THE RELATION BETWEEN DEMOGRAPHICS AND GENETIC INTEGRITY OF WALLEYE *SANDER VITREUS* POPULATIONS IN NORTHERN WISCONSIN

by

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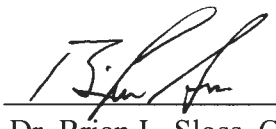

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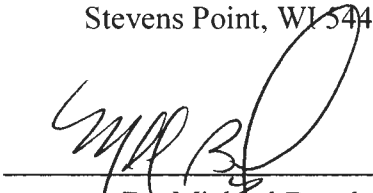

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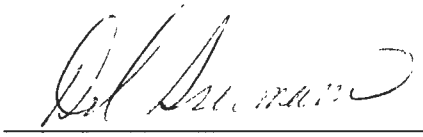

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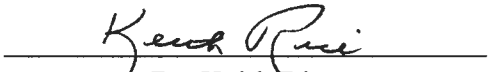

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ABSTRACT

Maintaining genetic integrity is a common objective in the management of both threatened and exploited fish species. A key aspect of genetic integrity is the temporal stability and geographic distribution of genetic diversity. Identifying and understanding the relations between demographics, management activities, and genetic diversity is critical in meeting objectives aimed at conserving genetic integrity. The objectives of this study were to determine if relations exist between the genetic characteristics and demographic variables of northern Wisconsin walleye populations and to determine if walleye growth characteristics were related to genetic or demographic variables in northern Wisconsin walleye. A set of 10 microsatellite loci were used to determine the genetic characteristics of 15 walleye populations by calculating expected heterozygosity (H_e), effective number of alleles (A_e), allelic richness (A_r), the inbreeding coefficient (F_{IS}), individual-specific internal genetic distance measure (mean d^2), mean relatedness (r), and pairwise estimates of genetic distance between populations (φ-st). Long-term monitoring data (1990- 2009) from the Wisconsin Department of Natural Resources and Great Lakes Indian Fish and Wildlife Commission were used to calculate demographic variables including sex ratio, lake surface area (proxy of total abundance), recruitment (YOY), and stocking intensity. Dorsal spine samples were used to determine age and early growth characteristics of each population. Relative condition factor (Kn) was used to measure the mean condition of fish in each population. Simple linear and forward stepwise regression modeling was used to determine the relation between each genetic characteristic and demographic variable. The results of this study showed significant relations between genetic and demographic characteristics of walleye populations.

Genetic characteristics were strongly related to demographic variables; for example, YOY and age predicted over 70% of the variance in inbreeding F_{IS} estimates (df = 12, F = 18.403, $p < 0.001$). An observed skew in the sex ratio correlated to decreased effective population size. This likely resulted in increased rates of genetic drift and subsequently, the observed lower genetic diversity measures. The genetic drift may have further impacted the population as demonstrated by a decrease in age-1 length in populations with lower levels of genetic diversity (df = 13, F = 6.109, $p = 0.028$). Stocking had significant impacts on both intra- and inter-population genetic diversity. Stocked populations had significantly higher levels of genetic diversity relative to non-stocked populations consistent with relations between stocking intensity and both H_e and A_e. Stocking also correlated to an observed disruption in the pattern of interpopulational genetic diversity. A Mantel test showed a significant interaction between geographic distance and genetic differentiation in non-stocked populations (1,000 permutations, Z = 5,154.65, $p = 0.019$) but no pattern was observed in stocked populations (1,000 permutations, Z = 1,117.89, $p = 0.068$) indicating stocking may disrupt the natural distribution of genetic diversity among walleye populations (genetic structure). Overall, results showed significant influences of demographics on the genetic integrity of walleye in Wisconsin. Low levels of recruitment, highly skewed sex ratio, and stocking intensity appeared to pose threats to the genetic integrity of walleye and should be considered in management programs aimed at conserving the genetic integrity of naturally recruiting populations.

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I am grateful to my friends and family who stood by me and by whose company I have been enriched. From my mother, I gained compassion. From my father, I gained an appreciation of critical thinking. From my sisters, Melinda and Christine, I have gained true friends that have always been there for me. From my friends, I have gained companionship and perspective. From nature, I have gained a sense of wonder and awe that motivates me beyond measure to seek knowledge and understanding.

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INTRODUCTION

Genetic integrity can be defined as the temporal stability of genetic diversity within and among the populations of a species. Genetic diversity provides the raw material needed for adaptive evolution to occur (Allendorf and Luikart 2008) thus ensuring the viability of a population (Quattro and Vrijenhoek 1989). Connections have been demonstrated between genetic diversity and fitness in a broad range of taxa (Reed and Frankham 2003) including numerous fish populations (Kartavtsev 1998; Thelen and Allendorf 2001; Wang et al. 2002). Because of the importance of genetic diversity, the protection of genetic integrity is often a primary goal in the management of fish species (Vrijenhoek 1998; Wang et al. 2002).

In 1998, the Wisconsin Department of Natural Resources (WDNR) developed a statewide Walleye (*Sander vitreus*) Management Plan to better guide management decisions (Hewett and Simonson 1998). The Walleye Management Plan includes population, genetic, and management specific goals. The genetic goal is to maintain the genetic integrity of naturally reproducing populations. The objectives of this goal are to determine and utilize any performance benefits of genetically distinct stocks, to examine the influence of historical stocking on population genetics, and to ensure stocking does not have a negative impact on lakes with naturally reproducing walleye populations. To meet these goals and ensure the genetic integrity of walleye, as well as other managed fish species, it is important to understand factors influencing the genetic diversity of fish populations. The explicit goal of conserving walleye genetic integrity presents not only a significant management consideration of the WDNR but also a framework to study the genetic effects of demographics in a managed fish species.

There is a need to better understand how demographic variables affect the genetic integrity of managed fish species. Wisconsin's abundant walleye resource presents an opportunity to couple long-term demographic data with polymorphic genetic data to determine potential connections between genetic and demographic characteristics of a managed fish species. The goal of this project was to identify factors influencing the genetic integrity of walleye populations to ensure the conservation of genetic resources. The first objective of this study was to determine if a relation exists between the genetic characteristics and demographic variables of walleye populations in northern Wisconsin by comparing the genetic diversity within and among populations with varying total abundances, sex ratios, recruitment levels, stocking intensities, and mean ages. The second objective of this study was to determine if walleye growth characteristics were related to genetic or demographic variables in northern Wisconsin walleye populations.

In general, genetic diversity has two main components, the diversity among individuals within a population (intrapopulational diversity) and diversity among populations within a species (interpopulational diversity or genetic structure; Allendorf and Luikart 2008). Intrapopulation diversity is important for ensuring the adaptability and, subsequently, the viability of a specific population. Cumulatively, populations of a species, each with their own unique genetic composition (interpopulation variation), exhibit genetic structure across the landscape largely influenced by migration routes and connectivity (Manel et al. 2003). This structure is influential in predicting the probability of shared, local (i.e., population-level) adaptations among populations. Stepien et al. (2009) found strong evidence of genetic structure in walleye across their native range corresponding to geographic isolation on a range-wide scale and emphasized the

importance of conserving this genetic diversity. Identifying and understanding this structure is a central tenet of the stock concept in fisheries (Berst and Simon 1981) and a foundation of contemporary fisheries management.

An initial step to better understand the genetic integrity of Wisconsin walleye was to determine the current levels of intra- and inter-population genetic diversity. Hammen (2009) assessed the genetic diversity of 26 naturally recruiting walleye populations in the ceded territories of northern Wisconsin using a standard set of microsatellite loci. Levels of intrapopulation genetic diversity in Wisconsin walleye were higher or comparable to populations in northern Minnesota (Borer et al. 1999; Eldridge et al. 2002), Quebec (Wirth et al. 1999) and Ontario (Cena et al. 2006). Furthermore, interpopulation levels of genetic divergence were significant in most pairwise comparisons consistent with an underlying pattern of hierarchical genetic structure. Using the pattern of genetic divergence observed among populations, Hammen (2009) identified eight genetic units that were largely congruent with geographic location (Figure 1). These data indicated walleye have a natural genetic structure consistent with isolation caused by landscape resistance to gene flow and, coupled with the high levels of intra- and inter-population genetic diversity, suggest local adaptations likely exist in Wisconsin's walleye populations.

To ensure the genetic integrity of naturally recruiting walleye populations, it is important to consider factors influencing the genetic structure found in Wisconsin. A primary management technique known to impact the genetic structure and integrity of fish populations is hatchery propagation or stocking (Halbisen and Wilson 2009; Marie et al. 2010). Genetic concerns with fish stocking include the use of inappropriate genetic

sources (Englbrecht et al. 2002), insufficient numbers of broodfish resulting in increased levels of relatedness (Aho et al. 2006), and overrepresentation of a small number of family groups resulting in lowered genetic diversity (Ryman and Laikre 1991; Ryman et al. 1995). All of these factors can result in the disruption of genetic integrity and, thus, potentially conflict with the stated desire to conserve walleye genetic integrity in Wisconsin.

Evidence exists that stocking may have impacted the genetic structure and integrity of some of Wisconsin's naturally recruiting walleye populations. The geographic boundaries of genetic units delineated by Hammen (2009) were not consistent with the current walleye management units (Fields et al. 1997) that are largely based on watershed boundaries. If stocking has occurred across the boundaries of genetic units, it is possible the natural genetic structure of Wisconsin walleye has been disrupted. The genetic impact of stocking has been proposed as an explanation for many of the anomalies observed in the genetic structure of walleye over broad geographic regions (Billington et al. 1992; Stepien and Faber 1998; Hammen 2009; Stepien et al. 2009). Currently, ongoing research is addressing the apparent disjunct between watershed boundaries and genetic structure of Wisconsin walleye.

Stocking may also be influencing the genetic integrity of walleye by adding genetic material to populations. Cena et al. (2006) determined there was a positive relation between stocking intensity and genetic diversity in Ontario walleye populations. A similar pattern has been seen in Wisconsin walleye; Franckowiak et al. (2009) showed stocking affected the genetic integrity of the walleye population inhabiting Escanaba Lake. Their study examined 50+ years of genetic diversity data using archived scale

samples and microsatellite genetic markers to reveal an influx of non-native alleles into Escanaba Lake associated with stocking events. The episodic influx of alleles led to a temporary increase in the genetic diversity of the population but ultimately replaced the original gene pool with a composite of stocked and original genes. Similar disruptions of genetic integrity in other fish species have been observed in Atlantic salmon (*Salmo salar*; Finnegan and Stevens 2008), brook charr (*Salvelinus fontinalis*; Marie et al. 2010), arctic charr (*Salvelinus umbla*; Englbrecht et al. 2002), and lake trout (*Salvelinus namaycush*; Halbisen and Wilson 2009). For example, Marie et al. (2010) showed a loss of genetic integrity was correlated with stocking intensity indicating that increasing the number of stocking events may increase the genetic impact of stocking. While Franckowiak et al. (2009) showed stocking disrupted the genetic integrity of one Wisconsin walleye population, the impact may not be uniform in all populations. In fact, other factors such as ecological integrity may moderate or negate the detrimental genetic influence of stocking (Englbrecht et al. 2002).

The genetic integrity of a population is largely determined by its effective population size (N_e) (Allendorf and Luikart 2008). The effective population size is defined as the size of an idealized population with the same level of genetic drift as the population in question (Waples 1989) and thus, N_e determines the rate of genetic drift, inbreeding, and the fixation of deleterious alleles in the population (Schwartz el al. 1998). In essence, this measure can be thought of as the 'genetic-size' of the population and is nearly always less than the census size (N_c), in some cases significantly less (Vucetich et al. 1997; Turner et al. 2002; Shrimpton and Heath 2003). Vucetich et al. (1997) suggested a ratio of N_e:N_c around 0.25 may be common in animal populations, however,

previous research has shown walleye populations in Wisconsin exhibit even lower N_e:N_c ratios (Franckowiak et al. 2009; Hammen 2009). Franckowiak et al. (2009) showed the ratio N_e:N_c of the Escanaba Lake walleye was surprisingly low, approximately 0.04 and suggested this may have affected the rate of genetic drift in the population. This low N_e:N_c ratio may be common for many naturally reproducing walleye populations in northern Wisconsin. Hammen (2009) reported the majority of 26 sampled walleye populations in northern Wisconsin had a low N_e compared to inferred population sizes (N_e:$N_c \leq 0.20$). Three primary demographic characteristics strongly influence N_e: fluctuations in population size (Vucetich et al. 1997), variance in family size (Frankham 1995; Hedrick 2000; Allendorf and Luikart 2008), and skewed sex ratios (Nunney 1993).

Extreme population fluctuations lead to decreases in N_e since, in general, the generation with the smallest N_c will have the greatest effect on N_e (Allendorf and Luikart 2008). The census size of many fish populations is highly variable. For example, Hutchings and Reynolds (2004) document a median reduction of 83% in the breeding population size of 230 marine fish populations. Vucetich et al. (1997) showed fluctuations in N_c could be the prevalent cause of a low N_e in most animal populations. For instance, Chinook salmon (*Oncorhynchus tshawytscha*) population fluctuations were shown to have a powerful effect on the N_e potentially decreasing the viability of small populations (Shrimpton and Heath 2003). In walleye, population size is largely a function of lake surface area; larger lakes are able to support larger populations of walleye (Nate et al. 2000). Since fluctuations in population size are inversely related to the total size of the population (Lande 1993), it follows walleye populations inhabiting larger Wisconsin lakes would have lower fluctuations in population size. This trend

would explain the observed relation between lake surface area and N_e in northern Wisconsin walleye (Hammen 2009) suggesting lake surface area is an important determinate (as a surrogate of census size) of the N_e of walleye populations.

The reproductive characteristics of walleye likely influence the N_e of Wisconsin populations (Franckowiak et al. 2009). The high fecundity (Malison and Held 1996) and juvenile mortality (Hartman 2009) of walleye may subject populations to elevated variances in reproductive success among individuals and thus influence N_e. Franckowiak et al. (2009) observed this interaction and suggested negative relation between the variance in reproductive success among individuals and the N_e of Escanaba Lake walleye. This finding complements the conclusions of Gaggiotti and Vetter (1999) that stressed the importance of both the total reproductive output of the population and the generational overlap to the N_e of pelagic fish populations. Their findings indicated populations with a higher reproductive output had values of N_e that were closer to the census population size. This study also showed a large generational overlap, similar to walleye, mitigated environmental perturbations to N_e. Walleye recruitment is highly variable both within (Hansen et al. 1998) and among populations (Beard et al. 2003a) signifying that variance in reproductive success could be playing an important role in the genetic integrity of walleye in Wisconsin.

Some mating systems lead to a natural bias in sex ratios that can influence N_e (Nunney 1993). A skewed sex ratio limits the evenness of the genetic contribution of individuals to the next generation thereby reducing N_e (Allendorf and Luikart 2008). In addition to natural biases in sex ratios, sex ratio biases in a hatchery setting have strong influence on genetic diversity when supplemental stocking occurs (Bartley et al. 1992;

Turner et al. 2002). Furthermore, sex-biased exploitation can lead to changes in the sex ratio and dramatic reductions in genetic diversity because of a restricted N_e (Ryman et al. 1981). Walleye commonly exhibit a skewed sex ratio during spawning periods (Schneider et al. 2007), are extensively stocked in Wisconsin (Kerr 2008), and are exploited for recreational and tribal harvest (Beard et al. 2003b). These factors increase the likeliness that sex ratios are influencing the genetic integrity of walleye in Wisconsin.

Genetic diversity may provide fitness-related benefits to populations (Hansson et al. 2001; Thalen and Allendorf 2001; Wang et al. 2002) that may help to ensure the long-term sustainability of walleye populations. A major factor linking genetic diversity to fitness is the negative influence of inbreeding on both genetic diversity and fitness (Hansson and Westerberg 2002). Inbreeding can cause serious losses of fitness due to inbreeding depression (Crnokrak and Roff 1998) where deleterious recessive alleles are expressed as homozygotes. There is evidence inbreeding affects walleye. Cena et al. (2006) found higher genetic diversity was associated with increased somatic growth in Ontario walleye populations. They concluded this pattern was likely due to the negative impacts of inbreeding on both fitness and genetic diversity. While several walleye populations with significant genetic indications of inbreeding have been documented in northern Wisconsin (Hammen 2009), it has not been resolved if the observed levels of inbreeding or associated losses of genetic diversity have had any negative biological impacts (i.e., inbreeding depression).

To maintain the genetic integrity of walleye populations it is necessary to determine possible threats to genetic diversity and the genetic impacts of management practices. Establishing a connection between genetic diversity and demographic

variables can help identify demographic trends that could be threatening the genetic integrity of walleye populations. Informed responses to genetic threats in walleye (and other fish species) will be improved by assessing the genetic impact of management practices.

METHODS

Experimental Design

Three primary determinants were considered when selecting walleye populations for this study: genetic structure, availability of demographic data, and recruitment category. To minimize the genetic and demographic variance induced by sampling a broad geographic region that may contain several genetic units of walleye (Stepien and Faber 1998; Dupont et al. 2007; Hammen 2009; Stepien et al. 2009), study sites were restricted to a cohesive genetic unit located in Oneida and Vilas counties (Hammen 2009). To be considered for inclusion in this study, lakes were required to have a minimum of 10 annual young-of-the-year (YOY) estimates and one population estimate after 1990 with the most recent estimate of each during or after 2005. Walleye YOY and adult population estimates were conducted by the Wisconsin Department of Natural Resources (WDNR) and the Great Lakes Indian Fish and Wildlife Commission (GLIFWC) as described by Cichosz (2009). Recruitment classification codes (Table 1; U.S. Department of Interior 1991) are used, in part, to manage walleye in Wisconsin. Three commonly used recruitment codes represent populations with some level of natural recruitment (Nate et al. 2000) and thus, the potential to show genetic integrity. These codes are naturally recruiting (NR), stock augmented (C-ST), and primarily stocked (C-ST) populations. Efforts were made to equally represent these three categories.

Fifteen lakes (Table 2; Figure 2) were sampled for walleye during the spawn (late April to mid-May of 2010 and 2011). To collect an unbiased sample from each population the majority of appropriate shoreline spawning habitat (see Raabe 2006) in each lake was electrofished (Rogers et al. 2005). A target sample size of 50 was used to represent the genetic diversity of each population (Ruzzante 1998). Each sample was

measured for total length (nearest mm) and weight (nearest 5g). Sex was determined, when possible, by the extrusion of gametes. A small anal fin clip (~15mm) was taken for subsequent genetic analysis in accordance with the University of Wisconsin-Stevens Point, Molecular Conservation Genetic Laboratory's standard operating procedure and preserved individually in labeled tubes containing 95% non-denatured ethanol. The third spine from the anterior end of the dorsal fin was taken for age and growth analysis by cutting the spine as close to the body as possible. All spines were stored in labeled coin envelopes.

The purpose of this study was to connect current genetic characteristics with demographic variables in a managed fish species using polymorphic genetic data and long-term monitoring data on walleye. Demographic variables included: population estimates, population sex ratios, a recruitment index, stocking intensity, and the mean age of the population. To determine if these demographic variables related to key aspects of genetic integrity each variable was compared to measures of intrapopulation genetic diversity using simple linear and forward stepwise regression modeling. This approach allowed the discrimination of salient demographic variables with respect to potential consequences on the genetic integrity of walleye. The influence of stocking on interpopulation genetic diversity was assessed by estimating the natural genetic differentiation among populations and determining if this pattern was disrupted in stocked populations. Potential impacts on population-specific walleye growth characteristics were assessed by determining if there were connections between demographic and genetic variables and either the growth rate or condition of the sampled populations.

Demographic Characteristics

Primary demographic characteristics used in this study were estimates of population size, sex ratio, recruitment, measures of stocking intensity, and the mean age of the adult spawning population (3+ years old). Long-term monitoring data from the WDNR and GLIFWC was used to generate estimates of population size and recruitment. In many lakes, the WDNR estimates walleye populations for adult spawning densities and young-of-the-year (YOY) recruitment to ensure sufficient information is available to make informed management decisions (Hewett and Simonson 1998). Lakes are selected annually for assessment using a stratified random design that incorporates lake size, historic level of harvest, and primary recruitment source (Cichosz 2009). Population assessments consist of a spring mark-recapture based estimate using fyke netting for marking and electrofishing for recapture. Population estimates were calculated based on the Chapman modification of the Petersen estimator (Chapman 1951).

The relation between walleye census population size (N_c) and lake surface area (Nate et al. 2000) is commonly used when making management decisions because of the lack of direct population estimates (Staggs et al. 1990). To determine the relation between lake surface area and N_c in the sampled populations, a linear regression was performed using the $\log_e$ lake surface area and the $\log_e$ mean walleye population estimates from 1990 to 2009. A strong relation was found ($R^2 = 0.768$, df = 13, F = 43.110, $p < 0.001$; Figure 5); therefore the $\log_e$ lake surface area was used as a proxy of walleye N_c to allow a more standardized assessment across populations with varying availabilities of demographic data.

Since the sex ratio of a population can drastically influence the effective population size (Ryman et al. 1981; Frankham 1995), sex ratios were calculated for each sampled population. The sex ratio was calculated by using the mean ratio of males to females reported from WDNR and GLIFWC population estimates from 1990 to 2009. This method of determining the sex ratio of walleye populations results in a male bias due to behavioral differences of the sexes (Schneider et al. 2007). However, this bias would likely be systematic across all populations and therefore the relative differences in the sex ratios among populations still held biological relevance.

The WDNR and GLIFWC estimate walleye YOY recruitment using a catch per mile (CPUE) index of fall electrofishing (Cichosz 2009). Hansen et al. (2004) concluded YOY density is nonlinearly related to CPUE and can be significantly influenced by water temperature. To better estimate the mean density of YOY for each lake, CPUE data was transformed into a density metric using the relation of Hansen et al. (2004):

$$\frac{C}{f} = 31{,}101\left(\frac{N}{A}\right)^{0.686} * Temp^{-2.045}$$

where *C/f* refers to the CPUE, *N/A* refers to the density (numbers/acre) of YOY, and *Temp* is the recorded water temperature ($^{\circ}$F) during the survey. Mean YOY densities were calculated for each population using all available estimates from 1990 to 2008. Mean YOY density was then multiplied by lake surface area to give a mean YOY abundance for each lake.

Stocking records from 1998 to 2009 reported from the WDNR were used to measure the intensity of stocking in each population. The hatchery effort index used by Cena et al. (2006) was modified to account for the differential survival of size classes of

walleye used for stocking in Wisconsin (Table 3; WDNR 1999). The stocking index (SI) used in this study was calculated as:

$$SI = \frac{\sum surv_i \times YOY_i}{log_e(SA) \times t}$$

where $surv_i$ refers to the expected survival to age-3 of the *i* size class as reported by WDNR (1999), YOY_i refers to the number of *i* size class walleye stocked, *t* indicates the number of years from first recorded stocked event to the most recent event, and *SA* refers to the lake surface area (ha).

Relative Condition Factor

Relative condition is measured using the ratio of a fish's weight to its standard weight based on growth coefficients established from the length-weight relation (LeCren 1951; Blackwell et al. 2000). Walleye growth coefficients were determined using length (*L*) and weight (*W*) data from all samples to solve the following length-weight model:

$$log_{10}(W) = log_{10}\alpha + \beta * log_{10}(L)$$

The standard weight (*W'*) was then calculated for each sample by:

$$W' = \alpha L^{\beta}$$

Relative condition factor (*Kn*) was calculated for each sample as follows:

$$Kn = {}^{W}/_{W'} * 100$$

A mean Kn was then calculated for each population.

Age and Growth Analysis

To estimate early growth and the age of each fish, back-calculation techniques were applied to dorsal spine samples as described in Borkholder and Edwards (2001). A

Dremel®,[1] 300 series cutting tool (Dremel Company, Racine, WI) with a 7/8" fine toothed blade (5231-1189220, Pfingst and Company, Inc., South Plainfield, New Jersey) was used to cut thin sections from each spine sample. The proximal end of each spine was cut to expose a clean portion. Three thin sections of each spine were cut and mounted on a glass microscope slide with cyanoacrylic cement. The cement was allowed to dry before the thin section was wetted and sanded with 1,000 grit wet-dry sandpaper. These sections were examined under a Nikon® SMZ 1500 (Nikon Instruments Inc., Melville, NY) dissecting microscope with a mounted Spot™ 14.2 Color Mosaic digital camera (Diagnostic Instruments, Sterling Heights, MI). A drop of Type B immersion oil (R.P. Cargille Laboratories, Inc., Cedar Grove, NJ) was used on the thin section that had the most discernable annuli. A digital image was taken for later analysis and the magnification of the image was recorded. Age was estimated by two independent observers. In cases where initial age assignment disagreed between readers, a consensus age was agreed upon by both readers. Once the age analysis was complete, fish < 3 years of age were excluded from all subsequent analyses.

The total radius and the distance to the outside edge of each annulus were measured on spine images using SPOT™ software (Diagnostic Instruments, Sterling Heights, MI). These measurements were primarily conducted using the anterior elongated spine transect; however, alternative transects were used when the anterior elongated transect was not clearly resolved (Figure 3; Borkholder and Edwards 2001). The length at which walleye developed dorsal spines was estimated by determining the relation between length at capture (mm) and total spine radius (mm). The intercept of

[1] The use of trademark names does not imply endorsement by the U.S. Government, State of Wisconsin, and/or the University of Wisconsin-Stevens Point.

this linear regression was used as an estimate of the mean length (K) walleye in this study developed dorsal spines. The length at capture (L_c), K, total spine radius (S_c), and the distance between the spine focus and the i^{th} annuli (S_i) were used to back-calculate length at age (L_i) using the Fraser-Lee formula (Fraser 1916; Lee 1920):

$$L_i = K + (L_c - K) * (S_i/S_c)$$

The mean L_i at each of the first three annuli was calculated for each population.

Genetic Analysis

Genetic methods followed standard lab protocols of the Molecular Conservation Genetics Laboratory at the College of Natural Resources, University of Wisconsin-Stevens Point (Appendix A). DNA was extracted using the Promega Wizard® Genomic DNA purification kit (Promega Corp., Madison, WI) following a standard protocol for 96-well plate extractions. Extracted DNA was quantified using a Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and normalized to a standard 20 ng/µL concentration. Polymerase chain reaction (PCR) was used with 10 previously designed primers to amplify and isolate microsatellite loci (Table 4; Borer et al. 1999; Wirth et al. 1999; Eldridge et al. 2002) using three multiplex reactions (Table 5). Genotyping was done on an ABI 3730 DNA Analyzer (Applied Biosystems, Inc., Foster City, CA) using an in-lane standard (Geneflo™ 625, Chimerx, Inc. Milwaukee, WI). Resulting genotypes were identified using GeneMapper® 4.0 (Applied Biosystems, Inc., Foster City, CA) and compiled using Microsoft Office Excel© 2010 v.14.0.6 (Microsoft Corporation, Redmond, WA). For a genotype to be included in the data set, a

minimum of seven successfully genotyped loci were required. To ensure consistency of the genotype data, 10% of the samples were genotyped a second time.

All loci were tested for deviations from Hardy-Weinberg expectations (HWE) in each population using a Chi-square test implemented in GenAlEx v6.4 (Peakall and Smouse 2006) with a sequential Bonferroni correction (Rice 1989). To reduce the effects of highly polymorphic loci (Pamilo and Varvio-Aho 1948) rare genotypes (expected frequency < 1) were pooled into one observed and expected frequency value as recommended by Hedrick (2000). Linkage disequilibrium was tested between all pairs of loci in each population using the exact test of Guo and Thompson (1992) implemented in GENEPOP 4.0 (Raymond and Rousset 1995; Rousset 2008) with a Markov chain method of 10,000 dememorization steps, 100 batches, and 10,000 iterations/batch. Evidence of null alleles, sequence stutter, and typographic errors was examined using MICRO-CHECKER v.2.2.3 (Oosterhout et al. 2004).

Genetic Characteristics

The genetic diversity of each population was assessed using expected heterozygosity (H_e), effective number of alleles (A_e), allelic richness (A_r), the inbreeding coefficient F_{IS}, d^2, and mean pair-wise relatedness (r). Heterozygosity is a commonly used metric of genetic diversity (Allendorf and Luikart 2008) and is often linked to population fitness (Reed and Frankham 2003; Wang et al. 2002). Effective number of alleles is a related metric of genetic diversity defined by the number of equally frequent alleles that, in an ideal population, would result in the actual H_e of the sampled population (Allendorf and Luikart 2008). Microsatellite Toolkit v3.1 (Park 2001) was

used to calculate H_e and GenAlEx v6.4 (Peakall and Smouse 2006) was used to calculate A_e for each population. Allelic richness provides an estimate of the mean number of alleles per locus and reflects the long-term adaptability of a population (Petit et al. 1998). HP-RARE v1.0 (Kalinowski 2005) was used to estimate A_r using a rarefaction method described by Leberg (2002) to account for biases caused by unequal sample sizes.

Inbreeding was assessed in each population using F_{IS} and mean d^2. The coefficient, F_{IS}, measures the relative increase in observed heterozygosity relative to expected heterozygosity (Wright 1922) and is expressed as a metric that increases from 0 to 1 with increasing levels of inbreeding. The inbreeding coefficient was calculated for each population and tested for statistical deviations from zero using ARLEQUIN v3.11 ($\alpha = 0.05$). Mean d^2 is measure of genetic diversity known for its sensitivity to historic inbreeding events in the population (Coulson et al. 1998). Fitness-related traits have been shown to be positively related to mean d^2 (Coulson et al. 1998; Hansson et al. 2001; Rossiter et al. 2001) and, specifically in walleye, mean d^2 was positively related to female early growth (Cena et al. 2006). Mean d^2 was calculated as the average squared differences in repeat units between alleles at each locus of an individual:

$$d^2 = \sum_{i=1}^{n} \frac{(i_a - i_b)^2}{n}$$

where i_a and i_b are the number of repeat units of alleles *a* and *b* at locus *i*, and *n* was the total number of scored loci for the sample. Individual mean d^2 values within a population were averaged to generate population specific mean d^2 values.

The coefficient of relationship between pairs of individuals can be estimated using genetic markers to approximate relatedness (r) in lieu of pedigree information (Lynch and Ritland 1999). This is an important consideration for the conservation of

genetic diversity since r measures the probability two alleles in separate individuals are identical by descent and therefore is related to N_e and inbreeding in the population. Estimates of r were calculated between all pairs of samples in each population using a maximum likelihood method implemented in ML-RELATE (Kalinowski et al. 2006) and mean r values for each population were calculated.

Statistical Analysis

Prior to statistical analysis, all variables were tested normality using a Shapiro-Wilk test in SYSTAT® v.11.0 (SYSTAT Software, Inc., Chicago, IL). Non-normally distributed variables were $\log_e$ transformed and retested for normality. Co-linearity among all variables was determined using a correlation matrix constructed in Minitab® v.15.1.1 (Minitab Inc., State College, PA). To relate demographic variables to genetic characteristics it was important to establish if demographic variables differed among populations. An ANOVA was used in PASW® Statistics v18.0.0 (SPSS Inc., Chicago, IL) to determine if sex ratios, recruitment, age, condition, and length at ages-1, -2, and -3 varied by population.

A series of statistical analyses were performed to determine the relation between genetic characteristics and demographic variables. First, differences in genetic characteristics (H_e, A_e, A_r, F_{IS}, d^2, and r) between stocked and non-stocked lakes were tested using a series of two-tailed T-test implemented in PASW® Statistics v18.0.0. Second, simple linear regressions were constructed relating each genetic characteristic to each demographic variable ($\log_e$[SA], sex ratio, YOY, SI, and age). All models were tested for significance using PASW® Statistics v18.0.0 both at the $\alpha = 0.05$ and following

sequential Bonferroni correction ($\alpha_1 = 0.01$). Scatter plots were visually examined for potential nonlinear relations and residuals of significant models were tested for normality using a Shapiro-Wilk test in SYSTAT® v.11.0. Trends between the model residuals and the independent variable were inspected using both a linear and a second order polynomial function. Any function explaining a significant portion of the variation in the residuals ($R^2 > 0.25$) was flagged and the model was checked for non-linear trends. Confidence intervals were calculated for each significant linear model. Forward stepwise regression analysis was used to consider more complex interactions between genetic characteristics and demographic variables. The best-fit demographic model was selected for each genetic characteristics using PASW® Statistics v18.0.0 ($\alpha = 0.05$ to enter, $\alpha = 0.10$ to remove).

A similar approach was used to test the relation between the growth characteristics to genetic and demographic variables. First, simple linear regression models were constructed between growth variables (length at age-1, -2, -3, and Kn) and all demographic and genetic variables. These models were tested for significance at the 0.05 α-level and after sequential Bonferroni corrections ($\alpha_1 = 0.0045$). Next, forward stepwise regression analysis was conducted relating either mean length at age-1, -2, and -3 or Kn of each population with genetic and demographic characteristics.

Genetic and Geographic Distances

Populations are expected to diverge genetically across the landscape due to landscape resistance to gene flow (Manel et al. 2003). Isolation-by-distance (IBD) is a common model used to describe this process and indicates the genetic differentiation of

individuals and populations increase with respect to geographic distance. To determine if walleye genetic divergence across the sampled populations was consistent with IBD expectations, a Mantel test was performed. Genetic distances were determined between all pairs of non-stocked populations using φ-st (Excoffier et al. 1992) as implemented in GenAlEx v6.4 (Peakall and Smouse 2006) with 10,000 permutations to determine significant deviations between populations. Pairwise geographic distances were calculated between the centers of each non-stocked lake. The genetic and geographic matrices were compared for significant interactions using a Mantel test with 1,000 iterations in GenAlEx v6.4 (Peakall and Smouse 2006). To test if this pattern was disrupted in stocked populations, this process was repeated for all pairwise combinations of stocked populations.

RESULTS

Demographic Characteristics

A total of 849 walleye were sampled from 15 lakes (Table 2; Figure 2). Sample sizes varied from 44 to 88 with a mean sample size of 57 fish. Eight populations were sampled in the spring of 2010, all Pelican Lake samples and half of Kawaguesaga Lake samples were collected in the fall of 2010, and five populations were sampled in the spring of 2011. Populations had a mean of four adult population estimates between 1990 and 2009 with two populations having only one estimate and one population with seven estimates (Table 6). Populations had a mean of 17 YOY estimates between 1990 and 2008 with a minimum of 10 and a maximum of 22 estimates. A Shapiro-Wilk test indicated YOY and SI were the only demographic variables not normally distributed (SW statistic = 0.847, $p = 0.015$ and SW statistic = 0.807, $p = 0.005$, respectively). A $\log_e$ transformation normalized YOY abundance but not SI (SW statistic = 0.899, $p = 0.092$ and SW statistic = 0.772, $p = 0.002$, respectively), therefore the $\log_e$ of YOY was used in subsequent analyses and no further efforts were made to normalize SI.

Demographic characteristics varied among populations (Table 6). The mean $\log_e$ of lake surface area was 6.70 with a minimum of 5.15 and a maximum of 7.84. The mean sex ratio (M:F) was 4.26 (SE = 0.72). An ANOVA of populations with > 2 population estimates showed a significant difference in the sex ratios of populations (df = 12, F = 2.121, $p = 0.038$). The $\log_e$ mean YOY abundance was 9.24 (SE = 0.53). An ANOVA indicated the difference in YOY abundance among populations was significant (df = 14, F = 2.576, $p = 0.002$). Two C-NR and all five NR lakes had no record of stocking from 1998 to 2009 while the remaining eight lakes had a mean SI of 59.55 with

a minimum of 13.09 and maximum of 132.15. The mean age of all samples in this study was 5.98 (SE = 0.37) and age significantly differed among populations (df = 14, F = 23.107, $p < 0.001$).

Relative Condition Analysis Results

There was a significant relation between $\log_{10}$ length and $\log_{10}$ weight (df = 808, F = 38,476.4, $p < 0.001$; Figure 4). Based on this relation, the most appropriate value for α and β, used in calculating relative condition, were 2.051×10^{-6} and 3.236, respectively. Therefore, the model:

$$W' = (2.051 * 10^{-6} * L)^{3.236}$$

was used to estimated W' when calculating Kn. The mean population relative condition factor was 100.58 (SE = 1.09; Table 7). The mean relative condition factor differed significantly among populations (df = 13, F = 16.498, $p < 0.001$). While sampling Pelican Lake, the scale used to determine fish weight malfunctioned; therefore, Pelican Lake fish were not included in the relative condition analysis.

Growth Analysis Results

The relation between length at capture and the total spine radius using the AE transect (df = 824, F = 2450.68, $p < 0.001$; Figure 6) indicated walleye first develop dorsal spines at 29.75 mm in length. The equation used to back-calculate length was:

$$L_i = 29.75 + (L_c - 29.75) * (S_i/S_c)$$

Mean length at age-1, -2, and -3 was 142.0 (SE = 3.3 mm), 232.4 mm (SE = 4.8) and 304.0 mm (SE = 6.3), respectively (Table 8). Back-calculated length at age-1, -2, and -3

differed significantly among populations (df = 14, F = 12.715, p = < 0.001; df =14, F = 16.062 p < 0.001; df = 24.477, p < 0.001, respectively).

Genetic Characteristics

The genetic data used in this study was collected with a high degree of fidelity and met all the assumptions of neutral genetic data. Re-genotyping 10% of the samples showed a low genotyping error rate (0.73%). Initial Chi-square analysis showed 10% (15/150) of all comparisons were significantly different from HWE expectations (α = 0.05). Following the binning of rare genotypes and sequential Bonferroni correction (α_1 = 0.000313) no comparisons were significantly different from HWE expectations. No evidence of linkage disequilibrium was observed. Three populations and two loci showed evidence of null alleles (TWS for Svi-L9 and THU and PEL for Svi-20). However, expected frequencies of null alleles were low (< 0.1) and no evidence of null alleles at a given locus was observed in more than two populations. Therefore, no correction was performed.

Genetic diversity was mostly high with mean H_e of 0.767 (SD = 0.009), mean A_e of 4.49 (SD = 0.13), and mean A_r of 9.34 (SD = 0.41; Table 9). The inbreeding coefficient (F_{IS}) averaged 0.003 (SD = 0.243). The inbreeding estimate for Thunder Lake (0.051) was the only significant F_{IS} estimate at the 0.05 α-level; this estimate was not significant after a sequential Bonferroni correction. The mean d^2 across all sampled populations was 24.94 (SD = 2.53). The mean relatedness (r) was 0.053 (SD = 0.003). A Shapiro-Wilk test indicated each genetic diversity measure was normally distributed.

Significant genetic divergence occurred among all sampled populations (Table 10). Estimates of φ-st between populations had a mean of 0.025. Approximately 55% (58/105) of these comparisons were significant after sequential Bonferroni corrections (α_1 = 0.00047). The geographic distance between populations had a mean of 33.3 km (Table 11). Tests of isolation-by-distance (IBD) showed non-stocked populations were mostly consistent with an IBD model but stocked populations were inconsistent with an IBD model (Figure 7). The Mantel test showed a significant interaction between geographic and genetic distance in non-stocked populations (1,000 permutations, Z = 5,154.65, p = 0.019) but not in stocked populations (1,000 permutations, Z = 1,117.89, p = 0.068). The linear regression between genetic and geographic distance for non-stocked populations fell outside the 95% confidence intervals for the same regression in stocked populations.

Comparison between Genetic Characteristics and Demographic Variables

Stocked and non-stocked populations differed significantly in several genetic measures (Table 12). Both H_e and A_e were significantly higher in stocked populations than in non-stocked populations (df = 13, t = -2.620, p = 0.021; and 1 df = 13, t = -3.145, p = 0.008, respectively). Mean relatedness (r) was significantly higher in non-stocked populations (df = 13, t = 2.715, p = 0.018).

The correlation matrix showed significant relations among genetic, demographic, and length variables (Table 13). The low degree of correlation suggested all genetic parameters presented mostly independent measures of genetic characteristics. Two significant correlations were found between demographic variables, YOY–M/F and Age–SI, indicating demographic variables were mostly independent. Significant correlations

existed between all back-calculated lengths indicating these were not independent measures of growth.

The results of the simple linear regression analyses showed significant relations between genetic characteristics and stocking intensity, YOY, and the sex ratio (Table 14). Both H_e and A_e were positively related to SI (Figure 8 and Figure 9, respectively). There was a negative relation between A_r and the adult mean sex ratio (Figure 10). There was a negative relation between levels of inbreeding (F_{IS}) and YOY abundance (Figure 11); the residuals of this linear regression were not normally distributed (SW statistic = 0.779, p = 0.002) with one lake producing a large residual value (Lake Tomahawk; -2.98 SDs from the mean). Removing this lake did not alter the slope of the relation and the residuals of the resulting model were normally distributed and met all other expectations of linear regression (Figure 12). There was a positive relation between r and sex ratio (Figure 13). With the aforementioned exception, all models had no outlying residual values (> 3 SDs from the mean), no patterns in the residuals as compared to the predictor variable, and had normally distributed residuals.

The forward stepwise regression analysis between genetic characteristics and demographic variables indicated a significant demographic model for each genetic characteristic except mean d^2 (Table 15). Consistent with the linear regression analyses, stocking intensity (SI), YOY, and sex ratios remained critical factors influencing genetic characteristics. Additionally, mean age of the population (Age) and the surrogate for population abundance (SA) were significant components of the resolved models. The best-fit demographic model for H_e showed a positive relation to SI. Similarly, the best-fit demographic model for A_e showed a positive relation to SI, and negative relation to both

SA and Age. The best-fit demographic model for A_r showed a negative relation to M/F. The stepwise regression modeling for F_{IS} indicated the negative relation between with YOY and age was the best-fit model. No demographic model was significant for d^2. Finally, the stepwise regression analysis indicated r was positively related to M/F. The coefficient of determination (R^2) suggested more than half the variation in each genetic parameter was accounted for by the resulting demographic model, with an exception of A_r that had an R^2 of 0.471. The demographic model for both A_e and F_{IS} had notably high R^2 values of 0.859 and 0.713, respectively.

Comparison between Growth and Condition to Genetic and Demographic Variables

Walleye growth and condition were related to genetic and demographic variables in walleye populations (Table 16). Length at age-1 was positively related to A_r at the 0.05 α-level (Figure 14), whereas length at age-2 and length at age-3 were not related to any genetic or demographic variables. Mean condition factor (Kn) was negatively associated with both A_e (Figure 15) and SI (Figure 16). All relations had normally distributed residuals with no outliers and no patterns in the residuals against the predictor variable. No relation was significant after sequential Bonferroni corrections (α_o = 0.0045).

The forward stepwise regression analysis between growth characteristics and demographic and genetic variables indicated a well-supported model for both length at age-1 and Kn (Table 17). The stepwise analysis for length at age-1 showed a positive relation with A_r as the best-fit model (Figure 14). No significant model was resolved for length at age-2 and length at age-3. The model for Kn showed a negative relation with

A_e as the best-fit model (Figure 15). These models accounted for a significant proportion of the variation among populations, 31.7% and 33.4% for length at age-1 and Kn, respectively.

DISCUSSION

The purpose of this study was to first determine if the genetic characteristics of walleye in Wisconsin were related to demographic variables and second, to determine if growth characteristics were related to genetic or demographic variables. Three key demographic variables showed relations to the genetic characteristics of walleye populations that were consistent with theoretical expectations and previous studies (Felsenstein 1971; Cena et al. 2006; Franckowiak et al. 2009; Marie et al. 2010). Specifically, low levels of recruitment, skewed sex ratios, and high stocking intensities appeared to pose a threat to the genetic integrity of walleye in Wisconsin. Inbreeding coefficients were negatively correlated with recruitment levels, and significant levels of inbreeding were observed in Thunder Lake. Skewed sex ratios were positively correlated to populations with high relatedness suggesting elevated levels of genetic drift could be negatively impacting genetic diversity (Allendorf and Luikart 2008).

The likely effects of stocking on the genetic integrity of walleye were consistently observed through increased intrapopulation genetic diversity and decreased interpopulation genetic diversity. Both of these outcomes were consistent with genetic introgression induced by stocking (Englbrecht et al. 2002; Finnegan and Stevens 2008; Marie et al. 2010). Walleye growth was related to genetic and demographic variables consistent with previous studies (Li et al. 1996; Cena et al. 2006). A significant decrease in walleye early growth rates was associated with low levels of genetic diversity, suggesting a potentially detrimental impact of genetic drift. A negative relationship between the relative condition of walleye and stocking intensity suggested either outbreeding depression or increased competition induced by stocking could be

contributing to low relative condition in some systems. The results of this study illustrate the importance of demographics and propagation on walleye genetic integrity and the potential benefits of genetic diversity in walleye populations.

Recruitment and Genetic Integrity

A primary challenge of walleye management is the difficulty of predicting recruitment and its subsequent impact on the population (Hansen et al. 1998; Beard et al. 2003a). Therefore, understanding the potential genetic implications of varying levels of walleye recruitment is critical. This study showed a negative relation between inbreeding and young-of-the-year (YOY) abundance among the 15 sampled walleye populations. The variability in YOY accounted for nearly half of the variability in inbreeding values. However, Lake Tomahawk seemed to fall outside of this relation. This lake had the highest stocking value, signifying a potentially biased estimate of YOY production. Overall, this relationship suggests recruitment strongly influenced the population's retention of genetic diversity and, subsequently, is a critical component in maintaining genetic integrity. Similarly, lower levels of recruitment may be resulting from inbreeding and/or other causative factors.

The reproductive output of a population (i.e., YOY abundance) is expected to strongly influence the effective population size (N_e; Felsenstein 1971). Therefore, as YOY abundance increases, the N_e of the population should also increase and, likewise, lower YOY abundance should lead to a depressed N_e. This is relevant because inbreeding and its detrimental effects (inbreeding depression) are negatively correlated to N_e (Newman and Pilson 1997). This pattern has been observed in Pacific sardine

(*Sardinops sagax*) and northern anchovy (*Engraulis mordax*), where the level of recruitment was highly predictive of the ratio of N_e to the census population size (Gaggiotti and Vetter 1999). Therefore, the elevated inbreeding levels observed in the current study are consistent with a depressed N_e as predicted by low levels of YOY abundance. A similar conclusion was reported by Franckowiak et al. (2009) when they observed a large increase in the N_e of Escanaba Lake walleye associated with lower variances of individual reproductive output. These results illustrate the importance of both the evenness of reproduction among individuals of the population and the total reproductive output of the population in maintaining the genetic integrity of walleye.

The N_e of the population can be influenced by several other factors, including generational overlap. Gaggiotti and Vetter (1999) showed populations with longer generation overlaps exhibited increased temporal stability of N_e compared to populations with shorter generation overlaps. In the current study, there were significant differences in the mean age of breeding fish among sampled populations. Venturelli et al. (2010) showed the age of maturation for walleye was strongly correlated ($R^2 = 0.95$) with growing degree-days across the walleye native range. Given the narrow geographic range of this study, growing degree-days between populations were not likely to differ. Therefore, differences in mean age of breeding fish observed in the current study likely represent differences in the generational overlap of the population. The stepwise regression model showed negative interactions between mean age of breeding fish and inbreeding consistent with a predicted decrease in the temporal stability of N_e associated with lower generational overlaps. Temporal perturbations of N_e lower the long-term N_e

of populations and, similarly, lead to increasing levels of inbreeding as seen in the sampled walleye populations (Allendorf and Luikart 2008).

Alternatively, inbreeding and its subsequent negative effects could explain a portion of the variability in recruitment and age. If the measures of inbreeding were consistent with long-term trends then decreases in recruitment and longevity via inbreeding depression would be expected (Wang et al. 2003b). For instance, Wang et al. (2003b) summarized evidence of inbreeding depression occurring in at least five salmonid species. They observed phenotypic impacts including decreases in egg weight, increased fry mortality, and increased adult mortality. The high mortality of walleye during their first year of development (Serns 1982) may make walleye recruitment sensitive to inbreeding depression by elevating already high mortality levels.

Despite the potential impacts of inbreeding on recruitment and age, this causative connection is unlikely for two main reasons. First, the level of inbreeding in a majority of sampled populations was not significantly different from zero (i.e., no inbreeding) and no other observed negative morphological conditions (i.e., deformities, etc.) were observed, suggesting inbreeding depression was not likely occurring. Secondly, no significant trend was observed between any demographic variable and d^2, a parameter known to be sensitive to historic levels of inbreeding and fitness-related traits (Coulson et al. 1998; Hansson et al. 2001; Rossiter et al. 2001; Cena et al. 2006). Therefore, it is more likely the observed low levels of recruitment and generation overlap were resulting in increased measures of inbreeding in some populations and not the alternative that inbreeding depression was leading to depressed recruitment and longevity.

Low levels of recruitment have been documented in walleye populations across Wisconsin (Serns 1983; Hansen et al. 1998; Beard et al. 2003a). However, the amount of time low recruitment takes to produce an inbreeding response is not clear. Short periods of low to no recruitment have been documented in some walleye populations (Hansen et al. 1998) and could be affecting the genetic integrity of these populations. It is possible to determine the temporal vulnerability of genetic integrity to low recruitment by studying populations with long-term DNA archives, such as Escanaba Lake (see Franckowiak et al. 2003). Regardless, recruitment and inbreeding levels, as measured, were highly correlated indicating inbreeding may be present in other walleye populations and may be associated with low levels of recruitment. However, the levels of inbreeding present in the current study suggest that inbreeding is not a likely chronic concern for Wisconsin walleye but instead is a likely acute problem associated with local (lake-level) conditions. Only Thunder Lake had significant genetic indications of inbreeding and this value was not significant after sequential Bonferroni corrections.

Skewed Sex Ratios and Genetic Integrity

Significant relations between the sex ratio and genetic diversity suggest the sex ratio is an important demographic consideration of walleye genetic integrity. Sex ratios were significantly different among populations indicating a male skew in most sampled populations. As this ratio increased, a similar increase in the mean relatedness within the population was observed. This result was consistent with the expected impacts of a skewed sex ratio on the N_e of the population. Allendorf and Luikart (2008) concluded that highly skewed sex ratios act to decreases N_e with respect to the total population size

so that N_e in most populations will seldom be greater than two times the rarer sex. As N_e decreases, the level of inbreeding increases in a population and thus, the level of relatedness among its members increases (Aho et al. 2006). This decrease in N_e should also result in increased genetic drift in turn decreasing genetic diversity (Waples 1989; Schwartz el al. 1998) and especially allelic richness (Luikart et al. 1998).

Differential levels of allelic diversity among populations is a strong indicator that genetic drift is occurring in the populations with low levels of allelic diversity (Luikart et al. 1998). Highly skewed sex ratios have likely decreased the N_e in the sampled walleye populations, thereby leading to losses of allelic richness by genetic drift (Ryman et al. 1981). Theoretically, losses occur in relation to the degree of skew in the sex ratio and thus, more highly skewed populations would have higher losses of diversity. As expected, there was a negative relation between allelic richness and the sex ratio indicating both relatedness and genetic diversity were impacted by the sex ratio, presumably from a depressed N_e. These results show genetic drift may be operating to decrease the genetic diversity in some walleye populations. This is why a common prescription for conserving genetic diversity in propagation programs is to maintain a 1:1 sex ratio if possible (Fiumera et al. 2000; Miller and Kapuscinski 2003). The goal of this approach is to minimize the relatedness of offspring and subsequently, maximize the N_e of a given cohort, thus maximizing genetic diversity.

Biases in sex ratio estimates are known to exist in walleye populations because of natural causes and gear-selection issues. Walleye spawning sites commonly have a sex ratio skewed towards males because males spawn earlier and stay on the spawning bed longer than females (Schneider et al. 2007). This behavior may increase the catchability

of male walleye resulting in a biased sex ratio estimate (Rogers et al. 2003). However, assuming the range of sex ratios observed in this study to be wholly caused by inherent bias does not explain the strong relations observed between the sex ratio and genetic characteristics. Furthermore, the male skew caused by sampling bias is likely systematic across populations so biologically relevant information can still be inferred from the sex ratio data.

An alternative explanation for the observed sex ratio differences in walleye populations is size selective exploitation. Skewed sex ratios have been reported in other exploited fish species (Buxton 1993; McGovern et al. 1998) and can be the result of size selective harvest (Fenberg and Roy 2007). Walleye exploitation is in Wisconsin is substantial. The mean mortality caused by exploitation is 11.83% in northern Wisconsin and occasionally exceeds 35% (Beard et al. 2003b). The sexual size dimorphism of walleye, where females are generally larger at age than males (Henderson et al. 2003), may disproportionately expose females to exploitation. Serns and Kempinger (1981) reported a higher mean female exploitation for walleye in Escanaba Lake from 1958 to 1979 and, in several years, the female exploitation was double that of male walleyes. A t-test failed to show significant differences in sex-specific exploitation rates. However, the inherent variability in exploitation may have masked any differential exploitation of female walleye if it was occurring in this study. Sex-specific harvest would skew the sex ratio of the population, reduce N_e, and subsequently result in a rapid loss of genetic diversity through genetic drift (Ryman et al. 1981). In the current study, the strength of the observed relation between the sex ratio and allelic richness ($R^2 = 0.471$, $p = 0.004$; Figure 10) and between the sex ratio and relatedness ($R^2 = 0.575$, $p = 0.001$; Figure 13)

suggests the sex ratio, as measured, had important genetic implications to walleye genetic integrity and further consideration of sex selective harvest may be warranted. Assumptions that the measured walleye sex-ratio imbalance represents a methodological artifact should be challenged in future studies.

Stocking and Genetic Integrity

Intrapopulational genetic diversity.—Stocking was a major factor in explaining the variance of genetic characteristics within walleye populations. Both the effective number of alleles and expected heterozygosity were significantly greater in stocked populations and were positively correlated to stocking intensity. Likewise, mean relatedness within stocked populations was lower than non-stocked populations. When a population is stocked, a source population with its own unique genetic characteristics is added to the receiving population. Previously, Hammen (2010) showed high levels of intrapopulation genetic diversity across walleye populations in northern Wisconsin. Therefore, even if closely-related walleye populations from the same genetic unit are used as a brood source, increased diversity in the recipient population is virtually unavoidable. In time, if stocked fish interbreed with resident fish, the gene pools become mixed resulting in higher diversity measures and overall lower mean relatedness. This observation was consistent with Cena et al. (2006) who showed stocking-induced genetic introgression was likely causing increased genetic diversity in Ontario walleye populations. These results were also consistent with the genetic introgression documented previously in one Wisconsin walleye population (Franckowiak et al. 2009). The current study expands on these previous findings and suggests the genetic impacts of

stocking may be directly related to stocking intensity over a broad geographic range. Genetic introgression has also been associated with high stocking intensities in other fish species (Englbrecht et al. 2002; Finnegan and Stevens 2008; Marie et al. 2010). For example, Marie et al. (2010) documented significant genetic introgression in brook charr associated with stocking intensity that resulted in an overall increase in genetic diversity within stocked populations.

This pattern of increased genetic diversity associated with stocking intensity is a threat to the genetic integrity of naturally recruiting walleye populations because it could lead to the disruption of the natural level of genetic diversity that would occur in the population. The dynamic equilibrium between genetic drift and mutation maintains a natural level of genetic diversity within populations (Nei 1975). When local adaptation (natural selection) is factored into the process, genetic structure is expected across widespread and numerous populations (Adkison 1995). Therefore, disruptions to the equilibrium by either an artificial increase or decrease in genetic diversity could have detrimental impacts on the adaptability and viability of the population (Hansen et al. 2002; Ayllon et al. 2006).

Interpopulational genetic diversity.—Stocking had a noticeable impact on the spatial distribution of genetic diversity, a major component of genetic integrity. Genetic diversity is naturally distributed across the landscape such that populations in close geographic proximity to one another have higher rates of migration and thus, are genetically more similar than populations more geographically isolated (isolation-by-distance; Manel et al. 2003). The relation between geographic distance and genetic differentiation in non-stocked walleye populations supported an isolation-by-distance

model (Manel et al. 2003) consistent with decreased gene flow in conjunction with increased geographic separation. This is an important pattern because it fulfills one of the major population requirements for harboring of local genetic adaptations (Hansen et al. 2002). Alternatively, the same relationship between geographic and genetic distance was not observed when comparing only stocked walleye populations. In these populations, the low levels of genetic differentiation, and the lack of a relation between geographic distance and genetic differentiation suggested stocking had resulted in a loss of interpopulational genetic diversity and a subsequent disruption of genetic integrity. Moreover, this disruption in the pattern of natural genetic differentiation increases the risks of losing naturally occurring local adaptations (Hansen et al. 2002; Ayllon et al. 2006). These results are consistent with previous studies suggesting stocking as an explanation for many of the anomalies in the genetic structure of walleye (Billington et al. 1992; Stepien and Faber 1998; Hammen 2009; Stepien et al. 2009). For example, Stepien and Faber (1998) proposed the prevalence of Lake Erie haplotype mtDNA in Ohio River walleye was due to extensive stocking with Lake Erie walleye. The results of this analysis show, even at a fine geographic scale, stocking can disrupt the genetic structure of walleye.

Growth, Genetic Diversity, and Demographics

Potential benefits of genetic diversity to early development in walleye were observed throughout this study. The first year is a critical developmental period for walleye since it encompasses several ontogenetic diet shifts (Galarowicz et al. 2006). The positive relation between allelic richness and length at age-1 show walleye grew

faster during their first year of development in populations with higher levels of genetic diversity ($R^2 = 0.320$, $p = 0.028$; Figure 14). Neutral genetic diversity is controlled by ecological processes (e.g., inbreeding and genetic drift) that could have biological impacts on or be correlated with fitness-related traits (Hansson and Westerberg 2002; Reed and Frankham 2003). In fact, Cena et al. (2006) found correlation between neutral genetic diversity and early growth in walleye populations across Ontario (Cena et al. 2006). As discussed previously, allelic richness is likely being lost from some Wisconsin walleye populations because of increased genetic drift associated with depressed N_e. The decrease in early growth in populations with decreasing levels of allelic richness suggests genetic drift could be resulting in potential losses of adaptive genetic diversity in conjunction with the observed loss of neutral genetic variation. A similar result was documented by Johansson et al. (2007) who showed habitat fragmentation increased genetic drift in populations of the common frog (*Rana temporaria*) resulting in lower genetic diversity and negative impacts on both body mass and survival. These results imply the low N_e of walleye observed in other studied walleye populations (Franckowiak et al. 2009; Hammen 2009) may be consequential to adaptive genetic diversity.

It is unclear if the documented decreases in body condition were from adverse stocking effects, outbreeding depression, or underlying ecological issues. The observed negative relation between condition, genetic diversity, and stocking intensity could be explained two ways: 1) explicit negative effects of stocking such as outbreeding depression and/or increased competition, and 2) stocking is a symptom of larger ecological issues in some systems that, although correlated, is not causative of the lower condition factor. There were negative relations between relative condition and both the

effective number of alleles and stocking intensity in sampled populations. The positive relationship between genetic diversity and stocking intensity made it difficult to differentiate between genetic and stocking effects. The effective number of alleles explained a slightly higher amount of the variation in condition, suggesting factors associated with outbreeding depression could be occurring. Outbreeding depression occurs when the increase in genetic diversity associated with hybridization disrupts local genetic adaptations thus decreasing fitness (Allendorf and Luikart 2008). The high level of intrapopulation genetic diversity and direct connection between the genetic diversity and stocking observed in this study indicates stocking is mixing genetically distinct groups of walleye consistent with requirements for outbreeding depression. Outbreeding depression has been shown to negatively impact the growth rate of fish such as the study of Granier et al. (2011) where genetically distinct strains of brook trout (*Salvelinus fontinalis*) were experimentally crossed. Nevertheless, given the close geographic proximity of all sampled populations and the previous research showing these populations to likely be from a single genetic unit, outbreeding depression is not a likely factor.

Increased competition for limited resources is a likely factor in the negative relationship between condition and stocking in walleye populations. Li et al. (1996) showed stocking decreased the mean weight of walleye in populations with natural recruitment suggesting systems with natural recruitment had already achieved a density close to the carrying capacity of the system so stocking increased competition thereby decreasing growth. This increase in competition would be consistent with the observed negative relationship between stocking intensity and condition in sampled walleye

populations. A further impact of this connection is that the observed decrease in condition associated with stocking could create a negative feedback loop affecting recruitment. In systems experiencing supplemental stocking, increased competition could result in lower lipid concentrations in female walleye. Lower lipid concentrations have been reported to decrease female fecundity in some walleye populations (Mole et al. 2008). This would presumably lead to a decrease in recruitment and increase in a given populations' dependence on supplementation, in turn increasing competition and further decreasing condition. Eventually, the management action may negatively impact the long-term sustainability and viability of the previously naturally recruiting population.

Alternatively, the observed relations with condition could portend larger ecological issues in the stocked systems. Some systems could both necessitate high levels of stocking and exhibit a low condition factor due to lake-specific factors, such as limited habitat and/or highly complex fish community structure or broad-scale factors such as climate and/or watershed development. For example, Beard et al. (2003a) showed yellow perch (*Perca flavescens*) densities and climate strongly influenced walleye recruitment in Wisconsin. Quist et al. (2002) established a connection between walleye condition and prey consumption and climate. These two studies showed similar ecological factors such as climate and the fish community affect both recruitment and condition in walleye populations. This suggests underlying ecological issues that decrease the overall vitality of a walleye population, as measured by low condition and poor recruitment, could explain the results of the current study. The observed connection between condition and genetic diversity would be an indirect connection mediated by

stocking. Therefore, delineating more specific interactions between walleye condition, stocking, and genetic characteristics warrants further investigation.

Summary

The primary objective of this study was to determine the connection between genetic characteristics and demographic variables of walleye in northern Wisconsin. This objective was evaluated by comparing contemporary estimates of genetic characteristics using microsatellite analysis to demographic variables generated from long-term walleye monitoring data gathered by the Wisconsin state agencies. The results of this study delineated several demographic variables salient to the management of walleye genetic integrity. Specifically, low levels of recruitment, highly skewed sex ratios, and high stocking intensities posed a threat to the genetic integrity of walleye and likely other managed fish species. The strength of the connection between these demographic variables and the genetic characteristics illustrates the importance of demographics to the genetic integrity of walleye in Wisconsin. Genetic characteristics were shown to have a measurable impact on walleye growth parameters. A connection was observed between genetic diversity and early growth suggesting genetic drift may have reduced fitness and genetic diversity in some populations. The relative condition of walleye was negatively related to both genetic diversity and stocking intensity suggesting increased competition, outbreeding depression, or other underlying ecological issues. The success of using demographic data from state monitoring methods showed these methods could be used in conjunction with contemporary data to produce new insights into the demography and genetic trends of naturally recruiting fish populations. Further assessments of walleye

populations exhibiting these demographic trends could expand the geographic scope of these findings and determine if these threats to genetic integrity are present throughout the native range of walleye.

One of the major limitations of this study was it only encompassed neutral genetic diversity. While neutral genetic markers may best measure underlying ecological processes, such as gene flow (Holderegger et al. 2006) and inbreeding (Coltman and Slate 2003), these loci are not functionally expressed genes and therefore cannot be directly used to study natural selection and adaptation (Bekessy et al. 2003). The genes of the major histocompatibility complex (MHC) can be used as informative genetic systems to study the effects of natural selection (Bernatchez and Landry 2003). The MHC gene complex encodes cell surface proteins responsible for binding with non-self peptides thereby eliciting an immune response against pathogens (Oosterhout et al. 2006). Initial studies of walleye MHC class I receptors have shown promising results for the study of natural selection in walleye (Fujiki et al. 2001; Christei et al. 2007). Studies using MHC markers have successfully identified pathogen resistance associated with specific MHC alleles in Atlantic salmon (*Salmo salar*; Dionne et al. 2009) and coupling MHC markers with neutral markers such as microsatellites has permitted the assessment of natural selection in maintaining the genetic diversity of guppy (*Poecilia reticulata*) populations (Oosterhout et al. 2006). Future research could incorporate MHC genetic markers to determine if important genetic adaptations occur in Wisconsin walleye and to assess the role of natural selection in maintaining the genetic integrity of walleye.

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Table 1. Recruitment classification code descriptions used for walleye population management in Wisconsin (U.S. Department of the Interior 1991).

Code	Description
NR	Natural reproduction only; consistent enough to result in multi-year class adult populations.
NR-2	Natural reproduction only; inconsistent, results in missing year classes.
C-NR	Natural reproduction is adequate to sustain the population even though the lake is being stocked.
C-	Natural reproduction and stocking provide more or less equal recruitment to the adult population.
C-ST	Stocking provides the primary source of recruitment but some natural reproduction occurs and may augment the adult population.
ST	Stocking provides the only source of recruitment and is consistent enough to result in multi-year class adult population.
REM	Stocking provides the only source of recruitment but was discontinued; the population will disappear in the future.
O-ST	Stocking provides the only source of recruitment but was initiated recently and has not yet yielded a harvestable population of adults.

Table 2. Walleye populations used in this study, the abbreviation, Water Body Identification Code (WBIC), and current recruitment classification code (Code; U.S. Department of the Interior 1991).

Lake	Abbreviation	WBIC	Code
Big Arbor Vitae	BAV	1545600	NR
Big St. Germain	BSG	1591100	C-ST
Eagle Chain	EC	1603700	NR
Kawaguesaga	KL	1542300	NR
Little Arbor Vitae	LAV	1545300	NR
North Twin	NTL	1623800	C-NR
Papoose	PAP	2328700	C-NR
Pelican	PEL	1579900	C-NR
Plum	PLU	1592400	C-NR
Thunder	THU	1618100	C-ST
Tomahawk	TOM	1542700	C-ST
Trout	TRO	2331600	C-ST
Two Sisters	TWS	1588200	C-NR
White Sand	WSL	2339100	C-ST
Willow Flowage	WIL	1528300	NR

Table 3. Estimated survival rate for the size classes of walleye used in Wisconsin stocking programs (WDNR 1999). Survival rates indicate percent of age-3 walleye expected to recruit to the population per number of fish of that size class stocked.

Size class	Survival rate
Fry	0.0024%
Small fingerling	0.81%
Large fingerling	1.62%
Extended growth fingerlings	5.7%

Table 4. Walleye microsatellite loci, primer sequence, allele size range, and references.

Locus		Primer Sequence (5'-3')	Size (bp)	Reference
Svi-2	F:	CAACCAGACCCAATCCCTTG	192-208	Eldridge et al. 2002
	R:	GGGCCGAGTATATCAGTTAAC		
Svi-4	F:	ACAAATGCGGGCTGCTGTTC	102-118	Eldridge et al. 2002
	R:	GATCGCGGCACAGATGTATTG		
Svi-L5	F:	CATATCCTATGTAGTATGG	177-225	Wirth et al. 1999
	R:	CAAATCCCATTTACACCCAC		
Svi-6	F:	AGTCGACATATTATGTAGAGTGC	136-173	Eldridge et al. 2002
	R:	GATCAACTGTGGAGGATGAGC		
Svi-7	F:	GAAACCTTACAAAAGCCTGG	163-173	Eldridge et al. 2002
	R:	TTATCTGCACTTCTACAGGC		
Svi-L9	F:	TACTGTTCACTTATCTATCC	243-297	Wirth et al. 1999
	R:	TGTATGTGTGTGTGTTCATGT		
Svi-17	F:	GCGCACTCTCGCATAGGCCCTG	101-113	Borer et al. 1999
	R:	CGTTAAAGTCCTTGGAAACC		
Svi-20	F:	CAAGTGCGCAATGGTGCATTAC	144-193	Eldridge et al. 2002
	R:	GAATGAAGAAATGCACCCATGC		
Svi-26	F:	CGAACTACTTATCTTCTGGC	156-189	Eldridge et al. 2002
	R:	GTAAGTGTGAATCAGCCAGAC		
Svi-33	F:	CAGGACTGCTGTGTATAGACTTG	90-102	Borer et al. 1999
	R:	GATATAGCTTTCTGCTGGGGTC		

Table 5. PCR reaction conditions, fluorescent labels, and thermocycler temperature profiles for all multiplexes. Multiplex refers to loci that were co-amplified in PCR with the temperature profiles below table, dNTPs and $MgCl_2$, refers to the final PCR concentration of each reagent (mM), Primer refers to the final concentration (µM) of each primer in the final PCR, and Label refers to the fluorescent label on the Forward primer of each locus. All reactions contained 1X PCR Buffer B (ThermoFisher Scientific, Inc., Waltham, MA) 0.5U of *Taq* DNA polymerase (New England Biolabs, Inc., Ipswich, MA).

Locus	Multiplex	dNTP	$MgCl_2$	Primer	Label
Svi-2	A	0.6mM	1.50mM	0.08µM	6FAM
Svi-4				0.06µM	6FAM
Svi-6				0.17µM	NED
Svi-7				0.20µM	HEX
Svi-L5	B	1.00mM	1.90mM	0.30µM	HEX
Svi-L9				0.25µM	6FAM
Svi-20				0.08µM	HEX
Svi-17	C	1.00mM	1.50mM	0.30µM	NED
Svi-26				0.30µM	6FAM
Svi-33				0.30µM	HEX

A 94°C for 2.0 min. 31 cycles each at 94°C for 30 s, 60°C annealing for 1.0 min., then 72°C for 2.0 min and a final elongation of 72°C for 15.0 min.

B 94°C for 2.0 min. 35 cycles each at 94°C for 45 sec, 53°C annealing for 45 sec, then 72°C for 45 sec. Final elongation of 72°C for 45.0 min.

C 94°C for 5.0 min. 35 cycles each at 94°C for 1.0 min, 52°C annealing for 1.0 min, then 72°C for 1.0 min. Final elongation of 72°C for 15.0 min.

Table 6. Summary of demographic variables for all sampled populations including abbreviations, $\log_e$ lake surface area in ha (SA), the stocking index (SI), the number of YOY estimates from 1990 to 2008 (N_{YOY}), the $\log_e$ mean YOY estimates (YOY), the number of population estimates from 1990 to 2009 (N_{PE}) used to generate the mean sex ratio (M/F), the number of spines aged (N_{Age}), and the mean age of the population (Age) where stated values are ± 1 standard error.

Lake	SA	SI	N_{YOY}	YOY	N_{PE}	M/F	N_{Age}	Age
BAV	6.09	0.00	17	11.05 ± 2.90	6	9.32 ± 2.41	88	3.72 ± 0.09
BSG	6.48	65.99	19	10.37 ± 2.51	6	5.89 ± 2.52	48	7.16 ± 0.41
EC	6.46	0.00	19	10.95 ± 2.57	2	7.00 ± 0.26	55	4.71 ± 0.22
KL	5.60	0.00	20	7.12 ± 1.75	3	2.97 ± 1.05	58	5.67 ± 0.29
LAV	5.38	0.00	19	9.17 ± 2.21	4	3.08 ± 0.68	61	6.52 ± 0.27
NTL	7.03	51.10	19	11.54 ± 2.83	1	3.80	62	4.85 ± 0.20
PAP	5.15	13.09	15	8.23 ± 2.14	4	4.33 ± 2.12	63	5.21 ± 0.23
PEL	7.28	0.00	19	11.04 ± 2.66	6	8.92 ± 2.47	49	5.22 ± 0.31
PLU	6.05	0.00	20	10.60 ± 2.42	6	3.67 ± 1.31	50	6.25 ± 0.37
THU	6.57	77.24	10	4.24 ± 1.59	1	0.18	49	7.20 ± 0.47
TOM	7.22	132.15	22	8.81 ± 2.10	4	1.84 ± 0.94	46	9.81 ± 0.47
TRO	7.34	98.52	20	9.20 ± 2.11	7	1.65 ± 0.56	70	6.21 ± 0.31
TWS	5.67	33.92	16	6.84 ± 1.86	5	1.73 ± 0.47	44	5.75 ± 0.43
WSL	5.69	30.48	10	8.22 ± 2.72	2	7.17 ± 0.77	59	6.54 ± 0.33
WIL	7.84	0.00	10	11.29 ± 3.37	2	2.40 ± 0.30	49	4.92 ± 0.18
Mean	6.70	33.50	17.00	9.24 ± 0.53	3.93	4.26 ± 0.72	56.73	5.98 ± 0.37

Table 7. Summary of relative condition factor (Kn) ± 1 standard error and sample size (N_{Kn}) for each population. Condition estimates were not available for PLU.

Lake	N_{Kn}	Kn
BAV	88	100.57 ± 0.87
BSG	49	103.73 ± 1.07
EC	56	106.91 ± 1.34
KL	58	98.73 ± 0.87
LAV	62	106.28 ± 1.39
NTL	62	96.92 ± 0.77
PAP	63	96.07 ± 1.11
PEL	52	108.10 ± 1.13
PLU	–	–
THU	50	97.98 ± 1.27
TOM	47	97.10 ± 1.00
TRO	70	94.57 ± 0.79
TWS	44	98.70 ± 1.24
WSL	59	97.50 ± 1.47
WIL	50	104.96 ± 0.93
Mean	58	100.58 ± 1.09

Table 8. Summary of back-calculated early growth estimates including mean ± 1 standard error for length (mm) at age-1 (L-1), age-2 (L-2), and age-3 (L-3), and sample sizes (N).

Lake	N	L-1	L-2	L-3
BAV	88	151.4 ± 1.7	244.3 ± 2.4	317.2 ± 2.2
BSG	48	146.1 ± 4.7	242.7 ± 6.3	321.2 ± 7.8
EC	55	131.8 ± 2.5	198.8 ± 3.2	252.6 ± 4.0
KL	58	140.7 ± 3.2	231.0 ± 4.5	309.7 ± 4.8
LAV	61	131.0 ± 2.7	225.2 ± 3.6	289.6 ± 4.0
NTL	62	150.0 ± 2.6	238.1 ± 3.9	294.6 ± 3.4
PAP	63	124.5 ± 3.6	215.8 ± 4.0	282.0 ± 4.2
PEL	49	125.7 ± 4.2	222.3 ± 4.2	304.6 ± 5.0
PLU	50	121.8 ± 3.1	203.7 ± 3.7	280.9 ± 4.2
THU	49	148.8 ± 5.6	232.9 ± 6.0	304.3 ± 6.2
TOM	46	159.4 ± 3.7	259.7 ± 5.6	335.2 ± 6.2
TRO	70	135.7 ± 2.5	223.5 ± 3.3	292.0 ± 3.5
TWS	44	151.6 ± 4.4	265.1 ± 7.2	354.2 ± 7.2
WSL	59	150.4 ± 3.1	235.1 ± 3.6	300.6 ± 4.5
WIL	49	161.7 ± 5.8	247.2 ± 5.3	321.1 ± 4.3
Mean	56.7	142.0 ± 3.3	232.4 ± 4.8	304.0 ± 6.3

Table 9. Genetic summary statistics for all 15 sampled populations including genetic sample size (n), expected heterozygosity (H_e), effective number of alleles (A_e), allelic richness (A_r), F_{IS} values, *p*-values for F_{IS} (*p*), mean d^2, and mean pair-wise relatedness (r) with their associated standard deviations.

Lake	N	H_e	A_e	A_r	F_{IS}	*p*	d^2	r
BAV	88	0.770 ± 0.020	4.57 ± 0.42	9.24	-0.002	0.534	23.80 ± 13.22	0.057 ± 0.091
BSG	48	0.778 ± 0.018	4.57 ± 0.32	9.17	-0.004	0.761	26.49 ± 13.29	0.051 ± 0.081
EC	56	0.762 ± 0.022	4.48 ± 0.50	8.98	-0.004	0.531	27.61 ± 15.19	0.057 ± 0.091
KL	55	0.758 ± 0.026	4.41 ± 0.44	9.23	0.018	0.218	24.28 ± 11.40	0.053 ± 0.088
LAV	62	0.757 ± 0.022	4.33 ± 0.40	9.65	0.019	0.208	22.67 ± 12.35	0.053 ± 0.088
NTL	62	0.761 ± 0.030	4.68 ± 0.59	9.85	0.001	0.494	26.80 ± 15.35	0.050 ± 0.083
PAP	60	0.759 ± 0.029	4.50 ± 0.47	8.90	0.020	0.178	26.09 ± 12.10	0.052 ± 0.084
PEL	49	0.756 ± 0.028	4.43 ± 0.49	8.44	-0.023	0.665	28.89 ± 13.62	0.056 ± 0.090
PLU	50	0.761 ± 0.022	4.31 ± 0.32	9.22	-0.031	0.855	22.19 ± 11.41	0.052 ± 0.088
THU	50	0.783 ± 0.015	4.63 ± 0.30	10.00	0.051	0.016	25.90 ± 11.11	0.048 ± 0.084
TOM	49	0.775 ± 0.020	4.55 ± 0.35	9.92	-0.046	0.976	28.07 ± 14.00	0.051 ± 0.087
TRO	67	0.779 ± 0.022	4.71 ± 0.36	9.50	0.015	0.228	26.03 ± 11.55	0.054 ± 0.096
TWS	44	0.770 ± 0.023	4.54 ± 0.44	9.40	0.030	0.119	20.25 ± 11.96	0.050 ± 0.082
WSL	60	0.766 ± 0.018	4.38 ± 0.34	9.35	0.003	0.430	23.14 ± 12.24	0.055 ± 0.090
WIL	49	0.766 ± 0.015	4.27 ± 0.26	9.32	-0.002	0.554	22.05 ± 10.30	0.053 ± 0.091
Mean	56.7	0.767 ± 0.009	4.49 ± 0.13	9.34 ± 0.41	0.003 ± 0.243	–	24.95 ± 2.53	0.053 ± 0.003

Table 10. Genetic distance matrix showing φ-st values (below diagonal) between all pairs of populations and corresponding *p*-values (above diagonal) based on 10,000 permutations. Bold indicates significant φ-st values following sequential Bonferroni correction ($\alpha_o = 0.00048$)

	BAV	BSG	EC	KL	LAV	NTL	PAP	PEL	PLU	THU	TOM	TRO	TWS	WSL	WIL
BAV	*	**0.0002**	**0.0001**	0.0017	0.0041	0.0018	0.0040	**0.0001**	**0.0001**	**0.0001**	0.0079	**0.0001**	0.1649	0.0005	**0.0001**
BSG	0.0168	*	**0.0001**	0.0090	0.0028	0.0106	0.0005	**0.0001**	0.0114	0.0397	0.4632	0.0020	0.1241	0.0012	0.0313
EC	0.0535	0.0419	*	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**
KL	0.0129	0.0117	0.0619	*	0.0107	0.0076	0.0695	**0.0001**	**0.0003**	**0.0001**	0.0092	**0.0001**	0.0022	0.0013	**0.0002**
LAV	0.0096	0.0135	0.0528	0.0108	*	0.0056	0.0035	**0.0001**	**0.0001**	**0.0001**	0.0043	**0.0001**	0.0547	0.0015	**0.0002**
NTL	0.0110	0.0106	0.0492	0.0105	0.0103	*	**0.0001**	**0.0004**	**0.0001**	**0.0001**	0.0006	**0.0001**	0.0005	**0.0003**	**0.0001**
PAP	0.0101	0.0178	0.0423	0.0061	0.0119	0.0171	*	**0.0001**	**0.0002**	**0.0001**	0.0026	**0.0001**	0.0009	0.0199	**0.0001**
PEL	0.0351	0.0404	0.0656	0.0517	0.0291	0.0185	0.0443	*	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**
PLU	0.0241	0.0119	0.0331	0.0198	0.0262	0.0279	0.0201	0.0738	*	**0.0001**	0.0005	**0.0003**	**0.0001**	0.0006	0.0022
THU	0.0227	0.0085	0.0513	0.0284	0.0319	0.0339	0.0314	0.0603	0.0280	*	0.0739	0.1182	0.0009	0.0013	0.0012
TOM	0.0100	0.0000	0.0445	0.0117	0.0123	0.0158	0.0139	0.0346	0.0191	0.0067	*	0.0010	0.1780	0.0115	0.0350
TRO	0.0296	0.0142	0.0478	0.0281	0.0381	0.0342	0.0285	0.0636	0.0185	0.0046	0.0153	*	**0.0001**	**0.0001**	**0.0001**
TWS	0.0036	0.0054	0.0504	0.0165	0.0073	0.0182	0.0167	0.0324	0.0360	0.0192	0.0040	0.0259	*	**0.0002**	0.0012
WSL	0.0146	0.0170	0.0382	0.0160	0.0140	0.0195	0.0087	0.0395	0.0179	0.0157	0.0110	0.0207	0.0216	*	**0.0001**
WIL	0.0224	0.0093	0.0416	0.0247	0.0218	0.0286	0.0268	0.0517	0.0158	0.0164	0.0089	0.0275	0.0191	0.0230	*

Table 11. Geographic distance matrix showing the geographic distance (km) between each pair of populations used in this study.

	BAV	BSG	EC	KL	LAV	NTL	PAP	PEL	PLU	THU	TOM	TRO	TWS	WSL
BSG	10.2													
EC	36.1	25.9												
KL	9.1	18.2	43.1											
LAV	2.7	8.5	34.0	9.8										
NTL	42.0	32.2	14.6	50.4	40.7									
PAP	30.8	35.3	55.9	35.1	33.2	53.6								
PEL	58.8	54.0	46.7	58.5	56.2	61.0	89.0							
PLU	13.2	7.7	27.4	22.3	13.0	30.0	29.7	60.9						
THU	37.1	28.5	14.7	41.5	34.6	29.3	63.1	32.1	33.4					
TOM	11.3	15.6	37.4	8.5	9.7	46.7	41.5	50.1	22.1	33.9				
TRO	12.9	17.1	40.3	19.6	15.0	41.7	18.5	70.5	12.8	45.4	24.2			
TWS	19.5	17.9	31.5	19.3	17.0	43.3	50.2	39.6	25.6	24.7	10.8	32.0		
WSL	17.2	26.1	51.4	17.5	19.9	54.3	19.2	75.2	24.6	54.2	25.3	12.9	35.6	
WIL	29.7	37.1	58.3	20.9	29.6	68.4	52.9	57.2	42.6	52.0	21.7	40.0	27.4	33.7

Table 12. Results of pairwise t-tests (df = 13) comparing genetic characteristics between non-stocked (n = 7) and stocked (n = 8) populations, the test statistic (t), and *p*-value. Expected heterozygosity (H_e), effective number of alleles (A_e), allelic richness (A_r), F_{IS} values, mean d^2, and mean pairwise relatedness (r) are shown as means of either non-stocked or stocked lakes with associated standard error. An asterisk indicates a significant difference between the genetic characteristics of non-stocked and stocked populations at the 0.05 α -level.

	H_e	A_e	A_r	F_{IS}	d^2	r
Non-stocked	0.761 ± 0.002	4.40 ± 0.04	9.15 ± 0.14	-0.004 ± 0.007	24.50 ± 1.03	0.055 ± 0.001
Stocked	0.771 ± 0.003	4.57 ± 0.04	9.51 ± 0.14	0.009 ± 0.010	25.35 ± 0.88	0.052 ± 0.001
t	-2.620	-3.145	-1.813	-0.990	-0.632	2.715
p	0.021*	0.008*	0.093	0.340	0.539	0.018*

Table 13. Matrix of correlation coefficients between all genetic, demographic, length, and condition variables. An asterisk refers to a significant relation at the $\alpha = 0.05$ level and bold indicates a significant relation at the $\alpha = 0.01$ level.

	A_e	H_e	A_r	F_{IS}	d^2	r	YOY	SI	SA	M/F	Age	L-1	L-2	L-3
H_e	0.59*													
A_r	0.36	0.52*												
F_{IS}	0.25	0.21	0.24											
d^2	0.47	0.08	-0.15	-0.31										
r	-0.26	-0.39	-0.60*	-0.33	0.13									
YOY	-0.19	-0.39	-0.40	**-0.67***	0.18	0.51								
SI	**0.68***	**0.76***	0.62*	-0.07	0.39	-0.48	-0.31							
SA	0.23	0.33	0.12	-0.39	0.41	0.01	0.40	0.40						
M/F	-0.15	-0.37	**-0.69***	-0.38	0.25	**0.76***	0.57*	-0.48	-0.10					
Age	0.10	0.49	0.51	-0.23	0.19	-0.49	-0.41	**0.75***	0.11	-0.49				
L-1	0.21	0.51	0.57*	0.04	-0.18	-0.29	-0.13	0.43	0.36	-0.24	0.24			
L-2	0.24	0.43	0.44	0.05	-0.25	-0.39	-0.22	0.43	0.18	-0.26	0.31	**0.85***		
L-3	0.13	0.39	0.25	0.01	-0.31	-0.38	-0.27	0.34	0.14	-0.25	0.320	**0.69***	**0.94***	
Kn	-0.58*	-0.39	-0.51	-0.27	0.07	0.43	0.48	-0.58*	0.15	0.460	-0.27	-0.27	-0.29	-0.20

Table 14. Summary of simple linear regression analyses between genetic and demographic characteristics (Demo; df = 13) including, the slope and associated R^2 for each analysis, an asterisk refers to a significant relation at the 0.05-α level, bold indicates a significant relation following sequential Bonferroni corrections ($\alpha_o = 0.01$).

	H_e		A_e		A_r		F_{IS}		d^2		r	
Demo	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2
YOY	-0.002	0.151	-0.012	0.036	-0.079	0.092	**-0.008**	**0.447***	0.224	0.033	0.001	0.263
SI	**1.58×10^{-4}**	**0.584***	**0.002**	**0.456***	0.006	0.387*	-3.80×10^{-5}	0.004	0.023	0.151	-2.86×10^{-5}	0.227
SA	0.004	0.110	0.041	0.060	0.061	0.014	-0.012	0.154	1.352	0.185	4.99×10^{-5}	<0.001
M/F	-0.001	0.140	-0.007	0.023	**-0.101**	**0.471***	-0.003	0.142	0.226	0.062	**0.001**	**0.575***
Age	0.003	0.236	0.010	0.01	0.145	0.258	-0.004	0.052	0.342	0.037	-0.001	0.236

Table 15. Results of forward stepwise regression analysis to determine the best-fit demographic model for each genetic characteristic. The R^2 was adjusted (R^2_{adj}) when more than one variable was present in the model. Degrees of freedom (df), F-value, and *p*-value are shown for each model.

Variable	Model	R^2_{adj}	df	F	*p*
H_e	0.0002(SI) + 0.761	0.584	13	18.277	0.001
A_e	0.005(SI) - 0.047(SA) - 0.097(Age) + 5.208	0.859	11	29.524	<0.001
A_r	-0.101(M/F) + 9.775	0.471	13	11.547	0.005
F_{IS}	-0.101(YOY) - 0.010(Age) + 0.165	0.713	12	18.403	<0.001
d^2	No Model	–	–	–	–
r	0.001(M/F) + 0.050	0.575	13	17.609	0.001

Table 16. Summary of linear regression analyses between back-calculated length at age-1, -2, and -3 and the condition factor (Kn) versus each genetic or demographic characteristics. The slope and associated R^2 are shown for each analysis; an asterisk refers to a significant relation at the 0.05 α-level. No values were significant after sequential Bonferroni corrections ($\alpha_o = 0.0045$).

	Length at age-1		Length at age-2		Length at age-3		Kn	
Variable	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2
A_e	20.46	0.045	33.00	0.057	23.72	0.017	-20.27	0.334*
H_e	739.68	0.258	891.96	0.182	1078.50	0.087	-193.39	0.150
A_r	17.65	0.317*	19.63	0.190	14.82	0.062	-5.31	0.250
F_{IS}	19.27	0.001	36.59	0.002	9.28	< 0.001	-53.13	0.075
d^2	-0.92	0.033	-1.82	0.063	-2.96	0.094	0.12	0.004
r	-1.41×10^3	0.079	-2.76×10^3	0.147	-3.55×10^3	0.138	0.71×10^3	0.176
YOY	-0.77	0.015	-1.89	0.045	-3.07	0.067	1.02	0.229
SI	0.13	0.176	-0.19	0.183	0.19	0.113	-0.06	0.328*
SA	5.75	0.133	4.15	0.034	4.08	0.019	0.77	0.021
M/F	-1.09	0.056	-1.71	0.066	-2.13	0.059	0.71	0.207
Age	2.01	0.050	3.89	0.091	5.27	0.095	-0.79	0.068

Table 17. Results of model selection between length or condition variables and genetic or demographic variables using forward stepwise regression. The R^2, degrees of freedom (df), F-value, and *p*-value are shown for each model.

Variable	Model	R^2	df	F	*p*
Length 1	$17.652(A_r)$ -22.907	0.317	13	6.027	0.029
Length 2	No Model	–	–	–	–
Length 3	No Model	–	–	–	–
Kn	$-20.269(A_e)$ + 191.872	0.334	12	6.012	0.030

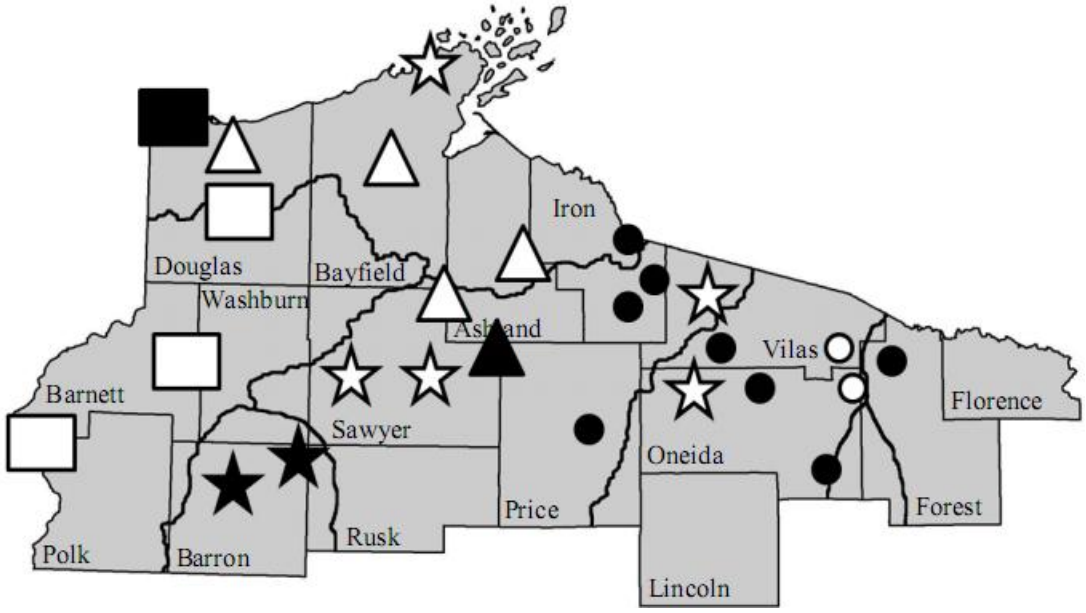

Figure 1. Eight genetic units of Wisconsin walleye described in Hammen (2009). Bold lines represent contemporary management units based on Fields et al. (1997). Black squares represent a St. Louis River genetic unit, white squares represent a St. Croix River genetic unit, black stars represent a lower Chippewa River genetic unit, white stars represent a 'central ceded territory' genetic unit, black triangles represent a Blaisdell Lake genetic unit, white triangles represent a Lake Superior genetic unit, black circles represent an upper Wisconsin genetic unit, and white circles represent an Eagle River genetic unit.

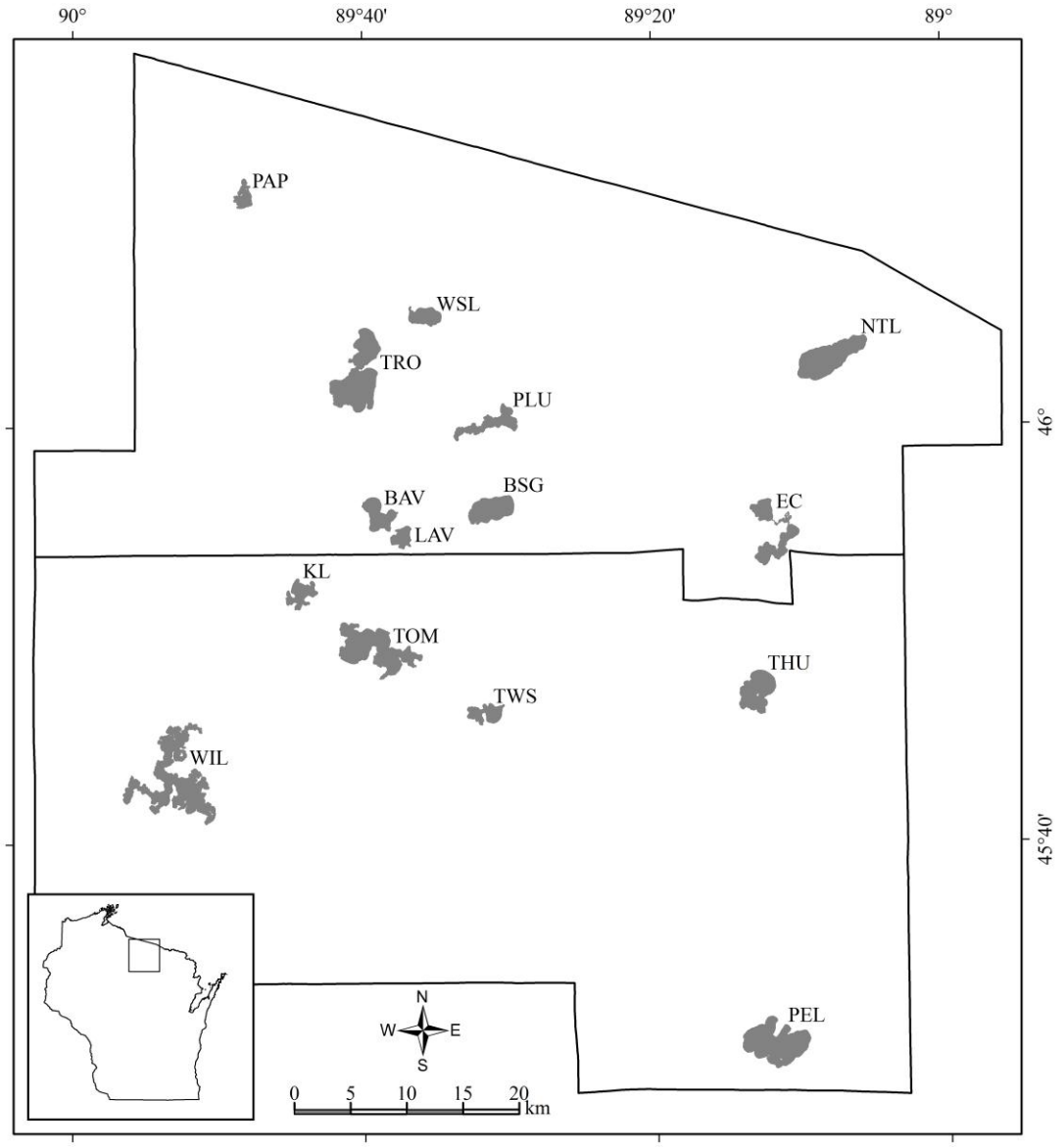

Figure 2. Map showing Vilas County (upper) and Oneida County (lower) in the state of Wisconsin with the 15 study sites. Lake names and Water Body Identification Codes (WBIC) corresponding to abbreviations can be found in Table 2.

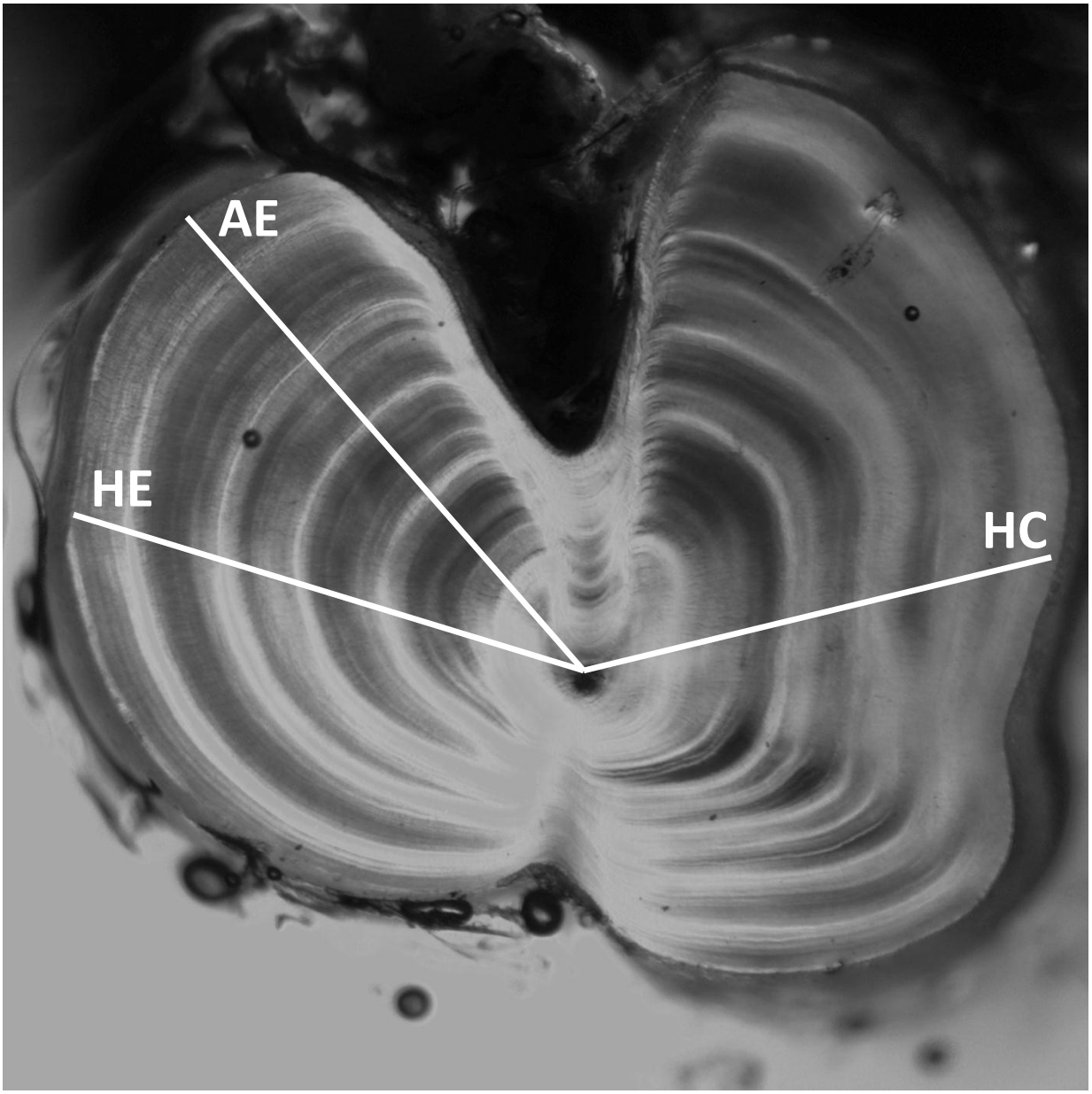

Figure 3. Walleye spine cross-section showing the anterior transect (AE), horizontal elongated transect (HE), and the horizontal compressed transect (HC; Borkholder and Edwards 2001).

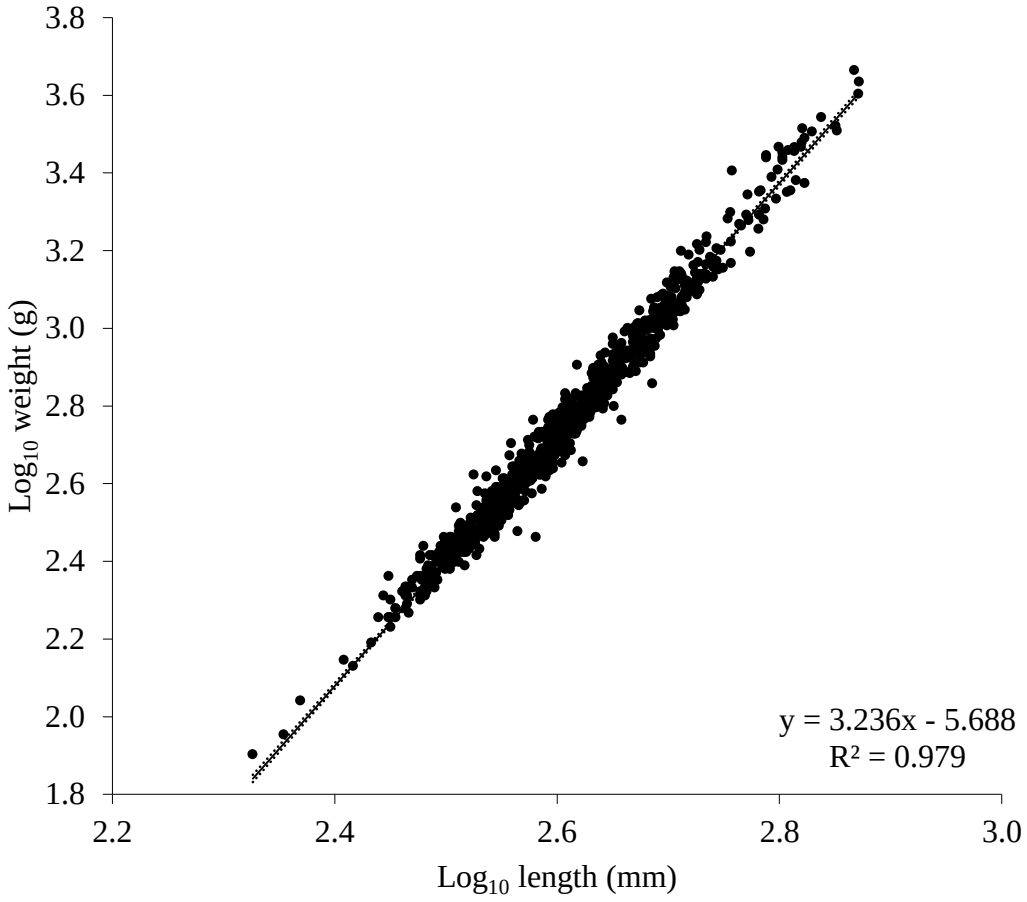

Figure 4. Relation between the length and weight of walleye samples used in this study. Weights were not obtained for PLU samples. Solid line and equation shows the best-fit model using linear regression (df = 808, F = 38,476.4, $p < 0.001$) and dotted lines show the 95% confidence intervals of the regression. Results were used to determine the value of growth parameters (α and β) used in the relative condition calculations.

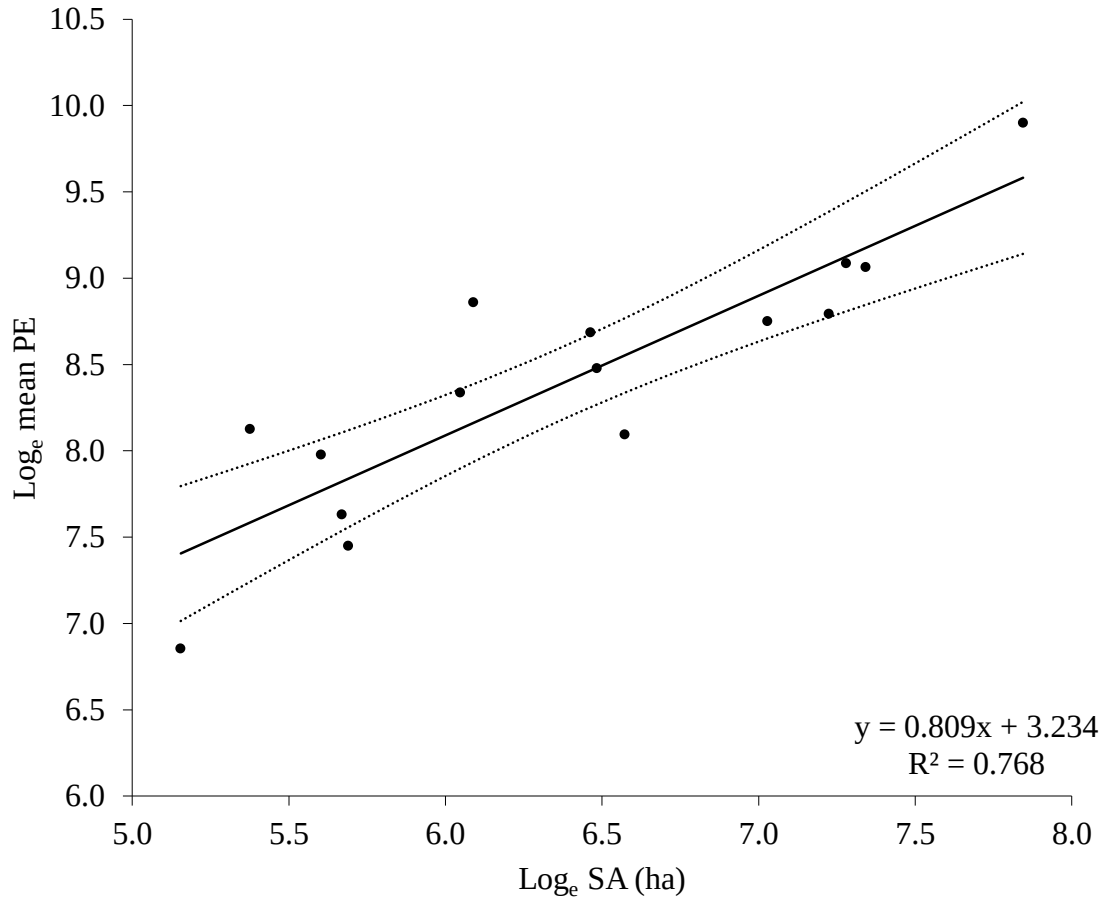

Figure 5. Relation between log$_e$ lake surface area (SA) measured in hectares and log$_e$ mean population estimate (PE). Solid line and equation shows the best-fit model using linear regression (df = 13, F = 43.110, $p < 0.001$) and dotted lines show the 95% confidence intervals of the regression.

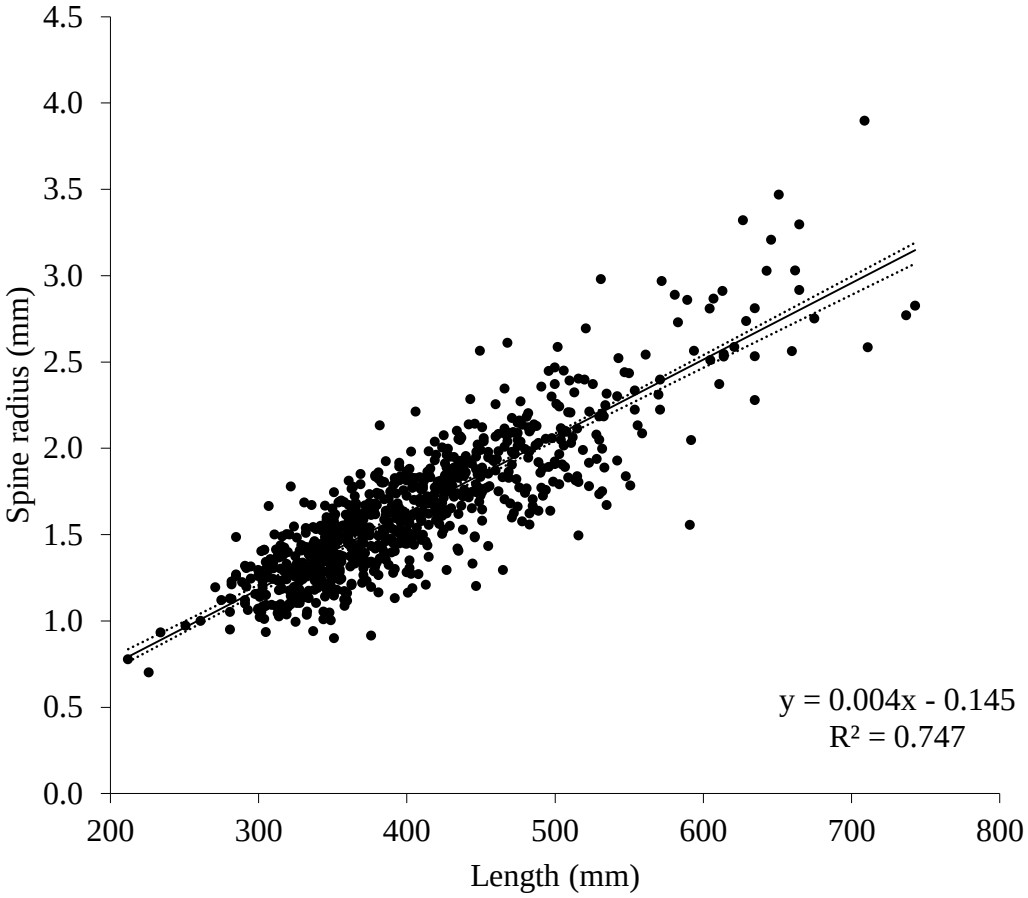

Figure 6. Relation between sample length at capture (length) and dorsal spine radius using the AE transect**.** Solid line and equation shows the best-fit model using linear regression (df = 824, F = 2,450.7, $p < 0.001$) and dotted lines show the 95% confidence intervals of the regression. Results were used to predict the average length walleye first developed dorsal spines.

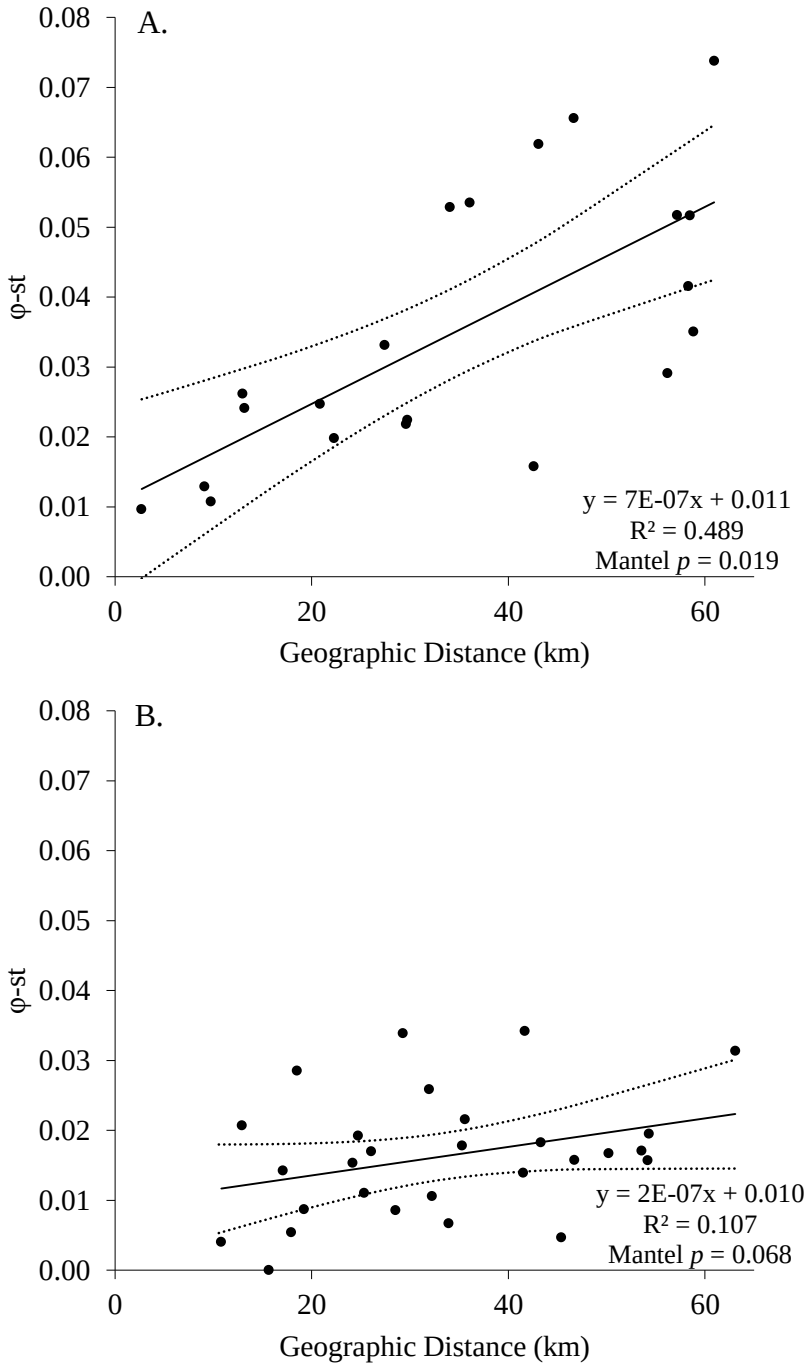

Figure 7. Relation between genetic distance (φ-st) and geographic distance between all pairs of non-stocked (A) and stocked (B) populations. Solid lines and equation shows the best-fit model using linear regression populations and dotted lines represent the 95% confidence intervals of the regression. Significance between geographic and genetic distance was calculated using a Mantel test with 1,000 permutations and is shown under the equation (Mantel *p*).

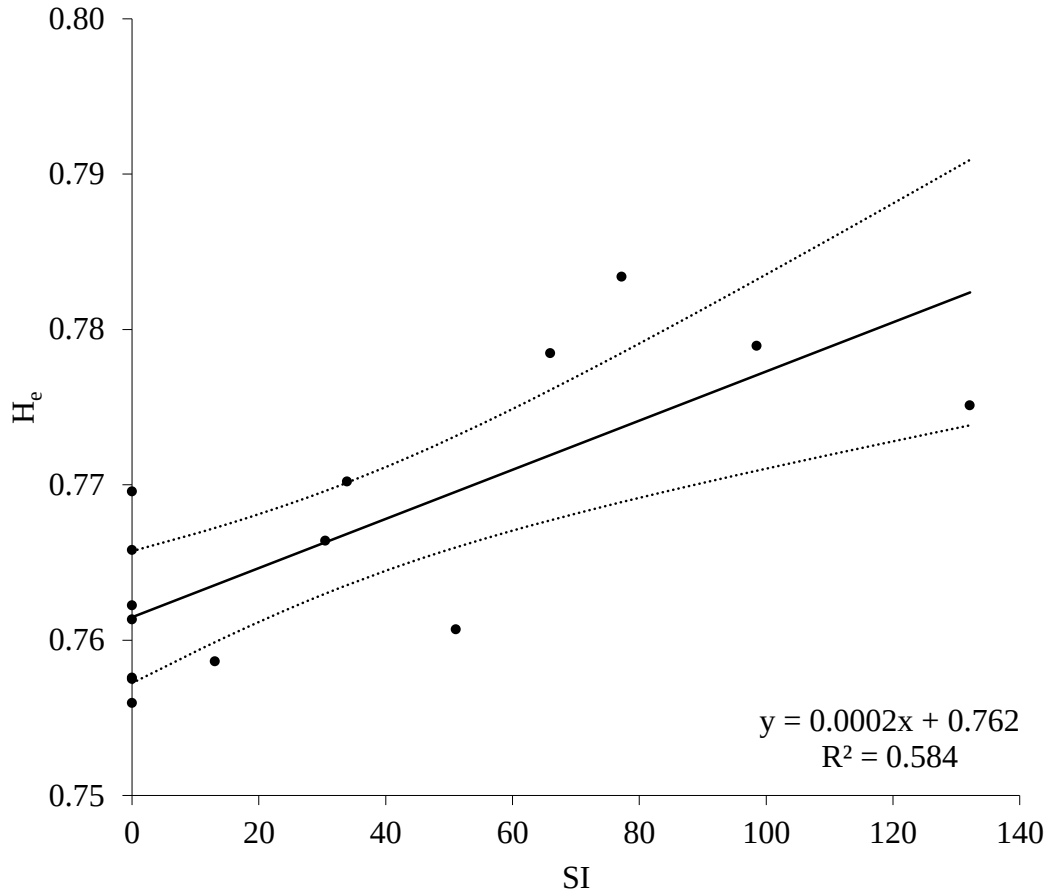

Figure 8. Relation between stocking index (SI) and expected heterozygosity (H_e). Solid line and equation shows the best-fit model using linear regression (df = 13, F = 18.277, $p < 0.001$) and dotted lines show the 95% confidence intervals of the regression.

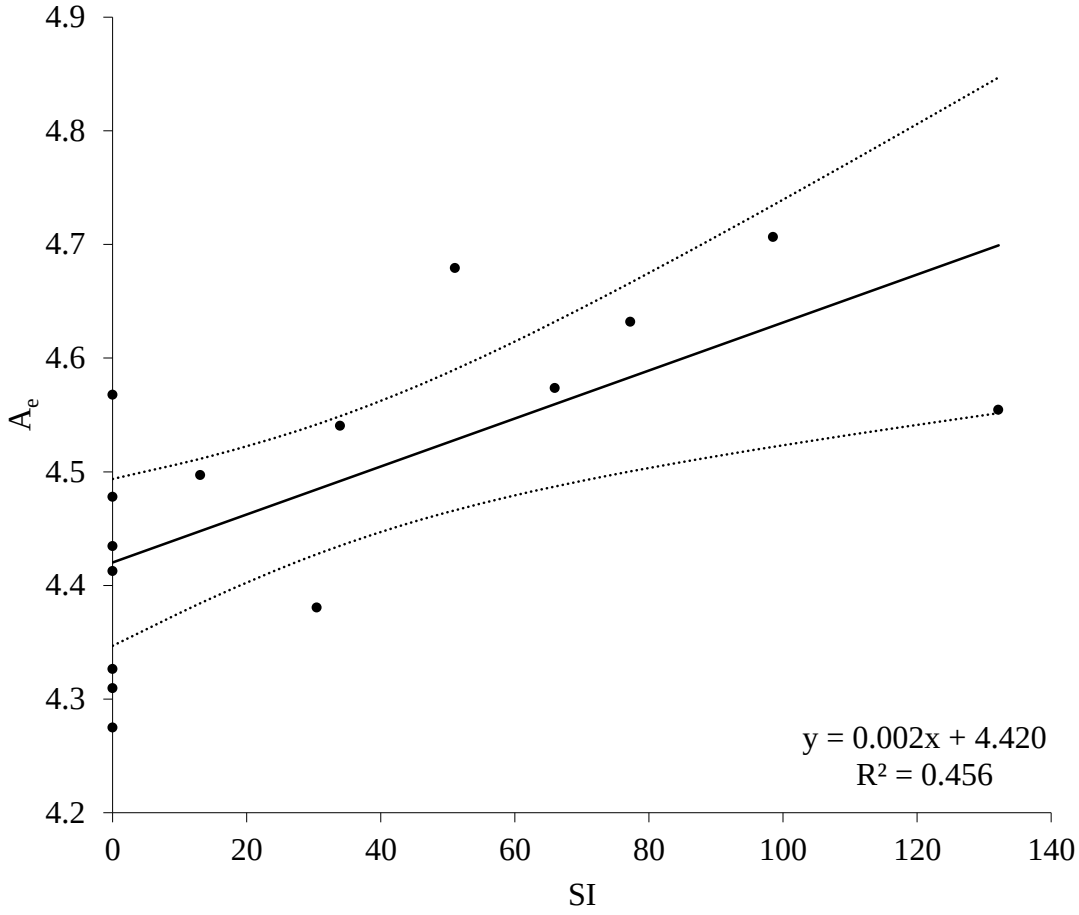

Figure 9. Relation between stocking index (SI) and effective number of alleles (A_e). Solid line and equation shows the best-fit model using linear regression (df = 13, F = 10.904, p = 0.006) and dotted lines show the 95% confidence intervals of the regression.

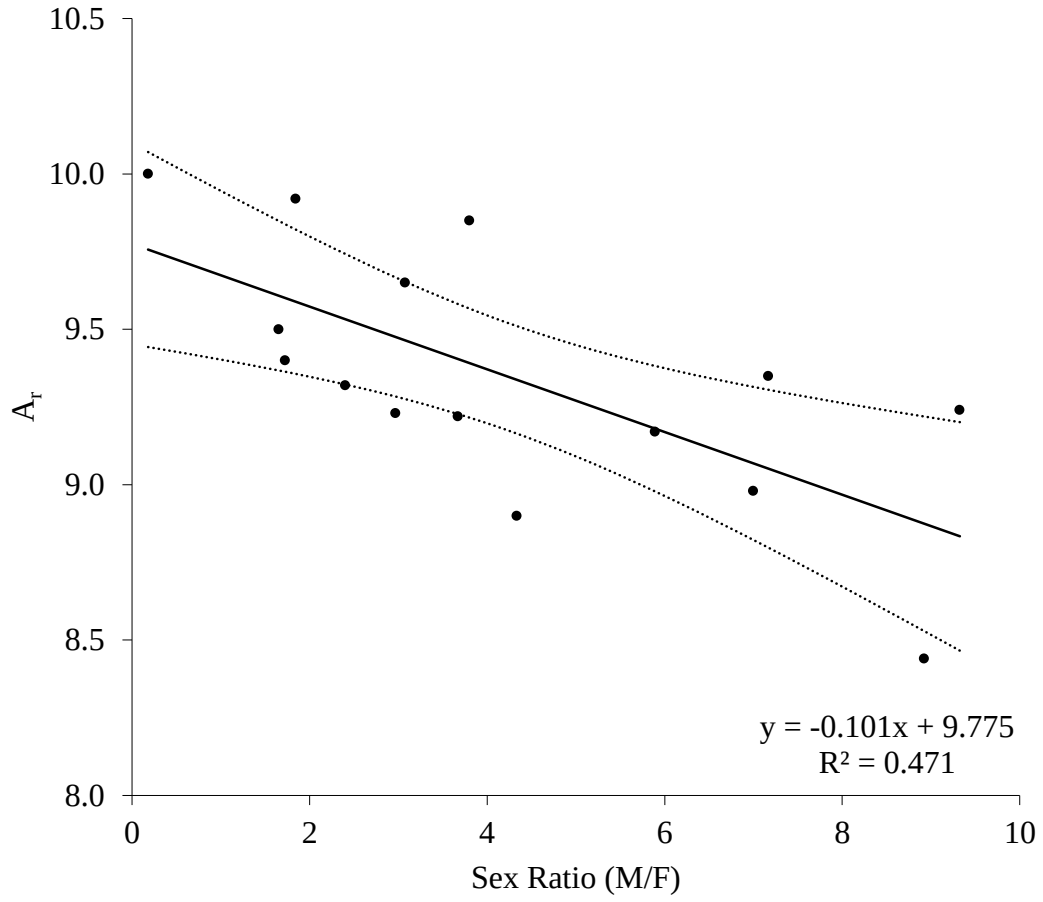

Figure 10. Relation between the sex ratio and allelic richness (A_r). Solid line and equation shows the best-fit model using linear regression (df = 13, F = 11.560, p = 0.004) and dotted lines show the 95% confidence intervals of the regression.

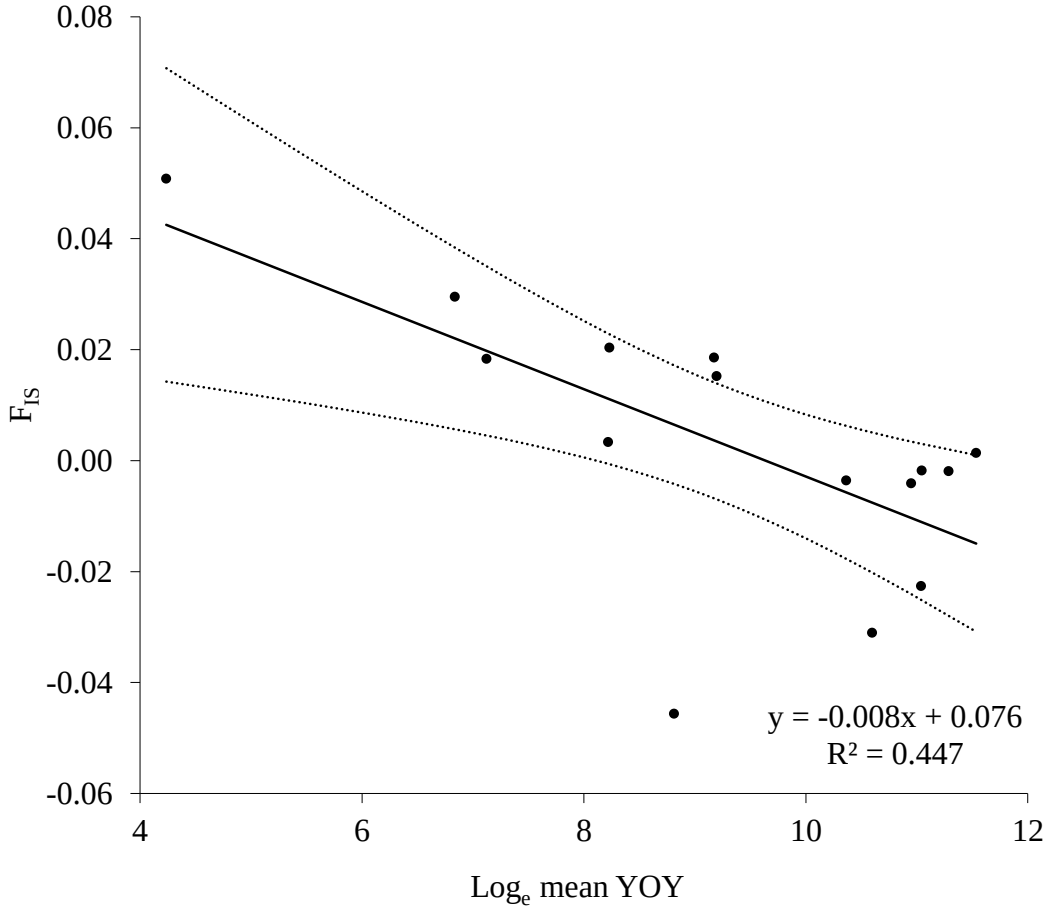

Figure 11. Relation between log_e mean YOY abundance and F_{IS}. Solid line and equation shows the best-fit model using linear regression (df = 13, F = 10.519, p = 0.006) and dotted lines show the 95% confidence intervals of the regression.

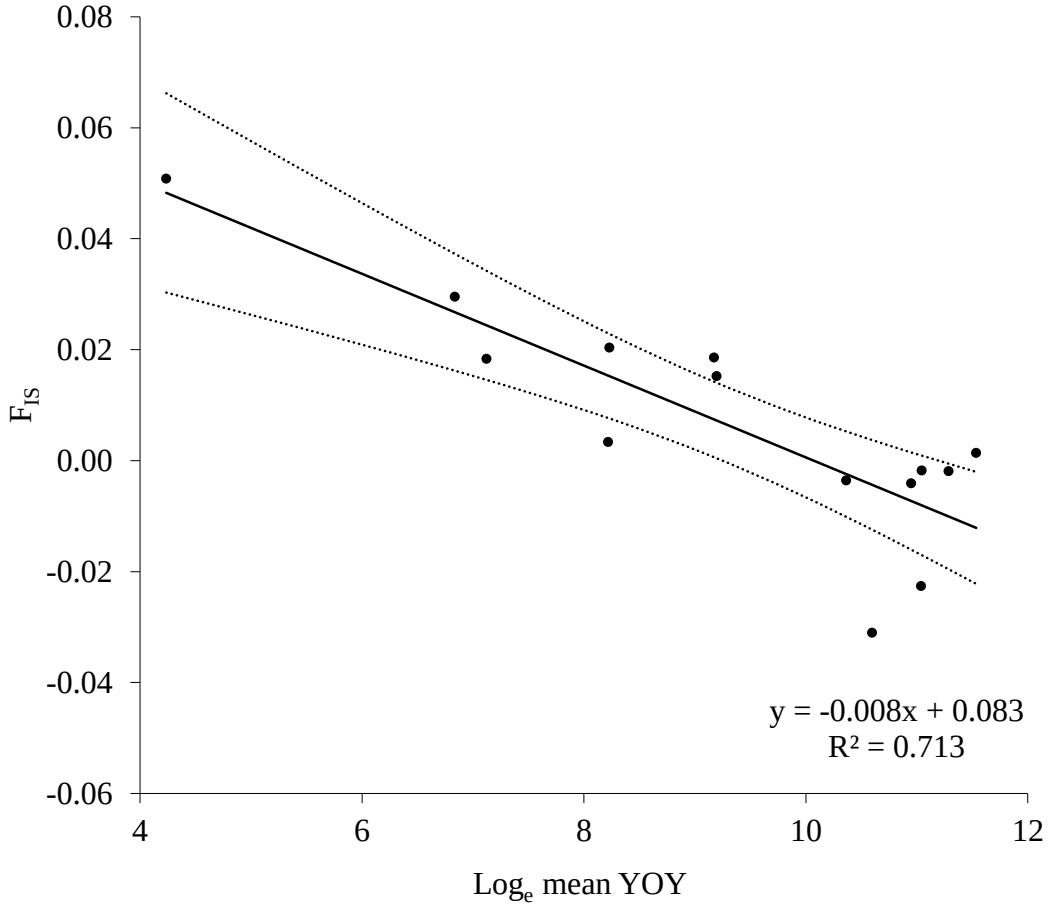

Figure 12. Relation between log_e mean YOY abundance and F_{IS} excluding the outlying value from TOM. Solid line and equation shows the best-fit model using linear regression (df = 12, F = 29.839, $p < 0.001$) and dotted lines show the 95% confidence intervals of the regression.

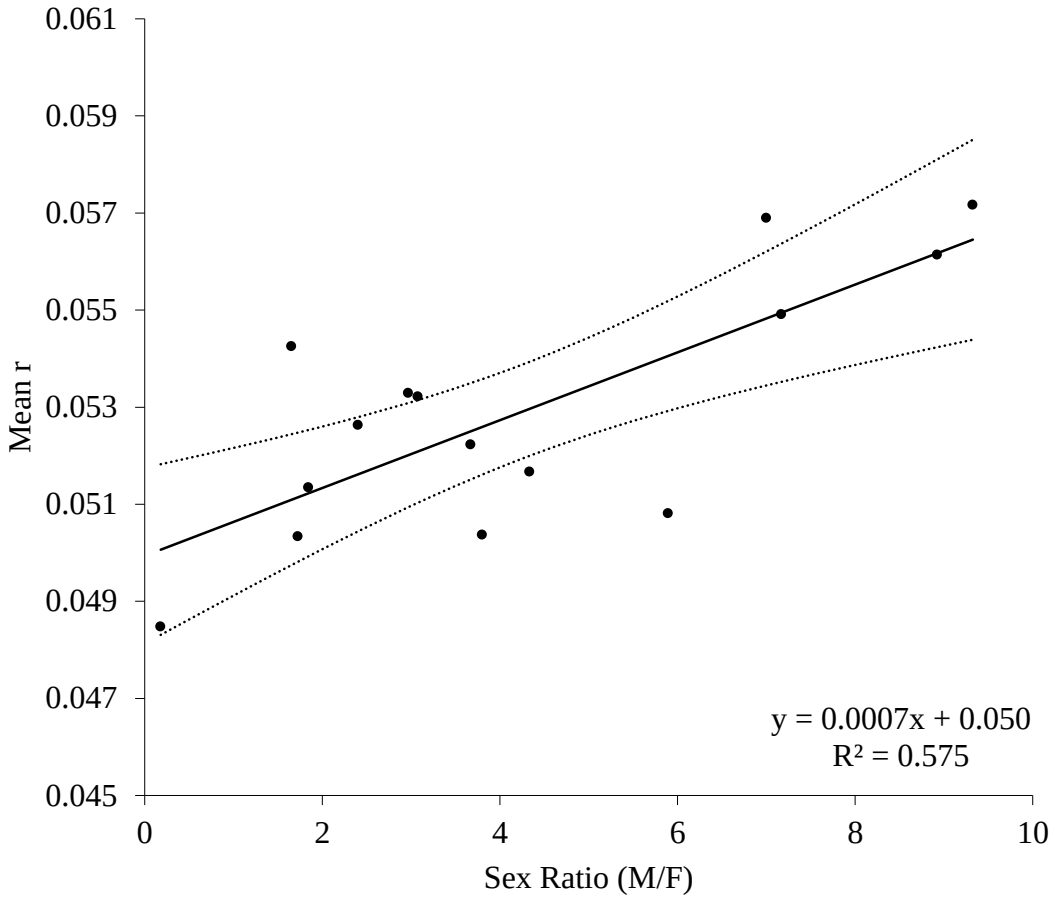

Figure 13. Relation between the sex ratio and mean relatedness (r). Solid line and equation shows the best-fit model using linear regression (df = 13, F = 17.609, p = 0.001) and dotted lines show the 95% confidence intervals of the regression.

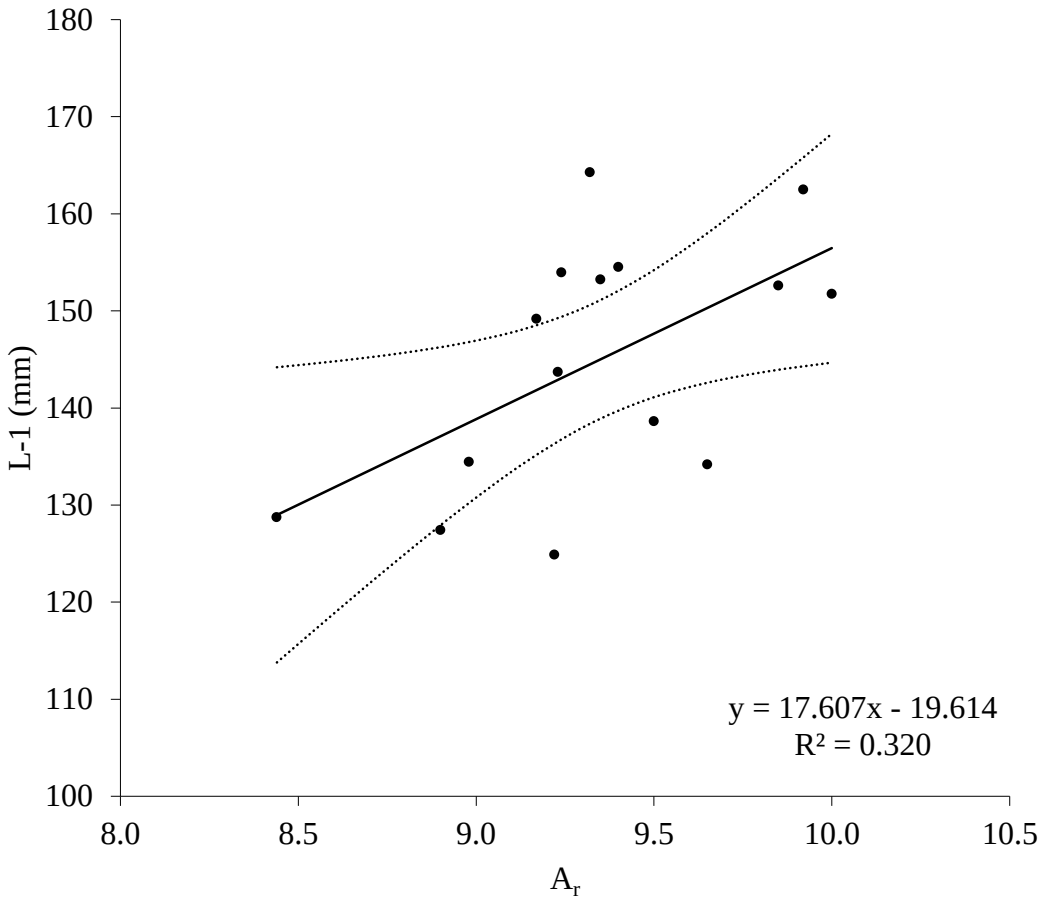

Figure 14. Relation between the length at age-1 (L-1) and allelic richness (A_r). Solid line and equation shows the best-fit model using linear regression (df = 13, F = 6.109, *p* = 0.028) and dotted lines show the 95% confidence intervals of the regression.

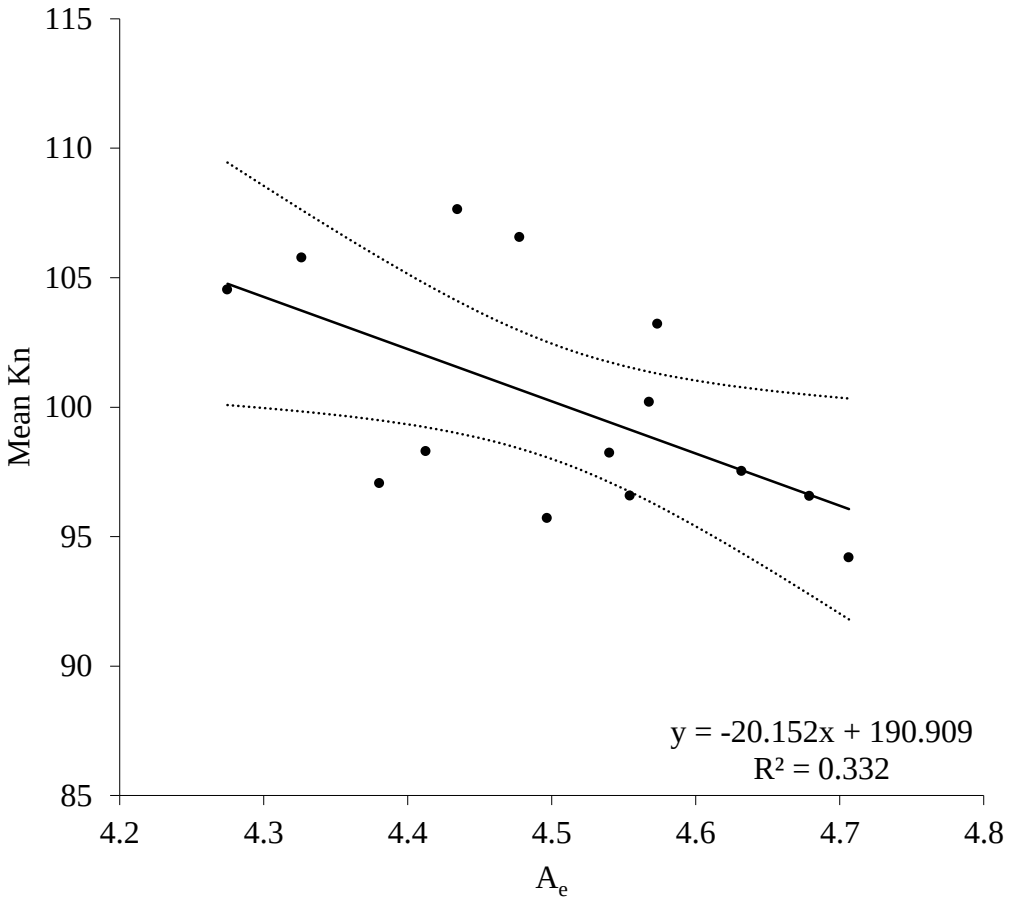

Figure 15. Relation between mean condition factor (Kn) and effective number of alleles (A_e). Solid line and equation shows the best-fit model using linear regression (df = 12, F = 5.959, p = 0.031) and dotted lines show the 95% confidence intervals of the regression.

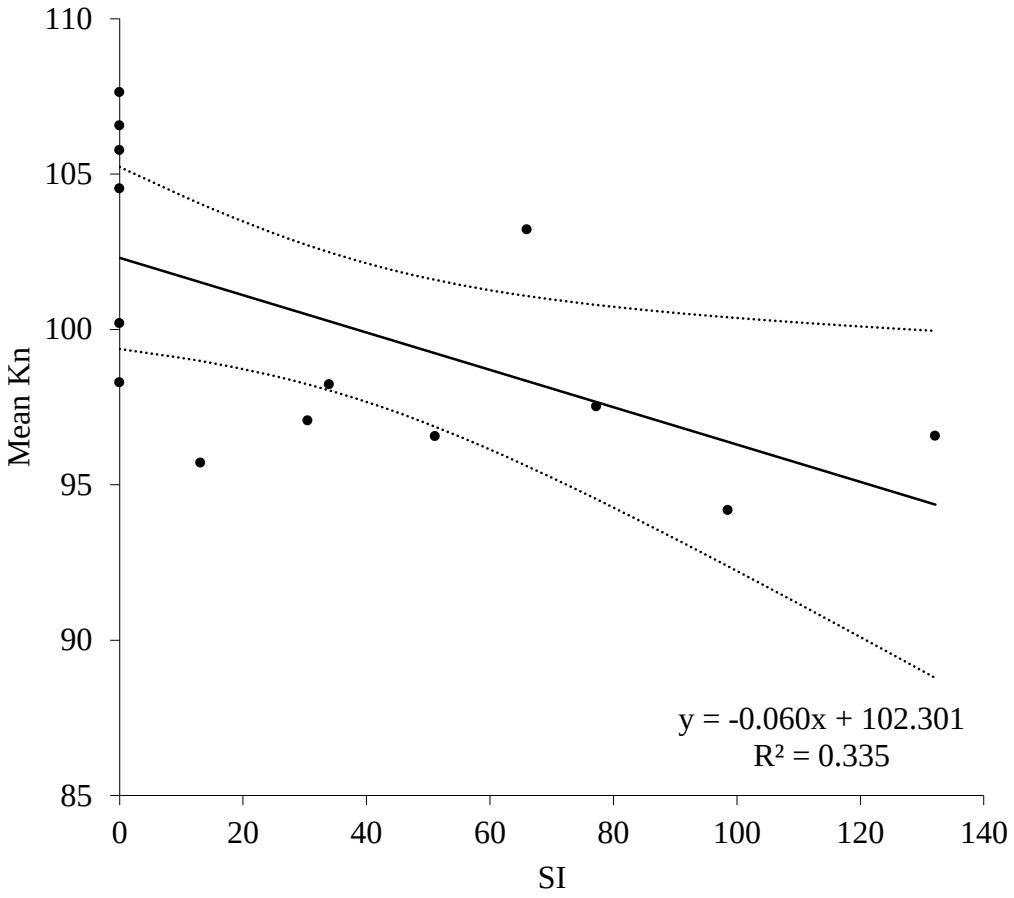

Figure 16. Relation between mean condition factor (Kn) and the stocking index (SI). Solid line and equation shows the best-fit model using linear regression (df = 12, F = 6.032, p = 0.030) and dotted lines show the 95% confidence intervals of the regression.

APPENDIX A

Molecular Conservation Genetics Laboratory

USGS Wisconsin Cooperative Fishery Research Unit
University of Wisconsin, Stevens Point

QUICK PROTOCOL

DNA Extraction from Tissue, Promega, 96 Well Format
Procedure #: MCGL-003

Supplies required:

Nuclei lysis solution	Proteinase K (25mg/mL)	0.5mM EDTA
70% ethanol	Ice bucket	50mL Falcon tube
Multi-channel basin plate	96-well PCR plate	96-well round bottom
Adhesive film	Heat seal foil	1-200µL tips
Cutting board	Biopsy punch (3mm or 5mm)	Kimwipes
5% bleach solution	Protein Precipitate Solution	Isopropanol
300 µL tips		

Equipment:

Eppendorf Thermosealer	WELCH multi-channel aspirator
Thermomixer R	Eppendorf Centrifuge 5804
Tweezers	Scissors
Multi-channel pipet	LABCONCO Centrivap Concentrator

Things to do before starting:

- Turn on Eppendorf Thermosealer
  - CAUTION: default temperature at 170°C
- Turn on Thermomixer R
  - Set temperature to 55°C, 800 rpm, and 3 hours
- Fill Styrofoam ice bucket with ice
  - Ice machine on 1st or 4th floor

<u>TISSUE PREPARATION</u>

1. Clean surface area of the cutting board using 5% bleach followed by 70% ethanol.
2. Prepare cutting board using tape to grid eight areas that correlate with the 96 well plate.
3. Label 96-well non-raised PCR plate:
   - Project code

- Date performed
- Initials
- Tissue (sample)

4. Place the first row of 8 sample tubes in a separate rack. Take off all caps.

5. Using clean tweezers, transfer tissue sample (1-8 sample tubes) to each grid area for biopsy punch.

6. Cut sample using a 3mm or 5mm biopsy punch and place in correct well of the 96 well non-raised PCR plate.

NOTE: If cutting fin clip, flatten out sample just before using the biopsy punch

Important: Wash tweezers and biopsy punch with 5% bleach and 70% ethanol between samples.

7. Place sample back into original tube and add 95% ethanol to fill tube.

8. Clean grid surface area with 5% bleach followed by 70% ethanol before cutting the next eight samples.

9. If tissue samples will be extracted another day, place 96-well plate with tissue punch in -20°C freezer until ready to use.

<u>EXTRACTION (See procedure for details of extraction)</u>

10. When all samples are cut, prepare digestion mixture (per sample) using a 50mL conical tube.

11. Calculate the total volume of Nuclei Lysis solution and 0.5 M EDTA based on number of samples.

- Add 120µL Nuclei Lysis solution
- 30 µL 0.5M EDTA

12. Place digestion mixture on ice
    - Digestion mixture will become milky when chilled
    - **Important:** Digestion mixture must be chilled
13. Add 12µL Proteinase K (20mg/mL) to digestion mixture just before aliquoting into 96-well plate and vortex digestion mixture 5-10 seconds.
14. Add 162 µL digestion mixture to each well using a multi-channel pipet and basin.
15. Cover 96-well plate with heat seal foil
    - Blue dot (or label) indicates top of heat seal foil
    - Check for proper orientation of heat seal foil to prevent melting or sticking to Thermosealer
    - Place an X on upper left hand corner of the heat seal foil to prevent mix up of A1 location and reduce sample error
16. Seal plate using the Eppendorf Thermosealer (See procedure for instructions)
    CAUTION: Default setting 170°C
17. Place 96-well plate on the Thermomixer R to incubate and shake for 3 hours.
18. Quick spin 96-well plate to remove moisture from surface of foil

19. **IMPORTANT:** Allow digestion mixture/samples to cool to room temperature.
20. Add 55µL Protein precipitate solution to each well of a new 96-well plate using a multi-channel pipette and basin.
21. Using the multi-channel pipette with tips, carefully pierce the heat seal foil with tips.
22. Carefully transfer the supernatant to the protein precipitate 96-well plate using the multi-channel pipette.
23. Mix the supernatant and protein precipitate solution by pipetting.
24. Seal the plate with a NEW piece of Secure Seal™ adhesive film and secure film with roller.
25. **IMPORTANT:** Place on WET ice for at least 10 minutes.
    - Supernatant/protein precipitate solution will turn a milky color
26. Centrifuge 96-well plate at 3700 rpm for 4 minutes using the Eppendorf Centrifuge 5804.
    - Remember to balance plate(s)
27. Prepare isopropanol plate while plate during centrifugation.
28. Add 150µL isopropanol to a round bottom (Fisher) plate using a multi-channel pipette and basin.
29. Remove plate from centrifuge without disturbing the pellet.
30. Carefully remove adhesive film by holding plate and slowly pulling off film. Discard adhesive film.
31. Carefully transfer the supernatant to the 96-well round bottom plate containing 150µL of isopropanol for DNA precipitation.
    - Do not disturb pellet
32. Mix the supernatant and isopropanol by pipetting at least five times.
33. Seal plate with a NEW piece of Secure Seal™ adhesive film and secure film with roller.
34. **IMPORTANT:** Incubate isopropanol and supernatant for 10 minutes at room temperature.
35. Centrifuge 96-well plate a 3700 rpm for 4 minutes using the Eppendorf Centrifuge 5804.
36. Carefully remove adhesive film by holding plate and slowly pulling off film. Discard adhesive film.
37. Using the WELCH multi-channel aspirator, carefully aspirate the supernatant. (See procedure for details)
38. Add 150 µL 70% ethanol to pellet and mix by pipetting.
39. Seal plate with a NEW piece of Secure Seal™ adhesive film and secure film with roller.
40. Centrifuge 96-well plate at 3700 rpm for 4 minutes using the Eppendorf Centrifuge 5804.

41. Using the WELCH multi-channel aspirator, carefully aspirate the supernatant.
42. Dry samples using LABCONCO Centrivap Concentrator or air dry samples on bench top with a Kim wipe covering plate.
43. Elute sample with 100µL Tris-low-EDTA (TLE). Note: small pellet elute with lower volume
44. Store DNA samples at 4°C (Refrigerator C) if normalizing same day or next day. Store DNA samples at -20°C (Freezer B) for long term storage.