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Genetic and expression changes in Lactate dehydrogenase as potential mechanism for promoting adaptation

by

Bora Demiri

A Thesis
Submitted to the Faculty of Graduate Studies
through the Great Lakes Institute for Environmental Research
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Windsor, Ontario, Canada

2011

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Genetic and expression changes in Lactate dehydrogenase as potential mechanism for promoting adaptation

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Bora Demiri

APPROVED BY:

_____

Andrew Hubberstey, External Reader
Department of Biological Sciences

_____

Daniel D. Heath, Internal Reader
Great Lakes Institute for Environmental Research

_____

Teresa J. Crease, Co-Advisor
Department of Integrative Biology, University of Guelph

_____

Melania E. Cristescu, Advisor
Great Lakes Institute for Environmental Research

_____

Aaron Fisk, Chair of Defense
Great Lakes Institute for Environmental Research

October 14, 2011

DECLARATION OF ORIGINALITY

I hereby declare that this thesis incorporates material that is the result of joint research. My data chapter was co-authored with my supervisors, Dr. Melania Cristescu and Dr. Teresa Crease. My advisors provided valuable feedback, helped with the project design, and provided editorial input during the writing of my manuscript. However, the primary contributions have been by the author and no part of this thesis has been published or submitted for publication.

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ABSTRACT

Understanding the genetic basis of adaptation is a central task for evolutionary biology. Here, I examined how *Ldh* gene expression contributes to the adaptation of *Daphnia pulex* and *Daphnia pulicaria* by using a controlled acclimation experiment. There were no statistically significant changes ($p>0.1$) in gene expression due to changes in temperature, dissolved oxygen. However, both *Ldh*A and *Ldh*B expression were significantly different in each type of *Daphnia* (pond, lake or hybrid) suggesting that these genes could contribute to adaptation or could be linked to loci that do. *Ldh*A was expressed significantly more than *Ldh*B ($p<0.005$), in all clones. Analysis of the protein structures of LDHA and LDHB revealed that both are functional LDH proteins, with very similar secondary structures. These results confirm that while the protein structures of *Ldh*A and *Ldh*B where very similar, expression of these genes could contribute to the adaptation of pond and lake *Daphnia*.

DEDICATION

To My Mom and Dad

ACKNOWLEDGEMENTS

I would like to thank my advisors, Dr. Melania Cristescu and Dr. Teresa Crease for taking me on as their graduate student and encouraging me throughout these two years. Both Melania and Teresa have shown me support and valuable guidance throughout my studies. I would like to thank Melania for being my mentor and for always finding the time to help me with my research, and Teresa for all her ideas, continuous feedback and stimulating discussions. I also thank the members of my graduate committee, Dr. Andrew Hubberstey and Dr. Daniel Heath, for their guidance, insightful discussions and suggestions.

Albana Pecani and Pulkit Sahota helped me tremendously with *Daphnia* culturing during the acclimation and gene expression experiment. In addition, I would like to thank Rebecca Williams for all her help and valuable advice on qPCR procedures. I would like to thank my lab mates, Ianina Altshuler, Sen Xu, and Anna Constantin for providing valuable knowledge and assistance during this study and to the rest of the Cristescu lab for their friendship, and encouragement throughout these two years. I am also grateful to Mary Lou Scratch for being so helpful.

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INTRODUCTION

Natural selection increases the frequency of genotypes that improve the survival and reproduction of an individual in a population (Dobzhansky and Pavlovsky, 1957). Mutations are introduced into the population randomly and they are preserved through genetic drift or natural selection (Dobzhansky and Pavlovsky, 1957). Natural selection works on pre- existing mutations and results in the adaptation of populations of a species to a particular niche and may eventually lead to the emergence of new species (Dobzhansky and Pavlovsky, 1957). While there are many examples of adaptation in natural populations (Crawford and Powers, 1992; Oleksiak *et al*., 2002; Dalziel *et al*., 2009) the mechanism of adaptation is an essential unsolved problem in evolutionary biology. In an attempt to understand this mechanism, allozyme analysis, DNA sequencing, and heritable differences in gene expression have been used to identify individual genes that contribute to adaptation (Nuzhdin *et al*., 2004).

Lactate dehydrogenase and adaptation

Molecular changes that contribute to the adaptation of a species to its local environment are a central aspect of evolutionary studies (Karl *et al*., 2009). Adaptation to a new environment is accomplished evolutionarily either by molecular changes in the genome that alter intrinsic enzymatic rate constants or by a heritable change in the control region of the genome influencing transcript level and consequently enzyme concentration (Crawford and Powers, 1992). Enzymes involved in the metabolic pathways of an organism are likely to contribute to the organism's adaptation to its *environment* (Eanes, 1999). Studies investigating the genetic basis of fitness differences in natural populations

are often based on a category of genes that encode for proteins involved with glucose metabolism, the energy production cycle (Dahlhoff and Rank, 2000; Haag *et al*., 2005; Ellegren and Sheldon, 2008). One such group of enzymes are the NAD-dependent L-lactate dehydrogenases (LDH), which are required by most organisms in order to reduce pyruvate to L(-) lactate during glycolysis and oxidize L(-) lactate to pyruvate during the process of gluconeogenesis (Everse and Kaplan, 1973). Temperature and oxygen levels have been shown to effect the expression of lactate dehydrogenases and their enzymatic function (Crawford and Powers, 1992; Weider and Lampert, 1985). Furthermore, LDH has been shown to contribute to the adaptation of natural populations in different habitats (Crawford and Powers, 1992; Powers *et al*., 1991; Oleksiak *et al*., 2002; Schulte *et al*., 2000; Johns and Somero, 2003). For instance, populations of *Fundulus heteroclitus* in Maine and Georgia are fixed for two different alleles at the heart-type *Ldh*B locus. The isozymes encoded by this locus have different temperature-dependent kinetic characteristics due to a few amino acid replacements making these two populations more adapted to their different thermal environments (Powers *et al*., 1991). Also, the expression of *Ldh* in natural populations of one species or sister species adapted to different environments, changes with variation of temperature across a species geographic range (Crawford and Powers, 1992) and is the same in sister species that inhabit the same thermal environment (Oleksiak *et al*., 2002). In addition, an increase in the activity of lactate dehydrogenase-B (LDH-B) in the killifish, *F. heteroclitus* is observed during prolonged exposure to hypoxia (Rees *et al*., 2001).

A strong association between *Ldh* genotype and habitat has also been observed in members of the *Daphnia pulex* species complex. The pond species, *D. pulex* and the

closely related lake species, *D. pulicaria* are fixed for two different alleles at the *Ldh* A locus. The fixation of different *Ldh* variants in these two sister species found in different aquatic habitats suggests that LDH variation is possibly adaptive or linked to loci that are (Pfrender *et al*., 2000; Crease *et al*., 2011). These studies collectively suggest that LDH plays an important role in the adaptation of a species. However, very little is known about the manner in which LDH effects adaptation. Therefore, it is of interest to identify changes in amino-acid-coding regions of *Ldh* sequences from closely related species found in different environments or look at the natural variation in gene expression that exists among these sister species in a controlled lab experiment.

Daphnia pulex & *Daphnia pulicaria*

The specious and cosmopolitan genus *Daphnia* is ideal for studying molecular changes that contribute to adaptation because it inhabits multiple freshwater habitat types with distinct ecological challenges (Pfrender *et al*., 2000; Dudycha, 2004; Lynch *et al*., 1999). One group of *Daphnia* receiving particular attention is the *Daphnia pulex* species complex, an assemblage of 14–15 described species that inhabit both lake and pond habitats in the Holarctic (Pfrender *et al.*, 2000). Two widespread members of this group include a pond-dwelling form called *Daphnia pulex* and a lake-dwelling form, referred to as *Daphnia pulicaria* (Pfrender *et al*., 2000). The two sister species, *D. pulex* and *D. pulicaria*, are estimated to have diverged ~82,000 years ago but still experience significant levels of gene flow (Omilian and Lynch, 2009). In laboratory settings, *D. pulex* and *D. pulicaria* interbreed creating hybrids (Heier and Dudycha, 2009). However, naturally occurring hybrids tend to be found in different niches from the parents,

particularly disturbed habitats, and have unique life histories (Heier and Dudycha, 2009). For example, in Ontario and Michigan's lower-peninsula, hybrids tend to occur in areas where forests have been cleared. Commonly, these hybrids are known as ''urban'' *Daphnia*, and generally reproduce by obligate parthenogenesis (Hebert and Crease, 1983). This mode of reproduction of hybrids, in contrast to the normal cyclic parthenogenesis of *D. pulex* and *D. pulicaria*, is one of the strongest pieces of evidence indicating that *D. pulex* and *D. pulicaria* are two species being driven toward separate evolutionary trajectories by ecological pressures (Heier and Dudycha, 2009).

Several studies have found that *Daphnia* in lakes and ponds differ considerably in their life history traits (Dudych and Tessier, 1999; Dudycha, 2003, 2004) since these habitats are characterized by different physical and biotic conditions (Wellborn *et al*., 1996). *Daphnia pulex* inhabits fishless, ephemeral ponds for a short period of time in the spring and early summer where it is exposed to invertebrate predation, anoxia, and complete freezing (Colbourne *et al*., 1997). Conversely, *D. pulicaria* populations can be found year-round in most stratified lakes (Cáceres and Tessier 2004). Furthermore, they inhabit the cold hypolimnetic region of the lake to avoid fish predation (Wright and Shapiro 1990). It is likely that these two species have diverged due to different environmental pressures in their habitats such as different temperatures, different dissolved oxygen concentration, selection regimes, biotic interactions, and food sources. However, despite advances in understanding the ecology of *Daphnia* and the genomic tools available we lack the understanding of the molecular mechanism used by these species for adaptation in ephemeral ponds and permanent lakes.

Lactate dehydrogenase in *Daphnia*

North American *D. pulex* and *D. pulicaria* are nearly morphologically indistinguishable but they are presently recognized as both ecologically and genetically distinct species (Pfrender *et al*., 2000; Hebert and Finston, 2001; Dudycha, 2004). Since morphological discrimination is difficult, these species are often distinguished on the basis of diagnostic alleles of the L-Lactate dehydrogenase A (*Ldh*A) locus (Heier and Dudycha 2009). Populations occurring in temporary ponds are most of the time homozygous for the slow allele (S) of *Ldh*A and are classified as *D. pulex*, whereas populations in permanent lakes are homozygous for the fast allele (F) and classified as *D. pulicaria* (Crease *et al*., 1997; Pfrender *et al*., 2000; Hebert and Finston, 2001; Heier and Dudycha, 2009). Even though their geographic range overlaps, they dwell in distinct habitats (Hebert and Crease, 1983). Hybrids of the two species, with the heterozygous SF (Slow/Fast) genotype for the *Ldh*A locus, can be found in nature in disturbed, deforested ponds (Hebert and Crease 1983).

Although *D. pulex* and *D. pulicaria* in North America have distinct mitochondrial lineages, there are many lake populations of *Daphnia* that have a *D. pulex*-like mitochondrial DNA while maintaining the *D. pulicaria Ldh*A profile (FF) (Crease *et al*., 2011). The maintenance of the *Ldh*A-F allele in lake populations of *Daphnia*, regardless of maternal origin, suggests that *Ldh*A genotypes differ in physiological performance, which may affect fitness (Cristescu *et al*., 2008). The predominance of the *Ldh*A-F allele in lake populations of *Daphnia* and the *Ldh*A-S allele in pond populations suggest that each allele may increase the organism's fitness in its corresponding habitat. In addition to *Ldh*A, the genomes of *D. pulicaria* and *D. pulex* code for a second Lactate

dehydrogenase isoenzyme, LDHB. Based on phylogenetic studies, the two loci (*Ldh*A and *Ldh*B) seem to be the product of a relatively recent gene duplication event in the *Daphnia* genome, after the divergence of *Daphnia* from other crustaceans (Cristescu *et al*., 2008).

This study seeks to understand whether the two Lactate dehydrogenase loci (*Ldh*A and *Ldh*B) contribute to the adaptation and speciation of *D. pulicaria* and *D. pulex*. More specifically, my work includes two main approaches to studying the significance of LDH proteins to the adaptation of these sister species; a protein structure study and a gene expression study. The protein structure study is based on LDHA and LDHB amino acid sequences from *D. pulex* and *D. pulicaria*, collected from 58 sampling sites in Canada and the United States. The gene expression study examines the expression of *Ldh*A and *Ldh*B of pond, lake and hybrid *Daphnia* that were acclimatized to 4 different environmental settings, each characterized by different temperatures, and a different concentration of dissolved oxygen (DO).

MATERIALS AND METHODS

Sample collection

Pond samples were collected with a dip net from shore, while lake samples were obtained by towing a plankton-net vertically through the deepest part of each lake. Following sampling, individual female *Daphnia* were allowed to reproduce parthenogenetically and establish clonal lines in separate 250 ml beakers. The isolates were maintained in filtered Detroit river water at 15 - 18 °C with a 14-h light, 10-h dark photoperiod and fed every 3-4 days with a combination of the microalgae species *Nannochloropsis* and *Tetraselmis* (Reed Mariculture) diluted in ddH_2O.

Daphnia pulex and *Daphnia pulicaria* were collected from a total of 9 habitats (3 lakes and 6 ponds) in Michigan and southern Ontario (Figure 1). *D. pulicaria* isolates were collected in July 2008 and May 2009 from three permanent lakes; Lawrence, Warner, and Three Lakes II, all located in south-western Michigan, USA (Figure 1). All three lakes are thermally-stratified hard-water lakes with small surface area (<30 ha), more than 10 m deep (Leibold and Tessier 1991), and have similar zooplankton communities (Haney and Hall 1975; Leibold and Tessier 1991). The *D. pulex* isolates were collected in the spring from three ephemeral ponds, Disputed and Canard 1 located in southern Ontario, and Solomon, located in south-western Michigan (Figure 1). The lake-pond hybrids were collected in the spring and early summer of 2009 from three ephemeral ponds, Canard 2, Canard 3 and Disputed, all located in southern Ontario (Figure 1).

Temperature and dissolved oxygen measurements

The temperature and dissolved oxygen concentration of all ponds located in southern Ontario was measured, every week during spring and early summer of 2010.

These measurements were taken to better comprehend the temperature and dissolved oxygen conditions that *Daphnia* experience in ephemeral ponds. As temperature increased throughout the season, dissolved oxygen concentration decreased. The last week of March was characterized by low temperature and relatively high dissolved oxygen (5.52 °C; 5.19 mg/l), while high temperatures (19.96 °C) and low dissolved oxygen concentrations (1.11 mg/l) were recorded for the third week of June.

Lactate dehydrogenaseA Survey

PCR and gel electrophoresis was used to confirm that the lake *Daphnia* had a *Ldh*A-FF genotype, pond *Daphnia* had the *Ldh*A-SS genotype, and that the pond-dwelling hybrid *Daphnia* were characterized by the *Ldh*A-SF genotype. The primer sets used span the single nucleotide polymorphism that differentiates between the two alleles at the *Ldh*A locus (Crease *et al*., 2011). The primer sequences are as follows: *Ldh*A slow allele Forward 5'-GAG CGA TTT AAC GTT GCG CCC-3', *Ldh*A slow allele reverse 5'-GGA CGA CTT GTG TGT GAA TTT G-3', *Ldh*A fast allele forward 5'-GAG CGA TTT AAC GTT GCG CCT-3', *Ldh*A fast allele reverse 5'- GGA CGA CTT GTG TGT GAA TTT C-3'. *Daphnia* genomic DNA was used as template to amplify 248 bp sequences using Top Taq DNA polymerase (cycling conditions: 94 ºC hot start, followed by 35 cycles at 94 ºC for 30 s, 58 ºC for 30 s, 72 ºC for 30 s, and 4 ºC hold).

Acclimation Experiment

D. pulex and *D. pulicaria* individuals were isolated and cultured as clonal lines under standardized conditions, as described previously, for a period of at least one year in

order to minimize maternal effects. Females from three clonal lines from each environment were selected to establish the mother generation of the acclimation experiments. Each female *Daphnia* was placed in a separate aquarium chamber.

The four treatments were characterized by a different temperature, 10 °C or 20 °C, and a different concentration of dissolved oxygen (DO), 6.5-7 mg/l or 2-3 mg/l. The aquaria were placed in two different environmental chambers that had an ambient temperature of 10 °C and 20 °C respectively. The high DO concentrations in the aquaria were obtained by pumping air in the water through an air pump, Topfin Airpump 8000, for 30 min twice a day. The low DO concentration in the other two aquaria was obtained by pumping Nitrogen gas in the aquaria for 30 min twice a day. The DO concentration and the temperature of each aquarium were measured twice daily. Each aquarium contained 27 chambers, 3 cm in diameter and 9.5 cm long. Each chamber had a 60 micron plankton-net floor that allowed the water and the food to circulate freely within the aquarium (Figure 2). The *Daphnia* in each aquarium chamber were fed daily with 1 ml of the unicellular green algae *Scenedesmus* cultured by the Lynch Lab at Indiana University. The *Daphnia* were exposed to 14 h light: 10 h dark cycle. The animals were checked daily, and the number of neonates and the time that the female produced offspring was recorded for every clone. The first clutch of offspring that the female produced was removed, as it is smaller and developed previous to the start of the treatment, and the female was placed back in the chamber. Once the female produced the 2^{nd} and 3^{rd} clutch, the female was removed and the offspring were left in the chamber to mature. When the *Daphnia* reached maturity and eggs could be seen in their brood pouch, they were collected and placed in 400 μL of RNA later (Ambion), and frozen instantly in liquid nitrogen to

prevent RNA degradation. The samples were then stored in -80 °C freezer until their total RNA was extracted.

RNA isolation and cDNA synthesis

RNAlater was decanted from the specimens and RNA was extracted using the RNA extraction and purification protocol provided by the *Daphnia* Genomic Consortium (Lopez and Bohuski, 2007). The total RNA of 4 to 6 individuals from each chamber was extracted. Homogenization of the *Daphnia* was done with a disposable plastic pestle in TRIZOL Reagent (Invitrogen) in order to facilitate the dissociation of nucleoprotein complexes in the tissues. Chloroform was added to each tube in order to isolate the total RNA of the samples. RNA purification was done using the RNeasy Mini Kit (QIAGEN). During RNA purification the samples were exposed to RNase-free DNAse I (QIAGEN) for 15 min prior to RNA elution. The purified nucleic acids were re-suspended in 30 µl of RNAse-free water. The concentration and purity of the RNA samples was checked with the NanoVue spectrophotometer (GE Healthcare Technologies). An amount of 40 ng of RNA from each extraction was reverse-transcribed with Sensiscript Reverse Transcription Kit (QIAGEN) according to the manufacturer's protocol. The *Oligo* (*dT*) *Primer* (50 µM) from Ambion was used for the reverse transcription reaction.

Quantitative PCR

Primers for quantitative PCR (qPCR) of *Ldh*A and *Ldh*B were designed against sequences downloaded from the DOE Joint Genome Institute's *Daphnia pulex* database (http://genome.jgi-psf.org/Dappu1/Dappu1.home.html) using Integrated DNA

Technologie's PrimerQuest^{SM} program that is based on the Primer3 code (Rozen and Skaletsky 2000). The primer sequences (Table 1) for the reference genes TATA box binding protein (*Tbp*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were developed by Spanier *et al.*, (2010). The primers were designed to generate 90 – 216 base pair (bp) products and the primer pair efficiency ranged from 94.6% to 96.6%. The efficiency of the primer pairs was obtained by exporting the optical raw data (not baseline corrected) from the 7500 ABI software after a qPCR run into MS Excel (Microsoft) and processing the data with the program LinRegPCR v. 11.0 (Ramakers *et al.*, 2003; Ruijter *et al.*, 2009). LinRegPCR determines a mean PCR efficiency corresponding to a primer pair by a linear regression fit of the data in the exponential phase of the qPCR reaction. Quantitative PCR reactions were performed on an ABI 7500 SDS Real-Time PCR System (Applied Biosystems, Fosterm City, CA) using SYBR Premix Ex Taq^{TM} (Perfect Real Time) master mix (Takara Bio USA, Madison, WI) according to the manufacturer's instruction. The qPCR reactions were performed in triplicate in a total volume of 20 µl that contained 10 µl of SYBR Premix Ex Taq^{TM} (2x), 2 µl of cDNA template, 0.4 µl of ROX^{TM} Reference Dye, 6.8 µl of H_2O, and 0.4 µl (10 µM) each of the forward and reverse primers. Negative control reactions without cDNA template were also included to check for non-specific signal due to PCR artifacts such as primer dimerization. Amplification was performed on an ABI 7500 SDS real-time thermal cycler (Applied Biosystems, Foster City, CA) using the SYBR Premix Ex Taq^{TM} (Perfect Real Time) TaKaRa recommended parameters for thermal cycling are 95 °C for 10 sec, followed by 40 cycles of 95 °C for 5 sec and 60 °C for 34 sec.

Relative Quantification of Gene Expression Analysis

The threshold for the qPCR was set manually at 0.028, which is in the region of exponential amplification across all of the amplification plots and above the background florescence. The threshold cycle (C_T) value reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold (Livak and Schmittgen, 2001). The average of triplicate C_T-values were used to calculate the difference in average C_T between the genes of interest (*Ldh*A or *Ldh*B) and the internal reference gene (*Gapdh*). The value of $2^{-\Delta CT}$ was used to determine the *Ldh*A and *Ldh*B gene expression in the four different treatments relative to *Gapdh* gene expression, which is assumed to be constant in all the different treatments (VanGuilder *et al.*, 2008). The expression data was corrected in order to fit a normal distribution by using $\ln(2^{-\Delta CT})$. A univariate analysis of variance (ANOVA) was done on the corrected data using IBM SPSS STATISTICS 19.0.0, to determine significant differences in gene expression in samples from different treatments (Table 2).

The $2^{-\Delta\Delta CT}$ method, as described in Applied Biosystems User Bulletin No. 2 (P/N 4303859), was used to calculate relative changes in gene expression of *Ldh*A and *Ldh*B in clones of one environment relative to another environment. The samples used as the basis for comparative results were chosen depending on the effect I was interested in. I looked at four different scenarios: (1) the effect of an increase in temperature at low DO by measuring gene expression of clones from aquarium 6 (Low DO, 20°C) relative to the gene expression of clones in aquarium 4 (Low DO, 10°C); (2) the effect of an increase in temperature at high DO by measuring the gene expression of clones from aquarium 5 (High DO, 20°C) relative to the gene expression of clones in aquarium 7 (High DO,

10°C); (3) the effect of an increase in DO at 10°C by measuring the gene expression of clones from aquarium 7 (High DO, 10°C) relative to the gene expression of clones in aquarium 4 (Low DO, 10°C); and (4) the effect of an increase in DO at 20°C by measuring the gene expression of clones from aquarium 5 (high DO, 20°C) relative to the gene expression of clones in Aquarium 6 (Low DO, 20°C). The average gene expression values were taken for 6 clones of each environment. Significant changes in the gene expression of *Ldh*A and *Ldh*B in each type of clone, due to changes in temperature or DO, were estimated by using a t-Test (t-Test: two sample assuming equal variances in Excel). In addition, the expression of *Ldh*B was compared to the expression of *Ldh*A in each clone from all treatments using the $\Delta\Delta C_T$ method.

The Conserved Domain Database (CDD) Analysis

LDHA and LDHB amino acid sequences from 70 *D. pulex* and *D. pulicaria* isolates (Crease *et al*., 2011) collected from different regions in North America were analyzed using the Conserved Domain Database (CDD) version 2.14 from NCBI (Marchler-Bauer *et al*., 2005). The CDD looks at protein domains within the primary amino acid sequence of interest (Marchler-Bauer *et al*., 2005). The protein domains are distinct units of molecular evolution associated with particular aspects of molecular function such as catalysis or binding (Marchler-Bauer *et al*., 2005). These sequences were analyzed in order to determine if they contained the amino acid residues that make up the functional domains (NAD-Binding site, 18 amino acids; Substrate binding site, 7 amino acids) and the structural domains (Dimer interface domain, 17 amino acids; Tetramer –interface domain, 17 Amino acids) typically present in LDH proteins. In

addition, ClustalW2 was used to align LDHA and LDHB sequences together, to take a closer look at the position of these conserved sites in each unique sequence.

Protein Structure Prediction using the Phyre Server

In order to predict the secondary structure of LDHA and LDHB proteins in the *D. pulex* species complex, I analyzed amino acid sequences from 70 *D. pulex* and *D. pulicaria* isolates from North America using the Protein Homology/analogY Recognition Engine (Phyre). The Phyre server uses a library of known protein structures in order to predict the secondary structure of user-submitted sequence (Kelley and Sternberg, 2009). The output produced by the Phyre server is in the form of a three-state prediction: alpha helix, beta strand and coil (Kelley and Sternberg, 2009).

RESULTS

Effect of temperature increase in *Ldh*A and *Ldh*B gene expression

When looking at the effect of temperature on gene expression, the relative gene quantification method is used to compare gene expression of clones in two aquaria that differ only in temperature. The clones at 10 ^{0}C are used as the basis for the comparative expression results, so the gene expression of the samples at 10 ^{0}C is set to 1.00. Under low DO, the expression of *Ldh*A increased at 20 ^{0}C for pond (1.361 ± 0.120) and lake (1.401 ± 0.163) *Daphnia* and decreased in hybrid *Daphnia* (0.655 ± 0.137) (Figure 3). The expression of *Ldh*B showed a similar pattern to *Ldh*A expression under the same conditions for pond *Daphnia* (1.569 ± 0.184) and hybrid *Daphnia* (0.508 ± 0.099, $p < 0.05$). However, a decrease in *Ldh*B gene expression at 20 ^{0}C was observed in lake *Daphnia* (0.587 ± 0.057). Under high DO (Figure 4), the expression of *Ldh*A was approximately equal in pond *Daphnia* at 10 ^{0}C (1.00 ± 0.118) and 20 ^{0}C (0.998 ± 0.422). However, there was a decrease in expression of *Ldh*A in lake *Daphnia* at 20 ^{0}C (0.645± 0.076), and an increase in *Ldh*A expression in hybrid *Daphnia* at 20 ^{0}C (1.379 ± 0.227, $p < 0.05$). The expression of *Ldh*B at 20 ^{0}C under high DO concentration showed a similar pattern for lake (0.241 ± 0.041, $p < 0.05$) and hybrid (1.338 ± 0.163) *Daphnia*, but a decrease in expression of *Ldh*B was observed in pond *Daphnia* (0.680 ± 0.078, $p < 0.05$).

Effect of dissolved oxygen increase in *Ldh*A and *Ldh*B gene expression

When looking at the effect of dissolved oxygen on gene expression, the relative gene quantification method was used to compare gene expression of clones in two aquaria that differ only in dissolved oxygen concentration. The clones at low DO were

used as the basis for the comparative expression results. Consequently, the gene expression of the samples at low DO is set to 1.00. At 10 ^{0}C (Figure 5), the expression of *Ldh*A in pond *Daphnia* at low DO (1.00 ± 0.126) and high DO (1.120 ± 0.132) were approximately equal. There was an increase in the expression of *Ldh*A in lake *Daphnia* at high DO (1.355 ± 0.136), and a decrease in *Ldh*A expression in hybrid *Daphnia* at high DO (0.560 ± 0.124, $p < 0.05$). The patterns of expression of *Ldh*B are similar to those seen for *Ldh*A under the same conditions for pond (1.01 ± 0.090), lake (2.548 ± 0.262) and hybrid (0.483 ± 0.058, $p < 0.05$) *Daphnia*. At 20 ^{0}C (Figure 6), the expression of *Ldh*A in pond *Daphnia* at low DO (1.01 ± 0.134) and high DO (0.924 ± 0.104) were approximately equal. There was a decrease in the expression of *Ldh*A in lake *Daphnia* at high DO (0.511 ± 0.052), and an increase in *Ldh*A expression in hybrid *Daphnia* at high DO (1.573 ± 0.276). The patterns of expression of *Ldh*B were similar to those seen for *Ldh*A under the same conditions for lake (0.589 ± 0.108) and hybrid (1.628 ± 0.199) *Daphnia*. However, there was a decrease in *Ldh*B gene expression in pond clones exposed to high DO relative to pond clones exposed to low DO (0.693 ± 0.064).

Statistical analysis of *Ldh* gene expression

The results of the ANOVA analyses showed that there was a significant difference in gene expression of both *Ldh*A and *Ldh*B associated with the type of clones (*D. pulex*, *D. pulicaria*, and *D. pulex/pulicaria* hybrids) (source = Type; $p < 0.005$; Table 2). Furthermore, there were significant differences in gene expression between *Ldh*A and *Ldh*B in all clones (source = Gene; $p < 0.05$; Table 2). However, the temperature that *Daphnia* was subjected to (10 ^{0}C or 20 ^{0}C), the dissolved oxygen concentration (high or

low), and the interaction of two or more sources did not produce significant difference in gene expression $p > 0.1$ (Table 2).

Gene expression of *Ldh*B relative to *Ldh*A

The averaged fold change in gene expression of *Ldh*B relative to *Ldh*A is 0.0546 ± 0.0272 in pond-lake hybrid *Daphnia* (Figure 7). This value indicates that *Ldh*B is expressed 12 to 37 times less than *Ldh*A in these clones. Also, the fold change in gene expression of *Ldh*B relative to *Ldh*A is 0.034 ± 0.005 in pond *Daphnia* suggesting, about 25 to 33 times less gene expression of *Ldh*B than of *Ldh*A. In *D. pulicaria* the fold change in gene expression of *Ldh*B relative to *Ldh*A is 0.164 ± 0.0179 indicating that *Ldh*B is expressed 5.5 to 7.1 times less than *Ldh*A (Figure 7).

Protein structural analysis

The LDHA and LDHB proteins are 327 and 324 amino acids long, respectively. All 11 unique LDHA amino acid sequences contain all the specific amino acids at particular sites in the primary protein-structure that make up the NAD-Binding site (18 amino acids), substrate binding site (7 amino acids), dimer interface domain (17 amino acids), and the tetramer (dimer of dimers)-interface domain (17 amino acids) (Figure 8 a). The Conserved domain database (CDD) analysis of the 37 unique LDHB sequences showed that LDHB had all the amino acids that make up the substrate binding site (7 amino acids) and dimer interface domain (17 amino acids) (Figure 8 b). However, the CDD analysis shows as absent two amino acids of the tetramer (dimer of dimers)-interface domain and three amino acids of the NAD-binding domain which are found

nearby to the N-terminus of the proteins (Figure 8 b). In contrast once I aligned the LDHA and LDHB sequences together (ClustalW), I found that the “missing” amino acids were present in LDHB sequences but were excluded from the CDD analysis of LDHB because of a "filter of low complexity" that blocks sequences that have low compositional complexity from being displayed. Therefore, LDHB has all of the binding sites that make LDH proteins functional but they are found closer to the N-terminus of the protein then in LDHA (Figure 9).

The same sequences were analyzed using the Protein Homology/analogY Recognition Engine (Phyre) server, in order to predict the secondary structure of LDHA and LDHB proteins in *D. pulex* and *D. pulicaria*. Despite the variability within alignments of LDHA and LDHB sequences, the two secondary structures obtained for LDHA and LDHB were very similar. The differences between the two structures were near the N-terminus of the peptide sequences. In the LDHA sequences the first 8 amino acids form a random coil and an α helical structure, whereas, in LDHB the first 10 amino acids form a random coil and a β sheet. The rest of the sequences form the same secondary structures (α helices, β sheets, random coil) in the exact same order in both LDHA and LDHB with the only difference being the number of amino acid residues that makes up each of these secondary structures (Figure 10).

DISSCUSSION

Ldh gene expression has been linked with adaptation of species to environments characterize by different dissolved oxygen concentrations and temperatures. Furthermore, *Ldh* sequence variation has been used to distinguish between *Daphnia pulex* and *Daphnia pulicaria*. In this study we look at how *Ldh*A and *Ldh*B gene expression and protein structure could contribute to the adaptation of these two sister species.

Effect of temperature on expression of *Ldh*A and *Ldh*B

Heritable variability in gene expression is a feature of eukaryotic genomes that provides a rich substrate for evolution and adaptation (Stamatoyannopoulos, 2004). For example, gene expression differences are greater between northern and southern populations of *Fundulus heteroclitus* than between southern *F. heteroclitus* and its sister species *F. grandis* (Oleksiak et al., 2002). These patterns of gene expression may be the result of these fish co-adapting to different environments: cold water for the northern *F. heteroclitus* population and warmer waters in the southern *F. heteroclitus* and *F.grandis* (Oleksiak *et al.*, 2002). My results analysis the values $2^{-\Delta CT}$ did not show any statistically significant differences in the expression of *Ldh*A or *Ldh*B between clones found in different temperatures (Table 2). However, the analysis of *Ldh*A and *Ldh*B expression data when temperature is the only variable shows that in some clones there is a significant difference in gene expression due to temperature (Figure 3, 4). The expression of these two genes may help the animals adapt uniquely to different temperatures depending on the type of the clone ($p<0.05$, Table 2). At high DO, the expression of *Ldh*A and *Ldh*B increased at 10 °C compared to 20 °C for the lake *Daphnia* (Figure 4). In

addition, the same pattern of expression was observed for *Ldh*B in pond *Daphnia* (Figure 4). Conversely, in hybrid *Daphnia* the *Ldh*A and *Ldh*B expression was higher at 10°C compared to 20 °C when individuals are found in water with low DO concentration (Figure 3). An increase in the expression of *Ldh* at low temperatures was also seen in a study done by Crawford and Powers (1992), which found that in order for populations of *F. heteroclitus* from colder waters to maintain the same LDH-B reaction velocities as their southern counterparts, twice as much enzyme is needed for each 10 °C decrease in environmental temperature. The northern population of fish had drastically greater concentrations of both LDH-B enzyme and it's mRNA than did the southern population (Crawford and Powers, 1992). My results also show that when the DO is low the expression of *Ldh*A is higher at 20 °C for both lake and pond *Daphnia* (Figure 3). Conversely, in hybrid *Daphnia* the *Ldh*A and *Ldh*B expression is higher at 20°C when individuals are found in water with high DO concentration (Figure 4). *Ldh* expression does not appear to be systematically up-regulated at a lower temperature as expected from previous studies, but depends on DO concentration and the origin of the clone.

Effect of dissolved oxygen on expression of *Ldh*A and *Ldh*B

LDH catalyzes the final step of the anaerobic metabolic pathway, glycolysis (Rossignol *et al*., 2003). In low oxygen conditions, glycolysis rate should be enhanced in order to compensate oxidative energetic metabolism decay, and pyruvate to lactate transformation must be efficient in order to keep a high NAD^+/NADH ratio and therefore allow continued glycolytic flux (Rossignol *et al*., 2003). In mammals (Semenza, 1999) and fish (Rees *et al*., 2001) genes coding for proteins involved in homeostatic response to

hypoxia, such as LDH are over-expressed in acute hypoxic conditions. Changes in dissolved oxygen concentration elicited opposite expression patterns of *Ldh*A and *Ldh*B in lake *D. pulicaria* and hybrid *Daphnia* (Figure 5, Figure 6). Hypoxic up-regulation of both *Ldh*A and *Ldh*B gene expression was seen in *D. pulicaria* at 20 °C (Figure 6) and hybrid *Daphnia* at 10 °C (Figure 5). However, *D. pulicaria* showed higher expression of *Ldh*A and *Ldh*B at high DO compared to low DO at 10 °C, while pond-lake hybrids showed higher expression of *Ldh*A and *Ldh*B at high DO compared to low DO at 20 °C. In contrast, pond *D. pulex* showed little to no change in the expression of *Ldh*A and *Ldh*B because of changes in DO concentration (Figure 5, Figure 6). The expression of these two genes may help the animals adapt uniquely to different DO concentrations depending on the type of the clone ($p<0.05$, Table 2). Furthermore, it appears from these observations that LDH does not seem to be systematically up regulated under low oxygen supply. This could be due to the fact that *Daphnia* is a hypoxia-tolerant invertebrate and while short responses to hypoxia include activation of glycolytic energy production, response to a prolonged period of hypoxia includes regulated metabolic depression (Paul *et al*., 1998; Gorr, 2004). Regulated metabolic depression is a stress-coping mechanism which relies on energy conservation and involves the suppression of every major ATP-utilizing functions in the cell (Gorr, 2004). *D. pulex* and *D. pulex*/*pulicaria* hybrids, inhabit shallow temporary ponds with fluctuating DO concentration that decreases (5.19 mg/l to 1.11 mg/l) while temperature increases throughout the spring and summer. Conversely, *D. pulicaria* inhabits the hypoxic, perpetually cold, hypolimnion layer of lakes to avoid fish predation (Threlkeld, 1979). Therefore, these *Daphnia* species dwell in environments characterized by long periods of low DO concentration and could be employing regulated

metabolic depression to survive in such environments. However, *Ldh* expression could also play a very important role in the response or detection of decreased dissolved oxygen in hypoxia-tolerant animals. Gorr (2004) exposed *Drosophila* to decreasing amounts of oxygen and found that the first glycolytic gene to be induced was *Ldh*. Therefore, similar pattern could be happening in *Daphnia*.

*Ldh*A vs *Ldh*B gene expression

LDHA and LDHB are isozymes that are found in both *D. pulex* and *D. pulicaria*. Previous studies have shown that both *Ldh*A and *Ldh*B are under purifying selection, but *Ldh*A is under stronger evolutionary constrain compared to *Ldh*B (Crease *et al*., 2011). This suggests that while both may produce functional enzymes, *Ldh*A is likely to be more important in *Daphnia's* metabolism. I analyzed the relative gene expression of *Ldh*B compared to *Ldh*A in order to determine the ratios of the two isozymes in each clone.

The results of the ANOVA showed that the expression of these two genes was significantly different in all clones (source = Gene, p = 0.000 Table 2) *Ldh*A is expressed 12 to 37 times more than *Ldh*B in *D. pulex/pulicaria* hybrids, 25 to 33 times more than *Ldh*B in *D.pulex*, and 5.5 to 7.1 times more than *Ldh*B in *D. pulicaria* (Figure 7). Despite the general low level of expression, *Ldh*B seems to be more important in lake individuals where the ratio of *Ldh*A to *Ldh*B expression is lower in lake *Daphnia* than in pond or hybrid *Daphnia*. My results also agree with previous research on *D. pulex* and *D. pulicaria* where preliminary gene expression results suggested *Ldh*A is expressed significantly higher than *Ldh*B (Cristescu and Egbosimba, 2009). The significantly different level of expression between the two genes could be due to mutations in

sequences regulating expression of *Ldh*B (Hughes, 1994). Immediately after gene duplication, the duplicate gene is redundant in function, and mutations in the control region of the gene will be selectively neutral and may drift to fixation (Hughes, 1994). These mutations could eliminate or reduce expression of one gene copy (Hughes, 1994; Lynch and Force, 2000).

Protein Structure

The analysis of protein structure showed that LDHA and LDHB isozymes in *D. pulex* and *D. pulicaria* code for two closely related proteins. While, both proteins have all of the amino acids that make up the functional and structural domains present in most LDH proteins, these amino acid sites in LDHB are much closer to the N-terminus than in LDHA. In addition, there are amino acid substitutions between the LDHA and LDHB in the conserved-domain-sites (Figure 9) that have similar amino acid side chains but could influence the overall efficiency of one of the allozymes. The placement of these binding sites within the protein structure and in relation to other amino acids in the protein could alter the kinetic properties of the two enzymes. However, more studies on the catalytic efficiencies of both isozymes are needed in order to assess what effect changes in the enzyme structure and animo acid sequence have on the function of the proteins.

LDHA sequences have very limited amino acid variation, with only 9 variable amino acids sites, while LDHB has considerably greater amino acid variation, with 36 variable amino acid positions (Crease *et al*., 2011). My PHYRE results suggest that even though there is ample variation in the primary protein structure of both proteins, all LDHA sequences code for one secondary structure and all LDHB sequences code for

another. These two secondary protein structures differ from each other at the N-terminus of the peptide sequences, where the first 8 amino acids of LDHA form a random coil and α helices and the first 10 amino acids of LDHB form a random coil and a β sheet. The rest of the sequences form the same secondary structures (α helices, β sheets, random coil) in the exact same order in both LDHA and LDHB with the only difference being the number of amino acid residues that makes up each of these secondary structures (Figure 10).

The LDHA allozymes that result from *Ldh*A –S in *D. pulex* and *Ldh*A-F in *D. pulicaria* have the same secondary structure according to PHYRE and the analysis of conserved domains revealed that both sequences contain all the amino acids that make up the functional and structural amino acid domains in LDHA. However, the few amino acid changes in these two sequences could affect the catalytic properties, or the thermo-stability of the enzyme. For instance, an amino acid substitution between LDHB in northern and southern populations of *F. heteroclitus* increases thermostability of the proteins found in southern populations (Bernardi *et al*., 1993). Therefore, to clearly elucidate the strong association between *Ldh*A S and F genotypes and habitat more studies are needed on the function of the proteins and the sequence of the control region of the genes.

*Ldh*A and *Ldh*B are likely the result of a gene duplication that occurred after the divergence of *Daphnia* from the other crustaceans (Cristescu *et al*., 2008). It is generally believed that a duplicated gene can either be silenced by mutations, adapt to a more specialized function, or continue to produce the same protein product (Hughes, 1994; Lynch and Conery, 2000; Innan and Kondrashov, 2010). If the duplicated gene continues

to produce the same protein product, it is usually under purifying selection, as non-synonymous mutations will likely have deleterious effects (Hughes, 1994). Several factors suggest that *Ldh*B could be retaining the same function as *Ldh*A in *Daphnia*; (i) it has been shown to be under purifying selection (Crease *et al*., 2011), (ii) *Ldh*B still maintains all of the amino acids that make up the functional and structural domains of the protein, (iii) it is expressed in the organism in a similar pattern as *Ldh*A, (iv) *Ldh*B codes for a very similar secondary structure to *Ldh*A despite all of the variability in the amino acid sequences.

Conclusions

The question of which genes or genomic regions facilitate the genetic adaptation of organisms to a new environment is central to ecological genetics. This study explores whether the expression and the protein structure encoded by the two L-*Ldh* loci (*Ldh*A and *Ldh*B) contribute to the adaptation of *D. pulicaria* and *D. pulex* to their respective environments. The gene expression study revealed that there were no statistically significant changes in *Ldh* gene expression due to changes in temperature, and dissolved oxygen ($p > 0.1$). This finding is in contrast to previous studies that associated *Ldh* expression with adaptation to a given environmental temperature (Crawford and Powers 1992) and DO concentration (Rees *et al*., 2001). However, studies in *D. magna* have shown that *Daphnia* uses a regulated depressed metabolic rate to survive long periods of hypoxia (Paul et al., 1998), which may prevent systematic up regulation of *Ldh* expression under low DO concentration and temperature. While the two genes might not contribute to the adaptation of the lake, pond and hybrid *Daphnia* to the thermal and DO

conditions that characterize their environments, my experiment showed that these two genes are expressed significantly differently in each type of *Daphnia* ($p<0.05$, Table 2). This suggests that these two loci could be linked to genes that contribute to the adaptation of each type of *Daphnia* to their respective environments, or both *Ldh*A and *Ldh*B expression could contribute directly to the adaptation of lake, pond and hybrid *Daphnia* to their respective environments.

My data shows a significant difference in the gene expression of *Ldh*B compared to *Ldh*A ($p = 0.000$), with *Ldh*A being expressed 6.25 to 33 times more than *Ldh*B times in all clones. In addition, the protein structure analysis found that LDHA and LDHB have all the amino acids that make up the structural and functional domains of most LDH proteins and their secondary structures are very similar. These results confirm that while both *Ldh*A and *Ldh*B code for functional proteins, *Ldh*A is most likely to contribute to the adaptation of pond and lake *Daphnia*. Future studies on the enzymatic activity, thermo-stability and protein synthesis rate of *Ldh*A in pond and lake *Daphnia* could help clarify the strong association between *Ldh*A S and F genotypes and habitat that have been observed in previous studies. Furthermore, the analysis of regulatory sequences of these genes would enhance our understanding of how these genes are regulated and how they contribute to the adaptation of these two sister species.

Table 1 Genes analyzed with qPCR in *Daphnia pulex* and *Daphnia pulicaria*. Here are shown the reference and differentially expressed genes with their respective putative function and gene ID from the Dappu V1.1 draft genome annotation, primer sequences, amplicon size and PCR efficiencies out of 2 as calculated for each primer set by LinRegPCR (11.0). GAPDH and TBP primer sets were developed by Spanier et al. (2010).

Gene Symbol	Gene Name	Function	Gene ID	Primer Sequences [5' to 3']	Amplicon size [bp]	PCR Efficiency
LDHA	L-Lactate dehydrogenase A	Glycolytic enzyme	Dappu-230172	ATC CAG ACT CCT GTT GCC CAT TCA		
				TTC GCC CTT GAG TTT GTC CTC CAT	216	1.898
LDHB	L-lactate dehydrogenase B	Glycolytic enzyme	Dappu-61140	TTG TCC AAT ACA GTC CCG ACA CCA		
				GCA ACC CAC TGA GTT TCC AAG CAA	90	1.931
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme	Dappu-302823	TGGGATGAGTCACTGGCATAC		
				GAAAGGACGACCAACAACAAAC	136	1.903
			Dappu-194512	CTACGATGCATTCGATAACATATACC		
TBP	TATA box binding protein	Transcription initiation		AGAACCAGCAATGAGTTAAACAAAG	144	1.891

Table 2 Analysis of variance of *Ldh* gene expression in *Daphnia*. The gene expression values were calculated using the $2^{-\Delta CT}$ method. The dependent variable is the fold difference in gene expression of *Ldh*A or *Ldh*B relative to *Gapdh* gene expression. The data was then corrected to fit a normal curve. Type refers to pond, lake or pond/lake hybrid *Daphnia*; Temperature is 10 °C or 20 °C; Dissolved oxygen is high or low; Gene is *Ldh*A or *Ldh*B. Interactions are indicated by an asterisk (*). Sources of variation that show a significant difference in gene expression ($p < 0.05$) are in bold-face type.

	Source	(p-value)
Gene		**0.000**
LDHB	Dissolved Oxygen (DO)	0.538
	Temperature (Temp)	0.095
	Type	**0.000**
	DO * Temp	0.829
	DO * Type	0.797
	Temp * Type	0.986
LDHA	Dissolved Oxygen (DO)	0.301
	Temperature (Temp)	0.547
	Type	**0.044**
	DO * Temp	0.519
	DO * Type	0.410
	Temp * Type	0.782

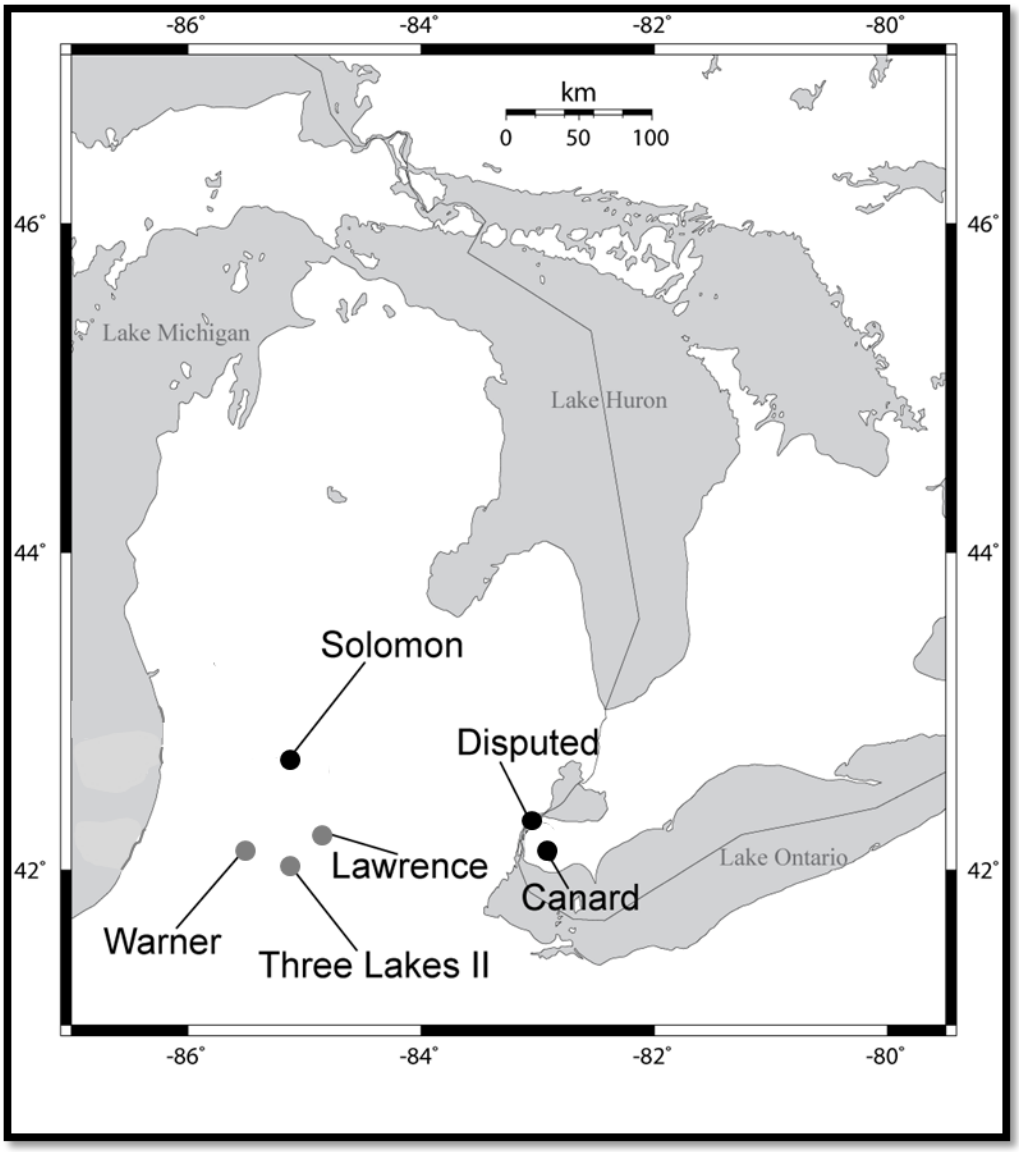
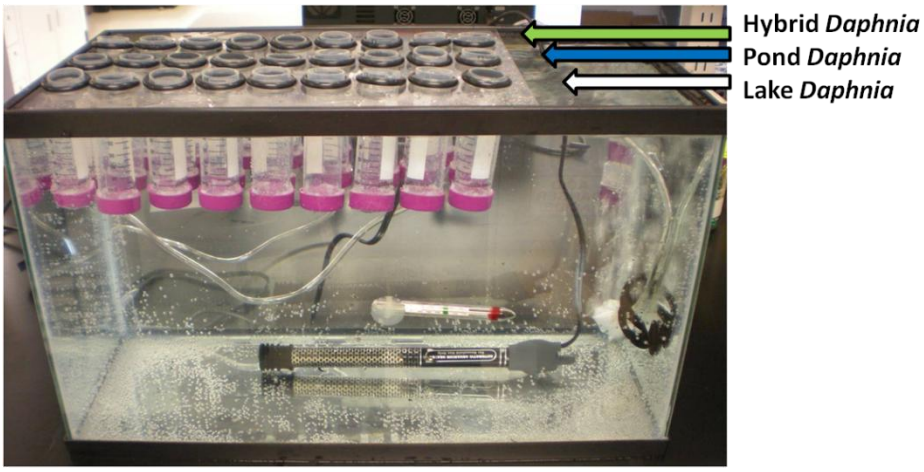


Figure 1 Distribution of *Daphnia* sampling sites in Michigan and Ontario. Pond and hybrid *Daphnia* were collected from five ponds, Disputed, Solomon, and 3 ponds from the Canard area. Lake *Daphnia* were collected from three Michigan lakes: Lawrence, Three Lakes II, and Warner.

a)



b)

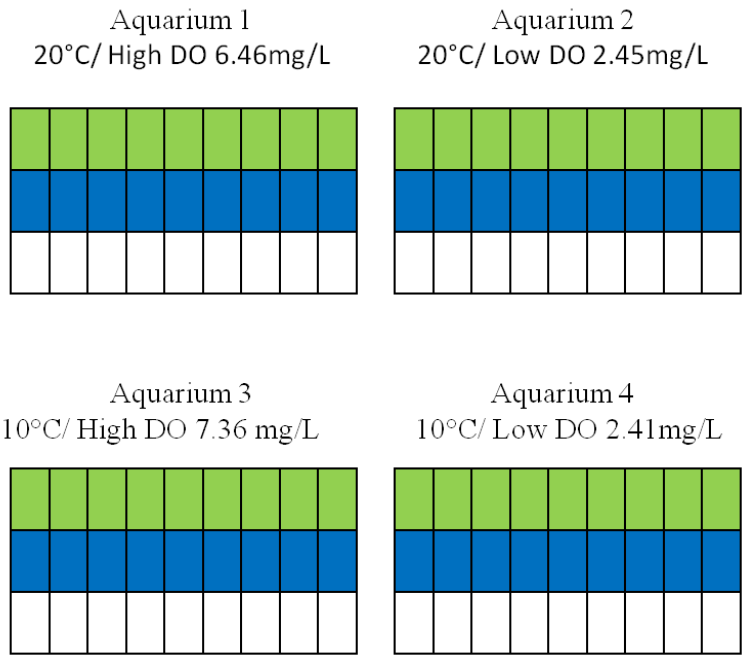


Figure 2 Aquaria set up. a) Each aquarium contained 27 chambers that were filled with 9 hybrid *Daphnia* clones, 9 pond *Daphnia* clones, and 9 lake *Daphnia* clones. Each chamber has a mesh bottom that allows the air/nitrogen and food to circulate freely within the aquarium while keeping the clones isolated from each other. b) There were four aquaria each characterized by different environmental conditions.

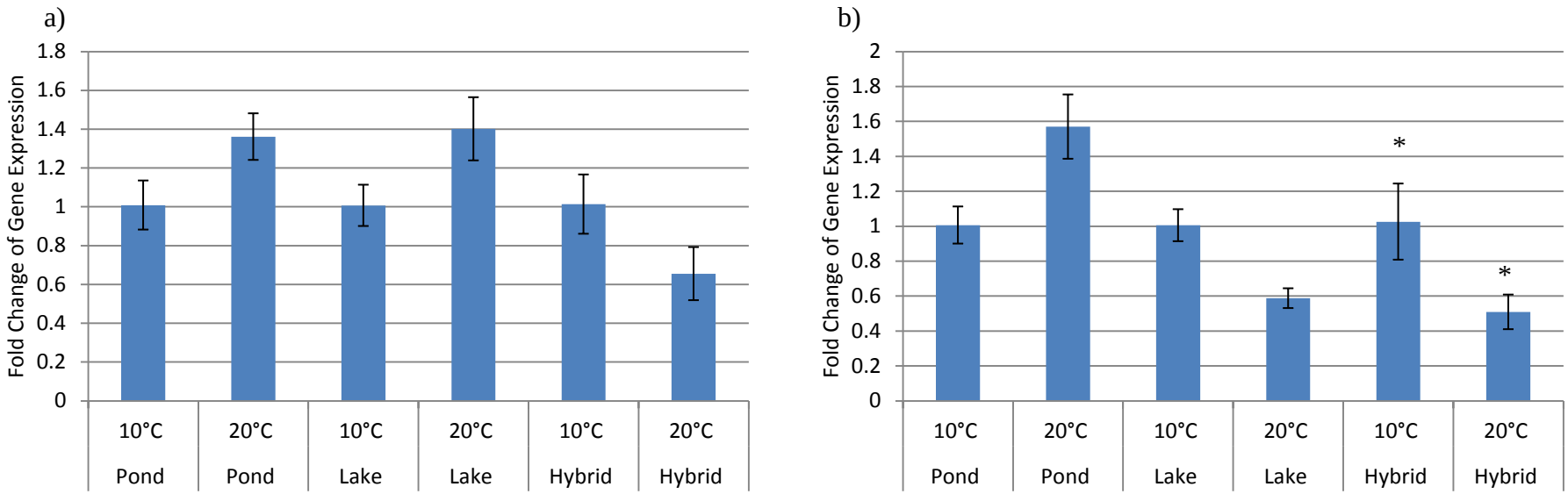


Figure 3 The effect of temperature on a) L*dh*A and b) L*dh*B expression in *Daphnia pulex*, *Daphnia pulicaria* and *D. pulex/pulicaria* hybrids at low DO. The bars indicate fold change of gene expression of clones subjected to 20°C and low DO relative to clones raised 10 °C and low DO. The average gene expression in each habitat at each condition is based on the average expression profiles of 6 clones. The fold change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ comparative C_T method. Significant changes in gene expression were estimated using a t-test and are indicated with * (t-test, $p<0.05$). The vertical bars in this graph indicate the average minimum and maximum values of fold difference in gene expression of *Daphnia* from each habitat in each condition.

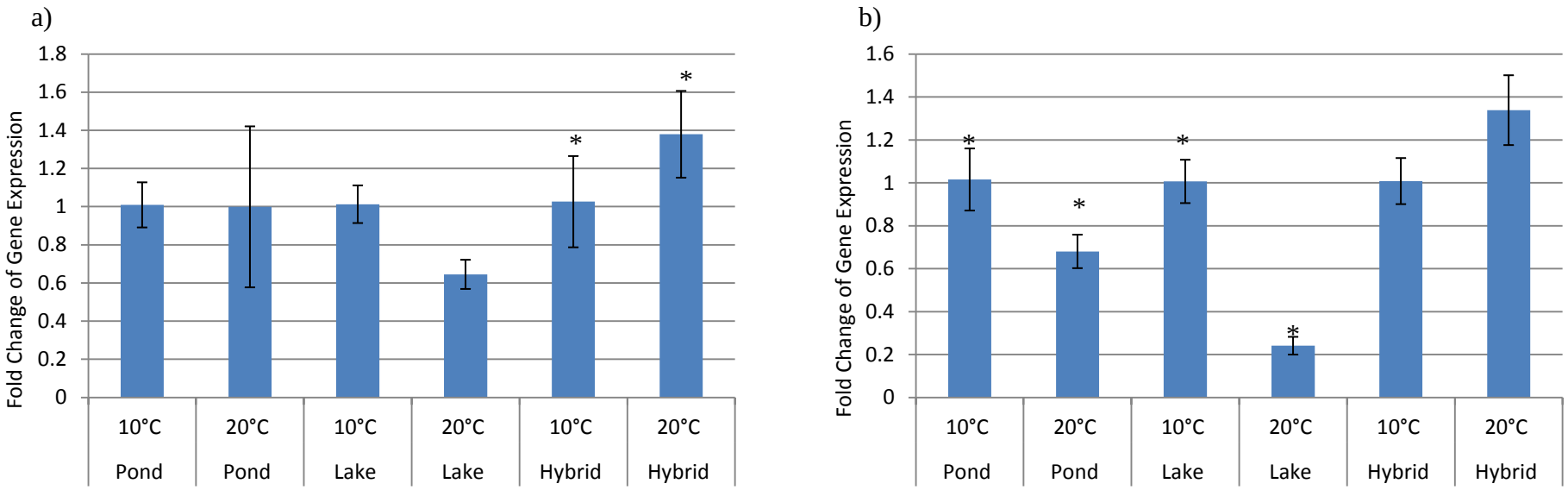


Figure 4 The effect of temperature on a) L*dh*A and b) L*dh*B expression in *Daphnia pulex*, *Daphnia pulicaria* and *D. pulex/pulicaria* hybrids at high DO. The bars indicate fold change of gene expression of clones subjected to 20 ºC and high DO relative to clones raised 10 ºC and high DO. The average gene expression in each habitat at each condition is based on the average expression profiles of 6 clones. The fold change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ comparative C_T method. Significant changes in gene expression were estimated using a t-test and are indicated with * (t-test, $p<0.05$). The vertical bars in this graph indicate the average minimum and maximum values of fold difference in gene expression of *Daphnia* from each habitat in each condition.

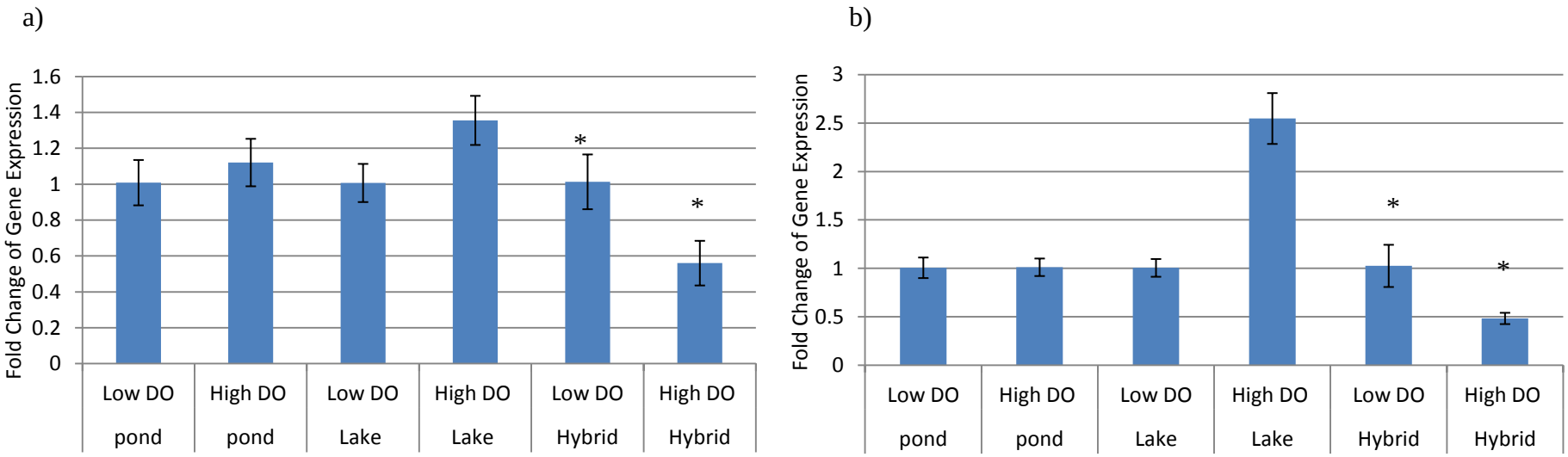


Figure 5 The effect of dissolved oxygen on a) *Ldh*A and b) *Ldh*B expression in *Daphnia pulex, Daphnia pulicaria* and *D. pulex/pulicaria* hybrids at 10 °C. The bars indicate fold change of gene expression of clones subjected to high DO and 10°C relative to clones raised low DO and 10 °C. The average gene expression in each habitat at each condition is based on the average expression profiles of 6 clones. The fold change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ comparative C_T method. Significant changes in gene expression were estimated using a t-test and are indicated with * (t-test, $p<0.05$). The vertical bars in this graph indicate the average minimum and maximum values of fold difference in gene expression of *Daphnia* from each habitat in each condition.

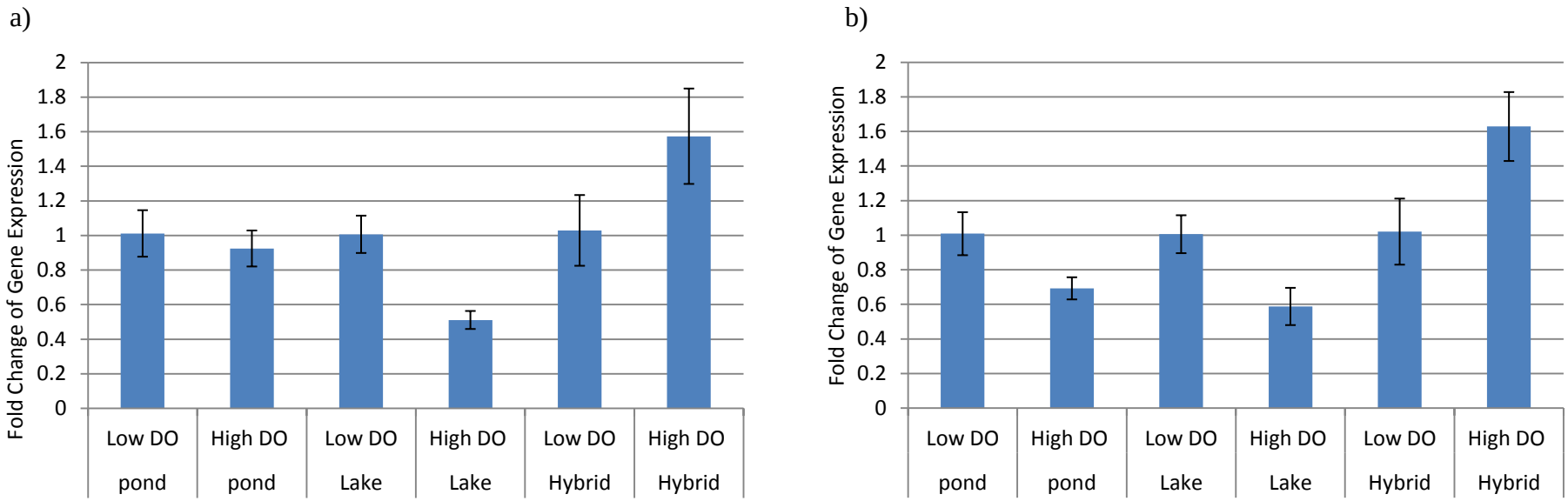


Figure 6 The effect of dissolved oxygen on a) *Ldh*A and b) *Ldh*B expression in *Daphnia pulex*, *Daphnia pulicaria* and *D. pulex/pulicaria* hybrids at 20 ºC. The bars indicate fold change of gene expression of clones subjected to high DO and 20 ºC relative to clones raised low DO and 20 ºC. The average gene expression in each habitat at each condition is based on the average expression profiles of 6 clones. The fold change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ comparative C_T method. Significant changes in gene expression were estimated using a t-test and are indicated with * (t-test, $p<0.05$). The vertical bars in this graph indicate the average minimum and maximum values of fold difference in gene expression of *Daphnia* from each habitat in each condition.

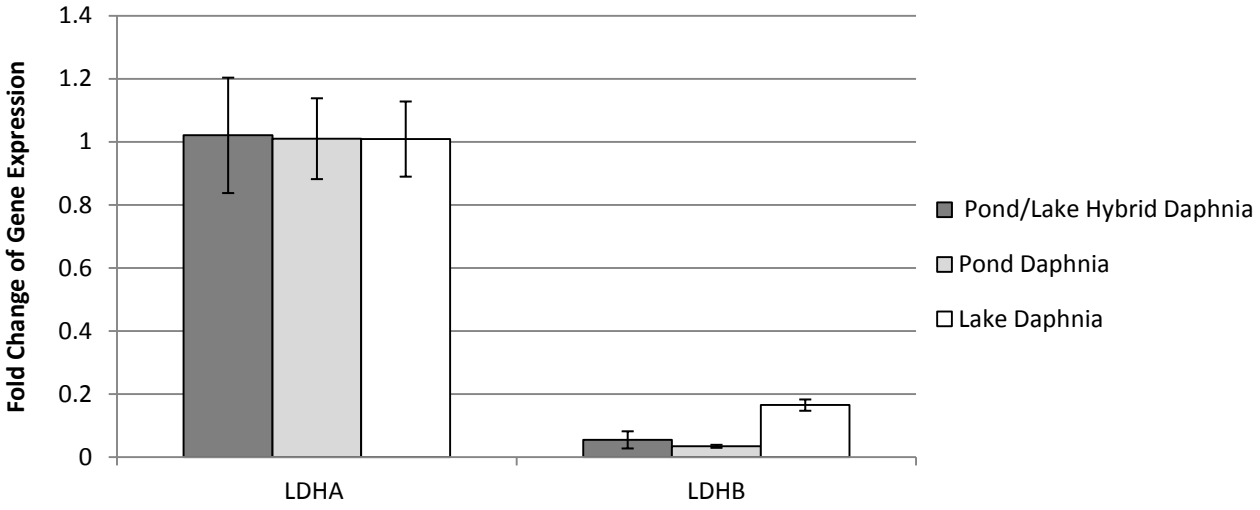


Figure 7 Gene expression of *Ldh*B relative to *Ldh*A in pond, lake, and hybrid *Daphnia*. The fold change in gene expression of *Ldh*B relative to *Ldh*A was calculated for each *Daphnia* clone using the $2^{-\Delta\Delta CT}$ comparative C_T method and then averaged for individuals in each environment. The black lines indicate the variance in gene expression based on standard deviation.

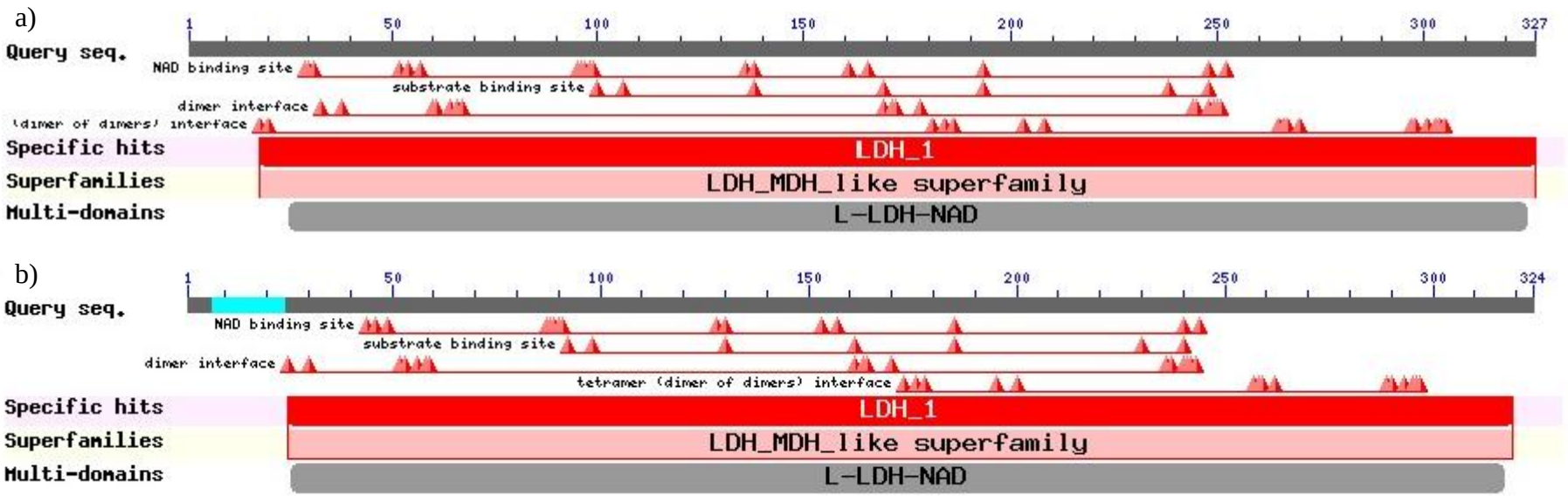


Figure 8 Graphical summary of conserved domain analysis for LDH in *Daphnia*. The analysis is based on a) 11 unique amino acid sequences of LDHA from *D. pulex* and *D. pulicaria* and b) 37 unique amino acid sequences of LDHB from *D. pulex* and *D. pulicaria*. The query sequences are represented by the upper grey bar except for the blue region near the N-terminal of LDHB which indicates a compositionally biased region not used in the domain database search. The four domains are denoted by the red lines with triangles. The point of each triangle shows the observed location of each residue that makes up the specific domain on each query sequence. The number of amino acids in each domain is as follows: NAD binding site = 18 amino acids, Substrate binding site = 7 amino acids, Dimer interface domain = 17 amino acids, Tetramer (dimer of dimer) interface domain = 17 amino acids. All 11 unique LDHA sequences contained all amino acids in each domain. All 37 unique LDHB sequences contained only 15 of 18 amino acids in the NAD-binding site, and 15 of 17 amino acid residues in the tetramer (dimer of dimers) interface domain.

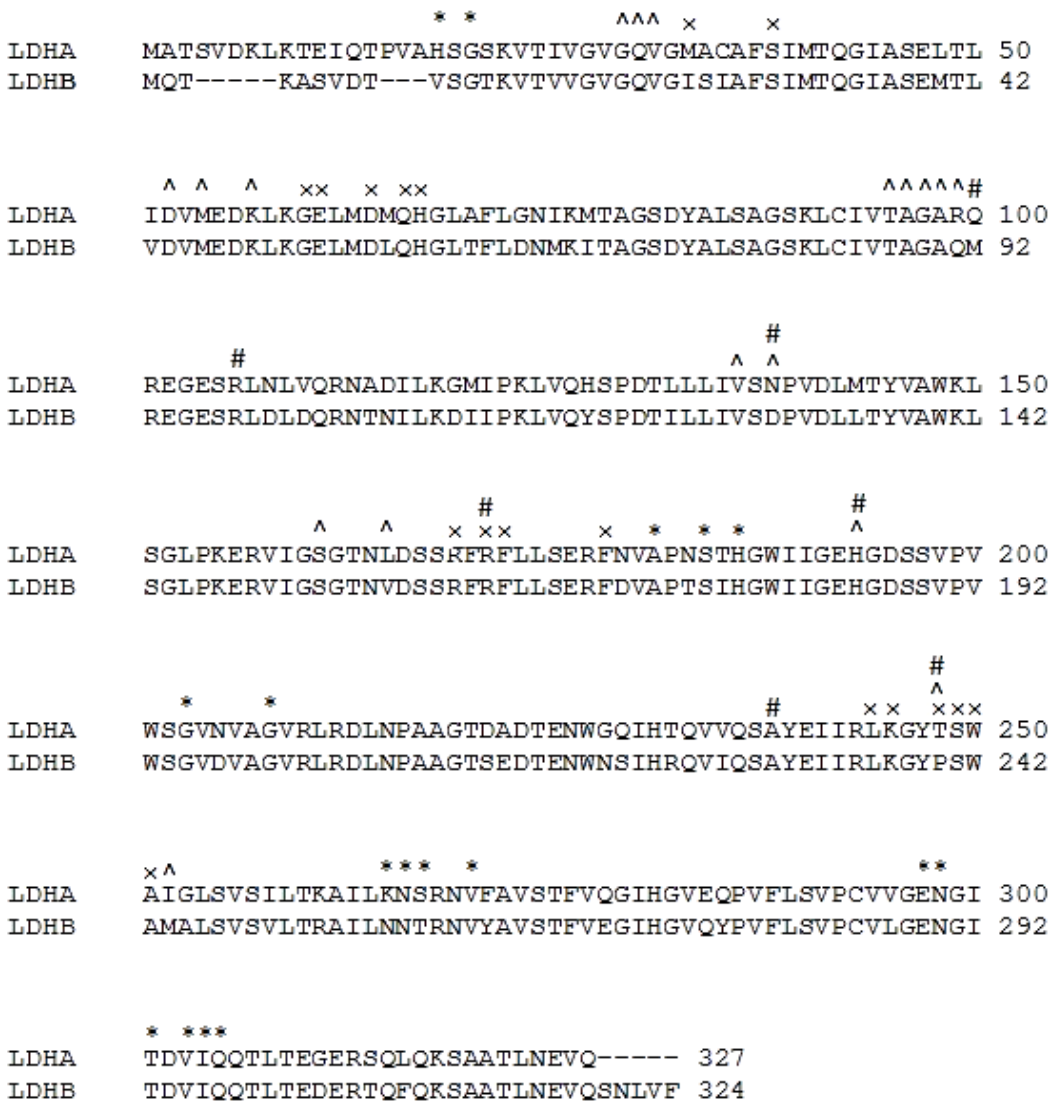


Figure 9 Alignment of LDHA and LDHB amino acid sequences. * represent the 17 amino acids that make up the tetramer (dimer of dimers) interface domain, ^ show the 18 amino acids that make up the NAD binding site, # show the 7 substrate binding sites, and × represents the 17 amino acids of the dimer interface in both proteins.

a)

b)

Figure 10 Graphical summary of secondary protein structure prediction by PHYRE. a) Protein structure obtained for all LDHA sequences in *D. pulex* and *D. pulicaria*. b) Protein structure obtained for all LDHB sequences in *D. pulex* and *D. pulicaria*. H and purple represent alpha helices C and white represent random coil, E and yellow represents beta sheets. The number under each structure represents the number of amino acids that make up these secondary structures in the LDHA and LDHB amino acid sequences.

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VITA AUCTORIS

NAME: Bora Demiri

PLACE OF BIRTH: Tirana, Albania

YEAR OF BIRTH: 1985

HOME TOWN: Windsor, Ontario

EDUCATION: Catholic Central High School, Windsor, Ontario

High School Diploma 2000-2004

University of Windsor, Windsor, Ontario

BSc. Honors Biochemistry and Biotechnology 2004-2009

Great Lakes Institute for Environmental Research

University of Windsor, Windsor, Ontario

M. Sc. Environmental Science 2009-2011