Genetic diversity of immune response genes and its effect on relative fitness in a polyploid salamander complex

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Summary

Amphibians are declining worldwide due to disease (IUCN, Daszak et al. 1999), with regional population collapses (UC Berkeley News 2009) spurring an interest in amphibian immune response genes, specifically within the Major Histocompatibility Complex (MHC) (Babik et al. 2008, Babik et al. 2009, Nadachowska-Bryzska et al. 2012). The ability of vertebrate populations to withstand novel disease exposure is mediated by the MHC genes as the genes play a major role in pathogen recognition (Hoglund 2009). As a result mammals, reptiles, and birds have high copy number and vast numbers of alleles (Ohta et al. 2006). Amphibians, however, appear to have very few copies (Ohta et al. 2006, Babik et al. 2008, Babik et al. 2009, Nadachowska-Bryzska et al. 2012) and very low genetic diversity (Bos and DeWoody 2005, Babik et al. 2009) on the species level, leaving them potentially susceptible to novel pandemics. Understanding disease risk, predicting the consequences of future exposure to pathogens in changing environments, and stemming the tide of amphibian declines requires a comprehensive understanding of the functional role of MHC diversity specifically in wild amphibian populations, a gap we intend to fill in this study.

There are several means of increased genetic diversity in an organism, such as increasing the number of copies of the gene in the individual (thereby increasing the number of variants present in any individual) or increasing the number of alleles (thereby increasing the combinations within an individual) either by access to different pools or recombination. Understanding how these routes to diversity influence disease response genes and improve fitness in the face of disease requires an amphibian study system that has naturally occurring variable copy number, varying access to alleles, and variable recombination. Additionally, a genetic system must be chosen that has direct, measurable ties between diversity of genotype and fitness.

The *Ambystoma* salamanders satisfy the above requirements of a model system. The *Ambystoma* are the dominant genus in the Great Lakes Region, and include a complex of polyploid unisexual females that are dependent upon five species as sperm donors (Bogart et al. 2009). The unisexual salamanders reproduce by kleptogenesis (Bogart et al. 2007), meaning they are dependent on the sperm for egg maturation, but do not necessarily incorporate the donated genome in their offspring (Bogart et al. 2007). This reproductive method leads to different populations of salamanders with different numbers of genomes from several species of Ambystomatid salamanders, each of which have unique pools of alleles. In any population, there are individuals with different gene copy number (due to increased copies of the full genome) and different pools of alleles (due to different species involved). Finally, the unisexual salamanders do not experience as much genetic recombination as the sexual Ambystomid salamanders and there are variable recombination rates within populations that depend on different sperm donors.

The MHC gene family similarly satisfies the requirements of a strong tie between diversity and fitness. Pathogenic disease necessitates diversity (Paterson et al. 2010) and is implicated in driving the development and maintenance the MHC genes, most diverse family of genes in vertebrates (Hedrick 2002). MHC genes identify antigens by binding to them and initiating a cascade of immune response (Hoglund 2009). Pathogens have short generation times and evolve quickly to evade host recognition. This in turn requires hosts to possess diversity in pathogen recognition and a means of regenerating that diversity over generations. More numerous and more divergent copies of MHC genes in an individual allow for the ability to identify more varieties of pathogens and facilitate more combinations within a population (Hedrick 2002).

Within the MHC gene family, MHC II is the most likely candidate for selection in the face of the current pandemic infections of amphibians. MHC II is the primary immunological presenter for extracellular infections (Savage and Zamudio 2011), such as chytridomycosis (Pounds et al. 2006). Unfortunately, amphibians not only have a limited number of MHC II loci, but suffer from low MHC II genetic diversity (preliminary data, Bos and DeWoody 2005, Richman et al. 2007, Babik et al. 2008, Nadachoska-Brzyska et al. 2012). Investigating MHC II exon 2 diversity in a system with relatively low copy number and allelic pool should make changes in fitness more apparent even with small changes in copy number (3-5N polyploidy) or allelic pool (variation between species).

This study seeks to describe interactions between routes to variability within diversity constrained amphibians. We will be using a polyploid complex of salamanders to investigate the relative effects of variable copy number, allelic diversity, and recombination upon fitness. In order to do address these goals, the following steps will be taken:

Aim 1) Establish measures of genetic diversity of individuals from several populations with different sperm donating species.

While it is assumed that the polyploid salamanders have increased genetic variability resulting from their hybrid origin and their increased genetic copies, the degree to which their individual diversity is increase is unknown. This information, as well as determining the number of loci, alleles present in populations, and any difference between populations with different sperm donors, is critical for further investigation.

We expect to find that there are identifiable alleles specific to each species and region and that polyploid salamanders have more individual variability due to the inclusion of these alleles from species not locally present. Though little investigation into this genus has been done, our preliminary results suggest the low diversity and limited loci extends to the diploid species of this genus.

Aim 2) Determine genetic diversity of subgroups within the populations.

As stated above, it is assumed that the genetic origins and nature of the polyploid salamanders will confer them an increase in individual genetic diversity. However, due to the reproductive system of the polyploids, it is unknown how this increase will translate to the subgroup of polyploids within a population. If the populations are dominated by few clonal types (i.e. gynogenesis dominates), the overall subgroup of polyploids may be highly heterogeneous. As these salamanders are such a large portion of the populations in which they occur (up to 85%), clonal dominance would significantly decrease the population wide diversity of disease response genes and leave the populations susceptible to disease spread.

It is our expectation that polyploids have increased subpopulation level MHC allelic richness and are not dominated by few clonal types. This expectation will be evaluated with the characterization of allele richness in each of the subpopulations. The increased subpopulation level MHC diversity of the polyploid salamanders significantly increases the allelic diversity of the overall population. This will be evaluated by the calculation of heterozygosity, allelic richness, and Fst of MHC loci with and without the inclusion of polyploid individuals.

Aim 3) Characterize the correlation between phenotype to genotype, as well as conflicts or synergistic interactions between alleles.

The potentially increased genetic diversity of the polyploid salamanders, particularly in immune response genes, is important to the stability and resilience of populations to disease, but only if the variability is functional. Having increased genomic copies causes complications in gene expression because of the mechanical issues involved with enlarged nuclei and increased chromosome density (Becak and Kobashi 2003, Osborn et al. 2003). Having increased copies does not help much if they are not expressed, nor does having increased individual diversity if the gene variants from different species conflict. In this study we plan to quantify the genetic diversity of both polyploid and pure breeding Ambystomatid salamanders and quantify their relative fitness in the face of disease challenge. As the system allows for variation in both copy number and allelic diversity, we will be able to address the importance of each factor and determine any synergistic or conflicting interactions between them.

We predict that the polyploid salamanders will survive and grow better than the sympatric sperm donor species in response to disease challenges. We also predict that the *Ambystoma* polyploids will have increased relative fitness in disease challenges, with an optimal diversity bounded by lowered ability to recognize pathogens due to low allelic diversity and antagonistic conflict between alleles due to conflicting alleles. Finally, we predict that survivability and growth will be correlated to MHC II diversity but not background diversity. If confirmed, these predictions would support the hypothesis that polyploid salamanders have higher disease response fitness due to their increased genomes and access to more alleles.

After we identify the patterns of individual variability (Aim 1) and subpopulation contributions to diversity (Aim 2), then evaluate the fitness effect of that variability (Aim 3), we will have a solid understanding of the effect of polyploids on the population level MHC diversity as well as general immune genetics of the region.

A. Significance and Further Background

A.1 Amphibians are declining worldwide and there is a gap in knowledge of salamanders of the Eastern United States.

Amphibians are declining worldwide (IUCN 2012, Daszak et al. 1999, Stuart et al. 2004), with 32% of species known to be threatened or extinct and 42% of all amphibians experiencing reductions in population, an indication that the percentage of threatened species will grow in the future (IUCN 2012). This level of threat is already significantly higher than any other vertebrate taxa and the declines appear to not only be widespread but recently increasing (IUCN 2012). Within amphibians, salamanders have the highest level of threat with 50% of the species listed as threatened, compared to the 32% of species in frogs (IUCN 2012). While habitat destruction is a major threat, disease spread has become tied to an increasing number of declines (Stuart et al. 2004, Lips et al. 2006, Skerratt et al. 2007, Wake and Vredenburg 2008, Rohr et al. 2008). Originally thought to be less susceptible to the effects of chytridomycosis, sudden catastrophic declines in populations of salamanders indicate a shift in disease susceptibility. Most recently, Central America experienced a 98%

decline in populations of salamanders in a period of a few years, with evidence of chytrid infection in the populations (UC Berkeley News 2009). Salamanders are important in terms of biomass, invertebrate predators, and as conduits of nutrients from aquatic to terrestrial woodland systems and the sudden loss of them is likely to have "a profound effect on the ecosystem" (Davic and Welsh 2004, UC Berkeley News 2009).

The 70% of the amphibian species found in the United States are endemic and one of the largest diversities of salamanders in the world exists within the border, yet the US is ranked 13th for highest percentage of threatened amphibians (IUCN 2012) and very little study of regional susceptibility or risk of disease decline has been conducted. Specifically, the Great Lake Region is understudied and is known to be a unique biological zone, peculiar in its numerous pulses of recolonization due to glaciation and high incidence of polyploid amphibian groups, such as *Ambystoma* which has 45% of its species threatened, with 29% at critical level (IUCN 2012). Currently, crucial information for conservation such as the number of immune responsive loci, the geographic diversity of the immune response genes, or how the gene variability is related to functional response is largely unknown (Babik et al. 2008).

We will fill this gap in knowledge by identifying the loci in the more *Ambystoma* species, describing the local and geographical allelic diversity, and tying this diversity to relative fitness. Additionally, primers that will be developed for this project are necessarily useful across multiple *Ambystoma* species, facilitating future investigations into species and populations not covered in this study.

A.2 Variability is critical in disease response and understanding depauperate regions is necessary to prevent further collapse.

The Great Lakes Region may suffer from lowered genetic diversity, having been subjected to periodic recolonization after glacial pulses (USGS 2008) and recent recolonized of patches of abandoned agricultural land. The recolonization of large regions often results in founder effects, where genetic diversity is reduced as a result of pioneering populations carrying only a subsample of the source population's total diversity. Increased distance from glacial refugia has been tied to reduced allelic diversity in Alpine Newts (Babik et al. 2008) and founder effects have been tied to reduced diversity in Tiger Salamanders as a result of post Pleistocene recolonization (Spear et al. 2005, Noel et al. 2011), indicating that amphibian genetic diversity may be low in the similarly post-glacier portions of the Eastern United States. Additionally, reductions in diversity have been found in other salamander species as a result of recolonization of agricultural lands (Zamudio and Weiczorek 2007), droughts, and possibly disease (Spear et al. 2006); all factors that affect the Great Lakes Region. These bottleneck and founder effects can outweigh the heavy balancing selection and dramatically reduce MHC variation (Hoglund 2009). With recent region wide collapses of salamander populations (UC Berkeley News 2009), evaluating the disease resistance of a likely depauperate region is critical to targeting conservation efforts.

However, critical information such as variation in the number of immune responsive loci, geographic diversity of the immune response, or gene variability's relationship to functional response is lacking in the Great Lakes. The ability of vertebrate populations to withstand novel disease exposure is mediated by immunity loci in the Major Histocompatibility Complex (MHC) (Hoglund 2009). Amphibians exposed to novel pathogens may be especially challenged as a result of their relatively low diversity at MHC loci (Otto and Whitton 2000, Bos and DeWoody 2005, Richman et al 2007, Babik et al. 2008, Nadachowska-Brzyska et al. 2012). Understanding disease risk, predicting the consequences of future exposure to pathogens in changing environments, and stemming the tide of amphibian declines requires a comprehensive understanding of the functional role of MHC loci and distribution of genetic diversity in immunity loci in natural populations of amphibians, a gap we intend to fill in this study.

A.3 MHC genes are a likely place to look for disease response and adaptive variability differences.

Pathogenic disease necessitates diversity (Paterson et al. 2002) and is implicated in driving the development and maintenance the MHC genes, most diverse family of genes in vertebrates (Hedrick 2002). MHC genes identify antigens by binding to them and initiating a cascade of immune response (Hoglund 2009). Pathogens have short generation times and evolve quickly to evade host recognition. This in turn requires hosts to possess diversity in pathogen recognition and a means of regenerating that diversity over generations. More numerous and more divergent copies of MHC genes in an individual allow for the ability to identify more varieties of pathogens and facilitate more combinations within a population (Hedrick 2002).

As a result of the strong tie of genetic diversity to disease response, this gene family shows patterns of balancing selection (Hughes and Yeager 1998, Hedrick 2002). There is a general trend toward more loci as lineages age, with amphibians having few copies, reptiles having several more, and mammals having nine (Hughes and Hughes 1995). These genes are the most diverse in alleles as well (Hughes and Hughes 1995), with humans having over 1700 different alleles (EMBL-EBI IMGT/HLA Database 2012). The high allelic diversity and the increased copy number results in 1700^{18} combinations of pathogen recognition within humans, more combinations than there are people, dramatically reducing the percent of the

population vulnerable to any particular pathogen at a given time. High diversity, in both genetic diversity and copy number, provides a stable mosaic of resistance that increases the stability of a population against wide spread disease.

Within the MHC gene family, MHC II is the most likely candidate for selection in the face of the current pandemic infections of amphibians. MHC II is the primary immunological presenter for extracellular infections (Savage and Zamudio 2011), such as chytridomycosis (Pounds et al. 2006). Unfortunately, amphibians not only have a limited number of MHC II loci, but suffer from low MHC II genetic diversity (preliminary data, , Bos and DeWoody 2005, Richman et al 2007, Babik et al. 2008, Nadachowska-Brzyska et al. 2012). We will characterize the individual, population level, and regional diversity of this gene that, while critical to disease response, has largely been uninvestigated in conservation genetics.

A.4 The Great Lakes Region is home to a large contingency of polyploid unisexuals that are critical in understanding disease risk and response of the area.

The dominant genus in the Great Lakes Region is *Ambystoma* (Michigan Herp Data Entry Framework 2012), which includes a complex of polyploid (3-5N) unisexual females that are dependent upon five bisexual, diploid species as sperm donors (Bogart et al. 2009). The unisexual polyploids reproduce by kleptogenesis (Bogart and Bi 2007), meaning they are dependent on the sperm for oocyte maturation, but do not necessarily incorporate the donated genome in their offspring (Bi and Bogart 2009; Figure 1). This process leads to different, possibly related, clonal lines of polyploid unisexuals that have different numbers of local sperm donor genomes (Figure 1, Gynogenesis Path). Additionally, polyploids have much lower individual fecundity than the donor species but paradoxically constitute up to 85% of some populations (Bogart and Klemens 2008).

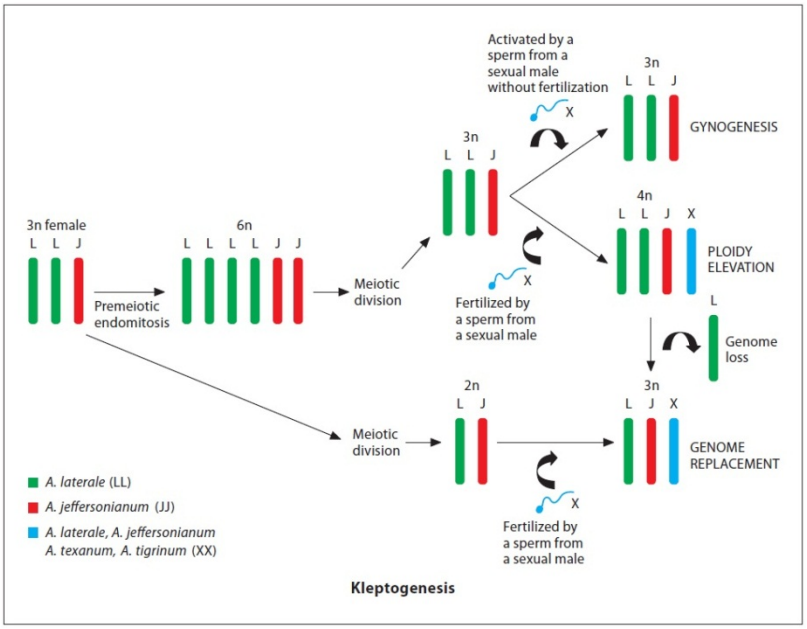

Figure 1. Polyploid reproduction via kleptogenesis, leading to one of three genotypes for offspring. Polyploids can create clonal lines via gynogenesis, incorporate the donor genome and elevate ploidy level, or replace a maternal genome with the donor genome and maintain ploidy level while creating genetically distinct offspring. The relative frequency of these paths is unknown. Taken from Bi et al. 2009.

The *Ambystoma* polyploids are unique in the fact that they have a single originating hybridization event and thus share a monophyletic mitochondrial lineage that has persisted for five millions years (Bogart et al. 2009). However, over this time period, *Ambystoma* as a whole have experienced numerous range expansions and contractions due to the glacial pulses that have intermittently covered the majority of the Great Lakes Region (Harding 1997). This expansion has served to put the polyploid salamanders in contact with several sperm donor species. As a result, contemporary polyploid salamanders not only partially overlap the separate ranges of all sperm donating species, but polyploids can harbor full genome copies from several diploid species that do not generally overlap in range (Harding 1997; Figure 2). This leaves populations of polyploids dependent upon a single species for reproduction but also results in full genomes of non-local species being represented in polyploid populations. The unisexual complex consists of three subpopulation categories: polyploid salamanders that have three to five copies of genomes from up to five different species, the single local sympatric sperm-donating diploid species that sometimes contribute genetic information to the polyploids, and parapatric diploids of the sperm-donating species that exist outside of the polyploid range.

Numerous *Ambystoma* species that contribute to the polyploid complex are of conservation concern (IUCN 2012). In the Great Lakes region, the stability of these populations is made even more precarious by the prevalence of the polyploid unisexuals. The heavy unisexual dominance skews *Ambystoma* populations to be greatly female biased to an average of 87% and up to 98% in some populations (Wilbur 1971, Bogart and Klemens 2008). While the males only constitute a small minority of the population, they are required for the production of the entire next generation, either in sexual reproduction with the diploids or spermataphore activation in the polyploids (Figure 1). This population structure puts the Great Lakes salamanders in a precarious position, as even small reductions in population will reduce the likelihood of rare males encountering the uncommon bisexual females. Reduced sexual reproduction creates a scenario that can quickly lead to population crash (Wilbur 1971), which could result in a massive loss of biomass in the ecosystem as well as an important venue of nutrient transport from aquatic to terrestrial systems. Understanding the dominance of polyploid

unisexuals is an important factor in evaluating the stability of this system.

However, kleptogenetic reproduction makes predicting connections between phenotype and genotype and between individual variability and population diversity difficult. Kleptogenesis may confer the salamanders an advantageous allelic diversity on an individual scale, the reproductive system complicates the prediction of population level diversity. Polyploid salamanders often reproduce without incorporating the spermatophore of the sperm donor male, creating clonal lines (Bi and Bogart 2009, Bi and Bogart 2010; Figure 1). Because the relative rates of unisexual cloning are uninvestigated, it is similarly unknown if the polyploid subpopulations are dominated by a few clonal lines. If this is the case, populations will be dominated by large numbers of genetically similar individuals and disease to which they are susceptible would infect vast portions of the populations.

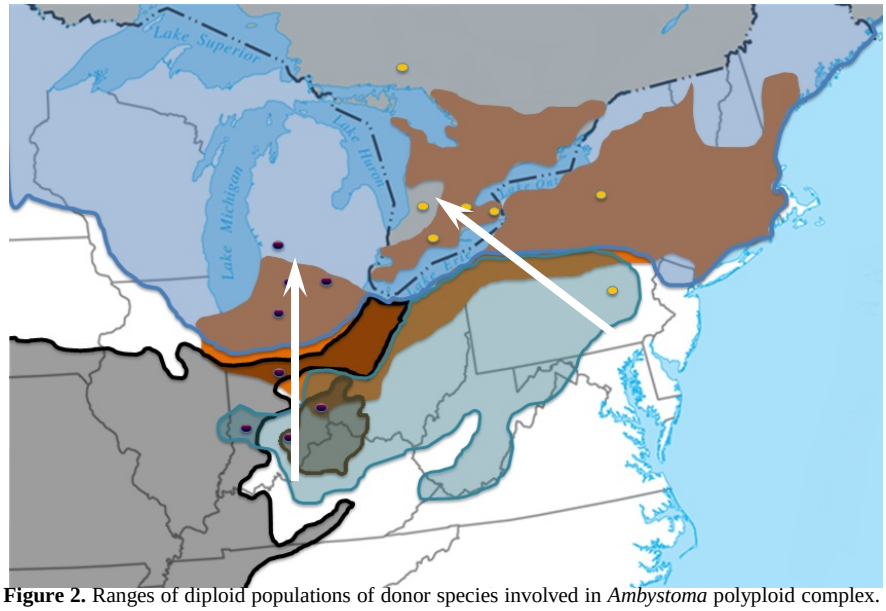

Figure 2. Ranges of diploid populations of donor species involved in *Ambystoma* polyploid complex. The polyploid zone (solid orange) is overlapped by the two major sperm-donor species (light blue = Blue Spotted Salamander, teal = Jefferson's Salamander), two less common sperm-donor species (Black = Small Mouth Salamander, Tiger Salamander range not shown, sperm donation by this species is restricted to a small region in south eastern Michigan), and the original mitochondrial donor species (Brown = Streamside Salamander). Sperm donor species do not significantly overlap in range, leaving polyploids dependent on a single species. Blue dots are points at which field sampling will be completed, yellow dots are populations where samples have already been obtained from the University of Guelph collection. White arrows indicate rough transects of increasing distance from glacial refugia.

Additionally, polyploidy may decouple genotype from phenotype in unexpected ways. Allelic conflict results in alleles that variably expressed based on the other alleles in the individual, making it of particular interest in polyploid organisms. In a survivorship experiment with autopolyploid *Xenopus* species, highly divergent alleles within the same individual resulted in an apparent shut down of the immune system as evident by the absence of an immune response and poor survivorship and growth. Too low of internal diversity results in the inability to identify various disease, too high results in the inability to express any of the alleles. Both cause reduction in fitness in the face of disease.

The biology of the *Ambystoma* polyploids creates conflicting hypothesis about the diversity conferred by copy number and allelic introgression levels by this subpopulation. In order to understand this effect, as we cannot extrapolate from previous studies, we will directly investigate the effects of increased copy number and allopolyploidy on individual fitness and population level diversity.

A.5 Broader significance outside study system

A.5.1 Polyploids create a general tie between genomics and ecology that has not been investigated in vertebrates and will be applicable to other systems.

Polyploidy is much more common in plant evolution than in animals, particularly vertebrates (Otto and Whitton 2000). Much work has been done on the effects of transient plant polyploidy upon disease resistance (Paterson et al. 2002, Oswald and Nuismer 2007) but the little work has been done in the persistent hybrid polyploids that are more typical of vertebrate genera. There are approximately 28 species of polyploid amphibians, 48 species of fish, 15 species of reptile, and even one recent finding of polyploidy in mammals (Otto and Whitton 2000). Some of these polyploid species are of commercial value, such as triploid salmon (Leggatt and Iwama 2003), or ecological significance, such as the amphibians (see A.1). Understanding the effect of increased copy number on response to disease is important to management. The understanding of the novel effect of genomic composition on the disease ecology in *Ambystoma* salamanders will provide useful insight into other systems. The interaction of factors such as strength of drift (distance from source populations), copy number, introgression and expression have not been outlined for vertebrate polyploids, and the information from investigating salamanders will be applicable to other systems. *Ambystoma* simply provide an ideal system for this investigation because of the number of interactions that can be investigated and while avoiding the complications of multiple hybrid origins.

A.5.2 While the main function of this project is to understand polyploids, much knowledge is gained about the sexual species, which are of conservation interest.

Half of the species of the *Ambystoma* are threatened and almost a third are classified as critical (IUCN 2012), yet almost no information exists on their genetic structure, geographic distribution of alleles, or their susceptibility to disease. This study will result in the design of primers applicable to these systems for future investigations into any member of this genus, but more importantly will provide a preliminary assessment of allelic variability and population structure of the diploid populations of these species throughout the northern portion of the *Ambystoma* range.

A.5.3 Regional studies provide a platform for international and community collaboration.

As the Great Lakes Region and its inhabiting polyploid salamanders encompass territories beyond the Canadian border, international collaboration is integral to obtain samples and local expertise in the northern portions of the system. The major work in describing the *Ambystoma* polyploid system has been done by Dr. James Bogart of the University of Guelph (Bogart and Bi 2007, Bi and Bogart 2009, Bi and Bogart 2010). Initial collaboration between this study's primary investigator and Dr. Bogart has already begun with a month long visit to the University of Guelph to leverage his lifetime collection of specimens from across the entire polyploid range, his expertise in identifying the genetic origin of the polyploids, and his general insight into the complexity of the system. As he is an asset to the understanding of this system, continued collaboration will occur in the future.

In addition to this international involvement, sample acquisition in the United States will make use of the network of herpetology expertise. Several contacts have been made with regional zoos, contract herpetologists, and other Universities to provide synergistic sample collection for all related investigations into this system, which range from surveys to effects of polyploidy on migration.

B. Innovation

B.1 While typically avoided due to their complexity, we are leveraging polyploid individuals to increase efficiency of data collected and to investigate a novel role.

Typically hybrids unisexuals are considered evolutionary dead-ends because of their semi-clonal reproduction and low fecundity. Counter intuitively, *Ambystoma* has persisted for longer than any other vertebrate unisexual yet described (Bi and Bogart 2010) and are unexpectedly numerous (Bogart and Klemens 2008), indicating some fitness advantage. To date, no project known to the principal investigators has been conducted addressing the possible population diversity elevating role of polyploids, a hypothesis that addresses both the fitness and influence of the polyploids.

In addition to posing the unique role of polyploids as a possible stop gap for low population variability, we will leverage the presence of polyploids hybrids to efficiently obtain information critical to the analysis of this system. Including polyploids in sequencing efforts increases the likelihood of capturing rare alleles, as more alleles present in the population will be recovered per individual sequenced. Polyploids also allow for more robust testing as the number of variant is not limited to two per loci. Statistical validation of number of loci is more easily confirmed with a smaller sample size due to the more continuous nature of copy number and allelic variability in polyploids. Preliminary data indicates one locus with limited variability; however we expect that polyploids will harbor more alleles per individual, including alleles foreign to the local diploid population. By sequencing polyploids we not only capture more alleles per individual, but important information about the allelic mosaic of introgression is obtained.

Finally, while polyploid and diploid sperm donor species will be included to ensure the correlation is universal, the unisexual nature of the polyploids allow for genetically identical individuals (clonal replicates) to be present in each disease challenge condition, increasing the power of the analysis be removing genetic variability as a factor.

B.2 We will leverage a large repository of data that spans decades and the range, while filling in the gap with field research already being conducted.

This project requires the coverage of a number of populations through a large range, which would be impossible to manage in a reasonable amount of time without the use of Dr. Bogart's repository of 13,000 samples. These samples are the results of years of surveys requiring genetic identification of the *Ambystomid* salamanders from government agencies and academic research, but analysis has been mostly limited to simply identifying biotypes in populations. All these samples are pre-extracted and have microsatellite data already assigned to them, dramatically reducing the necessary work load to adequately meet the aims of this project. While field work will still be require to fill in the geographic gaps, such as western Indiana and Michigan, we will leverage this untapped source of genetic information to prefect the methodology and extend the scope of the project.

B.3 Investigation of llumina instead of 454 increases efficiency of this and future studies.

To date, MHC research has largely been done on Roche 454 technology (Babik et al. 2008, Babik et al. 2009, Nadachowska-Brzyska et al. 2012), as read length has been insufficient in most other high throughput technologies. However, recent developments in the Illumina platform have increased read length to sufficient length to be a candidate for such sequencing efforts. As a result of Illumina sequencing technology's higher coverage, lower indel read error, and lower cost per base pair (Harismendy et al. 2009), recovering statistically supported alleles in the highly variable gene will be more efficient than on the 454 technology providing an efficient means of investigating future populations.

C. Research Plan

We endeavor to fill the gaps in knowledge about the population and geographic genetic variability of salamander populations in the Great Lakes Region. *Ambystoma* salamanders have a unique complex of unisexual polyploids (Bogart and Bi 2007) that allows for direct investigation of the importance of introgression of novel alleles and the impact of gene copy number upon fitness while at the same time characterizing the genetic diversity and richness of the sexual species, many of which are of conservation concern.

The *Ambystoma* polyploids are of particular focus in this study as they have an increased capacity for heterozygosity due to their increase in genomic copies, have persistent access to the gene pools of numerous species due to their hybrid nature, and have occasional recombination (of genomes rather than genes) due to their reproductive method. Additionally, the validity of explaining the fitness advantage of polyploid *Ambystoma* as a function of disease resistance is consistent with the relevant fitness experiments in amphibians. There was no difference in larval survival to transformation was discovered in competition experiments with *Ambystoma* polyploids and diploids (Wilbur 1971). Polyploid individuals grew slightly larger and emerged slightly earlier on average, but their survivability was negatively impacted by the presence of diploid larvae. Diploids are less density sensitive and less sensitive to the presence of the polyploids. This experiment was carried out under normal conditions (i.e.: in the absence of disease) and showed no fitness advantage for the polyploids. However, in numerous amphibian survivorship studies (Barribeau et al. 2009, Zeisset and Beebe 2010, Savage and Zamudio 2011), MHC II diversity was linked to successful growth and survival of larvae. Background genomic similarity was ruled out in *Xenopus* cross survivorship experiments (Barribeau et al. 2009) and another *Xenopus* study found a link between hybridization, MHC diversity, and survival (Jackson and Tinsley 2003). All of this evidence implicates MHC II diversity as a putative fitness advantage.

Even though there is no gene flow of kleptogenically introgressed alleles into the sexual salamander subpopulations, a dominant polyploid population may reduce the incidence of disease spread to susceptible sexual species. If only a minority of the population is susceptible to a disease, sweeping epidemic infections are unlikely to occur as the probability of contact between susceptible individuals is vastly decreased. Since polyploids dominate many of the populations and may be less susceptible to diseases with their increased MHC diversity, they may serve as a buffer to the less diverse and less populous sexual subpopulation.

Our **central hypothesis** is that the increased genomic copy number and hybrid origin of *Ambystoma* polyploids increases their internal allelic richness, which allows them to identify and respond to more pathogens, and the population level allelic diversity, which buffers the otherwise limited diversity of disease recognition genes of the sympatric diploids. This hypothesis rests on several assumptions:

- Polyploids have increased individual MHC allele richness. This assumption will be evaluated with the characterization of individual allelic diversity of all three subpopulation types (Aim 1).
- Polyploids have increased subpopulation level MHC allelic richness and are not dominated by few clonal types. This assumption will be evaluated with the characterization of allele richness in each of the subpopulations (Aim 2).
- The increased subpopulation level MHC diversity of the polyploid salamanders significantly increases the allelic diversity of the overall population. This assumption will be evaluated by the calculation of heterozygosity, allelic richness, and Fst of MHC loci with and without the inclusion of polyploid individuals (Aim 2).
- Increased internal allelic richness increases relative fitness in disease challenges. This assumption will be evaluated with survivorship experiments that expose polyploid and diploid larvae to several disease conditions (Aim 3).

By evaluating the validity of each of these assumptions, we will provide considerable support to our theory that polyploid salamanders are more immunologically fit and are an important component to the total population stability in the face of

disease. This study will also help evaluate the potential importance of the polyploid *Ambystoma* in the stability and resilience of Great Lakes salamander populations. Polyploids are not currently protected or actively managed. Finally, this study will identify population risk factors - such as distance from glacial refugia and polyploid dominance - that may indicate the need for targeted conservation efforts in the face of amphibian decline and in the management of other vertebrate polyploids.

C.1 Characterize internal MHC allelic variability of individuals in multiple populations with different sperm donating species (Aim 1)

C.1.1 Core Concept: Clonally reproducing unisexuals may have more individual diversity than sexual reproducing bisexuals.

Understanding variation in MHC diversity in individuals is critical to the understanding of disease response in a species. Allelic richness and number of functional loci can differ between species (Bos and DeWoody 2005, Richman et al 2007, Babik et al. 2008, Nadachowska-Brzyska et al. 2012) and no current research exists on the genetic diversity of the majority of the species involved in the *Ambystoma* polyploid complex. In this portion of the study, we seek to characterize the individual MHC allelic diversity of the *Ambystoma* polyploids and the sperm donor species. Two major influential factors contribute to MHC diversity, allelic richness and copy number, which we believe give the hybrid polyploid salamanders an advantage in disease response.

It is our **hypothesis** that *individual polyploids have more internal allelic diversity in the MHC II locus than the sympatric sperm donor species. We also predict that the background (microsatellite) diversity does not share this same pattern, with no significant difference between the polyploids and the sperm donor species.* If true, this finding would support the theory that disease drives the necessitation for diversity that in turn gives the polyploid salamanders their fitness advantage.

C.1.2 Scientific Approach:

C.1.2.1 Data

Sampling: In order to make this project tractable, the primary focus of sampling will be on the two major sperm donor species, *A. laterale* (L) and *A. jeffersonianum* (J) (Bogart and Klemens 2008; Figure 20). Restricting analysis to these two species will allow for and understanding of the largest contributing genotypes to the complex and perfect methodology, hypotheses, and theory that can easily be extended to the other sperm donor species.

The importance of restricting the number of sperm donors becomes evident when defining analytical units for this portion of the study. Even with limiting the scope to the two most prevalent sperm donor species, six analytical groups emerge: polyploid subpopulations in both L and J donor areas, the sympatric L or J subpopulations of sperm donors, and parapatric populations of L and J.

Including further species would be needlessly expensive, as our hypotheses are not species dependent. Including only one sperm donor population would eliminate our ability to tell if the trends are indeed species specific as well as limit the sampling of the polyploid range as L and J are allopatric and the polyploid zone covers both.

Twelve to thirty individuals of each subpopulation category will be sequenced for at least fourteen populations (Figure 2). Statistical power analysis is not possible at this time, as it is unknown how many loci, alleles, and variation is present in the populations, however prior studies captured full population level diversity of MHC II alleles in salamander species with similar population coverage (Bos and DeWoody 2005, Richman et al 2007, Babik et al. 2008, Nadachowska-Brzyska et al. 2012). Our use of polyploids will also boost the likelihood that more allele varieties will be recovered per sample and we are sampling at twelve to thirty individuals for two subsets of the population (polyploids and bisexuals), totaling up to sixty individuals per population. We are relatively confident that this coverage will be sufficient, but as soon as preliminary analysis is performed, a power analysis will be conducted to pinpoint necessary sampling coverage to ensure efficiency and statistical significance.

Collection of salamanders will be accomplished via dip netting, pit fall traps, and minnow traps placed in and around temporary ponds (Heyer 1994). Dip netted salamanders will be kept in buckets with pond water to prevent dehydration, pit fall traps will be partly filled with water and a sponge to prevent both dehydration and drowning, and minnow traps will be placed so that at least 25% of the trap is above water to prevent drowning. All traps will be checked at least every twelve hours to prevent unnecessary stress or risk to the animals. Any animal that is fatally injured or sick will be euthanized by submersion in a 0.2% Benzocaine solution. Captured animals will be first submerged in a 30% ethanol / 70% distilled water solution until limp/anesthetized and then a 2 mm tissue sample from the tail or toe will be taken with sterile scissors. Salamanders will also be photographed, measured, and sexed. Animals will then be held in buckets

containing a half inch of pond water until normal speed of movement has returned, at which point they will be released at their point of capture.

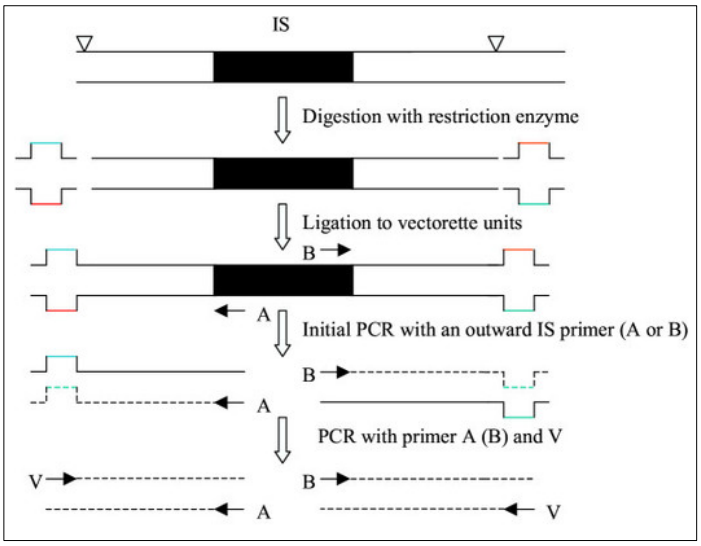

Figure 3. Vectorette PCR begins with restriction enzyme digestion on either side of the target gene (IS) to create points to attach pre-made vectorettes. PCR reactions are then performed from the middle of the gene outward (reactions A and B) to form two amplicons per product.

Field sampling will occur in populations from the western portion of the range, which has been largely unstudied (Figure 2: purple circles). These populations were chosen with consideration of known breeding populations, connections with local experts, and a desire to sample along a gradient from glacial refugia (west of the Appalachian Mountains) through the polyploid zone. Samples will be supplemented by the large bank of genomic extractions done at the University of Guelph, Ontario; a collection that spans most of the eastern range of *Ambystoma* and currently encompasses 13,000 extractions that span all three subpopulation categories and the majority of the polyploid range. These populations were chosen for similar reasons to our field selections; we are interested in sampling known breeding populations along a gradient from glacial refugia (east of Appalachian Mountains) through the polyploid zone with the inclusion of parapatric populations (Figure 2: yellow circles). Additionally, all samples selected from the collection were obtained within the last five years to ensure we are comparing contemporary populations.

Allelic Diversity: Genomic DNA will be extracted and purified from the small (~2mm) tissue clips using standard lab protocol (Qiagen DNeasy and Qiagen Minielute Kits). University of Guelph samples are previously extracted by Dr. James Bogart's Lab using similar methodology.

To access MHC diversity, the second exon of the MHC II gene will be sequenced in a multiplexed Illumina run. The second exon is the location of the antigen binding site, the most functional and diverse portion of the MHC gene product (Richman et al. 2007, Babik et al. 2008). Most studies in this field use this exon as a surrogate for the overall MHC II diversity (Bos and DeWoody 2005, Richman et al 2007, Babik et al. 2008, Nadachowska-Brzyska et al. 2012). However, the most variable portions of this exon lie in the extreme ends, making primer placement difficult (Babik et al. 2009). To solve this issue, genomic DNA will be digested with restriction enzymes and annealed to custom 53bp fragments of DNA called "vectorettes", following a modified version of the Ko et al. 2003 protocol (Figure 3). This step is required as up to 56% of the alleles recovered in other studies would have been lost if traditional methods of gene amplification were followed (Bos and DeWoody 2005, Richman et al 2007, Babik et al. 2008, Nadachowska-Brzyska et al. 2012), as primer placement on the exon is not possible in the variable regions and only the middle section of the exon could be recovered. However, by adding these vectorettes and using a combination of one vectorette specific and one internal gene specific primer, two amplicons can be obtained via traditional PCR (a center-to-5' and center-to-3' internal gene primer have been successfully developed for this purpose). These two amplicons overlap in the center of the gene (not shown in Figure 3), allowing full consensus sequences of the highly diverse gene to be obtained.

Sequencing will utilize the Illumina MySeq platform, as it is not sensitive to indels or homopolymer errors like the traditionally used 454 platform (Harismendy et al. 2009). Double stranded Illumina read length has recently been extended to over 150bp, which is sufficient to cover the 145bp and 105bp amplicons from the vectorette PCR. The low cost per base pair also allows for increased coverage (targeted at an average of 400x in this study), which increases the reliability of recovered alleles in this highly variable gene.

Ploidy and Genome Contribution Determination: Determination of ploidy of the organisms is only reliably done through microsatellite methods as morphological identification of polyploids has been shown to be unreliable (Greenwald and Gibbs 2012). Ploidy and identification of contributing genomes is done simultaneously through the use of species specific microsatellite primers developed by Dr. James Bogart's Lab. Seven well established loci (Greenwald and Gibbs 2012) are amplified through traditional PCR methods and run on a polyacrylamide gel to visualize small length differences. TotalLab 12 software (TotalLab Limited 2012) is used to determine allele size, which is referenced to known species allele lengths to determine genomic contributions. Ploidy becomes evident with the increased number of alleles present in the sample and running numerous loci accounts for homozygosity any single loci. If necessary, several other loci are known (Bogart, personal communication), but the seven commonly used are typically sufficient for the identification of ploidy and genotype.

Microsatellites: Microsatellites are included in this study to establish background genomic diversity, which will allow us to address if MHC II diversity is significantly increased above average background diversity and allow for a second measure of populations level diversity outside of a selectively active gene. Microsatellites will be run using primers from published papers (Babik et al. 2009, Noel et al. 2008) using acrylamide gel protocols.

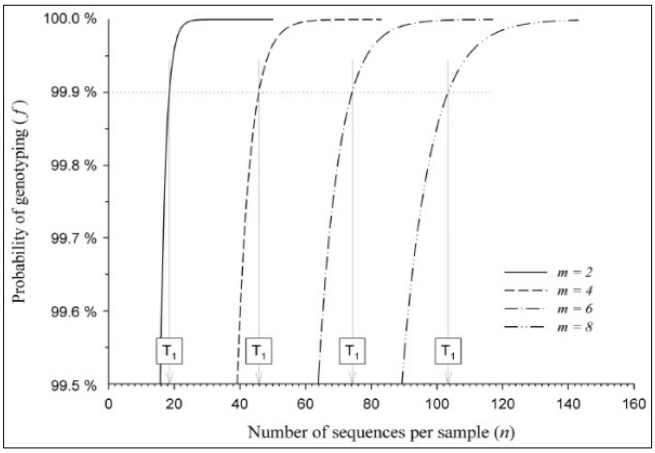

Figure 4. Probability of obtaining any allele a specified number of times (typically three), given the number of copies in the genome. This probability is used to determine the degree of coverage needed to obtain different levels of confidence in the allele. Taken from Galan et al. 2010.

C.1.2.2 Data Analysis

Sequencing of such a highly variable gene presents some analytical issues. Sequence errors can occur from either PCR artifact or pyrosequencing error. PCR can introduce error into the sequences in two ways, point mutations and chimeras. Point mutation error is random across the gene and results in alleles very similar to the parent genome. These errors are easily detected using established methods based on probabilities of multiple identical substitution errors (Galan et al. 2010, Nadachowska-Brzyska et al. 2012). While no published study uses the Illumina platform for MHC allele recovery, detailed algorithms exist for 454 analyses (Galan et al. 2010) that can be adapted to Illumina technology easily. These algorithms predict the probability of correctly obtaining the same exact allele a certain number of times (f, Figure 4), thereby statistically determining a confidence level of "true alleles" (Galan et al. 2010). The function predicts the minimal number of sequences required per individual (T_1, Figure 4) based on the incidence of base miscalling rate of the technology and assumed number of alleles present per individual (m, Figure 4). This probability is technology specific and can be adjusted to fit any platform. Note that the predicted 400x coverage will be more than sufficient to meet high accuracy needs.

PCR chimeras result in recombination of alleles that are also identified in the population. These errors will be addressed with established methods of identifying sequences that are vastly similar to two alleles with which they co-occur in an individual (Posada 2002, Zagalska-Neubauer et al. 2005).

Illumina technology is prone to higher levels of base pair error than the Roche 454 platform, and these point errors must be parsed from true point differences in alleles. This separation will be accomplished by modifying another analysis derived for Roche 454 technology (Galan et al. 2010). By plotting the distribution of the frequency of variants within individual samples, one expects to obtain multimodal distribution of allelic coverage based on the number of loci in the genome (Figure 5). The normally distributed peaks at ~30-60% and 80-100% correspond to heterozygosity and homozygosity (with allowance for normally distributed coverage bias). However, the large peaks at 1-4% frequency indicate rare variants recovered within an individual. Given the low gene copy number, rare variants are likely artifact and are removed from further analysis.

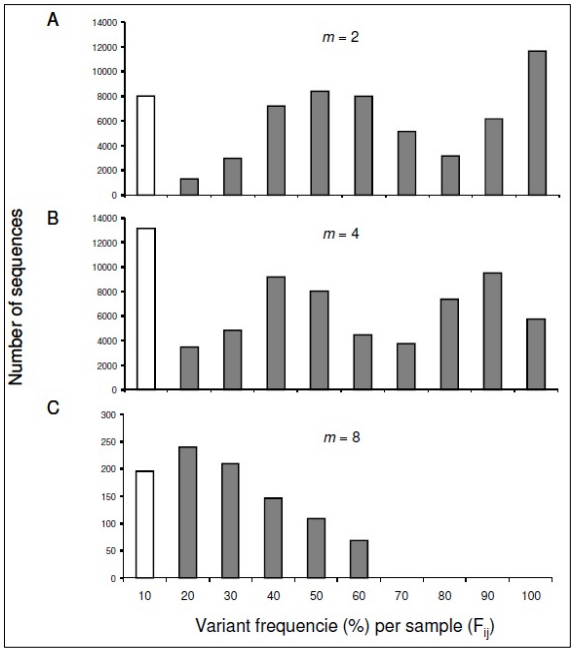

Figure 5. Recovered multimodal distribution of individual allele frequencies given different numbers of genomic copies (m). White bars are rare variants that are removed from the analysis. Modified from Galan et al. 2010.

Once alleles are recovered, heterozygosity and allelic richness for each locus (all microsatellites and MHC II exon 2) for each individual will be calculated using Arlequin software (Excoffier et al 2005). We will then compare the allelic diversity levels between these subpopulations to evaluate whether polyploids experience an increase in internal diversity of disease response genes, above that of the background genomic diversity and their sympatric sperm donor species but not more than the glacially unaffected parapatric sperm donor populations.

C.1.2.3 Alternatives

If Illumina sequencing proves incapable of discerning alleles from artifact due to an inability to compensate for amplification bias or unforeseen complications, traditional plasmid cloning of PCR products will replace next generation sequencing.

C.2 Characterize the effect of polyploid presence on total allelic distribution of populations within the polyploid range (Aim 2)

C.2.1 Core Concept: Kleptogenesis (Figure 1) complicates the scaling of internal variability to subpopulation level.

- If polyploids have low individual diversity, they may still have a large allelic pool due to numerous clonal lines and ploidy levels (3-5N).
- If polyploids have high individual diversity, they may still have a small allelic pool due to clonal reproduction.
- The diversity trends may not scale to the range level either, as evolutionary forces are not constant as distance from glacial refugia increases.

The increased variants and copies available to each individual are important to their ability to respond to disease, but are also important for increasing the number of different combinations possible in a population. Having population level diversity, particularly in MHC II genes, is crucial to the stability against widespread susceptibility to diseases. Understanding the factors the effect this population level diversity, such as founder effects and polyploid dominance, is important in predicting the disease risk and consequences of future pathogenic spread. In this portion of the study, we seek to quantify founder effects on the populations in the Great Lakes Region, characterize the genetic diversity of each subpopulation, and investigate how the polyploid dominance skews population level genetic diversity.

We predict that MHC II gene diversity will remain higher than the background genomic diversity in all subpopulations, though the diversity of both will likely dwindle with increased distance from glacial refugia. However, because of the advantages of being polyploid in light of diversity (Aim 1), we **hypothesize** that *the polyploid subpopulations will maintain higher levels of overall diversity compared to diploid subpopulations.* As a result of this increased diversity, we also predict that when considered at the whole population level, the inclusion of the polyploid subpopulation will significantly increase genetic diversity. Alternatively, as a result of clonal reproduction, an **alternative hypothesis** is that *polyploid subpopulations are heavily skewed to a few clonal lines, reducing overall population diversity.* The determination of which trend occurs will lead support or refute of our theory that the allelic diversity enjoyed by polyploid salamanders acts to buffer the low diversity of the sexual population.

C.2.2 Scientific Approach:

C.2.2.1 Data

The sequence data obtained in Aim 1 (C.1.2) will be used to address this aim; however, instead of obtaining individual levels of diversity, subpopulation level measures will be obtained. The populations chosen for investigation will reside along rough transects with increasing distance from two glacial refugia, one on either side of the Appalachian Mountains (white arrows, Figure 2). Both transects will begin south of the polyploid zone, to incorporate parapatric sperm donor populations and address populations unaffected by glacial pulses, and extend north into previously glaciated regions. The use of both transects allows for replication as well as more range-wide analysis of allelic diversity.

C.2.2.2 Data Analysis

See C.1.2.2 for recovery of allelic diversity.

The focus of this aim is to 1) address the subpopulation level diversity and the relative effects of such diversity upon total population and 2) identify founder effects at the range level. To address subpopulation genetic diversity, both microsatellite and MHC II exon 2 diversity will be calculated for each subpopulation category in each population along the transect. Specifically, divergence between subpopulations (Fst), heterozygosity, and allelic richness will be obtained for each group in Arlequin (Excoffier et al 2005). To address the effect of polyploid presence on the total population diversity, each population's measures of diversity will be calculated with and without the inclusion of the polyploid subpopulation in the data set. This analysis will determine if there is a significant increase in the genetic diversity at the full population level due to the presence of polyploids.

Distance from glacial refugia will be determined by geographic distance from the boundary of the last glacial progression (roughly the southern border of the polyploid zone in Figure 2). A regression of the distance to each diversity measure will be conducted for each locus (microsatellite and MHC II exon 2) to quantify any change in diversity correlated to distance from glacial refugia.

C.2.2.3 Alternatives

As this portion of the study is a different analysis of the same data obtained in the first aim, no alternative means of data acquisition are needed beyond those described above in C.1.2.3. Analysis programs are the most common used in the

literature, but if found to be unsuitable for our study because of inability to deal with polyploid characteristics or otherwise unforeseen complications, alternative analysis programs that address diversity will be investigated.

C.3 Determine fitness advantage and constraints to MHC variability using immune challenge experiments (Aim 3)

C.3.1 Core Concept – Polyploidy may decouple MHC genotype from immune response phenotype in unexpected ways.

The increase in genetic diversity, particularly in immune response genes, is important to the stability and resilience of populations to disease, but only if the variability is functional. Having increased genomic copies causes complications in gene expression because of the mechanical issues involved with enlarged nuclei and chromosome density (Osborn et al. 2003, Beçak and Kobashi 2003). Having increased copies does not help much if they are not expressed, nor does having increased individual diversity if the alleles conflict. In this portion of the study, we seek to investigate the bounds of functional allelic diversity in this system as well as quantify factors that may confound the connection between genotype and phenotype, specifically unexpressed pseudogenes, polyploidy, and allelic conflict.

We predict that the polyploid salamanders will survive and grow better than the sympatric sperm donor species in response to disease challenges. We also predict that the *Ambystoma* polyploids will have increased relative fitness in disease challenges, with an optimal diversity bounded by lowered ability to recognize pathogens due to low allelic diversity and antagonistic conflict between alleles due to conflicting alleles. Finally, we predict that survivability and growth will be correlated to MHC II diversity but not background microsatellite diversity. If confirmed, these predictions would support the **hypothesis** that *polyploid salamanders have higher disease response fitness due to their hybrid origin and polyploid make up and that disease and that the diversity found in the two above aims is functional*.

C.3.2 Scientific Approach:

C.3.2.1 Data

Sampling: In order to test the fitness effect of individual allelic diversity of MHC, polyploidy, and general genomic diversity upon fitness in the face of disease, survivorship experiments will be conducted. Only one population will be initially sampled for this portion of the study in order to reduce animal use, however, if the results from the above sequencing efforts indicate high diversity in populations, more populations will be added to the experiment. Due to the location of the investigators, the population selected will be an *Ambystoma laterale* dependent population, likely from Potato Creek State Park.

Ten egg masses (~30 eggs each) of each subpopulation type will be collected from the field via dip netting and reared in the lab as per Heyer 1994. These egg masses consist of at least half siblings (polyploids may be full clones). Polyploid egg masses are identifiably different from diploid egg masses (Bogart personal communication), allowing for visual identification and collection of sufficient numbers of both subpopulations. These will be housed into glass gallon jars and incubated in the existing culture room at University of Notre Dame until larval emergence.

Survivorship: Larvae will be separated into individual beakers to prevent cannibalism or resource competition. Once the larvae are free swimming at Gosner stage 26 (Gosner 1960, Jackson and Tinsley 2003), larvae will be exposed to known amphibian fungal parasites (A - *Aeromonas hydrophila* and B -*Batrachochytrium dendrobatidis*) by adding an inoculum of the pathogens at naturally occurring dosages ($1x10^6$ colony forming units (Barribeau et al. 2009) and $1x10^5$ zoospores (Savage and Zamudio 2011), respectively) to the water. These two pathogens were chosen for their proven record of differential infection of genotypes as well as their extracellular nature. One hundred and twelve individuals from each subpopulation will be randomly assigned to one of four groups with two replicates each (eight individuals per replicate per group): control with heat killed A, control with heat killed B, control with heat killed A + B, live pathogen A, live pathogen B, and live pathogen A + B. This sample size has been successful in previous studies of MHC diversity and disease fitness (Barribeau et al. 2009). A combined pathogen condition is included as multiple stressors are more likely to demonstrate the fitness of heterozygotes if present (Kurtz et al. 2005).

Larvae will be monitored every day. Survival and growth of the larvae will be measured every other day for 30 days. Survival is defined as responsive to touch and mobile. Growth will be measured via dissection microscope and defined as overall length from mouthparts to distal edge of muscular tail. Animals exposed to pathogens and lab conditions cannot be re-released due to concerns with introducing pathogens to native populations. As a result, all remaining larvae at the end of the experiment will be euthanized via submersion in 0.2% Benzocaine solution and tissue will be collected as described above. Tissue samples will be obtained post euthanasia for identification of ploidy and allelic diversity as in C.1.2. Additionally, the lab reared larvae allow for the sampling of high quality RNA without worry of sample degradation in transport back to the lab. Direct confirmation of expressed genes is needed to determine which copies of the genes sequenced from the tissue samples are functional.

Molecular: Genomic DNA will be extracted and purified as above using DNeasy and DNA Minielute kits (Qiagen). RNA extractions will be performed with standard lab protocol as described by the manufacturer of the RNA kits (Qiagen). Primers for the alleles obtained in DNA analysis will be used to test for presence of the RNA transcripts.

C.3.2.2 Data Analysis

MHC variability, ploidy, genomic composition, and microsatellite diversity will be obtained through the methods described in C.1.2. To determine the relationship between fitness and different measures of genetic diversity, an AMOVA will be performed regarding survivorship, growth, ploidy, and microsatellite and MHC diversity measures (as described in Aim 2). Additionally, regression analysis of fitness metrics versus diversity metrics will be performed for each test group.

All alleles recovered from Aim 1 will be clustered by lack of co-occurrence (Babik et al. 2008). Under-enrichment of transcripts from any loci cluster will be determined in order to identify putative pseudogenes.

C.3.2.3 Alternatives

As few studies of *Ambystoma*ta susceptibility to pathogens have been performed, it is possible that our chosen pathogens will not be suitable for this experiment. In this case other extracellular pathogens, such as monogenan flatworms (Jackson and Tinsley 2003) or *Saprolegnia* species (Romansic et al. 2008), will be substituted. Additionally, dosages may have to be adjusted.

C.4 Time Line

Sequencing of MHC II exon 2 loci in University of Guelph samples will be complete by early 2013. Field collections for the remaining required populations will begin April 2013, including collection of egg masses for rearing in lab. Survivorship trials will be complete by July 2013. Sequencing and analysis of Field collections and survivorship experiments will continue through March 2014. If further sampling is required, field sampling and retrieval of University of Guelph extractions will be completed between April and June 2014. Final write up and analysis is expected to be complete by December 2014.

C.5 Summary

Understanding disease risk, predicting the consequences of future exposure to pathogens in changing environments, is important in conservation efforts to battle the world wide amphibian declines. We seek to fill the gaps in knowledge in variation in the number of immune responsive loci, geographic diversity of the immune response, and genetic variability's relationship to functional response, specifically in the potentially at risk but understudied Great Lakes Region. This study will also help evaluate the impact of the polyploid *Ambystoma* on the stability and resilience of diploid salamander species, many of which are of conservation concern.

Ambystoma salamanders are especially well suited to the investigation of factors effecting functional diversity of disease resistance, being both common in a potentially at risk region and allowing for investigation of the impact of gene copy number, allelic richness, founder effects, and unisexual dominance on measures of fitness. Investigation into these interactions of these factors will be applicable to other amphibians, other vertebrate polyploids, and other species in the Great Lakes Region. For example, this study will identify population risk factors - such as distance from glacial refugia and polyploid dominance - that are applicable to other systems and may inform targeted conservation efforts. Finally, this project will support the collaboration between academic, governmental, and citizen scientists in the region as well as improve the understanding of vertebrate polyploidy and its effect on the immune system.

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