Using morphological and microsatellite analysis to investigate postglacial diversity in an isolated population of threespine stickleback (*Gasterosteus aculeatus*) in Nueltin Lake, Manitoba.

by

Bethany Sarah Schroeder

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Department of Biological Sciences

University of Manitoba

Winnipeg, Manitoba, Canada

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ABSTRACT

Gasterosteus aculeatus (threespine stickleback) is a well-known model for behavioral and evolutionary studies. Rapid evolutionary radiations in postglacial timeframes have promoted distinct local populations with remarkable variation in biological characteristics. This study examines genetic and morphological variation among populations from the Thlewiaza watershed, specifically an isolated freshwater population in Nueltin Lake. Statistically significant genetic differences were observed using 11 microsatellite loci; F_{ST} values ranged from 0.29 (within watershed) to 0.48 (between watersheds) in comparison with the Nueltin Lake population. Gene flow between populations was likely inhibited due to isostatic rebound following the recent deglaciation of North America, 8.5 kya. In comparison with similar freshwater populations, the retention of defensive structures in *G. aculeatus* from Nueltin Lake was unexpected, and may reflect strong piscivorous predation pressures. Levels of differentiation, both genetic and morphological, observed in the Nueltin Lake population are significant should be recognised as a Designatable Unit (DU) by COSEWIC.

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"Education is not the filling of a pail, but the lighting of a fire."

-William Butler Yeats

DEDICATION

To my mom

You are my role model

Thank you for your love, patience and endless support

I simply could not have done this without you

Now, let's "give it the berries".

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CHAPTER 1. GENERAL INTRODUCTION

The genetic diversity and dispersal history of a species is dramatically influenced by many processes, both historical and contemporary (Leinonen *et al.*, 2011). Understanding the evolutionary contribution of each of these processes to a species' history has many implications when considering species diversity and identification, biogeography and conservation or management needs (Leinonen *et al.*, 2011). In particular, species from recently deglaciated regions of the globe provide insight into rapid evolutionary processes and diversification on a contemporary time scale. Patterns of distribution and colonization routes provide insight into evolutionary adaptations, challenges for resource acquisition, and ecological stressors across closely related and disparate taxa.

Colonization, often following deglaciation, precedes the divergence of a variety of ecological traits including morphology, physiology and life history (McKinnon and Rundle, 2002). However, these postglacial radiations often experience high rates of extinction attributed to a rapidly changing landscape following a glacial retreat. As such, they are rarely able to persist long enough to establish widespread and distinct species, and are even more infrequently identified prior to extirpation (McKinnon and Rundle, 2002).

The threespine stickleback (*Gasterosteus aculeatus* Linnaeus 1758) is an anadromous fish species complex from the northern hemisphere which exhibits tremendous genetic and morphological diversity following repeated radiations into freshwater environments (Bell and Foster, 1994). Phenotypic diversity in *G. aculeatus* has stimulated significant interest and controversy among ichthyologists and evolutionary biologists (Bell, 1977). Evolution within the species complex is characterized by a

"raceme" model of evolution, where a highly conserved marine morphotype has repeatedly colonized freshwater systems which have evolved in sympatry and in parallel to exhibit a similar "freshwater" morphology across multiple independent populations (Bell and Foster, 1994; Bell and Andrews, 1997). Despite rapid evolution of morphologically and genetically distinct populations in recent history, extant marine sticklebacks are remarkably similar in morphology to fossil representatives that inhabited the oceans nearly 13 million years ago (Bell and Foster, 1994; Bell *et al.*, 2009; Leinonen *et al.*, 2011). Although repeated colonization events occur throughout the range of *G. aculeatus*, rapidly evolving freshwater populations, which provide incredible insight into questions of speciation and evolutionary processes, are at significant risk of extinction before further understanding of these evolutionary processes can occur.

Following the discovery of a single *G. aculeatus* in Nueltin Lake, a large postglacial lake in northern Manitoba (McKillop and McKillop, 1997), the potential exists for identifying evolutionarily significant findings comparable to the remarkable populations which are well-known from the western coast of Canada.

GENERAL BIOLOGY

Gasterosteus aculeatus is a well-known species complex of small, laterally compressed fishes, characterized by prominent dorsal and pelvic spines, which inhabit shallow areas of marine and freshwater in regions of North America, Europe, northern and central Asia and northern Africa (Scott and Crossman, 1973; Nelson, 2006). *Gasterosteus aculeatus* has been identified as a model system for exploring a variety of evolutionary processes

including speciation and adaptive radiation, sexual selection, parental investment and brood care (Mattern, 2007).

There are five genera within the Family Gasterosteidae found in north temperate climates, including three found in Manitoba: *Culaea, Pungitius* and *Gasterosteus* (Scott and Crossman, 1973; Stewart and Watkinson, 2004). *Culaea* is widely accepted as monotypic, nine species are recognized within *Pungitius*; however, considerable debate exists over the actual number of species within the species *G. aculeatus* due to widespread geographic distributions and tremendous morphological plasticity (Mattern, 2007). As a result, taxonomic recognition and resolution has been the focus of many studies on *Gasterosteus* spp. and, although taxonomic relationships within the genus are still disputed, monophyly at the familial level is well supported (Östlund-Nilsson *et al.*, 2007; Kawahara *et al.*, 2008).

Taxonomy

Complex patterns of morphological variation across the wide range of *G. aculeatus* have made taxonomic treatment difficult. About 50 synonyms are attributed to the species (Eschmeyer and Fricke, 2011), with even the noted French anatomist Georges Cuvier having described 11 species on the basis of morphological traits and geographical distribution that are now considered synonyms. Most authors now treat *G. aculeatus* as a species complex with many unique and reproductively isolated populations, subspecies or species (Bell and Foster, 1994). Species pairs of *G. aculeatus*, both benthic-limnetic as well as lentic-lotic, have been identified from a number of different locationsin North America and throughout its range. The tremendous diversity observed in *G. aculeatus* in

these remarkable species pairs is discussed in greater detail later in the chapter (Morphological variation, Chapter 1).

Studies of allozyme variation (Haglund *et al.*, 1992) in Asian, North American, and European populations recognized two primary clades of *G. aculeatus*: (1) European, North American, and some Japanese samples, which could be divided into an (1a) Atlantic basin clade comprising the eastern North American and European populations, and a (1b) basal Pacific basin assemblage comprising western North American and some Japanese populations; and (2) a divergent group of Japanese populations which may warrant additional taxonomic review (Mattern, 2007). Mitochondrial DNA sequences reveal a similar, but slightly different, structure; identifying 1) a primarily Japanese clade, 2) a widespread clade incorporating a diverse Atlantic basin grouping, and 3) a basal Pacific assemblage restricted to Alaska and British Columbia (Ortí *et al.*, 1994; Mattern, 2007). Questions remain regarding the mono- or paraphyletic status of the Japanese populations.

Distribution

Gasterosteus aculeatus exhibits a circumpolar distribution in the northern hemisphere and, while historically a marine species, is widely distributed throughout both estuarine and freshwater aquatic environments (Scott and Crossman, 1973; Wootten, 1984; Bell and Foster, 1994). In North America, *G. aculeatus* is known extensively from both the Atlantic and Pacific coasts, extending north from California through Alaska and from Chesapeake Bay to Hudson Bay and Baffin Island (Scott and Crossman, 1973). In Canada, it is reported frequently from coastal and estuarine waters of Hudson Bay and

the Atlantic provinces, and can be found in many streams and rivers in British Columbia. A few localized inland populations are known from the St. Lawrence River and the Lake Ontario basin but the species is not typically found throughout the central part of Canada (Scott and Crossman, 1973). *Gasterosteus aculeatus* is also known infrequently from coastal environments in northern Manitoba and is discussed further in subsequent sections of this thesis.

Life history (including habitat parameters)

Gasterosteus aculeatus is typically anadromous, spending most of its life in coastal marine waters and only returning to estuarine and nearshore environments for spawning and nest building activities. However, variations on this life history strategy are well documented throughout its range, and populations or individuals often employ an entirely marine existence (living and reproducing at sea), a typical anadromous strategy (breeding in freshwater, but overwintering in marine systems) or as freshwater residents (spending its entire life cycle in riverine or lacustrine habitat) (Wootten, 2009).

Spawning activity occurs most frequently in June and July, but can occur from April to September among more temperate populations of *G. aculeatus* located in southern regions of British Columbia (Scott and Crossman, 1973). Experimental thermal preferences of *G. aculeatus* from St. Lawrence estuary indicates optimal spawning temperatures of 9 to 12°C were preferred, although reproductive behavior was initiated over a wider thermal range. Significantly reduced spawning activity was noted at temperatures below 5°C and above 23°C (Lachance *et al.*, 1987). Reproductive activity has also been observed in bimodal distributions, with spawning activity peaking between 4 to 8°C (Roed, 1979) and then again at 15 to 18°C (Garside *et al.*, 1977). The varied

range for *G. aculeatus* may indicate that thermal preferences are a function of several abiotic variables including acclimation temperature, environmental salinity or genetic differences in thermal tolerances (Lachance *et al.*, 1987).

Male fish select breeding territories once water temperatures have reached the appropriate range, and barrel-shaped nests are constructed on firm, sandy substrate from plant material and small twigs which are held together by mucilaginous renal secretions (*Spiggin* protein complex) (Östlund-Nilsson, 2007). In a naturally occurring population from Vancouver Island, mean inter-nest distance was observed to be approximately 73 cm and not closer than 30 cm to that of a conspecific (Black and Wooten, 1970). Females are enticed into the nests through a series of complex behavioral patterns, including reciprocal courtship dances, where they then lay the eggs and rapidly depart. Parental care is well documented in *G. aculeatus* and is provided entirely by the male, who may care for eggs of one to several females at a time (Östlund-Nilsson, 2007). Female fecundity ranges from 30 to >1,000 eggs; however, most commonly 200 to 300 are laid (Morrow, 1980). Female stickleback have been known to lay eggs in nests of several males, and may be repeat spawners during a single breeding season if conditions allow (Bell and Foster, 1994). Eggs hatch between 6 and 10 days post-fertilization, though water temperature plays an important role in development time (7 days at 19°C (Breder and Rosen, 1966)). Slower development of eggs of *G. aculeatus* at lower temperatures (12°C) is accompanied by higher survival rates; whereas, eggs developing at higher temperatures with a faster hatching period may experience higher mortality (Heuts, 1956).

Adult fish usually spawn in the second year of life and do not typically live beyond four years of age (though, occasionally up to eight years, Morrow, 1980; Reimchen, 1992). Among limnetic-benthic species pairs in freshwater lakes of B.C. (discussed in more detail, *Morphological variation*, Chapter 1), limnetic forms reach sexual maturity earlier (around 1 year) and rarely live longer than 2 years; benthic forms mature around 2 years of age and may live to 7 years (Hatfield 2001a; 2001b; Hatfield and Ptolemy 2001). *Gasterosteus aculeatus* eat various invertebrates, fish eggs and fry with dietary preferences being strongly influenced by life history and habitat use (Wootten, 1984).

MORPHOLOGY

Phenotypic variation in fishes

Adaptive radiation represents the evolutionary diversification of lineages in response to increased competition for open niches in depauperate environments, such as following the colonization of a recently deglaciated region (Schluter and McPhail, 1993). Often promoting morphological divergence to maximize resource exploitation and reduce intraspecific competition, ecological character divergence is the driving force of adaptive radiation and diversification (Schluter and McPhail, 1993). Phenotypic variation (differences in body form, color, behavior) has been observed across many species of fishes, often in response to environmental exploitation of unoccupied ecological niches.

Postglacial landscapes provide the opportunity for replicate diversification events to occur in novel habitats and establish patterns of divergence across multiple species. In many disparate taxa, speciation and divergence events have promoted the evolution of

sympatric species pairs, with differing morphologies, to optimize resource use and promote localized adaptation. In some species, different morphotypes exhibit complete or partial reproductive isolation (e.g. dwarf and normal lake whitefish, *Coregonus clupeaformis*; Schluter and McPhail, 1993; Lu and Bernatchez, 1999a); whereas in other species, different morphotypes may exist sympatrically with no observed genetic differentiation between them (e.g. rainbow and steelhead trout, *Oncorhynchus mykiss*; Docker and Heath, 2003; Heath *et al.*, 2008).

Phenotypic differences arise, often in response to trophic specialization in the new niche. Variation between planktivorous and benthivorous diets of sympatric morphs of dwarf and normal whitefish has resulted in significant meristic, genetic and morphological differentiation; however, the level of genetic differentiation varies between systems (Lu and Bernatchez, 1999b). Increased competition due to reduced prey and habitat availability has also been suggested to promote reproductive isolation (Landry *et al.*, 2007). Salmonids, especially in postglacial lakes, have been shown to display high levels of phenotypic variation and may exhibit up to four sympatric morphs with varying life history, trophic preference, behavioral, and morphological traits (Jonsson and Jonsson, 2001). Selective breeding has been documented between such morphs, with individuals often choosing to spawn with similar morphs; however, all ecotypes are capable of producing fertile hybrids (Jonsson and Jonsson, 2001).

The divergence into localized "eco-morphs" may reflect the initial step towards diversification of fishes in postglacially fragmented landscapes, often characterized as having low species diversity, and demonstrates the influence of resource availability and interspecific competition on patterns of evolutionary diversification (Schluter and

McPhail, 1993). The relatively recent history of interactions of species in postglacial environments suggests that factors promoting divergence are still highly influential in those populations and examples of contemporary evolution may be observed through careful study of species from recently deglaciated regions (Schluter and McPhail, 1993[r1][B2]).

Morphology in Gasterosteus aculeatus

Morphological plasticity in response to isolation and population fragmentation in *G. aculeatus* is well documented, often as a result of a marine population colonizing a recently exposed or newly available freshwater environment (Bell and Foster, 1994; McKinnon and Rundle, 2002; Colosimo *et al*, 2005). Predictable patterns of body armor reduction and body shape variation accompanying a shift from a marine existence have been noted throughout their distribution and have been the focus of much research (Hagen and Gilbertson, 1972; Hagen and Moodie, 1982; Bell and Ortí, 1994; Kitano *et al*., 2008; Marchinko, 2009). *Gasterosteus aculeatus* is notable for its unique appearance as compared with other small fishes and the wide range of morphological variation exhibited throughout its range. Typically a small (often <100 mm), laterally compressed fish with large eyes and a narrow caudal peduncle, it is often silver to green to brown on the dorsal surface with silver sides and darker mottling (Figure 1). Nuptial coloration of the male fish during spawning season is characterized by bluish sides, a conspicuous red belly and bright blue or green eyes (Moyle 1976; Page and Burr 1991).

Marine morphotype

The marine morphotype of *G. aculeatus*, representing the putative plesiomorphic state, is characterized by prominent antipredator features which are strongly influenced by selection pressures across its freshwater range (Bell and Foster, 1994; Östlund-Nilsson, 2007). *Gasterosteus aculeatus* displays three (rarely two or four) prominent locking dorsal spines (the last spine is quite short) followed by a soft dorsal fin with 10 to 14 rays and the pelvic fins have been modified to a robust "pelvic complex" (Figure 2). The body scales have been modified as a series of overlapping lateral plates which terminate in a bony keel on the narrow caudal peduncle and can range in number (within and between populations) from 0 to 36 (Ziuganov, 1983). It has been demonstrated that articulation among armor elements acts to reinforce the locking mechanism of the dorsal spines (Reimchen, 1983; Wootten, 2009) (Figure 3). Such spine-locking effectively increases the "body" diameter of *G. aculeatus* for defense against gape-limited predators and promotes resistance to compression by the predator's jaws (Hagen and Gilbertson, 1972; Bell, 1988; Reimchen, 1983; 1991; 1994; Shapiro *et al*., 2004; Lescak and von Hippel, 2011).

The marine morphotype of *G. aculeatus* has been highly conserved both in geologic and evolutionary time as well as over broad geographic ranges. Fossil records indicate that fossilized marine *G. aculeatus* collected in California, from the late Miocene are virtually identical to modern marine forms of *G. aculeatus* (Bell, 1977). Other studies of presumably freshwater fossilized *G. aculeatus* have all exhibited reduced phenotypic expression of body armor and indicates that the hypothesized correlation between habitat and morphology was established nearly 10 million years ago (McPhail and Lindsey, 1970; Bell, 1977; Bell *et al*., 1989).

a) Dorsal spines

Dorsal spines of *G. aculeatus* vary in number, vertebral placement, length and degree of serration. Morphological variation in dorsal spine number is seen commonly in ninespine stickleback (*Pungitius pungitius*) and brook stickleback (*Culaea inconstans*) and is tremendously variable (observations of 5 to 11 spines are common in populations of *P. pungitius*, Herczeg *et al.*, 2010). In *G. aculeatus*, the numbers of dorsal spines are not as variable as dorsal spine length, which has been studied extensively (Hagen and Gilbertson, 1972; Moodie and Reimchen, 1976; Bell and Foster, 1994). Dorsal spines tend to be longer in populations that are sympatric with predatory fishes and are thought to impede ingestion by birds, fishes, or other gape-limited predators (Bell and Foster, 1994). The functionally significant measurement of dorsal spine length is the distance between the tips of the locked pelvic spines and the second dorsal spine located directly above (Reimchen, 1991).

b) Pelvic structure

The pelvic structure in *G. aculeatus* is comprised of multiple elements which include a bony pelvic plate formed of a ventral plane adjoining an ascending branch which often articulates with the lateral plates. Additionally, the pelvic fins have been modified into two robust, locking spines which extend away from the body at approximately 60^{o} to complete the pelvic girdle. When dorsal and pelvic spines are erect and locked, coupled with the pelvic girdle and the overlapping lateral plates, the armor complex of *G.*

aculeatus is an effective structure which encircles the vulnerable abdominal region and protects against predatory attack (Figure 3).

Reduction in pelvic structures amongst *G. aculeatus* is rare, but populations found in coastal Alaska, western Canada and northern populations in Scotland and Iceland have been documented as having partial, asymmetric or complete reduction of pelvic structures (Edge and Coad, 1983; Bell *et al.*, 1985; Campbell, 1985; Bell, 1987; Shapiro *et al*., 2004; Coyle *et al*., 2007). Incidences of pelvic reduction are nearly exclusively from regions that were recently deglaciated and represent significant examples of very rapid evolution (Bell and Ortí, 1994). Pelvic reduction occurs at low frequencies, typically occurs during ontogenetic development, and may proceed along variable trajectories of reduction (Bell *et al*., 1993; Kingsley and Peichel, 2007). This is thought to have evolved in response to a number of biotic and abiotic factors (as with all armor structures) including low ion availability in freshwater environments, reduced piscivorous predation in lake systems favoring a loss of defensive structures, and a high proportion of macroinvertebrate predators (Bell *et al*., 1993; Lescak and von Hippel, 2011). As compared to variation in lateral plate morphology, which has been shown to be correlated to the effects of selection on standing genetic variation (Discussed in greater detail, *Molecular toolkit*, this chapter), variation in pelvic expression is dependent upon a *de novo* mutation which may convey a particular advantage and may be selected for accordingly. Regardless of the benefit of this mutation to a population, if the spontaneous mutation does not arise, selection cannot act upon it.

It does appear that although correlated to reduced calcium levels, expression of reduced pelvic structures is contingent upon the absence of piscivorous predators

indicating that a single hypothesis is not sufficient to explain high levels of variability in phenotypic expression (Bell *et al*., 1993; Kingsley and Peichel, 2007). In systems with high rates of piscivorous predation (e.g. rainbow trout, *Oncorhynchus mykiss*), it has been shown that individuals with reduced or absent pelvic structures experience higher mortality rates than fish with a complete pelvic complex (Lescak and von Hippel, 2011). Pelvic reduction often proceeds asymmetrically, but reduction in elements is nearly always sequential (pelvic spines are lost first, followed by the posterior process, ascending branch and finally the anterior process/pelvic plate) (Bell and Foster, 1994).

c) Lateral plates

One of the most impressive examples of parallel evolution in nature is the rapid loss of lateral plate armor in separate freshwater populations of *G. aculeatus* (Colosimo *et al*., 2005). Variation in the number of lateral plates in *G. aculeatus* has formed the basis of a significant amount of research on the species (Bell, 1980; Bell and Baumgartner, 1984).

Developmentally, the anterior plates form first, followed by the posterior plates and bony keel (Mattern, 2007). The middle plates are the last to form (Colosimo *et al*., 2004). It was previously suggested that low and partially plated morphs may represent a retained paedomorphic state (Igarashi, 1970; Bell, 1981). Lateral plate development begins once juvenile fish reach approximately 12 mm standard length (SL: measured from snout to the tip of the caudal peduncle). Numbers of lateral plates increase with growth, and is developmentally complete once an individual attains 34 mm SL (Igarashi, 1970; Coad and Power, 1974; Bell, 1981; Hagen and Moodie, 1982; Bell and Baumgartner, 1984).

Variation in lateral plate morphs were historically classified as *leiurus*, *semiarmatus,* and *trachurus* (Cuvier and Valenciennes, 1829; Hagen and Gilbertson, 1973; Bell, 1976; Ziuganov, 1983; Bell and Foster, 1994) based on the number of lateral plates present on the individual, but has since been redefined as low, partial and complete morphs (Figure 3). The complete morph has a complete row of lateral plates which extend posteriorly from the operculum and/or insertion of the pectoral fin and run along the length of the body, terminating in a bony keel on the caudal peduncle (Wootten, 1984; Bell, 2001). Typically, the number of lateral plates observed in the complete morph ranges from 30 to 36 plates. Low morphs typically have only a few plates (0 to 9) clustered near the operculum and pectoral fin base (Barrett *et al.*, 2008). Some fish lack lateral plates completely and the keel along the caudal peduncle is fully absent in all members of this group (although see Ziuganov, 1983; Banbura, 1994). The intermediate form is represented by the partial morph that exhibits an intermediate phenotype and a variable number of lateral plates, typically 10 to 25 plates. This form often has a gap between the terminal lateral plate and the keel on the caudal peduncle; frequently only the thoracic plates have developed, leaving the abdominal and caudal regions unarmored (Bertin 1925; Bell 1976; Mattern, 2007; Wootten, 2009).

The relative frequencies of plate morphs are not always consistent within a population; marine populations are typically characterized as having a higher frequency of completely plated individuals, whereas most freshwater populations exhibit a low plate morph as the dominant phenotype (Hagen, 1973; Bell and Foster, 1994). However, polymorphic populations have been identified from both freshwater and marine systems (Wootten, 2009). Low plate morphs are virtually absent in marine populations, but are

retained as heterozygotes in the genetic diversity of a population, as lateral plate expression has been demonstrated to be under genetic control (Colosimo *et al*., 2005; Bell *et al.*, 2010). More commonly, complete plate morphs characterize marine populations; whereas, a reduction in lateral plate number has been observed consistently with a shift to a freshwater environment following a colonization event by a marine population [r3](Igarashi 1970; Coad and Power 1974; Bell 1981; Hagen and Moodie 1982). Selection for the low plate morph in freshwater populations is not well understood, and other possible correlations and expression of plate morphology may be attributed to decreased calcium availability in freshwater systems (Giles, 1983), predation pressures (presence or absence of various types of predatory stressors) (Hagen and Gilbertson, 1973; Reimchen, 1995; Kitano *et al*., 2008; Lescak and von Hippel, 2011), and stream gradients (Baumgartner and Bell, 1984). This expression of lateral plate morphs and further discussion of various hypotheses for variation in defensive structures in *G. aculeatus* represents much of the focus of Chapter 3 in this study.

Sexual dimorphism in Gasterosteus aculeatus

Sexual dimorphism also plays a role in divergent phenotypic expression. Divergent habitat usage has been shown to promote varying levels of defensive structures between male and female fish in a common waterbody based on life history characteristics (Huntingford and Coyle, 2007). Female fish tend to have a more pelagic life history and ecological selection favours the expression of longer dorsal spines to reflect the increased risk of gape-limited, piscivorous predation in the open water. Conversely, male *G. aculeatus* tend to occupy the benthic environment of a waterbody and express traits to

have a selective advantage against increased predation pressures from large macroinvertebrates. These traits, such as decreased spine lengths and a reduction in lateral plates, provide fewer grasping or holding points for predators and facilitate post-capture escape by fish (Bell *et al*., 2004; Reimchen and Nosil, 2006). Selection pressures can vary spatially and temporally based on seasonal faunal composition and relative predation stresses. Accordingly, divergent selection forces can favour certain morphs at different times of the year, such as decreased spine length in the summer when benthic invertebrate predators are most abundant. In the fall and winter months, avian predation is more frequent and consequently, larger spines are favoured which may suggest disruptive selection pressures in systems where predation is sufficient to warrant this (Reimchen and Nosil, 2002; 2004).

Sexual dimorphism has also been documented in head and trophic morphology, specifically that adult male individuals tend to have larger heads and mouths than do adult female fish and that females tend to have greater standard length and longer pectoral girdles (Kitano *et al*., 2007; Aguirre *et al*., 2008). Sexual dimorphism in dorsal spine length varied by population. Initially, it was thought that sexual dimorphism was only present in reproductively mature individuals; however, it has been determined that the relationship between trait expression and sexual dimorphism exists among sub-adults as well (Leinonen *et al*., 2011). The majority of known quantitative trait loci (QTL) for body shape are in regions of sex-determination which may reflect the role of sexual dimorphism in allometry and shape variation (Leinonen *et al*., 2011).

Molecular toolkit in Gasterosteus aculeatus

Based on the recent revolution in stickleback genomic resources, scientists now have the ability to identify and map genes responsible for promoting evolutionary change (Colosimo *et al*., 2005). This enhances the role of *G. aculeatus* as a model for studies on molecular mechanisms that underlie parallel evolution of phenotypic traits and for exploring the genetic basis of similar traits that have evolved independently in multiple, discrete populations (Peichel *et al*., 2001; Shapiro *et al*., 2004; Colosimo *et al*., 2005; Le Rouzic *et al*., 2011).

The recent development of many new tools and analysis methods has resulted in the production of an extensive library of genetic information for *G. aculeatus* (allozymes, mitochondrial (mt) DNA, microsatellites, complete genome sequencing, etc). More recent techniques include using sequenced restriction site associated DNA (RAD) markers to identify single nucleotide polymorphisms (SNP's) which are used to reveal precise information about sequence data (Hohenlohe *et al*., 2010). Of special consideration is a genetic linkage map for *G. aculeatus* which coordinates the location of approximately 250 previously identified microsatellites with known quantitative trait loci (QTL) for gill rakers, lateral plates, and dorsal or pelvic spines and are mapped in relation to particular chromosome regions (Peichel *et al.*, 2001; Appendix A). Including the ~350 microsatellites identified through Stanford's Center of Excellence in Genomic Science (CEGS), an estimated 80,000 microsatellites exist in the 670 megabase (Mb) *G. aculeatus* genome with a frequency of one marker every 5 centimorgans (cM) (Östlund-Nilsson, 2007). In addition to their value in QTL mapping, these selectively neutral markers (i.e. genetic variation which is not thought to influence the fitness of the

organism), are useful in the analysis of phylogenetic relationships, population structure, mating system, gene flow, parental assignment, introgressive hybridization, marker-aided selection and genetic linkage (Peichel *et al.*, 2001).

Despite these advances, the molecular mechanisms which promote parallel evolutionary change in phenotypes remain poorly understood; however, establishment of patterns of genomic variation are able to provide additional insight into the genes responsible for regulating changes in phenotype and the interrelationships between closely related and disparate taxa (Colosimo *et al*., 2005; DeFaveri *et al*., 2011). Recent studies in mapping, sequencing and transgenic studies have identified two genomic regions responsible for regulating most of the phenotypic variance in pelvic spine length and lateral plate expression between marine and freshwater populations in *G. aculeatus* (Colosimo *et al.*, 2005; Chan *et al*., 2010).

Lateral plates

Prior to the identification ofthe molecular regulatory mechanisms for the development of lateral plates, up to six separate models of inheritance had been suggested to account for variation in plate morphs in *G. aculeatus* (Colosimo *et al*., 2004; Cresko *et al*., 2004). Beginning with simple Mendelian models based on the expression of a single allele (*A* or *a*), it was suggested that dominant or recessive alleles could account for the presence of complete or low morphs; however, this could not explain the presence of partial morphs (Munzing, 1959; Cresko *et al*., 2004). Subsequent models suggested a two allele control mechanism, an additive allele model as well as epistatic interactions or modifier loci to

account for the variation observed in plate morphs between, and within, populations (Munzing, 1959; Colosimo *et al*., 2004).

The *Ectodysplasin* (*Eda*) gene has been identified among mammals in the development of a number of features which are derived from the ectoderm (e.g. teeth, hair, sweat glands, dermal bones) (Colosimo *et al*., 2005). In fishes, specifically *Oryzias latipes* (medaka), mutations in the *Eda* receptor have resulted in a reduction or total loss of many body scales, derivatives of the dermal skeleton. Many bony or calcified external features in fishes, including scales, lateral plates and other bony processes, have arisen from the dermal skeleton and have likely evolved from a common ancestral feature. This shared evolutionary trajectory has led to further analysis to identify whether variation at the *Eda* locus may underlie the molecular basis of phenotypic variation in lateral plate expression in *G. aculeatus* (Colosimo *et al*., 2005).

Two separate protein isoforms have been identified in the *Eda* gene which differ in length by two amino acids and bind to separate receptors (Yan *et al*., 2000). Both isoforms have been identified from low and completely plated fish (Colosimo *et al*., 2005). Further analysis was used to construct phylogenetic trees using a 1328 bp of exon and intron sequence data from the *Eda* locus and subsequently, was able to identify that *Eda* alleles of most low-plated populations share a common ancestry (with the exception of a population from Japan which is characterized by an independently derived allele of *Eda*) (Colosimo *et al*., 2005). This shared clade likely arose between 2 to10 million years ago, and greatly preceded the recent divergence of *G. aculeatus* (Colosimo *et al*., 2005). Despite this shared ancestry, it is not plausible that a single low-plate morph population

gave rise to all other low-plate morph populations throughout the circumpolar distribution of *G. aculeatus*.

Instead, using single-nucleotide polymorphisms (SNP's), it was identified that a single origin for all low-plate morph populations was not responsible for all subsequent populations. Rather, the alleles controlling the low-plate phenotype must exist, in some frequency, in marine populations (Colosimo *et al*., 2005). Microsatellites (Stn 380, 381) have been identified at intron 2 and 6 of the *Eda* gene which distinguish low and complete morph alleles among most populations of both marine and freshwater *G. aculeatus* (Colosimo *et al.*, 2005; Barrett *et al*., 2008; Kitano et al., 2008; Le Rouzic*et al*., 2011). This has provided an estimate of low-plate morph alleles in marine populations at a frequency of 3.8% (Colosimo *et al*., 2005) but may actually range up to 20% (Bell *et al*., 2010) or, as is more widely accepted, even lower (1%, Barrett et al., 2008; Bell *et al*., 2010; Le Rouzic et al., 2011). Eda_{complete} appears to be dominant over Eda_{low} and reciprocal introgression following hybridization can produce phenotypically low-plate morphs among newly colonized populations of fully plated individuals (Bell *et al*., 2010). Nearly 80% of the variation in plate morph observed in *G. aculeatus* is attributed to the influence of *Eda* on phenotypic expression (Colosimo et al., 2004; 2005; Bell *et al*., 2010).

In some anadromous populations, frequencies of the low-plate allele appear to be lower than expected and likely reflects localized adaptive stressors promoting complete morphs in Cook Inlet, Alaska (Bell *et al*., 2010). Additionally, low levels of the complete morph allele were detected among the freshwater population and may suggest a lack of hybridization among morphs, a lack of introgression or reproductive isolation which may

even suggest occurrence of separate biological species (Bell *et al*., 2010). This indicates that alleles for low-plate morphs are inherent within marine populations of potentially migratory *G. aculeatus*, and that following colonization of freshwater environments where low-plate morphs are suggested to be adaptive, recurrent localized selection occurs (Colosimo *et al*., 2005). This low frequency may be maintained by occasional occurrences of gene flow following contact between anadromous marine fish with low-plated freshwater populations or also by a fitness advantage in heterozygotes (Colosimo *et al*., 2005). The repeated fixation of Eda_{low} would suggest that the allele undergoes positive selection in freshwater populations, as the effects of genetic drift are unlikely to produce such a strong correlation between phenotype and environmental conditions (Barrett *et al*., 2008).

It has also been suggested that additional traits, which would promote successful colonization of freshwater by marine fish, are linked to *Eda* (Colosimo et al., 2005; Barrett *et al*., 2008; Bell *et al*., 2010). Although the mechanisms are not yet fully understood, low-plated populations appear to demonstrate correlated variation in salt tolerance, parasite susceptibility, and behavior, as well as variation in body shape (Colosimo *et al*., 2005). Further research is required to determine why Eda_{low} alleles are predictably acquired by freshwater populations of *G. aculeatus* following colonization from a marine environment despite their low frequency in the ancestral population (Bell *et al*., 2010). Variation in lateral plate morphology may not only be correlated to fitness advantages associated with the presence or absence of plates, but may also be correlated to pleiotropic effects of the *Eda* gene on one or many other traits under selection (Barrett *et al*., 2008; Le Rouzic *et al*., 2011).

Pelvic structure[B4]

One of the most well studied examples of vertebrate evolution and morphological variation is the extensive modification of paired appendages as observed in many species from different taxonomic groups (Chan *et al*., 2010). As previously discussed, the pelvic complex of *G. aculeatus* is comprised of both a set of locking spines which have been modified from the pelvic fins and a plate-like process which extends posteriorly from the pelvic spines and up the abdominal walls (Chan *et al*., 2010). Reduction in pelvic structures in *G. aculeatus* has been observed infrequently, although partial or complete loss of the pelvic complex has been noted in divergent populations throughout the species' range (Edge and Coad, 1983; Bell *et al.*, 1985; Campbell, 1985; Bell, 1987; Shapiro *et al*., 2004; Coyle *et al*., 2007). As with lateral plates, several factors may contribute to the repeated evolution of pelvic reduction, including an absence of gape-limited predators, calcium availability, and increased levels of insectivorous predation (Chan *et al*., 2010).

Genome wide linkage mapping has determined that pelvic reduction in *G. aculeatus* is controlled by a single major locus, pituitary homeobox 1 (*Pitx1*) (Shapiro *et al*., 2004). This gene is expressed in hindlimbs of many different vertebrates and is required for normal pelvic development (Coyle *et al*., 2007; Chan *et al*., 2010). *Pitx1* null-mutations have been observed to be lethal among other laboratory animals, but among *G. aculeatus* have been shown to disrupt expression only at specific sites in developing embryos (Shapiro *et al*., 2004; Chan *et al*., 2010). Despite a lack of variation in protein-coding observed in the *Pitx1* gene among pelvic reduced *G. aculeatus* as

compared with marine conspecifics, its expression in the region of pelvic development is almost completely lost (Shapiro *et al*., 2004; Chan *et al*., 2010). Accordingly, regulatory mutations at the *Pitx1* locus have been identified in the developmental variation of pelvic reduction among *G. aculeatus* (Coyle *et al*., 2007; Chan *et al*., 2010).

However, despite the obvious role of *Pitx1* in pelvic development, it appears that associated genes in the adjacent chromosomal region may actually be responsible for the levels of variation observed (Chan *et al*., 2010). Highly significant variation in allele frequencies among fish with opposing pelvic phenotypes were observed in microsatellite markers located approximately 30 kilobase (kb) upstream of the *Pitx1* locus (Chan *et al*., 2010). Further analysis suggests that the region immediately adjacent to the *Pitx1* locus, 23 kb upstream, is actually the regulating region for pelvic expression among *G. aculeatus*, and contains a tissue-specific enhancer for hind-fin expression. This region, identified as *Pel*, is shared among other fishes (zebrafish, *Danio rerio*, and other teleosts) and has been suggested to contain regulatory enhancers conserved among ancestral species (Chan *et al*., 2010). This indicates that pelvic expression in *G. aculeatus* is regulated by the synergistic effects of *Pitx1-Pel.* Deletions of varying length (757-1868 bp) in the enhancer region were identified in pelvic reduced fish from Paxton Lake, B.C., and confirm that this molecular deletion interrupts *Pel* enhancement function of the *Pitx1* gene (Chan *et al*., 2010). Further analysis of expanded regions of the genome indicate that nine separate haplotypes, with varying deletion positions, are consistently observed in populations of pelvic-reduced fish, and that the overlapping of haplotypes can significantly alter, or even completely remove, the enhancer region (Chan *et al*., 2010). The identification of an enhancer region to modify a regulatory mechanism for pelvic

expression has provided an opportunity to link major variation in vertebrate skeletal elements with site-specific DNA sequence changes[B5].

The integration of the influence of molecular mechanisms on phenotypic variation further promotes the understanding of adaptive evolution in specific traits among natural populations. However, it also identifies the further need for understanding of genes which underlie ecologically important traits and provides new opportunities for study of variation in phenotype during vertebrate evolution (Colosimo *et al*., 2005). Already, targeted genome scans have identified additional genetic signatures of parallel physiological evolution, including 24 loci (17 of which represent physiologically important genes) on a global scale (DeFaveri et al., 2011). These suggest that varying evolutionary pathways may underlie physiological adaptation to freshwater habitats in *G. aculeatus* following colonization and represents the significant research potential in this area for studies of divergent selection and phenotypic variation.

Morphological variation in **Gasterosteus aculeatus**

Gasterosteus aculeatus have been widely discussed in the literature regarding tremendous morphological plasticity, often associated with colonization of novel habitats (Hagen and Gilbertson, 1972; Bell, 1984; Reimchen *et al*., 1985; Schluter and McPhail, 1992; McPhail, 1993; Bell and Foster, 1994; Vamosi, 2003; Colosimo, 2004). Aside from the marine-freshwater variation, research into this species complex has identified a number of phenotypically distinct morphs of *G. aculeatus* which coexist sympatrically and are characterized by varying degrees of reproductive isolation (McKinnon and Rundle, 2002). Most well-known are the benthic-limnetic species pairs which have been studied

intensively from the western coast of British Columbia (Haas and McPhail, 1991; Taylor and McPhail, 2000[B6]). The "double-invasion hypothesis" for *G. aculeatus* in these lakes suggests the initial establishment of marine species in the lake adapts the limnetic morphotype, whereas subsequent colonizers become established as the benthic form as a result of resource competition (Schluter and McPhail, 1992). The limnetic forms are characterized as having longer, delicate and increased numbers of gill rakers, and a slimmer body form. Benthic morphs have fewer gill rakers that are shorter and stouter, and have a deeper, more robust body form (Schluter and McPhail, 1992; Taylor and McPhail, 1999; McKinnon and Rundle, 2002). Heritable morphological variation from the marine morphotype occurs rapidly, and reproductive isolation in *G. aculeatus* has been documented in as few as eight generations (Kristjansson *et al*., 2002).

A number of other morphological variations have been identified in *G. aculeatus* and have been shown to have a heritable basis. Lake-stream (lentic-lotic) pairs have been identified on Vancouver Island and the Strait of Georgia and are now under legislative protection (Misty Lake COSEWIC, 2006). Color variation with a heritable basis is also observed in differences of nuptial coloration expressed in populations from the Olympic Peninsula, WA. Spawning individuals exhibit either a red or entirely black color pattern as opposed to the more common red ventral surface and blue/green eyes of male fish (Hagen *et al*., 1980). A fully white morph has also been identified from the coast of Nova Scotia and varies considerably from the expected color pattern seen in marine conspecifics from nearby environments (Blouw and Hagen, 1990). Notable variation in body shape has been documented in Japanese populations from the Pacific Ocean as

compared to those found in the Sea of Japan and has been shown to have a heritable basis (McKinnon and Rundle, 2002).

Conservation efforts for localized variation in morphology

In Canada, 12 listings exist in the current Species at Risk (SARA) registry (www.sararegistry.gc.ca), the federal listing of endangered or at risk species, for *G. aculeatus*. Listing is achieved based on recommendations by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) which promotes awareness of the species' status and existing risk level but is unable to convey any formal level of protection. Following recommendation from COSEWIC and subsequent listing in SARA; legislative protection, to promote conservation or recovery efforts, may be identified. Species pairs representing benthic-limnetic pairings from four separate lakes have been listed on the SARA registry (Enos, Paxton, Hadley Lakes, Vanada Creek, COSEWIC 2002). Despite this listing, the species pair which existed in Enos Lake has since collapsed into a hybrid swarm due to the introduction of a species of non-native crayfish which altered turbidity in the aquatic environment, leading to a reduction of assortative mating behavior and a loss of reproductive isolation (Kraak *et al*., 2001). A second species pair in Hadley Lake has also become lost as a result of the introduction of an invasive predator, *Ameiurus nebulosus* (brown bullhead), to the lake (Hatfield, 2001; Foster *et al*., 2003; Wood, 2003,). Pairs from Paxton Lake and Vanada Creek are currently recognized as Endangered. A fifth species pair, representing the stream-lake (lentic-lotic) complex found in Misty Lake, was assigned the status of Special Concern (Reimchen, 1984; COSEWIC, 2005). In addition to the species pairs, two phenotypically

unique populations, a giant stickleback and an unarmored variant have also been recognized[B7]. These 12 listings all exist under the same species designation (*G. aculeatus*); despite a lack of formal taxonomic designation, they have been identified as being ecologically and evolutionarily relevant for protective measures.

MICROSATELLITES

The development and increased usability of DNA markers has enabled fisheries scientists to investigate processes of genetic variability as well as providing the opportunity to elucidate phylogenetic relationships of previously cryptic lineages and study mechanisms of inheritance and speciation (Liu and Cordes, 2004). Mutations in DNA can arise as a result of normal cellular processes or environmental interactions which promote genetic variation (polymorphism). Along with selection and genetic drift acting on genotypic expression, genetic variation accumulates at all levels of taxonomic classification and the interrelationship of such variability has been the focus of much scientific interest (Lui and Cordes, 2004).

Microsatellites consist of short (one to six nucleotides) tandem repeats of DNA which are found in virtually every species and are widespread throughout the eukaryotic genome (Chistiakov *et al.*, 2006). Microsatellites are single locus, co-dominant molecular markers which are used to assess recent population variation and structure and often exhibit high levels of allelic polymorphism. Allelic variants are inherited in Mendelian fashion and appear more frequently in non-coding regions of the genome (Selkoe and Toonen, 2006). As a result, they are not typically considered to be under pressure of selection (i.e. are selectively neutral), and expressed variation reflects

historical and contemporary population size, genetic drift, and mutation (Chambers and MacAvoy, 2000; Chistiakov *et al*., 2006).

The mean density of microsatellites varies significantly between taxonomic groups and even within closely related sister taxa (Estoup and Angers, 1998). In *G. aculeatus*, nearly 80,000 microsatellite loci are found throughout the genome and are ubiquitous in their distribution (Kingsley and Peichel, 2007). The highly polymorphic nature of microsatellites enables multiple loci to be assessed per individual to provide multiple comparisons per individual, and provides greater statistical power when comparing individuals and populations (Selkoe and Toonen, 2006).

Mutations

High mutation rates in microsatellite loci, ranging from 10^{-2} to 10^{-6} mutations per locus per generation, make them very useful when examining recent (10 to 100 generations) evolutionary patterns of change among populations (Ortí *et al*., 1997; Selkoe and Toonen, 2006). Mutations in microsatellites arise as a result of DNA slippage, polymerase slippage, slipped strand mispairing or unequal crossing over (Bennett, 2000). Slippage is thought to occur within the complex of proteins responsible for DNA replication as a consequence of mispairing (typically by one repeat unit although multiple units are possible) between the original template strand and the newly synthesized DNA strand. The resulting region of unpaired DNA is then forced to "loop out". If the loop occurs on the new strand, the net effect is the addition of a repeat unit; if it occurs on the template strand, this results in the loss of a repeat unit (Bennett, 2000). Mispairing occurs more frequently in pure repeat sections, making interrupted microsatellite segments less variable (Jarne and Lagoda, 1996). Other factors including microsatellite length, location

and sequence of repeat units are hypothesized to affect rate and direction of slippage although this has not yet been formalized (Bennett, 2000). Studies suggest that mono- and dinucleotide repeats occur most frequently in the genome and mutate faster than replicates with higher numbers (tri, tetra, pentanucleotides etc.) which is attributed to more efficient repair mechanisms at longer microsatellite arrays (Schlötterer and Tautz, 1992; Jarne and Lagoda, 1996; Chakraborty *et al*., 1997; Estoup and Angers, 1998; Chambers and MacAvoy, 2000). Levinson and Gutman (1987) also found evidence that microsatellites with a larger number of repeats mutate at a higher rate than those with a smaller number. Transitions (substitutions in basepairs involving purine-purine: adenine or guanine, or pyrimidine-pyrimidine: thymine or cytosine) occur more frequently than do transversions (purine-pyrimidine substitutions) (Hillis *et al.*, 1996); however, GT/CA appear to have the highest mutation rate, whereas AT/TA has the lowest (Bachtrog *et al*., 2000).

Mutation models

To understand differences in mutation rates and processes occurring in genetic variation, especially in microsatellites, understanding of the contrasting mutation models is important (Ortí *et al*., 1997). The infinite allele model (IAM) assumes a single mutational rate across all alleles (Kimura and Crow, 1964). It assumes that every mutation creates a novel allele and relies on a "same or different" approach to assess allelic variability (Jarne and Lagoda, 1998; Chistiakov *et al.*, 2006). In contrast, the stepwise mutation model (SMM) has been developed specifically based on microsatellite mutational tendencies, in which a mutation adds or subtracts (with equal probability) a

single unit to/from the current allele in a stepwise manner. The SMM infers that alleles with a single difference in repeat unit are more similar than those alleles with more repeat units that are different. Under the IAM, alleles differ equally from each other at any size (Luikart and England, 1999). A concern under the SMM is the occurrence of homoplasy, where alleles are considered equivalent (identical-in-size, IIS) yet does not share a common evolutionary history (identical-by-descent, IBD) (which can result due to back mutations etc.). Since most mutations seem to involve the gain or loss of a single repeat unit (Weber and Wong, 1993), the recognition of alleles which do not share a common evolutionary history leads to an underestimation of the total amount of variation and genetic distance, and to the overestimation of the similarities among populations. Under the IAM, homoplasy is not recognized (rather that each allele is unique); whereas, under the SMM, it is likely to occur frequently. However, in some studies (Estoup *et al.*, 1995), homoplasy was not observed on a large scale, and was suggested as being of low concern in future studies of population genetics, although this relationship is not yet fully understood (Jarne and Lagoda, 1996).

GEOLOGIC HISTORY OF THE STUDY REGION[r8]

The Laurentide ice sheet was a large, coalescent ice mass which occupied most of northern North America during the most recent glacial event, the Wisconsinan glaciation (existing 80 kya to 8 kya) (Klassen, 1983; Fulton and Prest, 1987). Glacial margins of the ice sheet extended from the Atlantic seaboard and continental shelf of eastern North America, across the continent to the western Cordillerans and south to approximately 40^{o} N in the United States. The combined masses of the Laurentide ice sheet are

hypothesized to have occupied an area between 10.2 and 11.3×10^6 km^2 and depressed the crust and surrounding terrain by at least 300 m (Fulton and Prest, 1987). Ice volume of the Laurentide ice sheet has been hypothesized to range from a minimum of 18.0×10^6 km^3 to a maximum volume of 34.8×10^6 km^3 and may account for 60 to 70% of glacial melt occurring during the Wisconsinan glaciation (Hughes *et al.*, 1981; Fulton and Prest, 1987).

The Wisconsinan glaciation is subdivided into early, middle and late substages. The Tioga period was the least severe and most recent event of the Wisconsinan glaciation, beginning approximately 23 kya, reaching its glacial maximum at 20 kya, and ending approximately 8 kya with the culmination of the Wisconsinan glacial period (Fulton and Prest, 1987). During the Tioga period, a large ice mass (remnants of the Laurentide ice sheet) occupied the majority of present day Hudson Bay and maintained Lakes Agassiz and Ojibway which acted as a refugium for many freshwater fishes (Hocutt and Wiley, 1986) (Figure 4). Approximately 8.5 kya, a massive melting of the Laurentide ice sheet occurred, and resulted in a rapid drainage of freshwater into the marine environment through the Hudson Strait and into the Labrador Sea (Barber *et al.*, 1999). This outflow of cold, freshwater ($>10^{14}$ m^3) altered sea surface salinity of the marine environment, affected ocean currents, and produced a climatic fluctuation of extremely low temperatures. This massive flushing of freshwater also enabled a large influx of marine waters into Hudson Bay and resulted in a rapid change from freshwater to an increasingly saline aquatic environment (Dyke and Prest, 1987). Glaciers receded entirely from Hudson and James Bay (HB-JB) region within 2000 years of the initial marine exchange and averaged a retreat rate of 300m/year (Klassen, 1983). Glacial

depression of surrounding regions was 100 to 300 m below present day levels (Fulton and Prest, 1987; Barber, 1999) enabling flooding of regions adjacent to the recently deglaciated areas to form the Tyrrell Sea (Figure 5). Due to isostatic rebound, recently deglaciated areas which became flooded began to uplift at a rate of 0.7 to 10 m per century in areas of tidal flats and produce a shoreline loss of up to 15 m annually in some regions (0.8 to 0.9 m per century in Churchill, MB) (Dredge, 1983). This resulted in probable isolation of populations on the margins of the rebounding terrain and likely restricted movement back to ancestral areas. The rebounding effect has continued through to recent time and has reduced the extent of the Tyrrell Sea to its current position as Hudson and James Bays (Figure 6).

NUELTIN LAKE

Nueltin Lake is a large, postglacial lake created by the recession of the Laurentide ice sheet nearly 8.5 kya (Dyke and Prest, 1987). Straddling the Manitoba-Nunavut border, Nueltin Lake is located approximately 450 km south of the Arctic Circle and 300 km (450 km by waterway) from the western shore of Hudson Bay ($60^{o}09'03"$N $099^{o}45'23"$W). Nueltin Lake is near the headwaters of the Thlewiaza River within the Thlewiaza watershed which is located predominantly in Nunavut, but also extends into northwestern Manitoba (Figure 7). The river system meanders through southern Nunavut and drains Nueltin Lake just south of Arviat at Hudson Bay. It is characterized by a series of waterfalls along its length that are presumably impassable, at least in the upstream direction, for *G. aculeatus* (McKillop and McKillop, 1997) (Figure 8, Chapter 2, this study). The drainage basin for Nueltin Lake ranges from 1851 km^2 to 2279 km^2,

and the maximum length of the lake is approximately 190 km; 2/3 of which is located in Nunavut (Dyke and Prest, 1987). Nueltin Lake exhibits a highly convoluted and irregular shoreline with many bays and side channels, as well as a prominent esker running in a north-southerly direction which separates the south-western third of the lake from the main waterbody. Current elevation is at 278 m above sea level, although the area continues to experience isostatic rebound at a rate of 0.8-0.9 m per century (Dredge, 1983).

A marine connection is presumed to have existed temporarily between Nueltin Lake and the ancestral Tyrrell Sea (present day Hudson Bay) (Dyke and Prest, 1987) (Figure 5). Rapid melting of the Laurentide ice sheet and a massive influx of marine water over previously glacially depressed terrain may have resulted in marine taxa being introduced to inland zones such as Nueltin Lake that became isolated along glacial margins (Dyke and Prest, 1987).

Piscivorous predators in the region include Arctic grayling (*Thymallus arcticus*), lake trout (*Salvelinus namaycush*), northern pike (*Esox lucius*), and various species of whitefish (*Prosopium cylindraceum, Coregonus* spp.) as well as numerous smaller species (See Fowler (1948) for listing of species as well as Scott and Crossman (1973)). A number of avian predators including *Sterna paradisaea* (Arctic tern) and *Larus* spp. (various gulls) are also present in the region in high numbers. A seasonal hunting and fishing lodge is the sole commercial occupant on Nueltin Lake, and has employed a strict catch-and-release fishing policy since 1978 (S. Gurke, Pers. Comm). This has maintained a healthy population of large predatory fishes in Nueltin Lake.

GASTEROSTEUS ACULEATUS IN MANITOBA

Previously unknown in Manitoba from regions outside the coastal environment of Hudson Bay, a single specimen of *G. aculeatus* was collected from Nueltin Lake, MB in 1996, at the base of a waterfall in a sandy backwater near Bagg Creek (59.86N 100.124W). The specimen was a fully plated, mature male individual, 41 mm total length with a caudal keel (McKillop and McKillop, 1997). The collection was made by minnow trap and no other individuals were collected. The fish was retained and deposited in the Manitoba Museum collection (catalogue no. MM 722, Appendix B).

Other historical collections have been made from both Ranger Seal Lake (CMNFI-1966-0224.8), located approximately 50 km inland from Hudson Bay in the Thlewiaza River system, as well as from the Caribou River (CMNFI-1966-0233.4), an adjacent watershed immediately south of the Thlewiaza. Both of these specimens represented fish of the low-plate morph, with accompanying reduction in body armor, as would be expected in fish collected in a freshwater environment. The retention of defensive structures (bony spines, pelvic complex, lateral plates etc.) by fish from the Nueltin Lake population, despite a significantly greater distance from the coast and consequent opposite expectations regarding armor, makes them particularly interesting.

Gasterosteus aculeatus are ranked as globally secure (G5), but it has been designated as rarely occurring within Manitoba and is considered to be vulnerable to extirpation (S1) (Manitoba Conservation Data Centre, 2010; Stewart and Watkinson, 2004). In addition to providing insight into a species of concern for the province of Manitoba, the finding of a single specimen in Nueltin Lake considerably extends the species distribution to the west in Manitoba, well beyond the coastal environment from

which it was previously known, and presents the opportunity to explore questions of migration, colonization and localized adaptation to a freshwater habitat (McKillop and McKillop, 1997).

Sample collection

A total of 103 *G. aculeatus* were collected from four locations in northern Manitoba and Nunavut (Table 1, Figure 8, and Appendix B). Dipnetting, kick seining and electrofishing efforts were used to successfully collect *G. aculeatus* from locations in Nueltin Lake, Caribou River, Rupert Creek and the mouth of the Thlewiaza River. In addition to *G. aculeatus*, other species were sampled and retained as voucher specimens for the Manitoba Museum collection (Appendix C).

Initial collection efforts were made at the reported site of the historical collection in Nueltin Lake near the base of a waterfall near Bagg Creek (McKillop and McKillop, 1997); however, subsequent collections at this location were unsuccessful. *Gasterosteus aculeatus* were collected in close proximity to the historic collection site, with the majority of collection localities occurring west of the prominent esker running north-south along the Manitoba portion of the lake (59.8738N 100.08006W). Successful collections of *G. aculeatus* (n=34) were made from four locations within the southwestern portion of Nueltin Lake in early July 2007. Despite having slightly different collection localities, all sites were within a similar location of the lake and samples were pooled for subsequent analysis by population.

Ice had recently melted in the northern portion of Nueltin Lake (late June) which is late for this region and resulted in colder water mixing with warmer waters from the

southern portion which had been ice-free for more than 30 days (S. Gurke, pers. comm.). Water temperatures at collection locations ranged in temperature from 9 to 22^{o}C. Female fish were fully gravid and were releasing eggs while being handled. Flow rates were variable depending on channel morphology but were characterized as low or medium-low velocity, and were slow enough not to damage nesting sites. Emergent vegetation appeared to be an essential attribute of successful collection locations for *G. aculeatus*, consistently two species: *Hippuris vulgaris* (common mare's tail), and *Sparganium hyperboreum* (northern bur-reed), and are attributed to spawning and nest-building requirements. Substrate was characterized as having a firm, sandy bottom, with small to medium sized cobble interspersed with medium to large sized boulders. *Gasterosteus aculeatus* were also collected in areas without boulders or coarse gravel; however, increased amounts of aquatic vegetation were observed in these locations. Collection water depth varied from 20 to 100 cm. Fish were collected both as single-species schools and also in multi-species communities. With the exception of the Esker South site, *G. aculeatus* were not collected sympatrically with northern pike (*E. lucius)* despite similar habitat preferences (Appendix C).

Forty-three fish were collected from the Thlewiaza River in Nunavut, near Hudson Bay (60.5000114N 94.950248W), approximately 20 km inland, in August 2008. All fish were collected via seine and dipnets from a side channel and backwater eddy of the Thlewiaza River. Habitat features were consistent with those found in Nueltin Lake, with the exception of having areas of high velocity flow in close proximity associated with the main channel of the Thlewiaza River. Other species were collected from this

location as well and were retained as voucher specimens for the Manitoba Museum collection.

Sixteen fish were collected in July 2008 from the Caribou River (59.650059N 95.400147W), approximately 20 km inland, in the Seal River watershed. All fish were collected using seine nets along shore at the base of a tall escarpment. Fish were collected in water of 75 to 100 cm depth and were associated with emergent or overhanging vegetation and firm, sandy substrate with randomly scattered small to medium sized rocks.

Ten fish were collected for tissue samples from Rupert Creek (57.538889N 92.556667W) in July (2005), along the west bank of a small tributary running perpendicular into the main stream about 75 m from creek flowing parallel to Hudson Bay in Wapusk National Park (Mooi and Klapecki, unpubl. data.) Fish were collected using dipnets along the shoreline and retained as specimens for the Manitoba Museum collection. All sampled fish were juveniles.

Pectoral fin clips were taken from all specimens and preserved in 95% ethanol (EtOH) for genotyping and microsatellite analysis. Following tissue collection, whole specimens were fixed in 10% formalin for one week and transferred to successively stronger concentrations of ethanol, finally being stored in 70% ethanol (EtOH) for further study. Specimens were identified and deposited into the collection of the Manitoba Museum for future use (Appendix B).

Fieldwork was performed under Manitoba Water Stewardship Aquatic Ecosystem Section Scientific Collection Permits 11-07 (for Nueltin Lake) and 22-08 (for the Caribou River) and Fisheries and Oceans Canada License to Fish for Scientific Purposes #S-

08/09-1037-NU (for the Thlewiaza River). Handling of fishes and retention of vouchers followed Animal Care Utilization Protocol F05-016/1 and F05-016/2 as approved by the Fort Garry Campus Protocol Management and Review Committee of the University of Manitoba.

STUDY GOALS AND HYPOTHESES[r9]

The goals of this study are to confirm and describe the persistence of an established, self-reproducing population of *G. aculeatus* in Nueltin Lake, Manitoba and to explore the differences and similarities of a presumed evolutionarily-young population with those found near Hudson Bay. Coastal populations are thought to be anadromous and may represent the genetic diversity and marine morphotype of the ancestral population. Genetic diversity and patterns of population relatedness will be examined between the Nueltin Lake population and adjacent populations from coastal regions of Hudson Bay. Predicted patterns of differentiation are expected in accordance with the known geologic history of the region and evolutionarily recent events of the Late Wisconsinan glaciation. It is expected that genetic differences will be observed based on hypothesized routes of postglacial dispersal and provide an excellent opportunity to examine processes of contemporary evolution on a previously unknown and unstudied population.

The species has been studied intensively in other regions of Canada and has provided tremendous scientific insight into studies of adaptive divergence and phenotypic plasticity. Divergent selection among coastal and inland populations provides an opportunity for the examination of localized adaptation to varying habitat characteristics

and a number of hypotheses exist to explain predictable patterns of morphological adaptation[r10].

This study will examine if an extant population has responded genetically and morphologically to apparent isolation in a novel environment. To address this idea, the study will incorporate a two stage process involving first, a molecular approach and secondly, a morphological approach to examine the diversity in the Nueltin Lake population and generate comparisons with populations from the coastal environment of western and southern Hudson Bay.

The molecular approach to this study uses nuclear DNA to investigate the genetic diversity of *G. aculeatus* in Nueltin Lake and compares it to other populations from within the Thlewiaza and adjacent watersheds. In Chapter 2, genetic variation within the Nueltin Lake/Thlewiaza River populations will be detected using 11 microsatellite loci and quantified to assess genetic diversity within sampled populations and provide estimates of gene flow between Nueltin Lake and Hudson Bay. Genetic structuring of the Nueltin Lake population will be examined for genetic bottlenecks and founder effects associated with isolation in a postglacial environment. Ongoing genetic contribution upstream (to Nueltin Lake) may be impeded as a result of significant geographic barriers along the river system and which restrict gene flow between the populations. [r11]This presents the opportunity for evolutionary diversification and interesting adaptive variations to accumulate. [r12] Genetic diversity and patterns of divergence will be examined within the accepted geologic and postglacial history of the region to identify probable colonization routes following declagiation. [r13]

In Chapter 3, this study will examine the morphology of *G. aculeatus* from Nueltin Lake and other coastal locations from western Hudson Bay to assess the role of geographic isolation influencing phenotypic expression in a postglacially fragmented landscape[r14]. Morphological characters, specifically defensive traits, will be examined and compared between estuarine and freshwater populations. Historical museum collections representing equivalent river-estuarine populations from the Hudson Bay region will also be used to investigate morphological variation. Predictable patterns of morphological change associated with a shift to a freshwater environment by a marine species are well documented in *G. aculeatus*; however, the ecological role in variation is not well understood. Various biotic and abiotic parameters influencing phenotypic expression in *G. aculeatus* will be discussed with respect to the isolated population from Nueltin Lake.

I hypothesize that Nueltin Lake represents a glacial relict population [r15]of *G. aculeatus* in Manitoba which has persisted since the end of the Wisconsinan glacial period nearly 8.5 kya and that [r16]genetic differentiation will be observed between inland and coastal populations. Associated with genetic variation across these populations, I predict that Nueltin Lake fish will exhibit the reduced phenotype for defensive structures as would be expected in a freshwater population and that the Thlewiaza and Caribou River populations will retain a “typical” marine morph.

Figure 1. An example of a threespine stickleback (*Gasterosteus aculeatus*) collected in Nueltin Lake, Manitoba, July 3, 2007 (59.8738N 100.08006W). Photo credit R. Mooi, 2007. Collection record: MM 2642.

Figure 2. Threespine stickleback (*Gasterosteus aculeatus*) cleared and stained following Dingerkus and Uhler (1977). Bone tissue indicated in red, cartilage in blue. Photo credit R. Mooi. Collection record: MM 1237 (Rupert Creek, Wapusk National Park, Manitoba).)

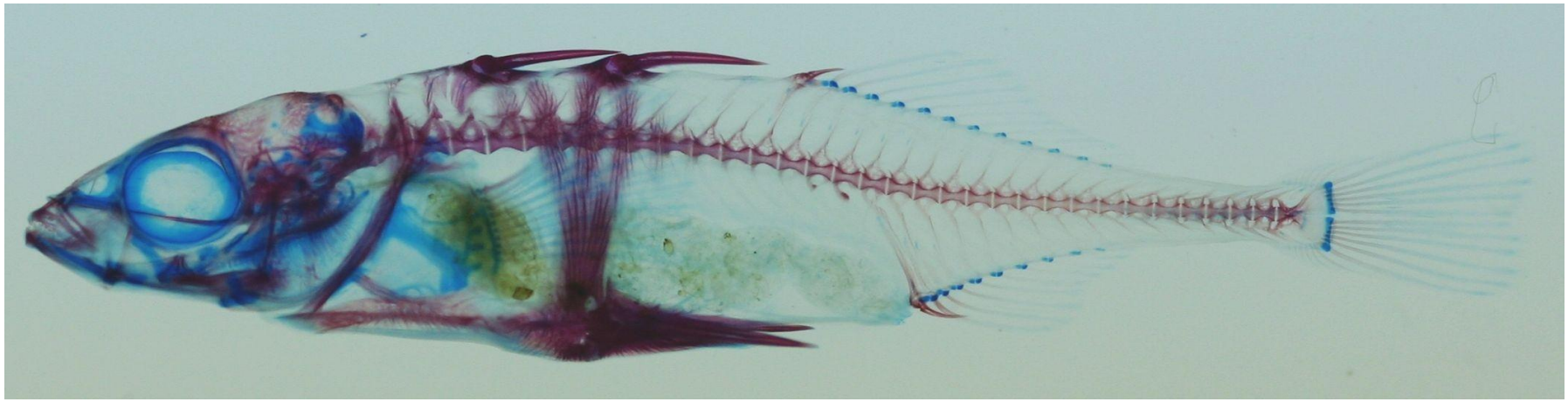


Figure 3. Phenotypic expression of defensive body armor structures and lateral plate morphs in threespine stickleback (*Gasterosteus aculeatus*). After Marchinko, 2009.

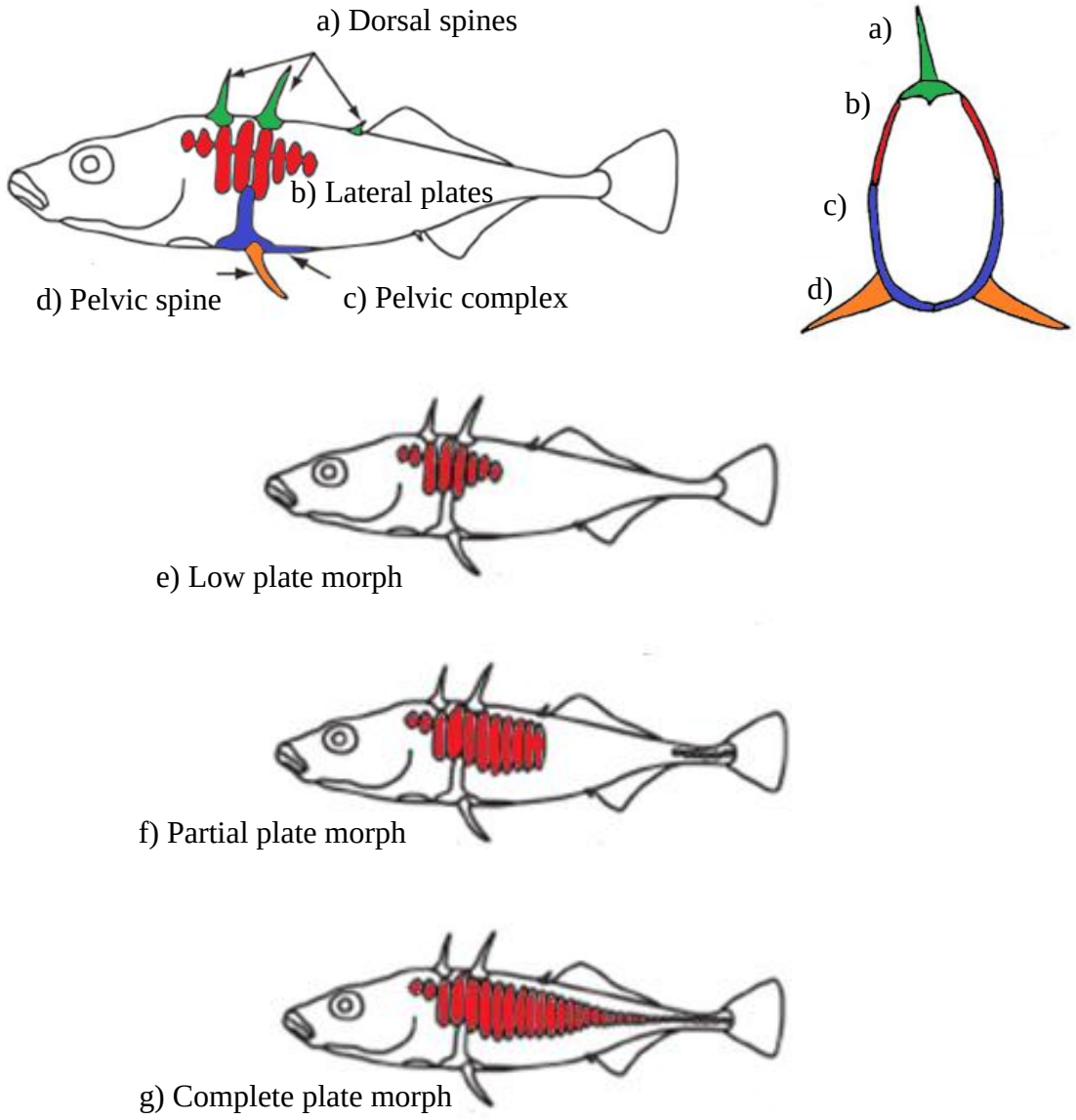
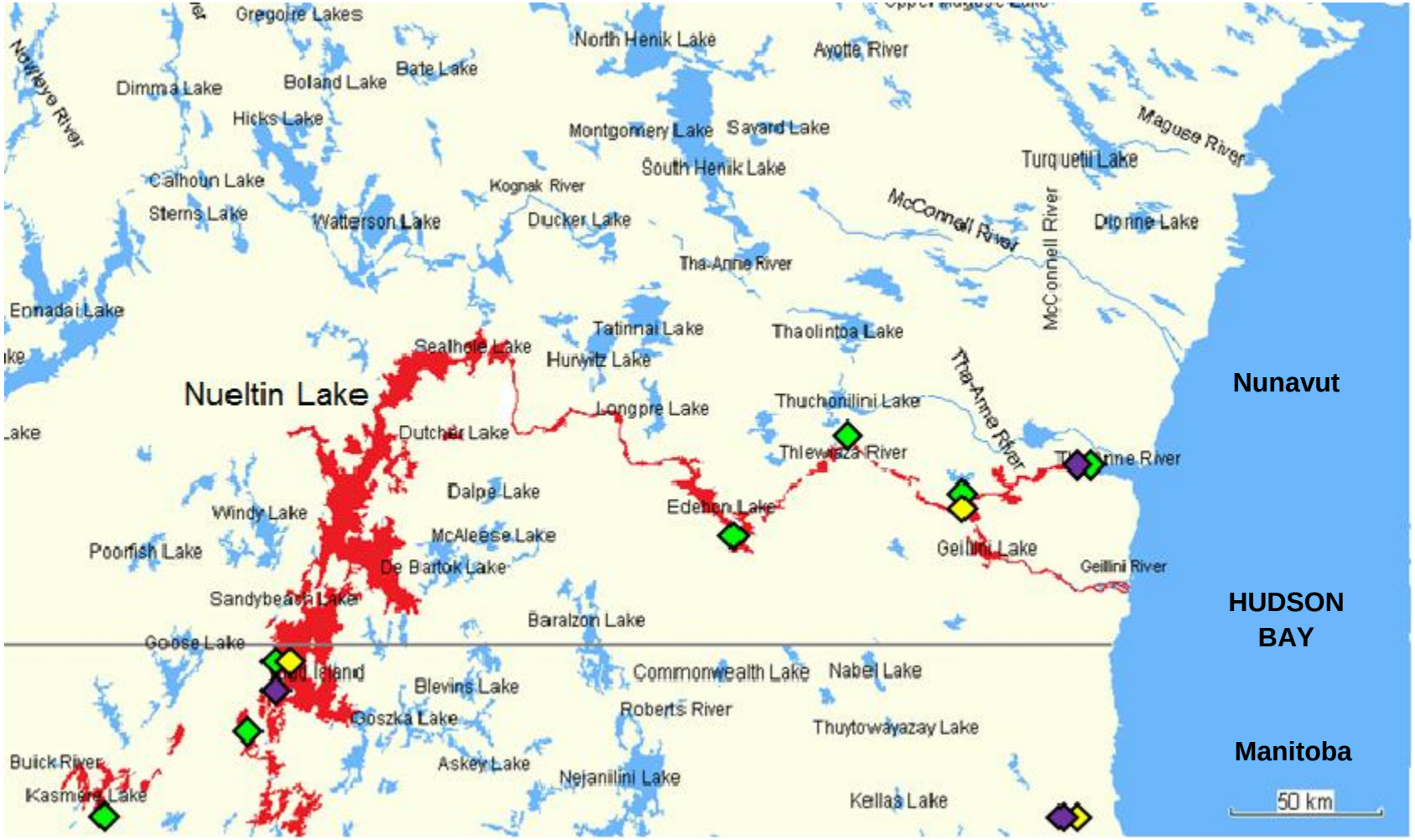


Figure 4. Map indicating the margins of the Laurentide ice sheet over North America near the end of the Wisconsinan glacial period, approximately 9.5 kya. Red star is approximate present-day location of Nueltin Lake. After Dyke *et al.*, 2003.

Figure 5. Map indicating the margins of the Laurentide ice sheet over North America near the end of the Wisconsinan glacial period, approximately 8.5 kya. Left point of open star marks Nueltin Lake precursor. After Dyke *et al.*, 2003

Figure 6. Map indicating the margins of the Laurentide ice sheet over North America following the end of the Wisconsinan glacial period, approximately 7.45 kya. Red star marks approximate location of present-day Nueltin Lake[MR17]. After Dyke *et al.*, 2003.

Figure 7. Map indicating the Thlewiaza watershed (red) comprised primarily of Nueltin Lake and the Thlewiaza River to Hudson Bay. Green: sampled - no collection, this study; Yellow: historical collection; Purple: collected, this study.



CHAPTER 2: MICROSATELLITE DIVERSITY AND PATTERNS OF POSTGLACIAL DISPERSAL BY *GASTEROSTEUS ACULEATUS* IN NUELTIN LAKE AND COASTAL HUDSON BAY.

ABSTRACT

The glacial recession which occurred over much of North America nearly 8.5 thousand years ago, and was accompanied by significant overland flooding over glacially depressed terrain, enabled the inland dispersal of aquatic species much further than normally possible in non-glaciated regions. Postglacial drainage changes have resulted in isolation of independent populations of *Gasterosteus aculeatus* throughout its native range and examples of convergent evolution. Presumed isolation following deglaciation at the end of the Wisconsinan period resulted in the establishment of a population of *G. aculeatus* in Nueltin Lake, a large postglacial lake in northern Manitoba and Nunavut. Analysis of 11 microsatellite loci indicates that the Nueltin Lake population is genetically differentiated from other populations in the same, and adjacent, watersheds. F_{ST} values in comparison with the Nueltin Lake population ranged from 0.29 (within watershed) to 0.48 (between watersheds), whereas F_{ST} values were observed to range from 0.08 to 0.48 across all populations. All pairwise comparisons of differentiation were statistically significant. Observed levels of heterozygosity were lower than expected in the Nueltin Lake population, and increased inbreeding was also detected (F_{IS}: 0.188). Allelic diversity ranged from 4 to 39 (mean 19.73) alleles per locus across all populations, with reduced numbers observed in Nueltin Lake fish (1 to 5, mean 3.18). Average allelic richness per locus ranged from 3.2 in Nueltin Lake, to 14.2 among fish from the Thlewiaza River population. Fewer private alleles were also detected among Nueltin

Lake *G. aculeatus* as compared with more coastal populations. AMOVA results indicate significant hierarchical partitioning of genetic variation as explained by coastal or inland populations (15.83%, $p<0.05$). Fairly high correlation ($r^2=0.7431$) was identified between genetic and geographic distance; however, results of genetic differentiation indicate statistically significant differences between the most proximate populations within the Thlewiaza watershed which are greater than those observed between populations from adjacent watersheds. Analysis also indicates the presence of a recent bottleneck event influencing the genetic structure of *G. aculeatus* in Nueltin Lake.

Statistically significant F_{ST} values observed between the Nueltin Lake population and conspecifics from within and adjacent watersheds have shown that *G. aculeatus* in Nueltin Lake represent a discrete and evolutionarily significant population which deserves recognition under COSEWIC as a Designatable Unit (DU).

INTRODUCTION

The Pleistocene glaciations, specifically the Wisconsinan glaciation have had significant impacts on the ecology and genetic structure of species in North America (Fulton and Prest, 1987; Wilson and Hebert, 1998). Repeated advance and retreats of glaciers over newly exposed terrains provided ecological opportunities for freshwater species along new dispersal pathways, but also created considerable challenges for species in a glacially-scoured, constantly shifting landscape (Pielou, 1974). However, as a result of this variable geography and transient aquatic connectivity, the large proglacial lakes and streams formed from glacial meltwater provided tremendous dispersal opportunities for many aquatic species, resulting in these taxa having much larger ranges than many species from nonglaciated regions (McAllister *et al*., 1986; Wilson and Hebert, 1998). In particular, rapid dispersal in postglacial timeframes has resulted in geographically widespread species ranges, but subsequent drainage changes have sometimes isolated portions of these ranges. Such isolation has occasionally driven rapid evolutionary radiations resulting in distinct localized populations with remarkable variation in morphological, physiological, and behavioral characteristics (Taylor and McPhail, 1999; Reusch *et al*., 2001).

Gasterosteus aculeatus is a predominantly marine fish species which is found regularly in coastal waters around Canada (Scott and Crossman, 1973; Stewart and Watkinson, 2004). It has been collected from the marine environment and have been found up to 110 km from the Atlantic coast of New York and 800km offshore in the Gulf of Alaska (Bell *et al*., 1994). In Canada, *G. aculeatus* has been collected from both the eastern and western coasts of the country, as well as from the Arctic Ocean in James Bay

and Hudson Bay (Scott and Crossman, 1973; McKillop and McKillop, 1997). In addition to a marine existence, variations in life history include an anadromous strategy requiring freshwater tributaries and estuarine environments for spawning activities as well as an exclusively freshwater residence, often as a result of vicariant barriers preventing the return to marine ecosystems (Bell and Foster, 1994, Östlund-Nilsson, 2007). Regardless of life history strategy, most populations are found in close proximity to coastal environments (Bell and Foster, 1994).

Gasterosteus aculeatus has been well studied throughout its range, often focusing on parallel and convergent evolution (Schluter and McPhail, 1992; Hatfield and Schluter, 1996; Bell *et al*., 2001; Bell *et al*., 2004). Multiple examples of independent invasions of marine fish into previously uncolonized freshwater lakes and river systems following deglaciation and habitat alteration has enabled the study of contemporary evolution of repeated events (Taylor and McPhail, 1999; 2000). Species pairs of benthic-limnetic forms of stickleback have evolved in parallel in four distinct lakes from the Strait of Georgia in western British Columbia. These evolutionarily significant pairs exhibit heritable variation for these adaptive traits and are known worldwide as examples of a natural model for speciation research (McKinnon and Rundle, 2002). Additional units have been identified as being lentic-lotic (lake-stream) pairs in similar glacially isolated habitats. Significant genetic differentiation has been observed in populations with very low geographic partitioning and indicates that adaptive and divergent selection based on habitat type promotes rapid diversification (Hendry *et al*., 2002; Hendry and Taylor, 2004). As a result of the evolutionary significance of these populations to studies of parallel evolution and ecological speciation, special conservation legislation has been

enacted to protect these species pairs from extinction. Despite these measures, two of the benthic-limnetic pairings have been lost (Hatfield, 2001; Kraak *et al*., 2001; Foster *et al*., 2003; Wood, 2003). This demonstrates the fragility of the genetic partitioning which supports the evolution of these species pairs and underlines the need for ongoing research to identify evolutionarily significant populations before we are left to ponder their extinction.

MICROSATELLITES

The genetic distribution and population structure of many species in Canada have been strongly influenced by glaciation; along with a reduction of range and population number as a result of glacial activity, strong founder effects characterize areas of recolonization in more recently deglaciated regions (Crossman and McAllister, 1986; Bernatchez and Wilson, 1998). As a result, northern populations are often characterized by shallow phylogenetic topologies, reduced genetic diversity, and discordant or conflicting phylogeographic patterns (Bernatchez and Wilson, 1998).

As with many taxa from glaciated regions that demonstrate reduced levels of intraspecific divergence and diversity (Bernatchez *et al*., 1989; Billington and Hebert, 1991), allozyme and microsatellite studies of *G. aculeatus* have revealed that freshwater populations are genetically less diverse and contain a subset of allelic richness of marine counterparts (Rafinski *et al*., 1989; Taylor and McPhail, 2000; Reusch *et al*., 2001). As a result of the most recent deglaciation and recolonization occurring in northern Canada in such an abbreviated geologic timeframe, sufficient variability in mitochondrial DNA (mtDNA) would not have accumulated in the studied populations (Nueltin Lake) due to

its recent divergence from the ancestral population (~8500 years) (Fulton and Prest, 1987). In these cases, more variable markers such as microsatellites become particularly useful over traditional mitochondrial DNA markers, as microsatellite loci offer higher resolution for detecting genetic variation among populations in contemporary timeframes (Chistiakov *et al*., 2006; Palsbøll *et al*., 2007).

Microsatellites were selected for use in this project due to their sensitivity to recent divergence and ability to distinguish variation at the population level. They are short (1 to 6 base pairs [bp] in length) tandem repeats that are abundantly dispersed throughout the genome (Chistiakov *et al*., 2006). They are widely considered selectively neutral, appearing more frequently in non-coding regions of the genome. As such, they are considered free from the pressures of selection, and observed variation reflects historical and contemporary population size, genetic drift, and mutation (Chambers and MacAvoy, 2000; Chistiakov *et al*., 2006).

The calculation of genetic distance between two populations gives a relative estimate of the time that has passed since the populations have existed as single cohesive units (Malone, 2003). Small estimations of distance may indicate population substructure (i.e. subpopulations in which there is random mating, but between which there is a reduced amount of gene flow). However, small estimates of distance may also be present because the populations are completely isolated but have only been separated for a short period of time. When two populations are genetically isolated, the two processes of mutation and genetic drift lead to differentiation in the allele frequencies at selectively neutral loci. The erosion of genetic diversity over time is a natural process in small

populations, as genetic drift causes the loss of alleles faster than mutation can create new diversity (Malone, 2003).

STUDY GOALS AND HYPOTHESES

The purpose of this study is to identify genetic diversity in a previously unknown population of *G. aculeatus* from Nueltin Lake and compare levels of genetic similarity to other populations from shared and adjacent watersheds. Reductions in levels of genetic diversity in postglacial populations have been well documented (Bernatchez and Wilson, 1998); however, patterns of genetic differentiation will be explored within the known glacial history of the Nueltin Lake region. Catastrophic melt and overland flooding following the glacial recession created temporary dispersal routes further inland than typically available and may have provided opportunities for eventual genetic isolation to have developed between inland and coastal populations when these connecting routes disappeared.

This study will use a suite of 11 microsatellite loci to investigate genetic differentiation in selectively neutral regions of the stickleback genome and compare patterns of genetic variability. It is hypothesized that fish in Nueltin Lake arrived via dispersal along margins of glacial floodwaters and became isolated inland as a result of isostatic rebound. This would suggest that Nueltin Lake diverged from the ancestral population in the Tyrrell Sea (Hudson Bay) nearly 7.2 kya, earlier than populations located nearer to the coast such as an estuarine population from the mouth of the Thlewiaza River near Hudson Bay, Nunavut, a riverine population from the Caribou River, and a population from Wapusk National Park located further south along the shore

of Hudson Bay. Based on the geography and topology of the region, it is hypothesized that dispersal to the present-day location of Nueltin Lake would not be likely, given the normal dispersal abilities of *G. aculeatus*. Isolation from conspecifics within the same and adjacent watersheds would enable high levels of genetic differentiation to be observed in the Nueltin Lake population and may be used to meet the criteria for a Designatable Unit (DU) under COSEWIC.

MATERIALS AND METHODS

Samples were collected as indicated in Chapter 1, this study. Collection records are listed in Appendix B, C. Genetic analysis of *G. aculeatus* was conducted using individuals from four populations which included Nueltin Lake, the mouth of the Thlewiaza River near Hudson Bay, the Caribou River, and Rupert Creek located in Wapusk National Park (Figure 7).

Microsatellite selection

Twelve microsatellite loci (Table 2) were selected from previously published studies to examine genetic relatedness and postglacial dispersal in *G. aculeatus* in Nueltin Lake and the Thlewiaza watershed (Taylor, 1999; Makinen *et al*., 2006). While microsatellite loci are considered selectively neutral markers, loci were selected from 12 separate linkage groups at a minimum distance of 0.25 cM from mapped quantitative trait loci (QTL) locations to prevent inheritance by proximity and to characterize genome wide variability (Peichel *et al*., 2001; Makinen *et al*., 2006; Appendix A). Polymorphism seen in loci near QTL may not be attributable to independent genetic mutation as a result of isolation,

but instead may be influenced by the shift to a freshwater environment and selective pressures on known morphological characters. Chapter 3 will examine morphological variability between coastal and inland populations; microsatellites associated with these loci may provide redundant information. In an effort to explore as much genome wide variability as possible, microsatellites were selected away from QTL regions in an effort to avoid duplicate sampling of information and increase the likelihood of neutrality among loci used in analyses.

For studies using microsatellite analysis, preferred sample size is approximately 50 individuals (Cena *et al*., 2006). Despite intensive effort in all locations, samples collected from each population ranged from 10 to 43 specimens. Due to the unknown population status of *G. aculeatus* in Nueltin Lake and the difficulty in obtaining the collected individuals, a smaller sample size and incorporation of more microsatellite loci into the study was implemented, although results would likely have been more robust using more individuals.

DNA extraction and microsatellite amplification

DNA was extracted from pectoral fin samples using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. Polymerase chain reaction (PCR) conditions from GenBank entries corresponding to selected loci were used as a starting point, but were modified for maximal amplification and yield. For visualization of PCR products, the forward primers were labeled with fluorescent dye (FAM, HEX, NED) and the 5'-end of the reverse primers were modified with a –GTTT

tail to enhance 3'-adenylation and reduce stutter (Brownstein *et al*., 1996; Makinen *et al*., 2006) (Table 2).

All amplifications were performed in 10 µl reaction volume containing 10x PCR manufacturer's buffer (20 mM Tris-HCl pH 8.4; 50 mM KCl), 1.5 mM $MgCl_2$, 0.2mM dNTP, 0.25 U Taq DNA polymerase (Invitrogen), 2 to 8 pmol of each primer and approximately 20 ng template DNA. Reactions were initially denatured at 95ºC for 3 minutes, followed by 30 cycles of 95ºC for 30 seconds, 53ºC for 30 seconds, and 72ºC for 30 seconds. PCR products were visualized on a 1.5% agarose gel electrophoresis with Tris/Borate/EDTA (TBE) solution prior to fragment analysis.

Following multiple unsuccessful attempts at amplification, likely due to a null allele (attributed to modification in one or both of the flanking regions of the microsatellite which inhibits proper primer annealing and thus, successful amplification), Stn 34 was eliminated from this study. The remaining 11 microsatellite loci were arranged into two groups with non-overlapping size ranges and individual fluorescent dye patterns and were resolved on an ABI 3130 Genetic Analyzer (Applied Biosystems Inc.) automatic sequencer.

Data analysis

Alleles were scored for size and genotyped using GeneMapper (Applied Biosystems, Inc.) and were visually proofread. Populations were examined for the presence of null alleles, scoring error and allelic dropout using the program Micro-Checker 2.2.3 (VanOosterhout *et al*., 2004). Nei's (1978) unbiased estimate of expected heterozygosity (H_e), observed heterozygosity (H_o) and allele frequencies were calculated for each of the

11 microsatellite loci for the four populations of stickleback using FSTAT v.2.9.3.2. (Goudet, 1995). An exact test for goodness of fit to Hardy-Weinberg Equilibrium (HWE) was conducted for each locus, as well as each locus within each population, using a Monte Carlo exact test for goodness of fit (20,000 permutations) in FSTAT v.2.9.3.2. (Goudet, 1995) and were adjusted for significance using the sequential Bonferroni correction (Rice, 1989) to account for multiple, simultaneous tests.

The number of alleles (N_a) and standardized allelic richness (A_r) were calculated by locus and locality in FSTAT v.2.9.3.2. (Goudet, 1995). To correct for differences in sample size among populations when estimating allelic diversity, allelic richness was calculated by using rarefaction sampling based on a minimum of 15 genes (HP Rare software; Kalinowski, 2005). Alleles that were detected in single populations only were identified as private alleles. The proportion of private alleles in each locus was calculated by dividing the total number of private alleles by the total number of alleles.

Selection of specific distance measures used to estimate genetic differentiation from microsatellite data is not consistent across the field of study (Makinen *et al*., 2008). Distances taking into account the stepwise mutation model (SMM) suffer from a higher variance when a small number of loci are used and distances based on the infinite allele model (IAM) might miss relevant biological information in highly divergent populations (Gaggiotti *et al*., 1999; Balloux and Lugon-Moulin, 2002). Variation among sample sizes is not thought to affect calculations of F_{ST} (F_{ST}, the amount of significant genetic differentiation in allele frequencies among populations, see Appendix D), as has been observed for R_{ST}, and suggests that when there are large differences in sample size, F_{ST} may be the preferred measure to estimate population structure. However, F_{ST} was not

specifically derived within the context of the stepwise mutational model, the assumed process at microsatellite loci (Weber and Wong, 1993; Goldstein *et al*., 1995; Slatkin, 1995; Di Rienzo *et al*., 1998); therefore, it does not reflect genetic distance between alleles but rather, allelic frequencies. Instead, F_{ST} assumes no mutational model and it is based on levels of genetic divergence resulting primarily from genetic drift (Heath *et al*., 2006). Implicit assumptions that arise when using F_{ST} to infer potential gene flow between populations are that population sizes are equal, sufficiently large and are in drift-mutation-selection equilibrium (Raeymaekers *et al*., 2007). Although these assumptions are rarely met, F_{ST} estimates have been found to be robust to violations of at least some of these assumptions and are preferred to other differentiation measures when sample sizes are small and fewer than 20 loci are scored (Ruzzante, 1998; Gaggiotti *et al*., 1999; Malone, 2003). Calculations of F_{ST} are improved when time following divergence from the ancestral population, relative to its size, is short as local variation may be attributed to genetic drift rather than mutation rates (Slatkin, 1995).

The program FSTAT (Goudet, 2001) was used to estimate pairwise genetic differentiation indices using Weir and Cockerham's (1984) θ statistic (hereafter referred to as F_{ST}), a maximum-likelihood estimator of F_{ST}. The significance of each pairwise comparison was tested with a G-statistic for genotypic differentiation (Raymond and Rousset, 1995), which weights loci according to allelic diversity (Goudet, 1995), and was corrected for multiple comparisons in all statistical tests using a sequential Bonferroni procedure (Rice, 1989).

Analysis of molecular variance (AMOVA, Excoffier *et al*., 1992), as implemented by ARLEQUIN (Excoffier *et al*., 2005, v 3.11 in Excoffier, 2007) is a nonparametric

equivalent to analysis of variance (ANOVA), which tests a given genetic structure by partitioning the total variance into covariance components due to differences within and among localities (Excoffier *et al*. 2005). Groupings based on coastal or inland populations, as well as by watersheds were tested. Significance of the AMOVA was based on 1000 permutations.

Regression analysis was used to examine the relationship between geographic distance and genetic differentiation between sampling locations. For each pairwise comparison, geographic distance was calculated using distance via best-estimate of fluvial connectivity (arbitrary assessment of migratory pathway by water) between sampling locations in Google Earth (2009). Scatter plots of the natural log of the geographic distance (ln(km)) and genetic differentiation (pairwise F_{ST} values plotted as (F_{ST} /(1-F_{ST})) (Rousset, 1997) at all sampling locations were graphed using XLSTAT (Microsoft, 2010). Linear regression was used to derive slope and r^2 values to determine if a linear relationship exists between geographic distance and genetic differentiation. A Mantel test was performed for all sampling locations using the program ISOLDE (as defined by GENEPOP, Raymond and Rousset, 1995; Rousset, 2008) to test for correlations between geographic distance and genetic differentiation.

The software program BOTTLENECK 1.2.02 (Cornuet and Luikart, 1996; Piry *et al*., 1999) was used to test for recent, drastic population declines. Analyses were based on 10 000 iterations using all three mutational models (IAM, SMM, two-phase model of mutation (TPM)). A Wilcoxon rank-sign test was used to examine whether the observed heterozygosity is larger than the heterozygosity expected from the observed allele number if the locus was at mutation-drift equilibrium (Hänfling *et al*., 2002; Wu and Hu,

2010). The distortion of allele frequency classes was tested for deviation from a normal L-shaped distribution (Luikart *et al*., 1998)

Genetic similarities and relationships among populations were visualized using principal components analysis (PCA) program PCAGEN (www2.unil.ch/popgen/softwares/) used to graphically represent the genetic variation among populations in two-and three-dimensional space.

RESULTS

In total, 103 *G. aculeatus* were genotyped at 11 microsatellite loci from four collection localities throughout the Thlewiaza River watershed and along the southwestern coast of Hudson Bay (Table 1, Figure 7).

Size ranges, observed and expected heterozygosities, inbreeding coefficient (F_{IS}: the probability that an individual has a pair of alleles that are identical by descent from a common ancestor; the proportion of the variance in the subpopulation contained in an individual) and allelic richness (A_r) were determined for each locus and locality and are presented in Tables 1 and 2. In general, observed levels of heterozygosity were lower than expected values at most loci among all populations. The observed heterozygosity across 11 loci varied between 0.19 and 0.81 (across all populations) with a mean of 0.48. The highest within-population gene diversity was observed at Stn 19 in the Caribou River population (0.91) and the lowest from the inland Nueltin Lake population (0.00 at both Stn 38 and Stn 174) indicating complete fixation of a single allele (Table 3). The inbreeding coefficient (F_{IS}) observed in Nueltin Lake was 0.19; the lowest value was observed in the Caribou River population (0.07) (Table 1). Rupert Creek exhibited the

highest F_{IS} value; however, this may be attributed to having a smaller sample size (0.26, n=10) or may also reflect collection bias. Many individuals from the Rupert Creek population were juveniles collected at one site and may represent a cohort of fish with the same parents; this might account for higher levels of shared genetic diversity.

Coastal populations exhibited higher numbers of both alleles detected, as well as number of private alleles, as compared with the inland population (Figure 9, a, b, c). The number of alleles detected ranged from 4 to 39 per locus, mean 19.73 (Table 3). Across all samples, 217 alleles were identified at 11 loci, 35 of which were found in the Nueltin Lake population (16.1%) and 156 in the Thlewiaza River population (71.89%). Average allelic richness per locus was 3.2 in Nueltin Lake and 14.2 in the Thlewiaza River population. Potential private alleles were observed in all localities and ranged in proportion from 0.171 to 0.513. Private alleles (alleles which are unique to a single population) (n=127) were observed across all populations, six of which were exclusive to Nueltin Lake and 80 were specific to the Thlewiaza River populations. The average gene diversity was higher among coastal/potentially migratory populations (mean 0.0.34 to 0.72) as compared with the inland, Nueltin Lake population (mean 0.22). Average allelic richness was higher among coastal populations (0.05 to 0.14) than was observed in Nueltin Lake (0.03) (Table 3).

Congruence with Hardy-Weinberg Equilibrium (HWE) at each sampling location, as well as at each locus within a sampling location, was tested and results are summarized in Tables 1 and 2. The Nueltin Lake population showed significant deviation from HWE after sequential Bonferroni correction. This deviation may be explained by Wahlund effects (the reduction in heterozygosity in a population due to subpopulation structuring,

(Waples and Teel, 1990)), obscure population structure or inbreeding effects (Castric *et al*., 2002). Using Microchecker 2.2.3 (VanOusterhout *et al*., 2004), null alleles were suggested at 8 of 11 loci; however, these may also be attributed to Wahlund effects. Failure to meet HWE is not sufficient for removing a locus from analysis, as such; all loci were retained for further examination (Selkoe and Toonen 2006).

Rarefaction was used to provide estimates of allelic diversity at equal sample sizes (Table 4). Relative allelic frequencies observed on transformed data identify a more proportional diversity among the Thlewiaza River, Caribou River and Rupert Creek populations; however, reduced diversity in Nueltin Lake is still observed in comparison. This suggests that by including greater numbers of individuals in the study, observed allelic frequencies may be quite different and re-emphasizes the need for a large number of individuals when conducting genetic analyses as unequal sample sizes appear to dramatically influence allelic diversity (Cena *et al*., 2006).

Estimates of genetic differentiation among sampling locations, θ values (an unbiased estimator of F_{ST}), were calculated using FSTAT (Goudet, 2001) for pairwise comparisons of each sampling location, adjusted for Type 1 error using a sequential Bonferroni correction (Rice, 1989). F statistics are summarized in Table 6. Genetic differentiation was found between all collection locations, with the greatest differences being observed between the inland Nueltin population and coastal samples from Rupert Creek, the most geographically disparate populations. F_{ST} values ranged from 0.0798 to 0.4756. Pairwise comparisons were tested for statistical significance using G-based exact tests for genotypic differentiation in GENEPOP (Goudet *et al*. 1996). All pairwise

comparisons between populations demonstrated genetic differentiation and were shown to be statistically significant ($p=0.05$).

Analysis of the hierarchical patterning of genetic variation between groups of sampling location using AMOVA revealed significant variation attributed to population structuring among *G. aculeatus* in Nueltin Lake as compared with coastal populations. Groups were defined as coastal or inland populations, as well as being grouped by individual watershed. Variation between coastal and inland populations was calculated to be 15.83% ($p<0.05$); variation among populations within these groupings was 13.53% ($p<0.05$). Variation within individuals accounted for 70.64% ($p<0.05$) of the total variation observed. When the Thlewiaza watershed was compared with adjacent coastal populations, variation among groups within populations was 25.77% ($p<0.05$) and variation within groupings accounted for 75.12% ($p<0.05$). When each watershed was compared individually, similar results were observed (variation among populations within groups: 27.78%, variation within populations: 75.94%, $p<0.05$).

Isolation by distance modelling, as proposed by Rousset (1997), was estimated using pairwise F_{ST} values between populations to examine the correlation between $F_{ST}/(1-F_{ST})$ and geographic distance. To test for correlations between geographic and genetic distances, Mantel tests were conducted using the program ISOLDE (as used in GENEPOP, Raymond and Rousset 1995a; Rousset, 2008). Linear regression analysis of the genetic ($F_{ST}/(1-F_{ST})$) and geographical (ln(Km)) distances resulted in an r^2 value of 0.4417 ($p<0.05$) (Figure 10). Despite having the greatest genetic distance being observed in the most geographically disparate populations (and the least differentiation in the most

proximate populations), populations with similar geographic distances exhibited genetic distances ranging from 0.201 to 0.622 (Figure 10).

Evidence of recent population bottlenecks was assessed against three mutation models for microsatellite data using the program BOTTLENECK. All locations exhibited a normal, L shaped distribution under a mode-shift curve. All loci fit the SMM at mutation-drift equilibrium, with p=0.001 for a one-tailed heterozygote deficiency, and p=0.003 for two-tailed heterozygote deficiency or excess to indicate the presence of a recent bottleneck event in Nueltin Lake.

Principal components analysis of 11 microsatellite loci on populations from Nueltin Lake and coastal western Hudson Bay using the program PCAGEN shows a clear distinction between the inland, Nueltin Lake population and the coastal populations (Thlewiaza River, Caribou River, Rupert Creek) (Figure 11: Axis 1=68.05%, F_{ST}=0.136). Further separation along the second axis appears to follow geographic patterning, with the Thlewiaza and Caribou River populations being grouped slightly closer together [MR18]than to the more geographically distant Rupert Creek population (Axis 2=19.79%, F_{ST}=0.039).

DISCUSSION

Dispersal patterns following the recent deglaciation of North America has been the focus of many recent molecular studies (e.g. reviewed in Brochmann *et al*., 2003; Hewitt, 2004; Schönswetter *et al*., 2005). Glacial isolation and postglacial range expansion are believed to have contributed significantly to present genetic population structure and diversity in species from these regions (Hewitt, 1999; Treier and Müller-Schärer, 2011). Change in

genetic variation among selectively neutral genetic markers such as microsatellites can be attributed not only to genetic drift [MR19]and mutation rates, but also as a result of historic events such as habitat fragmentation and bottleneck events often associated with range expansions and climatic or geographic fluctuations as a result of glacial processes (Hänfling *et al*., 2002).

Following dispersal in a postglacial environment, expected patterns of change among newly colonized populations include a dramatic reduction in genetic diversity as compared with their ancestral population (Hewitt, 1996; Comes and Kadereit, 1998; Treier and Müller-Schärer, 2011). It has been shown that *G. aculeatus* in Nueltin Lake represents an isolated population, and its presence there may be attributed to known geological events following the recession of the Laurentide ice sheet nearly 8.5 kya. Significant genetic differentiation in the Nueltin population as compared with other stickleback populations from western Hudson Bay indicates that fish in Nueltin Lake likely became isolated inland, along glacial margins, as a result of isostatic rebound following dispersal from the ancestral Tyrrell Sea. The demonstration of a discrete and evolutionarily distinct population of *G. aculeatus* located in Nueltin Lake, reflected by statistically significant, and high, F_{ST} values as compared to populations within and between other watersheds is supportive of being designated as a Discrete Unit (DU) under COSEWIC guidelines. An earlier divergence from conspecifics in the Tyrrell sea, coupled with a lack of gene flow upstream to Nueltin Lake has resulted in a shallow gene pool, as evidenced by reduced allelic diversity and increased levels of homozygosity, which decreases the ability of the population to withstand the effects of genetic drift and natural selection (Bernatchez and Wilson, 1998).

Following the most recent deglaciation of North America, melting of glacial ice resulted in eustatic sea level rises in excess of 100 m (Mann, 1986). Until isostatic rebound and tectonic uplift enabled glacially depressed terrain to emerge, low-lying coastal regions were submerged under marine waters (Johnson and Taylor, 2004). This process of submergence and rebound has been proposed as a mechanism for parallel freshwater colonization by *G. aculeatus* and other euryhaline fishes from the marine environment (McPhail and Lindsey, 1986; McPhail, 1993). Regional variation in the degree of isostatic depression and rate of tectonic uplifting promote slightly different rates of isolation and dispersal ability (Johnson and Taylor, 2004). Reductions in genetic diversity attributed to postglacial dispersal have been modeled (Ibrahim *et al*., 1996) as well as having been shown empirically, for example, in some populations of North American salmonid fishes (Bernatchez and Wilson, 1998). Freshwater populations of stickleback have been shown to exhibit reduced variation within, and greater variation among, populations than do marine populations (McKinnon and Rundle, 2002). Comparisons of genetic variation between Nueltin Lake and Thlewiaza River coastal population have confirmed reduced levels of genetic diversity, both in allelic frequencies and proportions of rare alleles.

Overall, fewer alleles were detected per locus in Nueltin Lake, higher levels of homozygosity are present and an ongoing lack of gene flowas a result of considerable environmental barriers along the river system (Table 1, 3). Similarly, higher genetic diversity in refugial or ancestral populations relative to their descendant populations is often observed (Treier and Müller-Schärer, 2011). The Thlewiaza River population exhibited much greater genetic diversity and lower levels of inbreeding than did Nueltin

Lake (Table 1, 3). Genetic variation is essential for both short and long-term persistence of populations and, when assessed using polymorphisms within segments of the genome, it has been shown to be influenced by a number of factors of critical importance to species conservation (Wu and Hu, 2010).

Populations undergoing sudden or longterm reductions in genetic diversity, such as a population bottleneck, may have a reduced capacity to adapt to changing environments that contributes to inbreeding depression, a reduction in fitness and increased probability of extinction for the population (Takamura and Mori, 2005; Wu and Hu, 2010). Smaller populations are more likely to experience reductions in allelic diversity as a result of random drift and may experience an increase in homozygosity as a consequence of mating between close relatives (Kimura and Crow, 1964; Nei *et al*., 1978).

In contrast with phylogeographic patterns observed among aquatic species from unglaciated areas (Bermingham and Avise, 1986; Avise, 1992), phylogeographic patterns among northern species show marked differences that can readily be interpreted in light of postglacial history and species-specific ecological characteristics [MR20](Wilson and Hebert, 1998). Overall, *G. aculeatus* in this study exhibits a level of genetic diversity comparable to sticklebacks across other postglacially fragmented landscapes (Hendry *et al*., 2002; Makinen *et al*., 2006). Observed heterozygosity in *G. aculeatus* from Nueltin Lake and coastal Hudson Bay (H_o, across all loci) ranged from 0.186 to 0.814, mean 0.481, with an average number of alleles detected per locus (N_a) of 19.73 (Table3). These values are comparable or slightly lower than those observed in similar studies of stickleback in postglacial environments [MR21](Table 5). Observed levels of

heterozygosity from the inland, Nueltin Lake population were characterized as being slightly lower (Ho: 0.00 to 0.765, Na: 1 to 5) than other studies of inland, anadromous species, but also higher than some (Table 5). Coastal populations from western Hudson Bay shared similar levels of allelic diversity with those observed in the anadromous smelt population (Na: 2 to 23); H_o values were slightly lower, although high levels of overlap exist(0.171 to 0.822 as compared with 0.431 to 0.930[MR22]).

Deviation from Hardy-Weinberg Equilibrium [MR23](HWE) can be attributed to a variety of causes. For a population to be in HWE, a number of assumptions must be met: infinite population size, random mating, and a lack of selection, migration, and mutation events (Crow, 1999). Understandably, these factors are rarely, if ever, achieved in natural populations. When an excess of homozygotes are detected, as in the Nueltin Lake population, it is likely to be attributed to the effects of selection acting on the loci (as has been shown for certain traits such as *Eda*, *Pitx1*¸Colosimo *et al*., 2005; Chan *et al*., 2010). Null alleles (caused by a mutation in the primer binding site leading to an allele that will not amplify) may also contribute to an excess of homozygotes by underrepresenting heterozygosity due to non-amplification of the second allele. Inbreeding may also promote the accumulation of excess homozygosity. Finally, a deficiency in the number of heterozygotes observed in a population may be attributed to Wahlund effects and population substructuring leading to varying levels of reproductive isolation within a population. Wahlund effects and inbreeding in the Nueltin Lake population may have contributed to the reduction in heterozygosity observed in that population; the inclusion of a large number of loci into a study will increase the ability to detect Wahlund effects as each locus represents an independent history of the population and reflects random

drift, mutation, and migration that have occurred in that location of the genome. Epistasis or genetic hitchhiking can obscure this information and results may be biased towards a linked grouping of loci; however, an assessment of linkage disequilibrium was included in this study.

F-statistics examine the correlation of alleles within individuals within a population and are related to levels of heterozygosity or inbreeding coefficients to describe the measure of non-random association of alleles within an individual. F-statistics describe the variation of allelic frequencies observed in a population. It can be more descriptively applied to identify the proportion of alleles by an individual within a subset of the total population (F_{IS}), among subpopulations from the total population (F_{ST}), and of individuals within the total population (F_{IT}) (Wright, 1951; Nei, 1986; 1987, Appendix D). If limited genetic exchange is occurring between two subpopulations, either as a result of isolation or due to inbreeding, alleles will eventually become fixed and F_{ST} will approach 1 indicating a complete lack of shared allelic diversity. F_{ST} values provide estimates of differentiation and may be compared broadly across all taxa (Table 7); however, divergence levels are often species specific and must be interpreted with caution.

All pairwise measures of genetic differentiation of sampling sites from Nueltin Lake and western Hudson Bay were tested using a G-statistic and were identified as being highly significant ($p<0.001$) (Table 6). As indicated by mean pairwise F_{ST} values, the most strongly differentiated populations were Nueltin Lake and Rupert Creek, $F_{ST}=0.4756$ and Nueltin Lake and Caribou River, $F_{ST}=0.3834$. These populations are the most geographically distant in this study and are separated by two different watersheds.

Rupert Creek and other coastal populations show a much lower, although still significant, level of genetic differentiation (0.0798 to 0.1749). Rupert Creek and the Caribou River population exhibit moderate levels of genetic differentiation (F_{ST} =0.1672). F_{ST} values of *G. aculeatus* from Nueltin Lake and western Hudson Bay are approximately comparable to populations from other recently deglaciated areas (Table 7); however, do indicate high levels of differentiation, exceeding those identified among species pairs from British Columbia. *Gasterosteus aculeatus* from Nueltin Lake have been shown to be more distinct from conspecifics in adjacent (and the same) watersheds than sympatric or parapatric species pairs which have been recognized as separate DU's.

Genetic bottlenecks can result in a severe reduction in genetic diversity in a population. They are often attributed to a catastrophic occurrence such as a population crash or vicariant dispersal event (Hallerman, 2003). Populations that survive a bottleneck without becoming extinct often experience lower levels of genetic variability upon which selection can act and may also experience greater effects of genetic drift. Founder effects are attributed to a subset of alleles becoming isolated along the margins of a species' range and, similar to bottleneck events, demonstrate reduced genetic variation from the original population. Several theories for population bottlenecks influencing speciation have been proposed for *G. aculeatus*; however, experimental and theoretical support for these models is weak (McKinnon and Rundle, 2002). Rather, divergent selection appears to be a more significant mechanism promoting speciation in this species. Population bottlenecks can certainly occur in populations, as has been demonstrated for Nueltin Lake, however its role in promoting future speciation remains unsupported. It is likely that *G. aculeatus* in Nueltin Lake is influenced by founder

effects as evidenced by the presence of low levels of shared allelic diversity with the ancestral population from the Tyrrell Sea. The result of such an event significantly reduces the population's ability to adapt to effects of selection and adaptive stresses, such as climatic changes or resource competition. Occurrence of genetic bottlenecks, as has been identified in Nueltin Lake, can have significant negative impacts on a population's ability to adapt under changing environmental conditions and often severely impacts reproductive fitness (Hallerman, 2003).[MR24]

Overall, reduced patterns of genetic diversity were observed in the Nueltin Lake population of *G. aculeatus* as compared with other populations from within the same watershed, as well as in populations from adjacent basins. Standard measures of genetic diversity used to elucidate population structure identified lower levels of allelic richness, increased homozygosity and highly significant genetic differentiation in Nueltin Lake from all other pairwise population comparisons. Genetic differentiation from the inland population in Nueltin Lake and a coastal population from the Thlewiaza River at the mouth of Hudson Bay yielded highly significant differentiation (F_{ST} =0.2854) despite both populations being from the same watershed and river system. Coastal populations from three adjacent watersheds shared significant genetic partitioning between the basins, but were less differentiated than Nueltin Lake is from the Thlewiaza population. This would support the conclusions that based on genetic differentiation and lack of gene flow, the Nueltin Lake population is extremely unlikely to be anadromous and should be considered an isolated, exclusively inland, population of *G. aculeatus* in Manitoba.

Nueltin Lake *G. aculeatus* may still contribute genetic information downstream to populations along the Thlewiaza River, and possibly entering Hudson Bay, if migrants

were able to disperse effectively over considerable elevational gradients along the length of the river system. *Gasterosteus aculeatus* is not a broadcast spawner and exhibits high levels of parental care (Bell and Foster, 1994); therefore, contribution of genetic information would require active migration by juveniles or adults to spawning locations downstream in the coastal environment. If this were attained, gene flow would be exclusively in the downstream direction.

Evidence to support a recent bottleneck event in Nueltin Lake, as a result of postglacial colonization far inland from the coast of Hudson Bay, was observed based on variation in allelic frequencies. Upon isolation from the ancestral population, the subset of alleles present in Nueltin Lake represented only a small proportion of the genetic diversity available to *G. aculeatus* in the Tyrrell Sea. Levels of genetic similarity would be expected to decrease as a result of prolonged isolation from the ancestral population, as confirmed by a lack of gene flowbetween the populations. Based on the effects of selection, drift, and mutation, variation in allelic frequencies in the fragmented populationand associated genetic differentiation builds [MR25]in comparison to the ancestral population. It is likely that a small proportion of the total alleles that occurred in the marine population were present in the migratory fish that colonized the peri-glacial, Nueltin Lake. This subset of alleles likely gave rise to the extant population of *G. aculeatus* found in Nueltin Lake. It is unlikely that genetic contributions will be made in the upstream direction, from coastal populations to Nueltin Lake, as a result of significant geographic and elevational gradients along the Thlewiaza River that would impede natural dispersal. Accordingly, the numbers of alleles in the Nueltin Lake population are expected to decrease over time, as the effects of mutation to generate new diversity will

not be sufficient to counteract effects of selection, genetic drift, and increasing homozygosity (Malone, 2003). A lack of gene flow[MR26]into Nueltin Lake identifies significant risk to the longterm success of the Nueltin Lake population, as declinesin genetic diversity are attributed to a reduction in the ability to adapt to changing environmental conditions. If limited genetic diversity exists upon which natural selection can act, populations may beunable to adapt successfully to ecological variation and may be at an increased risk of extinction. [MR27]

CONCLUSIONS

This study has confirmed the presence of a persistent, self-reproducing population of *G. aculeatus* in Nueltin Lake, Manitoba. Previously unknown from regions outside of the coastal environment of Hudson Bay in Manitoba, the Nueltin Lake population likely arrived with the recession of the Laurentide ice sheet during the rapid melting event which punctuated the end of the late Wisconsinan glaciation approximately 8.5 kya. Results of 11 microsatellite loci indicate significant genetic differentiation of the Nueltin Lake population as compared to conspecifics from within the same watershed and those from adjacent regions.Overall genetic diversity in the studied populations was comparable to that observed in similar studies of *G. aculeatus*; however fish sampled from Nueltin Lake were characterized as having decreased genetic diversity, increased levels of inbreeding and a lack of gene flow into the population was also identified.Coastal populations from three adjacent watersheds indicated significant levels of genetic divergence among the basins; however, comparisons of within-watershed

differentiation exceeded the differentiation observed among coastal populations between watersheds[MR28].

Loss of genetic diversity has been shown to influence the fitness of a population in the short term by, for example, increasing susceptibility to pathogens, and in the long term by decreasing evolutionary potential as a result of reduced genetic diversity for adaptation to changing environmental conditions (Franklin, 1980; Frankham 1995a; 1995b). In addition to the low levels of allelic diversity and loss of heterozygosity, levels of differentiation exceed those identified among federally listed endangered species pairs of *G. aculeatus* and are suggestive of the need for similar conservation measures for the Nueltin Lake fish.

Table 1. Sampling locations and summary statistics based on eleven microsatellite loci. n, sample size; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, inbreeding coefficient (as measured by θ_{IS}); N_a, number of alleles; N_{pa}, number of private alleles; P_{pa}, proportionof private alleles. Significant deviation from HWE is denoted with (*).

Gasterosteusaculeatus									
Collection Location	**GPS coordinates (decimal degrees)**	**Collection date**	**n**	**H_o**	**H_e**	**F_{IS}**	**N_a**	**N_{pa}**	**P_{pa}**
Nueltin Lake, MB	59.8738N 100.08006W	July 3-6, 2007	34	0.198*	0.243	0.188	35	6	0.171
Caribou River	59.650059N 95.400147W	July 4, 2007	16	0.631	0.685	0.073	88	24	0.273
Rupert Creek, MB	57.538889N 92.556667W	July 30, 2005	10	0.445	0.590	0.256	52	17	0.327
Thlewiaza River, NU	60.5000114N 94.950248W	August 12, 2008	43	0.588	0.649	0.096	156	80	0.513

Table 2. Locus name, batch number (1 or 2 for pooling), dye label, size range, linkage group, number of alleles, expected and observed heterozygosities, repeat motif, primer sequences, and accession numbers for eleven threespine stickleback (*G. aculeatus*) microsatellite loci used in this study.' Stn' prefix indicates the microsatellite was developed at CEGS[r29].

Locus	Batch	size range	LG	N_a	A_r	H_e	H_o	Motif	Forward primer	Reverse primer	Gen Bank
Stn 19[1]	1 (6-FAM)	157-201	II	39	0.379	0.358	0.118	$(CA)_{14}$	ACAGGCATGAATGACACTGG	GTTTGATGAGCACAACACCTGAGC	G72135
Stn 135[1]	1 (6-FAM)	108-116	XII	11	0.107	0.295	0.235*	$(CA)_{11}$	AAGTGGAATATCCCAATGGC	GTTTTCCAGTCTTCTTTATTGCGG	G72288
Stn 57[1]	1 (HEX)	107-117	V	15	0.146	0.255	0.118	$(CA)_{17}$	GATGGTGCCCATAAGACTCG	GTTTCATGTGTGGATGAAGGATGC	G72155
Stn 184[1,2]	1 (HEX)	202-232	XVIII	30	0.291	0.346	0.118	$(CA)_{17}$	ACTGCAGCTACATAGCAACG	GTTTACCGGATCGTCTTAATGGC	G72316
Stn 3[1]	1 (PET)	150-172	I	27	0.262	0.187	0.147*	$(GT)_{16}$	ACAGCGTCTCCGTAACATCC	GTTTAACCGTTGAACTCTGAAGGC	G72128
Stn 38[1]	2 (6-FAM)	208-216	IV	4	0.039	0.000	0.00*	$(CA)_{12}$	GCAGGTGACATCTTCAGGG	GTTTTTTCATTAGGACCCAGGACG	G72145
Stn 174[1]	2 (6-FAM)	97-111	XVI	21	0.204	0.000	0.000	$(CA)_{11}$	GGCTTTGTTGTTATGCTTACCG	GTTTTATCTGTCAGGAGCGTGTGG	G72310
Stn 61[1,2]	2 (HEX)	98-130	VI	19	0.184	0.506	0.765	$(CA)_{15}$	AGGAGGTCACCACAGGAGG	GTTTGACGAGTCAGCAGTTTGAGC	G72158
Stn 122[1]	2 (HEX)	179-197	X	17	0.165	0.165	0.176	$(CA)_{26}$	GCAACAGACTGGAGAAGCG	GTTTGCCGGTTATTGAATGTGGG	G72282
Stn 79[1]	2 (NED)	113-121	VII	5	0.049	0.115	0.118*	$(CA)_{11}$	GCAGTATAAGGCCTGGCTGG	GTTTACGCTGATGTCTCAGGTTCC	G72166
Stn 110[1]	2 (NED)	162-186	IX	29	0.282	0.445	0.382	$(CA)_{24}$	AGACAAACTCATGTAACAGCCC	GTTTACCTGGGTGCTTCAATGC	G72182

*Significant deviation from HWE

[1]Peichel et al., 2001

[2]Makinen et al., 2006

Table 3. Observed and expected heterozygosities (H_o and H_e) and number of alleles (N_a) with sample size (n) for eleven microsatellite loci in four populations of *Gasterosteus aculeatus*). Significant departures from HWE (after Bonferroni correction) are indicated by asterisks (*). Sampling locations include: Nueltin Lake (NL), Thlewiaza River (TR), Caribou River (CR) and Rupert Creek (RC).

Stn3					Stn19					Stn38				
Locus	**NL**	**RC**	**CR**	**TR**	**Locus**	**NL**	**RC**	**CR**	**TR**	**Locus**	**NL**	**RC**	**CR**	**TR**
Ho	0.15*	0.80*	0.56*	0.77	Ho	0.12	0.20*	0.63	0.51	Ho	0.00*	0.30	0.19*	0.19*
He	0.19	0.74	0.65	0.78	He	0.36	0.19	0.91	0.82	He	0.00	0.57	0.18	0.17
Na	3	7	8	20	Na	5	2	15	23	Na	1	4	2	2
n	34	10	16	43	n	34	10	16	43	n	34	10	16	43
Stn57					**Stn61**					**Stn79**				
Locus	**NL**	**RC**	**CR**	**TR**	**Locus**	**NL**	**RC**	**CR**	**TR**	**Locus**	**NL**	**RC**	**CR**	**TR**
Ho	0.12	0.30*	0.75*	0.63*	Ho	0.76	0.40*	0.75	0.79*	Ho	0.12*	0.50*	0.69*	0.35*
He	0.25	0.65	0.83	0.69	He	0.51	0.36	0.79	0.71	He	0.12	0.39	0.51	0.35
Na	3	6	11	13	Na	3	4	7	16	Na	4	2	3	3
n	34	10	16	43	n	34	10	16	43	n	34	10	16	43
Stn110					**Stn122**					**Stn135**				
Locus	**NL**	**RC**	**CR**	**TR**	**Locus**	**NL**	**RC**	**CR**	**TR**	**Locus**	**NL**	**RC**	**CR**	**TR**
Ho	0.38	0.70*	0.75*	0.81	Ho	0.18*	0.50	0.56*	0.35	Ho	0.24*	0.80*	0.81*	0.65*
He	0.44	0.74	0.66	0.82	He	0.17	0.77	0.69	0.58	He	0.30	0.72	0.76	0.67
Na	5	6	8	19	Na	3	6	7	11	Na	3	6	7	11
n	34	10	16	43	n	34	10	16	43	n	34	10	16	43
Stn174					**Stn184**									
Locus	**NL**	**RC**	**CR**	**TR**	**Locus**	**NL**	**RC**	**CR**	**TR**					
Ho	0.00*	0.20	0.50	0.77*	Ho	0.12	0.20	0.75*	0.65*					
He	0.00	0.65	0.76	0.72	He	0.35	0.70	0.79	0.82					
Na	1	4	10	15	Na	4	5	10	23					
n	34	10	16	43	n	34	10	16	43					

*Significant deviation from HWE

Table 4. Comparison of allelic richness in threespine stickleback (*G.aculeatus*) from Nueltin Lake and western Hudson Bay. Rarefaction standardized to 15 genes following Kalinowski (2005). Sampling locations are Nueltin Lake (NL), Rupert Creek (RC), Caribou River (CR) and Thlewiaza River (TR).

NUMBERS OF ALLELES OBSERVED IN EACH SAMPLE													
	AVG PER LOCUS		Stn3	Stn19	Stn38	Stn57	Stn61	Stn79	Stn110	Stn122	Stn135	Stn174	Stn184
NL	3.2		3	5	1	3	3	4	5	3	3	1	4
RC	4.7		7	2	4	6	4	3	6	6	6	4	5
CR	8		8	15	2	11	7	3	8	7	7	10	10
TR	14.2		20	22	2	13	16	2	19	11	11	15	23
NUMBERS OF PRIVATE ALLELES OBSERVED IN EACH SAMPLE													
	AVG PER LOCUS		Stn3	Stn19	Stn38	Stn57	Stn61	Stn79	Stn110	Stn122	Stn135	Stn174	Stn184
NL	0.55		1	0	0	0	0	2	2	1	0	0	0
RC	1.55		3	3	2	0	0	0	5	1	0	2	1
CR	2.18		3	3	0	0	3	0	3	2	0	4	6
TR	7.27		12	12	0	4	8	0	10	6	3	9	16
ALLELIC RICHNESS (15 genes) After Rarefaction													
	AVG PER LOCUS		Stn3	Stn19	Stn38	Stn57	Stn61	Stn79	Stn110	Stn122	Stn135	Stn174	Stn184
NL	1.97		1.84	3.03	1.00	1.93	2.00	1.84	2.61	1.95	1.96	1.00	2.48
RC	3.51		3.95	1.95	3.50	3.00	3.45	2.00	6.14	3.99	3.95	3.00	3.75
CR	4.70		3.92	9.07	1.86	5.54	5.05	2.00	5.10	3.71	3.98	5.27	6.23
TR	4.75		6.12	7.21	1.80	4.32	5.37	1.98	6.47	4.09	3.94	4.71	6.27
PRIVATE ALLELIC RICHNESS (15 genes) After Rarefaction													
	AVG PER LOCUS		Stn3	Stn19	Stn38	Stn57	Stn61	Stn79	Stn110	Stn122	Stn135	Stn174	Stn184
NL	0.24		0.26	0.81	0.00	0.00	0.00	0.62	0.24	0.72	0.00	0.00	0.00
RC	0.74		0.65	1.13	1.53	0.00	1.40	0.00	1.03	0.83	0.00	0.46	1.12
CR	1.08		0.47	3.72	0.00	0.59	1.77	0.00	0.84	0.37	0.01	1.53	2.56
TR	0.97		1.46	2.71	0.00	0.24	1.79	0.00	0.77	1.18	0.17	0.42	1.91

Table 5. Comparision of observed levels of heterozygosity (H_o) and average number of alleles per locus (N_a) among fishes using microsatellite loci.

Species	Common name	H_o	N_a	Reference
Gasterosteus aculeatus	threespine stickleback (Nueltin Lake)	0.00-0.77	3.2	This study
Gasterosteus aculeatus	threespine stickleback (Caribou/Thlewiaza)	0.17-0.82	9	This study
Gasterosteus aculeatus	threespine stickleback	0.00-0.80	6.8	Hendry *et al*., 2002
Gasterosteus aculeatus	threespine stickleback	0.35-0.96	29.5	Makinen *et al*., 2006
Cottus gobio	European bullhead	0.43	4.19	Hänfling *et al*., 2002
Esox lucius	northern pike	0.33	8.6	Jacobsen *et al*., 2005
Gadus morhua	atlantic cod	0.70	13.9	O'Leary *et al*., 2007
Sander vitreus	walleye	0.69	13.9	Strange and Stepien, 2007
Spirinchus thaleichthys	longfin smelt	0.27-0.93	10.05	Israel and May, 2010
Thymallus thymallus	European grayling	0.39	3.5	Gum *et al*., 2005

Table 6: F_{ST} values calculated using 11 microsatellite loci from *Gasterosteus aculeatus* populations in Nueltin Lake, MB, Thlewiaza River, NU, Rupert Creek, MB and Caribou River, MB. All values are statistically significant ($p \leq 0.001$).

LOCATION	Nueltin Lake	Rupert Creek	Caribou River
Rupert Creek	0.4756	-	-
Caribou River	0.3834	0.1672	-
Thlewiaza River	0.2854	0.1749	0.0798

Table 7. Comparison of observed F_{ST} values of fishes from comparable postglacial habitats or with similar anadromous life history characteristics to *Gasterosteus aculeatus*.

Species	Common name	Location	F_{ST}	Reference
Gasterosteus aculeatus	threespine stickleback	Nueltin Lake-Western Hudson Bay	0.29-0.48	This study
Gasterosteus aculeatus	threespine stickleback	Caribou/Thlewiaza rivers /Rupert Creek	0.08-0.18	This study
Gasterosteus aculeatus	threespine stickleback	Misty Lake-upper Misty Inlet, BC	0.29-0.35	Hendry *et al*., 2002
Gasterosteus aculeatus	threespine stickleback	Northwestern Europe (Finland)	0.17-0.37	Makinen *et al*., 2006
Gasterosteus aculeatus	threespine stickleback	Belgium, the Netherlands	0.03-0.12	Raeymaekers *et al*., 2005
Gasterosteus aculeatus	threespine stickleback	Scotland	0.00-0.78	Malhi *et al*., 2006
Gasterosteus aculeatus	threespine stickleback	Hraunsfijord, Iceland	0.01-0.14	Ólafsdóttir *et al*., 2007
Gasterosteus aculeatus	threespine stickleback	Benthic-Limnetic pair: Emily	0.34	Taylor and McPhail, 2000
Gasterosteus aculeatus	threespine stickleback	Benthic-Limnetic pair: Enos	0.21	Taylor and McPhail, 2000
Gasterosteus aculeatus	threespine stickleback	Benthic-Limnetic pair: Paxton	0.21	Taylor and McPhail, 2000
Gasterosteus aculeatus	threespine stickleback	Benthic-Limnetic pair: Priest	0.21	Taylor and McPhail, 2000
Gasterosteus aculeatus	threespine stickleback	marine	0.05	Taylor and McPhail, 2000
Pungitius pungitius	ninespine stickleback	Northern Sweden, Baltic coast	0.00-0.79	Mobley *et al*., 2011
Micropterus dolomieu	smallmouth bass	Lake Erie	0.02-0.18	Stepien *et al*., 2007
Oncorhynchus keta	chum salmon	Between central and north coast, BC	0.01	Beacham *et al*., 2008
Oncorhynchus keta	chum salmon	Between Fraser and Taku rivers	0.04	Beacham *et al*., 2008
Oncorhynchus tshawytscha	chinook salmon	Vancouver Island, BC	0.00-0.05	Heath *et al*., 2006
Oncorhynchus tshawytscha	chinook salmon	Fraser River, BC	0.01-0.13	Heath *et al*., 2006
Sander vitreus	walleye	Lake Erie: between east-west basins	0.01-0.06	Strange and Stepien, 2007

Figure 8. Map of western Hudson Bay and Thlewiaza River watershed. Arrows indicate elevational gradients along the Thlewiaza River, often associated with impassable waterfalls. Yellow indicators represent comparative populations of *Gasterosteus aculeatus* for this study. Image credit R. Mooi

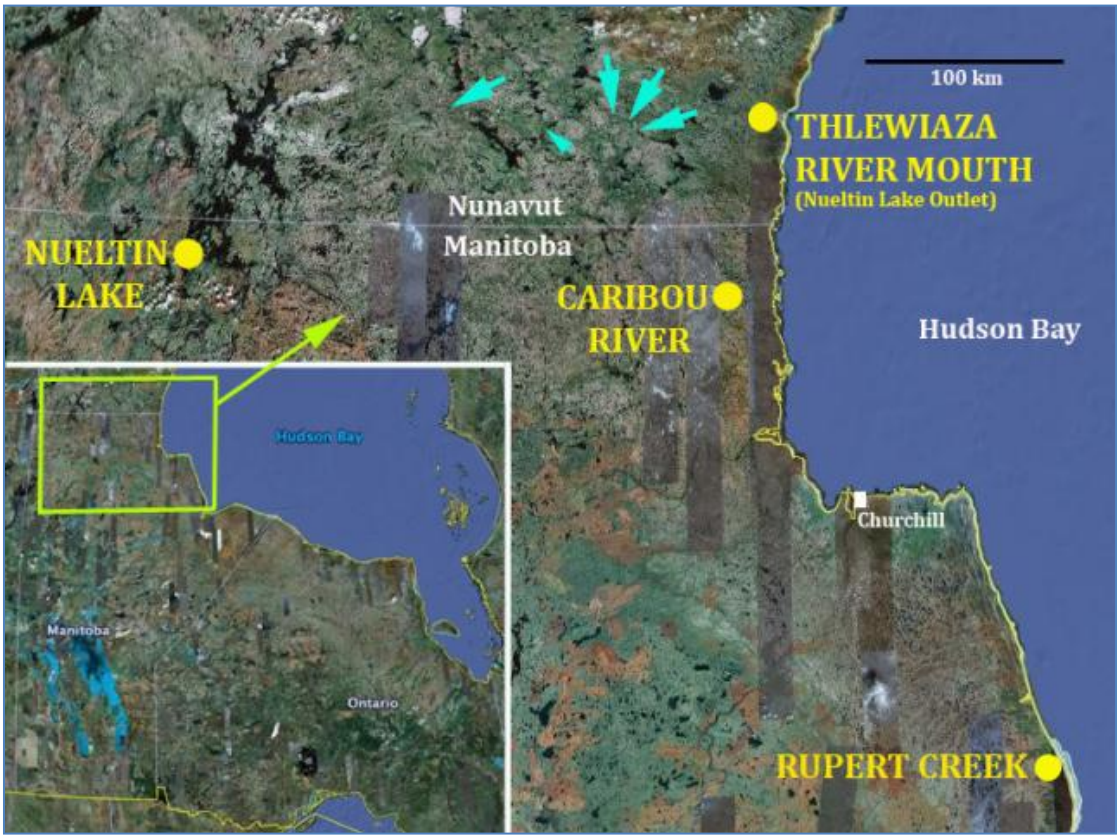


Figure 9. Allelic diversity of *Gasterosteus aculeatus* using eleven microsatellite loci, comparison by population. a) alleles as reported, b) with rarefaction for 15 alleles, c) with rarefaction, private alleles observed.

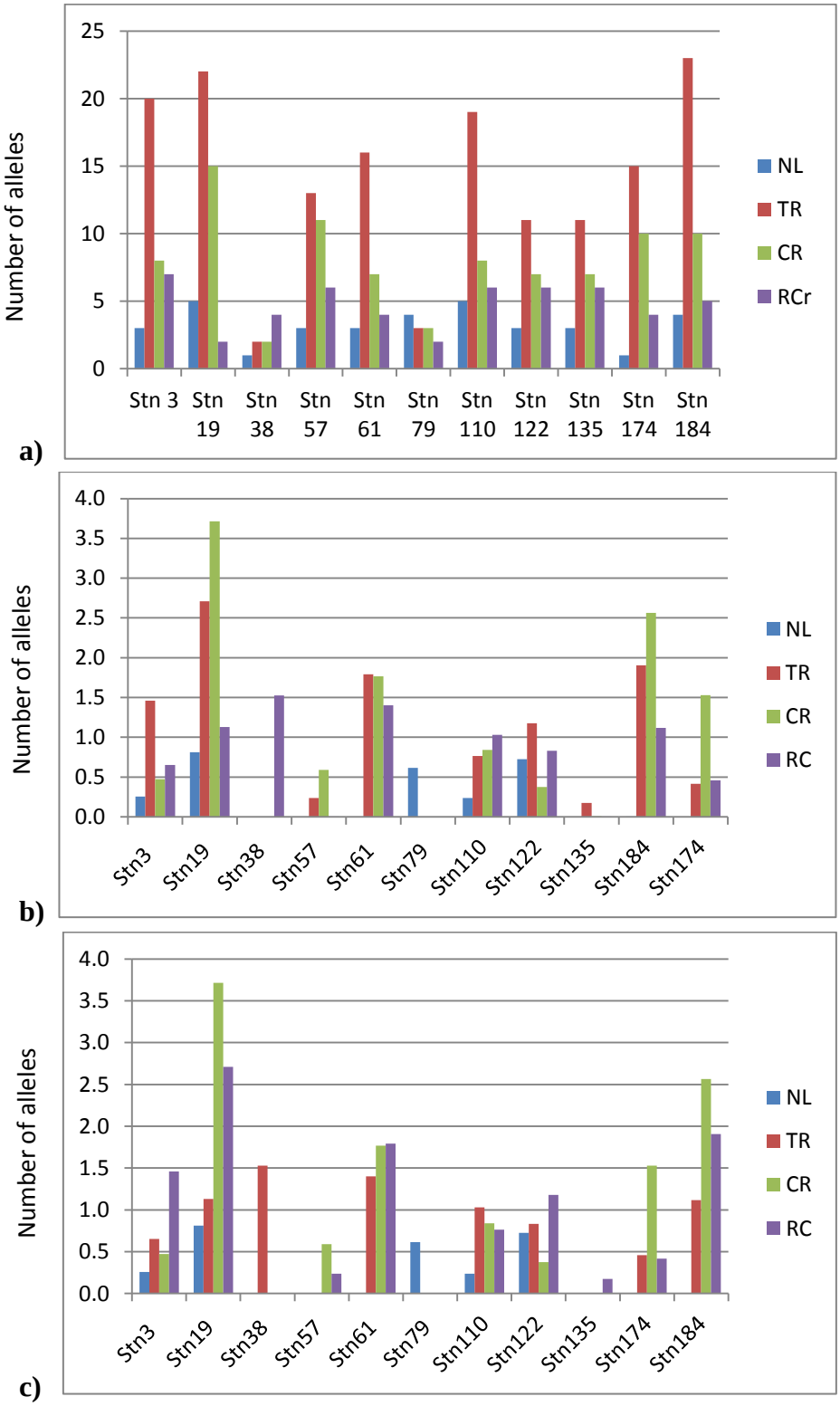
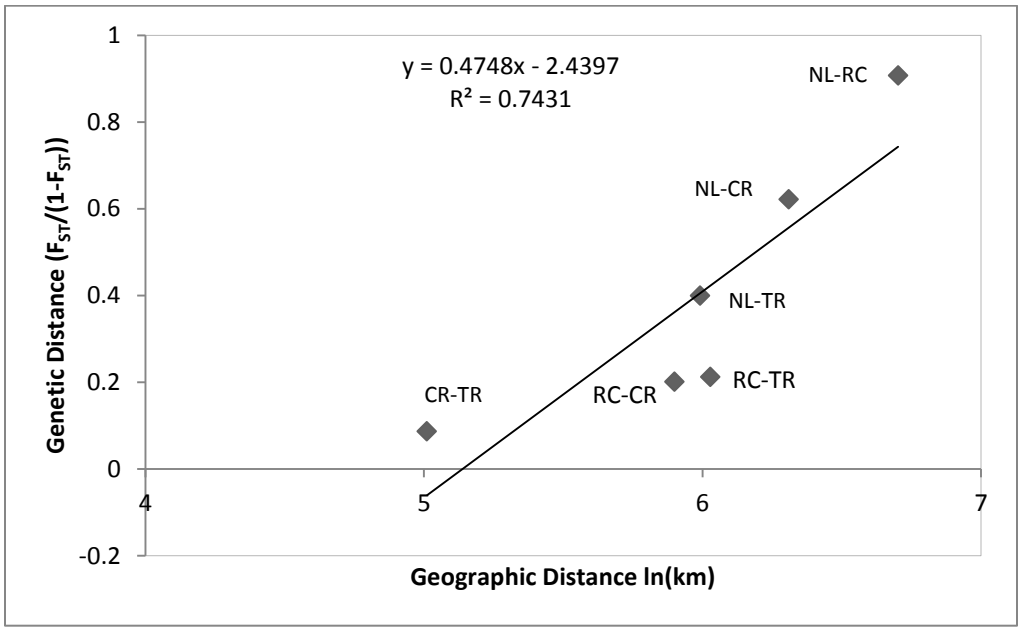


Figure 10. Correlation of pairwise measures of genetic differentiation ($F_{ST}/(1-F_{ST})$) and pairwise geographic distances (ln(km)) (Rousset, 1997). Distances (by waterway) were estimated between sampling locations based on GPS coordinates and Google Earth distance tool. NL: Nueltin Lake, CR: Caribou River, TR: Thlewiaza River, RC: Rupert Creek.



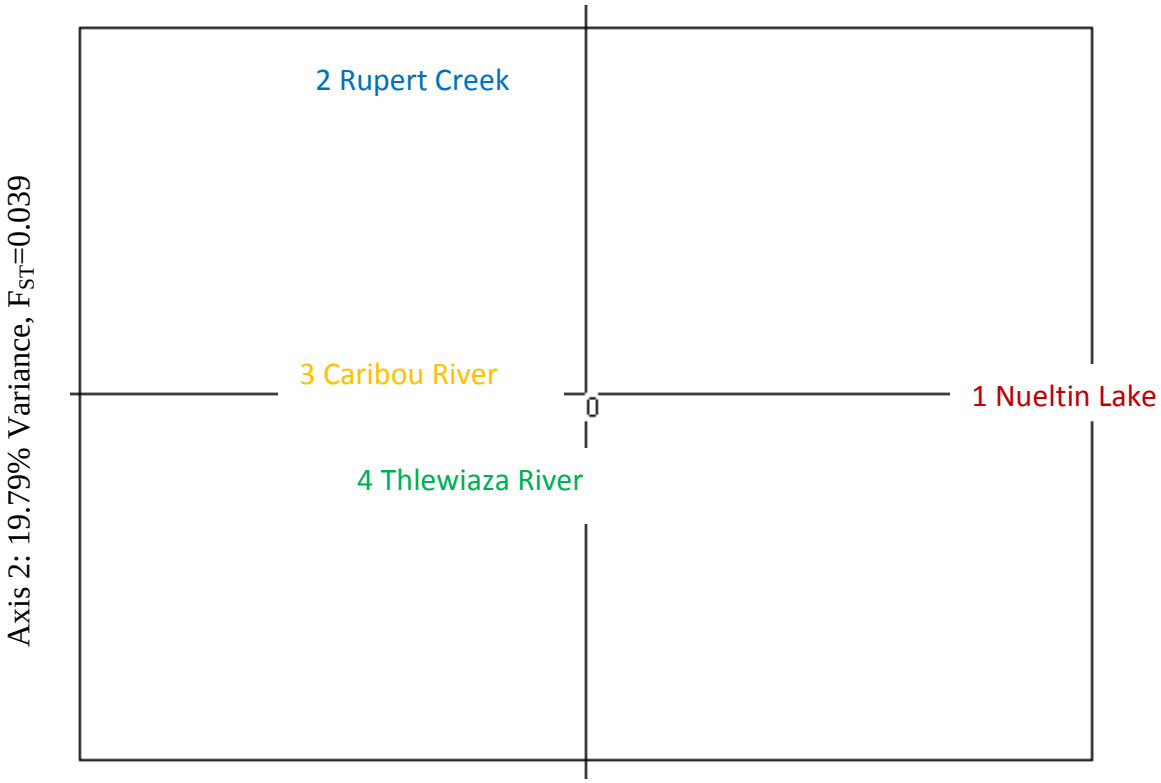


Figure 11.Principal components analysis of 11 microsatellite loci from Nueltin Lake, Rupert Creek, Caribou River and Thlewiaza River. The first axis (horizontal) explains 69.05% of the variance detected among *G. aculeatus* from Nueltin Lake and coastal Western Hudson Bay; whereas the second axis explains 19.79% of the variance.

CHAPTER 3: MORPHOLOGICAL DIVERSITY OF *GASTEROSTEUS ACULEATUS* OBSERVED IN NUELTIN LAKE, MANITOBA AND POPULATIONS FROM THE WESTERN COAST OF HUDSON BAY

ABSTRACT

Gasterosteus aculeatus is well-known for a tremendous array of morphological variation between, and within, populations that are thought to reflect divergent selective pressures. The ancestral "marine" morphotype exhibits high levels of defensive body armor, including a full set of overlapping bony plates, prominent locking dorsal and pelvic spines, and a pelvic complex which is comprised of a ventral plate and ascending process up the abdominal wall. However, following the colonization of freshwater environments, a shift in phenotype usually occurs and is characterized by decreased numbers of lateral plates, reduced lengths and robustness of spines and reduction of the pelvic complex. Despite being isolated in a freshwater environment, *G. aculeatus* from Nueltin Lake exhibits greater expression of body armor (longer dorsal and pelvic spines, robust pelvic complex) than do populations in closer proximity to the coastal environment. Fish in Nueltin Lake also exhibit a fully plated body form (mean plate number 19.36). It is suggested that the presence of several species of large piscivorous fish in Nueltin Lake may select for the retention of prominent spine lengths and a full series of lateral plates.

Fish from an equivalent estuarine-riverine system near Hudson Bay/James Bay in the Attawapiskat River system exhibit prominent dorsal and pelvic spines, but express the phenotype for a low-plate morph (mean plate number 6.8). This suggests that spine lengths, pelvic structures and lateral plates are expressed independently as would be expected given the known genetic basis of traits (role of *Eda, Pitx1* in phenotype, this study, Chapter 1). Populations nearer to the coast (Caribou River) exhibit reduced

defensive structures and an intermediate number of lateral plates (mean 13.4). Population differentiation based on structural morphology was not obvious using either of principal components analysis or non-metric multidimensional scaling, as significant overlap exists between phenotypes expressed in each population. Lateral plate number provided the most definitive distinction between populations, although it was not correlated with habitat type (coastal vs. inland). No difference in gill raker numbers between populations was observed in this study which suggests a lack of dietary variation depending on habitat type.

INTRODUCTION

The vast library of knowledge that exists on natural history and the widespread use and accessibility of genomic resources have identified *Gasterosteus aculeatus* as a "super model system" to examine questions of adaptive evolution and provides an opportunity for the synthesis of many disparate fields of biology and new insight into fundamental questions of evolutionary significance (Gibson, 2005). Combining more recent methods of genetic research with a traditional approach of morphological analysis can provide both complementary and opposing conclusions in taxonomic studies. For many species, adaptations to a wide range of habitats and ecologically variable niches have resulted in divergent morphologies which have been shown to correlate strongly with habitat specialization (Webster *et al*., 2011). Subtle variation in traits can maximize fitness levels by increasing foraging success, growth rate and body condition. However, adaptive diversification can introduce survival trade-offs for species, where localized specializations are costly in novel landscapes (Webster *et al*., 2011). In *G. aculeatus*, freshwater populations often exhibit parallel divergence in armor morphology from marine ancestors, most frequently observed as a reduction in the bony armor plates and spines which characterize this species. Multiple, independent colonizations of coastal, freshwater environments by marine ancestors is well supported by genetic information, notably sequence variation in mitochondrial DNA and microsatellite loci (Ortí *et al*., 1994; Thompson *et al*., 1997; Taylor and McPhail 1999, 2000; McKinnon *et al*., 2004; Colosimo *et al*., 2005; this study, Chapter 2).

The maintenance of genetic variation within populations, despite the influences of directional and stabilizing selection that often act to reduce variation within populations,

is of considerable interest to biologists and conservationists (Fisher, 1930; Kingsolver *et al*., 2001). In many situations, high levels of variation are observed to exist in populations despite significant evolutionary forces acting to homogenize intrapopulation diversity (Mitchell-Olds *et al*., 2007). Understanding the mechanisms which act to maintain variation in ecologically significant traits is essential to a comprehensive understanding of how populations persist, what factors influence biodiversity, and how populations may respond to rapid environmental change. Variation within a population may be maintained by balancing selection, high levels of mutation, or gene flow from adjacent populations (Mitchell-Olds *et al*., 2007).

Hypotheses for variation among defensive structures

Although the molecular mechanisms that contribute to variation in lateral plate expression in *G. aculeatus* (*Eda* gene, Colosimo *et al*., 2005; *Pitx 1* gene, Chan *et al*., 2010) are becoming better understood, the ecological mechanisms driving armor divergence remain unresolved (Raeymaekers *et al*., 2005; Colosimo *et al*., 2005; Cano *et al*., 2006; Marchinko and Schluter, 2007). Identifying the causative factors for variation in phenotypic expression has been confounded by multiple, interactive, ecological factors contributing to variation among populations (Bell, 2001). Although similar ecological conditions may have selected for reduction of armor in freshwater *G. aculeatus*, researchers have had little success explaining the mechanism(s) of armor divergence (Bertin, 1925; Bell, 2001). To explain the variation observed in *G. aculeatus* following the colonization of new lakes and streams by completely plated marine ancestors, several hypotheses have been proposed to explain the selective advantage of reduction in

defensive structures, including low calcium levels, increased body flexibility and maneuverability, thermal or latitudinal profiles, changes in swimming performance, and changes in predation regimes associated with a freshwater environment (Colosimo *et al*., 2004).

Predation pressures

Divergent selection is thought to promote variation in phenotypic expression of defensive structures in response to the risk of mortality associated with variation in predatory species found in marine and freshwater environments (Reimchen, 1980; Reimchen, 1994; Webster *et al*., 2011). This is directly correlated to the predator array in a specific environment and the trade-offs in armor expression and energetic costs. The defensive structures as a complex appear to interact and amplify the strength and effectiveness of the armor to withstand predation by piscivorous fishes and birds (Reimchen, 1983; 2000).

In general, *G. aculeatus* from marine and lake populations with large predaceous fish and bird population exhibited a trend towards longer dorsal and pelvic spines (Reimchen, 1994). In the presence of this predatory cohort, lake populations often exhibit fully plated morphs, and nearly all marine populations consist of the complete plate morph only (although polymorphic populations have been documented in both freshwater and marine environments). Trout more successfully preyed upon *G. aculeatus* with reduced pelvic structures which supports the hypothesis that high levels of piscivorous predation favor individuals with more robust defensive structures, and that spines likely facilitate post-capture defense and escape (Lescak and von Hippel, 2011). Strong selection for increased spine length is expected in marine populations as a result

of high predation pressures from piscivorous fish (Hagen and Gilbertson, 1972; Moodie and Reimchen, 1976; Ólafsdóttir *et al*., 2007b).

In comparison, *G. aculeatus* in lakes with fewer piscivorous fish and bird species often have short spines and few lateral plates (Reimchen, 1980; Bell *et al*., 1993). Predation risk by invertebrates, most often odonate naiads (Ólafsdóttir *et al*., 2007c), can contribute to selection for spine reduction and decreased numbers of lateral plates as well (Reimchen, 1980; Reist, 1980). Support for this hypothesis indicates that individuals with longer dorsal and pelvic spines experienced reduced mortality than did conspecifics with shorter spines when preyed upon by gape limited fish and birds (Reimchen, 1988). Additionally, lateral plates increase survivorship following predation by toothed predators. In structurally complex habitats, longer spines may inhibit maneuverability, and a reduction in spine length has been observed in populations from Iceland with lava formations as substrate (Kristjansson *et al*., 2002[r30]).

In freshwater habitats, insectivorous predation is often significant for juvenile and smaller-bodied fishes. Longer spines are not thought to offer any advantage; rather, these are hypothesized to increase mortality as they provide larger physical structures for insects to grasp while predating upon juvenile *G. aculeatus* (Reimchen, 1980). It has been shown that individuals with more lateral plates grew at a slower rate in freshwater than did conspecifics with fewer plates (Marchinko, 2009). Slower growth rates by juvenile *G. aculeatus* may increase predation pressures as predaceous insects have been shown to feed preferentially on the smallest individuals in a population (Foster *et al*., 1988). Accordingly, when confronted with higher rates of insectivorous predation, fish

that display the low lateral plate morph may experience decreased rates of mortality relative to those with higher plate numbers.

Ionic/water chemistry

Differences in aquatic chemistries between freshwater and marine environments, specifically variation in ion concentrations such as salinityand calcium, have been suggested as an alternate mechanism promoting the divergence of phenotypic expression of defensive structures in *G. aculeatus* (Heuts, 1947; Giles, 1983; Campbell, 1985; Bell *et al*., 1993). Under divergent forces of natural selection, variation suggests a role in fitness advantages between freshwater and marine environments. Essentially, freshwater populations that exhibit fewer lateral plates will experience higher fitness levels than do individuals with the complete lateral plate morph (Schluter, 2000). Although both salinity and calcium levels are hypothesized to influence plate expression, the mechanism which drives the phenotypic divergence differs between them.

An overall selective advantage of low plate morphs in freshwater may be attributed to variation in salinity tolerance correlated to the ecological mechanism of selection on lateral plate morphology. It has been determined that *Eda* is closely linked to three other genes including one possibly associated with salt secretion, *Gjb1* (Colosimo *et al*., 2005). If the ability to effectively osmoregulate and maintain ionic homeostasis at varying salinity concentrations is associated with lateral plate number, then divergent selection may favor the complete morph in marine environments, and the low plate morph in freshwater systems. However, the occurrence of polymorphic populations in both freshwater and marine systems does not support a strong association

between lateral plate number and water salinity (Hagen and Gilbertson, 1972; Baumgartner and Bell, 1984). As a result, no resolution exists on whether variation in salinity concentrations between marine and freshwater habitats results in selection on lateral plate number (Bell, 2001).

In most freshwater environments, aquatic calcium levels are reduced compared to those from the marine environment and studies have found a correlation between reduction in plate and spine number and pelvic girdle dimensions in a series of lakes along a gradient of calcium concentrations (Giles, 1983; Bell *et al*., 1993; Marchinko, 2009). Due to energetic costs associated with developing calcium carbonate rich skeletal components for defensive structures, selection should favor a reduction in armor development in environments with lower calcium levels (Giles, 1983). Under this hypothesis of calcium limitation, the occurrence of the low plate morph in freshwater with lower levels of dissolved low calcium would provide an advantage at lower calcium concentrations, but the inverse may not be true; that there may not be a selective disadvantage to lower plate morphs at higher calcium levels (Heuts, 1944; Francis *et al*., 1986; Bell *et al*., 1993; Bourgeois *et al*., 1994). However, no experimental work has directly investigated calcium limitation as a mechanism to promote a reduction in armor among populations of *G. aculeatus*. It may be difficult to elucidate the respective effects of low environmental calcium from variation among other ions in the aquatic environment, such as phosphate, when considering the influence on phenotypic variationand armor reduction (Bell *et al*., 1993).

However, it is important to recognize that competing hypotheses for phenotypic expression may not be mutually exclusive. To resolve the predator/ion hypothesis, it

must be recognized that the equilibrium between calcium carbonate and bicarbonate in freshwater systems often determines aquatic pH levels (Cole, 1994) which is directly correlated to fish community composition, as many salmonids do not tolerate water of low pH values (Bell *et al*., 1993).

Other hypotheses

Other hypotheses for variation in phenotypic expression include geographic and habitat attributes (populations from estuarine environments were polymorphic, with an increase in the frequency of complete morphs observed at increasing distance inland in lower salinity, high gradient stream habitats (Baumgartner and Bell, 1984)), a correlation between temperature and plate morph (increasing plate numbers in areas with lower winter temperatures and large annual fluctuations, (Hagen and Moodie, 1982)), and compensation for a reduction in water density accompanying a shift to freshwater (Mhyre and Klepaker, 2009). Despite many possible hypotheses, a widely accepted resolution to questions surrounding the mechanisms influencing phenotypic variation between freshwater and marine populations of *G. aculeatus* remains elusive for biologists. This study is not intended to provide such resolution, but rather, provide additional information to promote further understanding.

Gasterosteus aculeatus *in Nueltin Lake*

It has been determined that the population of *G. aculeatus* in Nueltin Lake represents an isolated, non-anadromous population that is genetically isolated from conspecifics in the same watershed (Thlewiaza River) and adjacent watersheds (Caribou River) (this study,

Chapter 2). The lack of gene flow between these populations provides the opportunity for localized adaptations ormutations to accumulate in Nueltin Lake and presents a unique situation for examination of divergent selection among closely related populations with shared evolutionary history. Phenotypic expression of selectively advantageous traits must be considered to impart a fitness advantage in the novel habitat and is expected to vary according to the biotic and abiotic factors that influence the population; reduced levels of genetic variability observed in Nueltin Lake *G. aculeatus* may influence the fitness of the population and promote additional difficulties for successful adaptation.

STUDY GOALS AND HYPOTHESES

This study will examine variation in morphological traits between inland populations of *G. aculeatus*, from both Nueltin Lake and historical collections from other inland populations, and compare those findings with populations from the western coast of Hudson Bay. The goal of this study is to investigate whether morphological differences exist between inland and coastal environments and compare the variation of phenotypic expression with known or expected patterns of variation. Additionally, the correlation and covariance of these traits will be examined within the context of habitat type to assess whether patterns of variation exist in the Nueltin Lake and coastal Hudson Bay populations. According to a number of hypotheses and widespread occurrences of parallel diversification, we would expect a reduction in defensive structures among fish from the inland population in Nueltin Lake and modifications of trophic structures to reflect a shift to a freshwater environment and variation in dietary preferences[r31].

MATERIALS AND METHODS

Samples were collected as indicated in Chapter 1, this study. Collection records are listed in Appendix B, C. Fish were collected from Rupert Creek (57.538889° N 92.556667° W) for inclusion in the genetic component of this thesis (Chapter 2), but sampled fish were juveniles of less than 20mm length. As a result of physical alteration due to tissue removal for genetic sampling, inclusion of these fish was prevented due to an inability to measure standard length.

Museum collections

Museum specimens from a river-costal system located further south in, and adjacent to, Hudson Bay were obtained on loan from the Royal Ontario Museum in Toronto, Ontario, (Figure 12, Appendix B). The location selected represents a similar dispersal opportunity inland along the Attawapiskat River from Hudson Bay following deglaciation at the end of the Wisconsinan period and for the purposes of this study, was considered an equivalent, or replicate, study environment. It was hypothesized that due to similar biotic and abiotic conditions, this river system would function as an experimental replicate for the Nueltin Lake population[r32] and the potential might exist to establish broad patterns of parallel variation. These museum collections were included in the subsequent study of morphological variation; however, gill raker numbers were not counted due to the potentially damaging effects of reflecting the operculum on fragile and historical specimens. Additional collections were sought; however, due to existing damage and desiccation, such collections were not suitable for inclusion in this study. More

specimens are available upon request from these institutions and may be included in future studies[r33].

Measurement techniques[r34]

Eleven morphological measurements were made per individual from each population using Fowler dial calipers (±0.01mm) and, where necessary, an occular micrometer. All measurements were made to the nearest 0.01 mm and rounded to the nearest 0.1 mm. For accuracy, all bilateral measurements and counts were made on both sides of the body to assess asymmetry; however, by convention, only measurements from the left side were used for analysis (Hubbs and Lagler, 1964). To further enhance accuracy, all measurements were repeated until the same, or a reasonably similar number, was obtained twice (Haas and McPhail, 1991). All measurements follow the protocol of Hubbs and Lagler [r35](2004). These measurements are presented in Table 8[r36]. Visualization of these measurements is provided in Figure 13. Lateral plates were scored according to Ziuganov, 1983. [r37]Fixation in ethanol produced a slight dehydration of the skin of the fish which enabled easy visualization of the lateral plates, especially when the fish were allowed to dry for one minute prior to examination. As a result, no staining of specimens was required for lateral plate counts. Meristic counts of lateral plate [r38]and gill raker numbers were also collected, except where noted above. As lateral plate development is incomplete prior to attaining a standard length of 34 mm (Coad and Power 1974; Hagen and Moodie 1982), following measurement of all individuals, a number of samples were rejected from analysis due to incomplete lateral plate development. Notably, only four of the possible 16 specimens were included from the

Caribou River population. For purposes of comparison, lateral plates were compared across Nueltin Lake, Thlewiaza River, Caribou River, Cochrane District[r39], and a pooled Kenora District [r40](Kenora District populations, Appendix B). The Kenora District was identified as an additional inland population to Nueltin Lake (Figure 12), whereas the other populations are initially identified as coastal representatives.

Genetic sex identification

Sex of each fish was determined by amplifying a portion of three prime untranslated region (3'UTR) of the isocitrate dehydrogenase (IDH) gene (Peichel *et al*., 2004). DNA, extracted from pectoral fin samples using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol, as specified in Chapter 2 of this study, was used for analysis. All amplifications were performed in 10 µl reaction volume containing 10x PCR manufacturer's buffer (20 mM Tris-HCl pH 8.4; 50 mM KCl), 1.5 mM $MgCl_2$, 0.2mM dNTP, 0.25 U Taq DNA polymerase (Invitrogen), 2 to 8 pmol of each primer (IDH exon II 37F and IDH exon II290R) and approximately 20 ng template DNA (Peichel *et al*., 2004). Reactions were initially denatured at 94ºC for 3 minutes, followed by 38 cycles of 94ºC for 30 seconds, 56ºC for 30 seconds, and 72ºC for 1 minute. A final extension was run for 5 minutes at 72 ºC. PCR products were visualized on a 1.5% agarose gel electrophoresis with 1X loading dye in TBE solution. Size fragment analysis was determined against a known size standard. Male fish exhibited two fragments (~280 and 300 bp) whereas females exhibited only one (~300 bp). Each PCR plate contained a positive male control as well as a negative control (ddH_20 rather than template DNA).

Sex ratio of *G. aculeatus* within a population has been suggested to vary according to lateral plate expression. In completely plated morph populations, the expected sex ratio ranges from 0.206 to 0.251 males, and among partially plated populations, is expected to range from 0.211 to 0.216 (Wootten, 1984). In the Nueltin Lake population, the ratio of males: females was 0.265 (9 male, 25 female), in the Thlewiaza River was 0.326 (14 male, 29 female), and in the Caribou River population was 0.313 (5 male, 11 female). The ratio varies with age and may be slightly skewed based on the ages of fish sampled (Caribou River fish had more juvenile fish than did other populations). The sex ratio thought to be influenced by sex-specific predation pressures as juveniles. Male fish tend to occupy a benthic environment throughout their lifespan and will experience higher levels of insectivorous predation as a result (Lescak and von Hippel, 2011). Insectivorous predation has been shown to favor reduced spine lengths and increased numbers of lateral plates due to abdominal puncture risk. Lateral plate development continues until approximately 34 mm SL (Igarashi, 1970; Coad and Power, 1974; Bell, 1981; Hagen and Moodie, 1982; Bell and Baumgartner, 1984) and therefore, reduced plate numbers in juvenile male fish who occupy a benthic environment and may experience more intense predation stresses may influence sex ratios beginning in early lifestages which may be attributed to varying sex ratios with age class.Alternatively, female fish tend to occupy a pelagic environment and experience higher levels of avian or piscivorous predation. Reduced lateral plate numbers in early lifestages would not confer a survival advantage in a pelagic environment in a similar manner to those fish in a benthic habitat.

Despite successful amplification and positive confirmation of sex in each individual, due to limitations in the collection phase of this project, visual confirmation of genetic identificationwere not consistentand subsequent analysis of sexual dimorphism in morphological traits was not possible. Sexes were not able to be determined reliably by visual inspection, so although the sex ratios of the populations were known, whole fish examination did not result in congruent ratios. Accordingly, data for all traits was pooled, irrespective of sex, to increase sample size and to enable inclusion of the museum collection specimens that were not able to be included in genetic analysis. Expected trends in sexual dimorphism may confound patterns of variation by underestimating differences between populations.

Data analysis

Morphometric characteristics were individually adjusted for body size both by division by, and regression against, standard length (Reist, 1985). Neither of the meristic variables (lateral plate number and gill rakers) were correlated with body size (the role of ontogeny in lateral plate development was accounted for, described in Methods section, this chapter) and were directly compared (Reist, 1985). To account for allometry, two ratio methods were employed. The first method involved dividing each character for each individual by that individual's standard length (Reist, 1985; Shea, 1985). The second method involved linear regression against standard length for each variable to identify the slope of the relationship (Hendry *et al*., 2002). Variables were transformed based on the slope for each trait. This is an equivalent method to $\log_{10}$ transformation (Reist, 1985; Shea, 1985). To confirm equivalency of these methods, when plotted

against each other, a perfect linear relationship exists (r^2=1.0). Standardized data using the above mentioned methods was used in all subsequent analysis and interpretation.

Descriptive statistics were calculated for all traits across all populations as well as by individual population. One-way ANOVA's were applied to each set of standardized variables by population to assess levels of variation among populations using XLSTAT (α=0.05). Assumptions of ANOVA include the independence of cases, normality of data, and homogeneity of variances (homoscedasticity). All traits were tested for normality using Shapiro-Wilk, Anderson-Darling, Lilliefors, and Jarque-Bera tests (α=0.05) in XLSTAT. Comparison of variances were tested with Levene's test for significance (α=0.05) in XLSTAT. Structural and meristic traits were analyzed individually and subjected to identical analyses.

Post-hoc Tukey's (HSD) tests were used to examine the significance of all pairwise variation for each population for each trait. Following the identification of covariate trends, additional Tukey's tests were applied to the representative trait for the group for each population to assess relative weight of each population to the overall trend.

Principal components analysis (PCA) is a method of linear transformation in which the total variance of the variables is repartitioned along orthogonal ordination axes and does not distinguish between dependent and independent variables (Kenkel, 2006). It is used to examine variation of traits among populations and within general groupings of coastal (Thlewiaza River and Caribou River, Cochrane District) or inland (Nueltin Lake and Kenora District) fish. PCA does not reduce dimensionality of the data but instead re-expresses linear variation along derived axes which are weighted by the respective

variables (Kenkel, 2006). Eigenvalues were used to identify the number of components to adequately describe variation in structural characteristics. Eigenvalues greater than 1 were included as relevant to the analysis. PC1 is the axis which maximizes the variance of points projected perpendicularly to it. PC2 is constrained as to be orthogonal to PC1; however, the variance of points projected onto it is again maximized. Additional component axes are included, added in the same method, until a satisfactory level of variance has been explained by the ordination and provides a reasonable representation of the underlying linear trends of coordinated variable response (Kenkel, 2006). Biplots were also used to examine the correlation of structural and meristic traits among populations of *G. aculeatus*. A correlation matrix was created to identify structural characteristics that were highly associated. The top 25% of correlations were identified (14/55 comparisons).

Nonmetric multidimensional scaling was used to represent pairwise variation Euclidean space in such a way that distance measures in ordination space reflect true rank to order distance measures among individuals in populations (Shepard, 1962; Kruskal, 1964; Kenkel, 2006). The program PRIMER (Plymouth Routines in Multivariate Ecological Research, Clarke and Warwick, 1994) was used to generate Figure 14. NMDS has advantages over PCA for representation of ordination space amongst groupings due to its flexibility and lack of assumptions, although many methods would be applicable to this study (Clarke and Warwick, 1994; McKune and Grace, 2002). NMDS was applied in addition to PCA to further explore the relationship amongst traits and attempt to discriminate between populations and provide resolution not identified using PCA.

RESULTS

All morphological traits examined on *G. aculeatus* in this study exhibited a normal distribution as tested above based on standardized data.

Fish from Nueltin Lake exhibited a full complement of defensive structures that included prominent dorsal spines, full pelvic structures, a bony keel, and were completely plated (mean 19.36) (Figure 14, Appendix E). Despite having flanks which were fully covered in lateral plates, Nueltin Lake fish did not attain the threshold value (>30) [r41]to represent a "complete morph" in accordance with the literature (Ziuganov, 1983) although space was not available along the abdomen for the development of additional plates. Questions of plate fusion or proportional coverage may be explored in future studies as a defining characteristic rather than solely based on plate counts.Nueltin Lake was expected to reflect a typical "freshwater" morphology,characterized as having reduced spine lengths and loss of lateral plates; however, the inverse was observed among all individuals collected.

Gasterosteus aculeatus from the Thlewiaza River also exhibited high levels of defensive structures as would be expected among fish collected in a coastal environment. However, mean values of dorsal spine lengths, pelvic complex, pelvic spine lengths and head morphology were all greater among fish from Nueltin Lake than those in the Thlewiaza River (Appendix E). No statistical difference was observed in mean lateral plate numbers between Nueltin Lake and Thlewiaza River fish; however, was significant in comparison with all other populations (Table 9).

Fish in the Caribou River population demonstrated the lowest mean values among all structural traits across all populations (Appendix E). Despite being adjacent to the coast of Hudson Bay, fish from Caribou River exhibited the lowest expression of spine lengths and represented a phenotype more usually associated with a strictly freshwater environment (as was expected among fish from Nueltin Lake). Caribou River fish also demonstrated significantly fewer lateral plates than fish from Nueltin Lake/Thlewiaza River (mean13.4 vs 19.36).

Fish collected from the estuarine region of the Cochrane District had the greatest mean values for all structural traits across all populations (Appendix E). Cochrane District fish exhibited prominent spines with a full pelvic complex which appeared much "thicker" or more robust than any bony elements seen among any other populations (pers. obs). However, despite this expression of conspicuous body armor, a significant reduction in lateral plate number was observed (mean 6.9) and this population was classified as a low plate morph despite being an estuarine population (located within a few kilometers from Hudson Bay and representing the most "marine" collection locality of any populations included in this study).

Kenora District fish were pooled from three populations, all collected over a small geographic distance (Appendix B). Hawley Lake and Spruce Lake are located within 20 km of eachother, both located within the Kenora District. This pooled population was not significantly different from the Caribou River population in any structural traits, but did differ in lateral plate number (Figure 24, Appendix E). As with the Cochrane District population, Kenora District fish exhibited low numbers of lateral plates (mean 6.8).

ANOVA analysis indicates that significant levels of differentiation exist among all traits across all populations with the exception of gill raker numbers which were not significantly different in any populations (Appendix E, F). Mean values of approximately 18.3 (18.09 to 18.55, Table 6, 7, 8) gill rakers were observed from Nueltin Lake, Caribou River and Thlewiaza River populations. Post-hoc Tukey's tests revealed that not all pairwise comparisons are significantly different, and the Caribou River and Cochrane population values frequently influenced the overall significance of the ANOVA (Table 9, Appendix F).

Results of principal components analysis did not provide clear distinction between populations (Figure 15). Four component axes were identified to account for 62.59% of the variation observed among populations (Table 11). The most visually obvious groupings were apparent when comparing PCA 3 and 4 (Figure 15) which were correlated with body depth (BD) and eye diameter (ED) (Factor plots, Figure 16). Additional variation is identified in PCA 1 and 4 which separates along the X axis and is correlated with spine lengths (first dorsal, second dorsal, pelvic spine lengths). Despite some visual distinction between groups, low levels of variation are explained by any one axis (9-28% per axis, Table 10) and therefore, the biological relevance of these results should be interpreted with caution.

Analysis of the correlation between morphological features identified a number of highly relevant linkages (Table 10). Several traits were shown to be correlated, most significantly were the defensive structures which included 1st and 2nddorsal spines, pelvic spines, pelvic complex, lateral plates (0.261-0.618).The highest correlation was observed between the second dorsal spine and pelvic spine lengths (0.681) which represents the

biologically relevant measurement for gape limited predation. Additional correlations were observed between the first dorsal spine and pelvic spine lengths (0.583) as well as the first and second dorsal spines (0.517). Some correlation among head morphology features were also identified, notably head length and jaw length (0.463), head length and eye diameter (0.269) as well as head length and snout length (0.262) (Table 10). Correlation was also observed between body depth and pelvic complex (0.275).

When variation among all traits across all populations was examined by nonmetric multidimensional scaling (NMDS), no definitive groupings were observed although more distinction between populations was apparent than was identified using PCA (Figure 17). Populations grouped together with loose association and straying between groups was observed across all populations, similar to the results observed in PCA (Figure 15). The Caribou River and Cochrane District (R1) populations grouped most tightly, yet all populations demonstrated a high degree of overlap. It is interesting to note that Nueltin Lake fish appear to cluster nearer to the estuarine population (Cochrane District, R1) in overall variation despite significant variation in lateral plate numbers (6.9 vs. 19.34, Appendix E). Kenora District populations appear to share slightly different overall variation, R3 clusters with Caribou River fish, whereas R2 and R4 represent an intermediate position. Despite shared proximity to the coastal environment of Hudson Bay, results of NMDS indicate that similar estuarine populations (Thlewiaza River and Cochrane District populations) do not group together (Figure 17).

DISCUSSION

Postglacial adaptive radiations of *G. aculeatus* provide the opportunity to explore the complex concepts of evolutionary diversification and selection (Bell *et al*., 2004). Exploring correlations between expressed phenotypic diversity and ecological factors influencing morphological differentiation enables increased understanding of some of the interesting questions in the study of evolutionary biology (Leinonen *et al*., 2006).

Gasterosteus aculeatus play a significant role in helping to uncover the interactive effects of genetics, adaptation and morphological plasticity attributed to repeated colonization of freshwater environments from marine populations and their subsequent diversification in the new habitat. Following the most recent deglaciation to affect northern North America and northern European regions, *G. aculeatus* likely dispersed through large regions of postglacial flooding and overland waterflow to colonize new habitats. Isostatic rebound is thought to have contributed to alteration of the hydrology of the region (Dyke and Prest, 1987) and likely contributed to the isolation of some fishes in a new environment. These recently isolated and replicated populations which occur throughout their global distribution provide multiple examples of independent adaptive divergence. In northern Manitoba and along the western coast of Hudson Bay, glacial retreats and dispersal opportunities enabled a population of *G. aculeatus* to become isolated in Nueltin Lake, Manitoba following deglaciation nearly 7.2 kya. This population of *G. aculeatus* represents the furthest inland report of the species from the province and provides an excellent opportunity to examine evolutionary processes in a contemporary time frame.

Patterns of morphological variation have been well documented throughout the range of *G. aculeatus* and, with a few exceptions, phenotypic expression follows a predictable reduction of defensive structures following the arrival into a freshwater environment. Various hypotheses have been advanced to explain this pattern of parallel divergence across a broad geographic range; however, exceptions have been observed and are of special interest to evolutionary biologists.

Phenotypic diversity was observed among all populations examined in this study, with varying levels of significance between populations (Table 8). Most significantly expressed however, was the lack of correlation between fish from predetermined "habitat types" and congruence with the expected phenotype. Despite selection of coastal/estuarine populations located within migratory range of Hudson Bay, analysis of the most geographically proximate populations to the marine environment (Caribou River Cochrane District, and Thlewiaza River) revealed a lack of a consistent pattern of morphology between them or with previously published studies (Bell and Foster, 1994; McKinnon and Rundle, 2002; Colosimo *et al*, 2005). Fish from the Cochrane District population, located in the estuarine zone of the Attawapiskat River at James Bay, exhibited prominent dorsal and pelvic spines, but were characterized as having few lateral plates, one of the most consistent attributes of marine/anadromous populations (Bell and Foster, 1994; Colosimo *et al*., 2005; Bell et al., 2010). Surprisingly, Caribou River fish did not share this phenotype, nor did they express morphology consistent with an expected marine morph. Rather than the prominent spines of the Cochrane District population, Caribou River fish appeared to be reducing their defensive armour against gape-limited predators. Spines were shorter and less robust than any other population

examined, although lateral plates were retained (although in lower numbers than in the Thlewiaza population). Fish from the Thlewiaza River, again located within migratory distance of the marine environment, expressed a third phenotype from the previous two "coastal/estuarine" populations. Thlewiaza River *G. aculeatus* exhibited high numbers of lateral plates, full dorsal and pelvic spines and did appear to reflect the expected marine morphotype.

In Nueltin Lake, a reduction in defensive structures would be expected following isolation in a freshwater lake, as has been documented in populations from western Canada and throughout its range (Schluter and McPhail, 1992; Bell and Foster, 1994; McPhail, 1994). However, this was not observed. Rather than a reduction in robustness and extent of body armor expression, Nueltin Lake fish have retained a completely intact defensive complex consisting of prominent dorsal and pelvic spines, a large pelvic girdle, a bony keel and a series of overlapping bony plates which covered the entire flank surface. In comparison, the Kenora District fish, selected to represent an additional inland population, demonstrated a more typical freshwater phenotype. Kenora District fish were characterized as having reduced spine lengths and pelvic structures and exhibited low plate numbers as would be expected from an inland population (Bell and Foster, 1994).

Many hypotheses exist to explain variation in defensive structures and lateral plates and have already been discussed in this study. Is it known that Nueltin Lake supports a recreational catch-and-release fishery for *T. arcticus* (arctic grayling), *E. lucius* (northern pike), and *S. namaycush* (lake trout) and so it may be suggested that the retention of prominent dorsal spines is a reflection of localized adaptation to increased

piscivorous and gape limited predation by larger-than-average lake species. However, no investigation of the relative abundance of these predatory fish was included in this study, and speculation is based on provincial angling records which indicate a high number of large fish in Nueltin Lake (Manitoba Water Stewardship, 2005). Also, no examination of effective predation by these piscivorous fish was conducted in this study to quantify actual predation pressures for *G. aculeatus* in this lake (i.e gut content analysis). The presence of large-bodied piscivorous predators of this magnitude is not characteristic of many lake populations which primarily identify *Perca flavescens* (yellow perch), *Salmo trutta* (brown trout) and *O. mykiss* (rainbow trout) as primary predatory species on *G. aculeatus* (Lescak and von Hippel, 2011). Marine piscivorous predators on *G. aculeatus* include various species of whitefish (*Coregonus* spp., *Prosopium* spp.), salmonids (*Salmo salar* - Atlantic salmon, *Salvelinus* spp. - charrs), and some species of cod (e.g. *Boreogadus saida*) (Scott and Crossman, 1973; Coad and Reist, 2004). Thus, the predation pressures on fish in the Nueltin Lake population might more accurately reflect that of a coastal/marine population, and accordingly, Nueltin Lake fish may be expressing a phenotype closer to a more typical marine morph which experiences similar levels of piscivorous predation. Additional comparisons with populations of *G. aculeatus* collected from a truly marine habitat (open water trawls with a seine net) would provide greater resolution to this question as significant difference in plate number was noted when comparing the estuarine Cochrane population with fish from Nueltin Lake[r42].

It has been shown that diversification of lateral plate morphs have an adaptive basis in *G. aculeatus* (Reimchen, 1994; 2000; Bell, 2001; Leinonen *et al*., 2011). The posterior lateral plates of completely plated individuals are thought to minimize injury

and increase the likelihood of escape following capture (Reimchen, 1992; 2000); however, they may increase the risk of capture as a result of decreased maneuverability and inability to avoid predation events (Bergstrom and Reimchen, 2002). Lateral plate morph frequencies rely on the ecological trade-offs and relative survival rates for evasion before capture versus escape potential and ability to withstand damage inflicted by piscivorous fishes (Reimchen, 2000). Accordingly, selection should favor low plate morphs in littoral habitats or structurally complex habitats which offer the opportunity for refuge. In pelagic zones of the marine environment, full lateral plating would be selected for due to the lack of structural refuge (Bell *et al*., 2004; Leinonen *et al*., 2011). No quantification of habitat was possible among fish from the Attawapiskat region; however, based on results of lateral plate expression, identification of habitat complexity among the estuarine region may be useful for further resolution of this question.

It has also been postulated that thermal gradients may influence lateral plate expression, both as an adaptive trade-off for increased growth rates among juveniles, as well as a physiological advantage conferred by increased plate numbers in colder climates (Klepaker, 1993). The pattern of higher plate numbers in the Nueltin Lake, Thlewiaza River and Caribou River group when compared to the Kenora and Cochrane District from a slightly more southern environment, and the lack of concordance with expected predation pressures, may indicate that thermal variation may promote adaptive divergence in lateral plate numbers.

Gill rakers, a trophic trait modified to maximize feeding efficiency in divergent habitat types, were also examined in this study. Surprisingly, no numerical difference was observed in any pairwise comparisons of populations in this study. Gill rakers have

been well documented as an adaptive trait in benthic-limnetic species pairs following a marine invasion into freshwater (McKinnon and Rundle, 2002). It was expected that variation in gill raker numbers would be observed between populations from various habitats. Longer, delicate gill rakers are observed among pelagic forms of *G. aculeatus*, often from marine environments with a selection for a planktonic diet (Day *et al*., 1994; Klepaker, 1993). Adaptive variation in gill raker number has even been observed between riverine and lacustrine populations (Gross and Anderson, 1984). The lack of difference among sampled populations (Nueltin Lake, Caribou River, Thlewiaza River) indicates selection for a common phenotype in gill raker and reflects similar dietary preferences and trophic niches. This reinforces the notion that the Thlewiaza and Caribou River populations, while in close proximity to the marine environment, exhibit localized adaptations that are not consistent with those optimized for a pelagic, anadromous life history.

Although often thought of as an armor complex, the variation among phenotypic expression of lateral plates and defensive structures observed in this study appears to reflect a tendency for different adaptations for different structures and indicates that the armor complex should not be expected to vary as a single unit. Fish in the Caribou River express reduced spine lengths with a partial plate morph, the Cochrane population demonstrates high levels of defensive structures but a significant reduction in plate number, and the Thlewiaza and Nueltin Lake fish exhibit high levels of spine and pelvic expression as well as high lateral plate numbers (Table 11, Figure 14. The lack of a consistent trend of increase or decrease may reflect the difficulties scientists have in explaining the causative mechanisms of divergence in these frequently investigated traits.

This can be explained based on the established role of molecular mechanism influencing phenotypic expression in *G. aculeatus* (Shapiro *et al*., 2004; Colosimo *et al*., 2005; Bell *et al.*, 2010; Chan *et al*., 2010). *Eda* is known to influence lateral plate expression as has been previously discussed. It is suggested that following colonization of freshwater environments by marine fish, strong selective effects result in an increase in the frequency of the low plate allele for *Eda* (Colosimo *et al*., 2005). The frequency of the low *Eda* allele has been estimated at only 1% among marine G. aculeatus (Barrett et al., 2008; Bell *et al*., 2010; Le Rouzic et al., 2011). The lack of a low plate morph among fish collected from Nueltin Lake fish would suggest selective pressures are acting on the population to promote the expression of the full plate morph. This provided further support of the role of increased piscivorous predation in Nueltin Lake as a driving force promoting complete lateral plate expression.

Alternatively, it may also be attributed to the absence of low *Eda* alleles among founding individuals following colonization and may be not be correlated with predation pressure as with spine lengths. Or, it may also be suggested that fish in Nueltin Lake initially expressed low plate morphs; however, strong selection for a completely plated morph may have resulted from high levels of piscivorous predation as has been demonstrated in other freshwater lakes (Kitano *et al*., 2008). Lack of concordance between populations for lateral plate phenotype, despite habitat type, suggests that influence of ecological factors is not be sufficient to explain phenotypic variation and that mutational mechanisms driving adaptation are a significant component of morphological variation in *G. aculeatus*. Genome mapping has identified QTL for dorsal spines, lateral plates, gill rakers and pelvic structures. With additional research, it is likely that similar

mechanisms will be identified to enable further resolution of the interactive factors which influence morphology. Based on the results of this study, it should be expected that armor traits vary independently, that spine lengths should not be used to assume lateral plate expression and that the conventionally described "freshwater morph" (or even "marine morph" as has been demonstrated for Cochrane fish) does not necessarily exist uniformly across all populations or habitats. The survival value of a given trait is highly dependent upon the context under which it has arisen (Leinonen *et al*., 2011).

CONCLUSIONS

Distinct morphologies associated with different freshwater habitats are influenced by selection and are derived repeatedly and independently under appropriate conditions (Hagen and Gilbertson, 1972; Moodie, 1972; Moodie and Reimchen, 1976; Bell, 1984; Bell and Foster, 1994; Ortí *et al*., 1994). This study sought to examine the occurrence of morphological variation among *G. aculeatus* from inland and coastal populations in northern Manitoba and western Hudson Bay. Differences among structural and meristic traits was identified among each population examined, but was not consistent across populations or by habitat type. Despite a number of hypotheses suggesting predictable patterns of variation and expected adaptive strategies associated with a shift to a freshwater environment, fish from Nueltin Lake did not express phenotypic variation in concordance with these theories.

Rather than a reduction in defensive structures expected with a shift from a marine environment, the inland population from Nueltin exhibited the highest levels of body armor of any of the examined populations in this study. Presumed coastal

populations, sampled from estuarine locations within 40 km of the coast of Hudson Bay, did not express morphological attributes consistent with those from a marine environment. Instead, both populations from the Cochrane District and Caribou River systems exhibited trends of phenotypic expression not typically correlated with those of an inland, freshwater morph. This supports the Caribou River population may be non-anadromous despite close proximity to Hudson Bay, and likely reflect adaptive traits for a riverine habitat. The population from the Cochrane district of northern Ontario near James Bay exhibits high levels of defensive structures as would be expected in a marine population, however significant reduction in lateral plate numbers as compared to the Nueltin Lake population indicates that divergent selection acts on plates and spine expression in different ways (Bell *et al*., 2001; Colosimo *et al*., 2005; Leinonen *et al*., 2011).

Deviation from predicted patterns of phenotypic expression reflects the complexity of ecosystems and the associated selection pressures faced by species undergoing adaptive diversification. The lack of concordance of the Nueltin Lake population to the multitude of hypotheses promoting a reduction in defensive structures in a freshwater environment reiterates the necessity for further understanding of the driving mechanisms of ecological speciation and phenotypic diversification. While it is likely, based on high levels of piscivorous and avian predators present in Nueltin Lake, that predation pressure would explain the unexpected morphology in these fish; other hypotheses may also play a significant role. Additional exploration of the causative mechanisms at work in this system would be beneficial to provide additional resolution

of this question. As with many phenomena in biology, there is not a "one size fits all" approach to evolution.

Table 8. Morphological measurements used to assess variation in *Gasterosteus aculeatus*.

Measurement	Abbv.	Description
Standard length	SL	Distance from the anterior tip of the upper lip to the middle posterior margin of the hypural plate
Head length	HL	Horizontal distance from the anterior tip of the upper lip to the posterior margin of the operculum
Body depth	BD	Depth of the body posterior to the base of the first dorsal spine measured vertically at 90° to the body axis
Jaw length (lower)	JL	Anterior tip of the lower jaw to the intersection of the maxilla and mandible
Snout length	SnL	Measured as the horizontal distance from the anterior tip of the upper lip to the anterior border of the eye orbit
Gape width	GW	Lateral measurement on the ventral surface from jaw corners
Eye diameter	ED	Maximum horizontal distance across the eye
1st dorsal spine length	1DS	Dorsal surface on the posterior edge of the first dorsal spine
2nd dorsal spine length	2DS	Dorsal surface on the posterior edge of the second dorsal spine
Pelvic spine length	PS	Lateral locking process of the pelvic spine to the tip
Pelvic complex length	PC	Measured ventrally, from the posterior fusion to the anterior margin

Table 9. Post-hoc Tukey's test (HSD) for pairwise analysis of the differences between the populations (C.I.=95%). (NL: Nueltin Lake, CR: Caribou River, TR: Thlewiaza River, KD: Kenora District, CD: Cochrane District). ** indicates significant difference.

ALL TRAITS				
Pop.	NL	TR	CR	KD
TR	0.383	~	~	~
CR	0.00**	0.020**	~	~
KD	0.296	0.00**	0.003***	~
CD	< 0.001**	< 0.001**	< 0.001**	< 0.001**

GILL RAKERS			
Pop.	NL	TR	CR
TR	0.917	~	~
CR	0.354	0.775	~

SNOUT LENGTH				
Pop.	NL	TR	CR	KD
TR	0.272	~	~	~
CR	1.000	0.224	~	~
KD	1.000	0.329	1.000	~
CD	< 0.001**	< 0.001**	< 0.001**	< 0.001**

HEAD LENGTH				
Pop.	NL	TR	CR	KD
TR	0.332	~	~	~
CR	< 0.001**	0.011**	~	~
KD	0.957	0.001**	0.813	~
CD	< 0.001**	< 0.001**	< 0.001**	< 0.001**

FIRST DORSAL SPINE				
Pop.	NL	TR	CR	KD
TR	0.060	~	~	~
CR	< 0.001**	0.002**	~	~
KD	0.341	0.000**	0.919	~
CD	0.001**	< 0.001**	< 0.001**	< 0.0001

STANDARD LENGTH				
Pop.	NL	TR	CR	KD
TR	0.070	~	~	~
CR	0.000**	0.05**	~	~
KD	0.946	< 0.001**	0.424	~
CD	< 0.001**	< 0.001**	< 0.001**	< 0.001**

BODY DEPTH				
Pop.	NL	TR	CR	KD
TR	< 0.001**	~	~	~
CR	< 0.001**	0.478	~	~
KD	0.088	0.007**	0.137	~
CD	< 0.001**	< 0.001**	< 0.001**	< 0.001**

JAW LENGTH				
Pop.	NL	TR	CR	KD
TR	0.686	~	~	~
CR	< 0.001**	0.004**	~	~
KD	0.662	0.001**	0.559	~
CD	< 0.001**	< 0.001**	< 0.001**	< 0.001**

GAPE WIDTH				
Pop.	NL	TR	CR	KD
TR	0.108	~	~	~
CR	< 0.001**	0.206	~	~
KD	0.891	0.016**	0.621	~
CD	< 0.001**	< 0.001**	< 0.001**	< 0.001**

PELVIC SPINE				
Pop.	NL	TR	CR	KD
TR	< 0.001**	~	~	~
CR	< 0.001**	0.004**	~	~
KD	< 0.001**	0.137	0.192	~
CD	< 0.001**	< 0.001**	< 0.001**	< 0.001**

PELVIC COMPLEX				
Pop.	NL	TR	CR	KD
TR	< 0.001**	~	~	~
CR	0.05**	0.018**	~	~
KD	0.120	0.021**	1.000	~
CD	< 0.001**	< 0.001**	< 0.001**	< 0.001**

SECOND DORSAL SPINE				
Pop.	NL	TR	CR	KD
TR	< 0.001**	~	~	~
CR	0.000**	0.003**	~	~
KD	< 0.001**	0.047	0.910	~
CD	0.001**	< 0.001**	< 0.001**	< 0.001**

LATERAL PLATES				
Pop.	NL	TR	CR	KD
TR	0.404	~	~	~
CR	< 0.001**	0.004**	~	~
KD	< 0.001**	0.000**	< 0.001**	~
CD	0.001**	< 0.001**	< 0.001**	0.998

EYE DIAMETER				
Pop.	NL	TR	CR	KD
TR	0.025**	~	~	~
CR	0.004**	0.738	~	~
KD	0.278	0.363	0.919	~
CD	< 0.001**	< 0.001**	< 0.001**	< 0.001**

Table 10. Eigenvalues (a) and eigenvectors (b) calculated using structural and meristic traits from *Gasterosteus aculeatus* populations in Nueltin Lake and western Hudson Bay. Eigenvalues greater than 1 are shaded in grey and were included in PCA.

a)

HL	0.29493	0.48012	0.16689	0.13166	-0.15401	0.03536	-0.08607	0.26868	-0.69371	0.20359	-0.10038
BL	0.23506	-0.02031	-0.60361	-0.17802	-0.21129	0.41658	-0.3384	0.40831	0.18705	0.08218	0.08447
JL	0.17885	0.53558	0.13737	-0.10447	-0.40572	-0.3714	0.07235	0.00036	0.51236	0.09814	0.26732
SNL	0.12097	0.30296	0.44315	-0.25399	0.54009	0.51619	-0.06621	0.06463	0.2383	-0.02553	0.09243
GW	0.18509	0.29313	-0.52727	0.07336	0.38486	-0.03118	0.66473	-0.03958	-0.0333	-0.04383	0.02938
ED	0.18228	0.11831	-0.00395	0.83576	-0.06154	0.27115	-0.16548	-0.23424	0.21689	-0.21487	-0.03874
1DS	0.43137	-0.17382	0.10566	-0.07168	0.06792	-0.26714	-0.04651	0.29453	-0.0779	-0.7578	0.14894
2DS	0.42359	-0.22493	-0.06335	0.00268	0.23261	-0.15209	-0.24457	-0.39365	-0.15256	0.34856	0.57374
PS	0.454	-0.118	0.01501	-0.01904	0.22772	-0.29769	-0.14373	0.0228	0.25196	0.27019	-0.6937
PC	0.34327	-0.05551	0.0426	-0.35481	-0.41815	0.35455	0.21984	-0.56008	-0.10383	-0.1777	-0.21554
LPN	0.2328	-0.43899	0.31193	0.20071	-0.19493	0.19366	0.51792	0.38022	0.11282	0.30511	0.14618

b)

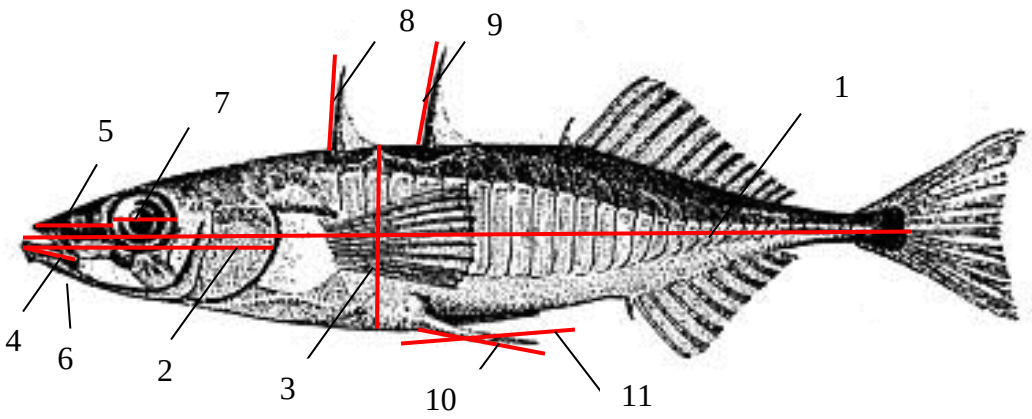
Component	Eigenvalue	Percent	Cumulative percentage
1	3.0477	27.707	27.707
2	1.5969	14.517	42.223
3	1.2	10.909	53.133
4	1.0407	9.461	62.593
5	0.9356	8.505	71.098
6	0.7444	6.767	77.865
7	0.6276	5.706	83.571
8	0.5777	5.252	88.823
9	0.4669	4.245	93.068
10	0.4297	3.907	96.974
11	0.3328	3.026	100

Table 11. Pairwise correlation matrix of structural characteristics in *Gasterosteus aculeatus*. Correlations which represent the top 25% of pairwise comparisons are indicated in grey (14/55 comparisons). Abbreviations as in Table 8.

	HL	BD	JL	SNL	GW	ED	1DS	2DS	PS	PC	LPN
HL	1.000										
BD	0.117	1.000									
JL	0.463	0.036	1.000								
SNL	0.262	-0.092	0.139	1.000							
GW	0.203	0.252	0.142	0.061	1.000						
ED	0.269	0.061	0.089	-0.002	0.133	1.000					
1DS	0.251	0.205	0.120	0.099	0.111	0.111	1.000				
2DS	0.171	0.237	0.000	0.064	0.170	0.175	0.517	1.000			
PS	0.254	0.232	0.150	0.135	0.203	0.162	0.583	0.618	1.000		
PC	0.222	0.275	0.204	0.095	0.062	0.029	0.351	0.361	0.315	1.000	
LPN	0.012	0.021	-0.122	-0.031	-0.130	0.146	0.355	0.270	0.295	0.261	1.000

Figure 12. Map showing collection locations of museum populations used for comparison with the Nueltin Lake-Thlewiaza River system. Collection data included in Appendix B. KD: Kenora District (Hawley Lake, Spruce Lake), CD: Cochrane District (Attawapiskat River).

Figure 13. Morphological measurements of *Gasterosteus aculeatus* used using dial calipers and occular micrometer. Images after Schluter (1993).



Gill rakers of *G. aculeatus*

Number	Abb.	Measurement
1	SL	Standard length
2	HL	Head length
3	BD	Body depth
4	JL	Lower Jaw length
5	SnL	Snout length
6	GW	Gape width
7	ED	Eye diameter
8	1DS	First dorsal spine length
9	2DS	Second dorsal spine length
10	PS	Pelvic spine length
11	PC	Pelvic complex length

Figure 14. Comparison of lateral plate expression in *Gasterosteus aculeatus* by population. Each bar represents an individual fish with SL>34 mm. Plate counts do not include keel scutes, if present. NL=Nueltin Lake, TR= Thlewiaza River, CR=Caribou River, KD=Kenora District, CD= Cochrane District.

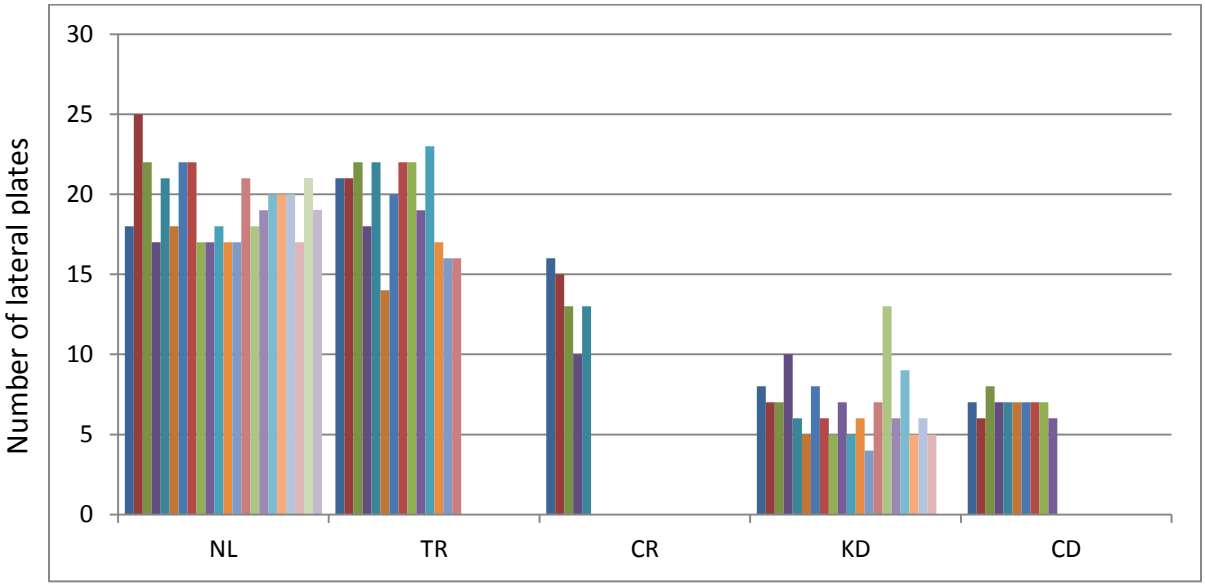


Figure 15. Principal components analysis of structural and meristic traits in *Gasterosteus aculeatus* from Nueltin Lake and western Hudson Bay. Four components are included in the assessment (62.57% of variation explained).

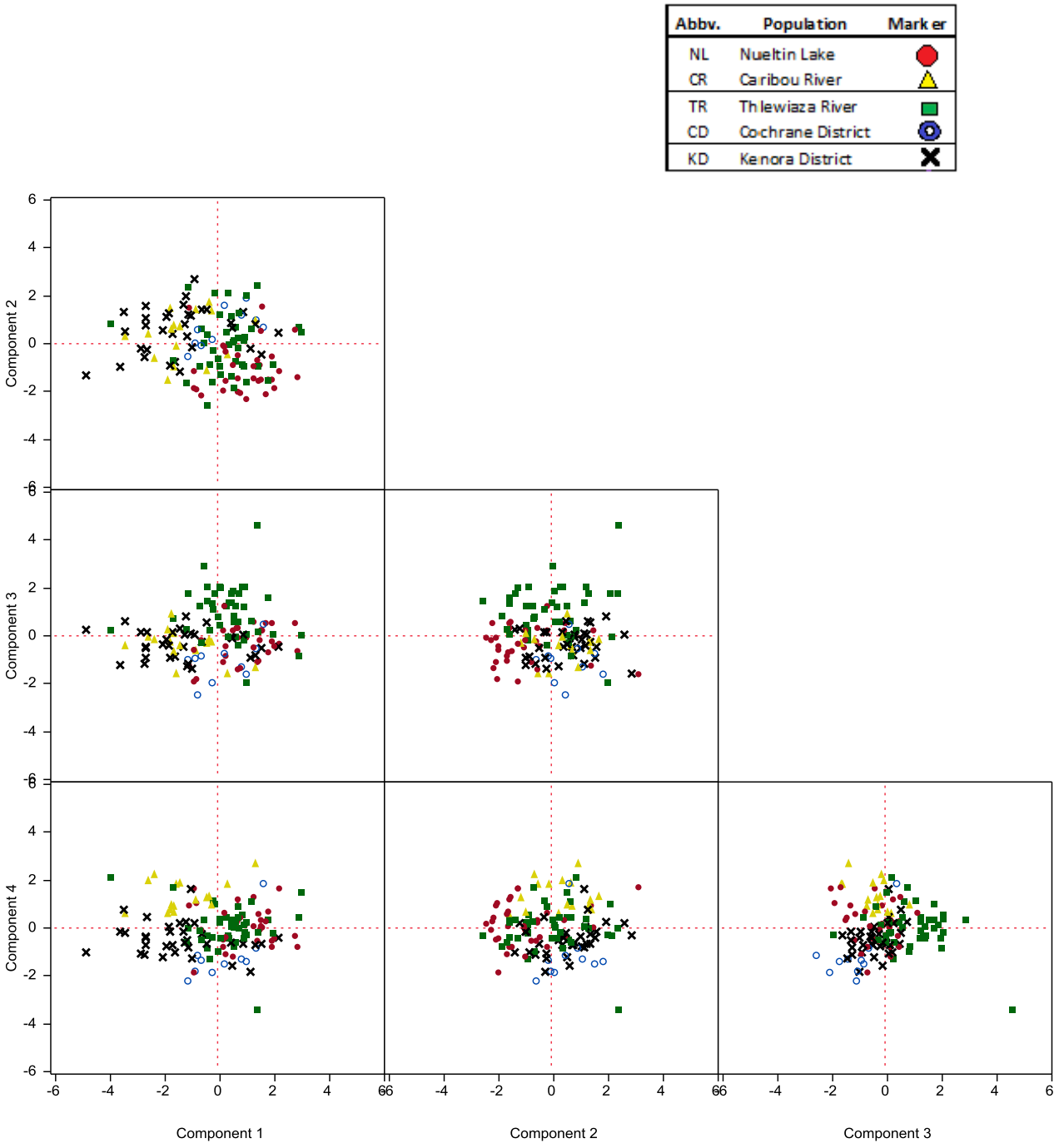


Figure 16. Factor analysis of structural and meristic traits in *Gasterosteus aculeatus* from Nueltin Lake and western Hudson Bay. Four components are included in the assessment (62.57% of variation explained). Variables correspond to Figure 9.

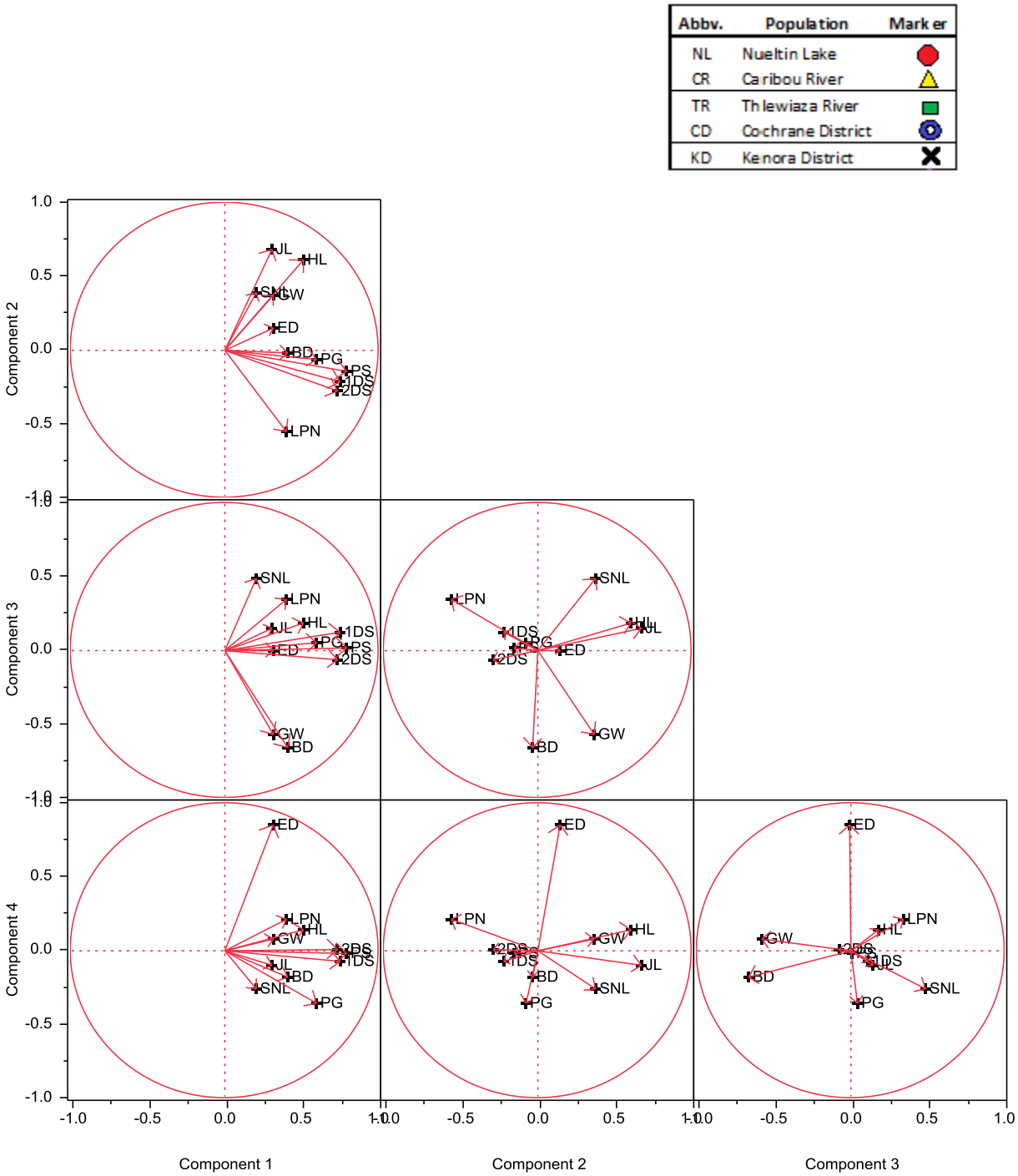
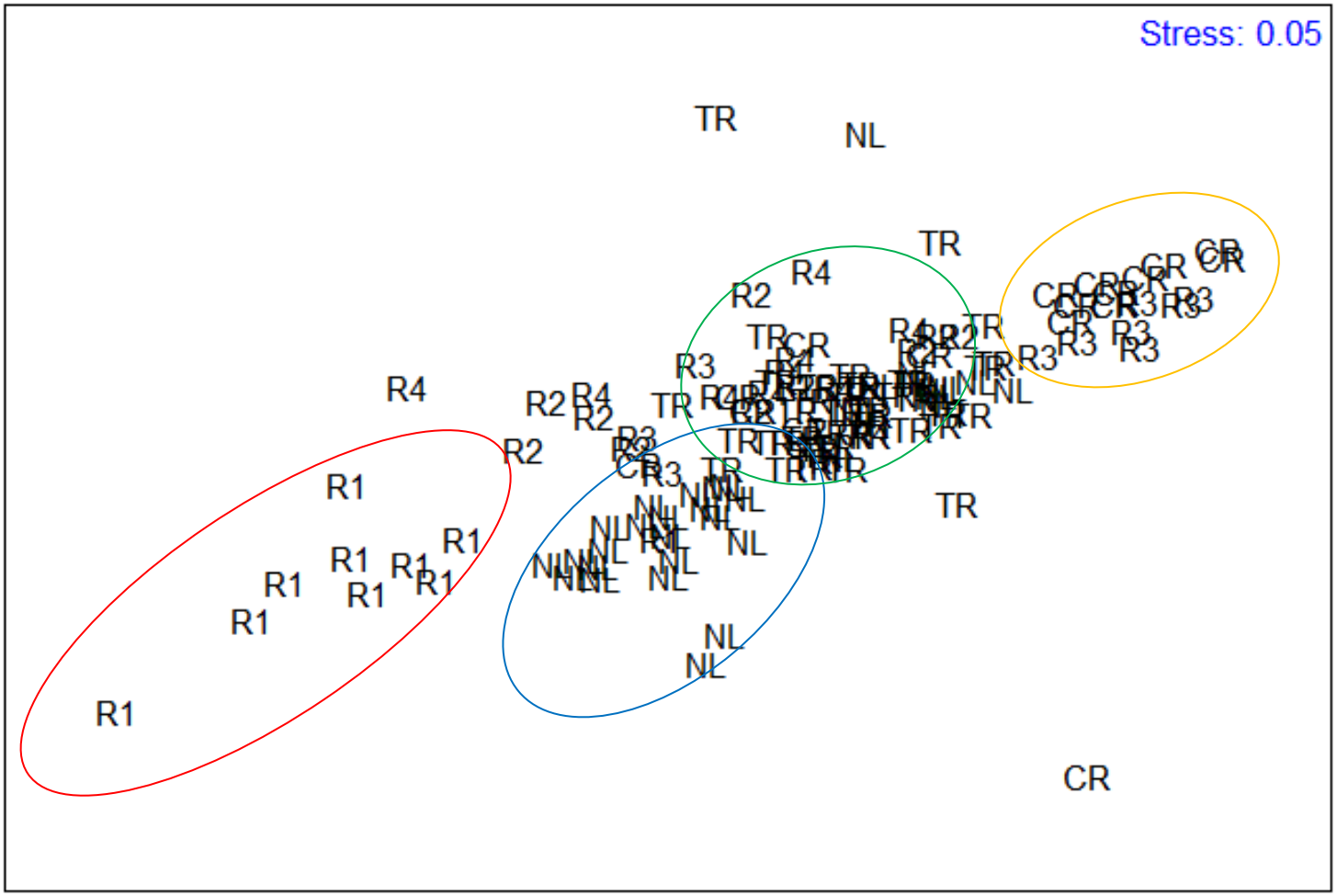


Figure 17. Non-metric multidimensional scaling plot of correlation of structural traits across all populations, stress:0.05. Ellipses represent general trends in groupings by population. (NL=Nueltin Lake, TR=Thlewiaza River, CR=Caribou River, R2 to 4=Kenora District, R1=Cochrane District).


Stress: 0.05
TR
NL
R4
R2
R3
R1
CR

CHAPTER 4: GENERAL CONCLUSIONS AND IMPLICATIONS FOR CONSERVATION

The fundamental goal of many evolutionary biologists is to explore the processes which give rise to new species and to interpret the significance of observed variation within populations (Taylor and McPhail, 2000). Postglacial divergence among populations of threespine stickleback, *G.aculeatus,* provides an exceptional opportunity to examine complex evolutionary processes as a result of repeated colonization of freshwater environments from an ancestral marine population (Bell and Foster, 1994). Many populations of *G. aculeatus* have rapidly adapted to diverse freshwater environments and exhibit unprecedented levels of phenotypic and genetic diversity (Bell and Foster, 1994). As a result, the species has been identified as a model organism in a wide range of studies and is an excellent subject for studies of parallel evolution (Colosimo *et al*., 2005; Kawahara *et al*., 2008).

This study has confirmed the presence of a viable, self-reproducing population of *G. aculeatus* in Nueltin Lake, Manitoba. Previously unknown from regions outside of the coastal environment of Hudson Bay, this represents the furthest collection inland of the species in the province. The occurrence of this typically anadromous species in Nueltin Lake provides an interesting opportunity to examine the environmental and evolutionary processes which influenced populations of fishes following the most recent deglaciation. Connected to the ocean by a highly convoluted river system with a series of presumably impassable waterfalls and elevational gradients, the presence of *G. aculeatus* so far inland provides insight into some complex biological questions of localized adaptation and morphological variation following a dramatic environmental shift.

Results of this study have confirmed that, based on selectively neutral genetic markers, highly significant differentiation exists between populations of *G. aculeatus* from Nueltin Lake and conspecifics downstream in the Thlewiaza River and with adjacent watersheds. Coastal populations indicated a significant level of genetic differentiation exists between groups despite only moderate levels of geographic partitioning. Genetic diversity within newly colonized populations in recently deglaciated regions is expected to be reduced from the ancestral population as a result of founder effects and genetic drift. This promotes the divergence of allelic frequencies among populations and provides the opportunities for mutations, which lead to localized adaptations, to accumulate rapidly. In Nueltin Lake, only a fraction of the observed allelic diversity present in the coastal population was detected. This can severely impact the ability of populations to adapt to new and changing environmental conditions by reducing the amount of standing variation on which selection can act (Leinonen et al., 2011).

High levels of gene flow constrain the abilities of populations to adapt to locally divergent selection pressures by continually altering the allelic frequencies of genes under localized selection for adaptation, reducing pressure of directional selection for beneficial mutations, and as a result, populations with ongoing genetic exchange with adjacent populations should remain morphologically conserved (Hendry *et al*., 2002). As Nueltin Lake has been shown to be significantly isolated from other populations of *G. aculeatus* from adjacent watersheds, decreased gene flow may account for increased divergence between populations than is observed in populations with ongoing genetic exchange (Hendry *et al*., 2002).

Divergent selection pressures between the ancestral marine population and those experienced by fish in an isolated freshwater environment have generated a number of hypotheses to suggest localized adaptation to the new ecosystem. Following the isolation of *G. aculeatus* in Nueltin Lake, most hypotheses would suggest a reduction in defensive structures as a result of reduced predation pressures, as well as variation in trophic characters to reflect different dietary preferences. However, prominent dorsal and pelvic spines, high lateral plate numbers and a robust pelvic complex were identified among fish from Nueltin Lake and are unexpected to be observed in fish from a freshwater environment (Bell and Foster, 1994). This indicates the fish in Nueltin Lake may be experiencing divergent selection mechanisms than similar postglacial populations, although, this difference may also be attributed to founder effects (or a combination of both). Variation in natural populations as a result of adaptive selection has been identified as the primary causative mechanism promoting divergence among phenotypically expressed traits in *G. aculeatus* (McKinnon and Rundle, 2002).

Conservation implications

Colonization following glacial recession promotes divergence in a number of traits including morphology, behavior, physiological attributes and life history characteristics and has been replicated in many populations of *G. aculeatus* throughout their natural range (Scott and Crossman, 1973; Stewart and Watkinson, 2004). These ephemeral populations appear to experience high extinction rates, often associated with glacial advance and retreats, and rarely persist long enough to disperse beyond the original environment to form discrete species with recognized distributions (McKinnon

and Rundle, 2002). Based on observed levels of highly significant genetic differentiation and unexpected morphological findings in this study, the question should be asked whether Nueltin Lake *G. aculeatus* may require conservation efforts to promote longterm population viability.

Criteria for designating species at risk or in need of additional legislative protection to promote ongoing population viability are variable; based on the level of management actions or risk evaluation required, different methods can be applied. The repeated, independent evolution of heritable morphological variation in *G. aculeatus* is characteristic of this species and highlights the taxonomic difficulties faced by biologists when trying to elucidate phylogenetic relationships and species distinction (Hatfield and Schluter, 1996).

The process to identify and determine necessity of conservation intervention varies between organizations based on relative importance of biological and socio-economic values (de Grammont and Cuaron, 2006). Regardless of the socio-economic impacts, an essential component for maintaining biodiversity and ecological resilience is the identification of biologically relevant conservation units. This is not a simple process as species vary considerably, even among closely related taxa, so establishing broadly applicable guidelines can be difficult (Fraser and Bernatchez, 2001; Moritz, 2002; Waples and Gaggiotti, 2006; Palsbøll *et al*., 2007).

Among evolutionary radiations of *G. aculeatus*, the question arises as to "what actually constitutes a species" and creates additional difficulties for identifying units of conservation in such a morphologically and genetically divergent species. The biological species concept put forth by Mayr (1942) suggests reproductive isolation between

populations as a determining criterion to delimit species boundaries. Heritable variation has been expressed among benthic-limnetic species pairs from British Columbia (McPhail, 1994); however, the documentation of a species pair collapse into a hybrid swarm following the breakdown of reproductive barriers and the complete extinction of another pair based on the introduction of a non-native predator, emphasizes the transient nature of these localized forms and questions the rigidity of pre- and post-zygotic isolating mechanisms for species identification in *G. aculeatus* (McKinnon and Rundle, 2002). Each species definition has a different emphasis, but the concepts they attempt to exemplify are virtually synonomous despite the respective name given (De Queiroz, 1998). However, many species concepts differ based on a single definition or minor detail in wording which can lead to different conclusions regarding the boundary and number of recognized species which leads to the fundamental problem of the species concept (De Querioz, 2007). While numerous methods and rationales exist for species delimitation, a full comparison and examination of their complexities are beyond the scope of this thesis. However, some of the most relevant concepts to this work are presented.

The application of the biological species concept may lead to the recognition of fewer species taxa than would be identified following an alternative concept such as the phylogenetic species concept (DeQuieroz, 2007). The latter concept suggests that a species is defined as the smallest identifiable cluster of organisms within which a parental pattern of ancestry and descent exists. It relies on monophyly of the group in question, specifically the possession of shared, derived character states. This concept, while applicable to *G. aculeatus*, would not likely be the most sensitive application of species

definition to some of the paired species observed in contemporary time frames and, as with the biological species concept, would underrepresent the diversity observed among. The genetic or genealogical species definition relies on delimitation of species by individuals sharing a common gene pool which forms a genetic unit. As previously identified, mitochondrial DNA groups *G. aculeatus* worldwide into only two separate clades, and represents a serious oversimplification of the complexities of this species. Comparatively, despite a shared ancestral gene pool in Hudson Bay, results of this study indicate significant genetic partitioning exists between Nueltin Lake and adjacent populations which suggests this is not the ideal application of a species concept. Based on this study, it appears that the most relevant definition of a species for this model system would be recognized under the evolutionary species concept. This concept relies on a common evolutionary trajectory among members of the "species" and recognizes unique evolutionary traits, tendencies and historical fate (DeQuieroz, 2007). This appears to be most relevant to the populations identified from Nueltin Lake and western Hudson Bay as well as the species pairs identified in British Columbia.

The concept of "evolutionarily significant units" (ESU) was introduced as a method for identifying and prioritizing species for conservation (Ryder, 1986). It has been determined that ESUs should be reciprocally monophyletic based on mitochondrial DNA and demonstrate significant divergence at nuclear loci (Moritz, 1994). In contrast, "management units" (MU) are characterized as having significant divergence of allelic frequencies at nuclear or mitochondrial loci, regardless of its phylogenetic distinctiveness (Moritz, 1994). MU's are relevant on a contemporary timescale, rather than historical timelines represented by ESU's. Accordingly MU's may be more relevant to

evolutionarily recent species such as the benthic-limnetic species pairs of British Columbia (Schluter and McPhail, 1992; Fraser and Bernatchez, 2001).

Enacted in 2003, the Federal *Species at Risk Act* (SARA) is the primary governing body to enact legislative protection for conservation and recovery of wildlife species in Canada. SARA is committed to protecting species which face the risk of extinction or loss of critical habitat. The Committee on the Status of Endangered Wildlife in Canada (COSEWIC), including representatives from federal, provincial and territorial governments, universities and non-government organizations, is tasked with the responsibility of assessing and recommending species' listing to SARA (COSEWIC, 2009). Below the species level, COSEWIC strives to identify Designatable Units (DU's) groups which are significant and irreplaceable when a single status designation does not reflect the probability of extinction of the species. DU's should be discrete and evolutionarily significant units of the taxonomic species, where "significant" is identified as being important to the species as a whole and, if lost, would not likely be replaced by natural dispersal. For *G. aculeatus* in Nueltin Lake, population discreteness has been demonstrated based on significant genetic differentiation and lack of gene flow between adjacent populations. It also represents a population which is unlikely to be replaced naturally following extirpation based on an unlikely occurrence of dispersal back to Nueltin Lake. For assessment of further conservation or legislative protection, it is suggested that results of this study satisfy the critera for demonstrating evolutionary significance of the population of *G. aculeatus* in Nueltin Lake and warrant its recognition as a Designatable Unit.

While genetics can be a useful tool in delineating conservation and management units for many species, specific thresholds for the amount of diversity and differentiation required for evolutionary significance is not explicitly defined (Palsbøll *et al*., 2007). Comparison of genetic variation will help identify phenotypically obscure yet still biologically significant units, and the incorporation of diverse and independent evolutionary lineages can provide additional ecological significance (Palsbøll *et al*., 2007).

Many Canadian taxa are problematic when being assessed against thresholds for divergence that were defined based on genetic studies of southern species (Bernatchez and Wilson, 1998). Glaciation has strongly influenced the genetic diversity of northern taxa as a result of population and range reduction associated by glacial advances and strong founder effects that characterize areas of recolonization (Bernatchez and Wilson, 1998).

Assessments of species conservation status enable government and management organizations to prioritize conservation efforts to conserve biodiversity within and among species (de Grammont and Cuaron, 2006). Local populations are increasingly the objects of conservation efforts for the purpose of preserving genetic variation in the hope of maintaining evolutionary potential (Rojas, 1992; Sites and Crandall, 1997; Malone *et al*., 2003). In general, the conservation status of a species is determined based on its vulnerability, irreplaceability or biological relevance, and the magnitude of the risk (i.e. probability of extinction over a given time frame). In the face of increasing habitat fragmentation, a significant risk for northern populations and regions are the expected dramatic effects of climate change.

As previously discussed, several populations of *G. aculeatus* have already been designated as being at risk and are listed on the SARA registry. Additionally, other populations have been identified in the Queen Charlotte Islands, B.C., in Scotland, Norway, and in the Cook Inlet region of Alaska for exhibiting parallel, unique and divergent characteristics. These populations may be threatened by habitat change and the introduction of predatory fish, and may also require protection (Reimchen, 1984; Bell and Ortí, 1994; Foster *et al*., 2003; Wood, 2003). In Nueltin Lake, reduced levels of diversity, and higher than expected levels of homozygosity, indicate the longterm persistence of this population may be limited due to reduced standing variation upon which selection can act, and which may promote extinction in the face of limited gene flow to introduce beneficial mutations to the population (Kitano *et al*., 2008). In addition to the genetic instability of the population, phenotypic expressions of morphological traits that do not follow expected hypotheses for freshwater variation present an evolutionary puzzle for biologists. Nueltin Lake may present many other interesting opportunities to study complex and synergistic effects of selection and adaptation; however, the risk exists that the population may be impacted by anthropogenic, climatic or biological factors for which it is not able to adapt. Despite the globally secure ranking of *G. aculeatus*, in Manitoba, the species has already been designated as rare and vulnerable to extinction due to its infrequent collection and limited known distribution within the province (Manitoba Conservation Data Centre, 2010; Stewart and Watkinson, 2004). As a result of this study, we have confirmed a population of *G. aculeatus* does exist in Nueltin Lake and suggest that glacial relict populations provide an interesting set of ecological

replicates in natural environments for further studies on adaptive selection and its role in speciation.

Gasterosteus aculeatus become negatively affected as a result of changes to water quality, increased turbidity, nutrient loading, loss of littoral habitat, introduction of non-native species and increased land use and resource exploitation (Hatfield, 2001). Increased turbidity and the introduction of an invasive species have already been identified in the loss of two federally listed species pairs from British Columbia. In Nueltin Lake, limited anthropogenic stressors are present in the immediate vicinity of the lake, however, considerable resource interests for mining and subsurface drilling pose serious threat to the watershed (MB Water Stewardship, pers comm.). Changes to watershed integrity as a result of alteration of habitat, riparian zone loss and sedimentation associated with resource extraction could severely impact the Nueltin Lake population. Limited genetic diversity identified as a result of this study indicates that this population would be unlikely to adapt to changes in environmental conditions. Nueltin Lake has recently been identified as a provincial park in Manitoba and encompasses a large area in northern Manitoba. While this is an excellent start to conserving the environmental integrity of the region, this does not preclude resource usage by companies and may still result in negative impacts to local populations.

Risk to the population as a result of non-native species introductions is another consideration for management. Use of live fish as bait, and the subsequent transportation of live bait is prohibited in Manitoba (MB Water Stewardship, 2005). Nueltin Lake Lodge, the only commercial industry to operate on the lake, has ensured that only trapping of bait from within the lake is permitted and restricts importation from locations

outside the watershed. Logistic constraints of importing boats and other fishing gear by patrons is limited and does not present a significant risk for non-native species introductions to Nueltin Lake.

It is important to note that maintenance of the biodiversity in Nueltin Lake should be the overall goal of any legislative protection, as aquatic ecosystems generally exhibit complex, interrelated dynamics and targeting a single species would likely not be effective. *Gasterosteus aculeatus* are successful in Nueltin Lake as a result of having sufficient nesting habitat, structural refuge, dietary options and well-adapted predatory defenses. By altering any of these parameters, *G. aculeatus* in Nueltin Lake may become significantly compromised and as a result, effective management and conservation efforts should target the wise stewardship of many biotic attributes present in Nueltin Lake

Nueltin Lake presents an opportunity to advance the theory of biogeography by identifying patterns of dispersal and colonization of many related and disparate taxa. Anecdotal evidence suggests the presence of freshwater harbour seals (*Phoca vitulina*) in Nueltin Lake (Beck, 1970). Without legislative efforts to protect this region from habitat loss and alteration, many other opportunities for study outside that of *G. aculeatus* may be lost before knowing they existed.

Further directions of study

Additional examination ofthe interplay between the complex history of deglaciation, varied physiological regimes, and patterns of molecular evolution may provide valuable new insights into mechanisms for speciation (Dooh *et al*., 2006). Ninespine stickleback, *P. pungitius*, exhibit similar levels of diversity to those seen in *G. aculeatus* with respect to a broad distribution throughout the northern hemisphere and high levels of

intraspecific variability in morphological features (Bell and Foster, 1994; Herczeg *et al*., 2010). *P. pungitius* are found throughout a range of aquatic environments of varying salinities (Östlund-Nilsson *et al*., 2007) and share many ecological similarities with *G. aculeatus*, but unlike this closely related taxon, is not characterized by an exclusively marine life history (Mattern, 2007). In Canada, *P. pungitius* is ubiquitous throughout the country in most rivers, streams and lakes extending into far northern environments (Scott and Crossman, 1973; Stewart and Watkinson, 2004). Typically a fresh- or brackishwater resident, the species is found from regions near Churchill and Hudson Bay (Stewart and Watkinson, 2004). Despite many shared biological attributes, *P. pungitius* likely exhibits a different postglacial dispersal strategy than does *G. aculeatus* due to the lack of an entirely marine lifestyle.

Morphological differentiation observed in *P. pungitius* has been attributed to isolation in different glacial refugia during the Pleistocene glaciations (Mattern, 2007). As a result of sympatric collection of *P. pungitius* in all locations where *G. aculeatus* were collected for this study, and a presumed different biogeographic history, patterns of genetic relatedness and inferences of dispersal history should be compared and contrasted between these closely related taxa in a postglacial environment. Morphological variation among isolated population of *P. pungitius* would be an interesting area of future study, as little research has been conducted in this area (Herczeg *et al*., 2010).

As this study forms the initial survey of the morphological and genetic diversity of the Nueltin Lake population of *G. aculeatus*, other research directions have been identified. To further explore some conclusions of this study, an indepth examination of sexual dimorphism as has been documented in other populations would be

complementary and would allow exploration of questions on trophic and structural characteristics and the role of habitat on variation (Kitano *et al*., 2007; Aguirre *et al*., 2008; Leinonen, 2011). Additionally, opportunities to examine the heritability of traits observed in this study are available for future research. Common garden experiments to examine the effect of habitat, predation and ionic limitations on phenotypic expression may provide some resolution to the hypotheses discussed in Chapter 3.

This study also forms the ground work for further studies of behavioral and physiological adaptation to a severe arctic environment. Such environmental conditions may present the opportunity for freeze avoidance behavior or mechanisms to develop (Barrett *et al*., 2011). Freshwater invasions from marine environments will likely reflect natural selection for genetic variation responsible for morphological traits, but also for genes which are involved in physiologically important functions, such as osmoregulation, thermal tolerance, and growth (Shimada *et al*., 2011). Following the conclusions of this study that *G. aculeatus* in Nueltin Lake are non-migratory, investigations into the ability to deal with hypoxia following an extended period of ice cover on the lake would present an interesting area of future study.

Gasterosteus aculeatus species pairs are widely regarded as a scientific marvel, representing a valuable contribution to the understanding of evolution and adaptive selection of the same level as Darwin's finches and the species flocks of cichlids in Africa's Great Lakes. The speed at which dramatic levels of genetic and morphologic diversity have evolved in *G. aculeatus* has garnered significant interest from scientists around the world. Remarkable variations in contemporary time frames enable unparalleled opportunities for studies of divergent selection, adaptive ecology and

evolutionary biology. This study forms the very beginning of hopefully many more studies on the glacially isolated population of *G. aculeatus* in Nueltin Lake, Manitoba. Tremendous research potential exists for this population and its contribution to science has barely begun. As with the world-renowned adaptive radiations of *G. aculeatus* on the West Coast of Canada, similar opportunities for evolutionary diversification in a contemporary timeframe exist for the Nueltin Lake *G. aculeatus*. Recognition of the evolutionary significance of this population as a Designatable Unit should promote interest in conservation assessment and other legislative protection strategies to ensure longterm viability of the population and enable further study opportunities. Proactive management of the watershed and additional research efforts will be vital to maintaining and furthering our understanding of this species, other northern taxa influenced by postglacial dispersal routes, and the interrelationship of habitat, selection and adaptive evolution.

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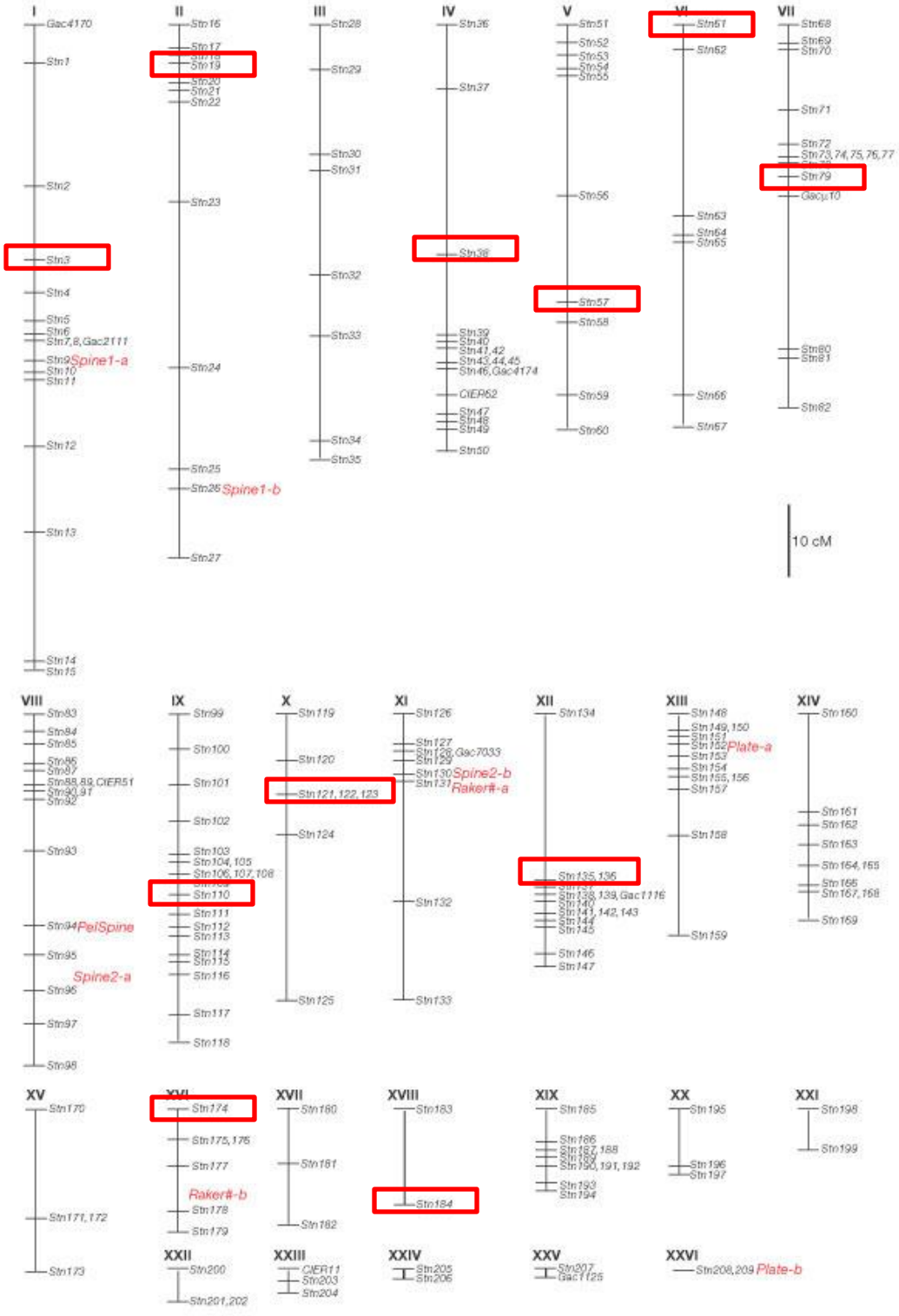
Appendix A: Linkage map indicating the location of approximately 255 *Gasterosteus aculeatus* microsatellites. Selected loci for this study indicated in red boxes (after Peichel *et al*., 2001)



Appendix B. Museum collection specimens used for structural and meristic analysis between populations from Nueltin Lake and western Hudson Bay.

Loaned from	Catalogue number	Location	Year coll'd	Species
Manitoba Museum	TMM 1231	Rupert Creek, Wapusk Nat'l Park, Manitoba	2005	G. *aculeatus*
Manitoba Museum	TMM1237	Rupert Creek, Wapusk Nat'l Park, Manitoba	2005	G. *aculeatus*
Manitoba Museum	TMM1241	Rupert Creek, Wapusk Nat'l Park, Manitoba	2005	G. *aculeatus*
Manitoba Museum	TMM722	Nueltin Lake, Manitoba	1996	G. *aculeatus*
Manitoba Museum	TMM2619	Nueltin Lake, Manitoba	2007	G. *aculeatus*
Manitoba Museum	TMM2641	Nueltin Lake, Manitoba	2007	G. *aculeatus*
Manitoba Museum	TMM2642	Nueltin Lake, Manitoba	2007	G. *aculeatus*
Manitoba Museum	TMM2643	Nueltin Lake, Manitoba	2007	G. *aculeatus*
Manitoba Museum	TMM2718	Thlewiaza River, Nunavut	2008	G. *aculeatus*
Manitoba Museum	TMM2734	Caribou River, Manitoba	2008	G. *aculeatus*
Manitoba Museum	TMM760	Caribou River, Manitoba	1966	G. *aculeatus*
Royal Ontario Museum	CID-22442-E	Ontario, Hawley Lake, Sutton River	1961	G. *aculeatus*
Royal Ontario Museum	CID-27823-E	Ontario, Attawapiskat River, Patricia portion	1971	G. *aculeatus*
Royal Ontario Museum	CID-57783-E	Ontario, Spruce Lake	1986	G. *aculeatus*
Royal Ontario Museum	CID-25364-E	Ontario, Hawley Lake	1959	G. *aculeatus*

GPS coordinates for Royal Ontario Museum Specimens:

CID-22442-E: Hawley Lake, Latitude: 54.5000000, Longitude: -84.6500000
CID-27283-E: Attawapiskat River, Latitude: 52.9500000, Longitude: -82.3000000
CID-57783-E: Spruce Lake, Latitude: 54.3333333, Longitude: -85.0166667
CID-25364-E: Hawley Lake, Latitude: 54.5000000, Longitude: -84.6500000

Appendix C: List of species collected July, 2008 in Nueltin Lake and coastal population of western Hudson Bay.

	Collection location															
Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Catostomus commersoni					X						X	X				X
Coregonus clupeaformis	X		X					X		X						X
Cottus cognatus	X								X			X				X
Culaea inconstans													X	X	X	
Esox lucius	X	X	X		X	X		X								
Gasterosteus aculeatus				X	X				X	X	X					X
Margariscus margarita			X		X			X	X	X						
Pungitius pungitius	X				X		X		X	X	X	X	X		X	X
Rhinichthys cataractae						X					X	X				
Thymallus arcticus	X		X													

Location			
1	Bagg Lake, channel leading from Nueltin to Bagg Lake	9	Nueltin Lake, Opposite Esker (tip), Wpt #14
2	Kasmere Lake, MB	10	Nueltin Lake, Low sand ridge, Wpt#15
3	Nahili Lake, near rapids, W shore	11	Caribou River, MB
4	Nueltin Lake, Esker south, Site 1	12	Churchill River, at culvert. Wpt 18
5	Nueltin Lake, Esker South, Site 2	13	Eastern Creek, N. At Hudson Bay
6	Nueltin Lake, East of lodge, East of Wpt #11 in main water b	14	Twin Lakes, Churchill, MB
7	Nueltin Lake, West of lodge	15	Landing Lake, Churchill, MB
8	Nueltin Lake, Sand bar, near to boat launch/airport	16	Thlewiaza River

Appendix D: Calculation of F Statistics

H_I=mean observed heterozygosity per individual within subpopulations
H_S=mean expected heterozygosity within random mating subpopulations
H_T=expected heterozygosity within random mating total population

Three different F coefficients:

F_{IT}= Correlation of genes within individuals over all populations
F_{ST} = Correlation of genes of different individuals in the same population
F_{IS} = Correlation of genes within individuals within populations

Overall fixation index – the mean reduction in heterozygosity of an individual relative to the total population

$F_{IT} = (H_T \text{ to } H_I)/H_T$

Inbreeding coefficient – the mean reduction in heterozygosity of an individual due to the effects of non to random mating within a subpopulation

$F_{IS} = (H_S \text{ to } H_I)/H_S$

Fixation index – the mean reduction in heterozygosity of a subpopulation (relative to the total population) due to genetic drift among subpopulations

$F_{ST} = (H_T \text{ to } H_S)/H_T$

F_{ST}, F_{IT}, and F_{IS} are interrelated so that:

$(1 - F_{IT}) = (1 - F_{ST})(1 - F_{IS})$

$F_{ST} = (F_{IT} - F_{IS}) /(1 - F_{IS})$

1

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Appendix E. Summary of transformed data of *Gasterosteus aculeatus* from all populations. Abbreviations follow Table 8. Data presented in centimeters (cm).

ALL POPULATIONS													
Statistic	SL	HL	BD	JL	SnL	GW	ED	1DS	2DS	PS	PG	LPN	GR
n	134	134	134	134	134	134	134	134	134	134	134	71	94
Min	1.918	0.166	0.093	0.010	0.014	0.008	0.015	0.000	0.000	0.045	0.049	4	14
Max	6.167	0.534	0.350	0.036	0.066	0.029	0.042	0.059	0.081	0.147	0.349	25	22
Mean	3.399	0.303	0.183	0.021	0.029	0.016	0.027	0.035	0.045	0.089	0.174	13.66	18.30
Var. (n-1)	0.565	0.004	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	41.74	1.99
St. dev (n-1)	0.751	0.066	0.049	0.005	0.007	0.004	0.006	0.009	0.011	0.019	0.049	6.46	1.41
NUELTIN LAKE													
Statistic	SL	HL	BD	JL	SnL	GW	ED	1DS	2DS	PS	PG	LPN	GR
n	35	35	35	35	35	35	35	35	35	35	35	22	34
Min	2.226	0.238	0.141	0.016	0.021	0.010	0.020	0.000	0.035	0.077	0.099	17	17
Max	4.246	0.368	0.256	0.025	0.034	0.022	0.038	0.049	0.064	0.122	0.244	25	21
Mean	3.531	0.312	0.202	0.021	0.028	0.017	0.029	0.040	0.051	0.101	0.187	19.36	18.55
Var. (n-1)	0.301	0.002	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	4.91	1.26
St. dev (n-1)	0.548	0.042	0.036	0.002	0.004	0.003	0.005	0.009	0.008	0.014	0.040	2.22	1.12
CARIBOU RIVER													
Statistic	SL	HL	BD	JL	SnL	GW	ED	1DS	2DS	PS	PG	LPN	GR
n	17	17	17	17	17	17	17	17	17	17	17	5	17
Min	1.918	0.166	0.093	0.010	0.014	0.009	0.017	0.016	0.023	0.050	0.076	10	17
Max	3.904	0.334	0.221	0.023	0.066	0.021	0.036	0.037	0.049	0.107	0.202	16	21
Mean	2.735	0.241	0.144	0.016	0.024	0.013	0.024	0.026	0.033	0.069	0.129	13.40	18.38
Var. (n-1)	0.430	0.003	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	5.30	1.32
St. dev (n-1)	0.655	0.055	0.040	0.004	0.012	0.003	0.007	0.007	0.009	0.016	0.042	2.30	1.15
THLEWIAZA RIVER													
Statistic	SL	HL	BD	JL	SnL	GW	ED	1DS	2DS	PS	PG	LPN	GR
n	43	43	43	43	43	43	43	43	43	43	43	30	43
Min	2.561	0.238	0.131	0.016	0.021	0.011	0.021	0.027	0.000	0.064	0.049	14	14
Max	3.704	0.340	0.184	0.026	0.038	0.020	0.031	0.044	0.053	0.102	0.201	23	22
Mean	3.189	0.290	0.161	0.020	0.028	0.015	0.026	0.035	0.043	0.085	0.164	19.50	18.09
Var. (n-1)	0.069	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	8.12	2.80
St. dev (n-1)	0.262	0.025	0.011	0.002	0.004	0.002	0.002	0.004	0.008	0.008	0.029	2.85	1.67
KENORA DISTRICT													
Statistic	SL	HL	BD	JL	SnL	GW	ED	1DS	2DS	PS	PG	LPN	
n	30	30	30	30	30	30	30	30	30	30	30	20	
Min	1.979	0.175	0.096	0.011	0.015	0.008	0.015	0.021	0.027	0.045	0.098	4.000	
Max	6.167	0.432	0.259	0.032	0.044	0.028	0.037	0.043	0.059	0.107	0.217	13.000	
Mean	3.794	0.303	0.180	0.021	0.028	0.016	0.027	0.031	0.041	0.078	0.165	6.750	
Var. (n-1)	1.005	0.006	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	4.408	
St. dev (n-1)	1.002	0.075	0.047	0.005	0.008	0.004	0.006	0.006	0.008	0.013	0.039	2.099	
COCHRANE DISTRICT													
Statistic	SL	HL	BD	JL	SnL	GW	ED	1DS	2DS	PS	PG	LPN	
n	10	10	10	10	10	10	10	10	10	10	10	10	
Min	3.804	0.331	0.219	0.024	0.033	0.019	0.028	0.040	0.055	0.114	0.212	6.000	
Max	6.167	0.534	0.350	0.036	0.048	0.029	0.042	0.059	0.081	0.147	0.349	8.000	
Mean	4.904	0.435	0.289	0.031	0.042	0.025	0.037	0.051	0.063	0.126	0.273	6.900	
Var. (n-1)	0.379	0.003	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.322	
St. dev (n-1)	0.616	0.053	0.037	0.004	0.005	0.003	0.004	0.006	0.007	0.010	0.034	0.568	

Appendix F. ANOVA values for all populations of *Gasterosteus aculeatus*. Degrees of freedom (df) =3, **indicates non significant value. (NL: Nueltin Lake, CR: Caribou River, TR: Thlewiaza River, KD: Kenora District, CD: Cochrane District).

STANDARD LENGTH							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	34	3.531	0.301	B/t group	32.669	24.83	<0.0001
CR	17	2.735	0.430	W/in group	42.439		
TR	43	3.189	0.069				
KD	30	3.424	0.667				
CD	10	4.904	0.379				

HEAD LENGTH							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	34	0.312	0.002	B/t group	0.250	25.16	<0.0001
CR	17	0.241	0.003	W/in group	0.321		
TR	43	0.290	0.001				
KD	30	0.303	0.006				
CD	10	0.435	0.003				

BODY DEPTH							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	34	0.202	0.001	B/t group	0.170	36.97	<0.0001
CR	17	0.144	0.002	W/in group	0.149		
TR	43	0.161	0.000				
KD	30	0.180	0.002				
CD	10	0.289	0.001				

JAW LENGTH							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	34	0.021	0.000	B/t group	0.002	32.68	<0.0001
CR	17	0.016	0.000	W/in group	0.002		
TR	43	0.020	0.000				
KD	30	0.022	0.000				
CD	10	0.032	0.000				

SNOUT LENGTH							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	34	0.028	0.000	B/t group	0.002	12.37	<0.0001
CR	17	0.024	0.000	W/in group	0.005		
TR	43	0.028	0.000				
KD	30	0.028	0.000				
CD	10	0.042	0.000				

GAPE WIDTH							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	34	0.017	0.000	B/t group	0.001	24.54	<0.0001
CR	17	0.013	0.000	W/in group	0.001		
TR	43	0.015	0.000				
KD	30	0.016	0.000				
CD	10	0.025	0.000				

GILL RAKERS							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	34	18.594	1.217	B/t group	4.613	1.13	0.3266***
CR	17	18.400	1.400	W/in group	174.930		
TR	43	18.095	2.869				

EYE DIAMETER							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	34	0.029	0.000	B/t group	0.001	14.060	0.00
CR	17	0.024	0.000	W/in group	0.003		
TR	43	0.026	0.000				
KD	30	0.027	0.000				
CD	10	0.037	0.000				

FIRST DORSAL SPINE							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	34	0.040	0.000	B/t group	0.005	29.645	<0.0001
CR	17	0.026	0.000	W/in group	0.006		
TR	43	0.035	0.000				
KD	30	0.031	0.000				
CD	10	0.051	0.000				

SECOND DORSAL SPINE							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	34	0.051	0.000	B/t group	0.007	26.963	<0.0001
CR	17	0.033	0.000	W/in group	0.009		
TR	43	0.043	0.000				
KD	30	0.041	0.001				
CD	10	0.063	0.000				

PELVIC SPINE							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	34	0.101	0.000	B/t group	0.030	50.030	<0.0001
CR	17	0.069	0.000	W/in group	0.019		
TR	43	0.085	0.000				
KD	30	0.078	0.000				
CD	10	0.126	0.000				

PELVIC COMPLEX							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	34	0.187	0.002	B/t group	0.145	27.457	<0.0001
CR	17	0.129	0.002	W/in group	0.171		
TR	43	0.164	0.001				
KD	30	0.165	0.002				
CD	10	0.273	0.001				

LATERAL PLATES							
Groups	Count	Mean	Var.	Sum of Squares		F value	P value
NL	22	19.364	4.909	B/t group	2605.446	135.854	<0.0001
CR	5	19.500	8.115	W/in group	316.441		
TR	14	13.400	5.300				
KD	30	6.750	4.408				
CD	10	6.900	0.322				