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Characterizing Winter Flounder (Pseudopleuronectes americanus) Nursery Areas Using Otolith Microstructure and Microchemical Techniques

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Otolith Microstructure and Microchemical Techniques

BY

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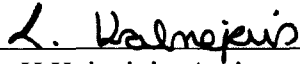
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ABSTRACT

CHARACTERIZING WINTER FLOUNDER (*PSEUDOPLEURONECTES AMERICANUS*) NURSERY AREAS USING OTOLITH MICROSTRUCTURE AND MICROCHEMICAL TECHNIQUES

by

David Bailey

University of New Hampshire, September, 2013

A preliminary study, using young-of-the-year winter flounder from 12 nursery areas from New Jersey to New Hampshire, evaluated indirect and direct measurements of nursery quality. Growth and condition indices (length d^{-1}, weight d^{-1}, Fulton's K and relative weight) were calculated from otolith microstructure to indirectly evaluate nursery quality. Boston Harbor, MA and Great Bay, NH were found to be the healthiest nurseries and the Niantic River, CT was found to be the least healthy nursery. In addition to these indirect indices, we conducted a study to determine the effectiveness of otolith microchemistry as a direct measurement of nursery habitat. Otolith elemental signatures were found to be site specific and vary on a small spatial scale (5-10km). Juveniles were classified back to natal nursery areas with 73% accuracy using otolith signatures. The indirect and direct measurements used in this study can be used to assess nursery habitat quality in the future.

INTRODUCTION

<u>Nurseries</u>

Estuaries and shallow marine coastal habitats have long been recognized as nurseries for fish. These nurseries supply adult fish to the offshore fisheries of many commercially important species (Beck et al. 2001). A nursery is a habitat where the growth and survival of juvenile fish is enhanced through favorable habitat quality. These habitats are favorable because they have abundant food sources, potentially lower predation, and higher water temperatures (Miller et al. 1991, Gibson 1994, Beck et al. 2001). It is in these nurseries where fish mature beyond early life stages eventually leading to recruitment into the adult population. It is important to note that a habitat is only a nursery area if its contribution of individuals that recruit into the adult population is greater, on average, than the contribution of other habitats (Beck et al. 2001). This means that not all juvenile habitats can be nurseries.

Even though estuaries and shallow marine coastal habitats are critical for sustaining fisheries, they are among the most threatened marine environments (Blaber et al. 2000). Anthropogenic disturbances to nursery areas have the potential to reduce juvenile fish growth and survival (Vasconcelos et al. 2007a). Anthropogenic disturbances including habitat alteration through agricultural, industrial, and engineering practices, pollution discharge, and heavy fishing pressure can all negatively affect the juvenile fish community within nurseries (Vasconcelos et al. 2007a). Reduced growth and survival in juveniles can have a cascading effects on the commercially important adult population (Désaunay et al. 2006, Hermant et al. 2010). Therefore, it is critical to maintain the

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highest quality nursery habitats to increase the probability of juveniles recruiting into the adult population. In order to maintain the highest quality nursery habitats a method for assessing nursery quality needs to be developed.

Winter Flounder

Certain stocks of winter flounder, *Pseudopleuronectes americanus,* a demersal, right-eyed flatfish, use estuaries and shallow marine coastal habitats as nurseries. Due to their large range, New Jersey to Nova Scotia (Perlmutter 1947, Scott and Scott 1988), winter flounder are separated into stocks, a common fisheries science identification technique. Stocks are groups of fish with similar life history characteristics that are essentially self-reproducing (Hilborn and Walters 1992). In the US, winter flounder are managed as three distinct stocks: the Gulf of Maine (GOM), Georges Bank (GB), and Southern New England/Mid-Atlantic (SNE/MA) stocks (Brown and Gabriel 1998). The two stocks that use estuaries and shallow marine coastal habitats as spawning sites and nurseries are the SNE/MA and GOM stocks (Pereira et al. 1999). However, recent studies have found some GOM adults utilizing deeper coastal waters for spawning (DeCelles and Cadrin 2010, Fairchild et al. 2013), which could mean juveniles are using offshore nursery areas too.

Despite these recent findings, adult winter flounder from the GOM and SNE/MA stocks typically migrate inshore in the fall and early winter, and spawn in late winter and early spring (Pereira et al. 1999). Winter flounder generally exhibit a high degree of site fidelity (Saila 1961, Phelan 1992), which is maintained by adults returning to natal areas and spawning adhesive demersal eggs (Pearcy 1962). Once hatched, larvae are pelagic, but due to their affinity to the benthos, are negatively buoyant and are able to regulate their vertical position in relation to the tide, resulting in high larval retention within

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estuaries and coastal habitats (Pearcy 1962). This suggests that spawning and nursery areas are closely linked (Pearcy 1962).

Winter flounder larval development is temperature-dependent and increases with increasing water temperature (Laurence 1975). After ~60 days in the water column, the larvae go through metamorphosis and settle to the benthos as juveniles (Chambers and Leggett 1987). Settlement typically occurs in the spring and early summer (Colette and Klein-MacPhee 2002). Juveniles remain in nursery areas for the first two years before moving offshore (Pereira et al. 1999, Fairchild et al. 2009). The time spent in these nursery areas is critical because it supports the development beyond the early life stages, a time period where the mortality rate reaches 99% (Pearcy 1962). The year class strength of winter flounder is determined primarily during these early life stages spent in the nursery (Sogard 1991).

As is the case with most commercially important fish species, winter flounder populations have declined dramatically over recent decades. Total commercial landings of winter flounder have fallen 89% from 1981 to 2010 ((NEFSC), 2011). Currently there is a federal winter flounder fishing moratorium for the SNE/MA stock and a 50 lbs. day^{-1} limit in state waters, rendering what was historically the largest stock commercially inactive. The GOM stock is doing slightly better, with federal and state trips each limited at 500 lbs. day^{-1}. The GB stock is currently the healthiest with federal trips limits at 1000 lbs. One reason for these declines in winter flounder populations could be from negative effects on their nursery habitats. Although fishing regulations are essential for the management of winter flounder, assessing the quality of the nursery habitat is also very

important. Assessing the quality of the nursery habitats will allow for better protection which could lead to increased recruits into the adult population.

Using Indices to Quantify Nursery Quality

The numerous factors influencing nursery health, and the complex relationship among all the factors, make it difficult to quantify nursery quality directly (Gibson 1994, Adams 2002). Thus, the quality of a winter flounder nursery is typically measured by comparing indirect metrics such as growth and condition indices. Growth has been the main metric for assessing quality because survivorship has been linked to growth rates (Houde and Hoyt 1987). For example, with rapid growth fish can achieve refuge size quicker when experiencing size-dependent predation (Taylor 2003), increasing survival and fitness. Fast growth also provides a survival advantage, with larger fish having a lower over-wintering mortality (Sogard 1997). Condition indices have been linked to fitness, with the assumption that fish in better condition have an increased fitness (Murphy et al. 1991). Therefore, high quality nursery areas are those in which juvenile fish have increased growth and condition indices, indicating optimal natural environmental conditions and low anthropogenic effects (Gibson 1994).

Using Connectivity to Quantify Nursery Quality

The only direct way to measure nursery quality is to quantify recruitment from indicidual nurseries into the adult populations. Unfortunately, the evidence to support successful recruitment has been indirect due to difficulties in obtaining juvenile movement data (Beck et al. 2001). Conventional tagging techniques to study juvenile movement suffer from multiple drawbacks including difficulty tagging small individuals, high mortality rates, and low tag return rates (Gillanders 2002). In the case of winter flounder, typical tags used for small (YOY) juveniles are coded wire tags and visible

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implant elastomer tags (Fairchild et al. 2005, Sulikowski et al. 2006). Though coded wire

and visible implant elastomer tags have been used successfully to track fish over months

in relatively small estuaries (Fairchild et al. 2005), their usefulness in tracking fish over

longer periods of time (years) in an expansive environment is questionable. Coded wire

tags are not externally visible so unless dedicated scientific surveys exist, tagged fish will

go undetected by fishermen. Visible elastomer tags fade over time or become occluded

by the fish's pigment making them hard to identity as the fish grow over time. Small t-bar

tags, disc tags, and acoustic transmitters are used on larger (age 1+) fish (Fairchild et al.

2013) but these tags also suffer from drawbacks. T-bar tag retention rates can be very low

and disc tags are subject to being over grown as the fish grows. Acoustic transmitters

may provide the most accurate movement data but they are expensive and unless

receivers are placed in the correct locations, the movement of the fish may not be

recorded. Transmitter signals may also be suppressed due to objects obstructing the

signals pathway to the receiver causing loss of data, which is common for a demersal

flatfish like winter flounder (Fairchild et al. 2013). Due in part to these difficulties, a new

technique to assess fish population connectivity using otolith microchemistry has been

developed.

Otoliths, found in the inner ear of fish, are structures composed of calcium

carbonate crystals. Otolith growth occurs daily and the newly deposited material creates a

pattern of daily growth rings. This newly deposited material also incorporates trace

elements from the environment. Once the new growth is deposited, it is metabolically

inert (it is neither reincorporated nor reworked), creating a pattern that is preserved for

the life of the fish (Campana et al. 2000). This pattern or otolith chemical signature may

provide a natural tag for tracking where fishes have been (Gillanders and Kingsford 1996, Campana 1999, Campana and Thorrold 2001). Quantification of connectivity using otolith elemental composition as a natural tag of a habitat enables the retrospective identification of nursery source(s) of adult fish (Thorrold et al. 2001, Hamer et al. 2005, Brown 2006, Vasconcelos et al. 2011). These chemical tags are useful only if nursery habitats impart distinct signatures. This study is the first to assess whether the nurseries used by winter flounder have distinct chemical signatures that can be traced into adult stocks.

Content

The first chapter indirectly evaluates the quality of winter flounder nurseries across a latitudinal gradient using both growth and condition indices. The growth indices are calculated using otolith microstructure analysis and the condition indices are calculated using morphometric measurements. This helps us begin to determine where the healthier nurseries are located and what characteristics of an estuary or shallow water environment make it a favorable nursery.

The second chapter gauges the effectiveness of otolith microchemistry as a method for quantifying nursery and adult population connectivity in winter flounder. In order to determine its effectiveness, an extensive suite of elements have been analyzed to determine: 1) if elemental signatures are site-specific; 2) the spatial scale at which elemental heterogeneity exists; and 3) the accuracy of classifying fish to natal nursery areas.

In future studies, the otolith chemical signatures determined in this study can be used to compare to adult populations of winter flounder to determine if the healthiest nurseries, as identified in Chapter 1, are actually recruiting the largest number of fish to the fishery.

CHAPTER I. GROWTH AND CONDITION OF YOUNG OF THE YEAR WINTER FLOUNDER (*PSEUDOPLEURONECTES AMERICANUS*) AS INDICATORS OF NURSERY QUALITY

Introduction

Winter flounder, *Pseudopleuronectes americanus,* a demersal, right-eyed flatfish, is an important commercial and recreational species. It is distributed along the northwestern Atlantic, ranging from Georgia, USA to Labrador, Canada, but is most common from New Jersey to Nova Scotia (Scott and Scott 1988). Due to their large range and ecological, behavioral, and growth differences, winter flounder are managed as three distinct stocks in the US: the Gulf of Maine (GOM), Georges Bank (GB), and Southern New England/Mid-Atlantic (SNE/MA) stocks (Brown and Gabriel 1998). As is the case with most commercially important fish species, winter flounder populations have declined dramatically over recent decades. Total commercial landings of winter flounder have fallen 89% from 1981 to 2010 ((NEFSC), 2011). Although fishing regulations are essential for the management of winter flounder, assessing the quality of the nursery habitat is also very important. With the exception of the GB stock, adult winter flounder migrate inshore to nursery habitats, estuaries and shallow coastal waters, in the fall and early winter to spawn in the late winter and early spring (Pereira et al. 1999). The juveniles remain in the estuaries and shallow coastal waters for their first two years before moving offshore (Pereira et al. 1999).

Estuaries and shallow coastal waters have been classified as essential fish habitat (EFH) for juvenile winter flounder. They serve as nursery areas that promote growth and survival because of their abundant food sources, potentially lower predation, and higher

water temperatures (Miller et al. 1991, Beck et al. 2001). These nursery areas support growth and survival beyond early life stages where the mortality rate is much higher, even up to 99% (Pearcy 1962). The year class strength of winter flounder is determined primarily during early life stages (Sogard 1991) which occur in these nursery areas. Unfortunately, due to their near-shore location these nursery areas are susceptible to anthropogenic effects which can lead to habitat alteration, potentially threatening fish populations.

Habitat quality of nursery areas depends on both the natural environmental conditions and the anthropogenic effects on the habitat (Gibson 1994). Natural attributes that affect nursery habitat quality include the physiochemical conditions (e.g. temperature, salinity, dissolved oxygen; (Phelan et al. 2000)), food availability (Vanderveer et al. 1990), predator density (Gibson 1994, Burrows and Gibson 1995), habitat structure (Gibson and Robb 2000), and competition (Rooper et al. 2006). Anthropogenic factors that influence habitat quality include pollution, habitat alteration, and physiochemical alterations.

The numerous factors influencing habitat health, and the complex relationship among all the effects, make it difficult to quantify habitat quality directly (Gibson 1994, Adams 2002). One approach to describing nursery habitat quality is by using indirect metrics to compare habitats such as juvenile condition and growth indices. These indices assume that increased growth rates enable juvenile fish to spend less time in the most vulnerable size ranges (Taylor 2003), thus increasing survival and fitness; high quality nursery habitats are presumed to be habitats where growth or juvenile condition is higher (Vanderveer and Bergman 1987, Sogard and Able 1992, Gibson 1994, Ellis and Gibson

1995). High quality nursery habitats, therefore, will contribute significantly more recruits into the adult population in comparison to nurseries of poorer quality (Power et al. 2007).

Both the condition and growth indices reflect the habitat in which the fish spends most of its time. If a fish moves between nurseries these indices will not reflect just one habitat, and can be less useful. In the present study, the probability of movement between nursery areas is minimized by using young of the year (YOY) winter flounder which remain inshore for their first two years before moving offshore (Pereira et al. 1999, Fairchild et al. 2009). Thus the growth and condition indices in this study represent the quality of the nursery habitat, in which the fish has spent its entire life.

Although it is necessary to determine the quality of nursery areas, it is also critical to understand the spatial differences in the timing of life history events (e.g. hatch date, length of larval phase, and time of settlement). This understanding can be used to promote successful recruitment by indicating when and where habitat protection is most critical.

The goals of this study were to evaluate winter flounder nurseries across a latitudinal gradient by using otolith microstructure analysis and morphometric measurements to: 1) calculate juvenile winter flounder condition and growth indices, and 2) determine the time of metamorphosis.

Materials and Methods

Field Sampling

Young-of-the-year (YOY) winter flounder were collected from 12 locations from Great Bay, NH to the Navesink River, NJ (Figure 1), between June and July 2012. Fish caught from the five Gulf of Maine and four Cape Cod sites were caught using a beach seine (17 m x 2 m; 6.35mm Delta mesh; swept area 550m^2) and/or beam trawls (1.0 m width, 6 mm mesh). Fish were measured (T_L; mm), weighed (g), and given individual sample names after landing. Total length measurements were used to ensure that the collected fish were from the YOY cohort. Based on existing age and size frequency data, fish were thought to be in the YOY cohort if T_L < 90mm, according to Massachusetts Division of Marine Fisheries winter flounder estuarine surveys (J. King, personal commun.). Actual age was later confirmed by counting daily otolith growth rings (see Results). Following initial measurements and cohort identification, fish were euthanized via cervical dislocation, transferred into individual labeled plastic bags, placed on ice, and transported back to the laboratory where they were kept frozen in a 0°C freezer until further analysis. Water quality parameters (salinity, temperature, and dissolved oxygen) were recorded prior to and upon the completion of collecting in a specific area using a YSI 6920 sonde. Habitat type and benthic composition also were recorded at each site. Fish caught from Narragansett Bay, RI, Niantic River, CT, and Navesink River, NJ were caught by third party researchers during routine surveys using various collection techniques. Fish were frozen after collection and transferred to the University of New Hampshire (UNH). Once at UNH, fish were thawed, weighed (g), measured (T_L; mm), and given individual sample names. Although the weighing procedure at these 3 sites

varied from the other 9 sites, weighing after freezing was found to have no effect on the weights. Therefore weights were comparable across all nursery areas.

Condition Indices

The general well-being of all fish was determined by calculating two similar morphometric condition indices. The first, Fulton's K, assumes that heavier fish at a given length are healthier. Fulton's K was calculated using the formula, $K = 100(W/L^3)$, where W is the weight (mg) of the fish and L is the total length (mm). A relative weight condition index also was calculated because it does not assume isometric growth (growth is the same throughout the organism) and is not size dependent (Suthers 1998). The relative weight condition index assumes that fish that are heavier than expected for a given length are in better condition. Relative weight was calculated using the formula $W_R = W/W_S * 100$, where W is the $\log_{10}$ weight (mg) and W_S is the $\log_{10}$ length-specific standard weight (mg) as predicted by a site specific length-weight regression representing all fish caught at a given location.

Growth Indices

Otolith Removal. Both sagittal otoliths were extracted using Teflon coated razors and plastic forceps, and right and left otoliths were separated based on position in the orbital. If there were any discrepancies as to which side they were removed from, they were separated according to the position of the sulcus and the rostrum (Secor et al. 1993). Once separated, otoliths were cleaned in distilled water and stored in individual 1.5 ml micro-centrifuge tubes. Left otoliths, previously found to provide the best correlation

between somatic and otolith growth (Sogard 1991), were used for microstructure

analysis, and right otoliths were used for microchemical analysis.

Approximately 10 winter flounder from each estuary were examined by

microstructure analysis (Table 1). The same fish also were used in a microchemical study

(see Chapter 2). At estuaries where more than 10 winter flounder were collected, the 10

fish with a length closest to the mean fish total length at the sampling site were chosen

for analysis.

Otolith Preparation and Measurement. Left sagittal otoliths were mounted sulcus

side up to glass microscope slides using clear Crystalbond™. Because of the concave

shape of the otoliths, mounting sulcus side up allowed the core to be polished before the

outer edge. This prevented over sanding of the thinner outer edge while enabling a

greater amount of the thicker core to be removed. Mounted otoliths were polished by

hand along the sagittal plane using a series of 800 to 2200 grit wet sand paper. If

polishing with the sulcus side up did not yield clear and visible daily rings, otoliths were

flipped and polished using the same series of sand paper. Once daily growth rings were

clearly visible, otoliths were photographed at 400x magnification using an Infinity

camera mounted to an Olympus CX41 compound microscope. Daily growth rings were

counted from the photographs using ImageJ with the cell counter add-in. Counts were

made, along the rostral axis when possible, from the anterior most accessory primordium

to the edge. Counting in this manner provided an estimate of the date of mid-

metamorphosis, because the accessory primordia appear at the midpoint of eye migration

(Sogard 1991). When daily rings were not clear along the rostral axis, counting took

place along the clearest axis. Daily growth rings were counted 3 times by the same

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reader; the final count was determined by the mean of the three counts. Otoliths were eliminated whenever the counting precision (coefficient of variation) was >5%. Daily rings from Narragansett Bay were not visible using this method or any other attempted methods, therefore Narragansett Bay fish were excluded from all analyses that required daily ring estimates (i.e. metamorphosis date and growth rate estimates).

Date of Metamorphosis and Growth Rate Estimation. Date of metamorphosis for each fish was estimated by subtracting the final daily ring count from the capture date. Since daily ring counts varied between fish within the same estuary, a mean metamorphosis date per estuary was calculated. Mean temperature experienced by the fish in the estuary post metamorphosis (i.e. the mean temperature from mean metamorphosis date per estuary to capture date) was calculated using a variety of temperature data (Table 3). Individual post metamorphic fish growth rates (weight and length day-1) were calculated by dividing fish weight or length by the final daily ring count. Because temperature is the major abiotic environmental factor controlling growth (Gibson 1994), weight and length day-1 calculations were standardized for temperature (values were divided by mean temperature experienced by the fish in the estuary post metamorphosis). This removed the effect of temperature on growth rate indices, allowing the indices to reflect other factors within the estuaries that control growth rates.

Statistical Analysis

An analysis of variance (ANOVA) with a Tukey's honestly significant difference (HSD) post hoc test was performed to identify differences in metamorphosis date, condition indices, and growth indices between estuaries. Principle component analysis

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(PCA) of the condition indices and both growth indices (non-standardized and temperature standardized) were used to rank winter flounder nursery areas. Rank also was calculated by scoring each location (1-11 or 1-9; 1 = highest, 11 or 9 = lowest) based on the value of condition or growth indices. An average of all four scores was taken and used as the overall quality ranking (1 = best, 11 or 9 = worst).

Results

Correlations among the different indices

The relationship among all the indices and the T_L was first examined as indices can be biased by size effects. All indices, except the relativized weight condition index, were significantly correlated to T_L (Table 2). Though significant, Fulton's K and length day^{-1} showed a relatively weak correlation (r^2= 0.21 and 0.14, respectively). There were also significant correlations among the different indices (Table 2). Though correlations were significant between indices from the two different types (growth and condition), they were weaker than the correlation within index type.

Condition Indices

The Fulton's K condition index varied between the 12 estuaries (ANOVA, df = 11, F ratio = 19.99, p < 0.0001); values ranged from 0.789 in the Niantic River to 1.10 in Boston Harbor (Figure 2). K values were higher in Boston Harbor, Great Bay, Navesink River, and Hampton-Seabrook than in Menemsha Pond and the Niantic River.

The relativized weight condition index varied between the 12 estuaries (ANOVA, df = 11, F ratio = 20.95, p < 0.0001); values ranged from 98.02 in the Niantic River to

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101.90 in Boston Harbor (Figure 3). Relativized weight values were higher in Boston Harbor and the Navesink River than in Cotuit Bay and the Niantic River.

Date of Metamorphosis

The date of metamorphosis varied between the 11 estuaries (ANOVA, df = 10, F ratio = 3.01, p = 0.0024), and ranged from 3/19/12 in the Navesink River to 4/14/12 in Beverly Harbor (Figure 4). Fish from the Navesink River went through metamorphosis at an earlier date than fish from all other locations. The date of metamorphosis did not significantly vary between sample locations north of the Navesink River.

Growth Indices

Growth in length (length day^{-1}) varied between the 11 estuaries (ANOVA, df = 10, F ratio = 6.91, p < 0.0001), and ranged from 0.54 mm day^{-1} in the Niantic River to 0.89 mm day^{-1} in Cotuit Bay (Figure 5). Fish grew faster in Cotuit Bay than fish in Beverly Harbor and the Niantic River.

Growth in mass (weight day^{-1}) varied between the 11 estuaries (ANOVA, df = 10, F ratio = 9.55, p < 0.0001), and values ranged from 0.01 g day^{-1} in the Niantic River to 0.03 g day^{-1} in Great Bay (Figure 6). Fish grew faster in Great Bay than fish in Menemsha Pond, Lagoon Pond, Beverly Harbor, and the Niantic River.

Growth Indices Standardized to Average Temperature

Length day^{-1} temperature^{-1} (mm d^{-1} T^{-1}) varied between the 9 estuaries (ANOVA, df = 8, F ratio = 13.54, p < 0.0001), and ranged from the 0.037 mm d^{-1} T^{-1} in the Niantic

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River to 0.069 mm d^{-1} T^{-1} in Waquoit Bay (Figure 7). Fish grew significantly faster in Waquoit Bay than fish in the Niantic River.

Weight day^{-1} temperature^{-1} (g d^{-1} T^{-1}) varied between the 9 estuaries (ANOVA, df = 8, F ratio = 9.90, p < 0.0001), and ranged from 0.066 mg d^{-1} T^{-1} in the Niantic River to 0.26 mg d^{-1} T^{-1} in Waquoit Bay (Figure 8). Fish gained mass faster in Waquoit Bay and Great Bay than fish in Menemsha Pond and the Niantic River.

Estuary Ranking

A 2-axis PCA ordination of the condition indices and growth indices explained 92.4% of the variation in the data, allowing the healthiest nurseries to be identified (Figure 9). Examination of the ordination revealed that Great Bay ranked as the healthiest nursery and the Niantic River as the least healthy nursery. Several other healthy areas were identified but either had high condition or high growth indices values, but not both. Boston Harbor and the Navesink River were identified as healthy nurseries because of high condition indices scores. Waquoit Bay and Cotuit Bay were identified as healthy nurseries because of high growth indices scores.

A 2-axis PCA ordination of the condition indices and temperature standardized growth indices explained 91.7% of the variation in the data, allowing only the worst nursery to be clearly identified (Figure 10). This was the Niantic River. The healthiest nurseries were not clearly identifiable because nurseries either had high condition indices or high growth indices but not both. The healthiest nurseries based on just condition indices were Boston Harbor, Great Bay, and the Navesink River. The healthiest nursery based on just growth indices was Waquoit Bay.

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The overall health of the nurseries was ranked using mean scores of the condition indices and growth indices (Table 3), the lower the mean score the healthier the nursery area. The healthiest locations in descending order were Boston Harbor, Great Bay, Waquoit Bay, and the Navesink River. The least healthy locations in ascending order were the Niantic River, Menemsha Pond, Lagoon Pond, and Little Harbor.

In addition, the overall health of the nurseries also were ranked using mean scores of the condition indices and temperature standardized growth indices (Table 4), the lower the mean score the healthier the nursery area. Using these indices, the healthiest locations were Boston Harbor, Waquoit Bay, Great Bay, and the Navesink River, in descending order, and the least healthy locations were the Niantic River, Menemsha Pond, Cotuit Bay, and Little Harbor, in ascending order.

Discussion
Using indices to determine nursery habitat quality

No single index is able to provide an accurate description of habitat quality (Gilliers et al. 2004). For instance, growth indices serve as long term indicators of environmental conditions, influenced by environmental changes from mid-metamorphosis to catch date. Alternatively, condition indices serve as short term indicators, measuring the well-being of the fish at the catch date, which reflect the current nutritional and energy status of the fish (Lambert and Dutil 1997). Used in conjunction, these two growth indices present a versatile indicator of habitat quality. In this study, the growth indices measured were length day^{-1} and weight day^{-1}. The two condition indices measured were relativized weight and Fulton's K. However, only the growth index,

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length day^{-1}, and the condition index, relativized weight, were used to determine nursery habitat quality in this study.

Though both growth indices showed a significant correlation to the total length, indicating a size effect bias, length day^{-1} was chosen over weight day^{-1} because of the weaker correlation. The relativized weight index was not significantly correlated to total length, indicating no size bias, therefore it was chosen over Fulton's K which exhibited a slight size effect bias. In addition, the lack of correlation between relativized weight and length day^{-1} further illustrates the differences in which the two indices reflect temporal environmental conditions. These differences result in much different habitat quality ratings. The only nursery location that was similar between the two indices was the Niantic River, receiving a very poor quality rating in both.

In this study the overall quality of the nursery was interpreted by two methods: 1) using a rating system or 2) based on the PCA ordination, both of which combine results from the two indices. The ranking system provides an ordinal habitat quality ranking, which does not indicate the strength of the scorer on both indices. It is the ranked average of both indices. The ordination displays habitat quality based on position on the axes. PCA ordination, therefore, is more exclusive, only defining areas of high or low quality based on the distance of separation from other locations, not just on integer ranking. Using both classification methods, Great Bay was classified as the one of the healthiest nursery areas while the Niantic River was classified as the least healthy nursery. Other nursery areas were classified as high or low quality but were more variable between methods of interpretation (Figure 9, Table 3).

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Environmental conditions affecting habitat quality

Though three distinct winter flounder stocks have been identified based on

tagging, meristic and life history data (DeCelles and Cadrin 2011) and genetic differences

have been found to occur on an estuary scale (Crivello et al. 2004), juvenile fish have

been shown to exhibit strong phenotypic plasticity to cope with variable environmental

conditions that overshadow parental effect of growth and condition (Fraboulet et al.

2010). Therefore in this study I assume that it is the environmental condition and not

genetics driving the differences in indices between nursery areas. However, these factors

driving the differences between nursery areas are not always clearly identifiable due to

the complex and variable nature of the areas (Vinagre et al. 2008). It also is unlikely that

the same factors are driving differences in different nursery areas.

Although the factors controlling habitat quality were not directly measured in this

study, several possible factors were identified. Potential important factors include

resource availability, predators present, sediment type, physiochemical conditions, and

anthropogenic factors. Prey quality and quantity are factors controlling growth (Neill et

al. 1994, Vanderveer et al. 1994), and also play a role in habitat quality between the

nursery areas (Sogard and Able 1992, Gibson 1994). Because juvenile flounder are

general and opportunistic feeders (e.g. (Beyst et al. 1999, Amara et al. 2001), the caloric

difference between prey may be a more influential factor affecting habitat quality than

quantity of prey in these highly productive nursery habitats. Predators have been found to

shape the size structure of winter flounder populations through size-dependent feeding

(Taylor 2003). In areas of increased predation it is therefore critical for the survival of the

fish that nursery areas are the best quality to promote growth through this critical size period. Sediment type, particle size, and color also may affect growth and condition (Yamashita and Yamada 1999), as well as cryptic behavior essential for survival (Fairchild and Howell 2004). Sogard (1992) observed faster growth on coarser sediments, possibly due to increased prey detection and capture.

If prey is not limited then temperature is likely the most important factor controlling growth. Juvenile fish grow faster in warmer waters (Gibson 1994). However, fish are likely to exploit variations in the local environment, achieving growth rates above that of the average temperature in the nursery area (Gibson 1994). This can lead to bias when accounting for temperature in the growth indices, as was done in this study, because the measured temperature of the nursery area is rarely the temperature experienced by the fish. This effect is increased if the fish undertake daily tidal migrations within the nursery area as winter flounder do (Tyler 1971). Variation in salinity affects growth and condition because energy is required to regulate in response to the change (Evans 1980, Moyle and Cech 2004). Although salinity is thought to have a smaller effect on growth and condition than temperature (Gibson 1994), both should be considered when comparing nursery areas with different hydrologies. Oxygen depletion is unlikely to affect growth and condition in well-mixed habitats such as coastal and river locations. However shallow nursery areas, such as bays and ponds, may experience oxygen depletion, particularly in areas that are polluted or highly vegetated (Vanderveer and Bergman 1986, Dorel et al. 1991).

Exposure to pollution is inversely related to fish growth and condition (Rowe 2003, Alquezar et al. 2006). This is a result of nutritional energy being devoted to

combating stress instead of maintenance, growth, and reproduction (Adams 2002). It also

makes fish more vulnerable to predation, physiological stress, and disease, potentially

affecting not only individual fish, but the population as a whole (Adams 2002).

Understanding how these potential factors vary between estuaries is critical in

identifying why the quality of nursery areas differs (Sogard 1991, Sogard and Able

1992). In order to link the quality of nursery areas definitively to specific factors, the

movement of the fish within the nursery must be understood. This allows the

environmental factors to be measured on a localized scale, thus accounting for the

discontinuities in the environmental conditions exploited by the fish within the nursery

areas. Relying on opportunistic environmental measurements may not actually reflect the

conditions experienced by the fish resulting in potentially biased conclusions. For this

reason, this study cannot definitively link the quality of the nursery area to specific

environmental factors; it can only compare nurseries and determine which are healthier.

Growth Rate Comparison

The growth rates observed in this study (0.53 to 0.89 mm day^{-1}) were at the higher

range observed in previous winter flounder studies (Sogard 1991, Sogard 1992, Meng et

al. 2000, Fairchild et al. 2005, Meng et al. 2008). In previous studies, growth rates were

calculated using either otolith increment measurements or from caging experiments

(Meng et al. 2000, Fairchild et al. 2005, Meng et al. 2008), whereas in this study, the

length of the fish was divided by the age of the fish. All of these methods have their

downfalls. The use of daily otolith increment measurements are justified only when there

is a linear relationship between the size of the fish and the size of the otolith (Campana

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and Neilson 1985); this assumes that all otoliths are the exact same shape from the core along the same axis. This assumption was not true in this study. It also proved difficult to polish all otoliths clearly along the same axis. Because otolith growth is less variable than somatic growth, estimating somatic growth from otolith growth yields inaccuracies during periods of rapid or slow somatic growth (Secor and Dean 1989). Growth rates calculated from cage experiments are slightly biased because fish movement is restricted and predators are excluded, both of which have the potential to affect growth rate. The growth rate calculated in this study (dividing age of fish by length) is subject to two errors: 1) the growth rate does not take into account growth prior to metamorphosis because daily increment counts were only made to the anterior most accessory primordium, thus overestimating the growth rate; and 2) since juvenile flounder growth is exponential, growth rate calculations are biased towards larger fish. These downfalls were somewhat remedied by conducting daily growth ring counts in the same manner at each estuary and by using fish of similar sizes. When assuming 5 weeks of growth prior to metamorphosis (Bigelow et al. 1953), growth rates ranged from $0.38 - 0.64$ mm day^{-1}. This range is right in the middle of growth rates calculated by previous studies (Sogard 1991, Sogard 1992, Meng et al. 2000, Fairchild et al. 2005, Meng et al. 2008).

Condition Comparison

The nursery areas sampled in this study had a slightly lower range of Fulton's K values (0.79–1.1) compared to other flatfish studies (Plante et al. 2005). This suggests that growth limitation may have occurred. In the common sole, *Solea solea,* a Fulton's condition factor of <0.9 is an indicator of starvation or food limitation in juveniles (Amara et al. 2007). Three nursery areas were below the 0.9 value - the Niantic River,

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Menemsha Pond, and Cotuit Bay - suggesting that fish may be suffering from starvation or food limitation. Whether starvation and food limitation was occurring at these sites could be clarified using gut content analyses or RNA/DNA ratio measurements. Since the relativized weight index is study specific, it cannot be compared to other studies in the same manner as Fulton's K, but the strong correlation between the two suggests that the nursery areas are behaving similarly.

Differences in Settlement Time

Only the Navesink River in New Jersey had a significantly different time of mid-metamorphosis; the timing of all of the other northern locations did not significantly vary. This variation likely is due to the increased warming of this southernmost site in relation to the northern sites. It was surprising that no differences were observed between the northern sites, even though there is a temperature difference along the latitudinal gradient. The lack of difference between the northern sites may be attributed to fish exploiting localized variations in their habitat (areas of higher or lower temperatures than average location temperatures). The time of metamorphosis is strongly influenced by temperature (Able and Fahay 1998), even more so than the size of the fish (Chambers and Leggett 1987). This suggests that even if spawning and hatch dates are similar in different nursery sites, settlement may still vary. To fully understand the differences in hatch date and length of pelagic larval stage, daily otolith core ring counts should be made. Unfortunately, daily otolith core rings were not counted in this study due to difficulties in polishing which led to imprecision in counting them.

Conclusion

Despite ever increasing fishing regulations, winter flounder populations are not rebounding. The collapse of the winter flounder fishery has dramatic ecological and economic consequences, which is why it is important to understand the role of nursery habitat quality variations and how these variations affect recruitment into the adult population. First, it will be important to determine measurable indices, which will most effectively and accurately determine the best nursery habitats. In this study we have explored four different indices and revealed differences in habitat quality results depending on each of these indices. Two of the indices proved better indicators of habitat quality because they were less biased by size. Once these indices are determined, we can determine more easily on which nursery habitats to focus management efforts. Additionally it will be important to determine if these areas are contributing more new recruits to the adult population by using other methods such as otolith microchemistry analysis. Relative contribution then can be linked to measurable nursery area variability. Once a link between contribution and variability is identified, the best measurements can be used by managers to assess nursery health.

Table 1. Sample size (n) and mean total length (L_T) and mass (M) ± one standard deviation of winter flounder juveniles used for otolith microstructure analysis.

Location	n	Mean Length (mm)	Mean Weight (g)
Great Bay, NH	11	66.5 ± 0.88	3.12 ± 0.13
Little Harbor, NH	9	64.4 ± 0.88	2.45 ± 0.11
Hampton-Seabrook, NH	9	60.1 ± 1.51	2.21 ± 0.21
Beverly Harbor, MA	7	49.0 ± 0.76	1.10 ± 0.06
Boston Harbor, MA	10	52.9 ± 3.58	1.80 ± 0.46
Cotuit Bay, MA	8	59.5 ± 1.99	1.96 ± 0.23
Waquoit Bay, MA	9	63.4 ± 1.49	2.4 ± 0.20
Lagoon Pond, MA	10	50.3 ± 0.78	1.23 ± 0.06
Menemsha Pond, MA	11	50.6 ± 0.65	1.18 ± 0.06
Niantic River, CT	12	48.1 ± 1.24	0.90 ± 0.08
Navesink River, NJ	11	54.4 ± 1.49	1.64 ± 0.13

Table 2. Determination coefficients (r^2) between Fulton's condition index (K; mg mm^3), relativized weight (RW), length day^{-1} (LD; mm day^{-1}), weight day^{-1} (WD; g day^{-1}), total length (TL; mm), and weight (W; g).

	K	RW	LD	WD	TL	W
K	1	0.74*	0.09**	0.36*	0.21*	0.15*
RW	0.74*	1	0.03***	0.06**	>0.01	>0.01
LD	0.09**	0.03***	1	0.54*	.14*	.15*
WD	0.36*	0.06**	0.54*	1	0.71*	.80*
TL	0.21*	>0.01	.14*	0.71*	1	.71*
W	0.15*	>0.01	.15*	.80*	.71*	1

* $p < 0.001$, ** $p < 0.05$, *** $p < 0.01$.

Table 3. Temperature data from all sampling locations.

Location	Metamorphosis to Catch Date			Temp @ Metamorphosis	Source	Instrument	Interval
	Average	Min	Max				
Great Bay, NH	16.9	8.9	25.6	9.5	National Estuarine Research Reserve System	N/A	15 min data
Little Harbor, NH	11.9	6.6	17.8	6.8	University of New Hampshire	Hobo	1 hr data
Hampton-Seabrook, NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Beverly Harbor, MA	9.6	6.6	19.5	8.6	Massachusetts Division of Marine Fisheries	Hobo	15 min data
Boston Harbor, MA	13.6	7.7	20.3	8.1	NOAA Tides and Currents	N/A	1 hr data
Cotuit Bay, MA	16.5	8.7	24.6	9.6	Cape Cod Cooperative Extension	YSI Sonde	15 min data
Waquoit Bay, MA	17.7	10.3	26.6	11.3	National Estuarine Research Reserve System	N/A	15 min data
Lagoon Pond, MA	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Menemsha Pond, MA	15.7	9.5	23.5	12.1	University of New Hampshire	Hobo	1 hr data
Niantic River, CT	14.8	8.7	20.0	9.1	Millstone Environmental Laboratory	N/A	Biweekly
Navesink River, NJ	15.2	8.0	27.2	8.9	NOAA Tides and Currents	N/A	1 hr data

Table 4. Growth and condition rankings for each location. Ranking based on index score (1-11: best-worst). Overall rating based on the average of the four indices.

Location	Length Day^{-1}	Weight Day^{1}	Fulton's K	Relativized Weight	Overall Rating
Boston Harbor, MA	3	4	1	1	1
Great Bay, NH	4	1	2	3	2
Waquoit Bay, MA	2	3	4	5	3
Navesink River, NJ	5	7	3	2	4
Cotuit Bay, MA	1	2	9	9	5
Hampton-Seabrook, NH	7	5	5	6	6
Beverly Harbor, MA	10	10	6	4	7
Little Harbor, NH	9	6	8	8	8
Lagoon Pond, MA	8	9	7	7	9
Menemsha Pond, MA	6	8	10	11	10
Niantic River, CT	11	11	11	10	11

Table 5 Growth and condition rankings for each location. Ranking based on index score (1-11: best-worst). Overall rating based on the average of the four indices.

Location	Length Day^{-1} °C^{-1}	Weight Day^{-1} °C^{-1}	Fultons K	Relativized Weight	Overall Rating
Boston Harbor, MA	4	5	1	1	1
Waquoit Bay, MA	1	1	4	5	1
Great Bay, NH	8	2	2	3	3
Navesink River, NJ	6	6	3	2	4
Beverly Harbor, MA	2	7	5	4	5
Little Harbor, NH	5	3	6	6	6
Cotuit Bay, MA	3	4	7	7	7
Menemsha Pond, MA	7	8	8	8	8
Niantic River, CT	9	9	9	9	9

Collection Location	Gear Used	Fish	Tows	CPUE
Great Bay, NH	Trawl	36	3	12.0
Little Harbor, NH	Trawl	45	14	3.2
Hampton-Seabrook, NH	Seine, Trawl	14	9	1.6
Beverly Harbor, MA	Seine	37	7	5.3
Boston Harbr, MA	Trawl	23	12	1.9
Cotuit Bay, MA	Seine	35	7	5.0
Waquoit Bay, MA	Seine, Trawl	40	8	5.0
Lagoon Pond, MA	Seine, Trawl	44	10	4.4
Menemsha, MA	Seine	36	15	2.4
Narragansett, RI	Seine	43	-	-
Niatic River, CT	Trawl	27	-	-
Navesink, NJ	Seine	40	-	-

Figure 1. Sample locations of winter flounder from estuaries in the northeast United States. Inset table contains collection information for each location. Fish: total number of winter flounder caught, Tows: total number of tows, CPUE: catch per unit effort (# fish tow^{-1}).

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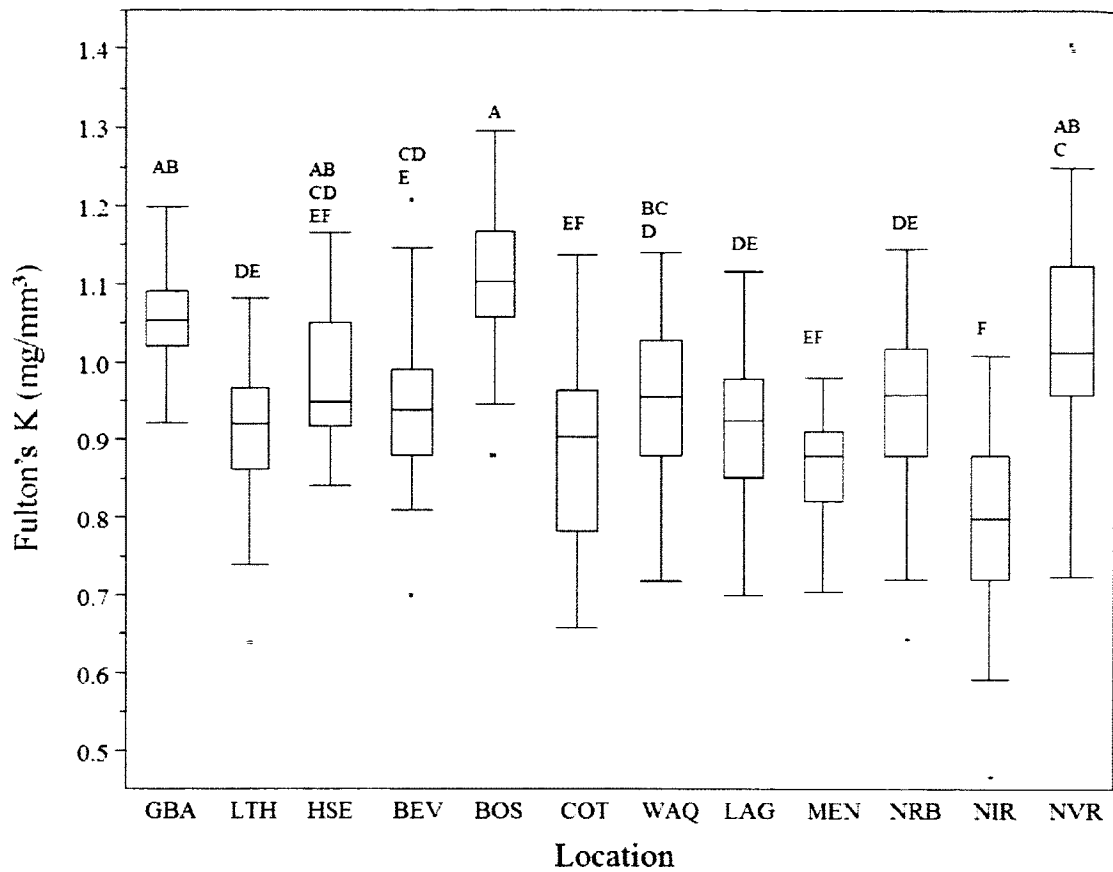
Figure 2. Box plot of Fulton's K values at each location. The center line of each box represents the mean, the top and bottom of the box indicate the 25th and 75th percentiles, respectively, and the whiskers indicate the range. Points denote outliers. Locations not sharing a letter are significantly different (p<0.05),(Tukey's HSD). GBA: Great Bay, LTH: Little Harbor, HSE: Hampton-Seabrook, BEV: Beverly Harbor, BOS: Boston Harbor, COT: Cotuit Bay, WAQ: Waquoit Bay, LAG: Lagoon Pond, MEN: Menemsha Pond, NRB: Narragansett Bay, NIR: Niantic River, NAR: Navesink River.
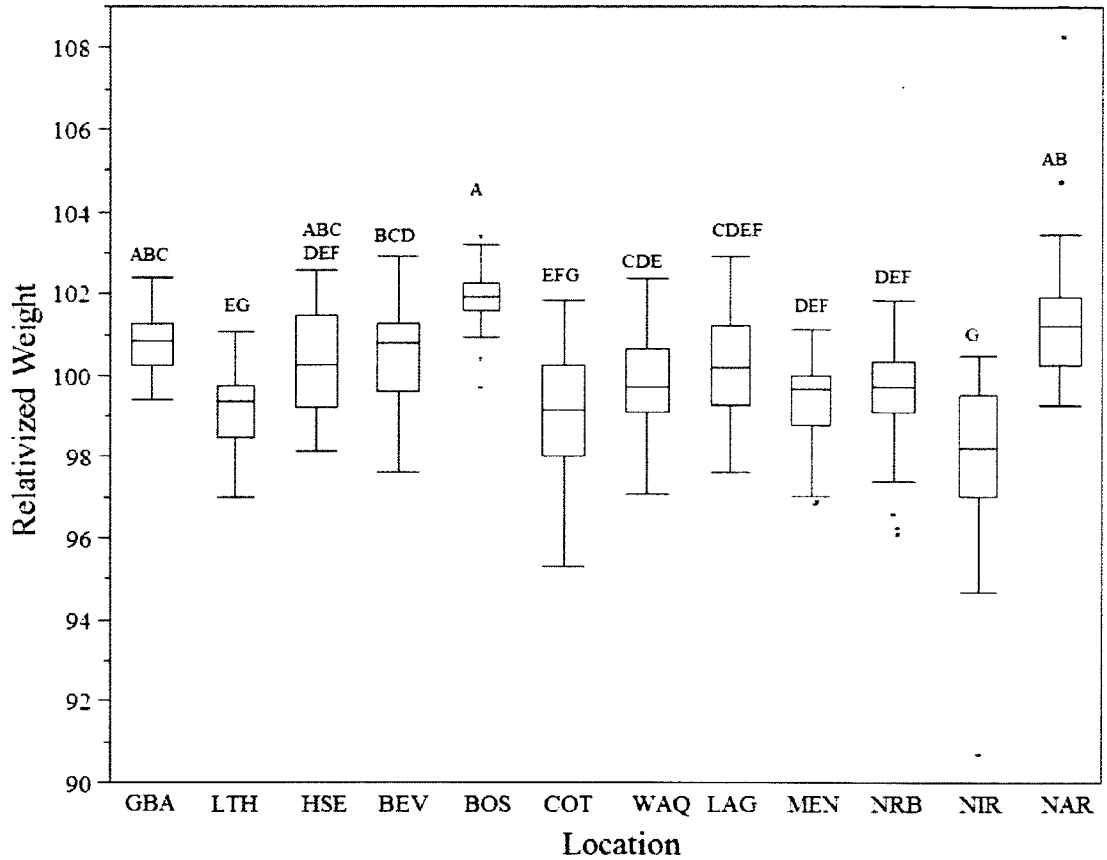
Figure 3. Box plot of relativized weight values at each location. The center line of each box represents the mean, the top and bottom of the box indicate the 25th and 75th percentiles, respectively, and the whiskers indicate the range. Points denote outliers. Locations not sharing a letter are significantly different (p<0.05),(Tukey's HSD). GBA: Great Bay, LTH: Little Harbor, HSE: Hampton-Seabrook, BEV: Beverly Harbor, BOS: Boston Harbor, COT: Cotuit Bay, WAQ: Waquoit Bay, LAG: Lagoon Pond, MEN: Menemsha Pond, NRB: Narragansett Bay, NIR: Niantic River, NAR: Navesink River.
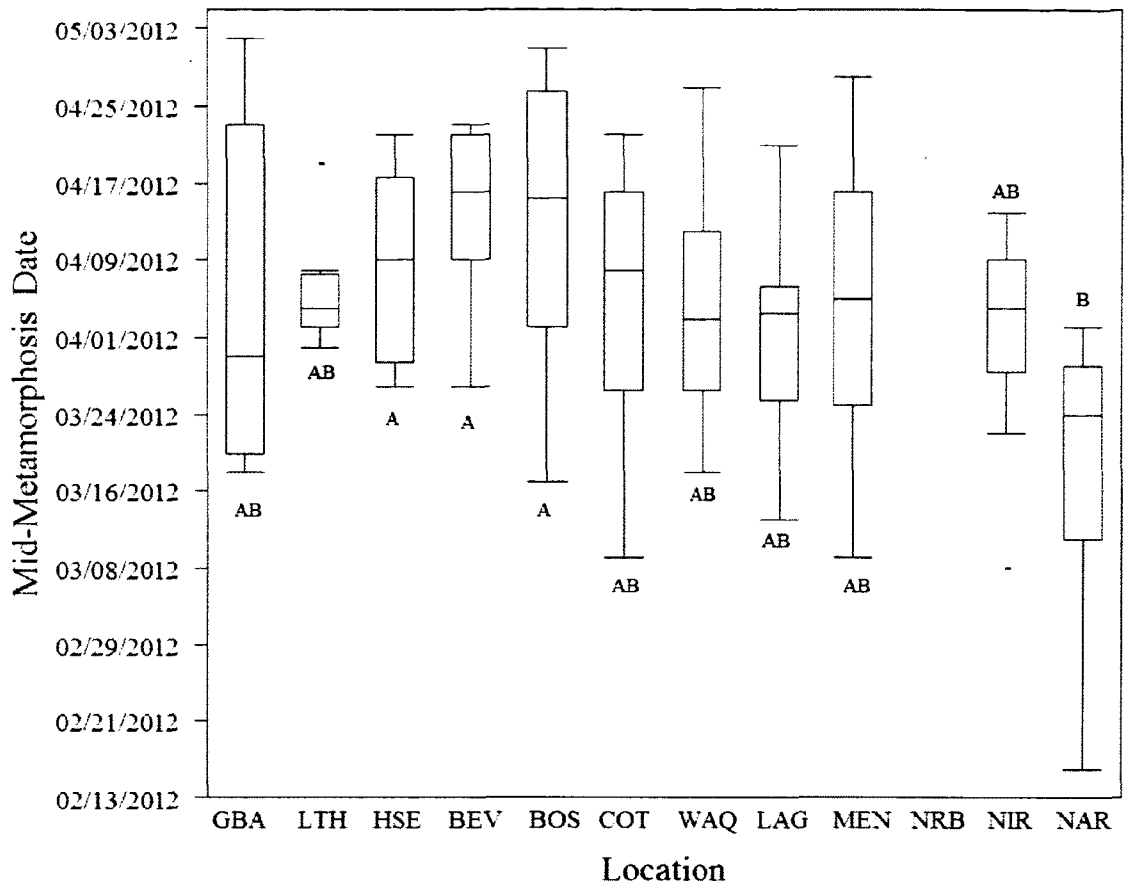
Figure 4. Box plot of mid-metamorphosis date at each location. The center line of each box represents the mean, the top and bottom of the box indicate the 25th and 75th percentiles, respectively, and the whiskers indicate the range. Points denote outliers. Locations not sharing a letter are significantly different (p<0.05),(Tukey's HSD). GBA: Great Bay, LTH: Little Harbor, HSE: Hampton-Seabrook, BEV: Beverly Harbor, BOS: Boston Harbor, COT: Cotuit Bay, WAQ: Waquoit Bay, LAG: Lagoon Pond, MEN: Menemsha Pond, NRB: Narragansett Bay, NIR: Niantic River, NAR: Navesink River.
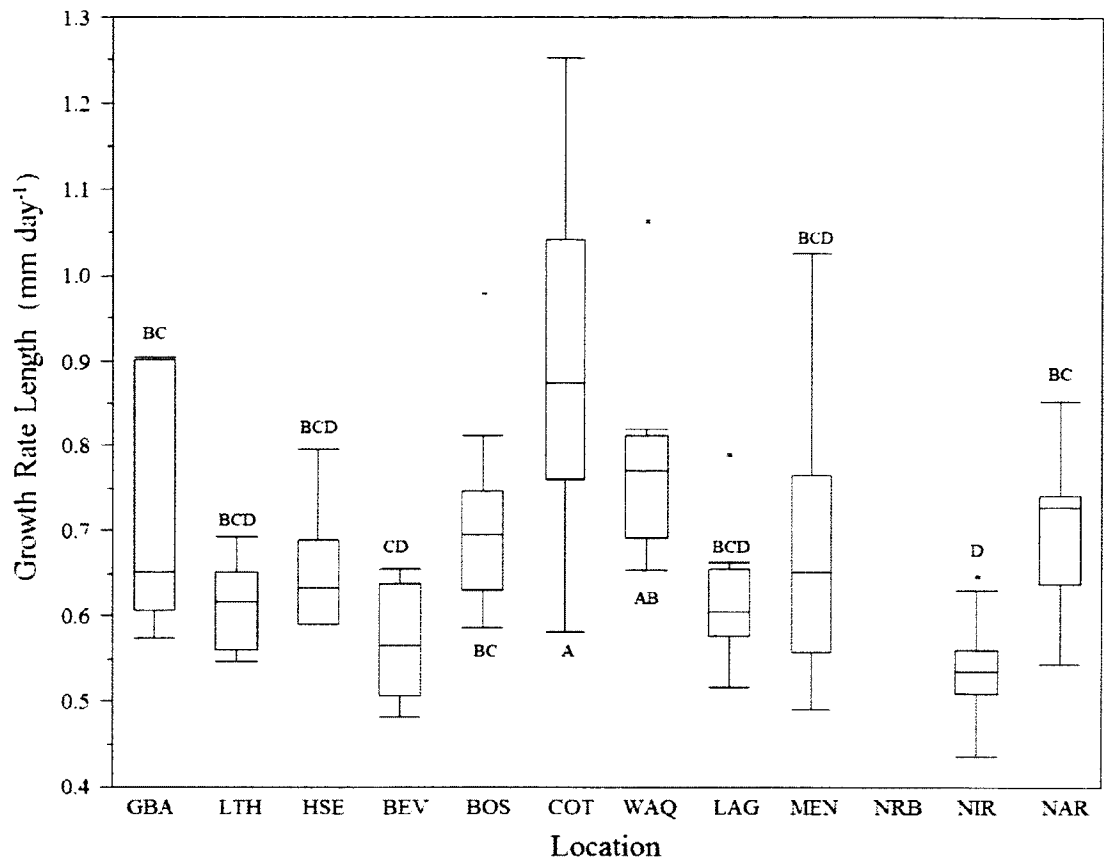
Figure 5. Box plot of growth rate length (mm day^{-1}) at each location. The center line of each box represents the mean, the top and bottom of the box indicate the 25th and 75th percentiles, respectively, and the whiskers indicate the range. Points denote outliers. Locations not sharing a letter are significantly different (p<0.05),(Tukey's HSD). GBA: Great Bay, LTH: Little Harbor, HSE: Hampton-Seabrook, BEV: Beverly Harbor, BOS: Boston Harbor, COT: Cotuit Bay, WAQ: Waquoit Bay, LAG: Lagoon Pond, MEN: Menemsha Pond, NRB: Narragansett Bay, NIR: Niantic River, NAR: Navesink River.

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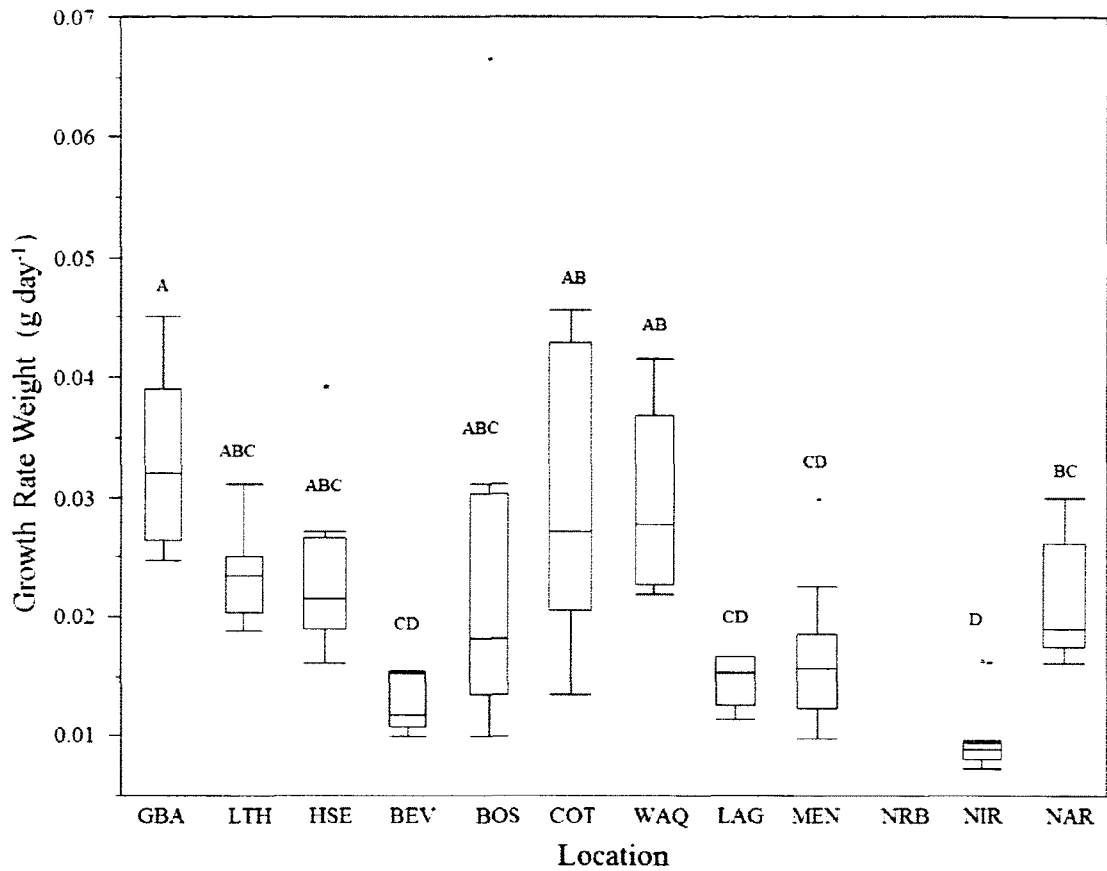
Figure 6. Box plot of growth rate weight (g day^{-1}) at each location. The center line of each box represents the mean, the top and bottom of the box indicate the 25th and 75th percentiles, respectively, and the whiskers indicate the range. Points denote outliers. Locations not sharing a letter are significantly different (p<0.05),(Tukey's HSD). GBA: Great Bay, LTH: Little Harbor, HSE: Hampton-Seabrook, BEV: Beverly Harbor, BOS: Boston Harbor, COT: Cotuit Bay, WAQ: Waquoit Bay, LAG: Lagoon Pond, MEN: Menemsha Pond, NRB: Narragansett Bay, NIR: Niantic River, NAR: Navesink River.
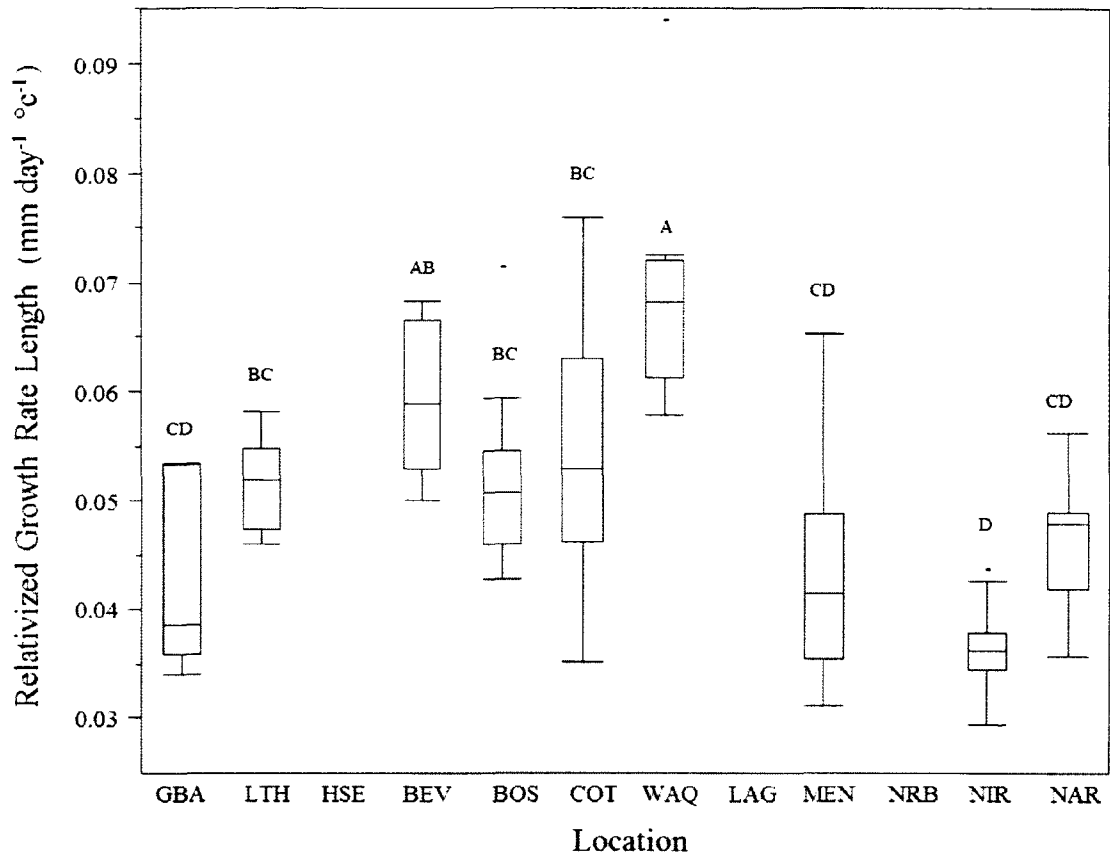
Figure 7. Box plot of temperature relativized growth rate length (mm day^{-1} °c^{-1}) at each location. The center line of each box represents the mean, the top and bottom of the box indicate the 25th and 75th percentiles, respectively, and the whiskers indicate the range. Points denote outliers. Locations not sharing a letter are significantly different (p<0.05),(Tukey's HSD). Locations without a box did not have temperature time series data available. GBA: Great Bay, LTH: Little Harbor, HSE: Hampton-Seabrook, BEV: Beverly Harbor, BOS: Boston Harbor, COT: Cotuit Bay, WAQ: Waquoit Bay, LAG: Lagoon Pond, MEN: Menemsha Pond, NRB: Narragansett Bay, NIR: Niantic River, NAR: Navesink River.

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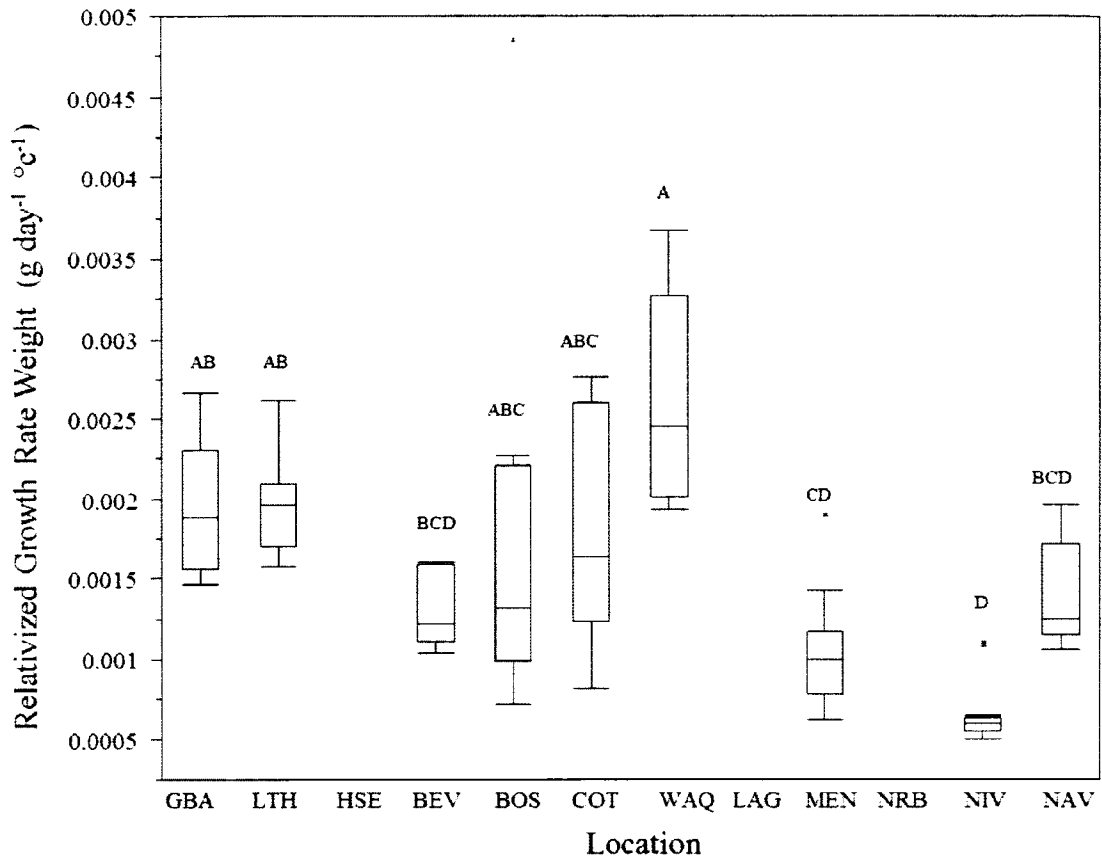
Figure 8. Box plot of temperature relativized growth rate weight (g day^{-1} $^\circ$c^{-1}) at each location. The center line of each box represents the mean, the top and bottom of the box indicate the 25th and 75th percentiles, respectively, and the whiskers indicate the range. Points denote outliers. Locations not sharing a letter are significantly different (p<0.05),(Tukey's HSD). Locations without a box did not have temperature time series data available. GBA: Great Bay, LTH: Little Harbor, HSE: Hampton-Seabrook, BEV: Beverly Harbor, BOS: Boston Harbor, COT: Cotuit Bay, WAQ: Waquoit Bay, LAG: Lagoon Pond, MEN: Menemsha Pond, NRB: Narragansett Bay, NIR: Niantic River, NAR: Navesink River.
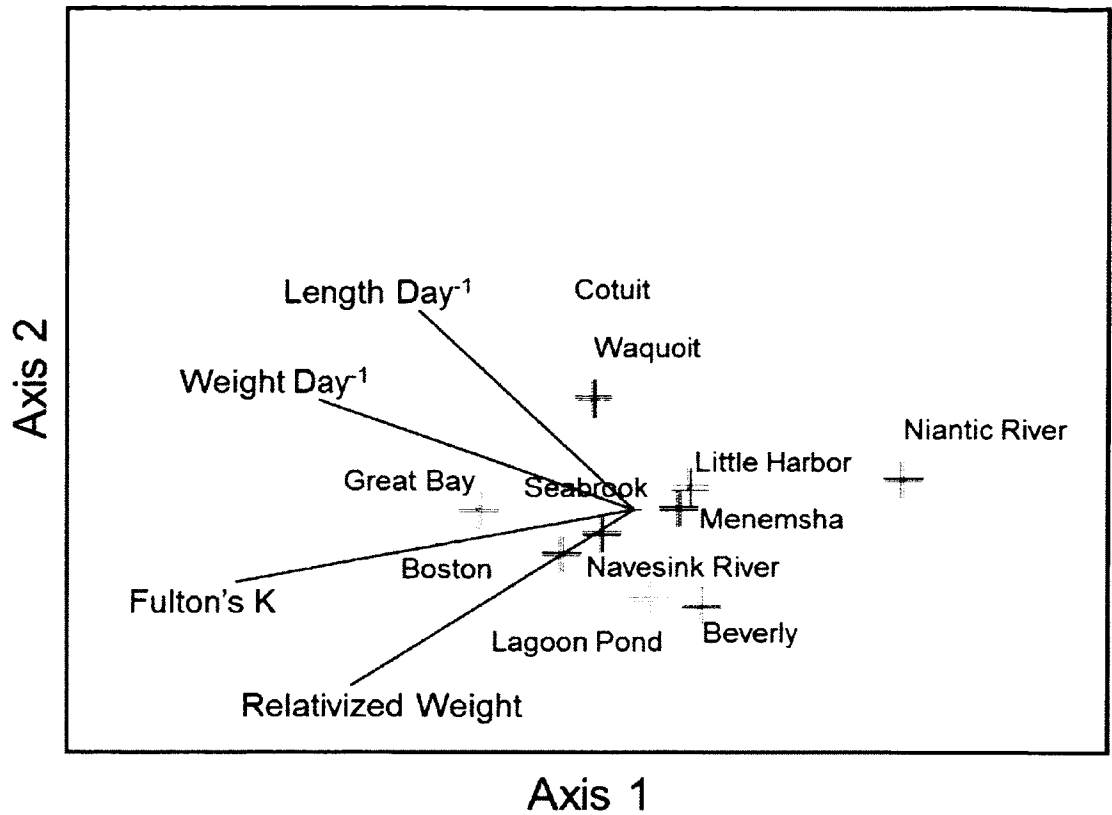
Figure 9. Principle components analysis ordination of locations using condition and growth indices. Crosshairs are location centroids. Axis 1 explains 62.48% of the variation and axis 2 explains 29.93% of the variation. Fulton's K r^2 = 0.823, Relativized weight r^2 = 0.583, Length day^{-1} = 0.444, Weight day^{-1} = 0.649. Healthier nursery areas a positively correlated with joint plot indices
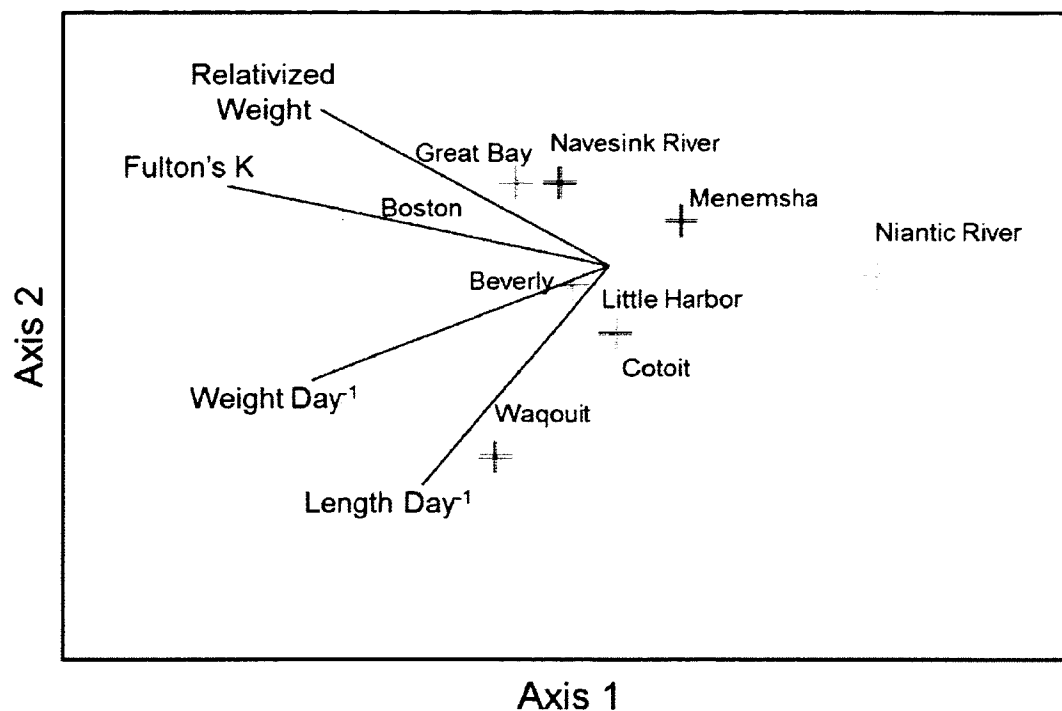
Figure 10. Principle components analysis ordination of locations using condition and growth indices. Crosshairs are location centroids. Axis 1 explains 62.48% of the variation and 2 and axis 2 explains 9.93% of the variation. Fulton's K $r^2 = 0.823$, Relativized weight $r^2 = 0.583$, Length day^{-1} = 0.444, Weight day^{-1} = 0.649. Healthier nursery areas a positively correlated with joint plot indices

CHAPTER II. ASSESSING VARIATION IN WINTER FLOUNDER (*PSEUDOPLEURONECTES AMERICANUS*) NURSERY AREAS USING OTOLITH MICROCHEMICAL SIGNATURES

Introduction

Winter flounder, *Pseudopleuronectes americanus*, a demersal, right-eyed flatfish, is an important commercial and recreational species. It is distributed along the northwestern Atlantic, from Georgia, USA to Labrador, Canada, but is most common from Nova Scotia, Canada to New Jersey, USA (Scott and Scott 1988). As with most commercially important fish species, winter flounder populations have declined dramatically over recent decades. Total commercial landings of winter flounder have fallen 89% from 1981 to 2010 (Murphy et al. 2011). Despite ever increasing fishing regulations, winter flounder populations are not rebounding. The collapse of this fishery has dramatic ecological and economic consequences, and merits research to better inform conservation policy.

Due to their large range and ecological, behavioral, and growth differences, winter flounder are managed as three distinct stocks in the US: the Gulf of Maine (GOM), Georges Bank (GB), and Southern New England/Mid-Atlantic (SNE/MA) stock (Clark 1998). With the exception of the GB stock, adult winter flounder migrate inshore in the fall and early winter, and spawn in late winter and early spring (Pereira et al. 1999). The juveniles remain inshore for their first two years before moving offshore (Pereira et al. 1999). These inshore habitats serve as winter flounder spawning and nursery areas and have been classified as Essential Fish Habitat (EFH). They are extremely important to

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population sustainability. EFHs are critical for winter flounder because they can support

the tenuous maturation beyond early life stages where the mortality rate can be as high as

99% (Pearcy 1962). The year class strength of winter flounder is determined primarily

during these early life stages (Sogard 1991). It is therefore critical to develop a method

for determining connectivity between adult populations and nurseries.

Understanding the connectivity between adult populations and nursery sources

can help lead to more effective management and rebuilding of the fishery. However,

determining population connectivity in the marine environment is difficult when using

conventional tagging techniques. These techniques suffer from multiple drawbacks

including difficulty tagging small juveniles, high mortality rates, and low tag return rates

(Gillanders 2002). These difficulties have resulted in the development of new techniques

to assess population connectivity.

Otoliths, found in the inner ear of fish, are structures composed of calcium

carbonate crystals. Otolith growth occurs daily and the newly deposited material creates a

pattern of daily growth rings. This newly deposited material also incorporates trace

elements from the environment. Once the new growth is deposited, it is metabolically

inert (it is neither reincorporated nor reworked), creating a pattern that is preserved for

the life of the fish (Campana et al. 2000). This pattern or otolith chemical signature may

provide a natural tag for tracking where fishes have been (Gillanders and Kingsford 1996,

Campana 1999, Campana and Thorrold 2001). These natural tags have been used to

differentiate individuals from a variety of systems: estuarine and riverine systems

(Thorrold et al. 1998a, Thorrold et al. 1998b, Gillanders and Kingsford 2000, Gillanders

2002), coastal and open ocean systems (Warner et al. 2006, Correia et al. 2012), and rocky reefs (Gillanders and Kingsford 2000).

When examining the otolith microchemical composition of the trumpeter, *Pelates sexlineatus*, Gillanders and Kingsford (2000) found differences in the elemental fingerprints (Sr, Ba, and Mn) within and among different Australian estuarine nursery habitats. These differences allowed juveniles to be classified back to their natal estuaries. By identifying differences in otolith signatures (Sr, Ba, Mg, Mn, Ni, Zn), Correia et al. (2012) were able to distinguish between blackbream, *Spondyliosoma cantharus*, from 3 fishing grounds off the Portuguese coast. Using the chemical composition of the otolith core they also were able to identify a common offshore spawning ground used by fish from all 3 fishing grounds.

Several otolith microchemistry studies have examined flatfish with similar life histories to that of winter flounder (i.e., fishes that spawn inshore and have a juvenile nursery phase before recruiting to the offshore adult populations) (Brown 2006, Vasconcelos et al. 2007b). Examining the otolith elemental composition of English sole, *Pleuronectes vetulus*, and the speckled sanddab, *Citharichthys stigmaeus*, Brown (2006) was able to classify fish to natal estuaries or coastal habitats along the California coast with 80% accuracy. The elements that were found to differ across the nursery areas were Sr, Li, Ba, and Mn. In a study conducted off the Portuguese coast, Vasconcelos et al. (2007b) were able to classify 3 different species of flatfish back to their natal estuary with 70-93% accuracy. An extensive suite of elements (Li, Na, Mg, K, Mn, Ni, Cu, Zn, Sr, Cd, Ba and Pb) was analyzed to achieve such high accuracy. The elements used in the

classification varied across flatfish species, with certain elements playing a more influential role in certain species.

Microchemical analysis has not been used for winter flounder to our knowledge except in an unpublished EPA study and a study by Pruell et al. (2011) in which natural tags were identified based on stable carbon and oxygen isotope ratios in the otoliths. They concluded that the otolith carbon and oxygen ratios were not site specific but rather correlated to salinity and freshwater flux, respectively. Because winter flounder are euryhaline, inhabiting estuarine areas with salinity ranging from 5 to 33 ppt (Pereira et al. 1999), it is difficult to use these isotopes; other elements need to be investigated. It is possible, however, that other elements may act as site-specific markers for winter flounder. If so, an elemental signature index of nursery areas based on otolith composition can be created. This index could be used for stock identification and to trace adults back to natal nursery areas. For winter flounder, this would allow for the increased protection of those nursery grounds that significantly contribute to the adult population, thereby potentially reducing early-stage mortality and increasing the resiliency of the adult population. Identifying the most successful nursery grounds is an important step in developing models of critical winter flounder nursery characteristics that may later be used in conservation policy.

In order to gauge the effectiveness of microchemistry as a management tool for winter flounder, we analyzed an extensive suite of elements to determine: 1) if elemental signatures are site-specific; 2) the spatial scale at which elemental heterogeneity exists; and 3) the accuracy of classifying fish to natal nursery areas.

Materials and Methods

Field Sampling

Young-of-the-year (YOY) winter flounder were collected from 12 locations from Great Bay, NH to the Navesink River, NJ (Figure 11) between June and July 2012. Fish caught from the five Gulf of Maine and four Cape Cod sites were caught using a beach seine (17 m x 2 m; 6.35mm Delta mesh; swept area $550m^2$) and/or beam trawls (1.0 m width, 6 mm mesh). Fish were measured (T_L; mm), weighed (g), and given individual sample names after landing. Total length measurements were used to ensure that the collected fish were from the YOY cohort. Based on existing age and size frequency data, fish were thought to be in the YOY cohort if T_L < 90mm, according to Massachusetts Division of Marine Fisheries winter flounder estuarine surveys (J. King, personal commun.). Actual age was later confirmed by counting daily otolith growth rings (see Chapter 1). Following initial measurements and cohort identification, fish were euthanized via cervical dislocation, transferred into individual labeled plastic bags, placed on ice, and transported back to the laboratory where they were kept frozen in a 0°C freezer until further analysis. Water quality parameters (salinity, temperature, and dissolved oxygen) were recorded prior to and upon the completion of collecting in a specific area using a YSI 6920 sonde. Habitat type and benthic composition also were recorded at each site. Fish caught from Narragansett Bay, RI, Niantic River, CT, and Navesink River, NJ were caught by third party researchers during routine surveys using various collection techniques. Fish were frozen after collection and transferred to the

University of New Hampshire (UNH). Once at UNH fish were thawed then weighed (g), measured (T_L; mm), and given individual sample names.

Approximately 15 fish from each estuary were examined by microchemical analysis (Table 6); the same fish also were used in a microstructure study (see Chapter 1). At estuaries where >15 fish were collected, the 15 fish with a total length closest to the average total length of fish for that location were chosen for analysis.

Otolith Removal

Both sagittal otoliths were extracted using Teflon coated razor blades and plastic forceps. Right and left otoliths were separated based on position in the orbital. If there were any discrepancies as to which side they were removed from, they were separated according to the position of the sulcus and the rostrum (Secor et al. 1993). Once separated, otoliths were cleaned in distilled water and stored in individual 1.5 ml micro-centrifuge tubes. Left otoliths, previously found to provide the best correlation between somatic and otolith growth (Sogard 1991), were used for microstructure analysis, and right otoliths were used for microchemical analysis.

Microchemical Sample Preparation

All sample preparation, except for otolith weighing, was conducted in a positive-pressure, trace metal clean room with HEPA filtered air. Equipment and consumables used during preparation were acid cleaned prior to use by soaking in 20% hydrochloric acid at 60 °C for 4 days, followed by copious rinsing with 18.2 MΩ/cm nanopure water. Otoliths were prepared in random order to eliminate any bias resulting from preparation (Hamer et al. 2003). Left sagittal otoliths were transferred into 2 ml microcentrifuge

tubes filled with nanopure water and vortexed for 3 min to remove any adhering tissue. The nanopure water was then siphoned off and replaced with 1 ml of 3% ultrapure hydrogen peroxide (Fisher Optima) to oxidize any remaining biological substances. The otoliths remained in hydrogen peroxide for 10 min at which point the hydrogen peroxide was siphoned off and the otoliths were triple rinsed with nanopure water. Otoliths then were stored in new 2 ml microcentrifuge tubes and were dried overnight in a laminar flow hood (Patterson et al. 1999). Once dry, otoliths were transferred to an acid cleaned weighing container (2 ml microcentrifuge cap) and weighed using a Mettler Toledo XP6 microbalance to the nearest 0.0000 mg. Following weighing, otoliths were transferred back to microcentrifuge tubes, triple rinsed with nanopure water to remove any contamination from weighing, and dried overnight in a laminar flow hood. Otoliths then were digested overnight using a volume of 25% ultrapure triple distilled nitric acid proportional to their weight (1 mg otolith: 259 μl microliters of acid). The volume of acid added was adjusted based on otolith weight to maintain a constant concentration of calcium in each sample so any matrix effect due to high calcium concentrations would be consistent between samples. Once completely digested, otolith samples were diluted to a final volume proportional to their weight (~1 mg otolith: 8.62 μl) with an ultrapure 2% nitric acid solution. Samples then were spiked with a ~2 ppb indium internal standard (Specpure) for determination of element recovery rates.

Microchemical Analysis

Otolith microchemistry was analyzed by solution based inductively coupled plasma mass spectrometry (SB-ICP-MS) at the UNH ICP-MS Lab, Morse Hall, Durham, New Hampshire. Otoliths were analyzed using a Nu AttoM® double-focusing high-

resolution magnetic sector mass spectrometer (Nu Instruments; www.nu-ins.com). The instrument was equipped with a micromist nebulizer, operating in self-aspirating mode (sample uptake rate 100 μl min^{-1}). Measurements were performed at either a low or medium resolution setting (m/Δm= 300 or 3200, respectively), depending on the spectral interferences for each element. Ag$^{(107)}$, Ba$^{(137)}$, Ca$^{(46)}$ Cd$^{(111)}$, Li$^{(7)}$ and Pb$^{(208)}$ were run at low resolution. Ca$^{(42,43)}$, Cu$^{(63,65)}$, Fe$^{(57)}$, Mg$^{(24)}$, Mn$^{(55)}$, Na$^{(23)}$, Sr$^{(88)}$, and Zn$^{(66,68)}$ were run at medium resolution. Instrument operating conditions are shown in (Table 7)

Initial trace element concentrations were quantified with an external calibration method with multi-element standards containing all of the elements of interest in the expected concentration range. Expected concentration ranges of elements were based on a previous unpublished Environmental Protection Agency winter flounder microchemistry study (B. Taplin, personal commun). Initial concentrations were calculated for each element by regression analysis based on the drift corrected values determined by comparing a monitoring standard. Calibration curves were run at the beginning and end of each session, though regression analysis was based on the calibration curve at the beginning of the day. Isotopes with a calibration curve r-squared value <0.950 were not included in subsequent analyses (Table 8). Most poorly calibrated isotopes likely suffered from an unresolved interference (e.g. Ca^{44}) or were concentrations too close to the detection limit (e.g. Zn^{66,68}). Instrument blanks of 2% HNO$_3$ were run prior to the calibration curves at the beginning and end of each day. The average detection limits were calculated for each element using the five sigma criteria (Table 8). Elements that had the majority of values below the detection limit also were not used in subsequent analyses.

Otoliths were analyzed randomly to eliminate any bias resulting from instrument drift (Hamer et al. 2003). Instrument drift was corrected off-line using linear interpolation between two multi-element standard monitoring solutions containing all elements of interest (Rosenthal et al. 1999). The monitoring solution was run after every 4 otolith samples. This bracketing method allowed for correction of each individual element. Determining the drift correction for each individual element is important because instrument drift does not occur at the same rate for each element. For quality control, precision, and accuracy checks, the NRC otolith certified reference material (CRM) FEBS-1 was analyzed after every 8 samples. Poor CRM recovery rates were observed when using initially calculated trace element concentrations; because of this, a matrix correction was used to calculate final concentrations. Because the multi-element standard monitoring solution used to calculate concentrations was a synthetic solution, with a less complex matrix than an otolith, it is not surprising that a matrix correction was needed. The matrix correction was calculated using the slope of the constant calcium standard additions that were run at the beginning of every run. Slope was calculated by plotting expected concentration vs. observed concentration. The matrix correction was not applied to any of the calcium values because calcium is the most abundant element and was within the certified range using the initial concentration values. Magnesium also was not matrix corrected because it was accidently omitted when preparing the constant calcium standard addition. Using the final concentration values with the matrix correction, many of the elemental concentrations (Li^7, Na^{23}, $Ca^{42.43,46}$, and Mn^{55}) determined in FEBS-1 were within the certified or 'informational' range; elements that were not within range (Mg^{24}, Sr^{88}, and Ba^{137}) gave consistent concentrations between runs (Table 9) and so

were included in the analysis. The accuracy of the drift correction was verified by examination of the CRM and indium recovery rates spaced throughout a run. Precision of replicate analyses over the course of all runs of the individual elements ranged from an average of 1.1 to 16.4% relative standard deviation (RSD) (Table 8), with elements with otolith concentrations closer to the detection limit giving the lowest precision.

Statistical analysis

Concentrations of trace elements were reported as μg element g^{-1} solution and then transformed to μg element g^{-1} otolith. Final elemental signatures were expressed as molar ratios (μmol element: mol^{-1} Ca^{43}) to account for fluctuations in the amount of material analyzed and the loss of material during the preparation process (Sinclair et al. 1998). All data were generalized log transformed ($B_{ij} = log(x_{ij} + log^{-1}Int(log(min(x)))) - Int(log(Min(x))))$ in an attempt to improve normality and relativize the variation in molar ratios between elements. Despite the generalized log transformation there was still a slight deviation from the assumptions of normality; because of this, Bray-Curtis distance measures were used for all subsequent analyses. The Bray-Curtis distance measurement also was used because the molar elemental ratios did not vary linearly, which is an assumption that must be meet when using other distance measurements.

One-way ANOVAs and t-tests were performed between dates of analysis and elemental molar ratios to account for any variations between run dates. No significant differences between dates were detected for any element at any of the 12 locations; therefore no adjustments were made to account for the sampling day. To ensure that the size of the otolith did not influence the variation in elemental chemistry (Fowler et al.

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1995), linear regressions between molar element:Ca ratios and otolith mass (g) were performed (Campana et al. 2000). A significant, albeit weak, relationship was found only between strontium concentrations and otolith mass ($r^2 = 0.0294$, n = 199, p< 0.05). Although this relationship was found to be significant, no adjustments were made to the strontium concentrations due to the risk of over correcting when using an adjustment based on a weak regression.

A non-metric multidimensional scaling (NMDS) ordination was used to examine similarities between fish. The appropriate number of dimensions was determined based on final stress results and the outcome of the Monte Carlo test. The Monte Carlo test evaluated if the ordination was stronger than expected by chance. Cluster analysis also was used to examine similarities and identify groupings of juvenile winter flounder based on elemental signatures; the flexible beta (b=-0.25) linkage method was used. Indicator Species Analysis (Dufrêne and Legendre 1997) was used to investigate which elements defined different estuaries. A perfect indicator would be an element that was always present in an estuary and exclusive to that estuary, never occurring in other estuaries. The indicator species analysis calculated indicator values for every element in each estuary, based on the standards of a perfect indicator. A Monte Carlo test was used to determine the significance of these indicator values.

To investigate if elemental signatures were estuary specific, a Multiple-Response Permutation Procedure (MRPP) with pairwise comparison was performed. This test was chosen for two reasons: 1) it does not require equal samples per estuary unlike a permutational multivariate analysis of variance; and 2) it does not require distributional assumptions. Groups were defined by natal estuary. To address the problem of increased

type 1 error when making multiple pairwise comparisons, a Bonferroni correction was used when interpreting results of pairwise comparisons. Therefore a $p<0.004$ was considered significant for all pairwise comparisons.

A quadratic discriminant function analysis (QDFA) with leave-one-out cross validation was used to test the ability of the otolith elemental signatures to classify juvenile winter flounder to their natal location. QDFA was chosen as the classification method because it does not require distributional assumptions (Krzanowski and Krzanowski 2000).

Results

The following elements were excluded because they failed to meet all of the quality control, precision, and accuracy checks: $Ag^{(107)}$, $Ca^{(44)}$, $Pb^{(208)}$, $Cu^{(63,65)}$, $Fe^{(56,57)}$, and $Zn^{(66,68)}$. The remaining elements, $Ba^{(137)}$, $Li^{(7)}$ $Ca^{(43,46)}$, $Cd^{(111)}$, $Mg^{(24)}$, $Mn^{(55)}$, $Na^{(23)}$, and $Sr^{(88)}$, met all of the quality control, precision, and accuracy checks, and were used in subsequent analysis. Ca^{43} was used to standardize all of the medium resolution elements instead of Ca^{42} or Ca^{44} due to its high r-squared calibration curve values (Table 8). Ca^{46} was used to standardize all the low resolution elements. Molar ratios of the elements included in the analysis were within the range of those reported in previous otolith microchemistry studies (Clarke et al. 2009) (Figure 12).

Otolith elemental signature grouping

A 2-dimensional NMDS ordination with a final stress of 13.48 explained 91.7% of the variation in otolith elemental signatures. The 1st dimension explained 60.5% of the variation and the 2nd explained 31.1%. The Cape Cod, Gulf of Maine, and New Jersey

fish separated from each other using the 2 dimensional ordination (Figure 13). The Narragansett Bay and Niantic River fish, however, were scattered amongst the Cape Cod and Gulf of Maine fish. Ba/Ca, Mn/Ca, and Sr/Ca had the most influence on the ordination, with r^2 values of 0.59, 0.64, and 0.375, respectively. Examination of the ordination using the location centroids allowed for greater discrimination (Figure 14). The centroid ordination revealed that Rhode Island fish were most similar to the Cape Cod fish, specifically the Cotuit Bay fish, and that the Niantic River fish were most similar to the Gulf of Maine fish, specifically those from Hampton-Seabrook. Fish from the same island, Martha's Vineyard (i.e. Lagoon and Menemsha Ponds), ordinated similarly. In the Gulf of Maine, Beverly Harbor and Great Bay were similar as were Little Harbor and Boston Harbor.

The cluster analysis identified five broad groups of fish based on elemental signatures, using only 30% of the elemental signature data (Figure 15). Two separate groups for both the Gulf of Maine and Cape Cod locations and one Navesink, NJ group were identified. The Narragansett Bay, RI and Niantic River, CT fish were distributed throughout the Cape Cod and Gulf of Maine groupings. Exact location groups were not discernible even when including more of the elemental signature data.

The Indicator Species Analysis found Ba/Ca, Mn/Ca, and Sr/Ca to be the only significant indicators of specific estuaries (Table 10) although all had relatively low indicator values (<10). Ba/Ca was an indicator for Menemsha Harbor, Mn/Ca for the Navesink River, and Sr/Ca for Great Bay.

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Spatial differences in otolith signatures

Juvenile winter flounder otoliths showed significant geographical differences in otolith signatures throughout specific New England estuaries. Otolith elemental signatures were significantly different between locations (MMRP, A= 0.38, p< 0.0001). Comparing 66 possible estuary pairs using the conservative Bonferroni correction, there was a significant difference between 76% of them (n=50; Table 11). Of the 16 estuary pairs that were not significantly different, twelve were locations within the same broad group (i.e. Cape Cod/Gulf of Maine) and generally were separated by a relatively small distance (<75 km). The four pairings that were not within the same broad location and were not significantly different were Hampton-Seabrook (NH) and the Niantic River (CT), Narragansett Bay (RI) and Waquoit Bay (MA), Narragansett Bay (RI) and Cotuit Bay (MA), and Boston Harbor (MA) and Cotuit Bay (MA).

Several geographic trends also were observed within the data (Figure 12). Mn/Ca values were highest in the Navesink River, NJ, with very little difference between all of the other locations. Ba/Ca values were the highest at the two Martha's Vineyard locations and relatively low at all other non-Cape Cod locations. Sr/Ca values were high at all Cape Cod locations and in Great Bay.

Classification of juvenile fish to natal habitat

Results of the QDFA with leave-one-out cross validation showed that fish could be classified back to their natal location with 73% accuracy (Table 12). Misclassifications generally occurred at locations that were not statistically different in the MMRP pairwise comparison.

Discussion

Natal Classification

In this study, microchemical signatures of juvenile winter flounder otoliths showed significant differences between nursery locations along the northeast coast of the United States. These microchemical signatures allowed juvenile winter flounder to be traced back to their natal nursery location with a classification accuracy of 73%. This accuracy suggests that there is sufficient chemical variation between nursery areas on a relatively small scale (~5-10 km).

The classification accuracy achieved in this study is similar to other studies that have examined fish from near coastal habitats. Clarke et al. (2009) reported 70% and 77% accuracy in the two years they examined Atlantic silverside, *Menidia menidia*, in similar, and in some cases the same, northeastern United States coastal habitats. While examining juvenile microchemical signatures of red drum, *Sciaenops ocellatus*, from southeastern United States estuaries, Patterson et al. (2004) reported a classification accuracy of 81%. Other studies have reported even higher classification accuracy, specifically in fish that have clear geographic areas in which various life-history stages take place, as is the case with winter flounder. A classification accuracy of 90% was reported by Thorrold et al. (1998b) when examining the estuary-dependent weakfish, *Cynoscion regalis*. A classification accuracy of 91% was reported by Walther et al. (2008) when examining the anadromous American shad, *Alosa sapidissima*. The classification accuracy reported in this study and the higher levels found in other studies is essential for using otolith microchemistry as an effective fisheries management tool.

Spatial Differences

In order to use microchemical signatures as a fisheries management tool there must be variation between sites (Thresher 1999). These microchemical variations occur because of environmental differences between locations. While these variations are essential for creating a distinct otolith signature, understanding the source of variation is not necessary for use as a fisheries management tool (Thresher 1999).

Though not necessary for use as a fisheries management tool, multiple studies have attempted to determine the causes of otolith microchemistry variation. There are many environmental characteristics which affect the variation of otolith microchemistry including concentration of the bioavailable forms of each element, salinity, temperature, fish age, ontogeny, physiology, growth rate, and metabolism (Elsdon and Gillanders 2003, Milton et al. 2008, Silva et al. 2011). Also, nursery habitat quality can affect many of these factors, which would lead to further variation. Fish also have been shown to adapt to their environment, leading to otolith microchemistry variations between nurseries (Conover et al. 2006). All of these factors make it difficult to know exactly how the environmental characteristics affect otolith microchemistry.

In our study we were particularly interested in Li, Ba, Mn, and Sr because they were significantly different between nursery locations. Multiple studies have measured the differences in these trace elements in relation to variations in environmental factors. For example, variation in incorporation of Ba and Sr has been clearly linked to concentration of the bioavailable forms of each element. Sr is influenced by water chemistry (Bath et al. 2000), diet (Kennedy et al. 2000, Buckel et al. 2004), and

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temperature (Martin et al. 2004). Also, the ambient concentration of Sr and Ba in near shore coastal habitats is largely dependent on river discharge, tidal stage, and the mixing pattern of the estuary (Coffey et al. 1997, Kraus and Secor 2004). Ba in inshore coastal habitats is linked to groundwater inputs as well (Coffey et al. 1997, Moore 1997, Shaw et al. 1998). The pH of the river discharge and extent of the salt marsh in the area also plays a role in ambient Ba concentration. Mn concentrations vary between the otolith core and surrounding material suggesting an endogenous or ontogenetic effect (Brophy et al. 2004, Ruttenberg et al. 2005). While these are possible explanations for the variations of these elements in our study, it cannot be confirmed without measuring each of these factors within each of our locations.

The small spatial scale differences (12 – 20 km) found in this study are similar to those identified in other studies. Thorrold et al. (1998a) identified small spatial scale differences within river systems when studying the American shad, *Alosa sapidissima*. Thorrold et al. (1998b) and Dorval et al. (2005) both observed small spatial scale differences within estuaries when studying weakfish, *Cynoscion regalis*. However, Clarke et al. (2009) did not observe such small-scale differences between locations in *Menidia menidia* otolith microchemical signatures when examining similar locations to this study. However, they did observe small spatial scale differences (5–10 km) within locations (Clarke et al. 2009). It is likely that the small-scale differences observed in this winter flounder study and others are due to differences in water chemistry. Small-scale differences within locations were not observed in this winter flounder study because fish were collected within a small area at each location (< 8 km), with the exception of the Narragansett Bay where fish were collected ~15 km apart. There was no variation in

elemental signatures between the 3 collection locations within Narragansett Bay despite this distance. The lack of variation between collection locations within Narragansett Bay could be a result of the small sample size analyzed at each collection location (~5 fish) or the homogeneity in water chemistry within Narragansett Bay. However, it is more likely that the lack of variation is due to the small sample size and not the homogeneity in water chemistry because Narragansett Bay is such a variable and large ecosystem with many different watersheds and dynamic tidal exchanges. Further testing is necessary to determine if there is within location elemental signature variability in Narragansett Bay.

Temporal Differences

Temporal differences in otolith microchemistry have been observed, on various scales, in several studies (Gillanders 2002, Clarke et al. 2009) resulting in the inability to classify fish to specific areas using previously established elemental signatures. Also, temporal differences can be distinct for different elements; Warner et al. (2006) found yearly differences in Mg, Sr, and Pb, but not in Mn, Zn, and Ba when examining open ocean differences in kelp rockfish, *Sebaster atrovirens*, off the California coast. Temporal differences have not only been found on an annual basis but also on an interannual basis (Gillanders and Kingsford 2000). Like *M. menidia* (Clarke et al. 2009), winter flounder might exhibit annual variation in elemental signatures given that both species inhabit similar habitats in the same geographic range. The scale of the temporal differences could have a drastic impact on the value of using microchemical analysis for natal nursery area identification.

Temporal variation in elemental signatures cannot be fully understood without a clear understanding of the factors influencing otolith incorporation in the first place, though the source of the variation is most likely due to environmental variation. If there is no temporal variation in microchemical signatures, then the signatures can be used to classify natal nursery areas of all year-classes. However, if the signatures vary temporally, then the signatures are limited and only year-class specific. Further annual and interannual microchemical testing is required to identify the temporal stability in winter flounder microchemical signatures. This would allow for the best method of analysis and implementation as a fisheries management tool. In this study, solution based inductively coupled plasma mass spectroscopy (SB-ICP-MS) was used but if temporal variation occurs on an interannual basis, laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) may be a more effective method to use. Unlike SB-ICP-MS, LA-ICP-MS does not require an entire otolith to be dissolved. Instead, a small portion of the otolith is ablated. This allows for elemental analysis to occur at a specific time, whereas solution based samples the elemental composition of the entire life of the fish.

Conclusion

Otolith microchemistry has the potential to be an effective tool to assess the connectivity among nursery areas and adult populations of winter flounder. Understanding this connectivity will provide information that is necessary for effective ecosystem-based management. In this study we found that otolith microchemistry of winter flounder has the potential to be a useful technique, based on a natal classification accuracy of 73%, but we also determined that there are some aspects that require further investigation prior to using it as a management tool. First, the temporal variation in the

elemental signatures needs to be identified and the analysis method needs to be standardized. SB-ICP-MS was found to work well for analyzing whole juvenile otoliths, but for adult otoliths, the preparation will be labor intensive because they will need to be micro-milled to the juvenile core prior to analysis. LA-ICP-MS may be the better method when examining both juvenile and adult fish, and should be investigated in the future. This technique allows for precise otolith locations to be sampled when using either juvenile or adult fish, enabling elemental signatures to be derived from specific life stages with relative ease.

With further refinement of methods, an elemental signature index of nursery areas based on otolith elemental composition should be possible. This index then can be used for stock identification and to trace adults back to natal nursery areas. For winter flounder, this would allow for the increased protection of those nursery grounds that significantly contribute to the adult population, thereby potentially reducing early-stage mortality and increasing the resiliency of the adult population. Identifying the most successful nursery grounds is an important step in developing models of critical winter flounder nursery characteristics that may later be used in conservation policy.

Table 6. Sample size (n), mean total length (L_T) ± standard error, and mean mass (M) ± standard error of winter flounder juveniles used for otolith microchemistry analysis from each sampling location.

Location	n	Mean Length (mm)	Mean Weight (g)
Great Bay, NH	15	67.0 ± 0.7	3.19 ± 0.12
Little Harbor, NH	16	64.2 ± 0.5	2.45 ± 0.07
Hampton-Seabrook, NH	14	61.3 ± 1.2	2.29 ± 0.14
Beverly Harbor, MA	16	55.1 ± 2.8	1.89 ± 0.38
Boston Harbor, MA	20	60.8 ± 2.8	2.89 ± 0.40
Cotuit Bay, MA	20	57.3 ± 1.4	1.77 ± 0.16
Waquoit Bay, MA	19	63.9 ± 1.1	2.53 ± 0.16
Lagoon Pond, MA	19	49.9 ± 0.6	1.17 ± 0.05
Menemsha Pond, MA	17	50.8 ± 0.6	1.16 ± 0.05
Narragansett Bay, RI	15	64.8 ± 2.2	2.68 ± 0.29
Niantic River, CT	15	49.0 ± 1.2	0.96 ± 0.08
Navesink River, NJ	12	55.0 ± 1.4	1.68 ± 0.13

Table 7. Typical operating settings of the ICP-MS for otolith analysis.

Instrumental Parameter	Set Point
Resolution	300, 3200
Forward Power	1200 W
Nebulizer Ar gas flow	26-30 psi
Auxiliary Ar gas flow	1 L/min
Coolant flow	13 L/min
Cones	Ni sampler and skimmer
Acquisition method	Magnetic jump with electric scan over small mass range
Channels per mass	20
Number of cycles	3
Number of sweeps	500
Dwell time	2ms
Data acquisition time	< 120 sec

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Table 8. Quality assurance values from all ICP-MS runs.

Element	Average RSD	Average Detection Limit (ug/L)	Average R-squared of Calibration Curve
Li(7)	1.7	8.57E-06	1.00
Na(23)	1.3	3.5E-04	1.00
Mg(24)	5.9	5.73E-05	1.00
Ca(42)	1.2	0.57	1.00
Ca(43)	1.1	0.56	1.00
Ca(44)	1.1	-0.53	0.89
Ca(46)	1.7	1.41E-01	1.00
Mn(55)	2.4	8.92E-06	1.00
Fe(56)	16.4	5.43E-05	0.91
Fe(57)	12.5	5.62E-05	0.93
Cu(63)	6.2	1.69E-05	0.96
Cu(65)	7.4	1.35E-05	0.97
Zn(66)	4.2	7.48E-05	0.98
Zn(68)	4.3	5.97E-05	0.98
Sr(88)	1.3	6.02E-03	1.00
Ag(107)	3.8	N/A	0.43
Cd(111)	4.6	6.3E-07	1.00
Ba(137)	2.7	7.91E-06	1.00
Pb(208)	2.7	2.19E-05	0.71

Table 9. Average CRM (FEBS-1) value and standard error for each run.

	Certified Value or Range (ppm)	Run 1 (n=8) Average CRM (ppm)	Standard Error	Run 2 (n=10) Average CRM (ppm)	Standard Error	Run 3 (n=12) Average CRM (ppm)	Standard Error
Li(7)	0.305 ± 0.044	0.23	0.00	0.27	0.00	0.25	0.00
Na(23)	2594 ± 161	2121.48	111.35	2380.52	40.13	2495.70	16.10
Mg(24)	23.6 ± 1.3	19.45	0.10	19.45	0.13	19.58	0.15
Ca(43)	383000 ± 14000	396997	3490	396159	1312	393680	2332
Ca(46)	383000 ± 14000	394471	1905	396924	1167	396538	4103
Mn(55)	0.686 ± 0.016	0.75	0.10	0.79	0.02	0.74	0.05
Sr(88)	2055 ± 79	1986.29	14.73	1920.63	8.37	1933.21	9.04
Ba(137)	5.09 ± 0.23	3.75	0.04	3.76	0.03	3.59	0.07

Table 10. Indicator Species Analysis. Asterisk denotes significant indicators.

Element	Location	Indicator Value	p-value
Li/Ca	Little Harbor	8.7	0.2641
Na/Ca	Hampton-Seabrook	8.5	0.0956
Mg/Ca	Beverly Harbor	8.5	0.1800
Mn/Ca	Navesink River	9.8	< 0.001*
Sr/Ca	Waquoit Bay	9.1	<0.001*
Ba/Ca	Menemsha Pond	9.0	<0.001*

Table 11. MMRP elemental signature pairwise comparisons. ** denotes significance using Bonferroni correction (p<0.004), NS denotes non-significant values (p>0.004).

	Great Bay	Little Harbor	Hampton-Seabrook	Beverly Harbor	Boston Harbor	Cotuit Bay	Waquoit Bay	Lagoon Pond	Menemsha Pond	Narragansett Bay	Niantic River	Navesink River
Great Bay		**	**	**	**	**	**	**	**	**	**	**
Little Harbor	**		**	**	NS	**	**	**	**	**	**	**
Hampton-Seabrook	**	**		NS	NS	**	**	**	**	**	NS	**
Beverly Harbor	**	**	NS		**	**	**	**	**	**	**	**
Boston Harbor	**	NS	NS	**		**	**	**	**	**	**	**
Cotuit Bay	**	**	**	**	**		NS	**	**	NS	**	**
Waquoit Bay	**	**	**	**	**	NS		**	**	**	**	**
Lagoon Pond	**	**	**	**	**	**	**		NS	**	**	**
Menemsha Pond	**	**	**	**	**	**	**	NS		**	**	**
Narragansett Bay	**	**	**	**	**	NS	**	**	**		**	**
Niantic River	**	**	NA	**	**	**	**	**	**	**		**
Navesink River	**	**	**	**	**	**	**	**	**	**	**	

Table 12. QDFA with leave-one-out cross validation. Rows are actual natal location of fish, columns are the predicted natal locations using the QDFA with leave-one-out cross validation.

	Great Bay	Little Harbor	Hampton-Seabrook	Beverly Harbor	Boston Harbor	Cotuit Bay	Waquoit Bay	Lagoon Pond	Menemsha Pond	Narragansett Bay	Niantic River	Navesink River
Great Bay	15	0	0	0	0	0	0	0	0	0	0	0
Little Harbor	0	14	0	0	1	0	0	0	0	0	1	0
Hampton-Seabrook	0	0	10	0	3	0	0	0	0	0	1	0
Beverly Harbor	1	0	1	9	3	0	0	0	1	1	0	0
Boston Harbor	1	2	0	0	15	1	0	0	0	0	1	1
Cotuit Bay	0	0	0	0	2	6	0	1	0	6	5	0
Waquoit Bay	0	0	0	1	0	0	14	1	1	1	0	0
Lagoon Pond	0	0	0	1	0	1	4	11	1	2	0	0
Menemsha Pond	0	0	0	0	0	1	1	0	15	0	0	0
Narragansett Bay	0	0	0	1	0	1	0	0	0	12	1	0
Niantic River	0	0	0	0	1	1	1	0	0	1	13	0
Navesink River	0	0	0	0	0	0	0	0	0	0	0	12

Collection Location	Gear Used	Fish	Tows	CPUE
Great Bay, NH	Trawl	36	3	12.0
Little Harbor, NH	Trawl	45	14	3.2
Hampton-Seabrook, NH	Seine, Trawl	14	9	1.6
Beverly Harbor, MA	Seine	37	7	5.3
Boston Harbr, MA	Trawl	23	12	1.9
Cotuit Bay, MA	Seine	35	7	5.0
Waquoit Bay, MA	Seine, Trawl	40	8	5.0
Lagoon Pond, MA	Seine, Trawl	44	10	4.4
Menemsha, MA	Seine	36	15	2.4
Narragansett, RI	Seine	43	-	-
Niatic River, CT	Trawl	27	-	-
Navesink, NJ	Seine	40	-	-

Figure 11. Sample locations of winter flounder from estuaries in the northeast United States. Inset table contains collection information for each location. Fish: total number of winter flounder caught, Tows: total number of tows, CPUE: catch per unit effort (# fish tow^{-1}).

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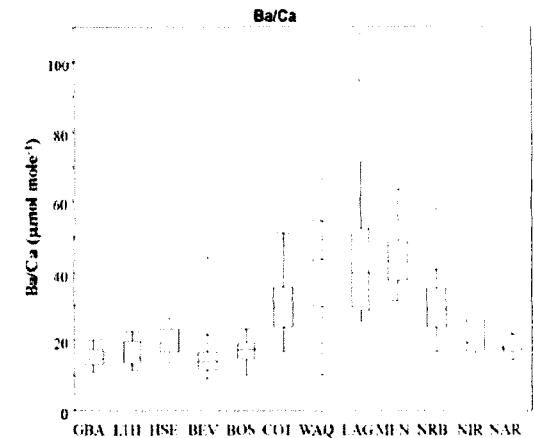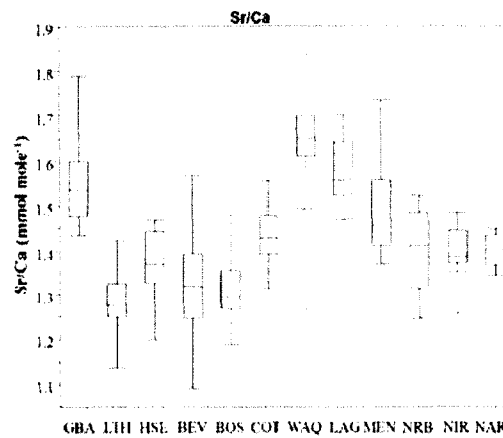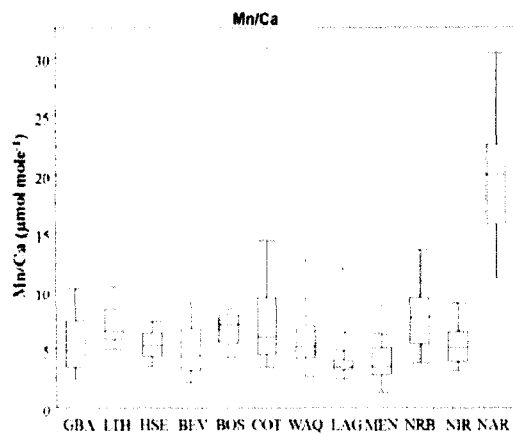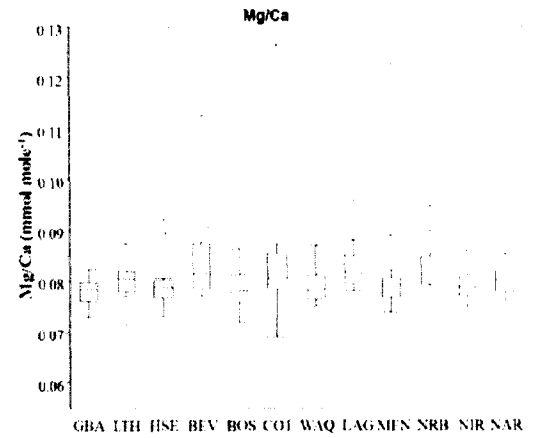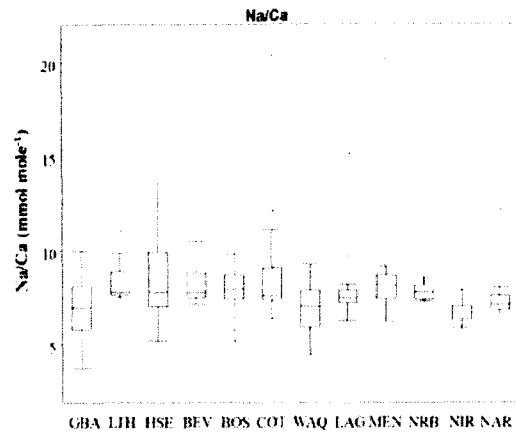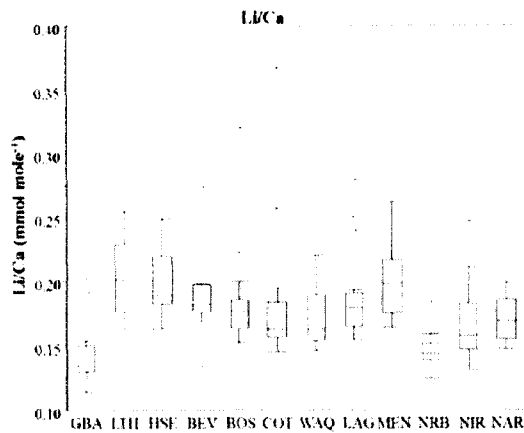
Figure 12. Box plots of each otolith chemical per location. The center line of each box represents the mean, the top and bottom of the box indicate the 25th and 75th percentiles, respectively, and the whiskers indicate the range. Points denote outliers.
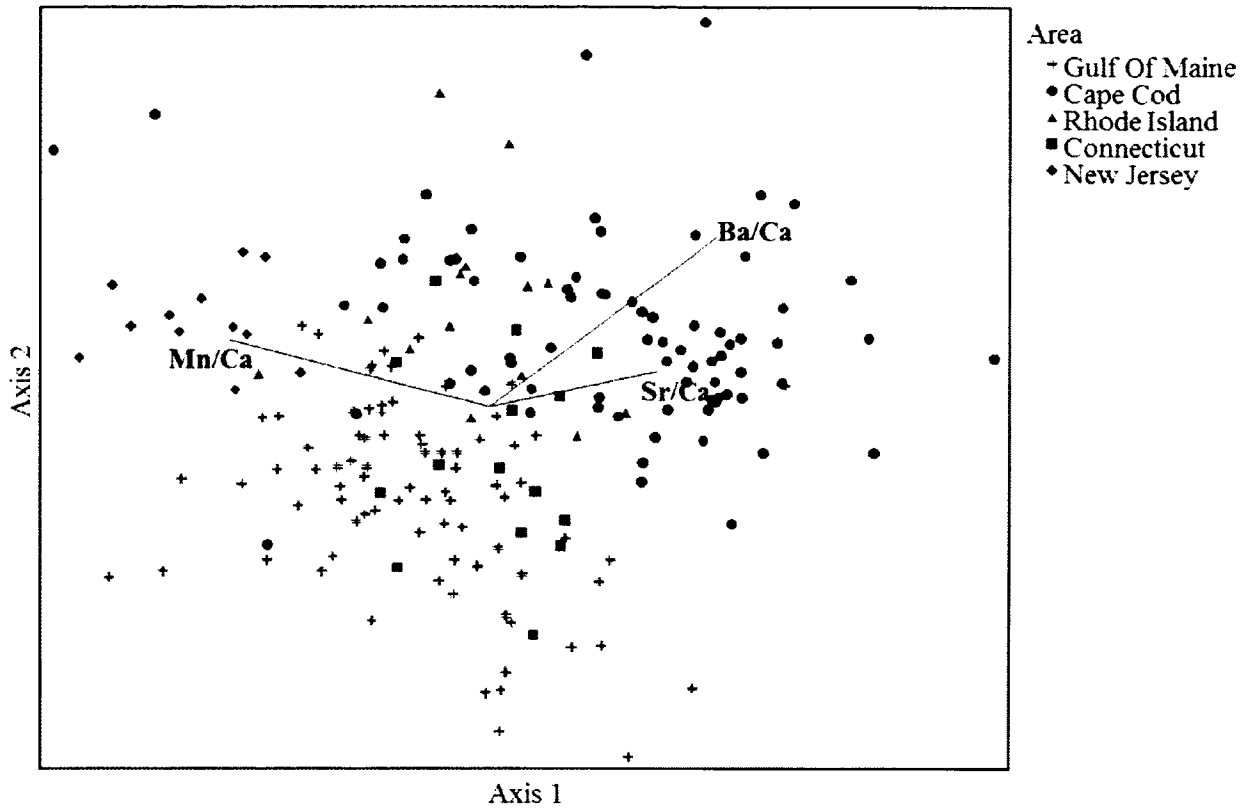
Figure 13. Non-metric multidimensional scaling ordination of winter flounder otolith microchemical signatures by area. Each point represents individual fish and symbols indicate area in which the fish were caught. Axis 1 explains 60.5% of the variation and axis 2 explains 31.1% of the variation. Joint plot contains elemental ratios that drive the ordination with r^2 values of: Mn/Ca= 0.64, Ba/Ca=0.59, Sr/Ca= 0.38.
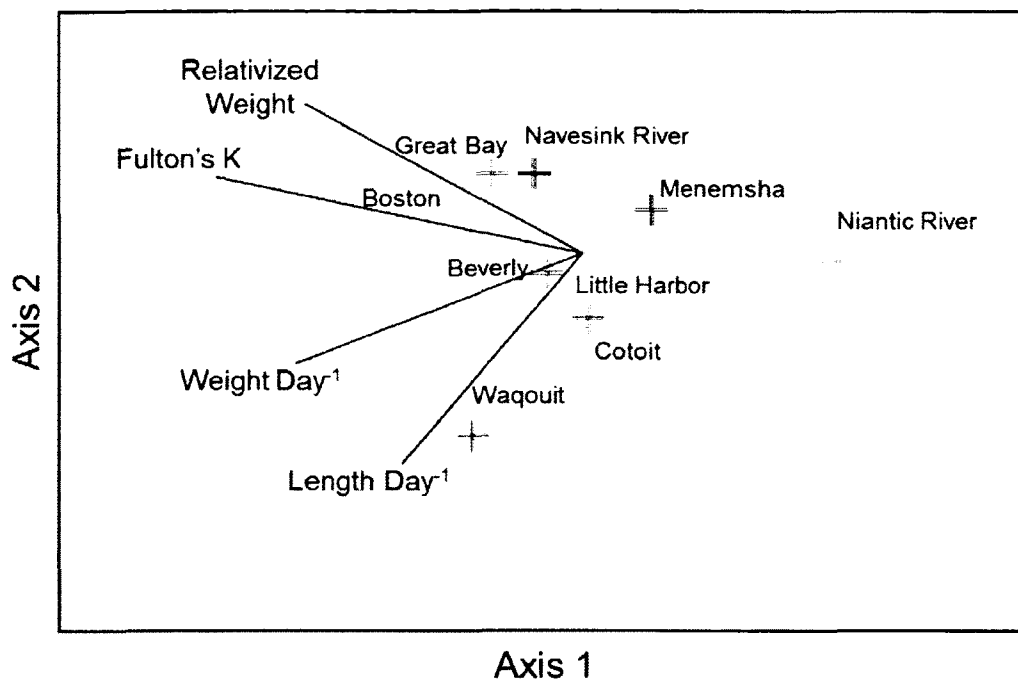
Figure 14. Non-metric multidimensional scaling ordination of winter flounder otolith microchemical signatures by collection location. Crosshairs represent the centroid of each collection location. Axis 1 explains 58.0% of the variation and axis 2 explains 32.1% of the variation. Joint plot contains elemental ratios that drive the ordination with r^2 values of: Mn/Ca= 0.64, Ba/Ca= 0.59, Sr/Ca= 0.38.
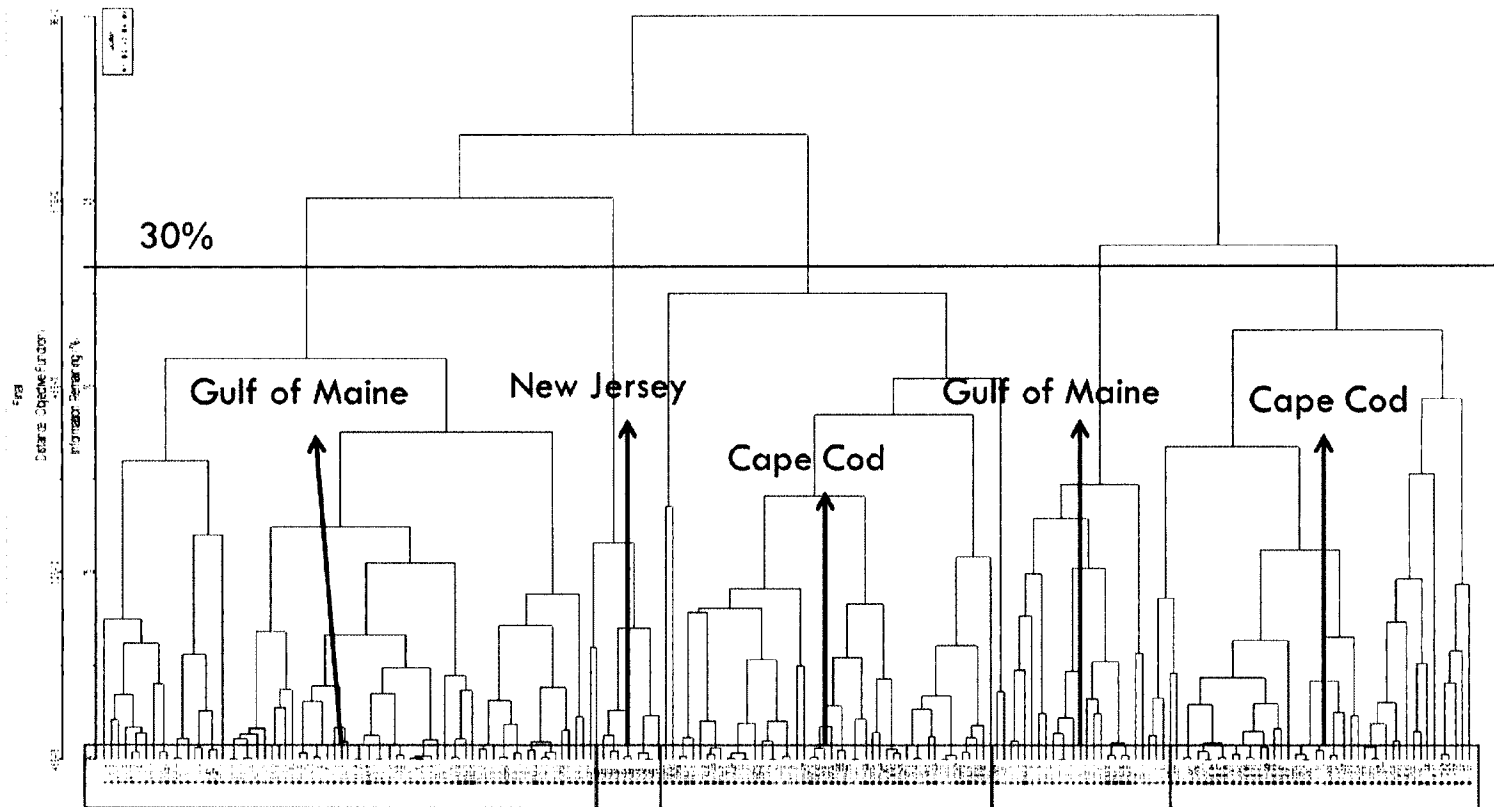
Figure 15. Dendogram from cluster analysis of individual winter flounder otolith microchemical signatures. Five primary clusters are identified based on 30% of the variation.

CHAPTER III. CONCLUSIONS

Despite ever increasing fishing regulations, winter flounder populations are not at sustainable levels. This could be because the decline in fish populations is not solely from fishing pressure but also from changes to essential nursery habitats. The collapse of the winter flounder fishery has dramatic ecological and economic consequences which is why it is important to understand the role of nursery habitat quality variations and how these variations affect recruitment into the adult population. This study evaluated the effectiveness of using indirect and direct measurements to determine nursery quality of twelve nursery habitats from New Jersey to New Hampshire.

When using indirect indices, growth and condition, it is important to determine which indices will most effectively and accurately determine the quality of nursery habitats. This thesis explored four different indices - length day^{-1}, weight day^{-1}, Fulton's K and relativized weight - and revealed differences in habitat quality results depends on each of these indices. Two of the indices proved better indicators of habitat quality because they were less biased by size. These indices were Fulton's K and length day^{-1} and they indicated Boston Harbor, MA and Waquoit Bay, MA as the best nurseries and the Niantic River, CT as the worst nursery. Not only were the best and worst winter flounder nurseries identified but also the most useful indices for determining which nurseries to focus manage efforts on in the future.

Otolith microchemistry has the potential to be an effective tool to assess the connectivity among nursery areas and adult populations of winter flounder. Understanding this connectivity will provide direct measurements to assess nursery habitat quality by determining which nurseries are contributing more new recruits to the adult population. Otolith microchemistry techniques can only be useful if nurseries show distinct chemical signatures. In this study the otolith chemical signatures of twelve nurseries were measured and differed enough such that fish could be classified with 73% accuracy to their natal nursery. This accuracy justifies further development of winter flounder otolith microchemistry as a tool to assess population connectivity.

Although otolith microchemistry will be a useful management tool, there are aspects of this method that require further investigation. First, temporal variation in the elemental signatures needs to be identified. If variations exist then nursery chemical signatures must be reevaluated at the scale of these variations. Regardless of temporal variations, otolith microchemistry can assess population connectivity as long as it is taken into consideration. In addition to determining temporal variations, otolith microchemistry also can be improved by using alternative methods of chemical analysis. For example, laser ablation inductively coupled mass spectrometry (LA-ICP-MS) would allow small scale finite time sampling of the chemical signature along otolith growth. This will be especially useful when analyzing adult flounder otoliths by eliminating the difficulty of isolating the juvenile core which is necessary for solution based ICP-MS.

In this thesis, indirect and direct measurements to measure nursery habitat quality of winter flounder have been identified. These measurements indicated differences among estuaries and coastal habitats in the Northeast. While ideally resource managers

would use both direct and indirect indices, because of the cost and time intensive requirements of direct measurements, such as otolith microchemistry, it is likely that managers will have access to indirect measurements only. Therefore future research needs to focus on establishing a relationship between the direct and indirect measurements identified here. This can be done by first determining the natal contribution of nurseries, and then comparing the most successful nurseries to the indirect indices to find which index most successfully classifies the best nursery. Establishing a relationship between recruitment and the indirect measurements will allow managers to make more accurate decisions with only indirect measurements.

APPENDIX IACUC

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15-May-2012

Fairchild, Elizabeth A
Zoology, Spaulding Life Sciences Ctr
Durham, 03824

IACUC #: 120403
Project: Characterizing Winter Flounder Nursery Areas Using Otolith Microstructure and Microchemical Techniques
Category: D
Approval Date: 25-Apr-2012

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category D on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *Animal use activities that involve accompanying pain or distress to the animals for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are used.*
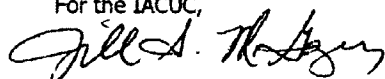
Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

cc: File
        Bailey, David

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