amn 3	–	–	5	179-197	–	–	–	–
Amn 16	2	105-107	1	111	–	–	3	105-113
Amn 34	2	187-195	3	195-199	–	–	–	–
Amn 41	–	–	3	122-156	4	124-132	2	119-131
Amn 42	3	145-149	5	145-159	–	–	2	145-147
Amn 43	2	152-154	–	–	5	146-158	2	140-152
Amn 44	3	149-153	3	137-143	6	143-153	–	–
Amn 46	3	131-135	5	131-149	3	148-150	3	131-143

REFERENCES

Baumann, P. C., I. R. Smith, and C. D. Metcalfe. 1996. Linkages between chemical contaminants and tumors in benthic Great Lake Fish. Journal of Great Lakes Research 22:131-152

Creer, D. A., and J. C Trexler. 2006. New polymorphic microsatellite loci in two fish species: bluefin killifish (*Lucania goodie*) and yellow bullhead *(A. meiurus natalis*) Molecular Ecology Notes 6:167-169

Galarza, J.A., S. Roques, J. Carreras-Carbonell, E. Macpherson, G. F. Turner, and C. Rico. 2007. Polymorphic microsatellite loci for the cardinal fish (*Apogon imberbis*). Conservation Genetics 8:1251-1253.

Hamilton, M.B., E. L. Pincus, A. Di Fiore, R. C. Flesher. 1999. Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. BioTechnicques 27:500-507.

Liu, Z., G. Tan, H. Kucuktas, P Li, A. Karsi, D. R. Yant, and R.A. Dunham. 1999 High levels of conservation at microsatellite loci among Ictalurid catfishes. Journal of Heredity 90:307-312

Murdoch, M.H., and P. N. D. Herbert. 1994. Mitochondrial DNA diversity of brown bullhead from contaminated and relatively pristine sites in the Great Lakes. Environmental Toxicology and Chemistry 13:1281-1289

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity 86:248-249

Rozen, S., and H. J. Skaletsky. 2000 Primer 3 on the WWW for general users and for biologist programmners In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp 365-386

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989 Molecular cloning: a Laboratory Manual, 2nd edn. Cold Sring Harbor Laboratory Press, New York

Scott, W.B., and E. J. Crossman. 1973 Freshwater fishes of Canada. Fisheries Research Board Canada Bulletin 184. 966 pp

Silbiger, R. N., A. C. Leonard, P. Dimsoski, S. Fore, S. I. Guttman, A. C. Roth, D. A. Gordon, T. Wessendarp, G. P. Toth, and M. K. Smith. 2001. Use of molecular markers to study the effects of environmental impacts on genetic diversity in brown bullhead (Ameirus nebulosus) populations. Environmental Toxicology and Chemistry 20:2580-2587.

Tan, G., A. Karsi, P. Li, S. Kim, X. Zheng, H. Kucktas, B. J. Argue, R. A. Dunham, and Z. J. Liu. 1999. Polymorphic microsatellite markers in *Ictalurus punctatus* and related catfish species. Molecular Ecology 8:1758-1760.

Van Oosterhout, C., W. F. Hutchinson, D. P. M. Willis, and P. Shipley. 2004 MICROCHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes, 4:535-538.

VITA AUCTORIS

NAME:	Linda I. Söderberg
PLACE OF BIRTH:	Stockholm, Sweden
YEAR OF BIRTH:	1975
EDUCATION:	Östra Reals gymnasium, Stockholm, Sweden, 1994
	Uppsala University, B.Sc., Uppsala, Sweden, 2003
	Uppsala University, M.Sc., Uppsala, Sweden, 2003