Transcriptomic and Physiological Variation as Mechanisms of Colonization Success in the Round goby (Neogobius melanostomus)

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Transcriptomic and Physiological Variation as Mechanisms of Colonization Success in the Round goby (*Neogobius melanostomus*)

By

Felicia Vincelli

A Thesis
Submitted to the Faculty of Graduate Studies through Great Lakes Institute for Environmental Research in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

2016

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Transcriptomic and Physiological Variation as Mechanisms of Colonization Success in the Round goby (Neogobius melanostomus)

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September 20, 2016
Author’s Declaration of Originality

I hereby certify that I am the sole author of this thesis. This thesis also incorporates the outcome of a joint research undertaken in collaboration under the supervision of professor Dr. Daniel Heath and Dr. Oliver Love. The collaboration is covered in Chapter 2 and Chapter 3 of this thesis. In all cases, the key ideas, experimental designs, data analysis and interpretation, were performed by the author, and the contribution of my supervisors was solely in an advisory capacity. I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis. I have obtained written permission from each of my supervisors to include the above material(s) in my thesis. I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office.
Abstract

The capacity to face and overcome environmental change when outside its native range is expected to ultimately determine the relative success of an invasive species. While mechanisms such as phenotypic flexibility and/or adaptation are expected to enable successful invasive species to adjust and succeed in these novel environments, there is still a general lack of empirical experimental data regarding the factors that facilitate the invasion success of taxa such as aquatic vertebrates. Combining a multi-scale (physiology and gene transcription) and experimental approach (water temperature) I examined the adaptation/acclimation to a thermal stressor as mechanisms to explain invasion success in the Round goby (*Neogobius melanostomus*) across populations varying in invasion stage within the Laurentian Great Lakes of North America. This thesis provides evidence of the Round goby showing transcriptional and physiological changes across thermal treatments, invasion categories, and functional responses across time since invasion. These results appear to be evidence of adaptation and are likely a result of plasticity. These combined results suggest that acclimation/adaptation in novel conditions are advantageous to Round gobies when colonizing novel environments and are likely an important factor in invasion success across species generally.
Dedication
To Mom and Dad
Acknowledgments

I would like to first thank my supervisors Dr. Daniel Heath and Dr. Oliver Love for giving me the incredible opportunities I have experienced throughout my graduate degree and providing me with immense support and encouragement. I would also like to acknowledge the members of my committee, Dr. Dennis Higgs, and Dr. Hugh MacIsaac for their guidance.

This project would not have been possible without the support of the Canadian Aquatic Invasive Species Network (CAISN) II for funding the project as well as support from the Ontario Ministry of Natural Resources, National Oceanic and Atmospheric Association and the University of Windsor. Additionally, I thank both the Natural Sciences and Engineering Research Council (NSERC) of Canada (D.D.H and O.P.L.) and the Canada Research Chairs (CRC) program for funding support. A special thank you to Kyle Wellband for taking the time out of his busy schedule to provide me with guidance and help me with laboratory protocols. I would also like to thank Chris Harris for his support and guidance with my cortisol assays. Sample collection (and my sanity) would not be possible without the generous help of Lida-Nguyen-Dang and Shelby Toews.

I would like to thank all of my friends at GLIER for always making me laugh. Our many hangouts and social gatherings made this time worthwhile. Finally, I would like to thank my Mom, Dad, and my brother Joe for their love, support, and patience.
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CHAPTER I
General Introduction

Invasive Species

The term ‘invasive’ is used to describe species that have been intentionally or unintentionally introduced outside of their native range and cause harm (Colautti et al. 2004; Hulme 2009; IUCN 2000). Invasive species are generally a growing economic and environmental problem (Bax et al. 2003) and have been linked to various ecological disturbances such as species extinction (Gurevitch et al. 2004), habitat loss (Gibbons et al. 2000) and coastal degradation (Lotze et al. 2006) to name a few. As species expand their invasive range, they may be faced with challenging environmental conditions such as change in temperature or other species that act as competitors, predators or prey (Lee 2002; Iwama 1999). As such, mechanisms enabling individuals to face and overcome environmental challenges are expected to increase the ability of a species to increase its range (Schneider 2008; Hoy et al. 2011). Short term acclimation (phenotypic plasticity) or genetic adaptation are two such mechanisms expected to play important roles in invasion success (Trussell and Smith 2000, Dzialowski et al. 2003).

Plasticity and Adaptation

Adaptation and acclimation are important processes that are correlated to the invasion success of many species (Chen, 2013; Gilad, 2006; Lee, 2002). Novel environmental conditions can initially drive changes in phenotype with no apparent genetic change (Piersma and Drent 2003). Environmentally-induced changes in phenotype associated with successful invaders have been studied in both aquatic and terrestrial species and include factors such as bioenergetics and nutrition (Liss et al. 2013), cardiorespiratory capacity (Lennox et al. 2015) and endocrine response (Moore et al. 2005). More generally, reproductive (Kirkpatrick et al. 2011), sensory (Wagner et al. 2006) and evolutionary physiology (Seebacher et al. 2011, Lee et al. 2002) have all been found to play a role in invasion success. However, as invasive species persist in novel environments, the new phenotype may become genetically fixed (Kocher, 2004). Furthermore, natural selection can change the genetic structure of invading species’ populations by selecting heritable
genes/biological traits which specifically enhance their invasion success (Stepien, 2005; Roman 2006; Bronnenhuber, 2011). Therefore, the study of the genetics and physiology of performance traits is important for understanding the evolution of successful invasive species.

The Stress Response

The stress response is a critical pathway for species to maintain their homeostatic state in the face of both biotic and abiotic stressors through physiological response (Barton, 2002). The physiological response to stress can be divided into three stages; primary, secondary, and tertiary responses (Iwama 1999) and has been linked to a broad range of environmental tolerances and adaptive capacities in fish (Trussell and Smith 2000, Dzialowski et al. 2003). Moreover, the broad environmental tolerance of stress via phenotypic flexibility is the mechanism whereby an organism changes its phenotype in response to novel or changing environments (Piersma and Drent 2003). Circulating stress hormones such as cortisol, have been used to quantify potentially flexible responses to chronic environmental stressors (Armario et al. 1986, Koolhaas et al. 1999, Mommsen et al. 1999, Barton 2002, Moncek et al. 2004, Rubenstein et al. 2007). The primary stress response is the initiation of neuroendocrine functions that release glucocorticoids (GCs) (such as cortisol) into the blood and can be elevated under chronically stressful conditions or during energetically-challenging life-history stages (Barton 2002, Auperin and Geslin 2008). The release of GCs from the inter-renal axis (fish) into the blood, not only plays a role in the acute response to stressors, but also acts as a metabolic regulatory hormone as it activates a number of metabolic pathways upon release (Iwama 1999; Basu, 2001). Given that cortisol has been characterized as a physiological response to stress in fish, it can be a valuable biomarker for measuring invasive species tolerance to altered habitat characteristics in novel environments (Seebacher and Franklin 2011, Kelley et al. 2014).
**Gene transcription**

Gene transcription is the first step in gene expression and is important in determining what proteins will be expressed, when they are expressed, where they are expressed, and ultimately dictating cell and tissue function. While gene transcription is only the first step for gene expression, it is believed to be a good proxy for gene expression as a whole (DeRisi et al. 1997). Gene transcriptional response happens when a cell is responding to a change in environmental conditions (or due to developmental programming). The response to an environmental signal will, in turn, adjust protein levels to compensate for the change in the environment to maintain homeostatic conditions. Most genes play multiple roles in various biological pathways that may display differential expression when exposed to environmental changes (e.g., temperature change in fish; Buckley, 2006; Smith 2013). Variation in gene transcription is important in determining the potential for evolution of gene expression (Chen, 2007), especially when determining whether natural selection plays a role in a successful invasion. Recently, gene transcription has become a powerful tool to study the molecular mechanisms underlying cellular and organismal responses to environmental variation (Roman 2006; Hodgins et al. 2013; Poelchau et al. 2013), and has improved our understanding of how invasive species respond to environmental stress (Carveth et al. 2006).

Recent studies have explored population-level expression variation in aquatic species and have also identified candidate genes that are part of conserved evolutionary pathways which underlie thermal stress responses (Kenkel et al. 2011, Kenkel et al. 2013). Moreover, gene transcription has been used to study the adaptive evolution of invasive species facing novel and changing environmental conditions (Prentis et al. 2008; Roberge et al. 2008; Henkel et al. 2008; Lockwood et al. 2010). Transcriptional responses to heat stress in the invasive blue mussel (*Mytilus galloprovincialis*) were found to differ at thermal-dependent genes compared to its native congener, *Mytilus trossulus* (Lockwood et al. 2010). These differential responses in gene transcription were used to predict and explain the adaptation and success of this invasive mussel on the central and southern coasts of California. The expression of protein coding genes also drives many critical biochemical and physiological processes, including performance-related traits such as metabolism (Goedkoop et al. 2011, Liss et al. 2013), growth (Power et al. 2001), reproduction
(Kirkpatrick et al. 2011), immune response (Lee et al. 2004) and stress response (Castro et al. 2011) These biologically relevant functional categories have previously been associated with invasion success and are crucial in overcoming novel environmental conditions including exploiting available food resources (growth/activity patterns), stress tolerance, and the ability to deal with novel parasites and pathogens (Gilman et al. 2005; Lardies et al. 2004; Eizaguirre et al. 2012). Overall, this can provide us with insight on which biological processes are involved in local adaption or acclimation as part on either natural range expansion or biological invasions.

**Quantitative Real-Time PCR**

Molecular genetic tools such as microarrays, next generation sequencing (NGS) and quantitative real-time PCR (qRT-PCR), have all been developed to help quantify messenger (mRNA) in tissue samples. Microarrays work by the competitive hybridization of cDNA synthesized from mRNA using gene-specific probes to give a quantitative estimate of the amount of mRNA. Next generation sequencing uses cDNA synthesized from mRNA to create whole transcriptome data (Jeukins et al. 2010). Finally, qRT-PCR quantitatively measures cDNA (in real-time) using gene-specific primers and fluorescent dyes (Tuomi et al. 2010). qRT-PCR has a greater sensitivity compared to other methods (Fleige et al 2006) and requires careful selection of candidate genes. The selection of candidate genes provides specificity and biological relevance to the scientific question.

**Round Goby**

The round goby (*Neogobius melanostomus*) is a globally successful invasive fish (Corkum 2004, Koris et al. 2012) and has been a particularly successful invader and disturbance in the Laurentian Great Lakes of North America. The species was first detected in Lake St. Clair in 1990 (Jude, 1992) and they are now found throughout the Great Lakes (Jude, 1995; Phillips, 2003; Schaeffer, 2005; Poos, 2010). The primary source of the Great Lake round gobies that invaded the St. Clair River is believed to be the Dneiper River (Kherson, Ukraine) on the Black Sea (Brown, 2009) via ballast water discharge (Jude, 1992).
Round goby is expanding its invasive range such that different geographical locations have a known history of colonization, and that this allows us to identify newly invaded versus established populations. Various phenotypic and life history traits of the Round goby have also been studied and proposed as facilitating their invasion success. Round gobies have been found to exhibit high fecundity and extended spawning season (MacInnis and Corkum 2000), high diet diversity (Carmen et al. 2006, Pettitt-Wade et al. 2015), high salinity tolerance (Kariotis et al. 2012) and aggressive behaviour (Dubs and Corkum 1996, Groen et al. 2012). Round gobies have also been found to have high levels of genetic variation in comparison to their native range (at neutral loci), which may reflect a greater buffer against founder effects and higher adaptive potential (Lee 2002, Brown and Stepieen 2009). Overall, the Round goby invasion of the Great Lakes provides an ideal study system for the role of adaptive responses to novel environments in successful aquatic invasions.

**Thesis Objectives**

This thesis investigates possible genetic and physiological adaptive mechanisms of colonization success in the Round goby within the framework of environmental variation. Specifically, it compares transcriptional and physiological differences among populations representing various times since establishment within the context of a water temperature manipulation designed to act as a biologically-relevant environmental stressor for this species in novel habitats. The goal of Chapter 2 is to examine the population variation in stress- and metabolically-mediated responses to temperature in the Round goby. To this end I measured baseline levels of circulating cortisol and the variation in transcription in four candidate genes across populations with variable time since invasion in temperature challenged fish. Chapter 3 utilizes a candidate gene transcription profiling approach to examine the acclimation and adaptation of the Round goby to novel environments. A transcription profile using genes associated with invasion success can help predict the spread of this invasive species and to examine the effect of novel habitat colonization on the basic cellular mechanisms of the invasive species. Together these chapters address whether there are functional linkages of key biological mechanisms which are expected to play a role in acclimation/adaptive responses in the invasive Round goby. I argue that Round goby in newly invaded regions exhibit physiological and genetic characteristics that
differ from individuals in established populations. To this end, these results demonstrate that Round goby appear to exhibit adaptive and environmentally relevant responses consistent with their successful invasion of the Great Lakes.

References


CHAPTER II

Flexibility in stress and metabolic responses to thermal challenges in established and recently invaded populations of round goby (Neogobius melanostomus)

Introduction

Biological invasions have a large ecological impact on a global scale and are important drivers of environmental and economic change (Vitousek et al. 1996, Sala et al. 2000, Sakai et al. 2000). Intentional and accidental introductions of invasive species are common in both terrestrial and aquatic ecosystems, and include plants, arthropods, amphibians, birds, fish and mammals (Ramstetter 2001, Lockwood et al. 2007). These nonindigenous species have caused substantial damage to native ecosystems, environmental degradation, even acting as vectors of human disease, and have been suggested as causes for the extinction of native species (Pimental et al. 2000, Ricciardi 2003, Clavero and García-Berthou 2005).

The invasion process has been well-documented and consists of a series of invasion stages that include uptake, transfer, release and establishment (Lockwood et al. 2005, Blackburn et al. 2011). Since each invasion stage acts as a potential barrier or limiting factor, a successful invasive species must possess mechanisms at each of these stages to overcome and persist, and then expand, within the new environment (Richardson et al. 2000, Colautti and MacIsaac 2004).

While the success of invading species has been studied broadly in plant invasions (Daehler 2003, Richards et al. 2006), there is a general lack of empirical data regarding the factors that facilitate the invasion success of other taxa such as aquatic vertebrates, fish, mammals and birds. Successful invasions have been linked to a broad range of environmental tolerances and adaptive capacities (Trussell and Smith 2000, Dzialowski et al. 2003). These include mechanisms that enable individuals to withstand and avoid environmental stressors to facilitate survival and reproduction (Matsumoto et al. 2000, Vinebrooke et al. 2004, Lenz et al. 2011). Another potential mechanism theorized to underlie a successful invader is a broad environmental tolerance via phenotypic flexibility. Phenotypic flexibility is the ability of an organism to change its phenotype (any physiological adjustments) in response to changes in the environment (Piersma and Drent...
This temporary phenotypic change is reversible and may have a selective advantage (Gabriel and Lynch 1992). To date, many studies have examined behavioural (Sagata and Lester 1999, Sol et al. 2002, Leal and Powell 2012) and morphological (Schweitzer and Larson 1999, Geng et al. 2006) aspects of flexibility, and some of these have been related to invasion success (Durand and Goldstein 2001, Niinemets et al. 2003, Thiébaut 2007, Peacor et al. 2012, Schultz and Dibble 2012). Given that physiology mediates the relationship between the individual and its environment (Ricklefs and Wikelski 2002), researchers have recently turned to physiological flexibility as an underlying mechanism for phenotypic variation, with the potential to mediate phenotypic responses under variable environmental conditions (Robinson and Dukas 1999, Sih et al. 2004, West-Eberhard 2005). Although considered a powerful approach to exploring the mechanisms behind successful invasions, little is known about whether the degree of physiological flexibility correlates with invasion success (Seebacher and Franklin 2011, Kelley et al. 2014). Researchers have recently turned to measures of circulating stress hormones as measures of potentially flexible responses to chronic environmental stressors (Armario et al. 1986, Koolhaas et al. 1999, Mommsen et al. 1999, Barton 2002, Moncek et al. 2004, Rubenstein et al. 2007).

Glucocorticoid hormones such as cortisol (fish, mammals) and corticosterone (birds, reptiles, amphibians) play two complementary roles in managing daily energetics and acute responses to stressors, enabling individuals to maintain homeostatic balance in response to both internal and external environmental variation, respectively (Chrousos 1998, Romero 2002, McEwan 2004). At baseline levels, glucocorticoids (GCs) can be elevated under chronically stressful conditions or during energetically-challenging life-history stages (Barton 2002, Auperin and Geslin 2008). During an acute stress response, the primary stress response is the initiation of neuroendocrine functions that releases GCs from the inter-renal axis (fish) or the hypothamic-pituitary-adrenal axis (birds, mammals, reptiles) into the blood, which activates a number of secondary metabolic pathways (Iwama et al. 1999, Basu et al. 2001). Measuring changes in circulating GCs within a population under various environmental conditions is thought to be a biologically useful and effective way to gauge flexibility in responses to novel or rapid changes in environmental quality.
(Schjolden et al. 2005, Ruiz-Gomez et al. 2011). For example, populations at the range edge in a species range expansion are often made up of individuals that exhibit heightened or hyper-responsive stress axes (Addis et al. 2011, Krause et al. 2015, Liebl and Martin 2012, Walker et al. 2015). Moreover, adaptive changes in hypothalamus-pituitary-adrenal/inter-renal (HPA/HPI) activity at the population level have been detected in vertebrates in as little as 10-12 generations (Atwell et al. 2012), making it plausible that range edge populations in an invasion may have evolved adaptive stress responses. Importantly, researchers have even begun to link the responsiveness of the HPA/HPI axes to individual fitness outcomes for invasive species (Jessop et al. 2013). Finally, research has suggested the importance (where possible) of accounting for variation in not only the native hormone, but also its receptor(s) (Greenwood et al. 2003; Moncaut et al. 2005). During the stress response, glucocorticoids act through the glucocorticoid receptor (GR), which regulates the transcription of target stress-related genes (Landys et al. 2006). The GR binds DNA at glucocorticoid response elements (GREs) in the promoter regions of corticosteroid responsive genes, inducing transcription (Hori et al 2011, Castro et al. 2011). Stress related genes enable individuals to manage energetic demands as well as to respond to stressful conditions by having the ability to cope with altered habitat characteristics (Romero 2002, Barton 2002, Auperin et al. 2008). This can be advantageous when acclimating and adapting to new environments and may provide invasive species with a competitive advantage for novel and changing aquatic environments.

The goal of the current study was to examine population level variation in stress- and metabolically-mediated responses to a thermal stressor in an invasive aquatic species of conservation concern, the Round goby (*Neogobius melanostomus*). The Round goby has been an extremely successful invader in the Laurentian Great Lakes of North America. The species was first detected in Lake St. Clair in 1990 (Jude 1992) likely introduced via ballast water (Jude 1992) with the primary source believed to be the Dneiper River (Kherson, Ukraine) on the Black Sea (Brown and Stepien 2009). The species can now be found throughout the Laurentian Great Lakes of North America (Phillips et al. 2003, Schaeffer et al. 2005, Poos et al. 2010, Kornis et al. 2012) (Figure 2.1). Various phenotypic traits known to have facilitated invasion success in Round gobies have been examined and include high
fecundity and extended spawning season (MacInnis and Corkum 2000), high diet diversity (Carmen et al. 2006, Pettitt-Wade et al. 2015), and aggressive behaviour (Dubs and Corkum 1996, Groen et al. 2012). Great Lakes Round goby have also been found to have high levels of genetic variation, which may reflect a greater buffer against founder effects and higher adaptive potential for environmental variation (Lee 2002, Brown and Stepiein 2009, Bronnenhuber et al. 2011). Although some examinations of their reproductive and stress physiology have recently been undertaken (Bowley et al. 2010, Marentette et al. 2012, Zeyl et al. 2013, 2014), to our knowledge no work has examined hormonal and gene transcriptional responses to environmental stressors in relation to invasion stage. The Great Lakes Round goby populations represent an ideal comparative model to examine the phenotypic mechanisms underlying invasion potential, given that sub-populations have well characterized times since colonization (Bronnenhuber et al. 2011). I combined an among-population study with both correlative and manipulative approaches to explore the role stress- and metabolically mediated mechanisms play in invasion success in the Great Lakes Round goby. To this end I measured baseline levels of circulating cortisol and variation in GR transcription across populations with variable time since invasion in ‘control’ fish (ambient water temperature). I also chose to examine three additional candidate genes (GLUT1, LDHB, and G6PD) given their close association with metabolic processes (such as glycolysis and gluconeogenesis), which are important for energy production and glucose homeostasis, especially during responses to environmental stressors. I combined this correlative approach with an experimental manipulation of water temperature (ambient, cooled and heated) to examine i) within-population flexibility in the cortisol response to this biologically relevant environmental (i.e., thermal) stressor and ii) whether the degree of flexibility varied with the time since invasion. The variation in time since invasion (9-25 years across sampled populations) combined with our experimental manipulation enabled myself to make scale-dependent predictions about the relationship between the time since invasion and circulating cortisol. Based on previous work in populations experiencing range expansion (see above), we predicted that Round gobies at the invasion front would exhibit higher resting cortisol levels and greater upregulation of stress- and metabolically-mediated genes compared to those in the established populations. Furthermore, given that expanding populations have been shown to be hyper-responsive to
standardized stressors (see above), I further predicted that individuals in the invasion front populations would show higher and greater upregulation of stress- and metabolically-mediated responses to the cooled and heated water treatments compared to those in the established populations.

Methods

Capture and Transport

I sampled a total of seven sites for Round goby collection that represented a variety of years since they were first detected (here we define it as ‘time since invasion’) (Figure 2.1; Table 2.1): Detroit River (42.3065.42”N, -83°07’56.75”W), Grand River (42°96’00.76”N, -79°87’27.08”W), Trent River (44°29’46.21”N, -77°98’38.73”W), Lake Erie (42°00’73.29”N, -82°56’97.31”W), Lake Huron (43°00’13.79”N, -82°41’31.90”W), Lake Ontario (43°27’03.99”N, -79°86’90.19”W), and Georgian Bay (44°50’90.88”N, -80°23’33.72’W). Since there is a break in the distribution of the time since invasion among sampled populations of the Round goby (between Lake Huron and Lake Ontario), this gap provided us with a distinct separation of ‘established’ and ‘invasion front’ populations. Each population was thus assigned to an invasion category based on the year in which they were first detected: ‘established’ population had been present for >22 years, while ‘invasion front’ had been present for <17 years (Table 2.1). All Round gobies were caught during the months of July-August, in 2014 and 2015 using a seine net between the hours of 08:00 - 11:00 AM. Individuals were held in aerated plastic coolers filled with lake water and kept in the shade until transport to the experimental setup once sufficient study animals had been collected.

Experimental Design

Fish from each site were randomly assigned to one of three biologically relevant experimental water temperature treatments: ambient (ambient lake temperature), heated (5°C above ambient) and cooled (15°C below ambient). Such departures from ambient
temperature can result in an acute stress response and they were chosen to allow us to quantify a physiological response to relevant environmental thermal variation (Donaldson et al. 2008, Kelley et al. 2014). The heated treatment (5°C above ambient) was chosen to ensure that it did not exceed the Round gobies thermal tolerance of 30°C (Cross and Rawding 2009). The cooled treatment (15°C below ambient) was chosen to mimic expected water temperature variation experienced during the fall-winter in the Great Lakes, which range from temperatures of 0.2-5°C in the winter (NOAA). Two covered tanks (to ensure a dark environment) were assigned to each temperature treatment, and each tank housed five (randomly assigned) gobies. The water for each temperature treatment was stored in a large cooler where the water was heated or cooled to the desired temperature whereupon water was pumped into smaller housing tanks. The set-up ensured a continuous flow of consistently oxygenated water at the present temperatures. All temperature trials began at approximately 12:00 pm to control for known diurnal variation in baseline plasma cortisol (Boujard and Leatherland 1992, Lorenzi et al. 2008) and all experimental fish were held for 20 hours after the experiment began. The water temperature for the cooled and heated challenges were adjusted approximately 2-3°C every hour until the final desired temperature was achieved (i.e., after approximately six hours for the cooled treatment and four hours for the heated treatment). Following these experiments, individual fish were humanely euthanized by immersion in a clove oil solution of 40 mg/L (Neiffer and Stamper 2009) for approximately 10 seconds, or until there was no movement from the fish. Individual blood samples were collected via caudal severance within four minutes of initial capture using 20 µL capillary tubes. The capillary tubes were then centrifuged for 10 min at 15 000 rpm and the resulting plasma was stored at -80°C until analysis. Gobies were measured for total length (to the nearest mm) and weighed (to the nearest 0.01g). Liver tissue was sampled within 30 minutes, stored in a high salt buffer solution (RNA-Later), and transported at approximately -20°C to the University of Windsor for subsequent gene transcription analyses.
Cortisol and Gene Transcription Assays

I determined the concentration of total cortisol in the plasma using a cortisol Enzyme-Linked-Immuno-Absorbent Assay (Enzo Scientific Inc., Michigan USA) with a 4-parameter logistic fit. Assay sensitivity is 156 through 10,000 pg/mL and all experimental values fell within this range. Raw, un-extracted plasma was assayed at a total volume of 100 µL (diluted 1:40 in assay buffer) in triplicate on 13 assay plates following previously in-house optimized protocols for this species yielding an intra- and inter-assay variation of 5.5% and 8.1%, respectively. For transcriptional analyses, total RNA was extracted from round goby liver tissue following the method of Chomczynski and Sacchi (1987). Complementary DNA (cDNA) was synthesized using a High Capacity cDNA Reverse Transcription (RT) kit and stored at -80°C until further analysis. For each gene of interest, primers and probes were designed to match conserved regions of sequences (closely related species to the Round goby) using Geneious Pro software v6.1.6 and Primer Express® software (v3.1). The specificity of each primer set was verified by PCR and amplicon visualization by gel electrophoresis as well as melt curve analysis (using SYBR Green). Assays were performed using the QuantStudio 12K Flex Real-Time PCR System in an Applied Biosystems thermocycler following manufacturer’s instructions (Applied Biosystems, Burlington, ON, Canada). Results were analyzed using the ExpressionSuite software (Applied Biosystems).

Sequences

The transcriptional response of GR was detected using the forward primer 5’-GGATGACCAGATGACGTGCT-3’, reverse primer 5’-AGCCCAGACTGAACGACATGA-3’, and probe sequence 5’-AGTGTTCGTGGCTCTT-3’. G6PD used the forward primer 5’-TGGTGACGCTGCTTAAA-3’, reverse primer 5’-CGGACCTGCTGTCTTGTGT-3’, and probe sequence 5’-AGGAGGATGTCTTTTCC-3’. GLUT1 used the forward primer 5’-AAGTGTTGAGAGATGTCACTGTAAC-3’, reverse primer 5’-CAGTCCCTTTGATGTGTCTGGAA-3’, and probe sequence 5’-TAATAAAGTGCTGCAGAGAA-3’. Lastly, LDHB had a forward primer of 5’-
TGGACAGTGCCTACGAAGTGA-3’, reverse primer 5’-CAGTCCGATGGCCCAGTT-3’ and probe sequence 5’-CAAGCTGAAGGCTACA-3’. All amplicon sizes were approximately 65bp.

Statistical Analysis

All data were tested for normality and non-normal data (cortisol data) were log-transformed (log(cort)) to meet assumptions of parametric statistical tests. All gene transcription data were normalized to an endogenous internal control (β-actin), which is represented by a ΔCt value (i.e., transcription relative to β-actin). I used general linear mixed models (GLMM) to analyze the response of log(cort) and gene transcription data (GR, G6PD, GLUT1 and LDHB) to invasion category (established and recently invaded) and experimental treatment (ambient, heated and cooled), as well as their interaction. Sampling year, tank number, and source population were all included as random effects. I also included Fulton’s condition factor K (Nash et al. 2006) as a covariate to examine whether a measure of individual condition influenced plasma cortisol or gene transcription either univariately or via more complex interactions with additional covariates. Non-significant interactions were backwards eliminated. Student’s T post-hoc tests were used to examine differences among the treatment-invasion category combinations. All statistical analyses were conducted using JMP (SAS Inc., Version 12.1).

Results

I detected no significant differences for sex, length, body mass or Fulton’s K index for fish assigned to any of the three temperature treatment groups or from the two invasion categories, or via the interaction between the two (all p > 0.48), indicating that all fish were assigned randomly to treatments with respect to these parameters within and between categories.
**Cortisol Responses**

I detected a significant effect of the experimental (thermal stress) treatment \( (F_{2, 272} = 8.59, p = 0.0002) \), invasion category \( (F_{1, 272} = 22.68, p < 0.0001) \) and the interaction between treatment and the invasion category \( (F_{2, 272} = 3.74, p = 0.025, \text{Figure 2.2}) \) on \( \log(\text{cort}) \), as well as a positive relationship between \( \log(\text{cort}) \) and Fulton’s condition factor \( K \) \( (F_{1, 224} = 4.59, p = 0.033) \). Post-hoc analyses revealed that fish from the invasion front populations had higher cortisol levels in the ambient and heated treatments relative to the established populations (Figure 2.2); however, fish exposed to the cooled water treatment were similarly high in both the invasion front and established populations (Figure 2.2). To explore the treatment by invasion category interaction effect, we further tested for relationships between “years since invasion” (Table 2.1) and mean cortisol levels across populations, within each treatment group. We detected a significant and strong negative relationship between years since invasion and \( \log(\text{cort}) \) within the heated water treatment only \( (\text{ambient: } R^2 = 0.38, \text{T-ratio} = -1.73, p = 0.14; \text{heated: } R^2 = 0.71, \text{T-ratio} = -3.47, p = 0.018; \text{cooled: } R^2 = 0.04, \text{T-ratio} = -0.45, p = 0.67; \text{Figure 2.3}) \). Finally, all the treatment groups showed significant population differences in \( \log(\text{cort}) \) levels \( (\text{ambient: } R^2 = 0.21, F\text{-ratio} = 3.74, p = 0.002; \text{cooled: } R^2 = 0.15, F\text{-ratio} = 2.57, p = 0.024; \text{heated: } R^2 = 0.17, F\text{-ratio} = -3.06, p = 0.009; \text{Figure 2.4}) \) and post-hoc analyses revealed complex sub-population patterns for variation in \( \log(\text{cort}) \) levels within each treatment group (Figure 2.4).

**Transcriptional Responses**

For the \( GR \) and \( G6PD \) gene transcription we detected a significant effect of both the experimental treatment \( (GR: F_{2, 294} = 12.5, p < 0.0001; G6PD: F_{2, 294} = 17.6, p < 0.0001; \text{Figure 2.5, 2.6a}) \) and the invasion category \( (GR: F_{1, 294} = 4.81, p = 0.03; G6PD: F_{1, 294} = 27.2, p < 0.0001; \text{Figure 2.5, 2.6a}) \) on gene transcription, but not for the interaction between treatment and the invasion category \( (GR: F_{2, 294} = 0.11, p = 0.89; G6PD: F_{2, 294} = 0.64, p = 0.53; \text{Figure 2.5, 2.6a}) \). For the \( GLUT1 \) gene transcription we detected a significant effect of the experimental treatment \( (F_{2, 296} = 28.9, p < 0.0001; \text{Figure 2.6b}) \), but not the invasion category \( (F_{1, 296} = 0.35, p = 0.56; \text{Figure 2.6b}) \) or the interaction between the two factors \( (F_{2, 294} = 0.35, p = 0.71; \text{Figure 2.6b}) \). Finally, for the \( LDHB \) gene transcription we detected
a significant effect of the invasion category ($F_{1, 294} = 9.25, p = 0.003; \text{Figure } 2.6c$), but not the experimental treatment ($F_{2, 294} = 2.93, p = 0.06; \text{Figure } 2.6c$) or the interaction between the two factors ($F_{2, 294} = 0.98, p = 0.38; \text{Figure } 2.6c$).

Discussion
My primary goal was to quantify the potential for stress- and metabolically-mediated mechanisms to affect the underlying invasion potential of Round gobies by taking both a correlative (i.e., an across invasion category) and a causal (i.e., experimental) approach. I focused initially upon glucocorticoid (cortisol) levels since (i) they are known to change in response to biologically-relevant changes in environmental conditions (both abiotic and biotic; Iwama et al. 1999), (ii) these changes are thought to be adaptive in that they enable individuals to flexibly respond to acute or chronic changes in environmental quality/state (Gabriel and Lynch 1992), and (iii) they have been implicated in population expansion across vertebrate taxa (Addis et al., 2011, Krause et al., 2015, Liebl and Martin, 2012, Walker et al., 2015). As such, I expected to detect higher baseline cortisol levels in the invasion front fish relative to the established populations, with even greater differences under thermal stress (exposure to cooled or heated water). As predicted, fish from the invasion front exposed to the heated water treatment showed higher cortisol levels than fish from the established range. Nonetheless, the cooled water treatment group generated a complex and unexpected treatment by invasion category interaction where both invasion front and established categories showed similar and elevated cortisol levels.

Significance of cortisol differences across invasion categories
Elevated baseline glucocorticoids (GCs) enable individuals to manage daily and seasonal energetic demands as well as to respond to stressful conditions and energetically-challenging life history stages (Romero 2002, Barton 2002, Auperin et al. 2008). Increased baseline GC levels may enable fish to expand their range by enhancing dispersal activity in combination with an elevated ability to cope with altered habitat characteristics. Such
flexibility can be advantageous when colonizing new environments and may provide invasive species with a competitive advantage for food and other resources. Our work therefore not only lends support for the GC-based expansion front hypothesis, but also points to variation in baseline GCs as a possible mechanism enabling invasive species to successfully manage novel environments. Previous studies have reported elevated baseline GCs in newly colonized populations of bird species. For example, a study by Addis et al. (2011) concluded that an individual may be able to respond faster to environmental stressors (such as predators and climatic conditions) when they have high baseline levels of GCs. Our work lends support for this idea, and specifically to the prediction that invasive species expanding into novel environments may benefit from altered cortisol management.

As predicted, fish in the invasion front category (populations established for less than 17 years) exhibited higher baseline cortisol levels than fish in the established category (populations established for greater than 22 years) within the ambient (i.e., unmanipulated) group (Figure 2.2). Moreover, when the invasion categories were further broken down to examine individual population differences, two recently invaded populations (Trent River and Lake Ontario) exhibited significantly higher baseline cortisol levels than the most established population (Lake Huron) in the ambient group (Figure 2.4a). Those results support the energy mobilization hypothesis proposed by Romero (2002), which states that GC concentrations are important for energy mobilization, and are at their highest during energetically costly times of the year. In newly arrived invasive individuals, elevated baseline GCs could result from the increased energetic demands associated with activities such as finding resources, shelter and breeding, which could enhance their long-term fitness through increases in growth, survival and reproductive output (Walker et al. 2015).

Moreover, elevated baseline GC levels may also reflect adaptation to stressors in a novel environment at the leading edge of a population invasion or distribution range (Krause et al. 2015).
Experimental evidence for cortisol flexibility as a mechanism for invasion success

Based on previous research examining GC variation in expanding populations, I predicted that both thermal stress treatments (heated and cooled water treatments) would result in elevated cortisol concentrations in the invasion front group relative to the established group. Interestingly, this prediction was correct for gobies in the heated, but not the cooled, water treatment. The differences in cortisol levels in the cooled treatment between invasion categories could be due to the range of seasonal temperature variation relative to the temperature treatments. The average summer water temperature at the seven sample sites ranges from 20-22°C and the water temperature in the cooled experimental treatment was decreased 15°C (to mimic winter water conditions). Therefore, the fish in the cooled treatment experienced a more dramatic change in temperature, which may have led to a hyper-responsive reaction of the stress axis in all fish, irrespective of their invasion status. Several studies have reported links between hyper-responsiveness of the stress axis under extreme conditions in invasive species. For example, Jessop et al. (2013) found that an invasive population of cane toads (Rhinella marina) in Australia showed high levels of plasma corticosterone during hot daily temperatures, which allows the toads to survive during extreme temperatures. Additionally, Liebl and Martin (2012) showed that GC hyper-responsiveness in an introduced population of house sparrows (Passer domesticus) could be cognitively advantageous by increasing the ability of individuals to solidify memories of novel resources.

Unlike the cooled water treatment group, the invasion front populations responded with higher cortisol levels than the established populations in the heated water treatment. This supported my prediction that the round gobies from the newly colonized populations would have a higher cortisol response than fish from established populations. The lower cortisol levels exhibited by fish in the established populations under the heated treatment could be evidence that established round gobies are more resistant to stressors which would enhance their ability to persist and to a greater extent, survive and succeed in new environments (Walker et al. 2015). The low response in the heated water treatment in the established population also agrees with findings from Jessop et al. (2013) who found that cane toads that exhibited a low response to an acute adrenocortical stress phenotype had a
longer survival period. If correct, the interpretation of the low response suggests that the established population may have less flexibility in response to a thermal stress than Round gobies in the invasion front. I also found a strong negative correlation in the heated water treatment between cortisol response and time since invasion (Figure 2.3b), indicating that individuals from newly established populations had higher cortisol response than those from the established populations. A previous study by Liebl and Martin (2012) also reported a link between elevated corticosterone levels and distance from a native habitat in invading population of house sparrows: populations further from the native range exhibited higher corticosterone levels. This supports my expectation that invasive species in a newly invaded range have increased scope for adrenocortical response to stress and that may enhance their invasion success (Walker et al. 2015). Thermal tolerance in invasive plants species has been widely studied (Rejmanek and Richardson 1996, Lee 2002, Rahel 2008, Hasanuzzaman 2000, Zerebecki and Sorte 2011, Colautti and Barrett 2013) and has been identified as a major factor facilitating invasion success. Broader evolutionary implications may suggest that fish in the established populations now exhibit a “canalized” or less reactive response to environmental stressors. This would agree with previous research suggesting that reactive GC responses to a thermal stress are only adaptive for individuals that are in the process of invading and colonizing new habitats (Liebl and Martin 2012; Jessop et al. 2013; Walker et al. 2015) Finally, I expected the heated and cooled treatments to further elevate cortisol above the ambient treatment within the invasion front category, but my results indicate no cortisol response to the thermal challenges. One possible explanation for this may be that the invasion front Round gobies were at their maximum GC stress response level and were unable to secrete additional cortisol. Findings by Marrentette et al. 2012 allow us to make comparisons between cortisol secretions in Round goby. They found that peak cortisol secretion of the Round goby to the chasing stressor at 10 and 30 minutes was 100 and 87.5 ng/ml respectively. In comparison, my results showed increased levels in the cooled and heated treatments by exhibiting peak cortisol levels of 127 and 143 ng/ml respectively. These findings may suggest that invasion front Round gobies are indeed secreting their maximum GC stress response level.
Transcriptional responses to environmental stressors

A second explanation for the lack of cortisol response to thermal challenge in the invasion front group could be that the Round gobies perceived the ambient water to be stressful as well. One explanation for this may be that the invasion front Round gobies are more metabolically active. To test this hypothesis, I analyzed the gene transcription profiles of the GR (glucocorticoid receptor) gene and three additional metabolic genes from the same fish examined for cortisol. The metabolic genes were chosen based in their direct correlation to glycolysis and gluconeogenesis. Glycolysis (a process which converts glucose to pyruvate) is an important cellular process, which supplies cells with sufficient energy that is associated with growth (Pelletier et al. 1995), reproduction (Comizzoli et al. 2003, Wang et al. 2012) and movement (Baldwin et al. 1989, Millar et al. 2009). On the other hand, the maintenance of blood glucose is also important for survival (Newsholme et al. 2003). The release of GCs act to restore glucose homeostasis by binding to GRs, thereby stimulating gluconeogenesis. My results indicate that although genes related to cortisol regulation (GR) and glycolytic pathways (Glucose transporter (GLUT1), Glucose-6-Phosphate Dehydrogenase (G6PD)) and Lactate Dehydrogenase-B (LDHB) exhibited inconsistent responses across temperature treatments, GR, G6PD and LDHB were nonetheless consistently upregulated within invasion front compared to established populations (Figure 2.5, 2.6). Upregulated levels of GR aid individuals in responding to stressful events via an increase in gluconeogenic pathways (Landys et al. 2008). Likewise, the upregulation of the G6PD gene is expected to stimulate the production of intermediate products in glycolysis pathway as well as the initiation of the pentose phosphate pathway (Buckley et al. 2006). Finally, upregulation of the LDHB gene activates the lactate dehydrogenase- B enzyme, which is responsible for the conversion of lactate to pyruvate in the gluconeogenesis pathway. Therefore, the significant upregulation of both GR and LDHB in the invasion front populations across water treatments provides evidence of upregulated gluconeogenesis. In short, these findings suggest that the Round goby in the invasion front are have higher baseline GCs and are more metabolically active (by means of glycolysis), but are not necessarily more responsive to thermal stressors compared with established populations. Previous studies have suggested that elevated levels of GC’s in
range edge species could facilitate a quicker response and a faster recovery when experiencing conditions that are not ideal (Addis et al. 2011, Walker et al. 2015). Again, although this line of reasoning requires further research, if correct it would support the idea that fish in the invasion front category exhibit higher cortisol levels, which allows them to manage life better in a stressful environment.

**Conclusion**

Invasive species are a growing economic and environmental problem on a regional and global scale (Bax et al. 2003). While the problem of invasive species is widely discussed, understanding their aggressive range expansion is crucial in determining the effects they have on biodiversity and ecosystem functioning (Crowl et al. 2008). Invasive species such as zebra mussels, and the Round goby, have rapidly spread throughout the Laurentian Great Lakes and have resulted in a variety of ecological impacts and concerns (Bossenbroek et al. 2001, Poos et al. 2010, Hinterthuer 2012). My work on gobies supports the idea that flexibility of the stress response can help a species exploit a novel environment and can be a factor that facilitates invasion success. This enhanced stress response may be due to an increase in energetic demands as well as a coping mechanism for new environmental conditions. Moreover, my finding of significant treatment by invasion category interactions also provides further support for cortisol flexibility, especially in the cooled treatment. The approach to quantify genetic and physiological dynamics of invading species can thus form a basis for future researchers to study the success and limitations of invasive species and their range expansion.
References


Table 2.1 History of Round goby invasion in seven targeted sites representing the times since invasion and their assigned invasion categories.

<table>
<thead>
<tr>
<th>Invasion Category</th>
<th>Population</th>
<th>Time Since Invasion (years)</th>
<th>Year Detected</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Established</td>
<td>Detroit River</td>
<td>25</td>
<td>1990</td>
<td>Jude 1992</td>
</tr>
<tr>
<td></td>
<td>Lake Huron</td>
<td>25</td>
<td>1990</td>
<td>Jude 1992</td>
</tr>
<tr>
<td></td>
<td>Lake Erie</td>
<td>22</td>
<td>1993</td>
<td>Charlebois 1997</td>
</tr>
<tr>
<td>Recently Invaded</td>
<td>Lake Ontario</td>
<td>17</td>
<td>1999</td>
<td>Owens 2003</td>
</tr>
<tr>
<td></td>
<td>Georgian Bay</td>
<td>16</td>
<td>1999/2000</td>
<td>EGBSC</td>
</tr>
<tr>
<td></td>
<td>Trent River</td>
<td>12</td>
<td>2003</td>
<td>Brownscombe 2012, Groen 2012</td>
</tr>
<tr>
<td></td>
<td>Grand River</td>
<td>9</td>
<td>2006</td>
<td>GRCA</td>
</tr>
</tbody>
</table>

GRCA- Grand River Conservation Authority

400 Clyde Road, PO Box 729, Cambridge ON, N1R 5W6

EGBSC- Eastern Georgian Bay Stewardship Council

833 Stisted Rd., RR #1, Burks Falls ON, P0A 1C0
Figure Captions

Figure 2.1  Map of the Round goby (*Neogobious melanostomus*) distribution and sampling sites at seven locations in southern Ontario: Detroit River (A), Lake Erie (B), Lake Huron (C), Grand River (D), Lake Ontario (E), Trent River (F), and Georgian Bay (G). Established and recently invaded sampling sites are also denoted by the solid and open dots, respectively. Bolded coastlines represent capture sites of the Round goby confirmed by the U.S Geographical Survey (USGS) (Kornis et al. 2012).

Figure 2.2 Experimental water temperature treatment by invasion category impacts on log cortisol in Round gobies: fish from the invasion front populations had higher cortisol levels in the ambient and heated water treatments, but not in the cooled water treatment, relative to the established populations. For ambient and heated water treatments “*” denotes significant effects across invasion categories.

Figure 2.3 Scatterplot of time since invasion and log cortisol responses of Round gobies for three experimental water temperature treatments: Panel A – Ambient; Panel B – Heated; Panel C – Cooled. A significant negative linear relationship was detected for the heated treatment (*P = 0.018 Y = a – bX; R = 0.71*).

Figure 2.4 Histogram showing mean log cortisol for Great Lakes Round gobies from the seven sampled populations for the three experimental water temperature treatments: Panel A – Ambient; Panel B – Heated; Panel C – Cooled. Different letters represent significant populations effects within each treatment.

Figure 2.5 Histogram showing relative transcription (ΔCt) for the *GR* gene in Round gobies exposed to different experimental water temperature treatments from two invasion categories (established populations and invasion front populations). Different letters represent significant invasion category effects within treatments, while different cases represent significant treatment effects across categories.

Figure 2.6 Histogram showing relative transcription (ΔCt) for three gene known to be involved in metabolism regulation in fish: *G6PD* (Panel A), *GLUTI* (Panel B) and *LDHB* (Panel C) in Round gobies exposed to different experimental water temperature treatments.
and from different invasion categories. For \textit{G6PD}, different letters represent significant invasion category effects within treatments, while different cases represent significant treatment effects across categories. For \textit{GLUT1} different letters represent significant treatment effects across categories. For \textit{LDHB} “*” denotes significant treatment effects across invasion categories.
Figures

Figure 2.1
Figure 2.2

![Bar chart showing log baseline cortisol levels for different experimental treatments. The chart compares two groups: Established and Invasion Front. The x-axis represents the experimental treatments (Ambient, Heated, Cooled) while the y-axis represents log baseline cortisol levels.]
Figure 2.3

A

Ambient

B

Heated

\[ r^2 = 0.71 \]
\[ p = 0.018 \]

C

Cooled
Figure 2.4

A

Ambient

Log Baseline Cortisol

Lake Huron Lake Erie Detroit River Georgian Bay Grand River Trent River Lake Ontario

Established Invasion Front

b b b

B

Heated

Log Baseline Cortisol

Lake Huron Lake Erie Detroit River Georgian Bay Grand River Trent River Lake Ontario

Population

ab ab a a

C

Cooled

Log Baseline Cortisol

Lake Huron Lake Erie Detroit River Georgian Bay Grand River Trent River Lake Ontario

Population

abcd bcd abc ab abc

d cd d
Figure 2.5

[Graph showing relative GR transcription (ΔCt) for different experimental treatments: Ambient, Heated, and Cooled. The graph compares established and invasion front conditions, with statistical letters indicating significant differences.]
Figure 2.6

A

Relative G6PD - Transcription (ΔCc)

Established Invasion Front

Ambient Heated Cooled

B

Relative GLUT1 - Transcription (ΔCc)

Ambient Heated Cooled

C

Relative LDHB - Transcription (ΔCc)

Ambient Heated Cooled
CHAPTER III

Transcription profiles of fitness related genes reflect time since establishment in the invasive Round goby (Neogobius melanostomus)

Introduction

Species may expand their range through short-term acclimation and/or long-term evolution of adaptive traits that maximize their fitness in their new environment. Species may initially respond to environmental stress associated with a novel habitat through phenotypic plasticity in response to the new environmental conditions (Piersma and Drent 2003). Indeed, an increased degree of phenotypic plasticity has been associated with colonization success by allowing colonizers to adopt phenotypes suited to local conditions (Brown et al. 2011a). This form of phenotypic acclimation is reversible and presumably provides a selective advantage (Gabriel and Lynch 1992). Thus, while phenotypic plasticity may enhance initial colonization, the successful phenotype may eventually become genetically fixed, reducing or eliminating the plasticity (Price et al. 2003; Pigliucci et al. 2006; Fitzpatrick 2012). Alternatively, natural selection can change the genetic structure of the colonizing population by selecting for heritable traits that specifically enhance their success by altering their sensitivity to environmental stressors (Stepien, 2005; Roman 2006; Bronnenhuber, 2011, Shulte 2014). The colonization success of a population can also increase by means of contemporary (rapid) evolution (Maron et al. 2004; Hendry et al 2007), which can result from strong selective pressures due to high levels of mortality in the novel environment (Stapely et al. 2015). While there is evidence that variation in physiological tolerances between established and range edge populations over latitudinal scales enhance invasion success (Osovitz et al. 2005, Sagarin et al. 2006, Place et al. 2008, to date it is unclear whether the differences resulted from acclimation to the different environments (plasticity) or from genetically-based differences. Some studies have reported genetic adaptation in newly invaded populations of mosquito (Aedes aegyptii) (Zhou et al. 2014), Japanese knotweed (Fallopia japonica) (Richards et al. 2012), and house sparrows (Passer domesticus) (Schrey et al. 2012).
Invasive species represent a special case of range expansion and are a growing economic and environmental problem on regional and global scales (Bax et al. 2003). The invasion process can be divided into a series of discrete stages and barriers which an invasive species must overcome to propagate in their new environment. These stages include: transport, introduction, establishment, and spread (Blackburn et al. 2011). Once the initial transport, introduction and establishment has occurred (via intentional or unintentional transport vectors), species may spread using long (aided by other species such as birds or humans) or short (diffusion dispersal) distance dispersal from their established range (Bronnenuber et al. 2011). Several factors such as dispersal rate (Spiegel et al. 2007, Leuven et al. 2009, Hulme et al. 2009), propagule pressure (Lockwood et al. 2005, Colautti et al. 2004) and dispersal mode (Sakai et al. 2001, Van Leeuwen et al. 2013) all affect the spread of invasive species. As invasive species expand their range, individuals and sub-populations are exposed to novel and potentially stressful environmental conditions. Perhaps not surprisingly, newly invaded populations have been found to exhibit phenotypic characteristics which differ from their more established or native region counterparts, presumably to compensate for the effects of the environmental stressors (e.g., Geng et al. 2006; Funk et al. 2008). Examples of key phenotypic traits include: elevated aggressive behaviour and competitiveness (Dubs et al. 1996, Groen et al. 2012), higher fecundity (Sousa et al. 2008), extended spawning season (Minchin et al. 2003), and broader diets (Carmen et al. 2006; Pettitt-Wade et al. 2015). The role of evolution in the invasion process is gaining much interest in recent literature (Perkins et al. 2013; Lande et al. 2015; Ziska et al. 2015). Broadening our understanding of the attributes that contribute to successful range expansions by means of acclimation and genetic adaptation can provide more accurate information on the risk status of current and potential new invasive species.

Variation in gene transcription at the individual level is driven by a combination of response to environmental signals and genetic effects (Westneat et al. 2015; Bonduriansky et al. 2015). Thus, variation in gene transcription can reflect both acclimation and adaptation, and indeed may be the proximate mechanism behind both acclimation and adaptation. Recently, gene transcription quantification has become a powerful tool for invasion biologists to characterize the invasion process at a subcellular level (Roman et al.
2006; Hodgins et al. 2013; Poelchau et al. 2013). Many candidate genes of known function or within known functional pathways have been identified as important for individual acclimation and adaptation to new environments (Kenkel et al. 2011, Kenkel et al. 2013). Many of these candidate genes are associated with performance and are correlated to biochemical and physiological processes such as; metabolism (Goedkoop et al. 2011, Liss et al. 2013), growth (Power et al. 2001), reproduction (Kirkpatrick et al. 2011) and immune response (Lee et al. 2004). However, while changes in the gene transcription of individual known-function genes can be important biomarkers of stress response (Wan et al. 2010; Zerebecki et al. 2011; Lyytinen et al. 2012), using functionally related groups of genes to generate a transcriptional profile can provide unique insight into the stress response as a whole, as well as acclimation and adaptation of individuals in a novel or changing environment (Gracey et al. 2008). Previous studies on invasive species have shown that differential gene transcription response at loci related to oxidative stress, proteolysis, energy metabolism, ion transport, cell signalling, and cytoskeleton reorganization may help to explain variation in invasion success (Lockwood 2010; Vein et al. 2014).

Examining the response of fitness-related genes to thermal challenges can provide us with insight on which biological processes are involved in local adaption or acclimation. Invasive species must be able to overcome many environmental factors to survive and persist in novel environments, including exploiting available food resources (growth/activity patterns), stress tolerance, and the ability to deal with novel parasites and pathogens. One way in which invasive species can maintain growth and activity patterns is by means of optimized metabolic processes (Gilman et al. 2005). The rate and efficiency with which animals are able to obtain, process, and allocate energy are important factors for organisms colonizing novel habitats, as is common in invasion biology (Sibly and Calow 1987; Lardies et al. 2004). Previous studies have shown that increased metabolic rates are correlated with adaptation of invasive species (Matthews and McMahon 1999; McCann et al. 2014). For example, high metabolic rates of two species of freshwater clams (Corbicula fluminea and Dreissena polymorpha) provide evidence of local adaptation that is associated with their capacity to colonize habitats requiring rapid burrowing due to high flow rates (Matthews and McMahon 1999). In more recent studies, lowered metabolic rates
have been shown to provide rapid acclimation to cold temperatures (Ibarz et al. 2010; Verbeek et al. 2012). For example, McCann et al. (2014) found that an invasive population of cane toads (*Rhinella marina*) in Australia are able to function at cool temperatures by adjusting their thermal tolerance (that occur via metabolic pathways) at their range edge. The tolerance of biotic and abiotic stressors in the newly invaded environment have also been linked to the distribution and success of invasive species (Schneider 2008; Hoy et al. 2011). For example, a study by Chen et al. 2013, found that an invasive wetland plant (*Alternanthera philoxeroides*) had a higher tolerance to waterlogging due to its higher photosynthetic capacity and non-soluble carbohydrate concentrations than its native counterpart (*Alternanthera sessilis*). Previous studies have also shown that the increase of transcription in stress related genes is correlated to local adaptation in invasive species (Wan et al. 2009; Zerebecki et al. 2011; Lyytinen et al. 2012). For example, a study by Zerebecki et al. 2011, found that the invasive and more thermo-tolerant species (*Diplosoma listerianum*) expressed higher levels of Heat shock protein 70 (stress correlated gene) than the less thermo-tolerant and native species (*Distaplia occidentalis*). Lastly, upon encountering new pathogens (which can potentially lead to high mortality or morbidity) successful invaders reduce their expression of costly inflammatory responses that are associated with metabolic and behavioural changes and rely more on antibody mediated immunity (Wessels et al. 1998; Lee et al. 2004). Since population bottlenecks are expected to occur at the invasion front, it is not surprising that, on average, there is a lower abundance and diversity of parasites at this stage (Phillips et al. 2010). However, in the absence of parasites or pathogens, invasive species may reallocate their energy to other mechanisms such as metabolism, growth and reproduction (Lee et al. 2005; White et al. 2012; Van der Most et al. 2011). A recent study by Llewellyn et al. 2012 found that invasion front populations of cane toads (*Rhinella marina*) have reduced investment in their immune function compared to long-established populations. This lowered immune response in invasion front populations has been interpreted as being adaptive (Horrocks et al. 2011; Eizaguirre et al. 2012). Studies in teleost fish have shown that high levels of stress hormones (glucocorticoids and catecholamines) result in a decreased expression of immune associated genes, which is believed to limit inflammation and reduce tissue damage. In a study done by Castro et al. (2011), the stimulation of cortisol (resulting in an upregulation
of stress related genes) in rainbow trout (Oncorhynchus mykiss) resulted in a suppression of immune related genes. Overall, the fitness related genes to a thermal stress can provide insight of the biological processes that are responsible for the adaptation and acclimation of invasive species colonizing novel environments.

The goal of this study was to examine the transcriptional response to the colonization of new habitats in a model invasive species: the Round goby (Neogobius melanostomus). In addition to the Round goby being an extremely successful invader in the Laurentian Great Lakes of North America, it is also a globally successful invader (Corkum 2004, Koris et al. 2012). In North America, the species was first introduced in Lake St. Clair in 1990, via ballast water discharge (Jude 1992). The primary source of the introduction is believed to be the Dnieper River (Kherson, Ukraine) on the Black Sea (Brown and Stepien 2009). The species can now be found throughout the Great Lakes (Phillips et al. 2003, Schaeffer et al. 2005, Poos et al. 2010). Phenotypic traits have been proposed as facilitating the invasion success in Round gobies (see Chapter I). Round gobies have also been found to have high levels of genetic variation in comparison to their native range which may reflect a greater buffer against founder effects and higher adaptive potential (Lee 2002, Brown and Stepien 2009). The invasive Round goby populations in the Great Lakes basin represent an ideal model for characterizing molecular genetic changes associated with different stages of the invasion process as establishment dates are generally well known and established and invasion front populations can be identified.

Here we examine the effect of novel habitat colonization on the Round goby transcription profile using twenty-six biologically relevant candidate genes. Our overall goal was to determine whether transcriptional profiling using genes associated with invasion success can help predict the spread of this invasive species and perhaps identify environmental limits to its spread. Candidate genes were selected from three functional categories: Metabolism, Immune Function, and Stress Response (Table 3.1) based on the most relevant fitness-related genes currently known for invasive species. We maintained groups of wild-caught gobies at ambient, elevated and reduced temperatures and used quantitative real-time PCR (qRT-PCR) to quantify relative gene transcription. In addition,
we compared these treatment-induced gene transcription profiles among populations with various times since establishment (9 to 25 years). Based on previous genomic work in successful invasive species, we predicted that the Round gobies in the invasion front populations would exhibit upregulation of metabolic and stress response genes relative to fish from established regions, but we predicted no such pattern for immune function genes. We also expected a greater transcriptional response to the cooled water treatment relative to the heated water treatment, as our cool water treatment was a greater change from summer ambient conditions and hence likely represents more of an environmental stressor. Additionally, we predicted that there would be a similar transcriptional response in Round goby from their native region (Dnieper River) to the established populations in the Great Lakes.

Methods

Capture and Transport
All Great Lake Round gobies were caught during the months of July and August, in 2014 and 2015. We targeted a total of seven sites for goby collection representing a variety of times since establishment (Table 3.1): Detroit River, Grand River, Trent River, Lake Erie, Lake Huron, Lake Ontario, and Georgian Bay (Figure 3.1). For temporal replication purposes three locations were sampled in both years: Detroit River, Lake Huron, and Grand River. Water temperature in all of the sites averaged about 22-24°C. In this study, individual sample sites are defined as a “population.” Each population was assigned to an invasion “category” representing the amount of time a given population had been observed at a site: ‘established’ populations have been present for ≥22 years, while ‘invasion front’ populations have been present for ≤17 years (Table 3.1). At each collection site, gobies were captured using a seine net between the hours of 8:00 am - 11:00 am. Individuals were held in 5L aerated plastic coolers filled with lake water and stored in the shade (for approximately one hour) until transport to a nearby location (approximately 2-3 km) where the experimental setup was located.
Experimental Protocol and Sample Collection

Fish from each site were randomly assigned to one of three experimental water temperature treatments: control (ambient lake temperature), heated (5°C above ambient) and cooled (15°C below ambient). A rapid increase or decrease from ambient temperature can result in an acute stress response (Donaldson 2008, Kelley 2014) and thus provides a quantitative physiological response. The magnitude of the heated water treatment (5°C above ambient) was chosen to ensure that it did not exceed the round goby thermal tolerance of 30°C (Cross 2009). The magnitude of the cooled water treatment (15°C below ambient) was chosen to mimic rapid Fall/beginning of winter water temperature declines in the Great Lakes where winter temperatures range from 0.2-5°C (NOAA 2015). Two covered 5 L tanks (to ensure a dark environment) were assigned to each temperature treatment, and each tank housed five gobies. The water for each temperature treatment was stored in a large 95 L cooler where the water temperature was controlled (ambient, heated or cooled) and water was pumped into the smaller 5 L housing tanks. Although pseudoreplication was taken into consideration, this experimental design was conducted for logistic purposes in the field (Hurlbert 1984; Hulbert 2009). The recirculating water set-up ensured a continuous flow of consistently oxygenated water. All experimental fish were held for 20 hours. The water temperature for the cold and warm challenges were altered approximately 2-3°C every hour until the final desired temperature was achieved (i.e., after approximately six hours for the cooled treatment and two hours for the heated treatment). Individual fish were humanely euthanized by an overdose solution of clove oil (approximately 40mg/L). Individual liver samples were collected and immediately preserved in a high salt buffer (used to protect and stabilize cellular RNA) and stored at -20°C until RNA extraction. Liver tissue was chosen because of its metabolic and immune importance, as well as its role in the response to stress (Cuesta et al. 2008; Gracey et al. 2008; Peatman et al. 2008). Liver has also been used in other studies pertaining to stress, glucocorticoid regulation, and organism-wide homeostasis (Aluru 2009).
Capture and Transport of Ukraine Round Goby

Fourteen round gobies were collected from the Dnieper River in Kherson, Ukraine on August 2013 (N 46°62746’, E 32°56882’). The Dnieper River is thought to be the native range of the Great Lakes invasive round gobies (Brown et al. 2009). Water temperature in the river was approximately 21°C – 24°C. The gobies were transported in a 6L container with fresh river water to the lab. Gobies were held in a cooler with aerated water consisting of 50% tap water, 50% river water. The experiment consisted of holding fish at ambient river temperature (control) and in heated water to elicit a temperature stress. Unfortunately, water cooling facilities were not available. The control experiment fish (N = 7) were held in a 6L container of tap water at 22°C. The temperature challenge fish (N = 7) were held in a 6L container of tap water at approximately 30°C (Cross, 2009). All experimental fish were held for 20 hours. After 20 hours, the fish were humanely euthanized in 50 ppm MS222 and liver tissue was sampled, stored in a high salt buffer solution, and transferred at -20°C to the University of Windsor.

Selection of Candidate Loci

Twenty-six genes were chosen for qRT-PCR analysis based on their relevance for invasion success (Table 3.1). The candidate genes were selected based on their roles in maintaining homeostasis upon organism exposure to temperature stress (Buckley et al. 2006, Buckley et al. 2008, Smith et al. 2013, Liu et al 2015) within three biologically relevant functional ontologies: Metabolism, Immunity, and Stress Response.

Metabolism

Genes in the metabolic pathway regulate the supply of energy (ATP) for several biological functions such as glycolysis, gluconeogenesis, protein synthesis, the electron transport chain, and the maintenance of membrane viscosity - all of these processes directly impact individual performance under stressful or competitive conditions (Buckley 2006). The candidate genes representing metabolic function in this study were: Glutamine synthetase (GLUL), Glucose-6-Phosphate Dehydrogenase (G6PD), Glucose transporter (GLUT1), Lipase (LPL), Lipin-1 (LPLN1), Transferrin (TF), Ceruplasmin (CP), Lactate
Dehydrogenase-B \((LDHB)\), Sodium-potassium ATPase \((NAK1ab)\), Beta-Globin \((HBB)\), and Cytochrome p450 1A3 \((CYP1A3)\).

Tissue-specific genes such as \(GLUL\), is a key enzyme for nitrogen metabolism in fish because of its active role in the conversion of glutamate and ammonia to form glutamine \((Walsh\ et\ al.\ 2003)\). Up-regulation of \(GLUL\) is speculated to be related to the movement and excretion of nitrogenous waste from the tissues of the fish \((Buckley\ et\ al.\ 2006)\). \(GLUT1\) and \(G6PD\) are both involved in energy metabolism. \(GLUT1\) transports glucose into cells from the blood or from other cells for metabolic needs \((Teerijoki\ 2000)\). \(G6PD\) is involved in the pentose phosphate pathway and thus in regulating metabolism. Up-regulation of \(G6PD\) has been previously reported to be related to cellular energy pools that are accessed to fuel the stress response and repair mechanisms \((Buckley\ et\ al.\ 2006)\). \(LPL\) and \(LPLN1\) are both involved in fatty acid metabolism. The changes in expression of genes related to lipid metabolism have been correlated with membrane fluidity during thermal acclimation \((Logan\ et\ al.\ 2010)\). Lipid metabolism was found to change during thermal acclimation because of shifts in membrane composition and in energy preferences for ATP production \((Logan\ et\ al.\ 2010)\). \(TF\) and \(CP\) are related to iron metabolism and work simultaneously to help maintain iron homeostasis in cells by controlling iron concentration \((Gracey\ 2000,\ Rojo\ 2007)\). \(TF\) has also been reported to have a role in immune function \((Go´mez\ et\ al.\ 2007;\ Neves\ et\ al.\ 2009)\), specifically, it plays a role in the innate immune response and macrophage activation \((Stafford\ et\ al.\ 2002)\). The main function of \(LDHB\) is to convert lactate to pyruvic acid. In a study by Segal \((1994)\), northern populations of killfish \((Fundulus\ heteroclitus)\) had higher expression of the \(LDHB\) gene than southern populations and this was attributed to an evolved difference between the two populations and not physiological acclimation. In a more recent study, \(LDHB\) was correlated to endurance swimming in cod \((Gadus\ morhua)\) \((Guderley\ 2004)\). \(NAK1ab\) is a sodium-potassium ATPase. It is part of a multigene family, with many of its transcripts associated with \(NA^+\) secretion in salt water. McCairns \((2009)\) studied differences in gene expression in salt- and freshwater sticklebacks and found that \(NAK1ab\) was expressed 1.7- 2.8 fold higher in freshwater sticklebacks relative to those from salt water. This suggests that \(NAK1ab\) alone can provide sufficient energy to promote sodium intake. \(HBB\) is a hemoglobin complex that is associated with oxygen transport and has been reported to have
a role in the response to prolonged heat, likely due to compromised oxygen supply associated with high water temperatures (Kassahn 2007). CYP1A3 is related to carbohydrate metabolism and has been shown to increase metabolism to meet the energy requirements associated with aspects of the cellular stress response (CSR), including chaperoning of proteins, the degradation of damaged proteins and the repair of damaged DNA (Buckley 2008). Together the selected metabolic genes provide a broad profile of metabolic responses to stress and they serve to characterize the metabolic transcription responses contribute to the acclimation and adaptation of invasive species.

**Stress**

Stress-response genes are involved in the regulation of processes such as cell survival, neuronal excitability, and renal sodium excretion. The candidate genes representing the stress response in this study are: glucocorticoid receptor (GR), Heat Shock Protein 90 (HSP90), Heat Shock Protein 70 (HSP70), Heat Shock Protein 47 (DNAJ), Steroidogenic Acute Regulatory protein (StAR), Serum/threonine protein Kinase (SGK1), and Urotensin (UI). During the stress response, glucocorticoid (‘stress’) hormones act through the glucocorticoid receptor, which regulates the transcription of target stress related genes. GR binds DNA at glucocorticoid response elements (GREs) in the promoter regions of corticosteroid responsive genes, inducing transcription (Hori et al 2011, Castro et al. 2011). HSP90 and HSP70 are chaperone proteins that maintain protein homeostasis during cellular exposure to thermal stressors by interacting with stress denatured proteins, preventing promoting damaged protein degradation (Parsell and Lindquist, 1993). Invasive *Diplosoma listerianum* have been shown to express higher levels of HSP70 when exposed to a thermal challenge, than the native *Distaplia occidentalis* (Zerebecki et al. 2011) suggesting that HSP70 transcription and broad thermal tolerance is associated with successful invasive species. DNAJ4 (also reported as HSP47) plays a role in regulating the ATPase activity of HSP70 (Liu 2015). StAR is a key enzyme in the rate-limiting step in steroidogenesis by which steroids are generated from cholesterol and converted into other steroids such as cortisol (Hori 2011). SGK1 encodes a serine/threonine protein kinase that activates specific potassium, sodium, and chloride channels. These channels are involved in the regulation of processes such as cell survival, neuronal excitability, and renal sodium excretion.
(Buckley 2008). UI works simultaneously with a corticotrophin releasing factor (CRF1). The upregulation of UI and CRF1 in Rattus rattus results in an increase in blood pressure and anxiety which in turn, are stress coping responses (Suda 2004).

**Immune**

Genes involved in the immune response are a part of complex interactions among cytokines, inflammation, and the adaptive response that promote homeostasis in response to pathogen exposure. The candidate genes representing the immune response in this study include: Tumor Necrosis Factor-a (TNF), Interleukin 20 (IL-20), Interleukin 6 (IL-6), Fibronectin (FN), Cannabinoid Receptors (CB), Complement 7 (C7), Cytochrome c oxidase subunit 1 (COX1), and Periplakin (PPL). TNF displays pro-inflammatory effects through activating pathways and promoting apoptosis in various cell types (MacKay et al. 1993; Wang et al. 2008). The induction of TNF transcription in immune challenged grass carp (Ctenopharyngodon idella) provided evidence for antiviral innate immunity (Wang et al. 2013). The transcription of IL-20 plays an important role in the cytokine network and has been reported to be modulated by pro-inflammatory cytokines and signalling pathway activators. The increased transcription of IL-20 in an immune challenged rainbow trout (Oncorhynchus mykiss), suggests that it may be manipulated by the rapid increase of other pro-inflammatory cytokines such as IL-1beta (Wang et al. 2010). IL-6 is involved in the control of immunoglobulin production, lymphocyte and monocyte differentiation, and the secretion and migration of leukocytes to inflammation sites (Hirano 1998; Kaplanski et al. 2003; Woo et al. 2005). Induction of IL-6 transcription in immune challenged rainbow trout (Oncorhynchus mykiss) increased macrophage growth and antimicrobial peptides, which suggests that IL-6 has a novel role in the immune system of fish (Costa et al. 2011). FN encodes a glycoprotein that forms at the cell surface and is involved in cell adhesion and migration including embryogenesis, wound healing, blood coagulation, host defense, and metastasis (Buckley 2006). CB and C7 are part of the innate immune response (Cuklev 2011) and have been shown to upregulate in response to increased temperatures in killifish (Austrofundulus limnaeus) (Podrabsky et al. 2004). COX1 has been studied for its role and association with electron transport and apoptosis (Wang et al. 2001). More recently, studies have shown the importance of COX1 in the protection of larvae against infectious agents.
before adaptive immunity has developed. COX1 was induced in zebrafish larvae (*Danio rerio*), which helped to prevent pathologies associated with excessive inflammation during development (Galindo-Villegas et al. 2012). PPL has a role in ligand binding, endocytosis, and antigen presentation (Beekman et al. 2004) and has increased transcription under thermal challenge in longjaw mudsuckers (*Gillichthys mirabilis*) providing evidence for PPL responding to temperature as well as having an important role in the immune response.

**Primer/Probe Design and Optimization**

For each candidate and control gene, gene sequences of Round goby related species available in Genbank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) were downloaded and aligned using Geneious Pro software v6.1.6. Primers and probes for ten of the selected genes were designed to match the most conserved regions of the sequences (averaging an amplicon size of 100 bp; Supplemental Table S1) with the use of Primer Express® software (v3.1). These genes were then referenced against a Round goby transcriptome. The remaining 18 gene primers and probes were designed using a preliminary Round goby transcriptome based on RNA-Sequencing data from five heat/cold/control paired samples in Round goby from the Detroit River (K. Wellband, unpublished data). The specificity of each primer set was verified by PCR and amplicon visualization by gel electrophoresis. In addition, a melt curve analysis (using SYBR Green) was conducted using the optimized primer sets to further verify the primer optimization. The primers are predicted to have 100% efficiency using pcrEfficiency software (Mallona et al. 2011). All primer and probe sequences are listed in Table S1 (Supplementary Material).

**RNA Extraction and cDNA synthesis**

Total RNA was extracted from round goby liver tissue following the method of Chomczynski and Sacchi (1987). RNA degradation was assessed using gel-electrophoresis and 28S and 18S RNA bands were identified. Purity and concentration of RNA was assessed using UV Spectrophotometry (Wellband 2013). Complementary DNA (cDNA) was synthesized using a High Capacity cDNA Reverse Transcription (RT) kit (Applied
Biosystems, Burlington, ON, Canada). The cDNA was stored at -80°C until qRT-PCR analysis.

Quantitative Real-Time PCR

OpenArray qRT-PCR was performed using a QuantStudio 12K Flex Real-Time PCR System following the manufacture’s instruction. A 5 µL mixture was prepared for each cDNA sample which contained 2.5 µL TaqMan® OpenArray® Real-Time PCR Master Mix (Applied Biosystems, Burlington, ON, Canada) and 1.2 µL cDNA. A 56×48 format OpenArray chip was used, which had 48 subarrays in each chip and each subarray contains 64 through-holes. Each chip was used to measure gene transcription for 48 individual Round goby cDNA samples for all 28 target genes (in duplicate). A total of 314 cDNA samples were used comprising of: ten sample sites each having thirty fish per site. Fourteen native site fish from the Dnieper River fish (7 control and 7 heated water) were also included. The 5 µL mixtures were prepared in 384-well plates and were then loaded into OpenArray® chips using the OpenArray® AccuFill System and each qRT-PCR reaction was performed in a 33 nL volume.

Endogenous Controls

Transcriptional stability of the raw Ct values was analyzed using GeNorm in R (Vandesompele 2002). B-actin was ranked as having higher stability than EF1a. In addition, two-way ANOVAs were conducted to test for transcriptional response of EF1a and B-actin to temperature, population and their interaction. EF1a showed a significant population effect (p = 0.0315). B-actin did not show significant effects for treatment, population or their interaction. B-actin was therefore chosen as the candidate endogenous control for ∆Ct normalization.

Normalizing and Calculating ∆Ct and ∆∆Ct values

First, all raw Ct (threshold cycle) values were normalized to the endogenous control (B-actin) to generate ∆Ct values for all genes for all samples. ∆∆Ct values (ΔCt values for heated and cooled treatments normalized to the mean ambient temperature treatment) were
calculated for only those genes that showed a significant treatment (heated and cooled) or interaction effect – see below.

**Statistical Analyses**
All statistical analyses were conducted using JMP (SAS Inc., Version 12.1) and gene transcription data was analyzed using the ExpressionSuite software (Applied Biosystems).

**Genes Responding to Temperature**
First, we used a general linear mixed model (GLMM) to test for treatment or treatment-by-population interaction effects (using ∆Ct values) to identify the genes responding to the treatments (heated and cooled versus ambient). Models included: temperature, population and the interaction between population and treatment for each gene. Sampling year and replicate tank were included as random effects. Genes that did not show a response to temperature were not analyzed further for invasion category effects (established and invasion front) in the challenged fish as the ambient temperature gene transcription data already captures gene transcription variation for the non-responding loci (Table 3.3). The fish collected from the native region (Ukraine) were not included in the statistical analyses due to slightly different challenge protocols in the field, rather those data were used for comparative purposes only.

**Invasion Category Effect**
A second GLMM was used to test genes for differences in transcription between invasion category groups (established and invasion front) using ∆∆Ct (heated and cooled treatments) and ∆Ct (ambient (control) treatment) values. Only genes that showed a significant treatment and or treatment-by-population effect (from the previous GLMM) were used to test for invasion category effects on challenge response (Figure 3.2; Supplementary Material: Table S2). The model included: the interaction between the established and invasion front populations. The sampling year and replicate tank were used in the model as random effects. A Benjamini-Hochberg procedure was conducted on all p-values to correct for multiple simultaneous comparisons (Yekutieli and Benjamini 1999). Once again, fish from the native region were not used in this statistical analyses (see above).
Functional Response

We correlated the estimated time since establishment and ΔCt and ΔΔCt to explore the possibility of a functional response of individual gene transcription to time since establishment. Only genes and treatments that showed a significant invasion category effect (from the GLMM) were used in the linear regression analysis to test for a linear functional response (Figure 3.3; Supplementary Material: S3). The native region data was used for comparison purposes to establish native region gene response to heated water challenges. Benjamini- Hochberg procedure was conducted on all p-values to correct for multiple simultaneous comparisons.

Gene Associations

Finally, a principal component analysis (PCA) using a Pearson correlation matrix was used to test for gene association patterns within each treatment group (ambient, cooled and heated). All genes across all ontology categories were used in the PCA using ΔCt values (Figure 3.4).

Results

Reference Genes

Two reference genes (EF1a and B-actin; Table 1) were chosen to act as endogenous controls (Zhong et al. 2011; Dheda et al. 2011). Only one endogenous control (B-actin) was chosen for normalization in our data analysis to reduce possible normalization errors. Gene stability and the effects of population, treatment, and their interactions were used as criteria for selecting the endogenous control for normalization.

Treatment Effect

We found more genes exhibited a response to the cooled temperature treatment than the heated treatment (and with higher significance), with twenty genes showing a significant transcriptional response in the cooled treatment and twelve genes showing a significant transcriptional response in the heated treatment at the P <0.05 alpha level (Table 3.3).
Commonly-expressed metabolic (GLUT1, TF, LPL, LPLN1 and HBB), immune (SGK1, HSP70, HSP90, DNAJ4, and STAR), and stress (IL20 and CB) genes showed a significant response relative to ambient temperature controls in both cooled and heated treatments. Furthermore, the transcriptional response to the cooled temperature involved eight genes that showed no response in the heated treatment, they included G6PD, LDHB, NAK1ab, GR, COX1, C7, TNF, and PPL. Relatively few genes exhibited a significant treatment-by-population interaction effect in either the heated or cooled water treatments (Table 3.3). Furthermore, most of the genes that responded to thermal stress had stress related function (Table 3.3). However, four genes did show a significant interaction effect (P < 0.05) when there was no significant treatment effect (Table 3.3). The primary purpose of this analysis was to identify genes that exhibited response to the treatment for inclusion in further analysis (ΔΔCt calculations), thus we used the 0.05 alpha value for the threshold for inclusion in further analysis, without an alpha-level correction for multiple simultaneous tests. No significant tank or year effect was observed at the P < 0.05 level.

Invasion Category Effects

Ambient (control): We found one stress related gene (HSP70) to have significant invasion category effects in the ambient water treatment. There were no significant invasion category effects for any of the metabolic or immune genes. No tank or year effects were significant for any gene (Figure 3.2; Supplementary Material Table S2).

Cooled and Heated Treatment: We found more genes exhibited an invasion category effect in the cooled treatment (ΔΔCt) than for the heated treatment, with five (out of twenty) genes showing a significant invasion category effect in the cooled and two genes (out of seventeen) showing a significant effect in the heated at P<0.05 (after Benjamini- Hochberg procedure was conducted) (Figure 3.2; Supplementary Material Table S2). Two genes (HSP70 and TNF) exhibited a significant invasion category effect in both heated and cooled treatments. Conversely, three genes (LPL, G6PD, an LDHB) had an invasion category effect in only the cooled treatment. Interestingly, all genes (which exhibited an invasion category effect) exhibited the same pattern of a significant down-regulation in invasion
front Round goby for both the heated and cooled water treatments. No tank or year effects were significant for any gene under either temperature treatment (Figure 3.2; Supplementary Material Table S2).

*Functional Response*

We used a correlation analyses to test for functional relationships between time since establishment and normalized gene transcription using only genes that exhibited a significant invasion category effect in the heated, cooled and ambient water treatment (Figures 3.3, Supplementary Material: S3). We detected a significant and positive relationship between years since establishment and *HSP70* in the cooled and heated temperatures (Figure 3.3). Conversely, there was also a significant and negative relationship between years since establishment and *HSP70* in the ambient (control) treatment. *LPL* was the only metabolic gene to exhibit a significant functional response in the cooled treatment. Comparatively, the native population seemed to respond similarly to the established populations in the heated and ambient treatments (Figure 3.3).

*Gene Associations*

We used a PCA to explore associations among the genes included in our transcription profile within each temperature treatment (ambient, cooled and heated; Figure 3.4). In the ambient treatment, the PCA revealed that 36.1% could be described by Factor 1 and 12.8% could be described by Factor 2 (Figure 3.4). In the cooled treatments, 25.7% and 9.31% of the variation could be described by Factor 1 and Factor 2 respectively (Figure 3.4). In the heated treatment, 33.7% and 10.1% of the variation could be described by Factor 1 and Factor 2 respectively (Figure 3.4).

Overall, the genes in the three functional groups (metabolism, immune and stress) do not show distinct clustering in the two treatment and ambient (control) conditions. However, there were other clusters of genes which were consistent across treatments, indicating possible correlated function or transcription. For example, among metabolic genes consistent associations of *LDHB* and *LPLN1* were evident in all three temperature treatments (Figures 3.4). However, no other associations of metabolic genes
were exhibited within the temperature treatments. However, associations between genes in different functional categories (NAK1ab and IL6 as well as G6PD and CP) were all found in the heated and ambient temperatures.

Discussion
The distribution of a species is limited by habitat requirements, specialized life histories and physiological tolerances, thus range expansion is predicated upon either a change in the environment at the range edge or a change in the biology of the organism (Hampe et al. 2005; Pfenninger et al. 2007; Svenning et al. 2008). One of the major challenges invasive species face is the dramatic environmental change they experience upon introduction, a process quite different from the gradual range expansion experienced by organisms expanding their range naturally. Successful invasive species must be able to rapidly adapt to novel environments to ensure not only individual survival but also species spread and persistence (Roman 2006; North et al. 2010; Peacor et al. 2012). Dispersing species that colonise novel environments, whether the dispersal is intentional or unintentional, must adjust to the novel environment through acclimation (plasticity) and/or genetic adaptation. However, assessing transcriptional measures (at one or two genes) or single phenotypic traits may not reflect complex adaptive responses. Alternatively, transcriptional profiles include the response of multiple genes across various biological pathways within the primary physiological responses to a novel environment (e.g., stress, energy management and immune response). Thus, transcriptional profiles are a powerful tool to study genetic adaption as well as acclimation. By examining the transcriptional profile responses of Round gobies from different populations exposed to different water temperatures, we gained insight into their ability to respond to novel and likely stressful conditions, such responses form the basis for acclimation and adaption to novel environments.

*Relative Gene Responses Across Biological Systems*
Transcriptional responses to thermal challenges have been reported in many aquatic species and suggests that organisms may exhibit either predictable plastic responses or evolutionary
adaptations to thermal stress (e.g., Smith et al. 2013; Lord et al 2015; Liu et al. 2015). We found that thermal stress challenges elicited a response across all three invasion critical gene categories, with the stress genes showing the most dramatic response. Additionally, in general, genes exhibiting a significant transcriptional response to one of the thermal challenges also showed a significant response to the other. Perhaps not surprisingly, the cooled water treatment tended to elicit a stronger transcriptional response than the heated water challenge. This is likely due to the fish in the cooled treatment experiencing a more dramatic change in temperature, which may have led to a hyper-responsive reaction of the stress, metabolic and immune axis in all fish. Transcriptional profiling at the individual gene level in response to a thermal stress has been reported in many studies (Swindell et al. 2007; Farcy et al. 2009; Lancaster et al. 2016). For example, a recent study by Komoro et al (2015) found limited thermal plasticity across multiple genes due to chronic thermal stress in the endangered delta smelt (Hypomesus transpacificus). Moreover, the response of metabolic, stress and immune genes to the cooled and heated challenges provides evidence that temperature is a critical environmental parameter for fish. As most genes did respond to a common environmental stressor (temperature), we are confident that a thermal challenge was a relevant choice for analyzing invasion and can be used as a powerful tool for invasion biology.

We found differences between the transcriptional profiles of fish from the established versus the invasion front populations under ambient (control) conditions as well as in their response to thermal stress. The differences included metabolic, stress-response and immune function related loci, highlighting the value of examining a profile of diverse genes with functions critical for novel habitat colonization success. Population-level differences in gene transcription have been previously reported and interpreted as adaptive, either through acclimation or genetic adaptation (Lucassen et al. 2006; Lancaster et al. 2016; Xu et al. 2016). For example, a study by Lucassen et al. (2006) showed that differential gene transcription of CS (citrate synthase) and COX (cytochrome c oxidase) contributed to adaptation and acclimation in northern (warm adapted) and southern (cold adapted) populations of cod (Gadus morhua L). Additional studies have also shown location-specific transcriptional responses to thermal stress in genes correlated with energy
and protein metabolism in response to stress (Polato et al. 2010, Fangue et al. 2006; Roberge et al. 2007). Moreover, Roberge et al. (2007) compared transcription differences between two subpopulations of Atlantic salmon (*Salmo salar*) and found that genes that promoted local adaptation in these two subpopulations, differed significantly in their transcription and were involved in biological functions such as metabolism and immunity. In our study, genes associated with metabolic function exhibited the most significant invasion category effects in response to thermal stress. The transcription of metabolic genes has been shown to exhibit adaptive evolution in fish (Elmer et al. 2010; Morris et al. 2014). For example, Morris et al. (2014) studied the plastic and adaptive responses of marine and freshwater populations of the threespine stickleback (*Gasterosteus aculeatus*) and reported that several genes (including those associated with metabolism) exhibited plastic and adaptive responses in ancestral and derived populations, respectively. They concluded that plastic responses can eventually evolve to meet the challenges of novel environments.

While transcriptional profiling provides an overview of the effect of the time since establishment on patterns of gene transcription, my data also allows specific gene responses between treatments to be explored. For example, *HSP70* and *TNF* exhibited a consistent significant invasion category effect in both heated and cooled treatments (Figure 3.2). The down-regulation of both genes in the invasion front (relative to established) fish under thermal stress might reflect reduction in the transcription of genes that are energetically costly to express (Krebs et al. 1998). That is, while rapid up-regulation *HSP70* and *TNF* is expected in response to environmental stress (Zerebecki et al. 2011; Wang et al. 2013; Quistad et al. 2014), high levels of those proteins can be harmful to individuals, therefore natural selection may act to limit the response in invasion front population which presumably experience chronic environmental stress (Krebs et al. 1998; Kollias 2005). I found a number of genes that exhibited a significant invasion category effect in only the cooled treatment, in all cases the established populations showed higher transcription than the invasion front fish. This suggests that these genes (involved in metabolism) are specifically responsible for cold water response in established populations of Round goby. One of the genes (*LDHB*), has been previously attributed to an evolved difference between southern and northern populations of killfish (*Fundulus heteroclitus*) (Segal 1994). The other two metabolic genes (*LPL* and *G6PD*) which exhibited an invasion category effect
are attributed to fatty acid metabolism and glycolysis; fatty acid metabolism and glycolysis are known to play a major role in growth, reproduction and movement (spread) (Tocher et al. 2003; Black et al. 2006). Only one stress gene (HSP70) showed a significant invasion category effect under ambient conditions; however, we cannot suggest that Round goby exhibit a lower resting stress response in invasion front populations based on a single gene.

Importance of Appreciating Invasion Stage

While an invasion category effect is indicative of a difference between invasion front and established populations in the transcriptional profile response to thermal stress, the nature of that response can be explored in more detail by examining the functional relationship between time since establishment and gene transcription (Figure 3.3). Furthermore, the shape of the relationship can provide information on whether the observed effect is due to a gradual change or a step function. Our study reveals an invasion category effect with time since establishment in metabolic, immune and stress categories in the cooled treatment. Specifically, HSP70 (and LPL in the cooled treatment) showed variation with time since establishment and exhibited an abrupt change in response in all temperature treatments. Interestingly, this abrupt change in response occurred at 17 (HSP70) and 16 (LPL) years since the date of detection in the cooled treatment. This abrupt change in response (step function) of these two invasion front populations (Lake Ontario and Georgian Bay) could be evidence of those populations being environmentally different from the others and therefore causing the Round gobies to acclimate. On the other hand, Round gobies could have genetically adapted in these sites which would make them genetically different from the other populations. The short generation time of the Round goby (~ 1-2 yrs) (Jude et al. 1992; Corkum et al. 1998) increases the likelihood of rapid adaptation. Alternatively, these functional relationships may be due to an environmental cline which can be due to acclimation (plasticity). Previous studies have interpreted transcriptional clines over latitudinal gradients as being evidence for adaptive responses (Powers et al. 1998; Poertner et al. 2008; Place et al. 2008). For example, Sezgin et al. 2004 reported a latitudinal cline in the transcription of three metabolic genes in ten populations of the common fruit fly (Drosophila melanogaster) spanning southern Florida to northern Vermont, thus suggesting temperature adaptation in these populations. Although studies have interpreted
transcriptional clines as being adaptive, genetic adaptation is unlikely in our case due to the limited number of generations between the established and invasion front populations in this study. Interestingly, when we compared the transcriptional response of Round goby from their native populations to the Great Lakes Round goby, we found they most closely corresponded with the Great Lakes established populations. Previous studies comparing native and invasive populations of aquatic species have found both physiological and transcriptional differences, and attributed their colonization success to adaptation and/or acclimation (Funk et al. 2008; Davidson et al. 2011; Daehler et al. 2003; Tepolt et al. 2015).

*Gene Interactions*

Genes interact in complex ways and many genes have multiple functions (Blencowe et al. 2006). Although the genes in this study were categorized into one of three functional groups (metabolism, immune, or stress), the functional category may not reflect all of actual gene functions. Thus, individual genes may be found in alternative functional group boundaries. For example, *HSP70* and *TF* (both categorised here as stress genes) have been reported to have a role in the immune system in mammalian species (Vabulas et al. 2002, Wallin et al 2002, Millar et al. 2003; Go´mez et al. 2007; Neves et al. 2009). This alternative biological relevance to the immune system may help to explain the high level of differentiation in *HSP70* transcription between invasion categories and treatments.

In theory, the three functional groups should cluster together in a PCA showing transcriptional response to a thermal challenge, with perhaps some overlap. Although there was clustering of some genes in the ambient temperature treatment, there was no apparent clustering of the genes in the functional groups (Figure 3.4). This is also true for the heated and cooled temperature treatments. The lack of functional group clustering in the PCA analysis suggests that there are alternative functions for many of the genes in this study. While some genes show highly conserved transcriptional associations, for example *LDHB* and *LPLN1* cluster across all temperatures treatments including ambient, consistent with their known related metabolic function, this is not a consistent pattern. For example, there are consistent transcriptional associations across temperature treatments that are not consistent with their putative functional categories, such as *NAK1ab* and *IL6*. While
NAK1ab was classified as a primarily a stress-response gene in this study, it has also been characterized as having immunological function (Delhase et al. 2011) which could explain its close association with IL6. Metabolic genes G6PD and CP cluster closely in the heated and ambient treatments; however, the lack of clustering of these two genes in the cooled treatment suggests that the coordinated transcription of these two genes may be disrupted under a strong temperature stress conditions. Transcriptional clustering was also evident between HSP90 and COX1 in the heated and ambient temperatures treatments. Mammalian studies have suggested that HSP90 has immunological function with direct association with COX1 due to its capability of converting COX1 into COX2 during tissue inflammation and damage (Murakami et al. 2003; Kudo et al. 2005). Overall, the transcriptional profile developed for this study includes genes that have correlated thermal stress responses and resting transcription, highlighting the value of transcriptional profiling across multiple genes with diverse putative function important for exploring the complex response of invasive species during the invasion process.

Conclusion
Range expansions in general, and biological invasions in particular, result in organisms having to rapidly adapt to novel environments, either through acclimation or genetic adaptation. Invasion biology has only recently accepted the critically important role environmental adaptation plays in successful (or unsuccessful) invasions (Bjorklund et al. 2010; Schulte et al. 2014). My goal in this study was to examine the adaptation and acclimation of Round goby in novel environments using transcriptional profiling. I predicted that invasion front populations that are actively adapting to a novel environment should exhibit an up-regulation of metabolic and stress response genes relative to fish from established regions. I also expected a greater transcriptional response to the cooled water treatment relative to the heated water treatment. We found a greater transcriptional response of fish in the cooled treatment relative to the heated treatment. Additionally, there was an overall up-regulation of metabolic genes in the established populations relative to fish from the invasion front populations. My results indicate that the invasion process, even on the scale of 20 years (6 generations) in the Round goby involves substantial transcriptional
changes that appear to be adaptive and are likely a result of plasticity, although we cannot rule out the possibly of rapid evolution. By characterization the adaptation process as involving metabolic, stress and immune responses, we can better predict the invasion potential of candidate invaders through transcriptional profiling their response to relevant environmental stressors.
References


Hoy, M., Boese, B. L., Taylor, L., Reusser, D., Rodriguez, R. (2012). Salinity adaptation of the invasive New Zealand mud snail (*Potamopyrgus antipodarum*) in the


Table 3.1 Twenty-eight biologically relevant candidate and endogenous control genes (with gene symbol and gene function citation) used for transcriptional profiling of the invasive Round goby in the Great Lakes. The genes are categorized into three ontology categories which include: Metabolism, Immune, and Stress.

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene Name</th>
<th>Symbol</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic</td>
<td>Cytochrome p450 1A3</td>
<td>CYP1A3</td>
<td>Carvan 1999</td>
</tr>
<tr>
<td></td>
<td>Glucose Transporter</td>
<td>GLUT1</td>
<td>Teerijoki 2000</td>
</tr>
<tr>
<td></td>
<td>Glutamine Synthetase</td>
<td>GLUL</td>
<td>Buckley 2006</td>
</tr>
<tr>
<td></td>
<td>Lipin-1</td>
<td>LPLN1</td>
<td>Buckley 2008</td>
</tr>
<tr>
<td></td>
<td>Transferrin</td>
<td>TF</td>
<td>Gracey 2008</td>
</tr>
<tr>
<td></td>
<td>Lipoprotein Lipase</td>
<td>LPL</td>
<td>Gracey 2008</td>
</tr>
<tr>
<td></td>
<td>Glucose-6-Phosphate Dehydrogenase</td>
<td>G6PD</td>
<td>McCairns 2009</td>
</tr>
<tr>
<td></td>
<td>Sodium-potassium ATPase</td>
<td>NAK1ab</td>
<td>Logan 2010</td>
</tr>
<tr>
<td></td>
<td>Lactate Dehydrogenase –b</td>
<td>LDBH</td>
<td>Kassahn 2007</td>
</tr>
<tr>
<td></td>
<td>Ceruloplasmin</td>
<td>CP</td>
<td>Liu 2015</td>
</tr>
<tr>
<td></td>
<td>Beta- Globin (HBB)</td>
<td>HBB</td>
<td></td>
</tr>
<tr>
<td>Immune</td>
<td>Complement 7</td>
<td>C7</td>
<td>Podrabsky 2004</td>
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<td></td>
<td>Cannabinoid Receptors</td>
<td>CB1</td>
<td>Rojo 2007</td>
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<td></td>
<td>Interleukin 20</td>
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<td></td>
<td>Interleukin 6</td>
<td>IL6</td>
<td>Castro 2011</td>
</tr>
<tr>
<td></td>
<td>Tumor Necrosis Factor</td>
<td>TNF</td>
<td>Laprairie 2012</td>
</tr>
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<td></td>
<td>Periplakin</td>
<td>PPL</td>
<td>Auguet 2014</td>
</tr>
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<td></td>
<td>Fibronectin</td>
<td>FN</td>
<td>Chervonsky</td>
</tr>
<tr>
<td></td>
<td>Cytochrome c oxidase subunit 1</td>
<td>COX1</td>
<td></td>
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<td>Stress Response</td>
<td>Glucocorticoid Receptor</td>
<td>GR</td>
<td>Bernier 1999</td>
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<td>Serum/threonine protein Kinase 1</td>
<td>SGK1</td>
<td>Suda 2004</td>
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<td></td>
<td>Urotensin</td>
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<td>HSP90</td>
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<td>EF1-a</td>
<td>EF-1a</td>
<td>Zhong et al. 2011</td>
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</table>
Table 3.2 History of Round goby detection at the seven sampling sites in this study giving the time since establishment based on reported first siting and the assigned invasion categories (Established versus Recently Invaded).

<table>
<thead>
<tr>
<th>Population</th>
<th>Invasion Category</th>
<th>Time Since Detected (years)</th>
<th>Year Detected</th>
<th>Coordinates of Capture Site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detroit River</td>
<td>Established</td>
<td>25</td>
<td>1990</td>
<td>42.3065.42”N, -83°07’56.75”W</td>
<td>Jude 1992</td>
</tr>
<tr>
<td>Lake Huron</td>
<td>Established</td>
<td>25</td>
<td>1990</td>
<td>43°00’13.79”N, -82°41’31.90”W</td>
<td>Jude 1992</td>
</tr>
<tr>
<td>Lake Erie</td>
<td>Established</td>
<td>22</td>
<td>1993</td>
<td>42°00’73.29”N, -82°56’97.31”W</td>
<td>Charlebois 1997</td>
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<tr>
<td>Lake Ontario</td>
<td>Recently Invaded</td>
<td>17</td>
<td>1999</td>
<td>43°27’03.99”N, -79°86’90.19”W</td>
<td>Owens 2003</td>
</tr>
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<td>Georgian Bay</td>
<td>Recently Invaded</td>
<td>16</td>
<td>1999/2000</td>
<td>44°50’90.88”N, -80°23’33.72’W</td>
<td>EGBSC</td>
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<tr>
<td>Trent River</td>
<td>Recently Invaded</td>
<td>12</td>
<td>2003</td>
<td>44°29’46.21”N, -77°98’38.73”W</td>
<td>Brownscombe 2012, Groen 2012</td>
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<td>Grand River</td>
<td>Recently Invaded</td>
<td>9</td>
<td>2006</td>
<td>42°96’00.76”N, -79°87’27.08”W</td>
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<td>Dnieper River</td>
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<td>-</td>
<td>-</td>
<td>46°62’74.60 N, 32°56’88.20 E</td>
<td>Brown et al. 2009</td>
</tr>
</tbody>
</table>

GRCA- Grand River Conservation Authority

400 Clyde Road, PO Box 729, Cambridge ON, N1R 5W6

EGBSC- Eastern Georgian Bay Stewardship Council

833 Stisted Rd., RR #1, Burk’s Falls ON, P0A 1C0
Table 3.3 Results of GLMM (P values) designed to test for the transcriptional response ($\Delta$Ct) to the cooled and heated treatments. The GLMM model consisted of population, Treatment and their interaction, with replicate tank and sample year included as random effects. I used the 0.05 alpha value as the threshold for inclusion in further analysis of transcriptional response. Significant P-values of $P<0.05$ are in bold text.

<table>
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<tr>
<th>Functional Category</th>
<th>Gene</th>
<th>Treatment Challenge</th>
<th>Treatment</th>
<th>Population</th>
<th>Treatment X Population</th>
<th>Tank Effect</th>
<th>Year Effect</th>
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<td>Metabolism</td>
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<td>0.083</td>
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<td>0.72</td>
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<td>0.70</td>
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Figure Captions

Figure 3.1  Map showing the round goby (*Neogobius melanostomus*) sampling sites in seven locations in southern Ontario. Det.R: Detroit River, L.E: Lake Erie, L.H: Lake Huron, G.R: Grand River, L.Ont: Lake Ontario, T.R: Trent River, G.B: Georgian Bay. Established and newly invaded populations are represented by solid and open dots, respectively.

Figure 3.2 Transcription at 20 genes in the Round goby showing differences between fish from established and invasion front populations under three temperature treatments (cooled, heated and ambient). Only genes that showed a significant treatment and or treatment- by population effect (from the previous GLMM) were used to test for invasion category effects and are shown. Transcription response relative to the control treatment ($\Delta\Delta Ct$) is shown for the cooled and heated treatments, while normalized transcription ($\Delta Ct$) is shown for ambient temperature. Significant P-values of $P<0.05$ are denoted by *. Benjamini- Hochberg procedure was conducted on all p-values to correct for multiple simultaneous comparisons. Striped bars represent the established populations while filled bars show the invasion front fish; error bars represent the standard error.

Figure 3.3 Scatterplots of Round goby relative transcription under heated, cooled and ambient water temperatures versus time since establishment for stress and metabolic genes that showed a significant linear correlation. Error bars represent the standard error.

Figure 3.4 Principal component analysis of all (26) genes responding to ambient, cooled and heated temperature treatments. Transcription responses ($\Delta Ct$) are shown for genes across all ontology categories. Circled shapes represent metabolic genes, while stars and triangles represent stress and immune genes respectively.
Figures

Figure 3.1
Figure 3.2

The graph illustrates the expression levels of various genes across different conditions: Cooled, Heated, and Ambient. The expression is represented by ΔCt. The categories are further divided into subcategories: METABOLIC, STRESS, and IMMUNE. The asterisks (*) indicate statistically significant differences.
Figure 3.3

Stress

Cooled

Heated

Ambient

Metabolism

HSP70

LPL

Time Since Establishment
Figure 3.4
Table S1. Primer (forward and reverse) and probe sequences of all twenty-eight genes designed for Quantitative Real-Time PCR. Amplicon size averaged 100bp.

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<tr>
<th>Gene Name</th>
<th>Symbol</th>
<th>Forward/Reverse Primers</th>
<th>Amplicon Size (bp)</th>
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| Glucose Transporter           | GLUT1  | F: AAGTGTTGAAGAGATGTCACTGTGAA  
                             |        | R: CACTGCTTTTGAATGTGTTTGA  
                             |        | Probe: TAAATAAAGTCTGGCAGAAGAG  | 75     |
| Transferrin                   | TF     | F: CAGGATAACAATAGACACCCAAA  
                             |        | R: GCCGTATATATAGGTCTGCTGTTG  
                             |        | Probe: ACAAAGCTCAAGCAAAGAC  | 74     |
| Lipoprotein Lipase            | LPL    | F: CTTGAGAGACGACGACAGT  
                             |        | R: CGGACGCCCCCTGCTGTTG  
                             |        | Probe: AGGAGGATGTTTCA  | 60     |
| Lipin-1                       | LPLN1  | F: TCCATGCTGCGGCACAF  
                             |        | R: GCCGCCAAATGAGATG  
                             |        | Probe: CTGGTCCTCCACAG  | 59     |
| Glucose-6-Phosphate Dehydrogenase | G6PD | F: TTGTCCTGACGCTT1AAA  
                             |        | R: CGGACCTGCTGCTGTTG  
                             |        | Probe: AGGAGGATGTTTCA  | 58     |
| Ceruloplasmin                 | CP     | F: CTCAGACTGAAITTTTTTGTTTGTAT  
                             |        | R: CGTACCTGACGCTTAAATTITTT  
                             |        | Probe: TCAAAAGTATCTCTAAAT  | 56     |
| Lactate Dehydrogenase –b      | LDHB   | F: TGGACAGTGCTGAGAAGTGA  
                             |        | R: CAGTCCTGAGGCCAGT  
                             |        | Probe: CAAGCTGAAGGCTACA  | 59     |
| Glutamine Synthetase          | GLUL   | F: TTTATTTACGCTATTGCTGCATGCT  
                             |        | R: TCGACTCCTTTCCACAGAAAGT  
                             |        | Probe: ACAGAGTATGTCGCACTAC  | 86     |
| Beta- Globin                  | HBB    | F: GCCGCTTACCCTGTTGAGT  
                             |        | R: ACCACCAAGCCCAACTCTC  
                             |        | Probe: CAAGCGCTTTTC  | 61     |
| Cytochrome p450 1A3           | CYP1A3 | F: CTTGCCCGCGTTTGC  
                             |        | R: CTGCACCCAGCGCACTCA  
                             |        | Probe: CTGGCAATTGCTGACC  | 59     |
| Sodium Potassium Kinase       | NAK1ab | F: CTCACCAGCGCGTTC  
                             |        | R: TCTCATGGCCAGCAGCTCA  
                             |        | Probe: CTTGCTTATTCTTTC  | 53     |
| Serum/threonine protein Kinase 1 | SGK1 | F: CTCACAAGCGGCGCAAGACA  
                             |        | R: TCTTGCTGGCTTATTTTGTA  
                             |        | Probe: CTGGCAATTGCTGACC  | 56     |
| Heat Shock Protein 70         | HSP70  | F: GGGGCGCTGGCCATCTGCTGCTGCT  
                             |        | R: GCCCGGACGCTGCTGCTGCT  
                             |        | Probe: CCACAAGTGTCAACAG  | 58     |
| Heat Shock Protein 90         | HSP90  | F: CAGGGCGCTGGCCCTACA  
                             |        | R: CAGAGCTGCTGCTGCTGCT  
<pre><code>                         |        | Probe: CTGGCAATTGCTGACC  | 54     |
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<td>R: TGACCTAAATGGACTCGTCCAA</td>
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Table S2. The effect of invasion category on gene transcription in the heated, cooled and ambient water treatments. Genes that revealed a significant treatment or treatment by population interaction were chosen for this analysis. Separate GLMM models were conducted for heated, cooled and ambient temperatures. Tank and Year were used as random effects. Benjamini–Hochberg procedure was conducted on all p-values to correct for multiple simultaneous comparisons. Significant P-values of P<0.05 is denoted by *.

- Represents the genes that did not have a significant treatment or treatment by population interaction.

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<td>GR</td>
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<td>0.60</td>
</tr>
<tr>
<td></td>
<td>CB</td>
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<td>-</td>
</tr>
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<td></td>
<td>COX1</td>
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<tr>
<td></td>
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<td>0.56</td>
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<tr>
<td></td>
<td>IL6</td>
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<td>C7</td>
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<tr>
<td></td>
<td>TNF</td>
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</tr>
<tr>
<td></td>
<td>PPL</td>
<td>0.10</td>
<td>0.38</td>
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</table>
Table S3. The functional response of time since establishment across genes by treatment group. Genes that revealed a significant invasion category effect were chosen for this analysis. Benjamini-Hochberg procedure was conducted on all p-values to correct for multiple simultaneous comparisons. Significant P-values of P<0.05 is denoted by *.

- Represents the genes that were not tested in all three treatment groups.

<table>
<thead>
<tr>
<th>(ΔΔCt) Cooled</th>
<th>(ΔΔCt) Heated</th>
<th>(ΔCt) Ambient</th>
</tr>
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<tbody>
<tr>
<td>Gene P-Value</td>
<td>Gene P-value</td>
<td>Gene P-value</td>
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<tr>
<td><strong>Metabolism</strong></td>
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<td>LPL 0.0095*</td>
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<td>LPL -</td>
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<tr>
<td>G6PD 0.080</td>
<td>G6PD -</td>
<td>G6PD -</td>
</tr>
<tr>
<td>LDHB 0.065</td>
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<td><strong>Stress</strong></td>
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<td>HSP70 0.00095*</td>
<td>HSP70 0.043*</td>
<td>HSP70 0.0095*</td>
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<tr>
<td><strong>Immune</strong></td>
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</tr>
<tr>
<td>TNF 0.11</td>
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CHAPTER IV
Conclusion

The importance of acclimation and adaptation in the colonization of new habitats is of growing interest for many ecologists (Chen, 2013; Gilad, 2006; Lee, 2002; Liss et al. 2013). One important application of that approach is quantifying the role of adaptation and acclimation in the success of invasive species (Gilad, 2006; Lee, 2002). Invasive species can maximize their fitness by initially responding to environmental stress associated with a novel habitat and expanding their range through short-term acclimation and/or long-term evolution of adaptive traits. Additionally, if the success of invasive species is indeed due to acclimation/adaptation, this suggests that the traditional approach of control or eradication of these species will likely fail. The Round goby are a particularly good model species for studying acclimation/adaptation in novel environments due to their global dispersal pattern and remarkably successful invasion of the Laurentian Great Lakes. Despite there being considerable literature regarding the physiological aspects of acclimation/adaptation in invasive species (e.g., Sih et al. 2004, West-Eberhard 2005; Rubenstein et al. 2007), the genetic mechanisms behind the physiological processes underlying acclimation/adaption in invasive species remain poorly studied.

The overall goal of this thesis was to study the physiological and molecular genetic mechanisms of adaptation and acclimation during the invasion process by investigating the transcriptional profile and circulating stress hormones of Round gobies from seven captures sites with different time since invasion in the Laurentian Great Lakes.

The goal of Chapter 2 was to examine stress- and metabolically-mediated responses to temperature in the Round goby by comparing levels of circulating cortisol and transcriptional variation in candidate genes associated with stress and metabolism with time since invasion. My results indicate significant differences of cortisol levels across invasion categories (established and invasion front populations) and provide experimental evidence for cortisol flexibility as a mechanism for invasion success. Increased cortisol levels in invasion front populations (including baseline cortisol in the cooled treatment) may enable fish to expand their range and providing them with the ability to cope with altered habitat characteristics. Transcriptional responses to temperature treatments suggest that the Round
goby in the invasion front are more metabolically active (by means of glycolysis), but are not necessarily more responsive to thermal stressors compared with established populations.

In Chapter 3, I developed a panel of known-function gene transcription assays (a transcriptional profile) and used it to examine the adaptation/acclimation of the Round goby in novel environments (invasion front). We found a greater transcriptional response of fish in the cooled treatment relative to the heated treatment. Additionally, there was an overall up-regulation of metabolic genes in the established populations relative to fish from the invasion front populations. Thus, the transcriptional responses to temperature and invasion category suggest that the colonization success of Round gobies are likely a result of plasticity.

While each of my chapters address specific aspects of the mechanisms behind the invasion process in RGs, the two chapters combined provide an integrated assessment of the role of adaptation/acclimation in the invasion process, specifically for the Round goby in the Great Lakes. Several studies have associated thermal tolerance with aquatic invasion success Swindell et al. 2007; Farcy et al. 2009; Logan et al. 2010; Zerebecki et al. 2011; Lancaster et al. 2016). I found that thermal stress elicited both a transcriptional response (across all three invasion critical gene categories) and a physiological response (cortisol) in both established and invasion front populations of Round goby. However, there was a stronger physiological (Chapter 2) and transcriptional (Chapter 3) response in the cooled treatment. This common response is likely due to the fish in the cooled treatment experiencing a more dramatic change in temperature. In turn, this may lead to a hyper-responsive reaction of the stress, metabolic and immune axis in all fish. Since there were higher cortisol levels in addition to a greater number of genes responding to the cooled treatment, I am confident that temperature is an effective stressor for my study, and temperature stress is an obvious and relevant environmental factor in invasion biology. Since transcriptional responses to thermal challenges have been reported in many aquatic species (e.g., Smith et al. 2013; Lord et al 2015; Liu et al. 2015) and has shown a response in both cortisol and gene transcription, this suggests that a thermal stress is appropriate for testing for adaptation/acclimation in newly invaded species.
Transcriptional profiling and physiological response also provided insight on the response to a thermal stressor across invasion categories. Our results from both chapters indicated that there were differences in cortisol and transcriptional profiles (metabolic, stress-response and immune function related loci) between fish from the invasion front and established populations. Population-level differences in gene transcription and physiological responses have been previously reported and interpreted as adaptive, either through acclimation or genetic adaptation as well as a degree of flexibility with the time since invasion (Gabriel and Lynch 1992; Lucassen et al. 2006; Lancaster et al. 2016; Xu et al. 2016). Since this study exhibits a distribution of samples representing time since invasion, it serves as a strong experiment approach to study acclimation/adaptation in comparison to solely conducting a survey of locally adapted populations to detect adaptive environmental responses.

Curiously, the two approaches of Chapter 2 and Chapter 3 resulted in outcomes that were not in agreement. Chapter 2 results indicated that invasion front Round goby are more metabolically active (by means of glycolysis). However, the transcriptional profile (Chapter 3) results did not agree with our expectation for an overall up-regulation of metabolic genes in the established populations relative to fish from the invasion front populations. Thus, these findings may suggest that invasion process (over 20 years of invasion) of the Round goby involves a great deal of transcriptional and physiological changes that are likely a result of flexibility and plasticity. Previous findings have provided evidence of variation in physiological tolerances among populations over latitudinal scales enhancing invasion success and have shown that there exists substantial and relevant physiological variation among populations (Osovitz et al. 2005, Sagarin et al. 2006, Place et al. 2008). To date it is unclear whether these types of differences result from acclimation to the different environments (plasticity) or from genetically-based adaptive differences. Together, previous studies and my findings reinforce the important role of sub-cellular (transcriptional) and organismal (physiological) acclimation/adaptation in newly colonized species.
Future Directions

The goal of this thesis was to examine the genetic and physiological adaptive mechanisms of colonization success in the Round goby. To do this, I explored the functional response of individual gene transcription to time since establishment. I observed an abrupt change in transcriptional response of genes at approximately 15 years post-colonization. This unexpected pattern highlighted two possibilities: 1) Round gobies had genetically adapted to these populations via a discontinuous transcription change, or 2) Round gobies had acclimated to the local habitats, but the habitats were geographically discontinuous in their environmental parameters. One way this can be tested is by rearing the fish in a common environment. This would eliminate differential geographical and environmental parameters and would provide a novel approach to reinforce the role of genetic physiological processes in the formation of local adaptation in invasive species. In addition, reciprocal transplant experiments could be conducted to assess the fitness consequences of fish in native and non-native environments in addition to analyzing the transcriptional patterns of particular genes. This method would allow me to further partition population level differences in gene expression and help quantify adaptive differentiation (Agren et al. 2012).

The approach of this thesis was to examine the acclimation/adaptation to a thermal stressor. Temperature was chosen to be a relevant environmental stressor because it could logistically be implemented for experimental challenges conducted in the field as well as its common use in invasive studies (eg. Cross 2009; Logan et al. 2010; McCann et al. 2014). Future studies can conduct experiments such as this using alternative stressors such as salinity stress, predation stress (such as a chasing stressor), and anthropogenic toxicants (PCBs).

The understanding of the range expansion of invasive species is crucial in predicting the effects they may have on biodiversity and ecosystem function (Crowl et al. 2008). My thesis work on gobies supports the idea that acclimation/adaptation can help a species exploit a novel environment and can be a factor that facilitates invasion success. These
novel approaches described here can help to reinforce the role of physiology and genetics in the colonization of novel species and can form a basis for future researchers to study the success and limitations of invasive species and their range expansion.
References


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