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EVOLUTIONARY TOXICOLOGY: IMPLICATIONS OF POLYCHLORINATED BIPHENYLS IN FISHES FROM THE LOWER GREAT LAKES

By

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Evolutionary toxicology: implications of polychlorinated biphenyls in fishes from the Lower Great Lakes

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DECLARATION OF CO-AUTHORSHIP

I hereby declare that this thesis incorporates material that is the result of joint research, as follows: Chapter 2 was co-authored with Dr. Trevor Pitcher, Dr. Daniel Heath and Dr. Ken Drouillard and has been submitted for publication to the peer-reviewed Journal of Great Lakes Research. Chapter 3 was co-authored with Dr. Trevor Pitcher, Dr. Daniel Heath and Dr. Ken Drouillard and has been published with the following citation: Farwell, M., Drouillard, K.G., Heath, D.D., Pitcher, T.E. Acclimation of life-history traits to experimental changes in environmental contaminant concentrations in brown bullhead (*Ameiurus nebulosus*). Environ. Toxicol. Chem. 31:863-869. Chapter 4 was co-authored with Dr. Trevor Pitcher, Dr. Oliver Love, Dr. Christina Seminiuk, Dr. Ian Butts and Dr. Ken Drouillard. Chapter 5 was co-authored by Dr. Trevor Pitcher, Dr. Stéphanie Doucet, Dr. John Hudson and Dr. Ken Drouillard. My collaborators provided valuable feedback, helped with project design, analytical techniques, and provided editorial input during the writing of each manuscript; however, primary contributions have been by the author.

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ABSTRACT

The ability to withstand a range of environmental contaminants has been documented in numerous taxa. Until recently, the field of ecotoxicology has focused, in part, on identifying adaptive traits of populations exposed to contaminants, with little emphasis on understanding mechanisms underlying individual-level variation in these traits. An emerging field, termed evolutionary toxicology, expands the scope of ecotoxicology by investigating such mechanisms and discussing their evolutionary implications. My thesis blends the fields of ecotoxicology and evolutionary toxicology by investigating novel avenues and addressing novel questions in these fields that remain to be explored, as discussed in my review article. To do this, I first used an ecotoxicological approach and identified associations between female reproductive traits and inorganic environmental contaminants in brown bullhead (*Ameiurus nebulosus*) in the Lower Great Lakes Region. I further used an experimental approach to support the hypothesis that acclimation is one possible mechanism underlying these associations. Next, I used an evolutionary toxicological approach to examine male primary and secondary sexual traits under sexual selection in wild populations of both Chinook salmon (*Onchorhynchus tshawytscha*) and Coho salmon (*Oncorhynchus kisutch*) with regards to individual measures of polychlorinated biphenyls (PCBs). I found that for Chinook salmon, PCB concentrations best describe variation in primary and secondary sexual traits in males with low 11-KT concentrations. This result highlights potential physiological mechanisms associated with PCBs that can explain variation in traits under sexual selection. It also supports the hypothesis that PCBs may have implications for the expression of sexually selected traits and suggests that future research focus on possible implications of this on

mating success and ultimately sexual selection. No significant associations were identified between PCBs and sexual traits in Coho salmon, potentially due to a number of sampling limitations. Overall, my thesis examines the bridge between ecotoxiolcogy and evolutionary toxicology and discusses how this novel research can be applied to management practices, such as habitat restoration.

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CHAPTER 1: GENERAL INTRODUCTION

Evolutionary toxicology: An overview

Populations from many taxa are able to withstand long-term environmental contaminant exposure, and in turn, often express different phenotypes compared to populations not exposed to the same conditions; which may be adaptive (Parker et al. 1999, Campbell and Campbell 2002, Medina et al. 2007, Nagajyoti et al. 2010) ,). Mechanisms underlying adaptive phenotypes, or traits, in regards to environmental contaminants have recently begun to be examined in wild populations (e.g. Wirgin 2011, Coutellec and Bara 2011). Currently, however, there is little experimental evidence for mechanisms underlying the presence of these adaptive traits. Questions regarding evolutionary causes and implications of adaptation to contaminants have encouraged ecotoxicologists to reassess past methodologies and focus on new ways to investigate adaptive mechanisms using wild populations in order to increase the ecological and evolutionary relevancy of their findings.

Evolutionary toxicology, a field only recently formalized (Shugart et al. 2010, Bickham 2011), is one vector through which rigorous experimental investigation of mechanisms underlying adaptive traits has occurred (e.g. Raquel et al. 2010, Coutellec et al. 2011, Lind and Grahn 2011). The aim of the evolutionary toxicology field is to identify and explain the ecological relevance and evolutionary implications of changes in population genetic structure due to environmental contaminant exposure (Bickham 2011). It applies evolutionary theories, concepts, and molecular tools to common ecotoxicological experiments, such as controlled experimental dose-response experiments, identifying physiological effects correlated with an increasing gradient of

contaminant exposure, and comparing phenotypes between organisms from reference and contaminated habitats.

One fundamental aspect of evolutionary toxicology is identifying and investigating adaptive traits; those that increase the fitness of an individual that possess them relative to one that does not (Klerks 1999). Adaptive traits typically arise when an individual or population is under stress, due to any number of factors, including contaminant exposure (Medina et al. 2007, Klerks 1999). Mechanisms underlying adaptive traits to environmental contaminants can generally be classified as one of three main types: tolerance, acclimation and genetic adaptation. Each of these mechanisms is unique, observed at a different biological scale, and has different evolutionary implications. Tolerance is an acute physiological response to contaminant exposure. This response occurs and is observed at the individual level and aims only to ensure the survival of that individual, thus increasing its fitness (Simms and Triplett 1994). It is not however, a mechanism that is typically sustainable over long periods of time, as the contaminant continues to be a physiological stressor (Woltering 1984). The amount of exposure to contaminants that an individual is able to tolerate is limited by an individual's physiological capability to do so (Woltering 1984). An individual experiencing long-term contaminant exposure or exposure greater than their physiology can withstand will either ultimately acquire adaptive traits through other mechanisms, or they will not survive. Tolerance to contaminant exposure does have a genetic basis yet it is environmentally induced and thus observed adaptive traits are not heritable. Acclimation is also a physiological response to contaminant exposure that occurs and is observed at the individual level. Acclimation differs from tolerance, and phenotypic plasticity in general, in that acclimation aims to maintain homeostasis when an individual is under stress

(West-Eberhard 1989). The benefit of maintaining homeostasis is that an individual is able to withstand either longer durations or higher degrees of contaminant exposure while maintaining typical physiological functioning (Beyers et al. 1999). An individual expressing an adaptive trait due to acclimation may therefore maintain this expression on a short or long-term basis. This maintenance is associated with an energetic cost which can be characterized using multiple metrics including phenotypic plasticity in traits linked to fitness (e.g. life-history traits, Rasanen et al. 2008), changes in immune function (Finkelstein et al. 2007), and changes in metabolic function (West-Eberhard 1989, Beyers et al. 1999). The specific costs of maintaining homeostasis can vary among individuals depending on other environmental stressors present (e.g. Lavado et al. 2011), as well as physiological and genetic constraints (e.g. reaction norms, West-Eberhard 1989). Acclimation mechanisms have a genetic basis but are also environmentally induced, thus observed adaptive traits are not heritable. Adaptive traits due to acclimation may also be reversible when contaminant exposure is removed (West-Eberhard 1989). Genetic adaptation occurs via selection favouring individuals with adaptive traits and is observed at the population level. Adaptive traits due to genetic adaptation can be characterized as either a change in average phenotype or in the plasticity of a trait (e.g. reaction norm) in a population; both of which must have a genetic basis and must be heritable. Changes in these traits take multiple generations to be observed at a population level, compared to short term physiological mechanisms, because of the selection process (natural or sexual). One benefit of genetic adaptation as an adaptive mechanism is that if contaminant exposure persists over a long period of time, as is true for many populations world-wide, then adaptive traits can be passed on to future generations. This may result in less physiological costs incurred for the maintenance of homeostasis, compared to acclimation

processes (Hoffmann and Parsons 1991). There are also downfalls to genetic adaptation; for example, fitness declines in contaminated populations may not be directly due to contaminants themselves, but due to decreased genetic variation at the population level caused by directional selection occurring when exposed to contaminants (Van Straalen and Timmermans 2002). It is important to note that for all three mechanisms, traits with adaptive potential in one individual or population are not necessarily the same as those in another population exposed to the same contaminant (Klerks and Weis 1987).

The main goals of this chapter are to review the ecotoxicology literature investigating tolerance, acclimation, and genetic adaptation as mechanisms underlying adaptive traits, to highlight trends in research focussing on each mechanism in terms of chronology and abundance, and, most importantly, to convince the reader of the novelty and relevance of incorporating evolutionary theories to ecotoxicological research. I will address these goals by providing a brief review of the ecotoxicological field to date, focusing on current research that will benefit from the identification of adaptive mechanisms. I will lastly focus more specifically on tolerance, acclimation and genetic adaptation, their application to current ecotoxicological research from an adaptive perspective and methods and examples of how to investigate these mechanisms.

Ecological Toxicology to Evolutionary Toxicology

In order to comprehend the novelty and relevance of the emerging field of evolutionary toxicology, one must first appreciate its historical development. The field of toxicology focuses on understanding the biochemical and physiological mechanisms underlying observed differences in sensitivities and relative toxicities of different contaminants in organisms (Chapman 2002). Research in this field is most often

conducted in laboratories, is highly controlled, and uses organisms that are successfully reared in a lab environment. It is from toxicology that contaminants and their chemical properties were identified and an understanding of how contaminants move through an organism and affect physiological processes was developed. It wasn't until the industrial revolution that noticeable phenotypic changes in organisms exposed to environmental contaminants were observed. Livestock were the first 'wild' organisms in which the effects of contaminants were examined as they are of economic and health interest (Hoffman 1995). At the same time, phenotypic changes in wild populations of undomesticated organisms were also being noticed, but were not investigated until decades later (Truhaut 1977).

Once environmental issues became of public concern in the 1960s, in large part due to Rachel Carson and her book *Silent Spring*, there was an urgent need to identify and understand phenotypic changes in wild populations exposed to contaminants to determine whether contaminants were in fact causing such changes. The field of ecotoxicology thus emerged as a way to apply toxicological principles obtained in laboratory research to ecological concepts, such as food web and population dynamics, with the goal of predicting, how and how much contaminants organisms are being exposed (e.g. bioaccumulation, biomagnifications, food sources), effects of exposure to environmentally relevant concentrations of contaminants on wild communities and ecosystems (e.g. reproduction, survival, growth, foraging, social interactions, distribution), not just at the individual level, and the development of management strategies to minimize the impacts of contaminants on ecological processes (e.g. persistence of contaminants, recommended environmental limits and regulations) (Truhaut 1977, Chapman 2002). The belnding of ecology into toxicological studies was

indeed gradual and initially occurred addressed during the 1970s and early 1980s via dose-response laboratory experiments to test for tolerance (see table 1.1). Tolerance tests were still conducted at the individual level in a variety of taxa, originating most often from laboratories and on occasion from field environments, ranging from bacteria to larger vertebrates, and aimed to determine lethal concentrations of contaminants that could then be applied to larger ecological scales and the development of management strategies for these ecological scales. These experiments used relatively high concentrations of contaminants, much greater than observed in wild habiats, to determine LC_{50} concentrations after a single, acute exposure. These concentrations would ultimately be used to determine what concentration of contaminants would result in the mortality (or other fitness trait depending on the test) of at least 50% of the individuals. The main benefit of these types of studies, especially at the time, was that they were the first attempts to link chemical exposure to a fitness measure (i.e. survival), thus providing support that contaminants were indeed affecting living organisms. There were, however, many limitations in the ecological application of these experiments which provided a basis for the field to grow upon in the future. One main limitation noted by multiple researchers in the early to mid 1980s is that there is substantial individual variation in responses both to contaminant type and concentration (e.g. Luoma et al. 1983, Levin and Kimball 1984). Another limitation of these experiments was that organisms from wild populations and ecologically relevant contaminant concentrations were often not used. There has been more recent data in the literature supporting the notion that organisms from wild populations with previous contaminant exposure can have different responses to contaminants than laboratory organisms because of adaptive traits previously established (Barata 2000, Medina et al. 2007). Generally, it was recognized that these

limitations needed to be addressed in order to understand the ecological relevance of research to date.

In the late 1980s to early 2000s, there was an explosion of ecotoxicological research coinciding with the improvement of analytical techniques and the inclusion of ecologically-trained researchers to the field. Research during this time was wide spread and focussed on topics such as identifying bioindicators of contamination, endocrine disruption, and human effects and contaminant food restrictions. From an adaptive perspective, ecotoxicological research mainly focused on identifying and quantifying adaptive traits in wild populations, and investigating whether and how populations vary in these phenotypes when exposed to different contaminant concentrations (e.g. Spies and Rice 1988 , DeVault et al. 1988, Hose et al. 1989). Initial descriptive research aimed to quantify differences in traits with adaptive potential between wild populations exposed to contaminants compared to reference populations. One benefit of this experimental design is that they have led to the identification of adaptive traits, including life-history traits and physiological processes, in wild populations (e.g. Phillips and Harrison 1999, Wirgin and Waldman 2004, Hewitt et al. 2008), a key step in understanding how populations are affected by contaminant exposure. The use of wild populations also ensures that adaptive traits that have been identified will have ecological relevance. One limitation to this comparative approach is that there is no experimental insight regarding mechanisms underlying the presence of these particular traits. Even when suites of biochemical or physiological assays underlying these traits are assessed as mechanisms (e.g. McMaster et al 1992, Greeley 2002) understanding why differences in these sub-organismal traits occur remains to be understood.

During this time, individual level research was also being conducted in laboratories to identify mechanisms underlying adaptive traits observed in wild populations. These studies used field-caught individuals to initially identify tolerance, but soon after to identify acclimation mechanisms as well. To investigate acclimation mechanisms, organisms would be exposed chronically, or over long periods of time, to lower and environmentally relevant concentrations compared to previous tolerance studies. Correlates between gradient exposures of contaminants and adaptive traits were then identified and common garden experimental designs using individuals previously exposed to contaminants compared to those from reference locations were often used to determine differences in fitness measures to examine their adaptiveness (see Table 1.1). In these experiments, the results were often applied to ecological risk assessment protocols organisms that are able to acclimate to changing environments are not optimal organisms to use as bioindicators (Via et al. 1995). These results were also applied to habitat rehabilitation practices where organisms that were able to acclimate to changing environments would likely benefit from contaminant removal compared to those that were not able to acclimate An obvious limitation to this application is that individuals, even when originating from wild populations, are not in their original habitat when experiments are conducted and therefore the legitimacy of using the results to set contaminant regulations in nature is somewhat unsubstantiated.

In the late 1990s, a new stream of research developed that focused on the genetic basis for adaptive traits which included quantifying changes in genome-wide diversity, in gene flow and migration, and in allele frequencies due to genotoxicity between populations (see Table 1.1). This inclusion of evolutionary theory in ecotoxicology originated from the realisation that wild populations exposed to contaminants may evolve

differently from those in similar environments but relatively less exposed, which could account for discrepancies not only between lab and field experiments, but between multiple field experiments as well (Coutellec et al. 2006). Shortly thereafter, empirical evidence supported the notion that environmental contaminants have the potential to elicit evolutionary processes, including changes in population genetics, as many contaminants are persistent, have genotoxic properties, and affect fitness-related traits (Klerks 1999, Medina et al. 2007). Evolutionary toxicology was conceptualized via the merging of ecotoxicology with conservation biology, a field which typically employs evolutionary theory and population genetics to investigate effects of environmental stressors on populations (Bickham 2011). Mechanisms of evolution elicited by environmental contaminants contain examples of both direct (e.g. mutations) and indirect (e.g. selection) effects at the individual level. There are multiple direct and indirect effects that can explain differences in the genetic structure of a population exposed to contaminants and are discussed in detail by Bickham (2011). Of the 'four cornerstones' to evolutionary ecotoxicology, the only adaptive mechanism discussed is changes in allelic or genotypic frequencies at loci related to life-history traits, due to selection. Because of the many possible mechanisms driving evolutionary change in populations exposed to contaminants, it is important for researchers to clearly define whether they are examining genetic consequences of contaminant exposure versus genetic mechanisms underlying the presence of adaptive traits in a population. The former being a descriptive study of the genetic make-up of populations in relatively more and less contaminated habitats and the later being an experimental study investigating the genetic basis of adaptive traits.

Adaptive traits: Identification and underlying mechanisms

It is clear from past and present ecotoxicological research that there is a need to investigate mechanisms underlying adaptive traits. To begin investigating these mechanisms, one must first determine whether the traits in question are in fact linked to individual fitness (Figure 1). While this first step may appear obvious, it is an assumption that is easily and often overlooked. Experimental testing of whether a trait is linked to fitness is especially important in wild populations as there are several environmental variables, other than contaminant exposure, that could also be affecting the trait of interest, as well as interpretations as to how and if the trait is linked to fitnesse (Levin and Kimball 1984, Klerks 1999). It is also important to keep in mind that adaptive traits are not limited to life-history traits, but can also include those that increase mating opportunities and success such as secondary sexual traits. It is because of this that it is important to investigate a carefully selected trait or suite of traits relevant to contaminant exposure to aid in determining which traits are contributing most to an individual's fitness level in a particular environment.

Tolerance has been one of the largest areas of research in ecotoxicology to date (see Table 1.1). Even though it is still somewhat studied today, it can be considered a starting point in ecotoxicological research investigating adaptive traits. One main benefit of studying tolerance, from an adaptive perspective, is that it often occurs as the initial response to contaminant exposure, prior to the onset of both acclimation and genetic adaptation. Individuals with greater tolerance to contaminants thus have a greater probability of acquiring alternative adaptive mechanisms (i.e. acclimation or genetic adaptation) allowing them to withstand long-term exposure. Tolerance, however, does not describe genetic mechanisms and therefore quantifying individual variation in tolerance has limited evolutionary relevance beyond, of course, initial selection upon introduction

of contaminants in the form of survival. Another limitation to studying tolerance as a mechanism underlying adaptive traits is that it focuses on survival rather than phenotypic differences between populations. It is more practical and ecologically relevant, however, to examine mechanisms underlying observed differences in adaptive phenotypic traits between populations, as all observed individuals must be considered tolerant to some degree as they are alive. Because of these limitations, the remainder of this paper will focus on acclimation and genetic adaptation mechanisms underlying the presence of adaptive traits.

Acclimation and genetic adaptation have been acknowledged as potential mechanisms underlying adaptive traits in the field of ecotoxicology for decades (Klerks and Weis 1987). What has been preventing the field from continuing in this direction, however, is a lack of understanding of these concepts from an evolutionary perspective and their limited application to wild populations. While acclimation and genetic adaptation are to some degree independent, as the former focuses on physiology and the later on population genetic structure to explain phenotypic differences, they still need to be experimentally distinguished as they ultimately can result in similar phenotypes (Figure 1). The most rigorous method to differentiate these two mechanisms is to test them as alternative hypotheses. This, or at least acknowledgement of alternative hypotheses, is evident in general evolutionary research (e.g. Bennett and Lenski 1997) and just beginning to be realised in the field of ecotoxicology, with the emergence of evolutionary toxicology (Bickham 2011, Klerks 1999). The following sections will describe current investigations of acclimation and genetic adaptation in ecotoxicology, suggestions for the improvement of methodologies aiming to identify those mechanisms, and the benefits and limitations of applying those concepts to management practices.

Acclimation

As described above, the majority of acclimation research in ecotoxicology occurred in 1990s, primarily using laboratory experiments. This area of research was popular among scientists during this time and had numerous publications from a variety of taxa (see Table 1.1). Despite having the knowledge, tools, and experience with testing acclimation responses, very few examples exist of field experiments providing empirical evidence supporting the presence of acclimation in wild populations exposed to environmental contaminants (but see Farwell et al. 2011). This is somewhat surprising as the potential presence of multiple environmental stressors other than contaminants, such as temperature, food availability, etc. may create a highly variable environment, which is generally thought to be more conducive to acclimation mechanisms, rather than genetic adaptation (Van Straalen and Hoffman 2000).

There are a number of reasons why acclimation may not be at the forefront of evolutionary ecotoxicology. One is that past acclimation experiments typically report mean phenotypic responses to varying contaminant concentrations rather than individual differences in plasticity (Forbes et al. 1995), something that is of importance when investigating adaptive traits in populations (Holloway et al. 1997). Another reason might be because of logistical issues surrounding experimental design of field-based acclimation experiments and the controlling of confounding environmental variables. This may be overcome using experimental mesocosms to create either a simulation of an original environment (e.g. Coutellec 2011) or the containment of a portion of the original environment (e.g. Rimet and Bouchez 2011). Both types of mesocosms allow for more rigorous design and ecological relevance, though contaminant causation may still be

difficult to determine. Finally, many contaminants are persistent in the environment and the notion that acclimation may still occur even though the stressor has been present over several generations is often not recognized. There are several traits, including many life-history traits linked to fitness, that have the potential to exhibit high degrees of plasticity, even after persistent environmental stress (Greeley 2002).

When testing acclimation as an alternative hypothesis to genetic adaptation, investigating acclimation first (Figure 1) might be a more efficient use of time and resources as hypotheses and methods for identifying acclimation are already prominent in ecotoxicology and no prior knowledge of population genetic structure is needed. Figure 1.2 outlines a hypothetical example of an experimental design testing for the presence of acclimation in a field environment. Once an adaptive trait (e.g. faster growth rate) has been identified in a contaminated habitat, individuals from that habitat are transplanted to a reference habitat where the trait is not observed in the resident population (e.g. slower growth rate). Depending on the trait, this method could require long or short term translocating (e.g. one reproductive season for reproductive traits). If the adaptive trait changes to resemble that observed in the transplanted habitat, then there is support that acclimation is occurring and further investigation into other metabolic costs may be necessary. If the trait does not change, then either sufficient time has not past for the trait to change or acclimation is not occurring. Investigation into the genetic structure of the populations can then commence knowing that differences in observed traits are not likely based on acclimation. A more rigorous method would be to not simply assume that plasticity in a trait linked to fitness results in an increased fitness and to therefore experimentally test this. This would be done by transplocaing acclimated individuals back from the reference habitat to their original contaminated habitat. The individuals that

were acclimated to the reference habitat should have lower fitness measures immediately after being placed in the contaminated habitat, even though it originated from that habitat, than those originating from the contaminated habitat and never relocated. There are certain traits that are not likely to exhibit acclimation mechanisms. Such traits are those that are constrained by biological and morphological limitations, such as number of limbs, jaw size, etc. and therefore testing for acclimation as an adaptive mechanism would not be relevant.

One main application of identifying acclimation as an adaptive mechanism in wild populations is for habitat rehabilitation and restoration efforts (Medina 2007, Crews and Gore 2011). In habitats where ecological risk assessments suggest that the health of a population will significantly benefit from a reduction in environmental contaminant exposure, managers may come to the conclusion that a complete and immediate reduction of contaminants from the area will provide an optimal habitat for this population to thrive in. While this line of thinking may, in some cases, prove to be true (i.e. those that can acclimate to the environmental change), in others where populations have genetically adapted to the contaminated environment, a sudden change in this metric could be just as stressful as the initial contamination exposure. Empirical evidence through experimental testing of acclimation processes in populations living in habitats suitable for rehabilitation will suggest whether rehabilitation efforts are predicted to increase, decrease, or have no effect on individual fitness measures.

Genetic adaptation

Investigating whether genetic adaptation is the mechanistic basis for the presence of adaptive traits in a contaminated population can be accomplished using a number of

different methods prevalent in the general evolutionary biology literature. Very few examples of experimental evidence exists supporting genetic adaption in wild populations in ecotoxicology despite the numerous studies characterizing their genetic structures (see Table 1.1). A recent study by Wirgin et al. (2011) used Atlantic tomcod (*Microgadus tomcod*) to identify genetic differences between populations exposed to relatively high (Hudson River and Hackensack River) and low (including St. Lawrence River and Miramichi River) environmental contaminants along the Atlantic coast of North America. They found evidence of a six-base pair deletion in the sequence of a functionally active aryl hydrocarbon receptor (AHR2) in tomcod originating from more contaminated populations. This deletion is suggested to have been selected for as a mechanism of tolerance to contaminants as indicated by declines in embryo mortality and malformations when the deletion was present in contaminated populations. This is the first study to use wild populations to identify changes at a specific locus linked to differences in fitness across habitats with varying contaminant concentrations. Evidence of genetic adaptation to contaminants is important to our understanding of how populations respond to persistent contaminant exposure over time. It also highlights the fact that contaminants may pose enough of a stress to a population already exposed to a suite of potentially confounding environmental stressors, so much that genetic adaptation can occur. The application of evolutionary theory may also improve regulations developed from ecological risk assessments, where changes in population genetic structure will be investigated as a mechanism underlying any adverse ecological effects due to contaminant exposure (Bickham 2011).

Another method that is increasingly being used to examine the genetic basis of adaptive traits to contaminants is quantitative genetics. In these studies, life-history traits

are typically used as metrics in quantitative genetics as they are often continuous variables and determined by many genes (Stearns 1992, Klerks et al. 2011). In evolutionary toxicology, the application of quantitative genetics is of value as it can identify whether traits are adaptive, when rearing offspring in different environments, it can provide insight into the genetic basis for variation observed in traits, and it can determine whether selection is acting upon adaptive traits once a genetic basis has been established (Klerks et al. 2011). There are considerably more examples of the use of quantitative genetics in ecotoxicology to date (e.g. Martinez and Levington 1996, Xie and Klerks 2003, Roelofs et al. 2006, Coutellec et al. 2011), though still limited, than those identifying and characterizing variation at specific loci linked to an adaptive trait. This is most likely due to the more controlled nature of quantitative genetics, which must be conducted in a laboratory environment to separate families and monitor offspring traits, and that no expertise in molecular genetics is required. One downfall of quantitative genetics is that even though wild organisms may be used, the true nature of their original environment is difficult to replicate, thus limiting ecological relevancy.

It is important to note that both natural and sexual selection can act upon adaptive traits with a genetic basis in relatively contaminated environments. Adaptive traits under sexual selection are often not considered from a toxicological perspective, and as a result, little is known about the effects of contaminants on these traits, let alone mechanisms underlying these effects. Traits under sexual selection are often described as elaborate male ornaments (Andersson and Simmons 2006) or armaments (Berglund et al. 1996) but may also include more cryptic traits such as sperm quality metrics (Eberhard 1985, Arnold 1994). In all cases, traits under sexual selection serve to increase reproductive fitness through increasing the number of reproductive opportunities for both the

individual and their offspring (Andersson 1994). There has been no known investigation into the genetic adaptation of sexual traits to environmental contaminants to date, however, a relatively small number of studies have investigated links between the two and discussed their results from an evolutionary perspective (e.g. Møller 1993, Quinn et al. 2002, Bortolotti et al. 2003, Hewitt et al. 2008). The benefit of those studies arises from the use of study organisms through which sexual selection had previously been demonstrated. Although these studies do not directly quantify changes in allele frequencies or the ultimate effect on fitness, they do begin to address these possibilities, something which is greatly lacking in the literature.

Summary

The field of ecotoxicology has identified the need to understand adaptive mechanisms underlying observed phenotypic differences between populations exposed to different levels of environmental contaminants. This issue has been addressed by the recent development of a new field, evolutionary toxicology. What can be considered lacking in the description of this field, however, is the acknowledgement of alternative adaptive mechanisms to genetic adaption. This is especially important for the interpretation of existing ecotoxicoloigcal research and for the application of this research to habitat rehabilitation and ecological risk assessments. Should sentinel organisms be able to acclimate to changing environments, they would not be relevant environmental indicators compared to those that are genetically adapted. The incorporation of environmentally and genetically induced adaptive traits to evolutionary toxicological research is possible through the combination of past ecotoxicological experimental designs and current genetic techniques. Conducting ecologically and evolutionary

relevant research is also necessary for research in evolutionary toxicology and thus studies should strive to use wild populations and field experiments to examine these mechanisms. Finally, identifying traits that increase fitness via reproduction, such as those potentially under sexual selection, and understanding mechanisms underlying variation in these traits will help to continue the forward progression of the evolutionary toxicological field.

Overview of the thesis

The objective of my thesis was to investigate novel avenues of the field of ecotoxicology. I did this by describing variation in adaptive reproductive traits associated with environmental contaminants, by identifying acclimation mechanisms underlying this variation and by discussing potential evolutionary implications of these associations in traits under sexual selection, in wild populations of fishes. For each chapter, I used sum concentrations of polychlorinated biphenyls (PCB) as an indicator of environmental contamination as PCBs are found across a range of habitat types, are persistent in the environment and have been associated with variation in the expression of reproductive traits (e.g. Bengsson 1980, Ankley 1991, Bortolotti et al. 2003). The majority of research examining relationships between PCBs and reproductive traits has been conducted in the lab. This presents issues when applying those results to wild populations as laboratory doses often exceed environmental concentrations, thus ecological relevance is lacking. Also, wild populations are rarely exposed to only PCBs, as they are in laboratory experiments, which is a concern as the combination of PCBs with other contaminants, such as pesticides, can alter biological responses (Njiwa et al. 2004). For these reasons, I quantified environmentally relevant concentrations of sum PCBs. I used the term ‘sum

PCB concentration' to indicate that my value is the sum of all quantifiable congeners measured in the analysed sample. I did not use the term 'total PCB concentration' as there may be PCBs present in the sample that are below detection limits or that are not accounted for due to laboratory error. Chapter Two used an experimental approach in a semi-natural environment to examine the acclimation of reproductive life-history traits of female brown bullhead (*Ameiurus nebulosus*) collected from the Lower Great Lakes Region to individual measures of PCB concentrations. Chapter Three is a progression of Chapter Two and describes similar associations between reproductive traits of female brown bullhead and contaminants, but instead uses a single population measure of sediment sum PCB concentration and examines these associations across a larger number of populations throughout the Lower Great Lakes Region. Chapter Four uses regression tree analysis to investigate whether individual measures of PCB concentrations and reproductive hormones can explain variation in primary and secondary traits under sexual selection. Data was collected from migrating male Chinook salmon (*Oncorhynchus tshawytscha*) in the Credit River, which flows into Lake Ontario, and have been identified as a source of PCB contamination in this river. Chapter Five investigates whether variation in individual-level PCB concentrations indicates the expression of male primary and secondary traits under sexual selection in a more elaborately ornamented salmonid species, Coho salmon (*Oncorhynchus kisutch*), also from the Credit River. Together, my thesis identifies mechanisms underlying variation in adaptive reproductive traits to environmental contaminants and discusses the implications of environmental contaminants for adaptive reproductive traits from an evolutionary perspective, in wild populations of fishes.

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Table 1.1: A summary of ecotoxicological literature describing attempts at identifying adaptive mechanisms as: tolerance, acclimation, or genetic adaptation. This is not by any means a comprehensive list of studies; however, it does provide major and unique examples and illustrates differences in chronology and abundance of research between each of the three mechanisms. Reviews are included where numerous papers exist on one topic. General descriptions of each paper are also included.

Adaptive Mechanism	Citation	Review (R) or Experiment (E)	Description and Notes
Tolerance	Klerks and Weis 1987. Environ. Poll. 45:173.	R	Ranging from bacteria to fish, many show evidence of tolerance to heavy metals, both field and laboratory organisms
	Chapman et al. 1982. Aquat. Tox. 2:47.	R	Tolerances of oligochaetes to a variety of pollutants, all field collected organisms
	Niinemets 2010. Forest Ecol. Manag. 260:1623.	R	Tolerance of forest tress to contaminant stress throughout ontogeny
	Cambell and Campbell 2002. Reptile Ecol. 21:894.	R	Tolerance levels of lizards and snakes to date, mostly in mid 1990s as research in these taxas was minimal until then.
	Snell and Janssen 1995. Hydrobiol. 313:231.	R	Tolerance in rotifers from lab cultures and field mesocosms
	Reuber. 1978. Sci. Tot. Environ. 2:135.	R	Tolerance of rats to pesticides for use by EPA.
	Andersson 1988. Water air and soil Poll. 39:439.	R	A review of aluminum tolerance in several plant species, lab experiments
	Grant 1976. Bull. Environ. Cont. Tox. 15:283.	R	Tolerance and toxicity levels in freshwater taxa to organic contaminant
	Walden and Howard. 1981. Pulp and Paper Canada. 82:115.	R	Tolerance of pulp and paper mill effluents on lab fish species.
	Woltering 1984. Aquat. Tox. 5:1-21.	R	Tolerance and toxicity of fish fry of metals, organics, complex effluents, 173 studies reviewed.
Acclimation	Klerks and Weis 1987. Environ. Poll. 45:173.	R	Ranging from bacteria to fish, many suggest either acclimation or genetic adaptation, but cannot distinguish between the two due to methodology
	Coutellec et al. 2011. Ecotox. 20:534.	E	Using mesocosm and freshwater snails in common garden experiment, found evidence of acclimation to pesticides
	Niinemets 2010. Forest Ecol. Manag. 260:1623.	R	Acclimation of forest tress to contaminant stress throughout ontogeny
	Grzes 2010. Euro. J. Soil. Biol. 46:350.	R	Table of studies demonstrating ecological and fitness trade-offs with carrying contaminant exposure and common garden experiments. Lab and field collections.
	Snell and Janssen 1995. Hydrobiol. 313:231.	R	Acclimation of rotifers in mesocosm experiments
	Zeng et al. 2009. Aquat. Tox. 93:1.	E	Acclimation to metals by cyanobacterium
	Bossuyt and Janssen 2005. Environ. Poll. 136:135.	E	Acclimation and homeostasis measurement to copper in daphnia
	Klerks 1999. Ecotox. 8:277.	E	Acclimation by grass shrimp to field sediments as well as known concentrations of metal and PAH, both individually and combined
	Forrester et al. 2003. Mar. Environ. Res. 56:423.	E	No evidence of acclimation or genetic adaptation of growth rates in fish exposed to sediment contaminants, example of alternative hypothesis testing and rigorous experimental design
	Grasman et al. 1998. Environ. Monit. Assess. 53:117.	R	Evidence of possible acclimation, suggestions for further methods to test and applications to management in fish-eating birds
	Wirgin and Waldman 2004. Mar. Res. 552:73.	R	Evidence and discussion of acclimation to PAHs in wild populations of fish, discussion of potential genetic adaptations, research in section below follows up on this
	Aziz et al. 1999. Pedobiol. 43:594.	E	Evidence of acclimation in earthworms to metals, collected from mines and reference locations, investigated for genetic adaptation, but no evidence to support this possibility

	Farwell et al. 2012. Environ. Toxicol. Chem. 31:863.	E	Experimental evidence of acclimation in brown bullhead to polychlorinated biphenyls
Genetic Adaptation	Wirgin et al. 2011. Science. 331:1322.	E	First to identify genetic basis for fitness differences in tomcod along east coast of North America
	Agra et al. 2010. Environ. Tox. Chem. 29:939.	E	Genetic basis for metal tolerance in daphnia
	Phillips and Hickey 2010. Aquat. Tox. 99:507.	E	Genetic basis of metal tolerance in freshwater clam
	Pease et al. 2010. Aquat. Tox. 99:10-16	E	Genetic basis for tolerance to copper in marine algae
	Lopez-Rodas et al. 2008. Bull. Environ. Toxicol. 80:158.	E	No genetic basis, evidence for evolution of microalgae to water pollution
	Medina et al. 2007. Chemosphere. 67:2105.	R	Genetic basis for tolerance mainly in aquatic species, list of quantitative genetic studies to date
	Klerks 2011. Ecotox. 20:513	R	Quantitative genetics studies to date, genetic basis for resistance discussed
	Sanchez-Fortun et al. 2009. Ecotox. 18:481.	E	Genetic basis for tolerance, uses acclimation as alternative hypothesis
	Waits and Nebert 2011. Tox. Sciences. 122:465.	E	Genetic basis for developmental defects in lab zebrafish to PCB126

Figure 1.1: Suggested experimental scheme to identify mechanisms underlying differences in observed traits in populations exposed to varying contaminant concentrations. First, differences in phenotypes need to be identified as adaptive. Next, if applicable, common garden or transplant experiments can identify or rule out acclimation as a mechanism. Finally, molecular tools can be used to identify any genetic mechanisms.

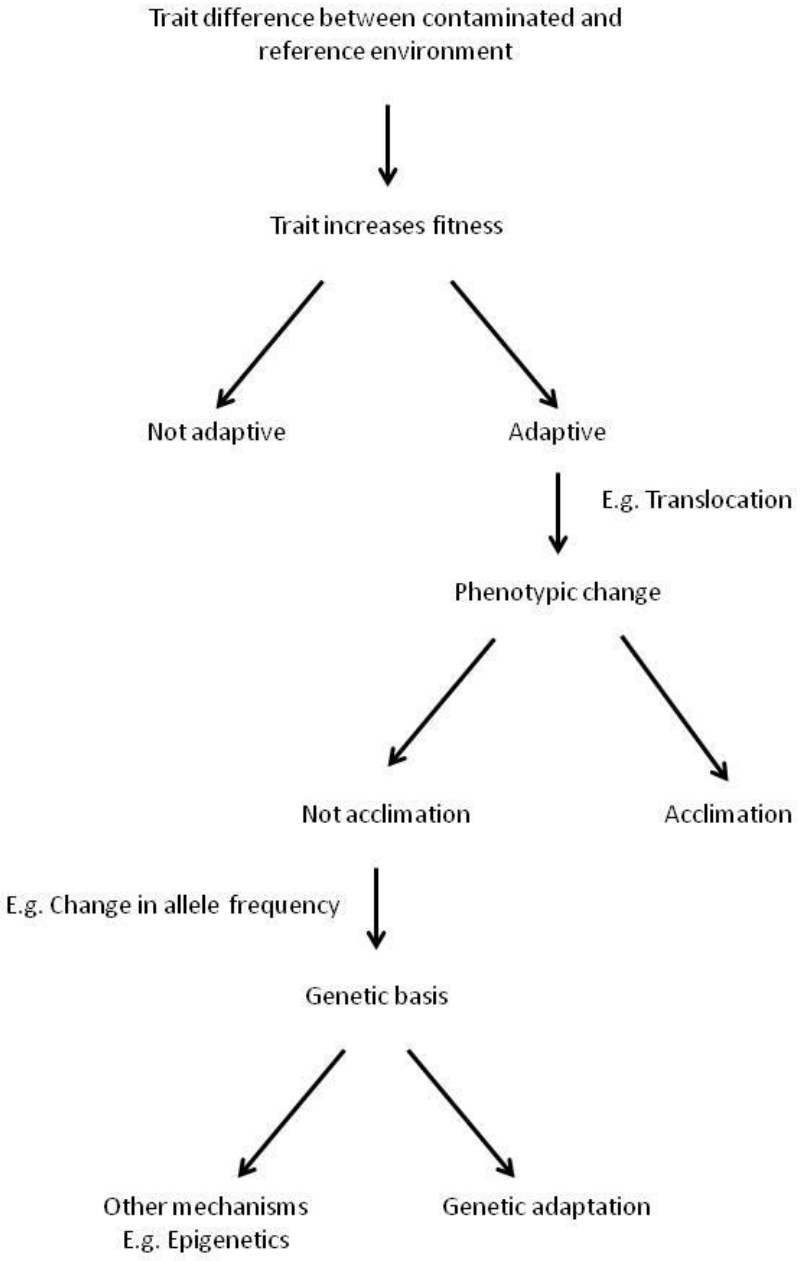
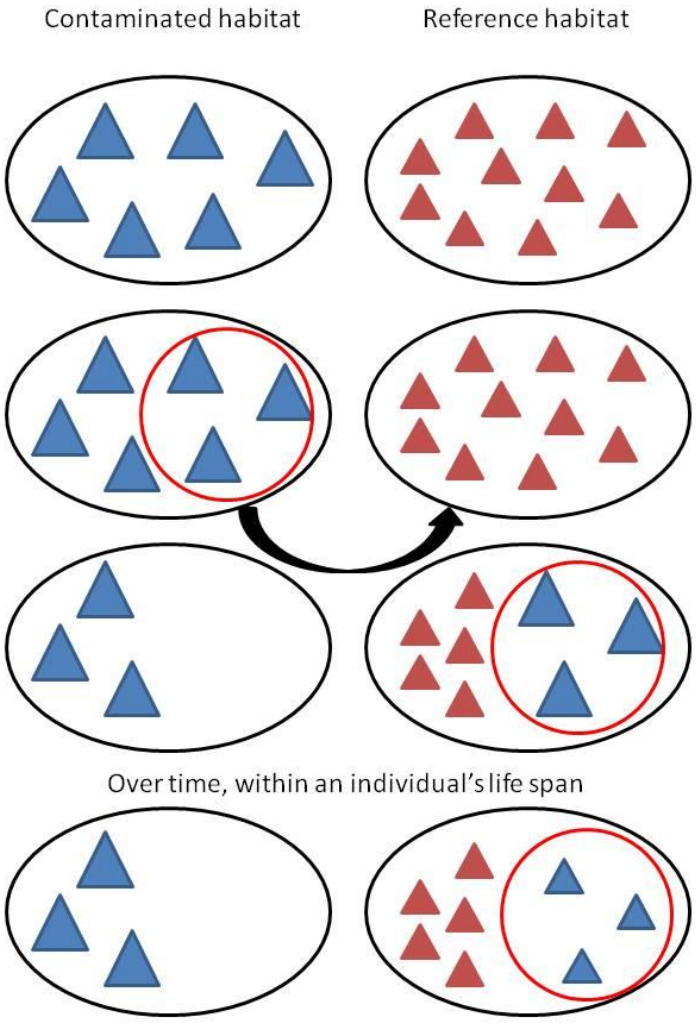


Figure 1.2: A transplant experiment aiming to identify acclimation as an adaptive mechanism. Blue triangles represent individuals from a contaminated habitat with relatively faster growth rates, indicated by triangle size, compared to red triangles which represent individuals from a reference habitat with relatively slower growth rates. If acclimation is underlying differences in growth rates, then when contaminated individuals are placed in the reference habitat, over time, their growth rate will resemble that of individuals originating from the reference habitat.



CHAPTER 2: ASSOCIATIONS BETWEEN FEMALE REPRODUCTIVE TRAITS AND POLYCHLORINATED BIPHENYL SEDIMENT CONCENTRATIONS IN WILD POPULATIONS OF BROWN BULLHEAD (*AMEIURUS NEBULOSUS*)[2]

Synopsis

Aquatic contaminants, specifically polychlorinated biphenyls (PCBs), a class of persistent organic contaminants, have been associated with sub-lethal effects on reproduction in fishes. I used female brown bullhead (*Ameiurus nebulosus*) to assess variation in reproductive traits across eight populations differing in sediment sum PCB concentrations in the Lower Great Lakes region. I also examined differences in maternal carotenoid allocation patterns among these populations. I found no significant associations between sediment sum PCB concentrations corrected for organic content (OC) and reproductive traits. However, I did find that egg diameter was negatively correlated with sediment PCB concentrations not corrected for OC ($r^2 = 0.39$, $p = 0.035$), suggesting observed relationships between sediment sum PCB concentrations and reproductive traits are driven by classes of environmental contaminants whose bioavailability are not predicted by OC. I also found an unexpected positive relationship between egg carotenoid concentrations and sediment PCB concentrations ($r^2 = 0.62$, $p = 0.01$). This positive relationship was explained by the maternal allocation of carotenoids based on a negative correlation between female muscle and egg carotenoid concentrations ($r = -0.91$, $p = 0.0018$), where females from less contaminated locations had lower egg and greater muscle carotenoid concentrations than those from more contaminated locations. This is expected to enhance offspring survival via increased antioxidant and immune function. The results of this study identify sub-lethal effects of environmental contaminants on reproductive life-history traits in female brown bullhead and, based on

results from Chapter Three, investigations of adaptive mechanisms underlying this variation is warrented.

[2] This chapter is the product of joint research with Dr. K.G. Drouillard, Dr. D.D. Heath and Dr. T.E. Pitcher and has been submitted to the Journal of Great Lakes Research.

Introduction

Aquatic contaminants have been associated with numerous sub-lethal effects on reproduction in fishes (e.g. see reviews Hewitt et al. 2008, Islam and Tanaka 2004, Kime et al. 1995, Kime and Nash 1999). The majority of research investigating relationships between contaminant exposure and reproductive traits has been conducted using acute laboratory exposure studies, however an increasing number of studies has focussed on reproductive traits in wild populations exposed to continuous and persistent contaminants. Identifying relationships between reproductive traits and environmental contaminants has implications for population dynamics and related management practices. This is because gamete quality, in particular egg quality, plays a significant role in determining embryo, and ultimately, larval fitness (Bobe and Labbé 2010, Brooks et al. 1997). In wild fish populations, exposure to contaminants has been linked to endocrine disruption and declines in gonadosomatic index (GSI) in yellow perch (*Perca flavescens*) (Hontela et al. 1995) and snakeheads (*Channa punctatus*) (Bhattacharya, 1993), decreases in fecundity in white croaker (Hose et al. 1989), decreases in egg diameter and fecundity in white suckers (*Catostomus commersoni*) (Munkittrick and Dixon 1988), and delays in egg development in fathead minnows (*Pimephales promelas*) (Kidd et al. 2007). These changes in reproductive traits have been demonstrated to reflect the relative degree of environmental contamination an individual is exposed to, with improvement in reproductive traits in locations where contaminant exposure was removed (Farwell et al.

2012, Munkittrick et al. 1994). The plasticity of female reproductive traits and their importance for offspring survival has made them common candidates for biomonitoring programs focussing on the effects of environmental contaminants on reproduction and population dynamics.

The chemical properties of eggs that reflect egg quality can also be affected by environmental contaminants. One class of such chemicals that has potential to be used as an indicator of contaminant exposure is carotenoids. Carotenoids are compounds found in plant and some bacterial and fungal material, and are assumed to be a limiting resource to the majority of animal species as they cannot be synthesized *de novo* (Olsen and Owens 1998). These compounds are found in most tissue types and maternal deposition results in egg yolks also containing carotenoids, causing their red to yellow colouration (Surai and Speake 1998, Blount et al. 2000). The amount of carotenoids deposited into the egg has largely been found to be positively related to the amount of carotenoids the mother possesses (Blount et al. 2002, Grether et al. 2008, McGraw et al. 2005). Carotenoid concentrations have been associated with increases in immune function and have antioxidant properties in both adults (Krinsky 2001, Blount et al. 2002) and embryos (Tyndale et al. 2008). Elevated egg carotenoid concentrations have been linked to decreases in oxidative damage and neural development deformities (Surai and Speake 1998) and increased immune responses in embryos (Sanio et al. 2003), ultimately having implications for offspring fitness (Tyndale et al. 2008). Metabolism of environmental contaminants, by both adults and embryos, can increase the amount of reactive oxygen species produced, potentially increasing oxidative stress (Di Giulio et al. 1989). Despite this connection, little research has focused on identifying relationships between environmental contaminants and egg carotenoid concentrations as it relates to offspring

fitness in wild populations, with none being conducted in fishes. Møller et al. (2005) found lower carotenoid concentrations in blood, liver, and eggs from wild female barn swallows (*Hirundo rustica*) exposed to radiation contamination compared to those from non-contaminated locations. This relationship was explained by a higher proportion of carotenoids in the contaminated birds being sequestered by the greater concentration of free radicals produced by radiation. This resulted in a lower proportion of remaining available carotenoids to contaminated birds compared to those not exposed to contamination. Similarly, adult birds living in more polluted urban environments had relatively lower liver carotenoid concentrations than those from rural environments (Møller et al. 2010).

In ecotoxicological studies conducted using wild organisms, average values of traits of interest are often compared among populations originating from locations expected to differ in their relative amounts of environmental contaminants. It can be beneficial to identify specific and biologically relevant contaminants in order to more rigorously compare the locations and assess the ecological implications of the contaminants. This notion assumes that measured contaminants are mechanistically linked to measured biological effects. One class of contaminants that has been linked to declines in reproductive trait development in fishes is polychlorinated biphenyls (PCBs) (Hewitt and Servos 2001). In a laboratory study, oral doses of PCBs resulted in significant declines in fecundity and offspring survival in minnows (*Phoxinus phoxinus*) (Bengtsson 1980). Similar results were found in a wild population of Chinook salmon (*Oncorhynchus tshawytscha*) from Lake Michigan where increases in sum PCB concentrations in muscle tissue were associated with declines in fecundity and hatching success (Ankley et al. 1991). These differences in reproductive traits have been suggested

to be due to changes in metabolic function (Beyers et al. 1999), toxic properties of planar PCB congeners on liver functioning and subsequent vitellogenin production (Johnson et al. 1997, Kime 1995) and changes to egg structure, including oocyte walls (Kime 1995). PCBs are also known to be correlated with several other organic contaminants in natal habitats (Szalinska et al. 2011) and are therefore a good metric to assess general environmental contaminant gradients.

Readily abundant and native to North America, brown bullhead (*Ameiurus nebulosus*) are tolerant of a variety of environmental stressors, including contaminants (e.g. Pyron et al. 2001). They are a philopatric species and therefore are likely to be exposed to contaminants from a localized region, compared to species with larger home ranges. A study by Sakaris and Jesien (2005) used ultrasonic telemetry to quantify the average home range of an adult brown bullhead to be 500 meters during reproduction and up to 3.1 km during the fall when the fish are retreating to deeper waters to overwinter. They are also benthic throughout the majority of their life and are therefore exposed to contaminants that accumulate in sediments. The majority of research on brown bullhead has focused on their prevalent tumour formation when exposed to environmental carcinogens; so much so that they are used by the International Joint Commission as an indicator species for contaminanted habitats (IJC 1989). In spite of this vast amount of research, little is known about the effects of contaminants on their reproduction and life-histories (but see Lesko et al. 1994).

I used brown bullhead to examine whether associations exist between female reproductive traits and sediment sum PCB concentrations found in the Huron-Erie Corridor. A recent study by Farwell et al. (2012), conducted in the Detroit River region of

the Huron-Erie Corridor, examined the acclimation of female brown bullhead reproductive life-history traits by experimentally manipulating environmental contaminant exposure. They found that females collected from contaminated locations that were subsequently allowed to clear their contaminants over a period of one year in semi-natural experimental ponds resulted in lower egg sum PCB concentrations, as well as greater egg diameters and GSI, compared to those examined directly after collection from the same contaminated locations. In the current study, we assess whether similar relationships between sediment sum PCB concentrations and reproductive traits are present across a broader range of wild populations, in terms of both geography and relative contaminant concentration. Sediment samples, rather than individual tissues as done by Farwell et al. (2012), were used to determine whether they may be useful for relatively rapid habitat assessment compared to lethal sampling of fishes.I predicted that females from locations with relatively lower sediment sum PCB concentrations would have greater measures of egg diameter, fecundity, GSI, and egg carotenoid concentration than females from locations with relatively greater sediment sum PCB concentrations.

Methods

Sample collection

Gravid female brown bullhead were collected using fyke nets and boat electroshocking from 10 May to 8 June 2010 from 8 locations in the Lower Great Lakes region: Belle River (BR; 42^{o}17’39”N, 82^{o}42’43”W), Turkey Creek (TU; 42^{o}14’43”N, 83^{o}6’19”W), Fox Creek (FC; 41^{o}59’48”N, 82^{o}50’56”W), Puce River (PR; 42^{o}18’8”N, 82^{o}46’50”W), Peche Island (PI; 42^{o}20’38”N, 82^{o}55’43”W), Cedar Creek (CC; 42^{o}0’42”N, 82^{o}47’13”W), Trenton Channel (TC; 42^{o}10’33”N, 83^{o}9’16”W), and Belle

Island (BI; 42°20’57”N, 82°58’31”W) (Fig. 2.1). These locations have been the focus of ecotoxicological studies (e.g. Farwell et al. 2012; Leadly et al. 1999), are a Great Lakes Area of Concern (IJC 1978), and have been demonstrated to vary in environmental contaminants (Drouillard et al. 2006).

Sediment samples were collected from each of the eight locations to assess sum PCB concentration in the environment. Three sediment samples of approximately 20 g each were obtained from the sediment surface of each location using a petite ponar. Each sediment sample was taken within 5 meters of the fish collection site. The sediment samples were homogenized to produce a single sample for each location and stored at -20°C prior to PCB analyses (Ali et al. 1993). Each sediment sample was taken at approximately 1 m water depth, except at Belle Island where they were taken at 2 m water depth. All sediment samples were similar in type (silt) and contained small amounts of organic debris which were removed prior to analysis.

Life-history trait assessment

Female bullheads were transported immediately after capture to Leadley Environmental Ltd. (42°6’14”N, 82°55’47”W) and euthanized using a lethal dose of MS-222. All egg samples for analysis were obtained and stored within less than 5 minutes after euthanasia. Total length and mass measurements were taken and used to calculate Fulton’s condition factor [$C = \text{body mass} / (\text{total length})^3$] (Ricker 1975), as PCB accumulation and egg development can depend on body condition (McDonald et al. 2002; Brooks et al. 1997). Ovaries were then removed, patted dry with paper towel and their mass (g) was recorded to estimate gonadosomatic index [GSI = ovary mass / (total mass – ovary mass)]. A sub-sample of eggs of known mass was taken to estimate fecundity; 50 of which were individually measured and used to calculate average egg diameter (mm).

The remaining eggs were divided into subsamples of a known mass, placed in 1.5 mL Cryovials® and snap frozen in liquid nitrogen. Samples for carotenoid analysis were stored at -80^{o}C and samples for lipid analysis were stored at -20^{o}C. Approximately 2 g of dorsal muscle tissue was excised and stored at -80^{o}C for carotenoid analysis. A pectoral spine was removed for age determination following methods in Blouin and Hall (1990) and visible annual rings were counted under a zoom stereomicroscope (SZX7 Olympus; www.olympusamerica.com).

Sediment polychlorinated biphenyl extraction and analyses

Sum PCB sediment concentration was determined from 20 g of sediment homogenate following the protocol outlined by Drouillard et al. (2006). Sediment samples were ground with 100 g anhydrous sodium sulphate (ACS grade, BDH, ON, Canada) to remove moisture and Soxhlet extractions for organic contaminants were conducted using 300 mL acetone:hexane (1:1) for 24 hours. Five samples were run concurrently, including a method blank to estimate percent recovery and an in-house dry sediment reference sample (SRM 1944) to estimate inter-assay variation. All samples were spiked with an internal recovery standard of 200 ng_{PCB34}/mL. After extraction, the extract was then condensed to approximately 2 mL using a rotary-evaporator. The extracts were then back extracted using a separatory funnel containing water and hexane over three solvent washings. Hexane from each washing was combined and dried over sodium sulphate. The extract was condensed again to approximately 2 mL and florisil chromatography was used for sample clean up. Fraction 1 was eluted with 50 mL hexane and fraction 2 with 50 mL dicloromethane:hexane (15:85). Fractions were condensed again and 0.2-0.5 g of activated copper was added to remove sulphur. Analysis of fractions was performed using a Hewlett Packard 5890 gas chromatograph (Avondale,

PA, USA) with an electron capture detector (ECD). The following PCB congeners (IUPAC numbers, coeluting congeners separated by slash: 18, 17, 28/31, 33, 52, 49, 44, 74, 70, 95, 101, 99, 87, 110, 82, 151, 149, 118, 153/132, 105, 138, 158, 187, 183, 128, 177, 171, 156, 180, 170, 199, 208, 195, 194, 205, 206, 209) were identified by retention time and referenced against an external PCB standard (Quebec Ministry of Environment Congener Mix; AccuStandard, New Haven, CT, USA). Sum PCB concentrations were calculated for each sediment sample as the sum of concentrations of each of the above congeners. Mean percent recovery of the PCB34 spike was 99.96 ± 6.74% (mean ± SD) and thus sample concentrations were not recovery corrected. All sum PCB in-house homogenate samples were in compliance (mean ± 2 SD) with the Great Lakes Institute for Environmental Research analytical laboratory quality assurance guidelines (Canadian Association for Environmental Analytical Laboratories Accreditation and ISO17025 certified).

Carotenoid extraction and analyses

Following Li et al. (2005), with minor modifications, carotenoid extractions from 1 g of ovarian (70-100 eggs) or muscle tissue were conducted under dim light and on ice. Thawed egg or muscle samples were homogenized and extracted in 2 mL of acetone. The mixture was then centrifuged at 3500 rpm for 5 min at 4 ^{o}C and the supernatant was removed. This was repeated two more times with 1 mL of acetone. Supernatants were pooled and 1 mL each of MtBE, hexane, and distilled water was added and centrifuged at 3500 rpm for 5 min at 4 ^{o}C to facilitate phase separation. The organic supernatant containing carotenoids was pipetted out and dispensed into a glass vial. Phase separation was repeated three times. Supernatants were homogenized, evaporated and sealed under nitrogen gas.

High-performance liquid chromatography (HPLC) was used to characterize egg carotenoids from 16 individuals across a subset of 4 locations (PI, BR, BI, TC) using a Waters 2695 HPLC coupled to a Waters 487 dual-channel UV-visible detector (following Chui et al. 2001). Samples were suspended in 200 μL of mobile phase containing methanol:acetonitrile:dichloromethane (42:42:16, v/v/v) and filtered through a Teflon membrane filter (0.45 μm) to remove particulates. A gradient elution of the same mobile phase concentration was used over 30 min and carotenoids were detected and quantified with an absorbance response at a maximum wavelength of 480 nm. Carotenoids were identified by comparison with responses of known carotenoid standards. Lutein and zeaxanthin (xanthophyll carotenoids) combined constituted, on average, 80 % ± 1.0 (mean ± S.E.) of all carotenoids in each egg sample (PI: 81.75 % ± 4.79, BR: 82.0 % ± 6.27, BI: 77.75 % ± 5.06, TC: 79.5 % ± 3.87; mean ± S.D.). All other carotenoids present were also identified as xanthophyll carotenoids and were present in relatively small and consistent amounts across the samples. As specific carotenoid identification was not required to assess the entire carotenoid concentration of each sample, a generalized method for quantifying total xanthophyll concentration using spectrophotometry was followed for all egg and muscle samples, as described below.

Total xanthophyll carotenoid concentrations were measured using a Beckman DU50 UV-visible spectrophotometer (McGraw et al. 2001). Each sample was individually suspended in 1 mL of ethanol and absorbance was measured at a wavelength of 448 nm (λ_{max} for xanthophylls). Quantification of carotenoid concentration in both egg and muscle tissues (μg carotenoid/gram of tissue) was determined using the following equation: [(A x volume of extract (mL))/(E x egg sample mass (g))] (McGraw et al., 2001), where A is the absorbance of the sample and E is the extinction coefficient at 1%/1

cm of the relevant carotenoids at λ_{max} (2550 for xanthophylls in ethanol). Each sample extraction was analyzed on the spectrophotometer in triplicate, with an average standard deviation of 0.53 %. Replicate extractions of 14 samples (7 BR, 7 TC) were also conducted to estimate extraction efficiency, with an average standard deviation of 1.3 %.

Total organic content analyses

The bioavailability of PCBs in sediment, and other non-polar organic contaminants, is dependent on the amount of organic content (OC) present in the sediment (Landrum and Faust, 1994) and therefore sediment PCB concentrations require OC correction when comparing to reproductive traits. While associations between sediment PCB concentrations and reproductive traits are still of value prior to the correction as they can indicate potential relationships with other inorganic contaminants such as heavy metals, they likely do not describe biologically relevant associations between the two. OC was determined using the loss on ignition procedure and using a Caarlo Erba Elemental Analyzer for total organic carbon (Drouillard et al. 2004). Pre-weighed sediment samples were combusted at 450^{o}C for 24 hours and the total organic carbon content was then determined gravimetrically.

Lipid extraction and analyses

Percent total lipids in eggs were measured as it has been linked to egg quality, carotenoid concentration, and concentration of lipophillic contaminants (Webster et al. 1999; Brooks et al. 1997; Goodwin 1980). Percent total lipids were determined gravimetrically for approximately 0.5 g egg tissue following the dichloromethane(DCM)/hexane extraction described in Drouillard et al. (2004). Briefly, egg tissue was ground with 15 g activated sodium sulphate and added to 20 mL glass syringes containing 15 mL DCM:hexane (1:1), each connected to 1 µm glass fibre

syringe filter, fitted to a solid phase extraction manifold (Phenomenex). Solvent extracts were collected and roto-evaporated to approximately 2 mL. Solvents were then reconstituted in hexane to 10 mL from which 1 mL samples were placed in pre-weighed aluminum weigh boats. Boats containing extracts were evaporated at room temperature and dried for 1 hr at 100 ^{o}C. Boats were then weighed and percent total lipid was calculated using the following equation: [((boat mass after drying – boat mass before drying)/sample mass)*1000].

Statistical analyses

Linear regression with multiple values of *Y* for each value of *X* (Sokal and Rohlf1995; p. 476) was used to examine variation in egg diameter (log transformed), fecundity, GSI, and egg carotenoid concentration in relation to the single sum PCB sediment concentration value I obtained for each location. Ie used this type of analysis because it is able to separate out the deviation from regression sums of squares and provide an independent estimate of error among *Y* values for each value of *X*, compared to regressions with a single value of *Y* for each value of *X* where there is only an unexplained sums of squares (Sokal and Rohlf 1995). Normality was tested for each variable using Shapiro-Wilk goodness-of-fit test and homogeneity of variance was tested for each variable using Levene's test.

Results

Sediment sum PCB concentrations across the eight locations ranged from 7.18 – 33.45 µg/kg dry weight (Table 2.1). There was no significant difference in percent lipid concentration of eggs ($F_{1, 65} = 1.28$, $P = 0.27$) or Fulton's condition factor of females ($F_{1, 65} = 0.78$, $P = 0.60$) across locations. There was no difference in mean age between

locations with the exception of Fox Creek where one female was 9 years of age (Table 2.1).

Using sediment sum PCB concentrations corrected for OC on a dry weight basis, there were no significant relationships between sediment sum PCB concentration and egg diameter ($r^2 = 0.16$, $P = 0.14$), fecundity ($r^2 = 0.02$, $P = 0.86$), GSI ($r^2 = 0.14$, $P = 0.18$), or egg carotentoid concentration ($r^2 = 0.04$, $P = 0.98$).

Using sediment sum PCB values not corrected for OC on a dry weight basis, there was a significant negative relationship between sediment sum PCB concentration and egg diameter ($r^2 = 0.39$, $P = 0.035$; Fig. 2.2a). There was no significant relationship between sediment sum PCB concentration and fecundity ($r^2 = 0.11$, $P = 0.12$; Fig. 2.2b) or GSI ($r^2 = 0.29$, $P = 0.073$; Fig. 2.2c). There was a significant positive relationship between sediment sum PCB concentration and egg carotenoid concentration ($r^2 = 0.62$, $P = 0.01$; Fig. 2.2d).Correlation analyses revealed statistically significant relationships between each permutation of the four reproductive traits ($r^2 = 0.35 – 0.65$, $P = 0.0038 – 0.0001$), except for that between egg diameter and fecundity ($r^2 = 0.22$, $P = 0.07$).

The positive relationship between egg carotenoid concentration and sediment sum PCB concentration was unexpected and a *post hoc* comparison between egg and muscle carotenoid concentration was conducted to investigate trends in maternal allocation of carotenoids. I found a significant negative relationship between egg and muscle carotenoid concentrations (r = -0.91, p = 0.0018), with females from less contaminated environments (sediment sum PCB concentrations) having lower egg carotenoid concentrations and greater muscle carotenoid concentrations compared to females from more contaminated locations (Fig. 2.3).

Discussion

I found that female brown bullhead reproductive traits are not associated with sum PCB concentrations in sediment corrected for OC from different locations in the Lower Great Lakes region. When using sum PCB concentrations in sediment not corrected for OC, negative relationships were identified with egg diameter and GSI, with only that of egg diameter being statistically significant. I also identified a significant positive relationship between egg carotenoid concentration and sediment sum PCB concentrations which can be explained by maternal allocation of PCBs across a PCB gradient.

Sediment sum PCB concentration values I obtained from the 8 locations in 2010 correspond with those reported in the literature for this region collected within the last 10 years (Drouillard et al 2006; Szalinksa et al. 2011). Previous studies reported PCB concentration as a mean value for a generalized area in the Lower Great Lakes region and therefore values specific to locations in this study were not reported. Values for locations originating from Lake Erie (FC, CC) have not been previously reported in the literature, however, discussion has suggested that sediment sum PCB concentrations have been in decline in the western basin since the 1970s (Heidtke et al. 2006). The range of sediment sum PCB concentrations I observed across the 8 locations was not as great as seen in the literature for the Lower Great Lakes region (e.g. Lower US; see Drouillard et al. 2006). This may be explained by the highly localized nature of contaminant 'hot spots' in this region (Drouillard et al. 2006; Szalinksa et al. 2011) not reflected in my sampling. My sediment sum PCB concentration values also fell between the range of values recorded from known heavily contaminated locations and from relatively 'pristine' locations where sum PCB concentrations are non-detectable (e.g. Upper CA; see Drouillard et al. 2006). Hamilton Harbour, classified as a primary area of concern (IJC 1978), has surface

sediment sum PCB concentrations recorded at 14260 µg/kg dry weight (Zeman and Patterson 2003), over two orders of magnitude greater than those observed from my locations. However, my sediment sum PCB concentration values do fall within a range of sum PCB concentrations that have elicited biological effects. The Interim Freshwater Sediment Quality Guidelines (IFSQG), outlined in the Canadian Sediment Quality Guidelines for the Protection of Aquatic Life, suggest that sediment sum PCB concentrations at 34.1 µg/kg dry weight or greater will result in up to a 40% likelihood of adverse biological effects (e.g. tumour growth, disease) on freshwater aquatic wildlife (CCME 2002). This is in comparison to the probable effect level, a minimum 50% likelihood of adverse biological effects, at a sediment sum PCB concentration of 277 µg/kg dry weight (CCME 2002). The most contaminated locations in this study (CC, TC, BI) have sediment sum PCB concentrations that fall just below the IFSQG concentrations. A previous study using brown bullhead originating from these same locations (TC, BI) demonstrated differences in life-history traits compared to those found at less contaminated locations that were also used in this study (BR, PI) (Farwell et al., 2012). This study, however, used individual measures of egg sum PCB concentrations, which exhibited a larger range of PCB concentrations, compared to those quantified in sediment samples in the current study. The differences in PCB ranges or sample type (i.e. tissue vs. sediment) may explain the lack of observed relationships between PCBs and reproductive life-history traits in the current study. The rank order of locations based on sediment sum PCB concentration also differed from that found by Farwell et al. (2012) where they found BI had lower PCB concentrations than TC, and PI had lower PCB concentrations than BR. While the relatively more and less (respectively) locations remained grouped together, these differences could also explain a lack of significant

relationships with reproductive traits in this study. These rank order differences could have resulted from a number of factors including differences in metabolic functioning (suggested by Farwell et al. 2012), food srouces or bioaccumulation rates, or sediment sampling protocol. I predicted that egg diameter, fecundity and GSI would decline with increasing contaminant exposure based on a previous study by Farwell et al. (2012) conducted in the same Lower Great Lakes region. My data do not support this prediction as I found no statistically significant relationships between sediment sum PCB concentration corrected for OC with any of the four reproductive traits. I did, however, find negative relationships with sediment sum PCB concentration not corrected for OC and egg diameter and GSI, with the later not being statistically significant.This finding suggests that sum PCB concentration is most likely not the contaminant metric that is driving the significant relationships observed between the sediment sum PCB concentration and the four reproductive traits. Sediment sum PCB concentration not only has a positive relationship to other groups of non-polar organic contaminants in the Lower Great Lakes region, but to polar inorganic contaminants as well such as heavy metals (e.g. Szalinksa et al. 2011). It is likely that the disparity in my results between the dry and OC weights for sediment sum PCB concentrations is a result of a positive correlation between sediment sum PCB concentration (dry weight) and polar inorganic contaminant concentration, of which bioavailability is not predicted by OC (Weltje 1998). Additionally, I do not account for interactions between contaminant classes on reproductive traits (see Bustness2006).Observations of smaller egg diameters and GSIs in locations with greater sediment sum PCB concentrations not corrected for OC may have also arisen due to unknown environmental quality characteristics (e.g. food availability), either independently or coinciding with contamination. Differences in food availability

across the eight locations were not expected to directly or indirectly impact reproductive trait development as Fulton's condition factor (body size metrics), percent total lipids in eggs, and age did not differ significantly across the eight locations.

I also predicted that egg carotenoid concentration would decline with increasing contaminant exposure due to an increase in antioxidant function (Di Giulio et al. 1989, Krinsky 2001, Tyndale et al. 2008). Contrary to my prediction, my data demonstrates a statistically significant positive relationship between egg carotenoid concentration and sediment sum PCB concentration, with a 50% increase in egg carotenoid concentration between my lowest and highest values. Few examples of this positive relationship between egg carotenoid concentration and environmental contaminants have been found in the literature. However, in laboratory experiments, female American kestrels (*Falco sparverius*) fed a mixture of a 1: 1: 1 Aroclor 1254, 1248, and 1260 also had greater plasma carotenoid concentrations (by 50%) and greater carotenoid concentrations in their offspring (by 50%), compared to control females (Bortolotti et al. 2003). The concentrations of the Aroclor mixture administered was designed to produce environmentally relevant body and egg burdens and were based on previously collected mammalian and avian muscle and egg samples (Fernie et al. 2003, Smits and Bortolotti 2001).

The positive relationship between egg carotenoid concentration and environmental contamination can be explained by two alternative hypotheses: the reproduction trade-off hypothesis (Alonzo-Alverez et al. 2004, Bertrand et al. 2006, Monaghan et al. 2009); or the maternal allocation hypothesis (Blount et al. 2000, Royle et al. 2001). The reproduction trade-off hypothesis suggests that relatively greater amounts of energy invested into reproduction, ultimately leading to increased egg size and/or fecundity, will

also cause an increase in oxidative stress in the female (Salmon et al. 2001, Wang et al. 2001) This increase in oxidative stress in turn increases the female's use of antioxidants, such as carotenoids (Finkel and Holbrook2000). The ultimate result of these processes leaves the amount of carotenoids available to the female for allocation to her eggs relatively lower, compared to females that invest relatively less into reproduction (e.g. Blount et al. 2002). In the present study, females from less contaminated locations produce larger eggs and may have an overall greater energy investment towards reproduction (GSI), than those from more contaminated environments. If the positive relationship between egg carotenoid concentration and sediment sum PCB concentration is explained by the reproduction trade-off hypothesis, I would predict that females from less contaminated environments (greater reproduction investment) would have lower carotenoid concentration in both their muscle and egg tissues compared to females from more contaminated locations. The maternal allocation hypothesis states that females will deposit relatively more carotenoids in her eggs in uncertain or detrimental environments to enhance offspring survival via increased antioxidant and immune function (Grether et al. 2008, McGraw et al. 2005). If the positive relationship between egg carotenoid concentration and sediment sum PCB concentration is explained by the maternal allocation hypothesis, I would predict that females from less contaminated environments would have lower carotenoid concentrations in their egg tissues and greater carotenoid concentrations in their muscle tissues, compared to females from more contaminated locations.

I used a *post hoc* analysis to further investigate these possible mechanisms underlying the positive relationship between egg carotenoid concentration and sediment sum PCB concentration (dry weight) by comparing carotenoid concentrations in egg and

muscle tissues. I found support for the maternal allocation hypothesis with a significant negative relationship between egg and muscle carotenoid concentrations ($r = -0.91$, $p = 0.0018$), with females from less contaminated environments (sediment sum PCB concentrations) having lower egg carotenoid concentrations and greater muscle carotenoid concentrations compared to females from more contaminated locations (Fig. 2.3). Little experimental research on the differential maternal allocation of carotenoids in contaminated environments has been conducted; however, Sanio et al. (2002) investigated this question in wild populations of barn swallows (*Hirundo rustica*) affected by radiation contamination. They found that females associating with males with manipulated short tails, an indication of radiation exposure, deposited greater amounts of carotenoids to their eggs compared to those associating with 'healthy' males. This suggests that females are presumably provisioning offspring expected to have greater radiation exposure with greater amounts of antioxidants to combat negative effects of the radiation. An alternative hypothesis that may explain variation in carotnoids is differences in dietary xanthophyll availability among populations. While there is no apparent spatial trend among populations, it is possible that differences in food quality could have occurred.

In summary, while my field survey does not provides evidence of associations between reproductive traitsand sediment sum PCB concentrations corrected for OC in brown bullhead, I do provide evidence supporting relationships between these traits and other classes of environmental contaminants whose bioavailability is not predicted by OC, such as heavy metals., with only egg diameter being statistically significant. I also found a positive relationship between egg carotenoid concentration and sediment sum PCB concentrations and provide evidence suggesting that this positive relationship is a result of differential maternal allocation of carotenoids to eggs, where females from less

contaminated environments had lower egg carotenoid concentrations and greater muscle carotenoid concentrations compared to females from more contaminated locations. The results of this study identify sub-lethal effects of environmental contaminants on reproductive traits in female brown bullhead. Further research on the ecological implications of between-population differences in the measured reproductive traits is needed to assess whether the observed differences in reproductive traits have an ultimate impact on individual fitness and population-level processes.

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Table 2.1: Means ± standard deviation (range) and sample sizes (*n*) for life-history and egg quality metrics of female brown bullhead (*Ameiurus nebulosus*) collected from eight locations in the Huron-Erie corridor. Sum PCB concentration (dry weight) of homogenized sediment samples is provided as a single measurement for each location (see Methods for details).

Location	*n*	Sum PCB (µg/kg dw)	OC (%)	Total Length (mm)	Total Mass (g)	Condition (mass / TL^3)	Age (years)	Egg Diameter (mm)	Fecundity (# eggs)	GSI (%)	Carotenoid Concentration (µg/g egg)	Carotenoid Concentration (µg/g muscle)
Belle River	9	7.18	3.46	313 ± 24.1 (217 – 425)	393.9 ± 35.2 (321 – 657)	1.94 ± 0.8 (0.86 – 3.14)	4 ± 0.3 (2 – 5)	2.51 ± 0.02 (2.4 – 2.59)	5677 ± 442 (5030 – 6390)	11.32 ± 0.89 (9.82 – 12.55)	2.07 ± 0.15 (1.91 – 2.33)	0.56 ± 0.02 (0.46 – 0.60)
Turkey Creek	7	8.96	4.07	236 ± 7.8 (203 – 266)	295.8 ± 11.2 (281 – 369)	2.03 ± 0.7 (1.96 – 3.36)	4 ± 0.2 (3 – 4)	2.56 ± 0.01 (2.51 – 2.6)	5723 ± 450 (4898 – 6200)	12.41 ± 1.4 (10.54 – 14.73)	2.00 ± 0.10 (1.86 – 2.14)	0.61 ± 0.01 (0.57 – 0.65)
Fox Creek	6	10.75	2.40	359 ± 34.6 (256 – 462)	420.4 ± 43.3 (366 – 634)	1.95 ± 0.9 (0.64 – 2.18)	6 ± 1 (3 – 9)	2.48 ± 0.03 (2.41 – 2.57)	6047 ± 556 (5299 – 6753)	11.38 ± 0.97 (10.24 – 12.61)	2.03 ± 0.11 (1.82 – 2.13)	0.59 ± 0.01 (0.56 – 0.62)
Puce River	9	17.09	3.64	276 ± 13.9 (212 – 351)	357.5 ± 17.8 (294 – 483)	1.56 ± 0.9 (1.17 – 3.08)	4 ± 0.3 (3 – 5)	2.49 ± 0.02 (2.41 – 2.56)	5299 ± 385 (4645 – 5720)	10.34 ± 0.95 (9.21 – 11.75)	2.22 ± 0.29 (1.96 – 2.87)	0.54 ± 0.01 (0.47 – 0.59)
Peche Island	8	17.64	14.71	282 ± 23.1 (197 – 397)	350.2 ± 27.9 (296 – 502)	1.62 ± 0.7 (0.80 – 3.87)	4 ± 0.2 (3 – 5)	2.58 ± 0.01 (2.51 – 2.6)	5866 ± 341 (5380 – 6390)	13.08 ± 1.6 (10.31 – 15.22)	2.05 ± 0.10 (1.90 – 2.16)	0.59 ± 0.02 (0.49 – 0.66)
Cedar Creek	10	22.16	9.33	292 ± 20.7 (216 – 387)	353.4 ± 30.5 (268 – 543)	1.63 ± 0.6 (0.94 – 2.66)	4 ± 0.4 (3 – 6)	2.40 ± 0.01 (2.34 – 2.57)	5646 ± 300 (5287 – 6753)	10.70 ± 0.90 (9.12 – 11.88)	2.89 ± 0.21 (2.12 – 3.13)	0.52 ± 0.01 (0.46 – 0.58)
Trenton Channel	11	28.71	9.87	266 ± 12.0 (201 – 322)	313.2 ± 13.2 (267 – 421)	1.65 ± 0.7 (1.26 – 3.29)	4 ± 0.2 (3 – 5)	2.40 ± 0.02 (2.33 – 2.5)	5225 ± 519 (4338 – 6001)	8.92 ± 1.4 (7.18 – 11.00)	2.86 ± 0.34 (2.99 – 4.30)	0.46 ± 0.01 (0.39 – 0.56)
Belle Island	6	33.45	3.97	292 ± 24.6 (192 – 369)	333.8 ± 20.3 (294 – 451)	1.25 ± 0.6 (0.90 – 4.15)	3 ± 0.3 (3 – 5)	2.42 ± 0.01 (2.38 – 2.46)	5520 ± 460 (5190 – 6412)	9.73 ± 0.97 (8.25 – 11.30)	2.86 ± 0.24 (2.55 – 3.11)	0.48 ± 0.01 (0.44 – 0.51)

Figure 2.1: Map of the eight female brown bullhead (*Ameiurus nebulosus*) collection locations in the Lower Great Lakes region. Locations are labelled in rank order from lowest (1) to greatest (8) sediment sum polychlorinated biphenyl (PCB) concentration (dry weight): Belle River (BR), Turkey Creek (TU), Fox Creek (FC), Puce River (PR), Peche Island (PI), Cedar Creek (CC), Trenton Channel (TC), and Belle Island (BI).

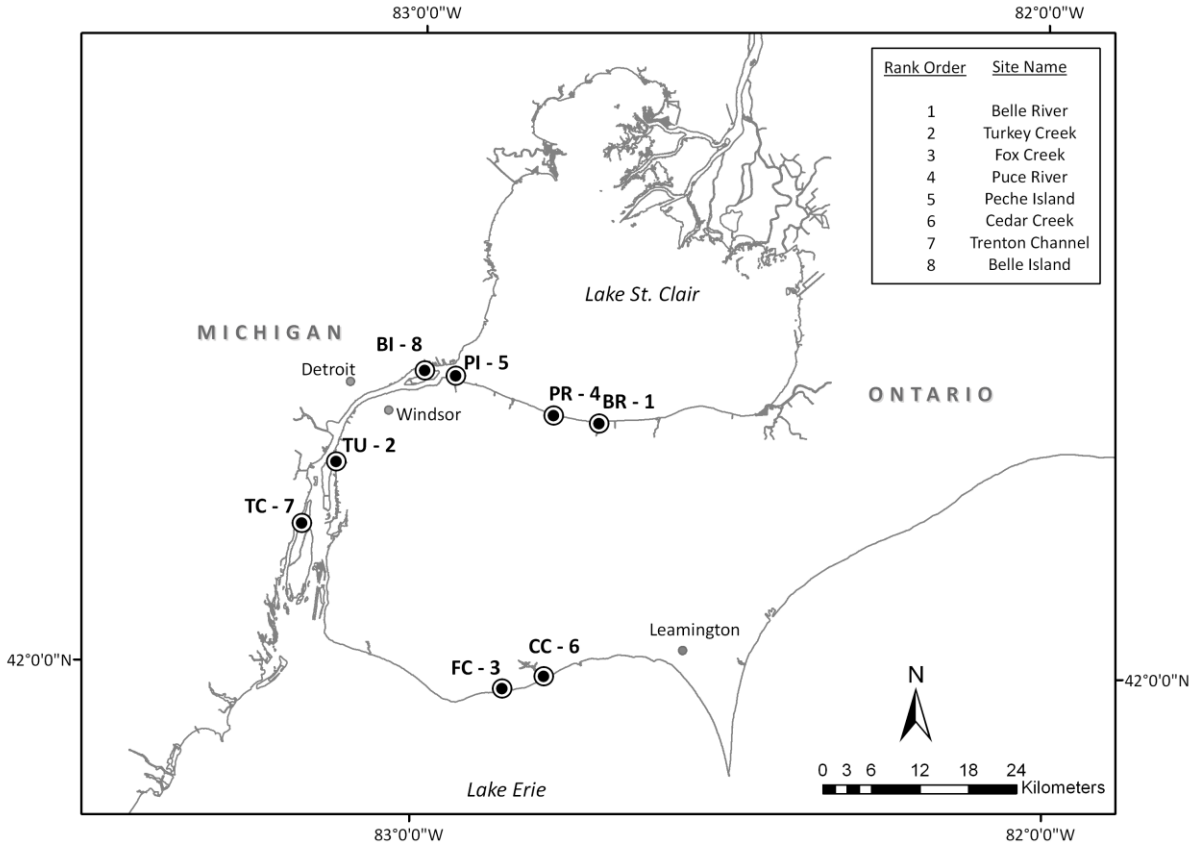


Figure 2.2: Relationships between sediment sum polychlorinated biphenyl (PCB) concentrations (dry weight) from the eight locations and (a) mean egg diameter (log_{10}), (b) mean fecundity (# of eggs) (c) mean gonadosomatic index (%), (d) mean egg total xanthophyll carotenoids (µg/kg), in female brown bullhead (*Ameiurus nebulosus*). Error bars denote 1 S.D.

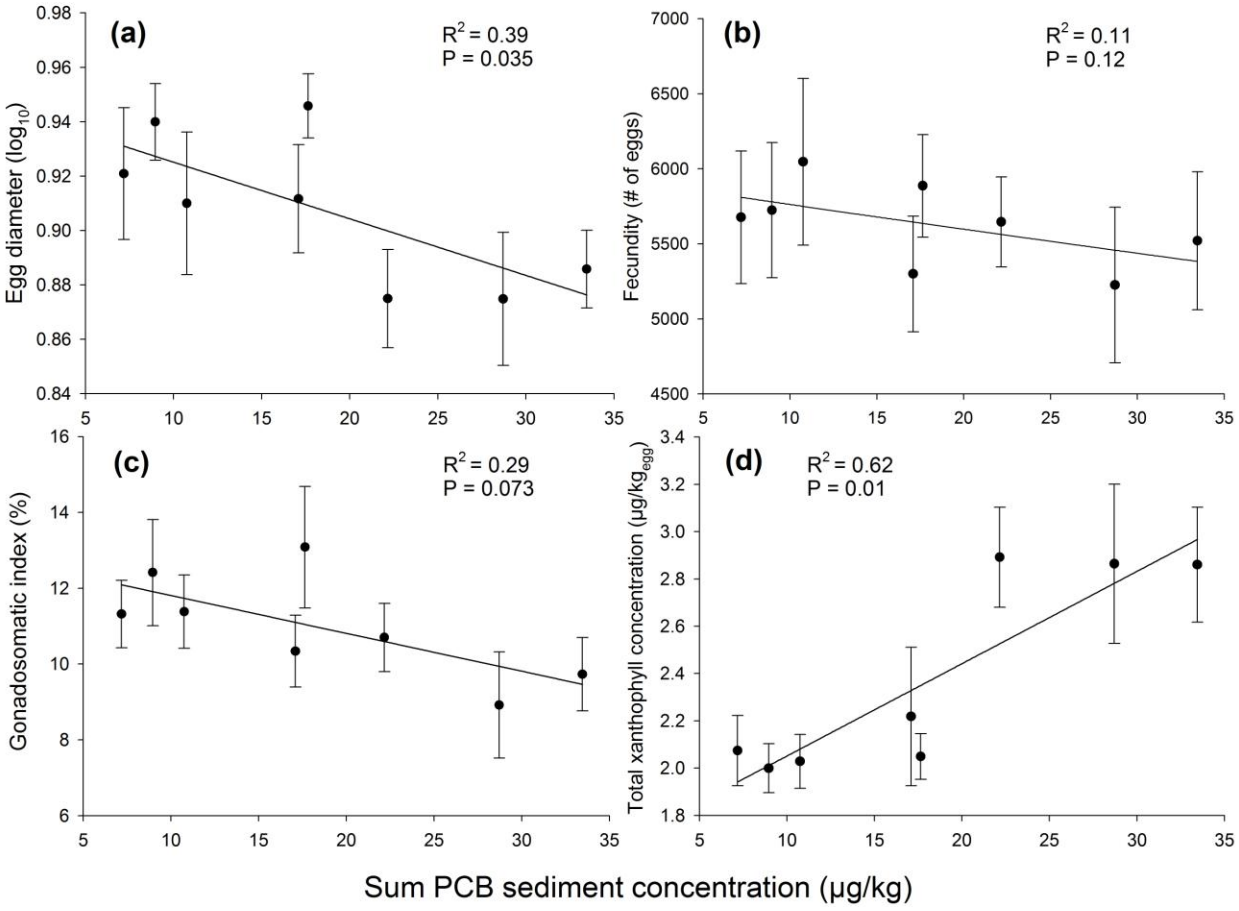
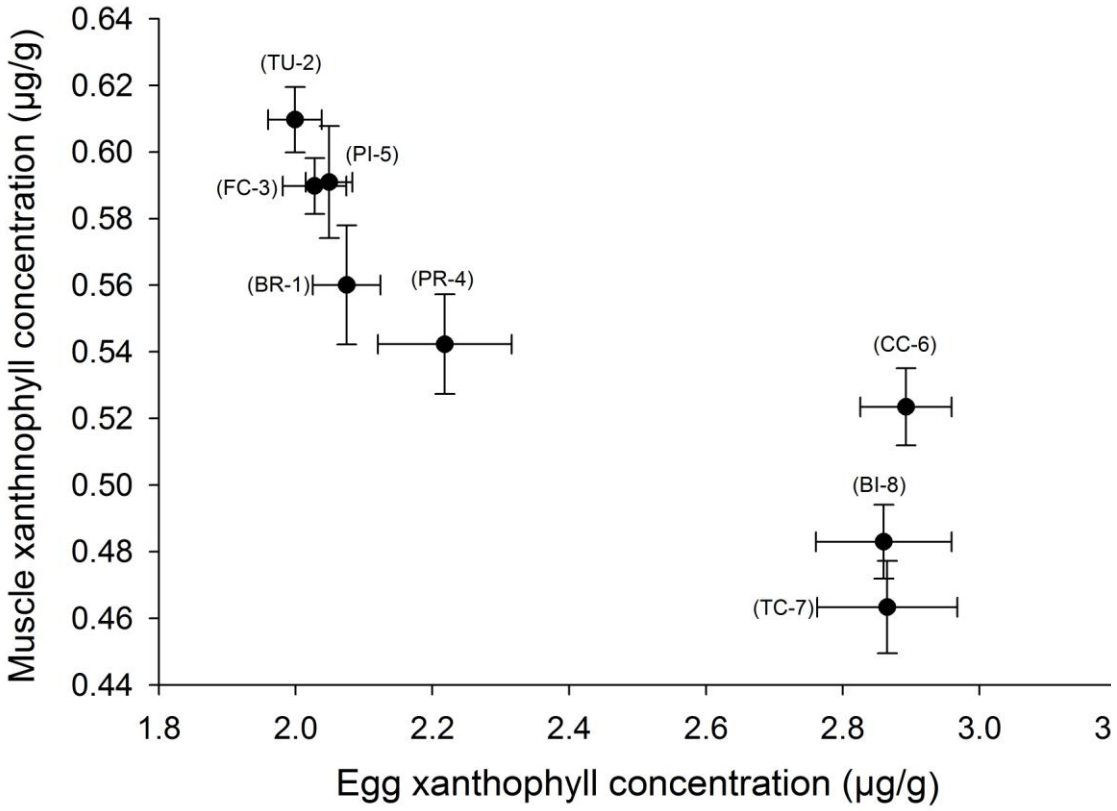


Figure. 2.3: Pearson correlation between mean muscle xanthophyll carotenoid concentration (µg/g) (± S.D.) and mean egg xanthophyll carotenoid concentration (µg/g) (± 1 S.D.) for each location. Locations are labelled in rank order from lowest (1) to greatest (8) sediment sum polychlorinated biphenyl (PCB) concentration (dry weight) corresponding to Figure 1: Belle River (BR), Turkey Creek (TU), Fox Creek (FC), Puce River (PR), Peche Island (PI), Cedar Creek (CC), Trenton Channel (TC), and Belle Island (BI).



CHAPTER 3: ACCLIMATION OF LIFE-HISTORY TRAITS TO EXPERIMENTAL CHANGES IN ENVIRONMENTAL CONTAMINANT CONCENTRATIONS IN BROWN BULLHEAD (*AMEIURUS NEBULOSUS*)[1]

Synopsis

One adaptive mechanism aquatic populations use to withstand environmental contaminants is acclimation. Polychlorinated biphenyls are a globally ubiquitous class of persistent organic contaminants and have been linked to reproductive impairments in fishes. I used female brown bullhead to test whether acclimation of reproductive life-history traits occurs in response to changes in sum polychlorinated biphenyl (PCB) exposure. I compared egg diameter, gonadosomatic index (GSI), and fecundity of fish directly caught from wild populations exposed to a range of contaminant concentrations (acute), to those collected from the same populations a year prior which were placed in a clean environment to clear their contaminants throughout that year (cleared). Sum PCB concentrations were also determined for each individual. Brown bullhead from acute treatments had significantly greater sum PCB concentrations compared to cleared treatments. Egg diameter and GSI metrics were greater in cleared treatments compared to acute treatments (by 6 and 14% respectively). Treatment effect (i.e. acute or cleared) accounts for 72 to 89% of the variation in the reproductive life-history trait variables, as opposed to location fish were collected from as indicated by intraclass correlation coefficients. There was no difference in fecundity between acute and cleared treatments. I found support that acclimation of reproductive life-history traits occurs to changes in sum PCB concentration. To my knowledge, the present study is the first experimental test of acclimation responses of female life-history traits to contaminants in wild populations.

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Introduction

Aquatic ecosystems are known to be exposed to a variety of environmental stressors, including contaminants, which have potential to disrupt population dynamics (Witters 1998). Regions such as Newark Bay-Hudson River (Wirgin et al. 2011) and several Great Lakes tributaries (Marvin et al. 2000) have become well-established study systems for investigating adaptive mechanisms underlying a population's ability to withstand exposure to contaminants. Populations in aquatic ecosystems such as those are able to survive and reproduce in environments where they are chronically exposed to contaminant concentrations well above those known to elicit lethal toxic effects in acute laboratory experiments (Wirgin and Waldman 2004). Adaptive mechanisms that elicit this resistance to environmental contaminants can be classified into two general categories: genetic adaptation and acclimation (Wirgin and Waldman 2004). Genetic adaptation describes genetically based changes in phenotype at a population level with selection occurring at the individual level thus facilitating phenotypes that maximize an individual's fitness to be passed on to future generations (Klerks et al. 1997). Genetic adaptations can only be observed over multiple generations once selection has occurred. A recent study by Wirgin et al. (2011) found evidence in Atlantic tomcod (*Microgadus tomcod*) of rapid evolutionary change at the locus of a functionally active aryl hydrocarbon receptor (AHR), AHR2, which is involved in the mediation of contaminant metabolizing enzymes. A six-base deletion in the sequence of the AHR2 locus in tomcod living in contaminated regions (Hudson River and Hackensack River), compared to reference locations (including St. Lawrence River and Miramichi River) is suggested to be a mechanism of polychlorinated biphenyl resistance and resulted from selection against embryos with a high incidence of mortality and malformations.

Individuals may also express resistance to contaminants via acclimation. Acclimation is plastic change in phenotype at an individual level in response to environmental change in order to maintain homeostasis (Klerks et al. 1997). Due to the link to homeostasis, acclimation is considered to be adaptive. Such changes in phenotype are largely based on the plasticity of a particular trait and the spectrum of alternate phenotypes an individual has the potential to express (West-Eberhard 1989). Acclimation responses resulting in similar phenotypes across individuals can occur both within and across generations and can only be observed in response to change in a particular stressor of interest (Wirgin and Waldman 2004). For example, mosquitofish from an industrially contaminated habitat, Bayou Trepangier, Los Angeles, with tissue concentrations of lead 550% greater than those from a reference habitat, showed decreased mortality after laboratory lead exposure compared to reference fish, yet showed no difference in mortality after 34 d of captivity in clean water (Klerks and Lentz 1998).

Life-history traits (i.e., those linked to growth or reproduction) have a complex and integrated genetic basis and thus, while having some degree of heritability, tend to express relatively higher plasticity across environmental gradients than traits that do not directly impact fitness (Stearns 1992). This plasticity and direct link to individual fitness explains why variation in life-history traits corresponding to differences in contaminant exposure has been a predominant focus in the literature for decades (Kime 1995, Mills and Chichester 2005). Despite the extensive laboratory research that characterizes variation in life-history traits, there is little evidence for acclimation in life-history traits in response to contaminant exposure using field experiments and wild organisms (Mills and Chichester 2005). This is most likely a consequence of complex population dynamics and interannual variation in life-history trait expression (Rose et al. 2003), complex

mixtures of contaminants (Klerks 1999), seasonal and annual environmental variation and logistical difficulties in experimental design (Paull et al. 2008). It is important to understand the mechanisms underlying patterns in life-history trait variation in wild populations for the design and implication of remediation processes and population-level management.

Life-history data collected from wild populations with respect to contaminants often compare traits between relatively clean and contaminated habitats in order to account for confounding environmental effects. Any observed differences in life-history traits should reflect their environment as a whole. However, it may be useful to focus on a single class of contaminants, relevant to the traits in question, in order to quantify differences in contamination among habitats. Polychlorinated biphenyls are a globally ubiquitous class of persistent organic contaminants and have been linked to reproductive impairments in fishes (Hewitt and Servos 2001). Declines in hatching success and fecundity have been reported for both increases in laboratory PCB-fed minnows (Bengtsson 1980) and increases of PCB concentration in egg tissue of wild-caught salmon (Ankley et al. 1991). In addition, exposures of many other organic contaminants often exhibit correlations with PCBs attributed to common exposure routes at contaminated locations. Thus PCB concentrations often provide a good set of readily detectable reference compounds for denoting gradients of highly exposed populations from reference populations.

Readily abundant and native to North America, brown bullhead are a philopatric (Sakaris and Jesien 2005) warm-water species that are tolerant of a variety of environmental stressors, including contaminants. They are also benthic throughout the majority of their life and are therefore exposed to contaminants that accumulate in

sediments. Due to these inherent qualities, brown bullhead have been a model study system for investigating non-lethal effects of aquatic contaminant exposure for decades. Much of the current research has focused on the prevalence and pathologies of tumors, genotoxic responses, and their importance as indicators of environmental toxicity Busch et al. 2004), while little is known about their reproduction and life history.

I used female brown bullhead to test whether acclimation of life-history traits occurs in response to changes in sum PCB exposure. For brevity, I call this the acclimation hypothesis. To test the acclimation hypothesis, I compared life-history traits of fish directly caught from wild populations exposed to a range of contaminant concentrations (acute), to those collected from the same populations a year prior which were placed in a clean environment to clear their contaminants throughout that year (cleared). The acclimation hypothesis assumes that sum PCB concentrations will be lower in cleared treatments compared to acute treatments, demonstrating an environmental change through which acclimation may occur. Following this assumption, the acclimation hypothesis predicts that reproductive life-history traits (egg diameter, gonadosomatic index [GSI], fecundity) from cleared treatments will be significantly greater than those from acute treatments, in accordance with changes in PCB concentration. Polychlorinated biphenyl analyses were conducted on egg tissue, as opposed to muscle or sediment, to reflect maternal offloading concentrations during egg development and to address concentrations that directly affect fitness via embryo survival and egg development (Daley et al. 2009). To my knowledge, the present study is the first experimental test of acclimation responses of female life-history traits to contaminants in wild populations.

Materials and methods

Sample collection

Female brown bullhead were collected using boat electroshocking from 20 April to 19 June 2008 (cleared treatment) and 14 April to 16 June 2009 (acute treatment) from four distinct locations in the Lower Great Lakes region (Fig. 3.1): Peche Island (PI; 42°34'N, 82°92'W), Belle River (BR; 42°28'N, 82°71'W), Belle Island (BI; 42°20'N, 82°59'W), Trenton Channel (TC; 42°51'N, 82°55'W). This region was chosen as it has been used in previous ecotoxicological studies (Leadly et al. 1999), it is a Great Lakes Area of Concern (IJC 1978), and has areas of localized contaminant exposure, concentrations of which vary geographically depending on hydrodynamics and local source inputs, but not on a small temporal scale (i.e. year to year) (Drouillard et al. 2006).

In 2008, field collected female brown bullhead were transported immediately after capture to Leadley Environmental Ltd. (42°6'N, 82°55'W) and placed in one of four semi-natural, aerated ponds, 6 m x 12 m x 3 m (L x W x D), corresponding to their original location, for a period of 1 year, until the next reproductive period. This translocation removed bullhead from their original sources of contaminant exposure and allowed contaminants in the fish to be depurated (Paterson et al. 2007a). The transplanted brown bullhead are hereafter referred to as 'cleared' as their life history traits were assessed after this clearing time period. Ponds were constructed 1 year prior to the experiment and had no prior fish in them. Each pond also had similar sun exposure, aeration, water temperature, insect communities and vegetation growth, suggesting that each pond experienced similar environmental conditions. Once brown bullhead were introduced, fish density was also similar between the ponds (~ 40 to 50 g/m^3 on average). Fish were fed floating pellets daily (Martin Mills Inc.), ad libitum, until November 2008

when the ponds began to ice over. Fish were not fed pellets in 2009, with naturally occurring insects and aquatic vegetation in the ponds, similar to that in wild habitat, available as food.

In 2009, female brown bullhead were immediately transported to Leadley Environmental Ltd. after capture. These brown bullhead are hereafter referred to as 'acute' as their life history traits were assessed after exposure to environmental contaminants present in their respective wild locations. Females that were sexually mature at the time of collection from two of the populations (PI, BI) were assessed immediately. Females collected from the other two populations (BR, TC) that were collected prior to vitellogenesis (i.e. large, yellow eggs) were held in covered, rubber lined, aerated ponds 2 m x 2 m x 0.75 m (L x W x D) until distended bellies and swollen genital pores were observed. Fish were held no longer than 2 weeks before processing. Elimination rates for the majority of PCBs are longer than 2 weeks (Paterson et al. 2007b) and egg development would have begun prior to collection, therefore potential clearing of PCBs during this time and its effect on egg development were considered to be minimal. Also at this time, sexually mature female brown bullhead from the cleared treatment were collected from their respective ponds and their life history traits were assessed. In both cleared and acute treatments, females with eggs that had not undergone vitellogenesis at this time were not included in the present study.

Life-history trait assessment

Cleared and acute females were euthanized using a lethal dose of MS-222 and total length (mm) and mass (g) were recorded. Ovaries were removed, patted dry with paper towel and their mass (g) was recorded to estimate GSI = ovary mass / (total mass – ovary mass). A sub-sample of eggs of a known mass was taken to estimate fecundity; 50

of which were used to calculate average egg diameter (mm). The remaining eggs were divided into subsamples of a known mass and preserved in 1.5 mL Cryovials at -20^{o}C for PCB analyses (see below). All egg samples were obtained and stored within 5 min after euthanasia. A pectoral spine was removed for age determination following methods in Blouin and Hall (1990). Briefly, spines were cut into 0.75 mm sections using a low-speed saw (Isomet, Buehler Inc.), mounted onto a slide using mounting medium (Flo-Texx, Lerner Laboratories), and annual rings were counted under a zoom stereomicroscope (SZX7 Olympus; www.olympusamerica.com).

Polychlorinated biphenyl extraction and analyses

Thawed egg samples 0.2 to 0.5 g (20 to 50 eggs) from each individual were homogenized, extracted using a micro-extraction technique following the protocol outlined by Daley et al. (2009). The extraction equipment consisted of 8, 20 mL glass syringes, each connected to 1 µm glass fibre syringe filter, fitted to a solid phase extraction manifold (Phenomenex). Egg samples were ground with 15 g of activated sodium sulphate and placed into the syringes containing 15 mL dichloromethane (DCM):hexane (1:1). An additional 15 mL of DCM:hexane was used to rinse the glass mortar and pestle used to homogenize the egg samples and was added to the syringes. Each syringe was then spiked with an internal recovery standard of 200 ng PCB30/mL. Six samples were run concurrently with each set, also including a method blank and an in-house homogenate fish sample (Detroit River common carp, *Cyprinus carpio*) as an inter-assay control. Extracts were then concentrated by rotary-evaporator to approximately 2 mL and florisil chromatography was used for sample clean up (Lazar et al. 1992). Fractions 1 (50 mL hexane) and 2 (50 mL DCM:hexane; 15:85) were collected. Extracts were then concentrated to 1 mL and placed in glass vials for analysis via gas

chromatography electron capture detection (GC-ECD). The PCB congeners in each sample were identified by retention time and referenced against an external PCB standard (Quebec Ministry of Environment Congener Mix; AccuStandard). The following congeners were present in the external standard and analyzed for detection in egg samples (IUPAC numbers, coeluting congeners separated by slash): 18/17, 31/28, 33, 52, 49, 44, 74, 70, 95, 101, 99, 87, 110, 151/82, 149, 118, 153, 105/132, 138, 158, 183, 128, 177, 156/171, 180, 191, 170, 201, 195/208, 194, 205, 206, 209. Sum PCB concentrations were calculated for each individual as the sum of each congener in µg/kg wet weight. Mean percent recovery of the PCB 30 spike was 78.45 ± 0.53% (mean ± S.E.). Four of 72 samples had recoveries less than the 70% threshold generally used for QA/QC (range 67.1 to 69.7%), however, all sum PCB in-house homogenate samples were in compliance (mean ± 2 S.D.) with the Great Lakes Institute for Environmental Research analytical laboratory quality assurance guidelines (Canadian Association for Environmental Analytical Laboratories Accreditation and ISO17025 certified). Samples were therefore not corrected for recovery.

Statistical analyses

Site-specific differences in sum PCB concentration between acute and cleared treatments were determined using independent t-tests. Variation in mass and Fulton's condition factor [$C = \text{body mass} / (\text{total length})^3$] across the four locations, and between acute and cleared treatments, were determined using an analysis of variance followed by a post-hoc Tukey test where applicable. This was done because PCB distribution and accumulation can be dependent on body condition (MacDonald et al. 2002).

Variation in life history traits between acute and cleared fish was analyzed using linear mixed-effects (or multilevel) models fitted via restricted maximum likelihood (*glht*

package multcomp, R Development Core Team, 2009). In these analyses, life-history traits (egg diameter, GSI, total lipids, fecundity) were entered as response variables. Because negative relationships were found between body mass and GSI and body mass and egg diameter, body mass was used as a covariate for both traits. Whether a population was acute or cleared was entered as a fixed factor and location entered as a random effect (treated as random intercepts). Random slopes were not applied as slopes between acute and cleared treatments, within locations, were not statistically different when models were fitted. Differences in the means of the dependent variables between acute and cleared treatments were evaluated using z-tests (Hothorn et al. 2008). To examine the between-group effect of location, I calculated intraclass correlation coefficients, reflecting the proportion of variance of dependent variables occurring among locations. The remaining proportion of variance is then assumed to occur within locations, including that of acute and cleared treatments (Raudenbush and Byrk 2002).

Results

Within locations, acute and cleared treatments differed in average sum PCB concentration, with cleared treatments having lower concentrations (PI: $t = -15.15$, $p < 0.001$, $df = 13$; BR: $t = -21.49$, $p < 0.0001$, $df = 16$; BI: $t = -26.64$, $p < 0.0001$, $df = 15$; TC: $t = -31.82$, $p < 0.0001$, $df = 18$). Compared to acute treatments, average sum PCB concentrations in cleared treatments declined by 43% in PI, 55% in BR, 61% in BI, 71% in TC (Fig. 3.2).

There were no differences in mean body mass among locations within acute ($F = 2.73$, $p = 0.06$, $df = 33$) and cleared ($F = 2.71$, $p = 0.06$, $df = 35$) treatments. Within locations, there was no difference in mean body mass between acute and cleared

treatments for PI ($F = 0.12$, $p = 0.74$, $df = 14$), BR ($F = 2.13$, $p = 0.16$, $df = 17$), and BI ($F = 3.85$, $p = 0.07$, $df = 16$). However, in TC, cleared fish had greater masses than acute fish ($F = 7.49$, $p = 0.01$, $df = 19$).

I also examined differences in Fulton's condition factor, which may better reflect overall condition than mass (Ricker 1975). There was no difference in body condition within three of four locations between acute and cleared treatments (PI: $F = 1.02$, $p = 0.33$, $df = 14$; BI: $F = 2.0$, $p = 0.18$, $df = 16$; TC: $F = 3.77$, $p = 0.07$, $df = 19$). In BR, cleared fish had greater body condition than acute fish ($F = 8.57$, $p = 0.01$, $df = 17$). Within acute treatments, there was a difference in body condition among locations with BI having a greater body condition than BR ($F = 3.49$, $p = 0.03$, $df = 33$). There was no difference within cleared treatments between locations ($F = 1.94$, $p = 0.14$, $df = 35$). There is also no apparent difference in mean age among locations or treatments, which can be related to condition (Table 3.1).

Differences in female reproductive life-history traits occurred among locations which were attributed to acute and cleared treatments. Combining the location treatments together, acute populations had significantly smaller egg diameters ($t = 4.87$, $p < 0.001$, $df = 68$; t-test) by an average of 6% and lower GSIs ($t = 2.07$, $p < 0.001$, $df = 68$) by 17% compared to cleared populations (Fig. 3.3a, 3.3b). While both egg diameter and GSI have a significant response to clearing, this response is more apparent and variable for GSI. There was no observable difference in the magnitude of change in these traits between acute and cleared populations and within locations. Combining location treatments together, no difference in fecundity was observed between acute and cleared fish. Intraclass correlation coefficients values (11–28%) also indicate that 72 to 89% of variation in the dependent variables occur within locations (e.g. between acute and

cleared treatments), rather than among locations (Table 3.2). No differences in fecundity were observed between acute and cleared treatments, both combined and across locations (Fig. 3.3c).

Discussion

My field experiment is to my knowledge the first to examine changes in life-history traits in response to contaminant clearing in a field setting. My results support the hypothesis that acclimation of life-history traits in female brown bullhead occurs in response to experimental changes in contaminant exposure. I found that females from cleared treatments had lower contaminant concentrations and had greater egg diameters and GSIs than fish from acute treatments.

A major assumption of the acclimation hypothesis is that fish from cleared treatments will have lower sum PCB concentrations in their eggs than those from acute treatments. My data supported this assumption; for each of the four locations, sum PCB concentrations in eggs from cleared treatments were found to be significantly lower than those from acute treatments. An overall clearing effect was predicted given that uptake of PCBs via ingestion and respiratory surfaces (Gobas et al. 1993) would have been limited in the aquaculture pond environment relative to the Detroit River and elimination routes of PCBs, due to loss by gills and/or fecal egestion would be maximized (Gobas et al. 1988). I also found that fish from more contaminated locations (e.g. TC) had cleared relatively more of their sum PCBs than fish from less contaminated locations (e.g. PI). This was not expected as metabolic rate, which strongly mediates both respiration and fecal egestion rates of fish, would not have been expected to differ across cleared treatments given similar temperature and feeding conditions were provided. Indeed,

metabolic rate has been demonstrated to be lower in fish exposed to contaminants in both lab and field studies (Beyers et al. 1999, Coghlan and Ringler 2005) which should have produced an opposite pattern to that observed, with higher relative clearance of PCBs from the least contaminated (Peche Island) site. My study suggests that clearing efficiency may be species or population specific or that local genetic adaptation of PCB clearing mechanisms may have occurred (Wirgin et al. 2011).

The acclimation hypothesis predicts that females from cleared treatments will have a greater output of reproductive life history traits compared to those from acute treatments. My data supported this prediction in that egg diameter and GSI were significantly greater in cleared treatments than in acute treatments, with GSI showing greater variability. However, my data showed no difference in fecundity between treatments. While there is some evidence for a response to contaminant exposure in egg size in fishes (Kime 1995), declines in fecundity and GSI have been observed for several species in contaminated habitats (Kime 1995, Jobling et al. 2002). Contaminant exposed populations have also exhibited fitness costs linked to changes in life-history traits, thus providing biological relevance. Munkittrick and Dixon (1988) found that white suckers (*Catostomus commersoni*) collected from lakes elevated in zinc and copper had lower growth, egg size, fecundity and higher rates of spawning failure compared to white suckers collected from reference lakes. Hose et al. (1989) found that white croakers from contaminated San Pedro Bay, California had a lower fecundity by 32% and an associated reduction in fertility of 14% compared to a reference location. Similar results were reported by Spies and Rice (1988) in starry flounder from San Francisco Bay where an increase in sum PCBs in eggs from 5 to 30 µg/g was associated with a decline in fecundity, fertilization success and embryo survival, which dropped from an approximate

average of 70 to 30%. Lesko et al. (1994) reported results contrary to ours where brown bullhead from more contaminated Lake Erie tributaries had greater fecundities and egg diameters than those from less contaminated tributaries. Those differences corresponded with greater total lengths and ages and were explained due to greater food availability and decreased predation in more contaminated tributaries, thus an indirect effect of contaminant exposure.

In the present study, overall increases in egg diameter and GSI of cleared fish may have been a response to changes in environmental quality characteristics (e.g. food and habitat of aquaculture ponds relative to the Detroit River) as well as contamination. Egg diameter has been shown to be affected by food availability and the presence of predators (Billman et al. 2011, Segers et al. 2011). However, the condition indices did not show consistent differences among locations in acute versus cleared fish which would have been expected had food quality effects strongly impacted life history traits. Gonadosomatic index metrics also appeared to retain a consistent association with PCB contamination both pre- and post clearance indicating that fish did not depurate all of their contaminant burdens. Although predation rates were not investigated, brown bullhead were subject to aerial predation in both wild and pond environments. While the exact mechanisms have yet to be discerned, there does appear to be a link between environmental contamination, life-history traits, and reproductive fitness.

My results have caveats that need to be acknowledged. First, I compared life-history traits using a quantified measure of environmental contamination (sum PCB concentration in eggs) instead of the more common method of comparing traits between two habitats known to differ in overall contamination with one relatively low in overall contaminants and one relatively high in overall contaminants. While there are benefits to

quantifying my environmental contaminant variable using a metric suitable to explain differences in life-history traits, it would be naive to dismiss potential impacts of the multitude of additional environmental contaminants, and their interactions, that are present in the present study region (for examples see Bustness 2006). Second, the PCB clearing rates appear to be different among the four locations; however no corresponding proportional difference in life-history traits was observed. This may be due to differences in maternal allocation of PCBs (Fisk et al. 1998), life-history traits responding to environmental variables other than sum PCBs (Bustness et al. 2008), or that differences in life-history traits cannot be observed beyond a certain PCB concentration threshold. Third, other contaminants which may have had more direct impacts on the life history traits measured here may have exhibited different clearance rates in aquaculture ponds relative to PCBs. Lastly, and most importantly, I assume that changes in life history traits are adaptive. This assumption was made as life history traits are directly liked to offspring fitness and that life history theory suggests that variation in these traits will maximize an individual's fitness. It is, however, possible, that observed differences in life history traits are not adaptive, and are instead due to other factors including deleterious effects of environmental contaminants. If observed changes in life history traits are not adaptive, then I have simply demonstrated phenotypic plasticity in these traits, rather than acclimation. Further research as to whether the changes in life history traits result in an increase in fitness of the individuals in their respective habitats is required.

In summary, my field-based experiment provides support for the acclimation hypothesis in that egg diameter and GSI can increase over the period of one reproductive season in response to a decrease in PCB exposure. My experiment also examines changes in life-history traits in response to contaminant clearing in a field setting, thus giving my

findings particular relevance to natural populations. This is especially important for habitat remediation programs and ecosystem managers as changes in life-history traits, with the potential to positively impact population dynamics, can occur with the removal of environmental contaminants. Even more so, these changes may occur within an individual's life span, resulting in measurable changes in a relatively short period of time. My results also highlight the potential benefits of using of multiple life-history traits, including those that are egg specific, as biological indicators of environmental change. Life-history traits can be relatively easy to measure, reflect overall environmental condition, and have population-level implications. Environmental contamination and remediation are issues of global concern and interest, and as such, more experimental research investigating individual and population-level responses to contaminant exposure and clearing, which is relevant to natural populations, is needed to aid managers in decision-making processes and predictions of future ecosystem health.

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Table 3.1: Means ± standard deviation and sample sizes for body length, body mass and age of brown bullhead (*Ameiurus nebulosus*) collected from four locations in the Detroit River (PI = Peche Island, BR = Belle River, BI = Belle Island, TC = Trenton Channel).

Location	Status	*n*	Total length (mm)	Total mass (g)	Age (years)
PI	Acute	9	266 ± 10.0	252.1 ± 18.67	3 ± 0.2
	Cleared	6	279 ± 10.6	260.8 ± 34.36	3 ± 0.3
BR	Acute	9	271 ± 9.5	227.8 ± 19.85	3 ± 0.2
	Cleared	9	272 ± 10.7	294.2 ± 40.97	3 ± 0.2
BI	Acute	7	272 ± 16.0	343.6 ± 52.13	4 ± 0.5
	Cleared	10	260 ± 5.2	248.4 ± 18.66	3 ± 0.3
TC	Acute	9	266 ± 7.7	253.0 ± 25.31	3 ± 0.2
	Cleared	11	289 ± 5.7	342.3 ± 21.06	3 ± 0.1

Table 3.2: Acute and cleared treatment comparisons of female bullhead (*Ameiurus nebulosus*) life-history traits (mean ± standard error). Samples sizes are those combined from all 4 locations for each treatment. Z-scores and associated p-values (z-tests) describe whether statistical differences occur between treatment means of each life-history trait. Intraclass correlation coefficients (ICC) were calculated to examine the between-group effect of locations, reflecting the proportion of variance of life-history traits occurring among locations. The remaining proportion of variance (1 – ICC) is then assumed to occur within locations, including that of acute and cleared treatments.

Life-history trait	Treatment	*n*	Mean ±SE	ƶ	*P*-value	ICC %
Egg Diameter (mm)	Acute	34	2.59 ± 1.01	6.08	< 0.001	11
	Cleared	36	2.74 ± 1.01			
GSI (%)	Acute	34	12.50 ± 1.20	3.8	< 0.001	28
	Cleared	36	14.60 ± 1.20			
Fecundity (# eggs)	Acute	34	5390 ± 145.6	0.36	0.72	13
	Cleared	36	5444 ± 148.7			

Figure 3.1: Map of the four brown bullhead (*Ameiurus nebulosus*) collection sites in the Lower Great Lakes region: 1 Peche Island (PI), 2 Belle River (BR), 3 Belle Island (BI), 4 Trenton Channel (TC).

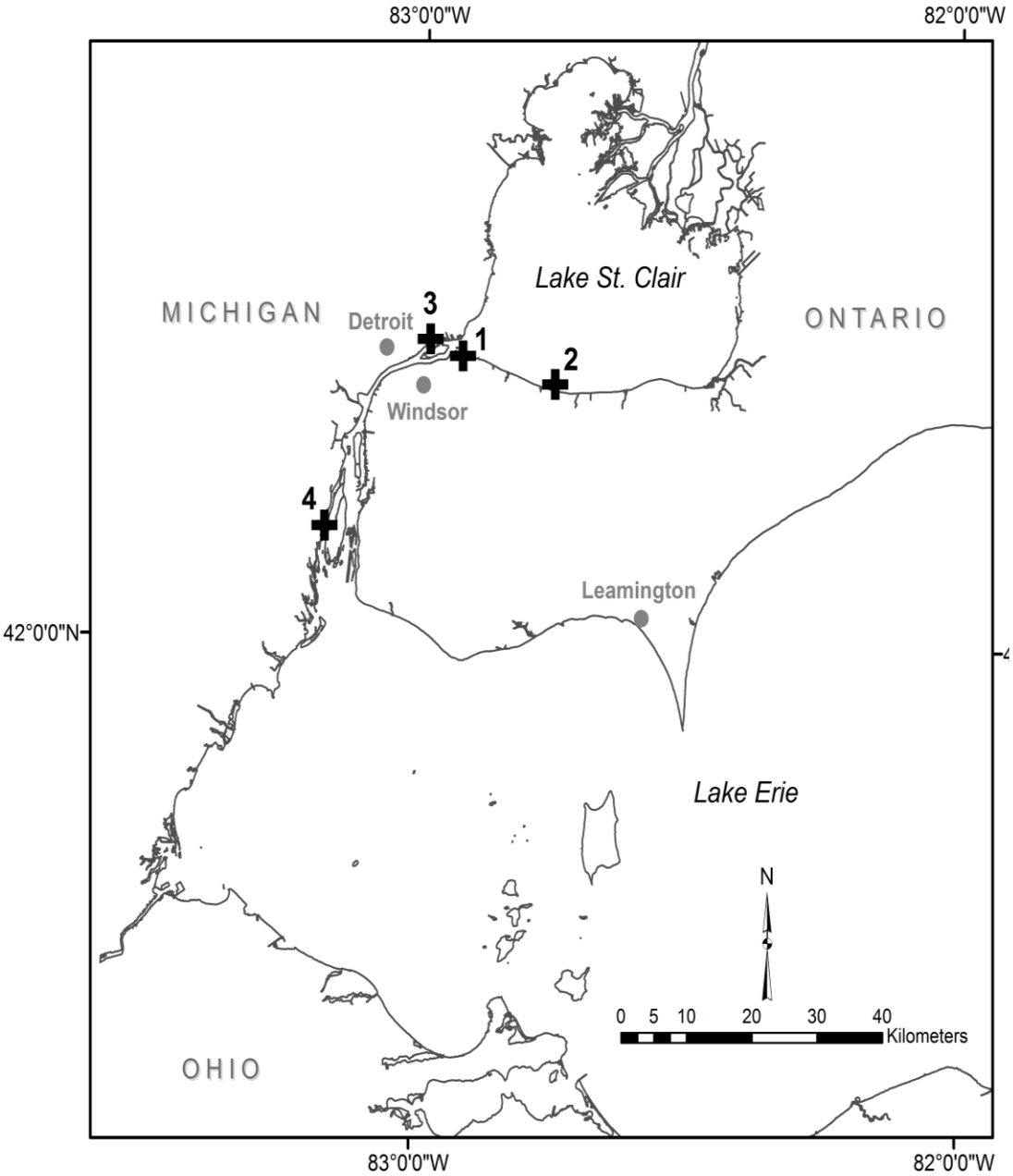


Figure 3.2: Sum polychlorinated biphenyl concentrations in female brown bullhead

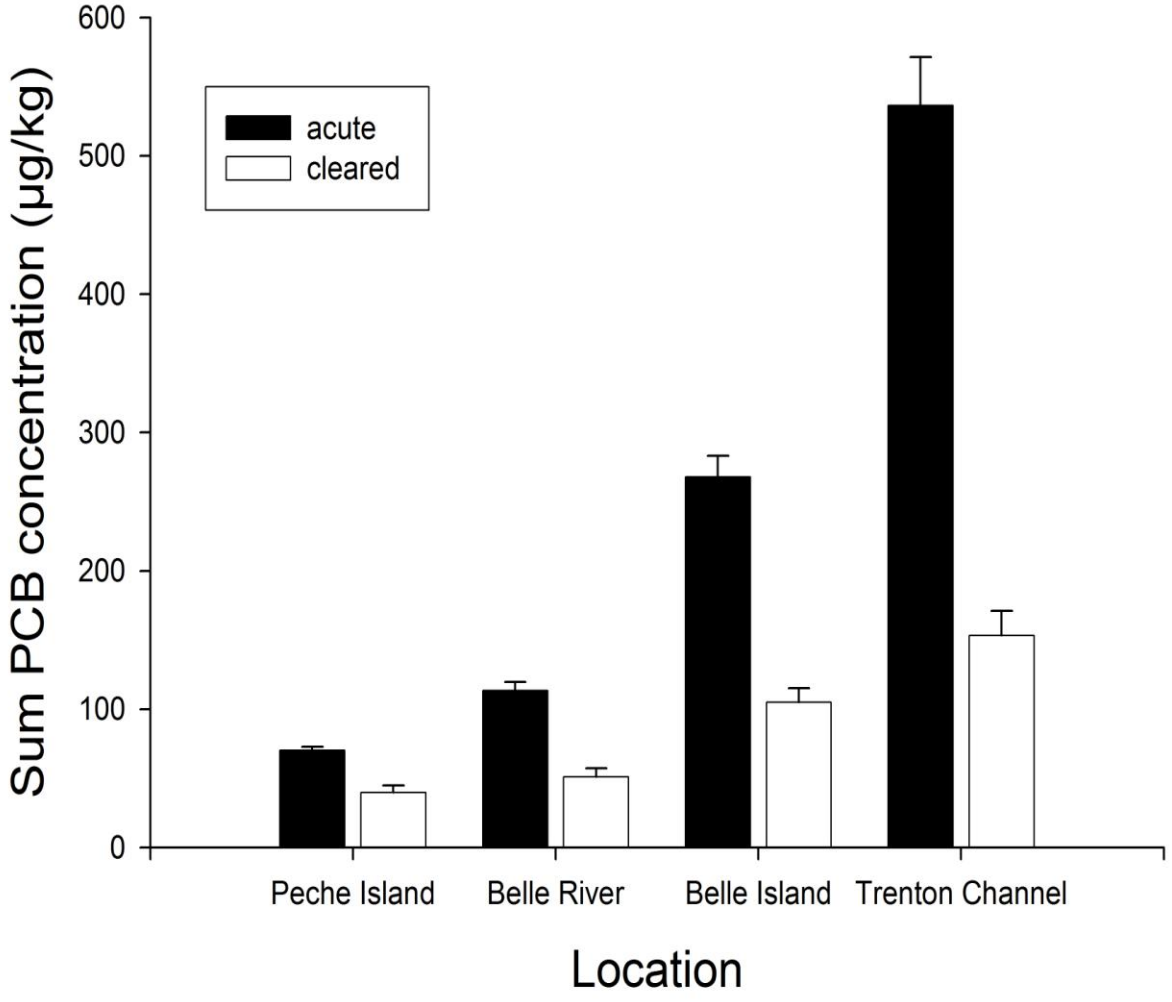
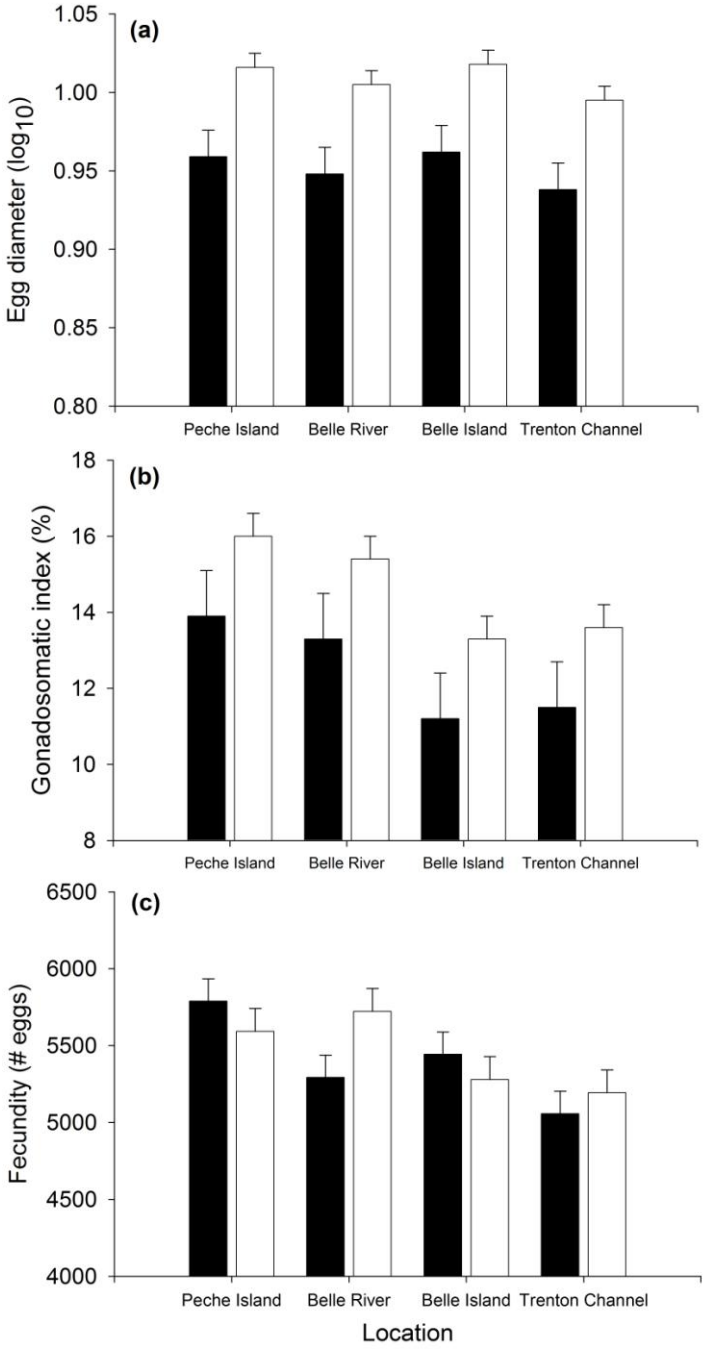


Figure 3.3: (a) Mean (±SE) egg diameter across eight populations of brown bullhead, paired by sampling location, comparing acute (black) and cleared (white) treatments. (b) Mean (±SE) gonadosomatic index across eight populations of brown bullhead, paired by sampling location, comparing acute (black) and cleared (white) treatments. (c) Mean (±SE) fecundity across eight populations of brown bullhead, paired by sampling location, comparing acute (black) and cleared (white) treatments.



CHAPTER 4: VARIATION IN PRIMARY AND SECONDARY SEXUAL TRAITS EXPLAINED BY POLYCHLORINATED BIPHENYL-MEDIATED ENDOCRINE FUNCTION IN MALE CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*) [3]

Synopsis

Individual variation in traits under sexual selection is often discussed only as a consequence of selection, with less emphasis on variation arising from physiological constraints in response to environmental factors, such as contaminants. Hormones are involved in the development and maturation of sexual traits and production and signaling of these hormones are also susceptible to environmental contaminants. I used a wild population of Chinook salmon (*Onchorhynchus tshawytscha*) from the Credit River, Ontario, to investigate whether individual measures of sum PCB concentrations and reproductive hormones can explain variation in primary (sperm velocity, percent of motile sperm, sperm density) and secondary (mid-eye-to-hypural-flexure length, caudal peduncle depth, hump height, kype length and total body depth) traits under sexual selection. Using a regression tree analysis, I demonstrate that 11-ketotestosterone (11-KT) concentrations best describes variation in primary and secondary sexual traits, with males with lower 11-KT having greater measures of primary sexual traits and lower measures of secondary sexual traits, and vice versa. I then demonstrate that PCB concentrations best describe variation in primary and secondary sexual traits in males with low 11-KT concentrations, where males with low 11-KT concentrations will have low PCB concentrations. Alternatively, I demonstrate that maturation inducing steroid (MIS) concentrations best describes variation in primary and secondary sexual traits in males with high 11-KT concentrations, where males with high 11-KT concentrations will have high MIS concentrations. This chapter highlights physiological mechanisms associated

with PCBs that can explain variation in traits under sexual selection and suggests future research to investigate resulting evolutionary implications.

[3] This chapter is the product of joint research with Dr. K.G. Drouillard, Dr. O.P. Love, Dr. C.A.D. Seminiuk, Dr. I.A.E. Butts and Dr. T.E. Pitcher.

Introduction

Males across several taxa exhibit variation in primary and secondary sexual traits as a consequence, in part, of sexual selection (reviewed in Andersson 1994). Secondary sexual traits are more commonly examined in sexual selection research where females assess elaborate male ornaments with the goal of obtaining a high quality mate (Andersson and Simmons 2006) or where males use armaments during bouts of male-male competition for gaining access to reproductive females (Berglund et al. 1996). Primary sexual traits are those which are required for reproduction, such as gametes, and also have the potential to be under sexual selection as differences in particular measures of these traits (e.g. pollen size, genitalia shape) can result in differences in successful mating attempts (Eberhard 1985, Arnold 1994). Sexual selection acting on primary sexual traits is often more cryptic than secondary sexual traits and may be less prevalent across taxa. For example, sperm velocity is the primary determinant for competitive fertilization success in some species, where males with faster sperm will obtain greater fertilization success compared to males with slower sperm (Hoysak and Liley 2001, Gage et al 2004). While variation in both primary and secondary sexual traits may ultimately be explained by sexual selection, understanding proximate mechanisms underlying this variation is also important and is less understood. Variation in both primary and secondary sexual traits has been associated with environmental factors such as resource availability (Grether et al. 1999, Bobe and Labbé 2010), climate shifts (Shine 1999, Rurangwa et al. 2004Møller et al. 2010) and contamination exposure (Kime 1995, Hewitt et al. 2008). When examining individual variation in sexual traits within a single population, males are often exposed to similar environmental conditions. Individual variation in the expression of sexual traits might therefore be a result of differences in the physiological capability of

expressing these traits (Andersson 1986, Hill 1995), different genotypes or epigenetic states (Hill 2011), or any resulting combination of the above.

One set of physiological mechanisms that control the development of both primary and secondary sexual traits and are also susceptible to disruption from environmental factors are reproductive hormones (Sonnenschein and Soto 1998, Gregory et al. 2008, Butts et al. 2012). In vertebrates, the hypothalamus-pituitary-gonadal (HPG) axis regulates a complex web of hormone signalling involved in the development of primary and secondary sexual traits and thus individual variation in HPG axis function is of particular interest from an evolutionary standpoint. In fish, simplified, the androgen 11-ketotestosterone (11-KT) is produced in the testes, via environmental stimulation of the HPG axis, which further signals for sperm cell proliferation, division, and spermatogenesis (Nagahama 1994). Once spermatogensis has commenced a subsequent increase in maturation inducing steroids, such as 17 alpha,20 beta-dihydroxy-4-pregnen-3-one (MIS), occurs which signals spermiation and sperm activation (Mirua et al. 1992, Nagahama 1994). A diagram of these hormones and their roles in sperm development can be seen in Figure 4.1. Plasma concentrations of 11-KT have also been associated with the development of morphological secondary sexual traits (Owens and Short 1995, Oliverira et al. 2008, Butts et al. 2012,), although signalling pathways involved in this development is unknown. Little is also known about the role of other hormones, aside from limited research on testosterone (e.g. Saraiva et al. 2010), in the development of secondary sexual traits. With multiple hormone pathways of the HPG axis involved in primary and potentially secondary sexual trait development, there are multiple opportunities for changes to normal hormone function to occur, often resulting in changes to sexual trait expression (Kime and Nash 1999).

Polychlorinated biphenyls (PCBs) have been linked to changes in endocrine function of the HPG axis across several taxa (e.g. Birnbaum 1994, Cooper and Kavlock 1997, Tyler et al. 1998, Singleton and Khan 2003) including fishes (e.g. Safe 1994, Monosson et al. 1996, Khan et al. 2001, Reiser et al. 2004, Nakata et al. 2005, Martyniuk et al. 2009). One system in which this link has been rigorously studied in is the Atlantic croaker (*Micropogonias undulates*). Khan and Thomas (1997) demonstrated that not only do PCBs act to impair neurotransmitter systems in the brain of this fish, and thus disrupt normal brain signalling and function, but also that direct PCB exposure has been linked to the impairment of luteinizing hormone (GtH II; see Figure 4.1) secretion and declined gonadal growth in this species (Khan and Thomas 2006). Based on this evidence, as well as those referenced above, PCBs do have the potential to alter endocrine functioning in a biologically relevant manner. While the links between PCBs, endocrine function and sexual traits are evident, the strength and direction of the relationships between PCBs, endocrine function and primary and secondary sexual traits do vary among studies, possibly due to differences in species, reproductive status or types and concentrations of congener(s) individuals are exposed to (see Gregory et al. 2008, Khan and Thomas 2006). Quantifying one contaminant metric that is biologically relevant for sexual traits, rather than comparing metrics between contaminated and reference populations as is commonly seen in ecotoxicological studies, can be useful from an evolutionary perspective (Chapter 1). Quantification of contaminants allows for individual level concentrations to be associated with individual measures of both sexual traits and underlying physiological mechanisms, through which contaminants have the potential to affect the expression of both primary and secondary sexual traits.

Male salmonids are biologically and evolutionary relevant systems to examine impacts of environmental factors, including PCBs, on endocrine function and primary and secondary sexual trait expression. Salmonids have a semelparous life-history strategy where spawning occurs at the end of their life cycle after upstream migration to their natal habitat from large freshwater lake or ocean systems (Hendry et al. 1999). During migration, males will develop a bony kype structure and a noticeable dorsal hump as secondary sexual traits (Fleming and Reynolds 2004, Butts et al. 2012). These traits, including an overall larger body size and caudal peduncle depth used for aggressive burst swimming (Webb 1984, Ehlinger 1991), are important for male-male competition during the formation of spawning hierarchies (Fleming and Gross 1994). They are also positively associated with more favorable positioning within these hierarchies, allowing males with relatively more elaborate and developed traits to gain advantaged access to females, often resulting in increased fertilization success (Fleming and Gross 1994, Jones and Hutchings 2002, Blanchfield et al. 2003). There has also been evidence to support that variation observed in these adaptive traits are heritable (Beacham and Murray 1987, Fleming and Gross 1994 , Hendry et al. 2000, Hendry 2001). Along with secondary sexual trait development, gonad development and sperm production is occurring during migration in preparation for the spawning event. Sperm metrics such as velocity, motility and density are key determinants of fertilization success, especially in external fertilizing species where sperm competition, post-copulation competition of ejaculates for fertilization (Birkhead and Møller 1998), is prevalent (Hoysak and Liley 2001, Gage et al. 2004, Yeates et al. 2007). The importance of sperm competition is great as up to 10 males may attempt to spawn with a single female, simulateously (Keenleyside and Dupuis 1988), with the lack of male competitors typically occurring only in artificial or experimental

settings (Gross 1991). Variation in these adaptive sperm metrics has also been demonstrated to be heritable, although relatively little work has been conducted in salmonids (see review and references therein; Evans and Simmons 2008).

While both primary and secondary sexual traits are ultimately important for maximizing fitness in salmonids, there is a limited amount of energy available to males for sexual trait development during migration as individuals do not typically eat once migration has commenced and migration itself is energetically demanding (Dingle 1996, Hendry and Berg 1999; but see Garner et al. 2009). Individual variation in secondary sexual traits in particular has been found to be the result of differences in energy allocation and availability (Beacham and Murray 1987, Quinn et al. 2001, Kinnison et al. 2003). Moreover, males with less pronounced kypes, humps and overall smaller body sizes, and therefore in less optimal ranks in the spawning hierarchies, have been found to have faster (Vladic and Jarvi 2001), longer-lived (Gage et al. 1995, Uglem et al 2001), or more (Gage et al. 1995, Neff et al. 2003) sperm to increase their fertilization success (Stoltz and Neff 2006, Knapp and Neff 2008). The energy available to males during migration is in the form of lipid stores throughout the body, which deplete over time (Kinnison et al. 2003). Persistent organic pollutants, such as PCBs, are typically found in lipid rich tissues throughout the body and during lipid depletion these pollutants can be mobilized to regions with greater lipid concentrations in response to fugacity gradients (Findlay and DeFreitas 1971, Mackay and Patterson 1982). While the specific chemicokinetics of PCBs are beyond the scope of this research, more generally, magnification and toxicity of PCBs has been observed in male and female muscle and gonad tissue in migrating salmonids (Debruyn et al. 2004, Kelly et al. 2007), thus having

the potential to alter typical endocrine function and ultimately primary and secondary sexual trait expression.

I used adult male Chinook salmon (*Oncorhynchus tshawytscha*), also known as "hooknoses", to investigate physiological mechanisms underlying individual variation in primary and secondary sexual trait expression. Chinook salmon were collected from the Credit River ($43^{o}35'$ N, $79^{o}42'$ W;), which flows into Lake Ontario. Chinook salmon have been stocked in Lake Ontario for over 40 years (Crawford 2001). Chinook salmon carcasses after spawning in the Credit River have been identified as a source of PCB contamination in this river system (O'Toole et al. 2006). It has also been found in Credit River Chinook salmon that 11-KT is positively related to body size and hump depth, while 11-KT and MIS were not related to various sperm metrics (Butts et al. 2012). Furthermore, Kinnison et al. (2003) demonstrated high heritabilities of secondary sexual traits associated with increased fertilization success in Chinook salmon populations from New Zealand, suggesting that these traits are adapative and may have evolutionary significance. I used a regression tree analysis to model relationships between gonad or muscle sum PCB concentrations and 11-KT and MIS concentrations as predictor variables to explain variation in primary (sperm velocity, percent of motile sperm, sperm density) and secondary (mid-eye-to-hypural-flexure length (L_{MEH}), caudal peduncle depth, hump height, kype length and total body depth) sexual traits. I predicted that variation in primary and secondary sexual traits would be best explained by 11-KT and MIS concentrations. I further predicted that variation in primary and secondary sexual traits already explained by 11-KT and MIS concentrations would be further explained by sum PCB concentrations. No predictions as to the direction of these associations (positive or negative) were made as the results are varied in the literature and little to no work has

been conducted in wild populations of fishes. This study is novel in that it highlights potential implications PCBs have on traits involved in sexual selection in a wild population of spawning salmonids.

Materials and methods

Sample collection

Adult male Chinook salmon (n = 24) were collected during the spawning season using backpack electrofishing from 3 to 6 October 2010. Within two minutes after capture, milt samples were collected, individuals were sacrificed, and blood samples were collected to minimize handling effects on hormone production. Milt was stored in Ziploc bags over ice (not in direct contact) in a cooler for no longer than 4 hours until sperm analysis was conducted. Blood was then obtained from the caudal vein using heparin-rinsed 3 mL syringes with 23-gauge needles. All blood samples were kept on ice in a cooler for 3 to 5 hours until they were centrifuged in heparin-rinsed 1.6 mL Eppendorf tubes for 10 min at 2500 rpm to obtain plasma for hormone assays. Plasma was snap frozen in liquid nitrogen and then stored at -80 ºC until hormone assays were conducted. After milt and blood sampling, body size measurements used to describe secondary sexual traits were taken; L_{MEH}(to avoid variation in length due to tail fin erosion), cadual peduncle depth, hump height (distance from the lateral line to the highest point on the dorsal surface), kype length (distance from middle of eye to the tip of the snout) and total body depth (Table 4.2; Figure 4.2) (Hendry and Berg 1999, Kinnison et al. 2003, Pitcher et al. 2009). Gonad and muscle tissue samples of 2 to 5 g were excised, wrapped in hexane-rinsed tin foil and stored at - 20 ºC until PCB extraction and analysis were conducted.

Sperm activity

To assess sperm velocity and motility, an aliquot of milt (< 0.2 μL) was pipetted into a chamber of a 2X-CEL glass slide (Hamilton Thorne, MA, USA) and covered with a coverslip. Sperm were then activated with 15 μL of river water collected from the Credit River at 11^{o}C. Sperm activity was video-recorded using a CCD black and white video camera (XC-ST50, Sony, Japan) module at 50 Hz vertical frequency, mounted on an external phase contrast microscope (CX41 Olympus, Melville, NY, USA) with a 10x negative-phase magnification objective (Pitcher et al. 2009). Analysis of sperm velocity and motility were analyzed using the HTM-CEROS sperm analysis system (version 12, CEROS, Hamilton Thorne Biosciences, Beverly MA, USA) set at the following parameters: number of frames = 60, minimum contrast = 11, photometer = 55 to 65, minimum cell size = 3 pixels. Sperm velocity and motility metrics were recorded at 5, 10 and 15 s post-activation as the majority of fertilization events in salmonids occur across this time frame, although fertilization success under sperm competition has been found to be lower at 15 s compared to 5 and 10 s in other salmonids (Hoysak and Liley 2001, Fleming 1996, Yeates et al. 2007). Velocity was analyzed using three metrics that reflect movement patterns observed in natural conditions; average path velocity (V_{AP}), straight-line velocity (V_{SL}), and curvilinear velocity (V_{CL}). Motility was defined as the percent of sperm motile at 5, 10 and 15 s post-activation. Values for these metrics are reported by the computer-assisted sperm analysis software as an average for each individual (Table 4.2).

Sperm density

Sperm density was assessed using 1.5 μL of milt diluted in 500 μL of Courtland's saline solution (7.25 g/L NaCl; 0.38 g/L KCl; 0.47 MgSO4 × 7H20; 0.4 g/L

Na2HPO4×H20; 1.0 g/L NaHCO3; 0.22 g/L MgCl2; 1.0 g/L C6H12O6; adjusted to pH 7.8; Pitcher et al. (2009)). A 10 μL volume of the suspension was placed onto a Neubauer haemocytometer and the suspension was allowed to settle for 10 min to ensure sperm were being counted on the same plane. Sperm were counted in 5 of the 25 squares on the haemocytometer under a Zeiss Axiostar compound microscope at 400x magnification. To determine sperm density as number of sperm per mL of milt (Table 4.2), the average number of sperm in the 5 squares was multiplied by 25 (number of squares), then by 10 (chamber depth, μm), then by 500 (sample volume, μl).

Hormone assays

Plasma levels of 11-KT and MIS were determined using commercial Enzyme-linked-Immunoabsorbent Assays (ELISA; Cayman Chemicals, Ann Arbor, MI, USA) following methods outlined in Butts et al. (2012). Briefly, optimal dilutions were determined as 1:12,000 dilution for 11-KT and 1:2,000 for MIS. For each hormone, plasma was assayed in triplicate across three (11-KT) or four (MIS) assay plates yielding an intra- and inter-assay variation of 3.93% and 14.38% for 11-KT, and 4.8% and 9.6% for MIS, respectively. Relevant cross-reactivity for the 11-KT assay included adrenosterone (2.9%), and for the MIS assay included 20ß-hydroxyprogesterone (0.1%).

Polychlorinated biphenyl extraction and analyses

Sum PCB concentrations were quantified in both gonad and muscle tissue as PCBs have been demonstrated to magnify in both tissue types at different rates, depending on fugacity gradients and nonlipid storage capacity (Mackay and Patterson 1982, Gobas et al. 1999). Thawed gonad and muscle samples, ranging from 0.2 to 0.5 g for both tissue types from each individual, were homogenized and PCB congeners and neutral lipids extracted using a micro-extraction technique following the protocol outlined

by Daley et al. (2009). A solid phase extraction manifold (Phenomenex, Torrance, CA, USA) was used for the PCB extractions, which consisted of eight, 20 mL glass syringes, each connected to 1 µm glass fibre syringe filter, . Tissue samples were homogenized, using a glass mortar and pestle, with 15 g of activated sodium sulphate and placed into the syringes containing 15 mL dichloromethane (DCM):hexane (1:1). An additional 15 mL of DCM:hexane was used to rinse the glass mortar and pestle. Each syringe was then spiked with an internal recovery standard of 200 ng PCB34/mL. Each set of extractions was run with a method blank and an in-house homogenate fish sample (Detroit River common carp, *Cyprinus carpio*) as an inter-assay control. Extracts were then concentrated by rotary-evaporator and neutral lipid content was assessed gravimetrically using 1 mL of the 10 mL sample (Drouillard et al. 2004). Extracts were again concentrated by rotary-evaporator to and florisil chromatography was used for sample clean up (Lazar et al. 1992). Fractions 1 (50 mL hexane) and 2 (50 mL DCM:hexane; 15:85) were collected. Extracts were then concentrated to 1 mL for analysis via gas chromatography electron capture detection (GC-ECD). The PCB congeners in each sample were identified by retention time and referenced against an external PCB standard (Quebec Ministry of Environment Congener Mix; AccuStandard). The following congeners were present in the external standard and analyzed for detection in egg samples (IUPAC numbers, coeluting congeners separated by slash): 18/17, 31/28, 33, 52, 49, 44, 74, 70, 95, 101, 99, 87, 110, 151/82, 149, 118, 153, 105/132, 138, 158, 183, 128, 177, 156/171, 180, 191, 170, 201, 195/208, 194, 205, 206, 209. Sum PCB concentrations were calculated for each individual as the sum of each congener in µg/kg wet weight. To account for differences in spawning condition between males and because percent lipid content is low (1.2 ± 0.7 %), all samples were lipid corrected as lipid content was a key factor in determining PCB

accumulation (Persson et al. 2007). Mean percent recovery of the PCB34 spike was 82.2 ± 2.7% (mean ± S.E.). All samples had recoveries greater than the 70% threshold generally used for QA/QC and all sum PCB in-house homogenate samples were in compliance (mean ± 2 S.D.) with the Great Lakes Institute for Environmental Research analytical laboratory quality assurance guidelines (Canadian Association for Environmental Analytical Laboratories Accreditation and ISO17025 certified).

Statistical analyses

Values for all metrics collected and used in the following analyses are summarized in Table 4.2. Normality and homogeneity of variance was examined for each variable using Shapiro-Wilk goodness-of-fit test and Levene's test respectively. Percent motility was arcsine square root transformed and both muscle and gonad lipid corrected sum PCB concentrations were log transformed to comply with normality.

Principal components analysis (PCA) was used to summarize variation in morphology metrics related to secondary sexual traits (L_{MEH}, caudal peduncle depth, hump height, kype length and total body depth). One informative PC axis was extracted that explained 69% of this variation and is referred to hereafter as body size. Males with greater body size scores had larger measures of all morphology metrics than those with lower body size scores.

Principal components analysis was also used to summarize variation in sperm velocity metrics (V_{AP}, V_{SL}, V_{CL}) at 5, 10 and 15 s post-activation. One informative PC axis was extracted for each time that explained 92.8 %, 86.3 % and 84.7% of the variation respectively. These PCs are referred to hereafter in as sperm velocity. At all post-activation times, males with greater velocity scores had greater values of V_{AP}, V_{SL} and V_{CL} than those with lower scores.

One final set of PCAs were used to summarize both primary and secondary sexual traits for each post-activation time of 5, 10, and 15 s. These traits included body size, sperm velocity, percent motility and sperm density. Two informative PC axes were extracted for 5 s post-activation. PC1 explained 37.2 % of the variation and captured a negative relationship between sperm density and body size. PC2 explained 31.4 % of the variation and captured a positive relationship between sperm velocity and percent motility. Two informative PC axes were extracted for 10 s post-activation. PC1 explained 40.8 % of the variation and captured a negative relationship between both sperm velocity and percent motility, and body size. PC2 explained 32.7 % of the variation and captured a negative relationship between sperm density and body size. Two informative PC axes were extracted for 15 s post-activation. PC1 captured all variables of interest and explained 43.3% of this variation. Males with greater PC1 scores had larger body sizes, slower sperm velocities, lower percent motilities and lower sperm densities than those with lower PC1 scores. PC2 explained 31.3 % of the variation and captured a positive relationship between sperm density and body size. PC scores for each post-activation time are referred to hereafter as sexual traits.

I then used independent regression tree analyses (RTA) to investigate relationships between sexual trait PCs (response variable) at each post-activation time, 11-KT, MIS and either gonad sum PCB concentration or muscle sum PCB concentration (response variables). RTA is useful to investigate relationships between predictor and response variables when there is no *a priori* assumption of the nature of these relationships and therefore statistical analysis does not provide support for a specific hypothesis (Prasad et al. 2006). Unlike many statistical analyses which provide statistical support for a specific hypothesis, RTAs partition, or split, each predictor variable into

binary categories determined by maximizing their between group sums of squares with respect to the response variable. Regression trees are typically read along their peripheries in order to maximize the amount of variation in the response variable that is explained by the model. A single r^2 value is produced from the model to describe the amount of variation that is explained and *k* fold cross-validation techniques are used to validate the resulting model. This validation technique uses a subset of the data ($k = 10$) to conduct the same RTA *k* times and reports an average r^2 value (Kohavi 1995). One benefit of this validation technique is that it uses all data points in the analysis. A root mean square error (RSME) value is also produced as an indication of model accuracy, with smaller values indicating less variation between the model and the data set. In order to compare models to determine that which best fits the data set in relation to the number of parameters in the model, I used Aikakie's Information Criterion (AIC) (Burnham and Anderson 2002), or AIC_c, as I have a finite sample size. The model with the smallest AIC_c value represents the best model within the model set, with the remaining models ranked relative to the best model using AIC_c differences (ΔAIC_c). Models with ΔAIC_c values < 2 indicate that there is substantial support for these models as being the best-fitting models within the set (Burnham and Anderson 2002). Models with ΔAIC_c values between 2 and 4 have less support (Burnham and Anderson 2002). Models with greater r^2 values and lower root-mean-square errors also provide an indication of the best model.

Results

Individual gonad and muscle sum PCB concentrations varied from 181.6 – 463.7 µg/kg wet weight and 175.6 – 372.1 µg/kg wet weight, respectively (Table 4.2). Although sum PCB concentrations were similar between the two tissue types, there was no

significant correlation between them ($r = 0.36$, $p = 0.09$). 11-KT concentrations ranged from 34.1 – 341.1 ng/mL and MIS concentrations ranged from 24.3.1 – 176.8 ng/mL (Table 4.2).

When identifying the best model to describe variation in Chinook salmon primary and secondary sexual traits, I found that the model with the lowest AIC_c value included sexual trait PC1 at 15 s using gonad sum PCB concentration. This model also had the greatest r^2 value, the k-fold r^2 value that most closely approached the overall r^2 value and the lowest RSME value. Of the remaining models, none had ΔAIC_c values < 2 and several models had ΔAIC_c values between 2 and 4, when compared to the best model. Furthermore, AIC_c values tend to be lower for those including gonad sum PCB concentrations compared to those including muscle sum PCB concentrations. Results from each model are reported in Table 4.3.

From my best model (Figure 4.4), I found that variation in primary and secondary sexual traits of spawning male Chinook salmon is best described by 11-KT concentrations as it was the first predictor variable split in the RTA. Here, with males with lower 11-KT concentrations have greater measures of primary sexual traits and lower measures of secondary sexual traits, and vice versa. Next, I found that PCB concentration best described variation in primary and secondary sexual traits in males with low 11-KT concentrations, where males with low 11-KT concentrations will have low PCB concentrations. This relationship is indicated along the left branch of Figure 4.4. I also found that maturation inducing steroid (MIS) concentrations best describes variation in primary and secondary sexual traits in males with high 11-KT concentrations, where males with high 11-KT concentrations will have high MIS concentrations. This relationship is indicated along the right branch of Figure 4.4. Overall, males with lower

gonad sum PCB concentrations and lower 11-KT concentrations also had lower sexual trait scores (smaller body sizes and greater sperm metrics) and males with greater MIS concentrations and greater 11-KT concentrations also had greater sexual trait scores (larger body sizes and lower sperm metrics) ($r^2 = 0.55$). This RTA was validated by *k* fold validation ($r^2 = 0.37$) and produced an AIC value of 61.3.

Discussion

My study has provided support, using RTA, that individual variation in primary and secondary sexual traits can be explained by plasma reproductive hormone concentrations and gonad sum PCB concentrations in spawning male Chinook salmon. Support for these associations was only demonstrated when using sperm metrics at 15 s post-activation. Through my analysis, I have identified relationships between PCB and hormone concentrations that can explain variation in sexual trait expression, therefore potentially having further impacts on higher level processes, such as sexual selection, in wild populations.

I investigated several RTA models, each of which initially had the potential to best describe variation in sexual traits in Chinook salmon. First, I found that the majority of models using gonad sum PCB concentrations had lower AIC_c values and explained a greater amount of the variation in the data compared to models using muscle sum PCB concentrations. In my study, gonad tissue is biologically relevant as 11-KT and MIS, as well as sperm, are produced in the testes. The differences observed between gonad and muscle tissue were not suprising due to the rapid development of the lipid-rich gonad tissue and the rapid loss of lipids in the muscle tissue. Also, a review by Monosson (1999/2000) describes incredible variation in PCB distribution among tissue types during

reproduction, depending on species, life histories, congeners of interest, etc. Second, my best model was found at 15 s post-activation, and no other substantially supported models were found at 5 s or 10 s post-activation. I did, however, find models with some support at both 5 s and 10 s using both gonad and muscle sum PCB concentration. All but one of the models with some support at 5 s and 10 s include sperm velocity metrics, which might indicate the importance of velocity at these time frames. Also, my sexual trait PC1 axis using sperm metrics at 15 s post-activation only explains 43.3 % of the variation of all primary and secondary sexual trait metrics. While this is considered to be relatively low, of all the sexual trait PC1 axes, it does explain the most variation, incorporates all sexual traits of interest and produces the best model. The sexual trait PC1 axis at 15 s also describes relationships between the metrics that follow expected patterns of differential energy allocation. I further discuss the trends based from my best model that incorporates gonad sum PCB concentrations and sperm metrics at 15 s post-activation and I caution readers to keep these caveats in mind for appropriate interpretation of my results.

The first splitting in the model (Figure 4.4) showed that males with smaller body sizes, faster sperm velocities, greater percent motilities and larger sperm densities also had lower 11-KT concentrations (< 147.78 ng/mL) than males with larger body sizes, slower sperm velocities, lower percent motilities and lower sperm densities, and vice versa. Compared to other salmonid species, my 11-KT concentrations were found to be an average two-fold greater than those previously reported in the literature during spawning season, for both wild and laboratory-reared individuals (Mayer et al. 1990, Miura et al. 1992, Cardwell et al. 1996, King and Young 2001,). Hormone concentrations are often associated with a high degree of variability, both within and between species, for reasons including variation among assay methodologies (e.g. RIA vs. ELISA),

endocrine biochemistry (Mayer et al. 1990, Hourigan et al. 1991), reproductive behaviour (Liley and Kroon 1995, Oliveira et al. 2002), reproductive state (Espinosa et al. 2001) and environmental conditions (Onuma et al. 2003, Kondic-Spika et al. 2011, O'Connor et al. 2012). Identifying and comparing general trends in hormone concentrations over different contexts or time can therefore be more informative than those with absolute hormone concentrations. I found a positive relationship between body size and 11-KT which is consistent with the literature indicating that androgens, such as 11-KT, play a role in development of secondary sexual traits in fishes (Borg 1994, Youson 2003, Oliveira et al., 2008,). I also found a negative relationship between sperm metrics and 11-KT which was unexpected. This hormone is often positively related to sperm metrics such as density and volume (Schulz et al. 2010) and is not the only hormone involved in determining sperm motility in salmonids (Miura et al. 1992), a primary determinant in fertilization success (Gage et al. 2004). Miura et al. (1991) found that male Japaneese eel (*Anguilla japonica*) injected with 10 ng/mL 11-KT more than doubled the number of mature spermatogonia, an indicator of viable sperm production, than males injected with 1 ng/mL of 11-KT. There was, however, no significant increase in the number of mature spermatogonia in males injected with 100 ng/mL 11-KT. While I are unable to obtain a precise 11-KT concentration threshold for sperm production in my Chinook salmon population, it is possible that my relatively high values of 11-KT compared to other salmonids are greater than what required to observed a functional difference in sperm traits. Assuming that the relatively high 11-KT concentrations are accurate and not due to sampling or methodological errors, this suggests that the negative relationship I found between sperm traits and 11-KT is likely more statistically driven than biologically relevant. From this perspective, and supporting sperm competition theory (Parker 1990,

Ball and Parker 1996) body size could be driving the relationship between sexual traits and 11-KT, where males with higher 11-KT concentrations allocate more energy into secondary sexual trait expression and subsequently less into sperm production and maturation, than males with lower 11-KT concentrations and smaller body sizes. Liljedal et al. (1999) also found a negative relationship between secondary sexual traits and sperm density in Arctic charr (*Salvelinus alpinus*), where spawning males with lighter red colouration had higher sperm densities than males with darker red colouration.

The next splitting in the model, along the left side of Figure 4.4, showed that males with sexual traits explained by low 11-KT concentrations also had low gonad sum PCB concentrations (< 9.82 log lipid transformed). This result was somewhat unexpected as the literature suggests that PCB exposure is negatively related, or unrelated, to 11-KT synthesis (Freeman and Idler 1975, Dabrowska et al. 2000, Milnes et al. 2006). My muscle and gonad sum PCB concentrations are comparable to those found by O'Toole et al. (2006) in Chinook salmon from the Credit River in 2003 with a mean muscle sum PCB concentration of 370 ± 111.92 µg/kg wet weight (S.D.), lower than those found by Jackson et al. (2001) in Chinook salmon from Lake Michigan in 1996 with a mean muscle sum PCB concentration of 1941 ± 200 µg/kg wet weight (S.D.), and up to 10 times greater than Sockeye salmon (*Oncorhynchus nerka*) from the Pacific coast in both 1995 and 2001, respectively (Debruyn et al. 2004, Kelly et al. 2007). While my RTA suggests that low gonad sum PCB concentrations (log transformed lipid normalized) best describes the variation in males with low 11-KT concentrations, the remaining greater gonad sum PCB concentrations next best describes low 11-KT concentrations. All of the concentrations that I recovered, both 'low' and 'high' are thought to be great enough to observe a relationship between gonad sum PCB concentration and 11-KT concentration if

one was present. For example, Baldigo et al. (2006) found that male smallmouth bass (*Micropterus dolomieui*), common carp (*Cyprinus carpio*) and brown bullhead (*Ameiurus nebulosus*) had muscle PCB concentrations (ug/glipid) approximately 10 times lower than those found in my Chinook salmon. In all species, muscle PCB concentrations were positively correlated with E2:11-KT ratios. It was uncertain, however, whether increases in E2:11-KT were due to increases in E2 or decreases in 11-KT. Freeman and Sangalang (1977) found *in vitro* exposure of grey seal (*Halichoerus grypus*) testes cells to 0.45 ppm Aroclor 1254 resulted in declines in 11-KT synthesis from 49% - 57% compared to control cells. This concentration is comparable to those measured in my study; however, any differences in tissue exposure method and environmental conditions could result in different 11-KT responses. It is likely that the general presence of PCBs in this system at these concentrations is enough to elicit lower 11-KT concentrations. That being said, it is also possible that the relatively small range of PCBs that I quantified is not large enough to elicit substantial variation in 11-KT concentrations. For example, Baldigo et al. (2006) quantified PCB ranges that represented 5- 10 fold differences, compared to my study which only had a 2.5 fold difference. It is also possible that specific PCB congeners might better explain variation in 11-KT concentrations. Khan and Thomas (2006) found that disruption of lutenizing hormone in Atlantic croaker (*Micropogonias undulatus*) only occurs when directly exposed to coplanar (more toxic), as opposed to *ortho*-substituted (less toxic) congeners. This would also be an area warranting future investigation.

The final splitting in the model, along the right side of Figure 4.4, showed that males with sexual traits explained by higher 11-KT concentrations also had higher MIS concentrations (≥ 107.70). This positive relationship was somewhat surprising as MIS is typically thought to increase during spermiation just prior to sperm release, while 11-KT

begins to decline during this time (Nagahama 1994). However, 11-KT has also been found to remain at high levels during spermiation until sperm release (Liley and Kroon 1995, Miura et al. 1992). Similar to my 11-KT concentrations, my MIS concentrations are generally greater than those in other salmonids during reproduction, although to a lesser degree than for 11-KT (Fitzpatrick et al. 1986, Mayer et al 1990, King and Young 2001, Onuma et al. 2003). This observation suggests that these salmon produce and sustain relatively high concentrations of both hormones prior to spawning, possibly due to the nature of their semelparous life-history strategy and their short spawning season. The maintenance of relatively high concentrations of other steroids, such as cortisol, during entire spawning seasons has been observed in other salmonid species (e.g. Carruth et al. 2000). More interesting is that gonad sum PCB concentration did not explain variation in high 11-KT concentrations, again supporting the notion that PCB concentrations found in these fish are all sufficient to result in low 11-KT concentrations.

In summary, I demonstrated that individual measures of sum PCB concentrations and reproductive hormones can explain variation in traits under sexual selection in a wild population of Chinook salmon.I found that variation in sexual traits was best explained by 11-KT concentrations, where males with greater 11-KT concentrations also have greater body sizes and lower sperm metrics. Subsequently I found that low 11-KT concentrations in my population of Chinook salmon could be explained by the presence of PCB concentrations, while high 11-KT concentrations were explained by high MIS concentrations. These relationships between PCBs, endocrine function, and sexual trait expression demonstrate that physiological processes, such as endocrine function, can explain links between environmental contaminants and the expression of traits under sexual selection. These findings also therefore provide support for mechanisms that may

impact the expression of these traits, other than sexual selection. Further research is required to investigate the evolutionary impact of these mechanismsMy study is unique not only in that it quantifies individual variation in contaminant concentrations in a wild population, but that it then attempts to use this variation to explain differences in sexual trait expression via underlying physiological mechanisms. This study highlights physiological mechanisms associated with PCBs that can explain variation in traits under sexual selection and suggests future research to investigate resulting evolutionary implications.

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Table 4.1: A summary of literature describing relationships between polychlorinated biphenyls and primary and secondary sexual traits. Each research article is described by the general location where their experiment took place and the origin of their study species (Wild (W) or Laboratory (L)), the specific organism used and their experimental design (experimental (E) or descriptive (D)). A summary of sexual traits examined and their findings are also provided for each article.

Location	Citation	Organism	Design	Sexual trait and findings
L	Campagna et al. 2009. J. Androl. 30:317.	Duroc breed boar	E	Sperm velocity, percent motility, viability; decreased when PCB mixture added to semen compared to control
L	Socha et al. 2008. Cybium. 32:197.	Common carp (*Cyprinus carpio*)	E	Sperm velocity; decreased when PCB mixture added to semen compared to control
L	Hsu et al. 2007. Toxicol. Appl. Pharmacol. 221:68.	Sprague-Dawley rats	E	Sperm count and percent motility; lower in adult male offspring whose mothers were injected with PCBs than controls
L	Khan and Thomas 2006. Mar. Environ. Res. 62:25.	Atlantic croaker (*Micropogonias undulates*)	E	GSI; lower in males fed PCBs than controls
L	Kuriyama and Chahoud 2004. Toxicology. 202:185.	Sprague-Dawley rats	E	Testes size, sperm density; lower in males injected with PCBs *in utero* than controls
L	Qin et al. 2003. Environ. Health Perspect. 111:553.	African clawed frog (*Xenopus laevis*)	E	Testes development, sperm density; lower in tadpoles developed in PCB environment than controls
L	Kim 2001. J. Vet. Med. Sci. 63:5-9.	Sprague-Dawley rats	E	Testes mass, sperm density; lower or no difference between males injected with PCBs *in utero* and controls, depending on number of dosages
L	Fielden et al. 2001. Reprod. Toxicol. 15:281.	C57BL/6J female mice DBA/2 breeder male mice	E	Sperm velocity, linearity, density, fertility; greater sperm metrics and lower fertility in males exposed to PCBs through lactation than controls
L	Huang et al. 1998. Arch. Environ. Contam. Toxicol. 34:204.	C57BL/6J female mice DBA/2 breeder male mice	E	Testis mass, fertilization success; greater testis mass and lower fertilization in males exposed to PCBs through lactation than controls
L	Faqi et al. 1998. Human. Exp. Toxicol. 17:365.	Sprague-Dawley rats	E	Testis and seminal vesicle mass; greater testis and lower seminal vesicle masses in males injected with PCBs *in utero* than controls
L	Gray et al. 1993. Fund. Appl. Toxicol. 20:288	Fisher 344 rats	E	Testes mass, sperm density, sperm motility; no difference between males injected with PCBs and controls
L	Cooke et al.1996. Toxicol. Appl. Pharmacol. 136:112.	Sprague-Dawley rats	E	Testes mass, sperm density; greater in males exposed to PCBs through lactation than controls
L	Sager et al. 1991. Environ. Toxicol. Chem. 10:717.	Holtzman rats	E	Sperm density, morphology, motility, fertilization success; no difference in sperm traits and lower fertilization in males exposed to PCBs through lactation and controls
L	Bustnes et al., 2007. Arch. Environ. Contam. Toxicol. 53:96	American Kestrel (*Falco sparvius*)	E	Colour; no difference between males fed PCBs and controls
L	Bortolotti et al. 2003. Funct. Ecol. 17:651.	American Kestrel (*Falco sparvius*)	E	Colour; duller colour in males during pairing season fed PCBs than controls
L	Fisher et al. 2001. Arch. Environ. Contam. Toxicol. 41:215	American Kestrel (*Falco sparvius*)	E	Behaviour; more aggressive displays and copulation behaviour in males fed PCBs than controls
W	Sol et al. 2008. Arch.	English sole	D	GSI; negative correlation between liver sum PCB

	Environ. Contam. Toxicol. 55:627.	(*Parophrys vetulus*)		concentration and GSI
W	Koch et al. 2006. Environ. Toxicol. Chem. 25:1689.	Shovelnose sturgeon (*Scaphirhynchus platorynchus*)	D	GSI; muscle PCB concentrations negatively correlated with GSI
W	de Solla et al. 2002. Environ. Toxicol. Chem. 21:922.	Common snapping turtle (*Chelydra serpentina*)	D	Precloacal growth rate; slow in males with greater average muscle tissue sum PCBs than those from reference locations
W	de Solla et al. 1998. Environ. Health Perspect. 106:253.	Common snapping turtle (*Chelydra serpentina*)	D	Precloacal length; smaller in males with greater average blood plasma PCBs
W	Reeder et al. 1998. Environ. Health Perspect. 106:261.	Cricket frogs (*Acris crepitans*)	D	Testes development; greater oocyte development in males from habitats exposed to PCBs than those from reference locations
W	Quinn et al. 2002. Environ. Toxicol. Chem. 21:1417.	Great Black-Backed Gull (*Larus marinus*)	D	Plasma carotenoids explaining plumeage colour used for reproductive communication; no correlation with plasma PCB concentrations and carotenoids

Table 4.2: Means, standard deviations (S.D.) and ranges of sum polychlorinated biphenyl (PCB) concentrations (wet weight) for gonad and muscle tissues, plasma hormone concentrations of 11-ketotestosterone (11-KT) and 17 alpha,20 beta-dihydroxy-4-pregnen-3-one (MIS), and primary (sperm) and secondary (body size) sexual trait metrics for Chinook salmon from the Credit River, flowing into Lake Ontario (n=24). Sperm metrics were assessed at 5, 10, and 15 seconds post-activation.

Metric	Mean ± S.D.	Range (min – max)
Contaminant metrics		
Gonad PCB (µg/kg wet wt)	285.9 ± 67.8	181.6 – 463.7
Muscle PCB (µg/kg wet wt)	251.9 ± 44.16	175.6 – 372.1
Gonad lipid (%)	1.20 ± 0.70	0.35 – 2.75
Muscle lipid (%)	1.23 ± 0.95	0.11 – 3.46
Hormones		
11-KT (ng/ml)	159.54 ± 76.24	34.1 – 341.1
MIS (ng/ml)	88.82 ± 43.40	24.3 – 176.8
Secondary sexual traits		
L_{MEH} (mm)	653.2 ± 57.0	565 – 750
Cadual peduncle depth (mm)	74.1 ± 12.4	54 – 110
Hump height (mm)	112.8 ± 15.9	81 – 145
Kype length (mm)	110.9 ± 15.5	82 – 140
Total body depth (mm)	217.6 ± 29.8	165 – 265
Primary sexual traits		
Sperm density per mL ($x10^7$)	5.18 ± 1.44	2.34 – 8.47
5 seconds		
V_{AP} (µm/s)	79.72 ± 21.97	36.0 – 121.1
V_{SL} (µm/s)	52.88 ± 22.10	24.1 – 108.5
V_{CL} (µm/s)	102.39 ± 27.23	44.8 – 153.5
Motility (%)	85.19 ± 15.54	31.5 – 97.9
10 seconds		
V_{AP} (µm/s)	53.48 ± 13.16	33.1 – 83.2
V_{SL} (µm/s)	32.85 ± 11.46	16.3 – 66.8
V_{CL} (µm/s)	67.71 ± 17.17	39.4 – 97.5
Motility (%)	80.57 ± 15.44	33.9 – 98.6
15 seconds		
V_{AP} (µm/s)	41.43 ± 9.74	22.9 – 58.6
V_{SL} (µm/s)	28.02 ± 7.62	14.6 – 45.4
V_{CL} (µm/s)	55.41 ± 15.45	28.7 – 88.9
Motility (%)	77.19 ± 14.85	36.5 – 99.7

Table 4.3: Regression tree analysis output for models describing variation in principal component scores (PC1 and PC2) for sexual traits (response variable) using sperm velocity and percent motility measures at 5, 10, or 15 s post-activation. Gonad (G) or muscle (M) sum PCB concentrations as well as plasma hormone concentrations of 11-ketotestosterone (11-KT) and 17 alpha,20 beta-dihydroxy-4-pregnen-3-one (MIS). The lowest AIC value is from the model that best describes the data and is in bold. Models with a Δ AIC_c value between 2 and 4 represent models that have less support and are marked with an asterisk. Root mean square error (RMSE) values are an indication of model accuracy, with smaller values indicating less variation between the model and the data set. Overall r^2 values describe the amount of variation in the data that is explained by the model and k-fold r^2 values describe that based on k-fold validation techniques.

Model	AIC	RMSE	r^2 Overall	r^2 k-fold
5 s				
PC1 G	63.40*	0.70	0.53	0.28
PC2 G	67.66	0.75	0.41	0.16
PC1 M	63.50*	0.68	0.52	0.29
PC2 M	70.48	0.85	0.24	0.03
10 s				
PC1 G	63.6*	0.69	0.51	0.27
PC2 G	63.31*	0.71	0.53	0.27
PC1 M	65.52	0.75	0.46	0.11
PC2 M	63.89*	0.66	0.54	0.31
15 s				
PC1 G	**61.29**	0.65	0.55	0.35
PC2 G	75.71	0.89	0.18	-0.32
PC1 M	63.9*	0.70	0.49	0.21
PC2 M	71.42	0.81	0.31	-0.01

Figure 4.1: A simplified diagram of the roles of 11-ketotestosterone and 17 alpha,20 beta-dihydroxy-4-pregnen-3-one (MIS), as well as gonadotropins (GtH I, GtH II) in the production and maturation of sperm in salmonids; adapted from Yaron and Sivan (2006).

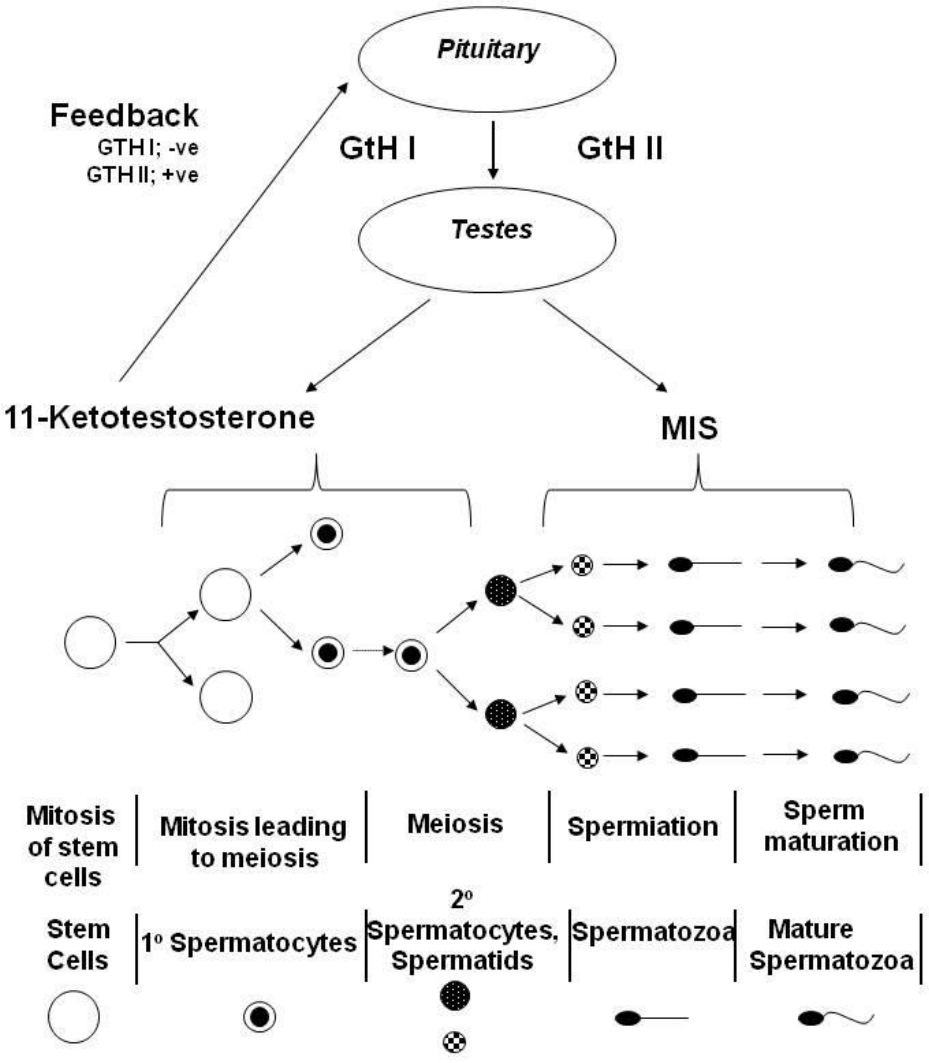


Figure 4.2: Measurements of secondary sexual traits recorded on Chinook salmon. *a*, kype length; *b*, hump depth; *c*, body depth; *d*, caudal peduncle depth; *e*, mid-eye to hypural flexure length (L_{MEH}).

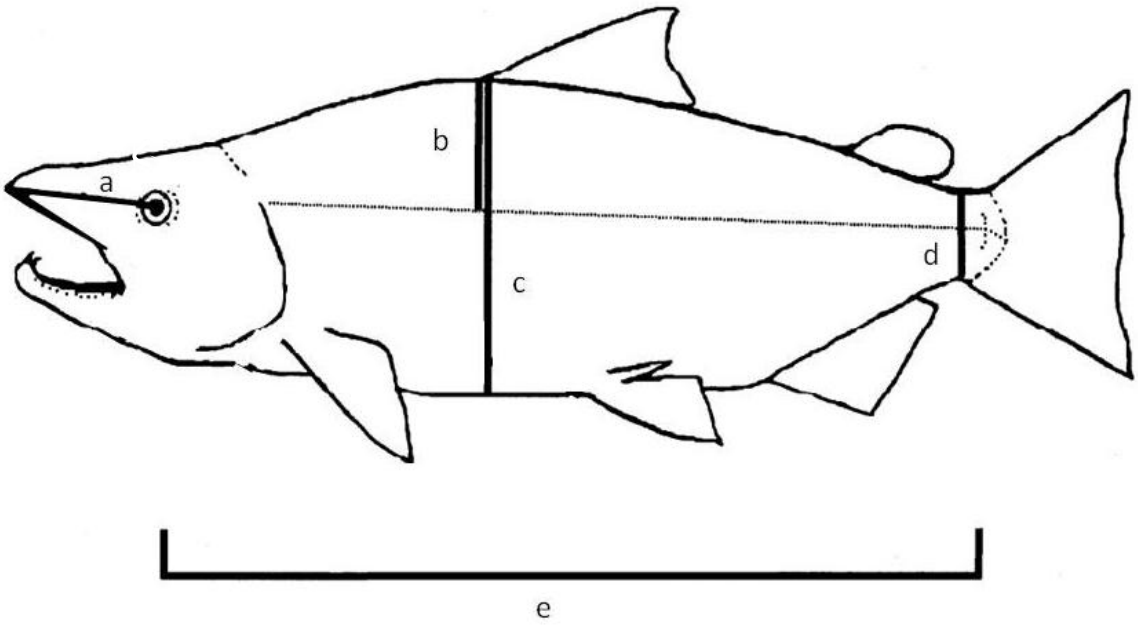
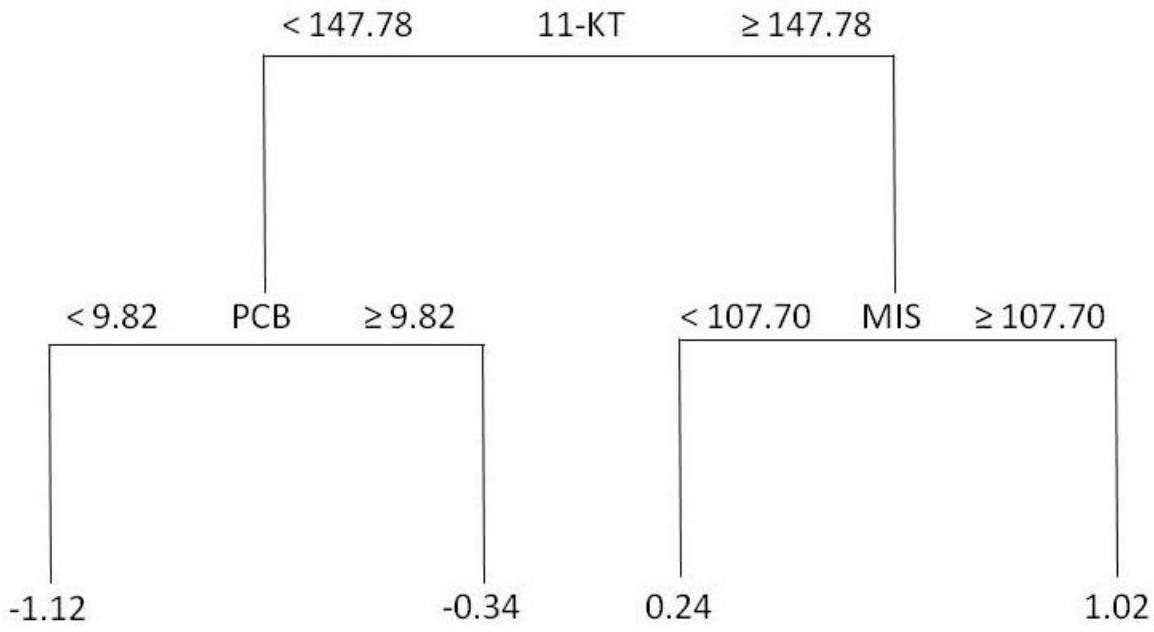


Figure 4.3: Regression tree analysis applied to the PC1 sexual trait principal component at 15 seconds post-activation as the response variable (n=24), where male Chinook salmon with greater sexual trait scores had larger body sizes, slower sperm velocities, lower percent motilities and lower sperm densities than those with lower sexual trait scores and vice versa. 11-ketotestosterone (11-KT), maturation inducing steroid (MIS), and gonad sum PCB concentration (PCB) were used as predictor variables. The values at the bottom of the tree represent the mean sexual trait PC scores for individuals meeting the splitting criteria for each predictor variable, high' and 'low' values of which are denoted at each node.



CHAPTER 5: PRIMARY AND SECONDARY SEXUAL CHARACTERS IN RELATION TO SOMATIC POLYCHLORINATED BIPHENYL CONCENTRATION IN SPAWNING MALE COHO SALMON (*ONCORHYNCHUS KISUTCH*) [4]

SYNOPSIS

Individual variation in primary and secondary sexual traits can arise from either evolutionary processes or physiological and genetic mechanisms. While numerous studies have identified sexual selection to be a driving force of variation in sexual traits, relatively few studies have focused on identifying environmental factors, such as contaminants, that could also be driving this variation. In Chapter Five, I used a wild population of Coho salmon (*Oncorhynchus kisutch*) from the Credit River, Ontario, to examine associations between primary and secondary sexual traits and individual measures of PCB concentrations. Multiple correlation analyses found no significant associations between primary and secondary sexual traits themselves, or between these traits and PCB concentrations. The lack of significance of these tests can be explained through a number of hypotheses. The range of PCB concentrations I quantified was relatively small and therefore even if these values were great enough to elicit a response, variation in these responses across the PCB gradient may have also been small and therefore not detectable. It is also possible that other contaminants, or suites of contaminants could better explain variation in sexual traits. Chapter Two demonstrates that inorganic contaminants correlated with PCB concentrations may better describe variation in female reproductive life-history traits. My sampling may have also not took place during peak spawning season or this season may have not been optimal for the fish compared to previous runs as males from this year had significantly lower values for all primary and secondary sexual traits. This chapter, due to the small sample size, lacked

statistical power (range: 0.05 – 0.84) which would explain the lack of any significant correlations. Finally, it is possibly that PCBs simply are not associated with variation in male primary and secondary sexual traits in this system. While I do not show associations between sexual traits and PCBs, it does not mean that they do not exist and future investigations with larger sample sizes and PCB ranges are suggested.

[4] This chapter is the product of joint research with Dr. K.G. Drouillard, Dr. S.M. Doucet, Dr. J.W. Hudson and Dr. T.E. Pitcher.

Introduction

Males across several taxa possess elaborate traits which exhibit variation among individuals as a consequence of sexual selection These are collectively known as secondary sexual traits (reviewed in Andersson 1994). Females use variation in secondary sexual traits to assess potential mates with the goal of obtaining a high quality male to ultimately maximize her own reproductive fitness (Andersson and Simmons 2006). Males can also use secondary sexual traits as armaments during male-male competition for access to females (Berglund et al. 1996). Individual variation can also be observed in male primary sexual traits, those that are required for reproduction to occur, such as sperm (Morrow and Gage 2001). Primary sexual traits also have the potential to be under sexual selection as differences in particular measures of these traits (e.g. pollen size, genitalia shape) can result in differences in successful mating attempts (Eberhard 1985, Arnold 1994). Variation among males in both primary and secondary sexual trait expression is not only dependent on sexual selection due to female mate choice and male-male competition (Sheldon 1994) but also on the individual's physiological capability to develop these traits (Emerson 2000). This may be especially true when individuals are under conditions where cellular processes may be compromised (Hill 2011), such as extreme temperature shifts (Shine 1999, Møller et al 2010), food availability and resource competition (Linville and Breitwisch 1997, Grether et al. 1999, Møller et al. 2010, Bobe and Labbé 2010, Vladić and Järvi 2001), disease (Folstad and Karter 1992, Zuk 1996, Bobe and Labbé 2010) and environmental contaminant exposure (Kime 1995, Reeder et al. 1998, Hewitt et al. 2008, Møller et al. 2010).

While numerous studies have demonstrated that primary and secondary traits under sexual selection are linked to measures of male quality as the mechanism for mate choice by females, such as immune function (Blount et al. 2003), reproductive success (Andersson 1982), offspring quality (Kempenaers et al. 1997) and genetic compatibility (Neff and Pitcher 2005), relatively few have focussed on identifying environmental factors that could also be driving changes in the expression of these traits through physiological mechanisms (see Pizzari et al. 2004). One such factor that has surprisingly received little attention from an evolutionary perspective is environmental contaminants (Bickham 2011). This is surprising as the impacts of environmental contaminants, such as sewage effluents (Jobling et al. 2002), radiation (Møller et al. 2005), pulp and paper mill effluents (Hewitt et al. 2008), synthetic estrogens (Kidd et al. 2007), and PCBs (Farwell et al. 2012) on wildlife have been a concern for decades (Truhaut 1977) and they have been associated with declines in measures of primary and secondary sexual traits. Studies of wild populations, with respect to contaminants, often compare average measures of sexual traits between populations from contaminated and reference locations, rarely with any individual-level measure of contaminant concentration. The lack of evolutionary context in toxicological research highlights the need to quantify individual level measures of both contaminants and sexual traits, along with specific and biologically relevant contaminant classes, within a single population, as evolutionary mechanisms occur at the individual level.

Polychlorinated biphenyls (PCBs) are a class of persistent organic contaminants that have been associated with variation in male expression of primary and secondary sexual traits. In these cases, generally, males exposed to PCBs, or greater concentrations

of PCBs, have lower sperm quality metrics, such as velocity, density and viability (e.g. Qin et al. 2003, Socha et al. 2008, Campagna et al. 2009, Pocar et al. 2012; but see Gray et al. 1993, Fielden et al. 2001) and duller coloured and less developed secondary sexual traits (Fisher et al. 2001, Quinn et al. 2002, Bortolotti et al. 2003; but see Bustnes et al. 2007), than males not exposed to PCBs, or to lower concentrations of PCBs. Mechanisms underlying these broad relationships are not well understood, although they have been associated with physiological processes such endocrine function (Chapter 4, McMaster et al. 1992, Saraiva 2010), oxidative stress (Møller et al. 2005) and have the potential to differ among sexual traits.

Coho salmon are a well-suited study system to investigate individual variation in the expression of traits under sexual selection from both an evolutionary and toxicological perspective. Coho salmon are external fertilizers and typically spawn in freshwater streams in North America stemming from either large freshwater lake or ocean systems (Crawford 2001). They also exhibit a semelparous mating system (Hendry et al. 1999) where females migrate up natal streams and compete for nesting sites, while males form dominance hierarchies and compete with each other for access to females (Fleming and Gross 1994, Quinn 2005). Males with larger body sizes, more developed kypes and humps, and redder colouration tend to be those positioned most favourably in these hierarchies (Fleming and Gross 1994) and therefore have greater access to females (Gross 1985) and increased fertilization success, as observed in other salmonids (e.g. Jones and Hutchings 2002, Blanchfield et al. 2003). There has also been evidence in Coho and other salmonids that suggest variation observed in these adaptive traits are heritable (Beacham and Murray 1987, Fleming and Gross 1994, Hendry et al. 2000, Hendry 2001, Kinnison

et al. 2003). Although primary access to females may be granted to those with greater measures of these secondary sexual traits, sperm competition, where ejaculates compete post-copulation for fertilization (Birkhead and Møller 1998), is still great within the hierarchies as several males may be attempting fertilization simultaneously (Gross 1985). Sperm metrics such as velocity, motility and density have been identified in other salmonid species as key determinants of fertilization success under these conditions (Hoysak and Liley 2001, Gage et al. 2004, Yeates et al. 2007), although they have yet to be investigated in Coho salmon. However, Pitcher et al. (2009) demonstrated a negative relationship in spawning male Coho salmon between the intensity of red colouration and sperm velocity, suggesting a trade-off between investment in primary and secondary sexual trait development to potentially increase reproductive success. Variation in these adaptive sperm metrics have also been demonstrated to be heritable, although relatively little work has been conducted in salmonids (see review and references therein; Evans and Simmons 2008). During migration, lipid stores are used as energy for the costly development of primary and secondary sexual traits, as well as for swimming upstream. The depletion of lipid stores allows for the mobilization of previously accumulated lipophillic compounds, such as PCBs, in response to fugacity gradients (Findlay and DeFreitas 1971, Mackay and Patterson 1982). Polychlorinated biphenyl concentrations and toxicities have also been found to magnify in muscle tissue of migrating Sockeye salmon (*Oncorhynchus nerka*) (Debruyn et al. 2004, Kelly et al. 2007) and thus have a greater potential to impact the development of primary and secondary sexual traits during this time. Recent studies have identified PCB concentrations in the muscle tissue of Coho salmon from the Great Lakes that were well above those found in reference pristine

habitats (Jackson et al. 2001, Madenjian et al. 2010), although those concentrations have been in decline since the 1970s (Stow et al. 1994).

I used a wild population of spawning Coho salmon (*Oncorhynchus kisutch*) from Lake Ontario to test the hypothesis that variation in individual-level PCB concentrations can indicate variation in the expression of male primary and secondary traits under sexual selection. I predicted that males with greater sum PCB concentrations would have lower measures of primary (sperm velocity, motility, longevity, density, viability) and secondary (red colouration, kype length and hump depth) sexual traits than those with lower measures of sum PCB concentrations. This is assuming that males with greater muscle sum PCB concentrations will have a decreased physiological capability to develop and maintain these traits than those with lower muscle sum PCB concentrations (Hill 2011). Identifying whether variation PCB concentrastions can explain variation in traits under sexual selection will provide evolutionary context to toxicological questions (Bickham 2011, Chapter 1), creating a novel combination of research fields that has been lacking in the literature.

Methods

Sample collection

Adult male Coho salmon (n = 32) were collected during the spawning season using backpack electrofishing on 30 October 2010 in the Credit River (43^{o}35’ N, 79^{o}42’ W), flowing into Lake Ontario, Canada. Coho salmon have been stocked in Lake Ontario for 45 years (Crawford 2001). Only males that produced viable sperm and had red colouration detected by reflectance spectrometry were used in this study to ensure that

they were reproductive (n=18). Non-red males were not used because they were not likely to be in peak spawning condition, even if they produced sperm. Any quantification of sperm metrics would therefore likely not be of ecological or evolutionary relevance. Within 2 min after capture, milt samples were collected and stored in Ziploc bags over ice (not in direct contact) in a cooler for no longer than 4 hours until sperm analysis was conducted (see below). Milt was transported on ice to the lab for viability analysis within 8 hours (see below). Individuals were then sacrificed and reflectance spectrometry was used according to Pitcher et al. (2009) to quantify average spawning coloration of three landmarked locations across the lateral line integument (see below). Total mass and length was then recorded for each individual, as well as hump depth (distance from the lateral line to the highest point on the dorsal surface) and kype length (distance from middle of eye to the tip of the snout) (Pitcher et al. 2009). Dorsal muscle tissue samples of 2 to 5 g were excised, wrapped in hexane-rinsed tin foil and stored at - 20 ºC until PCB extraction and analysis were conducted (see below). Muscle tissue was collected, rather than gonad tissue which has been demonstrated to better explain variation in primary and secondary sexual traits and reproductive hormones (Chapter 4), as it still can explain a significant amount of variation in sexual traits (Chapter 4) and it may be more greatly associated with colour.

Spawning colour assessment

Reflectance spectrometry was used to measure the intensity of male abdominal spawning red colouration as it applied to integument pigmentation (Pitcher et al. 2009). I used an Ocean Optics reflectance spectrometer (USB 4000; detector range: 200–1100 nm; www.oceanoptics.com) and a xenon pulse lamp (PX-2; illumination range: 220–750 nm;

Ocean Optics) where a bifurcated fibre-optic probe delivered light from the light source to the measurement area and delivered reflected light from the measurement area to the spectrometer (R-400-7-UV-VIS; Ocean Optics). The probe was encased in a matt-black holder, which maintained the probe at a fixed distance to the measurement area and excluded external light. All spectrometry readings were made relative to a white standard, which reflected >97% of light at the wavelengths used in the analyses (Labsphere WS-1; Ocean Optics). Before collecting reflectance readings, the surface of the scales was wiped to minimize specular glare that might result from the reflection of water. Reflectance was measured at three consistent land-marked locations across the span of the lateral line of each male, all containing red colouration. For each of the three landmarks, two readings were collected, each of which comprised 20 consecutive measurements averaged by the spectrometer operating software (OOIBase 32; Ocean Optics). These two readings were averaged for each landmark and were subsequently averaged to obtain a single mean reflectance spectrum at 1 nm bins for each individual as the three landmarks did not differ substantially in reflectance.

Sperm activity

To assess sperm velocity, motility and longevity (Pitcher et al. 2009), sperm was recorded using a CCD black and white video camera (XC-ST50, Sony, Japan) through a negative phase-contrast microscope (CX41 Olympus, Melville, NY, USA) with a 10x magnification objective. First, an aliquot of milt (< 0.2 μL) was pipetted into a chamber of a 2X-CEL glass slide (Hamilton Thorne, MA, USA) and covered with a coverslip. Sperm cells were then activated with 15 μL of river water collected from the Credit River. Sperm velocity, motility and longevity were analyzed using the HTM-CEROS sperm

analysis system (version 12, CEROS, Hamilton Thorne Biosciences, Beverly MA, USA) set at the following parameters: number of frames = 60, minimum contrast = 11, photometer = 55 to 65, minimum cell size = 3 pixels. Approximately 80 % of fertilization events occur within 15 s post-activation of sperm (Hoysak and Liley 2001, Yeates et al. 2007) and therefore I analzed sperm velocity, motility and longevity at 5, 10 and 15 s post-activation (PA). . For each individual, velocity was analyzed using three metrics ; average path velocity (V_{AP}), straight-line velocity (V_{SL}), and curvilinear velocity (V_{CL}). Motility was defined as the percent of sperm motile at 5, 10 or 15 s PA. Longevity was defined as the time PA that 95% of sperm were immotile. Values for these metrics are reported by the computer-assisted sperm analysis software as an average for each individual. Each video recording was manually checked for quality control.

Sperm Density

A Neubauer haemocytometer under 400x magnification was used to assess sperm density according to Pitcher et al. (2009) using 1.5 μL of milt diluted in 500 μL of Courtland's saline solution (7.25 g/L NaCl; 0.38 g/L KCl; 0.47 MgSO4 × 7H20; 0.4 g/L Na2HPO4×H20; 1.0 g/L NaHCO3; 0.22 g/L MgCl2; 1.0 g/L C6H12O6; adjusted to pH 7.8). A 10 μL volume of the suspension was placed onto thehaemocytometer And sperm were counted in 5 of the 25 squares under a Zeiss Axiostar compound microscope. To determine sperm density as number of sperm per mL of milt, the average number of sperm in the 5 squares was multiplied by 25 (number of squares), then by 10 (chamber depth, μm), then by 500 (sample volume, μl).

Sperm viability

I assessed percent of viable sperm (i.e. live cells with intact membranes) for each individual using a LIVE/DEAD® sperm viability kit (Molecular Probes, Eugene, OR). This sperm viability assay uses two stains to visibly identity viable and non-viable sperm; SYBR-14 penetrates the outer sperm head membrane of live cells and stains nucleic acids green while propidium iodide is unable to penetrate living cells and instead stains nucleic acids of degrading or dead cells red (Rurangwa et al. 2004). Following Niu et al. (2010), I created a 50x dilution of SYBR-14 in buffer (10 mmol/L HEPES, 150 mmol/l NaCl, 10% BSA). I then combined 5 µl of the diluted SYBR-14 (100 nmol/L) with 5 µl of propidium iodide (12µmol/L) and 1 mL of semen and incubated for 10 min. Flow cytometry was used to assess viability of 100 000 sperm cells per individual based on their stains using a Backman Coulter Cytomics FC 500 flow cytometer and Cytomics RXP Analysis software (Beckman Coulter). Standards using only SYBR-14 and only propidium iodide were also conducted to act as a live or dead control, respectively.

Polychlorinated biphenyl and lipid extraction and analyses

Thawed muscle samples, 0.2 to 0.5 g from each individual, were homogenized and PCB congeners and neutral lipids extracted using a micro-extraction technique following the protocol outlined by Daley et al. (2009). The extraction equipment consisted of 20 mL glass syringes fitted to a 1 µm glass fibre syringe filterand then to a 12 port solid phase extraction manifold (Phenomenex, Torrance, CA, USA). Using a mortar and pestel, tissue samples were ground with 15 g of activated sodium sulphate and wet packed into the syringes containing 15 mL dichloromethane (DCM):hexane (1:1). An additional 15 mL of DCM:hexane was added to the syringes after the mortar and pestel were rinsed. Each syringe was spiked with an internal recovery standard of 200 ng

PCB34/mL after one hour had elapsed to allow for the sample to extract in the solvent. Six samples were run concurrently with each set, along with a method blank and an in-house homogenate fish sample (Detroit River common carp, *Cyprinus carpio*) as an inter-assay control. Extracts were then concentrated by rotary-evaporator to approximately 2 mL and reconstituted to 10 mL in hexane in a 10 mL volumetric flask for gravimetric lipid content assessment (Drouillard et al. 2004). Extracts were again concentrated by rotary-evaporator to approximately 2 mL and florisil chromatography was used for sample clean up (Lazar et al. 1992). Fractions 1 (50 mL hexane) and 2 (50 mL DCM:hexane; 15:85) were collected. Extracts were then concentrated to 1 mL and placed in glass vials for analysis via gas chromatography electron capture detection (GC-ECD). The PCB congeners in each sample were identified by retention time and referenced against an external PCB standard (Quebec Ministry of Environment Congener Mix; AccuStandard). The following congeners were present in the external standard and analyzed for detection in muscle samples (IUPAC numbers, coeluting congeners separated by slash): 18/17, 31/28, 33, 52, 49, 44, 74, 70, 95, 101, 99, 87, 110, 151/82, 149, 118, 153, 105/132, 138, 158, 183, 128, 177, 156/171, 180, 191, 170, 201, 195/208, 194, 205, 206, 209. Sum PCB concentrations were calculated for each individual as the sum of each congener in µg/kg wet weight. To account for differences in spawning condition between males (4.63 ± 1.739 %; mean percent lipid content ± S.D) all samples were lipid corrected as lipid content is a key factor in determining PCB accumulation (Persson et al. 2007). Mean percent recovery of the PCB34 spike was 81.63 ± 1.4% (mean ± S.E.). All samples had recoveries greater than the 70% threshold generally used for QA/QC and all sum PCB in-house homogenate samples were within two standard

deviations of the mean laboratory database value derived from laboratory control charts from the Great Lakes Institute for Environmental Research analytical laboratory quality assurance guidelines (Canadian Association for Environmental Analytical Laboratories Accreditation and ISO17025 certified)..

Statistical analyses

Principal components analysis (PCA) was used to summarize variation in sperm velocity metrics at 5, 10 and 15 s PA (V_{AP}, V_{SL}, V_{CL}; Pitcher et al. 2009). One informative PC axis for each 5, 10 and 15 s PA was extracted that explained 76.8 %, 72.4 % and 95.1 %, respectively, of this variation and is referred to hereafter in as sperm velocity. At 5, 10 and 15 s PA, males with larger PC scores had greater sperm velocities than those with smaller PC scores. No single PC was able to explain the variation in reflectance spectra as commonly used in reflectance spectrometry analysis (Endler 1990). Instead, I used the lowest reflectance value between 475 – 500 nm, which represents the peak absorbance range of the dominant carotenoid involved in salmonid, including Coho salmon, red colouration, astaxanthin (Smith et al. 1992, Bjerkeng and Berge 2000). This lowest value was observed at 483 nm for 14 of the 18 individuals. The remaining 4 individuals also had their second lowest reflectance values at 483 nm with their overall lowest valleys at 380 nm. The differences in reflectance between the first and second lowest valleys ranged from 0.6 % - 2.3 %. I therefore used reflectance values at 483 nm for all males as a measure of red colouration, where males with lower reflectance values having more intense red colouration than males with higher reflectance values. Unlike Pitcher et al. (2009) and Chapter 4, no single PC axis explained a significant amount of the variation in body size and morphology metrics used to gain access to females and

therefore kype length and hump depth were analysed as independent secondary sexual traits.

Normality was tested for each variable using Shapiro-Wilk goodness-of-fit test and homogeneity of variance was tested for each variable using Levene's test. Pearson correlations were used to test the hypothesis that variation in expression of male primary and secondary traits under sexual selection can act as an indicator of individual-level PCB concentrations. Pearson correlations were also used to examine whether negative relationships between primary and secondary sexual traits exists as would be expected in reproductively mature male Coho salmon (e.g. Pitcher et al. 2009).

Results

Muscle sum PCB concentrations across the 18 males averaged 245.4 ± 54.7 µg/kg wet weight (S.D.) and ranged from 144.3 – 336.9 µg/kg wet weight. Lipid content of muscle tissue averaged 4.63 ± 54.7 % and ranged from 1.47 – 8.15 %. Means ± S.D. and ranges for all primary and secondary sexual traits are also listed in Table 5.1.

Total mass and length were significantly correlated ($r = 0.779$, $p = 0.0001$) and also correlated with kype length (total mass: $r = 0.534$, $p = 0.022$; total length: $r = 0.555$, $p = 0.017$) however, kype length, hump depth and percent reflectance were not correlated with each other: kype length and hump depth ($r = 0.358$, $p = 0.14$), kype length and percent reflectance ($r = -0.282$, $p = 0.26$), hump depth and percent reflectance ($r = -0.011$, $p = 0.96$). Of the sperm metrics, only motility at 10 s and 15 s ($r = 0.749$, $p = 0.0003$), velocity at 10 s and motility at 15 s (0.505, $p = 0.033$), and viability and motility at 5 s (r

= 0.644, p = 0.0039) were significantly correlated. Finally, there were no significant correlations between any primary or secondary sexual traits (Table 5.2).

There were no significant correlations between muscle sum PCB concentrations and male primary sexual traits: velocity at 5 (r = -0.175, p = 0.49, Figure 5.1a), 10 (r = 0.062, p = 0.81, Figure 5.1b) and 15 (r = -0.374, p = 0.13 Figure 5.1c) s PA, motility at 5 (r = 0.170, p = 0.50, Figure 5.2a), 10 (r = 0.198, p = 0.43, Figure 5.2b) or 15 (r = 0.219, p = 0.38, Figure 5.2c) s PA, longevity (r = -0.294, p = 0.24, Figure 5.3a), density (r = -0.036, p = 0.89, Figure 5.3b), viability (r = 0.357, p = 0.14, Figure 5.3c).

There were also no significant correlations between muscle sum PCB concentrations and male secondary sexual traits: percent reflectance (r = -0.002, p = 0.99, Figure 5.4a), kype length (r = -0.056, p = 0.83, Figure 5.4b), or hump depth (r = 0.186, p = 0.46, Figure 5.4c).

A post-hoc power analysis to examine the statistical power of correlations between muscle sum PCB concentration and primary and secondary sexual traits was conducted (GPower, http://www.psycho.uni-duesseldorf.de/aap/projects/gpower). The statistical power for each primary sexual trait was as follows: velocity at 5 (0.49), 10 (0.18) and 15 (0.84) s PA, motility at 5 (0.50), 10 (0.51) and 15 (0.68) s PA, longevity (0.73), density (0.12) and viability (0.84). The statistical power for each secondary sexual trait was as follows: reflectance (0.05), kype length (0.16) and hump depth (0.48). As the power for the majority of primary and secondary sexual traits would generally be considered to be low (Thomas and Juanes 1996), I determined the sample size required to obtain a power of 0.80. There are few studies which examine correlations between sum PCB concentrations and primary and secondary sexual traits, especially in fishes. I

therefore used an estimated effect size of 0.44 to determine samples sizes for all metrics, which was estimated from two studies examining correlations between sperm density and motility in human male sperm in relation to concentrations sum PCB metabolites in blood (Dallinga et al. 2002) and HCH and DDT in semen (Pant et al. 2007). This effect size is similar to the effect sizes found in my study (0.34 ± 1.9; mean ± S.D.). I found that the required sample size to obtain a power of 0.8 using an effect size of 0.44 was 34, approximately double my current sample size.

Discussion

I did not support the hypothesis that variation in individual-level PCB concentrations indicates variation in the expression of male primary and secondary traits under sexual selection. I found no significant correlations between primary (sperm velocity, motility, longevity, density, viability) and secondary (red colouration, kype length and hump depth) sexual traits with muscle sum PCB concentration. The lack of associations I found between sexual traits and PCBs can be explained a number of limiting factors that warrant future investigation

My muscle sum PCB concentrations are comparable to those found by O'Toole et al. (2006) in Chinook salmon (*Onchorhynchus tshawytscha*) from the Credit River in 2003 with a mean muscle sum PCB concentration of 370 ± 111.92 µg/kg wet weight (S.D.). Compared to other Coho salmon in the Great Lakes, my values are lower than those found by Jackson et al. (2001) from Lake Michigan in 1996 with a mean muscle sum PCB concentration of 1268 ± 77 µg/kg wet weight (S.D.) and than those found by Madenjian et al. (2010) also from Lake Michigan in 2004 and 2006 with a combined

mean muscle sum PCB concentration of 710 ± 110 µg/kg wet weight (S.D.). My muscle sum PCB concentrations were also up to 10 times greater than those from Sockeye salmon from the Pacific coast in both 1995 (Debruyn et al. 2004) and 2001 (Kelly et al. 2007) and where toxicity at these concentrations had the potential to increase egg mortality (Debruyn et al. 2004). While the absolute values of my PCB concentrations are relatively high, the range of these values is relatively small compared to the above studies. Having all high values of PCB concentrations could limit the ability to detect associations with individual variation in sexual trait expression. This is especially true as threshold responses of sexual traits and mortality to contaminants have been demonstrated (e.g. Kim 2001, Debruyn et al. 2004, Campagna et al. 2009)

I found no significant relationships between muscle sum PCB concentration and primary sexual traits. I compared my sperm metrics to those reported by Pitcher et al. (2009) who measured primary and secondary sexual traits in spawning Coho salmon in the Credit River 3 years previous using similar methodologies. I found that all of my sperm metrics were lower than previously reported. Our sperm density and velocity metrics were approximately an order of magnitude lower and our longevity was on average 5 s shorter. Pitcher et al. (2009) also report larger absolute ranges for these metrics. Percent motility was not measured by Pitcher et al. (2009), however, compared to Chinook salmon in this system, our values and range of these values were also significantly lower (Chapter 3, unpublished data).While there are no field experiments examining associations between PCBs and sperm metrics, associations between other environmentally relevant contaminants and sperm metrics have been observed in wild populations. For example, Jobling et al. (2002) found that wild populations of roach

(*Rutilus rutilus*) originating from habitats exposed to sewage effluents had reduced milt volumes and sperm densities than those from reference populations. Similarly, Franssen et al. (2007) found that sperm density of Atlantic mollies (*Poecilia mexicana*) was lower in populations living in more sulfidic environments. There has also been support from laboratory experiments of negative links between PCBs and sperm metrics. A vast amount of literature exists that describes declines in all sperm metrics used in this study correlated to PCB exposure in humans living in high risk areas (e.g. Longnecker et al. 1997, Ulbrich and Stahlmann 2004, Van Oostdam et al. 2005). Laboratory work has been conducted in mice and rats with the goal of applying these results to human populations (see Chapter 4: Table 4.1). For example, Pocar et al. (2012) exposed male mice to 1, 10, and 100 µg/kg of PCB 101 and 108 and demonstrated a 20-30% decrease in sperm viability for all treatments compared to control males as measured by a live:dead assay. One study to date has been conducted using fish. Socha et al. (2008) exposed milt collected from common carp (*Cyprinus carpio* L.) directly to Delor 103 and 106 at concentrations of 1, 5, 10, and 50 ng/mL and found that all concentrations but 1 ng/mL Delor 106 resulted in declines in sperm velocity. The difficulty with assessing biological relevance of PCB concentrations in wild populations of fishes arises not only from the lack of studies conducted in fishes, but also the drastically different methodologies used in mammalian studies, making comparisons and inferences from these studies difficult.

I also found no significant relationship between muscle sum PCB concentration and secondary sexual traits. My measures of body size, kype length and hump depth were within a similar range as those reported for Coho salmon by Pitcher et al. (2009). My measure of percent reflectance at 483 nm, however, is on average 15 times lower than

that reported by Pitcher et al. (2009), which suggests that overall our fish have much darker red colouration. Similar to our sperm metrics, we also have a smaller absolute range of values for percent reflectance compared to Pitcher et al. (2009).There are a limited number of studies, either field or laboratory, to date investigating associations between PCB concentration and secondary sexual traits, with none found using fish (see Chapter 4: Table 4.1). There have been, however, studies linking other environmentally relevant contaminants to secondary sexual traits in wild populations. For example, Møller and Mousseau (2001) found that male barn swallows (*Hirundo rustica*) living in radiation contaminated habitats in Chernobyl, had increased frequencies of albinism compared to reference populations. This is relevant as barn swallows use bright red and blue colouration to attract females for mating. Hewitt et al. (2008) describe lower frequencies and under developed tubricle formation in chub and sucker species living in habitats exposed to pulp and paper mill effluents compared to reference populations. The majority of research focusing on the effects of PCBs on secondary sexual traits has used laboratory-reared American Kestrels (*Falco sparvius*) as a study organism where female American Kestrels use the brightness of male colouration as a basis for mate choice (Negro et al. 1998). In several studies using American Kestrels, birds are exposed to Aroclors 1248:1254:1260, 1:1:1, and are fed 7 mg/kg body weight of this mixture once daily for 1 year, during which time various experiments were conducted. Males exposed to PCBs following the above methodology had duller and less optimal coloration for mate attraction and lower carotenoid concentrations when pairing occurs during the breeding seasons compared to control males (Bortolotti et al. 2003). Mating behaviour during courtship was also affected by PCBs, where males exposed to PCBs had more aggressive

displays and copulation behaviour and lower hatching success of their clutches compared to control males (Fisher et al. 2001). This decrease in hatching success was later thought to be due to lack of parenting behaviour and not physiological impact of PCB exposure (Fisher et al. 2006). When colouration of a different laboratory population of American Kestrels was examined, where males were exposed to 6 and 60 ppm Aroclor 1242 for 5 days, no difference in colour between experimental and control males (Quinn et al. 2002). This finding could suggest that long term exposure to relatively high concentrations of PCBs is more likely to elicit a response in colouration than lower and more environmentally relevant concentrations. I found only a single study that examined associations between PCB concentration and secondary sexual traits in a wild population. Bustnes et al. (2007) reported a range of PCB concentrations in Great Black-Backed Gulls (*Larus marinus*) between 110-178 µg/kg wet weight (the low end of my range for PCB concentrations) and found no significant relationship between plasma sum PCB concentration and plasma carotenoid concentrations. They did, however, report a significant positive relationship between PCB concentration and asymmetry in wing length, which they suggest could be an indicator of change in environmental conditions.

There are several reasons that might explain why I did not observe any significant correlations between primary and secondary sexual traits and muscle sum PCB concentration. First, it is possible that my range of muscle sum PCB concentrations was not great enough to solely elicit variation in primary and secondary sexual traits. However, the range of my PCB concentrations were almost identical to that found in Chinook salmon, also from the Credit River, where variation in primary and secondary sexual traits and hormone concentrations were explained by individual measures of gonad

sum PCB concentrations through a regression tree analysis and model comparison (Chapter 4). This issue could be addressed by collecting samples from nearby populations in the Great Lakes with relatively more and less PCB exposure, in order to create a larger range of PCBs to examine while keeping in mind issues surrounding local adaptation which could also impact sexual trait expression. If PCB concentration is not correlated with primary and secondary sexual traits across populations with a greater range of sum PCB concentrations, then it is less likely that individual differences in sum PCB concentrations within a smaller range would be correlated with primary and secondary sexual traits. Long term dose-response laboratory experiments would be able to identify threshold concentrations that elicit changes in primary and secondary sexual trait expression.Also, based on my results from Chapter 4, as lipid stores from muscle tissue are being directed to the gonads for development in order to reproduce, gonads, with their relatively high fat percentage, may contain greater concentrations of organic contaminants and may ultimately be more greatly associated with sexual trait expression than muscle tissue. The comparison between multiple tissue types would be able to identify which are most closely associated with sexual trait expression. These tests would be more practical for smaller and shorter-lived fish species, compared to salmonids. Second, it is possible that another environmental contaminant, or suite of contaminants, would be more likely to be associated with variation in primary and secondary sexual traits. Not only have PCBs been linked to declines in primary and secondary sexual traits, they are also positively correlated with several other organic contaminants in wild habitats (e.g. Szalinska et al. 2011). Further, mixtures of organic contaminants, such as pesticides and PCBs have also been found to magnify the detrimental effects on sperm

quality metrics (Njiwa et al. 2004). Alternatively, evidence supporting associations between inorganic contaminants and female life-history traits have also been found (Chapter 2). This issue could be addressed by quantifying additional contaminants that have a potential to elicit responses in primary and secondary sexual traits and correlating them to these traits. A more practical and informative approach would be to first identify contaminant(s) of interest in laboratory studies prior to examination in field organisms. Third, it is possible that my collections did not take place during peak spawning season. This is possible as the absolute values and ranges of both primary and secondary sexual traits were significantly lower than those reported for the same Coho run three years previous (Pitcher et al. 2009; Figure 5.5). These differences may also be seasonal or due to an unknown environmental factor. I also found no correlations between primary and secondary sexual traits, which were observed in Coho salmon by Picher et al. (2009), as well as in other studies of fishes, including salmonids, during spawning season (Stoltz and Neff 2006, Knapp and Neff 2008). If Coho salmon were sampled prior to optimum spawning time and development of primary and secondary sexual traits had not been completed, then my results would lose evolutionary and biological relevance. To address this issue, daily collections throughout the spawning season quantifying the development of primary and secondary could be conducted. Annual comparisons of primary and secondary sexual traits between Coho salmon runs, as well as knowledge of unique environmental events, would also be useful in quantifying long-term variation. It is noteworthy, however, that sexual trait measures in this study are comparable to those from Chinook salmon collected only weeks earlier from the same Credit River location where associations between sexual traits were found (Chapter 4). While they are different

species, these similarities suggest that sexual trait expression may vary annually and that potentially significant associations do exist, but were not detected. Fourth, it is possible that I did not find any significant correlations between muscle sum PCB concentrations and primary and secondary sexual traits, or just between primary and secondary sexual traits, due to a lack of statistical power for several of my metrics. My sample size is comparable or larger than those used in the few studies that investigate links between sexual traits and PCB concentrations and some of my metrics had a large enough statistical power to suggest that any relationship between muscle sum PCB concentrations and sexual traits would have been observed if they were present. To address this issue, future sample collections could be conducted to augment the data set in hopes of increasing my statistical power. Lastly, it is possible that PCBs are simply not associated with the expression of primary and secondary sexual traits. There have been a number of laboratory studies acutely injecting male rats with relatively high concentrations of PCBs having found no difference between experimental and control groups in various sperm metrics (e.g. Sager et al. 1991, Gray et al. 1993, Kim 2001). While experimental studies also exist that do demonstrate a link between sexual traits and PCBs in acute studies, it is possible that no response occurs at lower, environmentally relevant and more persistant exposures.

The aim of this study was to determine whether variation in individual-level PCB concentrations indicates variation in the expression of male primary and secondary traits, which was not supported by my data. This lack of support, however, could be explained by a number of limitations presented in this study that warrant further investigation. Identifying relationships, at the individual level, between primary and secondary sexual

traits and environmental change is important for understanding the potential for these changes to impact evolutionary processes such as sexual selection (Bickham 2011). While it is possible that environmental contaminants are not linked to the expression of sexual traits, and therefore have limited evolutionary relevance from a sexual selection stand point, further experimental research examining mechanistic links between contaminants and sexual traits is required. The use of wild organisms and the application of environmentally relevant exposure concentrations and durations to investigate these relationships will provide additional ecological and evolutionary relevance.

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Table 5.1: Means, standard deviations (S.D.) and ranges of primary (sperm metrics) and secondary (% reflectance, kype length, hump depth) sexual traits for Coho salmon (*Oncorhynchus kisutch*) from the Credit River, flowing into Lake Ontario (n=18). Sperm metrics were assessed at 5, 10, and 15 seconds post-activation.

Metric	**Mean ± S.D.**	**Range (min – max)**
Primary sexual traits		
Sperm density per mL ($x10^7$)	5.18 ± 1.44	1.90 – 8.47
Sperm longevity	23.55 ± 4.37	16 – 32
Sperm viability (%)	90.42 ± 3.27	84.81 – 94.47
5 seconds		
V_{AP} (µm/s)	86.45 ± 9.86	70.5 – 105.9
V_{SL} (µm/s)	52.47 ± 14.72	32.1 – 73.7
V_{CL} (µm/s)	97.24 ± 11.20	78.4 – 117.9
Motility (%)	81.97 ± 11.75	60.7 –100.0
10 seconds		
V_{AP} (µm/s)	43.9 ± 8.3	31.0 – 60.2
V_{SL} (µm/s)	24.4 ± 8.5	2.6 – 39.5
V_{CL} (µm/s)	49.3 ± 11.0	33.4 – 75.0
Motility (%)	75.6 ± 16.43	31.6 - 100
15 seconds		
V_{AP} (µm/s)	37.10 ± 20.00	0 – 81.6
V_{SL} (µm/s)	28.44 ± 20.32	0 – 81.3
V_{CL} (µm/s)	44.36 ± 23.85	0 – 96.8
Motility (%)	67.85 ± 19.53	29.3 – 94.4
Secondary sexual traits		
% Reflectance @ 483 nm	2.50 ± 0.91	0.95 – 4.48
Kype length (mm)	157.6 ± 10.97	139.7 – 177.8
Hump depth (mm)	99.8 ± 8.96	88.9 – 114.3
Total length (mm)	792.2 ± 35.67	736.6 – 868.7
Total mass (kg)	5.61 ± 0.93	3.67 – 7.08

Table 5.2: Correlation matrix of primary and secondary sexual traits in Coho salmon collected from the Credit River (n=18). Values not in brackets indicate r-values and those in brackets indicate p-values.

Primary sexual traits	**Reflectance (%)**	**Kype length (mm)**	**Hump depth (mm)**
Sperm density (#/mL)	0.067 (0.79)	0.057 (0.82)	-0.262 (0.29)
Sperm longevity (s)	0.180 (0.47)	0.301 (0.22)	0.150 (0.55)
Sperm viability (%)	-0.055 (0.83)	-0.368 (0.13)	0.185 (0.46)
Sperm velocity PC 5 s	-0.149 (0.55)	-0.008 (0.98)	-0.053 (0.83)
Sperm velocity PC 10 s	0.302 (0.22)	-0.270 (0.28)	0.264 (0.29)
Sperm velocity PC 15 s	0.312 (0.21)	0.114 (0.65)	-0.059 (0.82)
Sperm motility (%) 5 s	0.187 (0.46)	-0.324 (0.19)	0.308 (0.21)
Sperm motility (%) 10 s	-0.181 (0.47)	-0.100 (0.69)	0.304 (0.22)
Sperm motility (%) 15 s	-0.018 (0.94)	-0.250 (0.32)	0.081 (0.75)

Figure 5.1: Pearson correlations between log transformed lipid corrected muscle sum PCB concentrations and sperm velocity at 5 (a), 10 (b) and 15 (c) seconds post-activation from Chinook salmon collected from the Credit River (n=18).

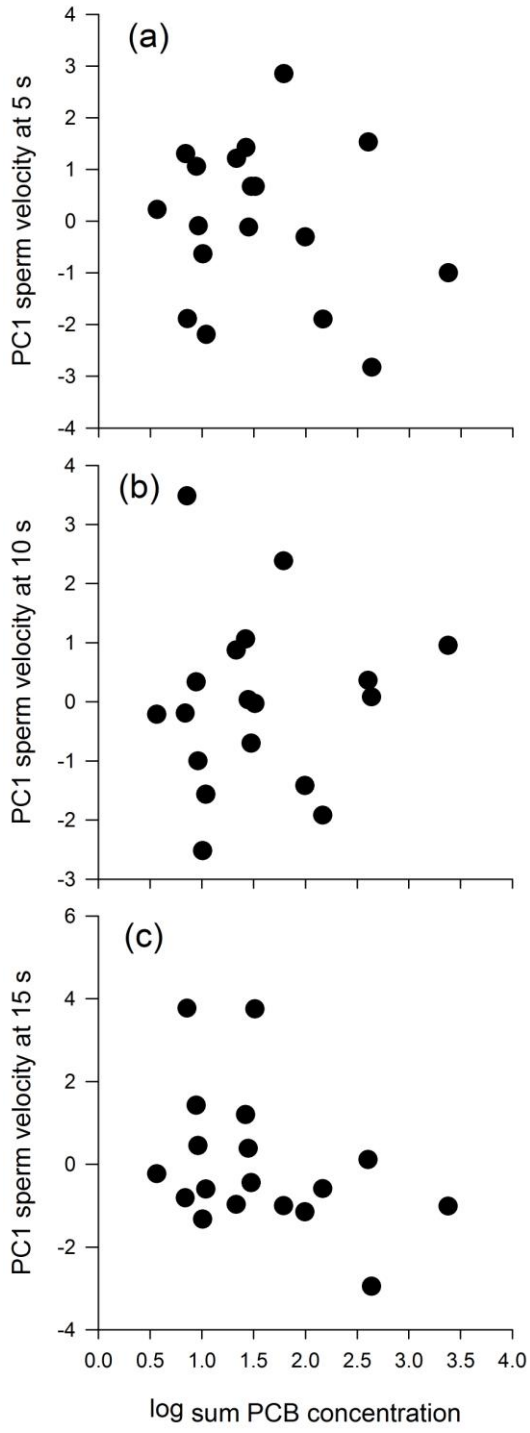

Figure 5.2: Pearson correlations between log transformed lipid corrected muscle sum PCB concentrations and sperm motility at 5 (a), 10 (b) and 15 (c) seconds post-activation from Chinook salmon collected from the Credit River (n=18).

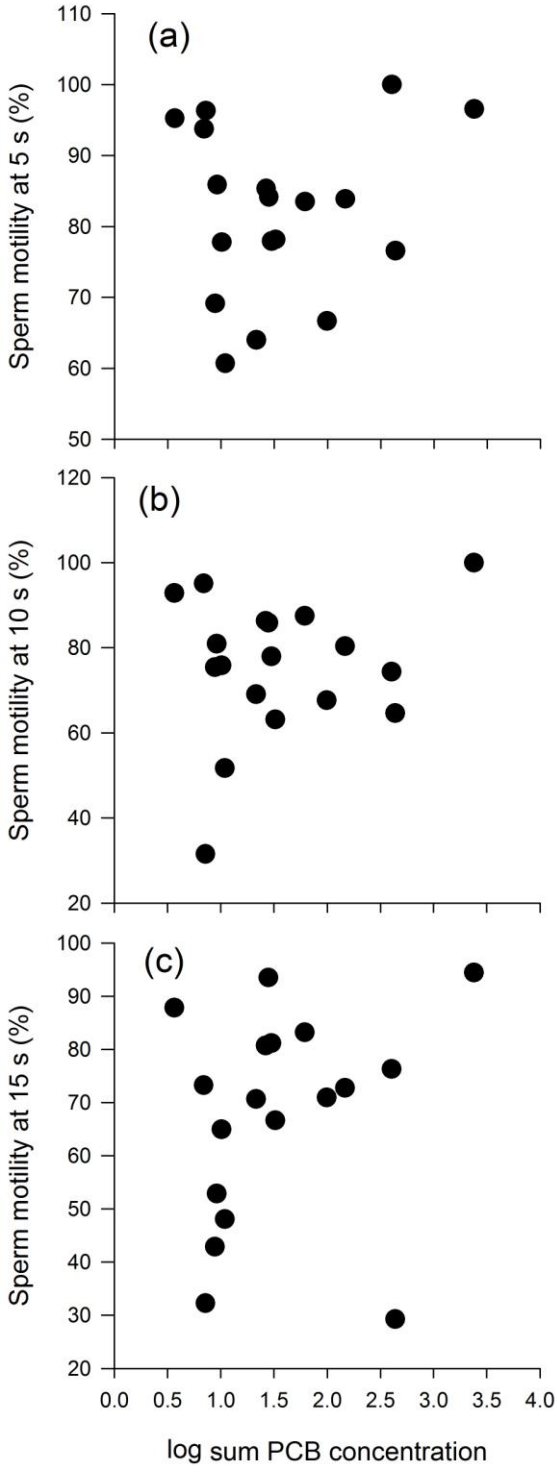

Figure 5.3: Pearson correlations between log transformed lipid corrected muscle sum PCB concentrations and sperm longevity (a), sperm density (b) and sperm viability (c) from Chinook salmon collected from the Credit River (n=18).

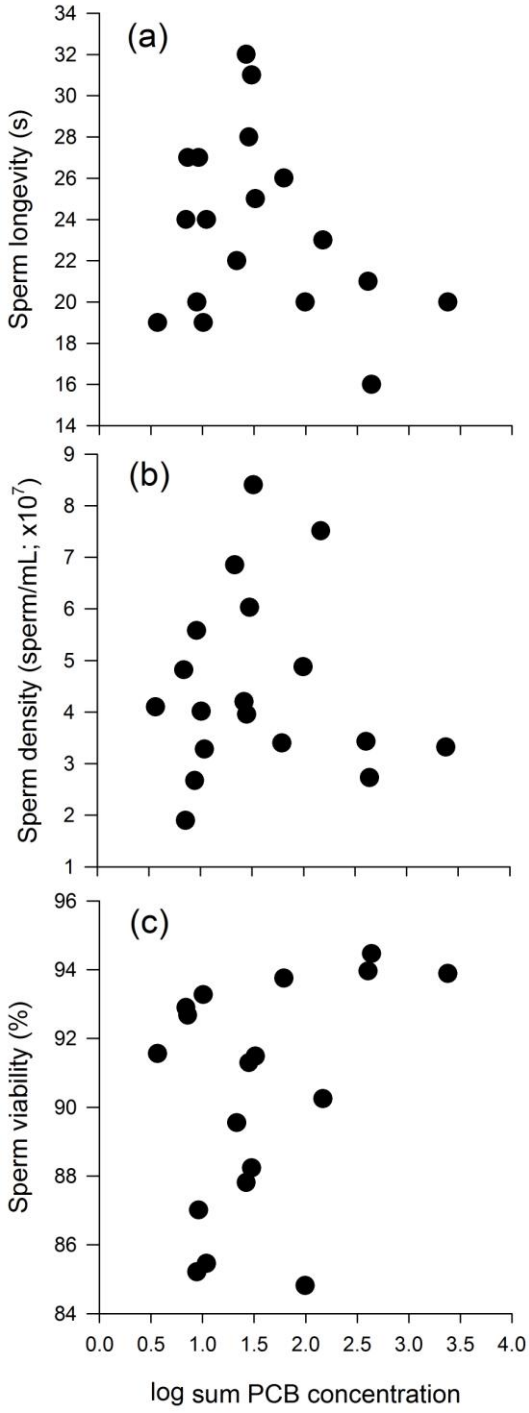

Figure 5.4: Pearson correlations between log transformed lipid corrected muscle sum PCB concentrations and male secondary sexual traits: percent reflectance (a), kype length (b), and hump depth (c) from Chinook salmon collected from the Credit River (n=18).

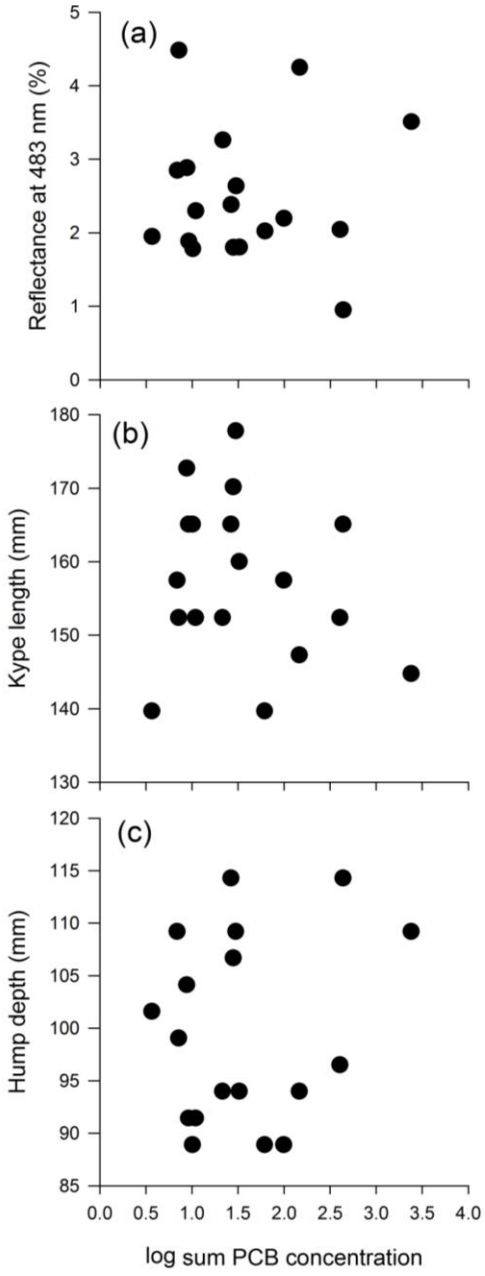

Figure 5.5: Photos of Coho salmon collected in 2007 by Pitcher et al (2009; a) and in 2010 for the current study (b), both from the Credit River. Note the darker and less red colouration and the less pronounced kype of the Coho salmon from my study. Both photos were taken at the same time of year during their respective spawning seasons.

(a) Pitcher et al. 2009

(b) Farwell et al. 2010

CHAPTER 6: GENERAL DISCUSSION

Thesis summary and implications

The ability to withstand a range of environmental contaminants has been documented in numerous taxa. Until recently, the field of ecotoxicology has, from a pre-evolutionary perspective, focused on identifying adaptive traits of populations exposed to contaminants with the ultimate goal of applying these results to various management practices. Those studies, while identifying differences in adaptive traits in wild populations between relatively more and less contaminated habitats, there was little emphasis on understanding mechanisms underlying variation in these traits or on their evolutionary implications. An emergent field, termed evolutionary toxicology, attempts to address these issues by examining the genetic basis for adaptive traits in wild populations exposed to contaminants. Those studies, similar to previous ecotoxicological studies mentioned above, often examine genetic differences between wild populations exposed to relatively more and less contaminants. The limitation of evolutionary toxicology research is that it often has a purely genetic focus and lacks an ecological understanding, which is important from a management perspective. My thesis aims to blend the fields of ecological and evolutionary toxicology by examining associations between life-history traits and environmental contaminants and acclimation, as an alternative mechanism to genetic adaptation, underlying these associations and by investigating relationships between traits under sexual selection and environmental contaminants. By doing so, I incorporate topics that are relevant to evolutionary theory that have yet to be examined in the field of evolutionary toxicology and I ultilize common ecotoxicological experimental design to provide relevance of my findings for management practices.

Chapter Two is the most ecotoxicologially based chapter where I examined associations between female reproductive life-history traits, including carotenoid allocation patterns, and sediment sum PCB concentrations among eight wild populations of brown bullhead (*Ameiurus nebulosus*). An extensive amount of laboratory research that characterizes variation in life-history traits across contaminant gradients (Mills and Chichester 2005, Kime 1995), however, less research has focussed on using several wild populations across a geographic range. By examining populations across the Lower Great Lakes Region, I can determine whether associations between life-history traits and contaminants are relatively wide-spread and whether management practices can be developed accordingly. I found no significant associations between sediment sum PCB concentrations corrected for organic content (OC) and reproductive traits. However, I did find that egg diameter was negatively correlated with sediment PCB concentrations not corrected for OC, suggesting observed relationships between sediment sum PCB concentrations and reproductive traits are driven by classes of environmental contaminants whose bioavailability are not predicted by OC. Carotenoids have been demonstrated to have antioxidant properties (Krinsky 2001) and therefore have the potential to decrease oxidative damage (Surai and Speake 1998, Tyndale et al. 2008) produced by contaminant metabolism (Di Giulio et al. 1989). There have been multiple studies demonstrating a negative association between carotenoid concentrations and contaminant exposure (e.g. Møller et al. 2005, Møller et al. 2010). My results demonstrate an unexpected positive relationship between egg carotenoid concentrations and sediment sum PCB concentrations. This positive relationship was explained by the maternal allocation of carotenoids based on a negative correlation between female muscle

and egg carotenoid concentrations, where females from less contaminated locations had lower egg and greater muscle carotenoid concentrations than those from more contaminated locations (Royale et al. 2001). This is expected to enhance offspring survival via increased antioxidant and immune function. The results of this study identify sub-lethal effects of environmental contaminants on reproductive life-history traits in female brown bullhead and, based on results from Chapter Three, investigations of adaptive mechanisms underlying this variation is warrented to provide insight to management implications. This study also highlights the importance of examining multiple reproductive life-history traits, especially when the relative effects of contaminants and the relatively contributions of fitness of each trait is unknown.Chapter Three is a continuation of Chapter Two, where a sub sample of four populations was used to experimentally test whether acclimation, an adaptive mechanism, of female life-history traits occurs in response to changes in sum PCB exposure. Aquatic ecosystems are known to be exposed to a variety of environmental stressors, including contaminants, which have potential to disrupt population dynamics (Witters 1998). Despite the extensive laboratory research that characterizes variation in life-history traits (Mills and Chichester 2005, Kime 1995) and an increasing number of studies assessing genetic adaptation [e.g. Wirgin et al. 2011), there has been little evidence for acclimation in life-history traits in response to contaminant exposure using field experiments and wild organisms (Mills and Chichester 2005). Through a translocation experiment, I found support for the acclimation hypothesis where translocated females had lower PCB concentrations in their eggs, as well as greater egg diameters and gonadosomatic indices compared to females from the original four populations. While I do not identify whether these phenotypic

differences are in fact adaptive, a distinguishing feature of acclimation, the observed plasticity in reproductive traits have been linked to increases in fitness in other fish species. Furthermore, these findings suggest that habitat restoration efforts of contaminated habitats may be warranted and successful as individuals will be able to demonstrate plasticity in fitness-related traits. Finally, with the support for acclimation occurring in egg diameter in addition to the negative association with egg diameter and environmental contaminants in the Lower Great Lakes Region (Chapter Two), it is possible that acclimation to contaminants is not simply a local phenomenon.

Chapter Four expands the breadth of evolutionary toxicology by investigating physiological mechanisms underlying variation in traits under sexual selection. While the links between contaminants, physiology, and resulting traits are not uncommon in ecotoxicological studies, demonstrating that these links have potential evolutionary implications, especially in traits under sexual, rather than natural, selection, provides a novel perspective currently lacking in this evolutionary toxicology. Chapter Four also adds to the field of evolution where relatively little research investigates physiological constraints underlying individual variation in traits under sexual selection, compared to that identifying variation in these traits as a consequence of sexual selection (see Pizzari et al. 2004). One set of physiological mechanisms that control the development of both primary and secondary sexual traits and whos typical functioning are to susceptible PCBs are reproductive hormones (Sonnenschein and Soto 1998, Gregory et al. 2008, Butts et al. 2012). I used male spawning Chinook salmon (*Oncorhynchus tshawytscha*) to investigate whether individual measures of gonad and muscle sum PCB concentrations as well as

plasma concentrations of reproductive hormones, 11-ketotestosterone (11-KT) and 17 alpha,20 beta-dihydroxy-4-pregnen-3-one (MIS), could explain variation in primary (sperm metrics) and secondary (body size) sexual traits under sexual selection. Using regression tree analysis, I demonstrate that 11-ketotestosterone (11-KT) concentrations best describes variation in primary and secondary sexual traits, with males with lower 11-KT having greater measures of primary sexual traits and lower measures of secondary sexual traits, and vice versa. I then demonstrate that PCB concentrations best describe variation in primary and secondary sexual traits in males with low 11-KT concentrations, where males with low 11-KT concentrations will have low PCB concentrations. Alternatively, I demonstrate that maturation inducing steroid (MIS) concentrations best describes variation in primary and secondary sexual traits in males with high 11-KT concentrations, where males with high 11-KT concentrations will have high MIS concentrations. These findings provide support for mechanisms that may underlie the expression of sexual traits, other than sexual selection. Further research is required to investigate the evolutionary impact of these underlying mechanisms. My study is unique not only in that it quantifies individual variation in contaminant concentrations in a wild population, but that it then attempts to use this variation to explain differences in sexual trait expression via underlying physiological mechanisms. This study highlights physiological mechanisms associated with PCBs that can explain variation in traits under sexual selection and suggests future research to investigate resulting evolutionary implications.Future studies could benefit from the use of regression tree model comparisons when physiological links between contaminants and reproductive traits are unknown. Laboratory studies to date tend to investigate single links between two

variables (e.g. contaminant exposure and hormone concentration, hormone concentration and sperm traits, contaminant concentration and sperm traits) rather than multiple links between multiple variables as in this chapter. Model comparison could therefore act as the basis for more complex laboratory experiments that have yet to be conducted. Also, as little is known regarding associations between contaminants and sexual traits, future studies are encouraged to analyze contaminants in multiple tissue types and sexual traits at multiple times if applicable (e.g. sperm velocity). This may be necessary until a more comprehensive data set exists in the literature on this topic.

Chapter Five continues the investigation of individual level associations between contaminants and male primary and secondary sexual traits in a more highly ornament salmonid; Coho salmon. Multiple studies describe negative links between PCB exposure and primary (e.g. Socha et al. 2008, Pocar et al. 2012) and secondary traits (e.g. Fisher et al. 2001, Quinn et al. 2002), however, few studies investigate these links in wild populations and discuss their implications in terms of sexual selection. Contrary to my general findings in Chapter Four, I found no significant correlations between any of the primary or secondary sexual traits measured and muscle sum PCB concentration. These results could be due to a variety of reasons. The range of PCB concentrations I quantified was relatively small and therefore even if these values were great enough to elicit a response, variation in these responses across the PCB gradient may have also been small and therefore not detectable. It is also possible that other contaminants, or suites of contaminants could better explain variation in sexual traits (e.g. Chapter Two). My sampling may have also not took place during peak spawning season or this season may have not been optimal for the fish compared to previous runs as males from this year had

significantly lower values for all primary and secondary sexual traits. This chapter, due to the small sample size, lacked statistical power which would explain the lack of any significant correlations. Finally, it is possibly that PCBs simply are not associated with variation in male primary and secondary sexual traits in this system. Identifying relationships, at the individual level, between primary and secondary sexual traits and environmental change is important for understanding the potential for these changes to impact evolutionary processes such as sexual selection (Bickham 2011). While it is possible that environmental contaminants are not linked to the expression of sexual traits, and therefore have limited evolutionary relevance from a sexual selection stand point, further experimental research examining mechanistic links between contaminants and sexual traits is required. Due to the lack of studies describing relationships between traits under sexual selection and PCB concentrations, future studies are advised to use study organisms with well defined and researched mating systems to ensure evolutionary relevance and to focus on relationships between PCBs and traits known to impact mating success. Future studies should also ensure that sampling of wild populations occurs at a time when sexual selection is likely to be great and when traits of interest are fully developed. Reproductive seasons, especially of salmonids, can be very short, and therefore prior knowledge of the specific study population, as well as the breeding system in general, is necessary.

Future research

My thesis has demonstrated that traits of ecological and evolutionary interest (i.e. life-history and sexually selected traits) can vary in their expression when exposed to

different concentrations of environmental contaminants. Individual variation in the response of these traits, let alone mechanisms underlying these different responses, has yet to be investigated. Individual variation is what selection acts upon and therefore the lack of research in evolutionary toxicology that identifies links between individual variation in traits and variation in contaminant concentrations is somewhat alarming. A first step to addressing this issue would be to identify whether individual variation in contaminant exposure exists within wild populations and to determine whether this variation is biologically significant. Along these lines, it is not enough to simply identify that variation in contaminant exposure exists, but it is important to ensure that this variation is great enough to elicit quantifiable variation in traits of interest. Finding populations that exhibit high individual variation in contaminant concentrations may prove difficult, suggesting why the majority of studies using wild populations compare traits at the population level.

The use of wild organisms in evolutionary toxicology, similar to ecotoxicology, is necessary to suggest evolutionary relevance of contaminant exposure. Genetic adaptation of wild populations to contaminants has been observed in multiple studies (Chapter 1). What is often not considered, however, is the role of physiological and ecological mechanisms that may also be influencing genetic adaptation. It is important for researchers to fuse the ecological aspect of ecotoxicology with the genetics aspect of evolutionary toxicology to understand both the general ecology and genetic structure of their study systems prior to investigation. This will provide ecological and management relevance to an evolutionary study. A common method in ecotoxicology that has been applied to evolutionary toxicology is to compare traits between populations from

relatively low and high contaminated habitats. This design allows the researcher to incorporate the effects of all contaminants in the system as well as any confounding ecological and environmental factors. While the use of single measures of contaminant exposure, as in my thesis, can provide more direct links between contaminants and traits of evolutionary interest, it also inherently excludes the possibility of other contaminants, or suites of contaminants, from also having links with these traits. An increasing number of studies have been finding synergistic effects of multiple contaminants on individual phenotypes compared to single contaminant exposures (e.g. Njiwa et al. 2004). Future research should investigate the effects of multiple contaminant exposures and further quantify these contaminants in wild individuals. Determining whether individual variation in traits of evolutionary interest is best described by single or multiple contaminants will provide a better mechanistic understanding of the association between contaminants and these traits.

The field of evolutionary toxicology is a merging of ecology, evolution, conservation biology and toxicology. As with any emerging area of research, there are initial logistical difficulties and steep learning curves. It is important that researchers in the field of evolutionary toxicology, and founding fields, are open to learning new concepts, ideas, frameworks, and techniques. While this may seem obvious, it is critical to the development of a new field and is often forgotten.

My thesis has begun to explore the blending of the fields that create evolutionary toxicology through novel avenues utilizing wild populations of fishes. I show that alternative mechanisms to genetic adaption exist to explain differences in adaptive traits to contaminant exposure and that these mechanisms can be widespread across

populations. I also describe individual level variation in traits under sexual selection related to individual differences in contaminant concentrations and physiological mechanisms mediating these relationships. My thesis suggests that environmental contaminants have the potential to impact adaptive and evolutionary processes and outcomes in ways that can be informative from a management perspective.

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