Exxon Valdez Oil Spill
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Evaluation of Two Methods to Discriminate Pacific herring (*Clupea pallasi*) Stocks Along the Northern Gulf of Alaska

Restoration Project 02538
Final Report

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Study History: A Detailed Project Description (DPD), "Evaluation of two methods to discriminate Pacific herring (*Clupea pallasi*) stocks along the northern Gulf of Alaska", was submitted to the *Exxon Valdez* Oil Spill Trustee Council (EVOS-TC) in December 2000. Following review by the Trustee Council, Project 01538 was approved in January 2001. During March, April and May 2001, Pacific herring were collected from the focal spawning areas of six major spawning aggregations in Alaska (e.g., Sitka, Prince William Sound [2], Kamishak, Kodiak, Togiak). Hearts and otoliths were removed from 156 fish and stored for subsequent fatty acid and elemental analysis, respectively. At the request of the EVOS-TC, we submitted a revised DPD in April 2001 for FY02 activities that included the objective to collect Fall 2001 samples from Prince William Sound (PWS) for comparison with the samples collected from pre-spawning herring in PWS during Spring 2001. The age, sex, and maturity of sampled herring were determined by Alaska Department of Fish and Game technicians in the field. Fatty acid analysis of heart tissue samples was conducted by the Auke Bay Lab (NMFS) and Mylnefield Research Services, Ltd; the elemental analysis of otoliths was conducted by Dr. Ken Severin at the Advanced Instrumentation Laboratory at the University of Alaska-Fairbanks.

Abstract: Understanding the stock structure of northern Gulf of Alaska (NGA) herring is relevant to the assessment and management of these exploited populations. We evaluated the capabilities of otolith microchemistry and heart tissue fatty acid profile to identify the stock of origin for herring sampled from four focal NGA spawning aggregations, and two outside NGA (Sitka and Togiak). Otolith microchemistries were measured using an electron microprobe equipped with four wavelength dispersive spectrometers. Fatty acid profiles were determined by performing trans-esterification and fatty acid chromatography on purified lipids from whole hearts. A MANOVA revealed significant regional differences among the mean fatty acid profiles of heart lipids from Sitka, NGA and Togiak herring and among the four NGA stocks. Cross-validation of discriminant functions demonstrated that fatty acid profiles could be used to correctly identify unknown samples more than 90% of the time. Otolith microchemistries were also significantly different between Sitka and Togiak and among some NGA stocks, but could not effectively discriminate between NGA and other regions, or between two of four NGA stocks. Further work is necessary to verify the temporal stability of the biomarkers before they can be used to determine stock contributions from mixed-stock herring fisheries in this region.

Key Words: *Clupea pallasi*, electron microprobe, fatty acid, Gulf of Alaska, mixed-stock fishery, otolith microchemistry, Pacific herring, stock identification, stock structure.

Project Data: *Description of data* - Data collected during the course of FY01 field activities include: herring age, sex, and size statistics, heart tissue fatty acid profiles, and otolith microchemistry profiles from mature, pre-spawning female herring aged 4 to 7 collected during March and April 2001 from focal spawning locations in Sitka Sound (n = 20), PWS (PWS-Montague Island [n = 38]) and Fairmont Bay (PWS-Northeast [n = 36]), Kamishak Bay (n = 33),

west-side Kodiak Island (n = 25), and Togiak Bay (n = 18). Similar data were collected in November 2001 from mature female herring aged 4 to 7 sampled from Montague Island (FY02). Heart tissues (n = 168) and otoliths (n = 150) that were analyzed for fatty acid profiles and elemental compositions, respectively, were necessarily destroyed during processing. However, we collected additional heart tissue samples from Sitka (18), PWS-NE (22), PWS-Montague (7), Kamishak (14), and Kodiak (10) to archive in frozen storage at the Auke Bay Lab (NMFS). *Format* - All electronic data (e.g., age, sex, size, fatty acid and otolith microchemistry profiles) are maintained in Excel spreadsheets and Word text documents. *Custodian*: Ted Otis, 3298 Douglas Place, Homer, Alaska 99603-8027, email: Ted_Otis@fishgame.state.ak.us). *Availability* – contact custodian.

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EXECUTIVE SUMMARY

Pacific herring (*Clupea pallasi*) are an important component of the marine ecosystem providing a trophic pathway for energy flowing from secondary producers to apex predators, including humans. Two related aspects of herring life history that remain poorly described are the degree to which herring return to natal areas to spawn and the scale at which population structure exists within large geographic areas. These key characteristics are directly relevant to how exploited herring stocks should be assessed and managed.

In this study, we evaluated two tools for discriminating herring stocks over relatively fine (≤ 100 km) spatial scales. Tools that can discriminate stocks at this scale are necessary to understand the underlying structure of herring populations in the Northern Gulf of Alaska (NGA). For instance, commercial fisheries that target herring that spawn in Kamishak Bay (Cook Inlet) and the northwestern side of Kodiak Island (Shelikof Strait) are managed separately, however, there is evidence suggesting these stocks mix in northern Shelikof Strait as rearing juveniles and over-wintering adults (C. Burkey, Alaska Department of Fish and Game, unpublished scale pattern analysis data). Despite a management strategy that treats these stocks as independent entities, the degree to which they are separated is unknown. Similar issues surround herring management in Prince William Sound (PWS) where temporally and spatially separated spawning aggregations are managed as a single stock. Previous efforts to identify population structure among PWS herring using microsatellite DNA variation found little genetic divergence among the four spawning aggregations sampled, with the possible exception of Port Chalmers (O'Connell et al. 1998).

Many diverse techniques have been proposed as stock identification tools (Pawson and Jennings 1996; Begg et al. 1999a). Two of the more promising methods recently developed include elemental analysis (EA) of otoliths and fatty acid analysis (FAA) of select body tissues. Because otoliths are acellular, their microchemistry is stable (Campana and Neilson 1985) and can help identify subtle differences in aquatic environments fish are exposed to during their life (Gunn et al. 1992; Radtke and Shafer 1992; Secor 1992). This record of environmental exposure relies on the assumption that otolith microchemistry is, in part, determined by the chemistry of the water occupied by the fish (Radtke and Shafer 1992; Campana and Gagne 1995). Recent reports have also suggested that the fatty acid composition of phospholipids in some body tissues (e.g., heart tissue, brain, eggs) have a stable genetic basis, making these tissues appropriate as stock identifiers (Joensen and Grahl-Nielsen 2000; Joensen et al. 2000).

In this study, we evaluated the capabilities of otolith microchemistry and heart tissue fatty acid composition to identify the stock of origin for herring sampled from four focal spawning aggregations within the NGA, and two outside the NGA (Sitka and Togiak). Specifically, our objectives were to 1) determine whether EA or FAA will allow discrimination among Alaska's three major herring stocks (Sitka, NGA, and Togiak); and if so, 2) determine whether EA or FAA can detect finer scale structuring of putative herring stocks inside PWS and elsewhere in the NGA.

In this pilot study, we maximized the resolution of these techniques by limiting our analysis to pre-spawning female herring aged 4 to 7. Given our expectation that Kamishak and Kodiak

herring resided within their respective natal spawning areas for only a short period, and probably occupied the same over-wintering areas, we focused our EA on the microchemistry of the otolith accreted prior to the first annulus. Otoliths were examined between the focus and the first annulus for the presence of nine elements- Na, Mg, P, S, Cl, K, Ca, Fe, and Sr. Lipids were extracted from the hearts of the same herring and examined for the presence of 39 fatty acids.

Differences in the chemical composition of tissues from different groups were examined by multivariate analysis of variance (MANOVA), univariate ANOVA, and discriminant function analysis (DFA). In addition, we cross-validated the DFA results using the leave-one-out method (Huberty 1994) to estimate the reliability of each stock identification method.

The elemental compositions of herring otoliths from Sitka, Togiak and NGA were significantly different (MANOVA; $P < 0.0001$). DFA indicated that elemental analysis could be used to reliably separate herring from Togiak and Sitka, however, there appeared to be little discriminating power between Sitka and NGA or Togiak and NGA. On a fine spatial scale, EA provided detectable differences among NGA stocks ($P < 0.0001$), but DFA indicated the existence of two groups (Kodiak/PWS-Montague and Kamishak/PWS-NE) that did not conform to our *a priori* stock designations. More detailed analysis indicated the group designations determined by EA derived from the Na and Cl content of the otoliths (ANOVA; $P < 0.004$). .

Our results suggests that fatty acid analysis of heart lipids may be a reliable method for discriminating herring stocks at fine spatial scales (i.e., $\leq$ 100 km). Analysis by MANOVA revealed significant differences between the mean fatty acid compositions of heart lipids from Sitka, NGA and Togiak herring ($P < 0.0001$). DFA revealed that the fatty acid data could be used to correctly identify unknown samples from these stocks more than 94% of the time. Heart lipids from the four NGA stocks- Kodiak, Kamishak, PWS-Montague and PWS-NE, were also easily discriminated by their fatty acid compositions. MANOVA results indicated significant differences among the average fatty acid compositions of the four stocks ($P < 0.0001$). Using DFA, erroneous stock identifications occurred less than 10% of the time and stock specific error rates ranged between 8% and 12%. Unlike the elemental data, our *a priori* stock identities appeared to best describe the fatty acid data structure.

Although these results are quite promising, follow up work is necessary in order to verify the utility of fatty acid analysis for discriminating herring stocks. This follow up work should include all members of each population (e.g., all cohorts, sexes, and maturity levels) and aim to satisfy Galvin et al.'s (1995) third criterion for successful application of a mixed stock analysis (MSA) technique by establishing the temporal stability of the stock specific chemical signatures we identified in our pilot study. The potential value of a tool for discriminating herring stocks over fine spatial scales cannot be overestimated. In the near term, this approach can be used to resolve a number of pressing management questions regarding stock structure in Prince William Sound, Kodiak/Kamishak, Togiak/Dutch Harbor, and in southeastern Alaska. Ultimately, the ability to identify the stock of origin for herring collected away from their natal spawning areas would provide a basis for understanding larval dispersal patterns, home ranges of individual populations, locations of stock specific over-wintering areas, and perhaps the degree to which Pacific herring home back to their natal spawning areas.

INTRODUCTION

Pacific herring (*Clupea pallasi*) are an important component of the marine ecosystem providing a trophic pathway for energy flowing from secondary producers to apex predators. Throughout their life, herring are prey to birds (Logerwell and Hargreaves 1997), marine mammals (Iverson et al. 1997), invertebrates (e.g. hydromedusae: Wespestad and Moksness 1989), other fish (Tanasichuk et al. 1991), and humans (Fischer et al. 1997). In 2002, the commercial sac roe herring fishery harvest in Alaska was approximately 29 thousand tonnes. To sustain viable populations of Pacific herring, and the species that prey on them, we must fully understand the role they occupy in the food web of marine ecosystems (Schweigert 1997).

Two related aspects of herring life history that remain poorly described are the degree to which herring return to natal areas to spawn and the scale at which population structure exists within large geographic areas. These key characteristics are directly relevant to how exploited herring stocks should be assessed and managed (Hourston 1982; Wheeler and Winters 1984; Hay and McCarter 1997; McQuinn 1997). Several recent studies highlight the behavioral complexity of herring and how it relates to stock discreteness (Stephenson 1999; Overholtz 2002; Hay and McKinnell 2002; Huse et al. 2002). The ability to manage stocks discretely is a principal component of sustainable fisheries management- one that requires an ability to accurately apportion the catch from mixed stock fisheries (Mundy 1996).

In this study, we were interested in identifying population structure of Pacific herring residing in the North Gulf of Alaska (NGA). For instance, fisheries that target herring spawning in Kamishak Bay and the Northwest side of Kodiak Island are managed separately, however, there is reason to believe that fish from the two areas mix in northern Shelikof Strait as rearing juveniles and over-wintering adults (C. Burkey, Alaska Department of Fish and Game, personal communication). We were also interested in the degree of population structuring that occurs in Prince William Sound (PWS), where temporally and spatially separated spawning aggregations are managed as a single stock. Previous efforts to identify population structure among PWS herring using microsatellite DNA variation found little evidence for genetic isolation among the four spawning aggregations sampled, with the possible exception of Port Chalmers (O'Connell et al. 1998).

Reported Atlantic and Pacific herring homing rates range from 66-94 percent (Tester 1949; Cushing and Burd 1957; Hourston 1982; Wheeler and Winters 1984). The corresponding stray rates of 6-34% indicate there is more than sufficient gene flow between neighboring spawning areas to compromise the ability of allozyme markers to discriminate between putative stocks (Smith and Jamieson 1986; Bembo et al. 1996; Waples 1998). Waples (1998) warned, "because the amount of migration necessary to obscure most genetic evidence of stock structure (only a handful of individuals per generation) is generally inconsequential as a force for rebuilding depleted populations on a time scale of interest to humans, there is no guarantee that genetic methods alone will provide sufficient precision for key management decisions involving marine species". Thus, NGA herring managers have continued to seek a tool that allows them to identify population structure within and among their respective management areas. Such a tool

would allow Kodiak and Kamishak Bay managers to accurately apportion the potentially mixed-stock harvest that occurs each fall/winter during the Shelikof Strait food/bait fishery.

Many techniques have been investigated to facilitate discriminating between fish populations including: nuclear and mtDNA analysis (O'Connell et al. 1998), enzyme electrophoresis (Schweigert and Withler 1990), multilocus genotype 'familyprinting' (Letcher and King 1999), parasite markers (MacKenzie and Abaunza 1998; Oliva and Ballón 2002), scale patterns (Rowell 1981; Ross and Packard 1990; Barros and Holst 1995), mass marking of otoliths using temperature manipulation (Hagen et al. 1995; Joyce et al. 1996; Courtney et al. 2000) and fluorescent markers (Beckman et al. 1990), stable isotope composition of otoliths (Gao et al. 2001) and the use of life history (Begg et al. 1999b), phenotypic (Swain and Foote 1999), meristic and morphometric characteristics (Schweigert 1990).

Recently, trace differences in otolith microchemistry have been used to discriminate between stocks of several marine fish species, including pink snapper *Chrysophrys auratus* (Edmonds et al. 1989), orange roughy *Hoplostethus atlanticus* (Edmonds et al. 1991), yellow-eye mullet *Aldrichetta forsteri* (Edmonds et al. 1992), Atlantic cod *Gadus morhua* (Campana and Gagne 1995; Campana et al. 1995), and salmonids (Kalish 1990). Thresher (1999) provides a comprehensive review of the use of otolith elemental composition as stock discriminators and offers some cautionary suggestions for researchers interested in employing this promising technique. Campana et al. (1997) cautions that there may be some potential for non-standardized lab equipment and procedures to contribute to differences in otolith elemental composition when comparing results among labs.

Because otoliths are acellular, their microchemistry is stable (Campana and Neilson 1985) and can help identify subtle differences in aquatic environments fish are exposed to during their life (Gunn et al. 1992; Radtke and Shafer 1992; Secor 1992). This record of environmental exposure relies on the assumption that otolith microchemistry is, in part, determined by the water chemistry occupied by the fish (Radtke and Shafer 1992; Campana and Gagne 1995). In controlled laboratory studies, Fowler et al. (1995a; 1995b) demonstrated that temperature, salinity, and ontogeny can influence otolith microchemistry. Successful application of otolith elemental analysis (EA) for stock discrimination is therefore likely influenced by the magnitude of disparity between aquatic environments occupied by each stock. However, it is generally when multiple stocks occupy the same environment and are harvested together in mixed-stock fisheries that the need for stock discrimination is greatest.

Fatty acid compositions of fish lipids have been investigated for decades (Ackman et al. 1963), however, much of the early lipid research was directed at determining the commercial value of fish oils (e.g. Ackman and Eaton 1966) and understanding how fat content relates to various life history functions (e.g. Rajasilta 1992). Because the composition of certain lipids can be closely related to the types of food recently ingested (Navarro et al. 1995; Kirsch et al. 1998), recent investigations have been directed at diet analysis and foraging distribution (e.g. Iverson et al 1997; Iverson et al. 2001; Budge et al 2002).

Many studies have established that fatty acid analysis (FAA) also has utility as a stock identification tool. As early as the 1930's it was demonstrated that different stocks of fin whale

Balaenoptera physalus could be distinguished by the degree of unsaturation of their oils (measured as iodine value: Lund 1934, as cited in Grahl-Nielsen et al. 1993). More recent research suggests that the composition of phospholipid fatty acids prominent in some body tissues (e.g., heart tissue, brain, eggs) have a genetic basis that makes analysis of these tissues appropriate for stock identification studies (Joensen and Grahl-Nielsen 2000, Joensen et al. 2000). Chemometry of fatty acids from heart tissue have been used to discriminate stocks of striped bass *Morone saxatilis* (Grahl-Nielsen and Mjaavatten 1992), Atlantic herring *Clupea harengus harengus* (Grahl-Nielsen and Ulvund 1990), and Atlantic cod *Gadus morhua* (Joensen et al. 2000). Fatty acid analysis of eggs has been used to discriminate between American lobster *Homarus americanus* populations (Castell et al. 1995), Baltic cod *Gadus morhua* stocks (Pickova et al. 1997), and even the wild/domestic origin of sturgeon ova (Czesny et al. 2000). Chemometry of fatty acids have also been used to distinguish between closely related species of the genus *Sebastes* from the Faroe Islands (Joensen and Grahl-Nielsen 2000) and juvenile chinook and coho salmon from the Fraser River (Mjaavatten et al. 1998).

While many of the aforementioned techniques have proven successful for certain applications, each has its own limitations that may reduce its effectiveness for specific stock identification situations. Where one tool cannot detect putative stock specific differences, another often can. Whether or not detection of discernable differences in arbitrarily selected variables constitutes ecologically significant, distinct populations is open to debate (Waples 1998). However, fishery managers still need to apportion mixed stock fishery harvests in order to discretely manage putative populations. Making the right choice among the increasing number of stock identification tools available is considered by some to be one of the greatest challenges fishery managers face today (Waldman 1999).

In this pilot study, we evaluated the capabilities of otolith microchemistry and heart tissue fatty acid composition to identify the stock of origin for herring sampled from four focal spawning aggregations in the NGA, and two outside the NGA (Sitka and Togiak). Otolith microchemistries were measured (ppt) using an electron microprobe equipped with four wavelength dispersive spectrometers. Fatty acid profiles were determined by performing trans-esterification and fatty acid chromatography on purified lipids from whole hearts. Otolith microchemistries were significantly different between Sitka and Togiak (MANOVA; $P<0.0001$) and among some NGA stocks (MANOVA; $P<0.0001$), however, otolith microchemistries could not effectively discriminate between NGA and other regions, or between 2 of the 4 NGA stocks. A MANOVA also revealed significant regional differences among the mean fatty acid profiles of heart lipids from Sitka, NGA and Togiak herring ($P < 0.0001$) and among the four NGA stocks ($P < 0.0001$). Cross-validation of discriminant functions demonstrated that fatty acid profiles could be used to correctly identify unknown samples more than 90% of the time. We concluded that heart tissue fatty acid profiles can be used to discriminate among NGA Pacific herring, but further work is necessary to verify the temporal stability of the biomarkers our study identified before they can be used to determine stock contributions from mixed-stock herring fisheries in this region.

OBJECTIVES

The detailed project description (DPD) we submitted to direct this research listed the following objectives for FY01 and FY02 activities:

FY01:

1. Collect herring samples from Sitka, PWS, Kodiak, Kamishak, and Togiak; extract lipids for fatty acid analysis to be performed in FY02.

FY02:

1. Determine whether EA or FAA will allow discrimination among Alaska's three major herring stocks, and if so;

2. Determine whether EA or FAA can detect finer scale structuring of putative herring stocks inside PWS and elsewhere in the NGA.

3. Collect otoliths and soft tissue from PWS herring during November 2001 and store them for future analysis.

METHODS

Study Area

Our approach was to examine the utility of EA and FAA by first using them to discriminate stocks that have been discriminated by other methods and then applying the new techniques to the NGA stocks. Consequently, our study area included sampling locations extending from Sitka Sound (~57º N Latitude, 136º W Longitude), north to Prince William Sound (~61º N Latitude) and west to Togiak Bay (~59º N Latitude, 161º W Longitude; Figure 1). Except for Togiak Bay, which is in the Bering Sea, all sampling locations were within the Gulf of Alaska (GOA), with most of the samples coming from locations in the Northern Gulf of Alaska (NGA). Two of the GOA's largest embayments provided three of our six sampling locations, Kamishak Bay in Cook Inlet, and Montague Island and Fairmont Bay in Prince William Sound (hereafter referred to as PWS-Montague and PWS-NE, respectively), while one of its largest island complexes, Kodiak/Afognak, provided another of our sample locations.

The coastal bathymetry of the GOA is characterized by a broad continental shelf, except in Southeast Alaska where the shelf narrows and deep fjords abound. The physical and biological characteristics of the coastal environment throughout our study area are largely dictated by the Alaska Coastal Current (ACC). The ACC originates in Southeast Alaska and follows the coast north across the entrances to PWS and Cook Inlet, past Kodiak and Afognak Islands via Shelikof Strait, around the Alaska Peninsula via Unimak Pass, and into the Bering and Beaufort Seas. The ACC is characterized by low salinity due largely to the tremendous volume of freshwater flowing into it from the hundreds of glaciers and waterways found throughout its length. Pacific herring can be found spawning at many locations along Alaska's ubiquitous coastline with commercially viable populations of interest to this study being located in Sitka Sound, Prince William Sound, Kamishak Bay (Lower Cook Inlet), Kodiak/Afognak Island (Paramanof Bay), and Togiak Bay.

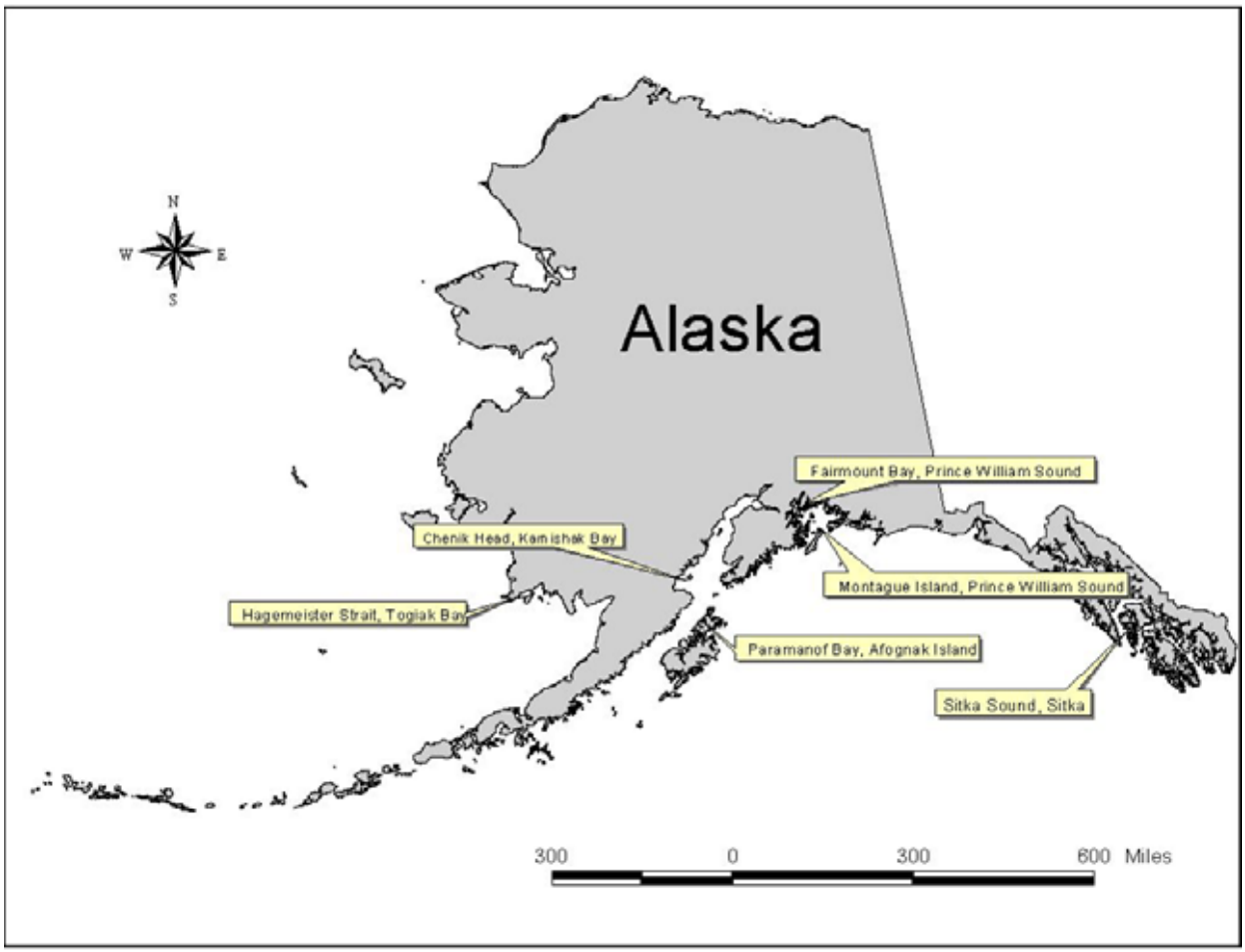

Figure 1. Map of Alaska illustrating the six locations from which Pacific herring were sampled to determine whether heart tissue fatty acid composition or otolith microchemistry could be used to discriminate stock structure.

Sample collection

Mature, pre-spawning female Pacific herring, aged 4 to 7, were collected from the focal spawning areas of six major spawning aggregations in Alaska (n = 241). For this pilot study, we controlled for age, sex, and maturity to minimize the effects of potential sources of variation and thereby maximize the resolving power of the two techniques we evaluated. Sample sizes from each area were as follows: Sitka 38 (20/18), PWS-NE 58 (30/36), PWS-Montague 45 (30/38), Kamishak 47 (30/33), Kodiak 35 (25/25), Togiak 18 (15/18). The first value in this list is the total number of fish collected from each area; the numbers in parentheses represent the number of fish processed for EA and FAA, respectively. The balance were archived in frozen storage (–70° C). Sitka Sound and Togiak Bay are well south and west of the NGA, respectively. Samples from Togiak and NGA were run first to verify that at least one of the stock identification tools we evaluated could distinguish between these geographically isolated spawning populations previously shown to be genetically divergent (Grant and Utter 1984; O'Connell et al. 1998).

For each specimen, standard length (SL in cm), sex, and gonad maturity was determined. When pre-spawning female Pacific herring between 190-250 mm SL were encountered, a scale was removed to determine the age of the fish. Heads from herring aged 4 to 7 were removed and stored frozen in individually labeled plastic bags for later laboratory processing of the otoliths. Whole hearts were removed, transferred to labeled vials, placed in liquid nitrogen, and stored at –70° C until analyzed (Ackman et al. 1969; Grahl-Nielsen and Mjaavatten 1992).

To avoid lipid deterioration and oxidation when fish could not be sampled immediately, we held them in totes of iced seawater. Anelich et al. (2001) found that no deterioration in lipid composition occurred in catfish fillets stored at 2º C for 13 days. Aidos et al. (2002) found very little change in percent free fatty acids of herring oil stored for 160 days at temperature ranging up to 50º C, however, the formation of primary oxidation products did occur gradually over time even when oil was stored at 0º C. In our study, hold time never exceeded 13 hours and generally less than 3 hours elapsed between a herring's harvest until it's heart tissue was placed in liquid nitrogen (-70º C). We monitored body core temperatures while samples were being processed and temperatures never exceeded 4º C.

Otolith preparation and elemental analysis

Left and right sagittal otoliths were dissected from each specimen, cleaned and rinsed with deionized water and then placed in cell culture trays. The otoliths selected for elemental analysis were mounted proximal side up on petrographic glass slides using thermal plastic cement. Placing the proximal side up ensured that the otolith center was close to the surface and could be exposed with gentle hand polishing using 9 and 3 micron diamond embedded lapping paper. During preparation, otoliths were viewed with both bright field and transmitted light microscopy to determine when fine scale patterns indicative of daily growth ring formation during the larval period were exposed to the surface. The otoliths were then rinsed in deionized water, air dried, and a digital image was captured and annotated to identify a target area for elemental analysis. The target area chosen was between 100 and 200 microns from the otolith center along the dorsal, ventral and posterior quadrants. This appeared to be a common location for observing daily ring formation and likely corresponded to early larval period. The prepared samples were labeled and shipped along with digital images to the Advanced Instrumentation Laboratory at the University of Alaska Fairbanks.

After polishing, the otoliths were cleaned with Formula 409 and coated with a 30 nm layer of carbon. They were analyzed in a Cameca SX-50 electron microprobe (EM) equipped with four wavelength dispersive spectrometers. A 15keV, 5 nA, 16 micron diameter beam was used for all analyses. Details concerning counting times, standards, detection limits, and analytical errors are summarized in Table 1. Five EM measurements were taken from each otolith and the mean value for each element detected was used in the statistical analysis. Preliminary analysis suggested nine elements [Sodium (Na), magnesium (Mg), phosphorus (P), sulphur (S), Chlorine (Cl), Potassium (K), Calcium (Ca), Iron (Fe), and Strontium (Sr)] were detectable in herring otoliths using EM and these elements were chosen for analyses. Although Mg was analyzed, in most cases it was below detection limits and was not used in the statistical analysis.

Chemical analysis
The lipids of whole hearts were extracted by homogenizing them in a solution of 33% methanol and 66% chloroform. The homogenate was subsequently liquid-liquid extracted with an aqueous solution of 0.88% potassium chloride (KCl) equal to one-quarter of the volume of homogenate. The bottom layer of the resulting biphasic solution was collected and combined with one-quarter of its volume of methanol in distilled water (1:1) in a separatory funnel. The bottom layer containing the purified lipid was withdrawn and evaporated in a water bath under nitrogen to a volume of 5.0 mL, transferred to a vial, topped with nitrogen and stored at -80°C.

The purified lipids were shipped on dry ice to Mylnefield Research Services Ltd., a component of the Scottish Crop Research Institute, for trans-esterification and fatty acid chromatography. At Mylnefield, 60 mg samples of purified lipid were mixed with 4 mL of internal standard (methyl tricosanoate C23:0) and 200 μL methanolic KCl and shaken for 30 seconds. Approximately, 1 g of sodium hydrogen sulfate monhydrate was added to neutralize the KCl and the solution was decanted into a clean test tube and mixed with 8 mL isooctane.

Fatty acids were identified by injecting 1.0 µL of trans-esterified sample into a temperature-programmed gas chromatograph. Fatty acids were initially separated with a Hewlett Packard model 5890 gas chromatograph equipped with a 25 m Chrompack CP_Wax 52CB column (0.25mm x 0.2µm film thickness). The temperature at injection was 170ºC where it remained for 3 minutes and then it was increased by 4ºC per minute until it reached a maximum of 220 ºC. Separated fatty acids were carried by hydrogen and detected by flame ionization and up to 39 fatty acid peaks were identified. Prior to each analytical run, a known standard (i.e. standard GLC-412-BS, Nu-Chek Prep. Elysian, MN) was processed and results compared with stated values to ensure agreement. Fresh dilutions of the standards were produced weekly. Concentrations of fatty acids were calculated relative to the concentration of the internal standard and corrected for differences in response. Duplicate analyses were performed for each sample, and analytical results compared. All reported values are means of duplicate analyses found to be within 7% of each other.

Statistical Analysis
Differences in the chemical composition of tissues from different groups were examined by multivariate analysis of variance (MANOVA). A one-way MANOVA with group as the main factor and percentages of analytes as the response variables was used to test the null hypothesis that tissues derived from different groups had similar chemical compositions. Significance of the MANOVA was evaluated with Wilk's lambda. In addition, we performed one-way univariate ANOVAs for each of the analytes to determine which analytes likely contributed most to the differences identified by the MANOVA. Null hypotheses of similarities among analytes compared by univariate ANOVAs were rejected if the resulting probabilities of Type I error were less than 0.05 divided by the number of tests performed.

We employed descriptive discriminant function analysis (DFA) to identify which groups differed. Like ANOVA, MANOVA identifies differences between groups, but does not reveal which groups differ. DFA resolves differences among groups by identifying a series of canonical functions, each of which is a linear combination of the response variables. These functions progressively reduce the error in the data set. Consequently, the degree of resolution offered by

the analysis can be viewed by fitting the data to the functions that account for the error in the data set and plotting these fitted data against each other. The number of functions that account for the error represents the dimensionality of the data set. We determined the dimensionality of the data set by partitioning Wilks' lambda (Huberty 1994) and testing the hypothesis that after fitting a given function the residual error was equal to zero. Fitted data for each function were plotted in bi-plots to examine how the functions separated the data. We also examined the pooled within group canonical structure to identify which analytes exerted the most influence on the separating functions. Analytes identified this way were further examined by one-way ANOVA using Tukey's method of pairwise testing to contrast percentages among groups with family error rates equal to 0.001.

In addition, we cross-validated the DFA results using the leave-one-out method (Huberty 1994). Group membership for the left-out sample was determined using the nearest neighbor method, with the number of neighbors considered equal to one less than the number of groups. The MANOVAs, ANOVAs and DFAs were performed in SAS release number 6.12 using the non-parametric DISCRIM procedure. All analyses used the within group co-variance matrices because the covariance matrices were not found to be homogenous. Tukey's pairwise tests were performed in Minitab version 13.

Prior to their being analyzed, the elemental and fatty acid data were used to develop four data sets. In the first two data sets, the data derived from samples collected at Kamishak, Kodiak, PWS-NE and PWS-Montague were combined into a single group herein referred to as northern Gulf of Alaska (NGA). The purpose for pooling was to validate the method using populations that are located over a broad geographic range- Sitka, NGA, and Togiak. One elemental data set and one fatty acid data set resulted from this organizational scheme. The third and fourth data sets comprised the unpooled elemental and fatty acid samples collected from Kamishak, Kodiak, PWS-NE and PWS-Montague. These sets were designed to test the utility of the discriminating methods on a finer spatial scale.

Elemental data submitted to the DFA represented the mean composition of each sample. All elements were used in the analysis because only nine elements were measured and this was less than the number of otoliths sampled from Sitka and Togiak. Initially, the average elemental composition of each otolith was transformed by taking the log of the ratio of each element to Ca (Aitchison 1992). The transformed values for K, Cl, P, S, Na, Mg, Sr and Fe were used in the MANOVAs, ANOVAs and DFAs for each of two data sets.

Preprocessing of the fatty acid data was more involved because only a subset of the fatty acids identified could be used. Only 14 fatty acids could be used in the data set comprising Togiak, Sitka and NGA because Togiak was represented by only 15 samples. However, these 14 fatty acids represented an average of 90% of the fatty acids observed. More samples were collected from fish representing the Kamishak, Kodiak, PWS-NE and PWS-Montague stocks, permitting inclusion of five additional fatty acids in that analysis. Prior to analysis, the fatty acid data in both data sets were transformed using the same approach used for the elemental data. In both cases, the denominator was the concentration of C16:0. Thus, the log ratios of 13 and 18 fatty acids were examined for the broad and fine scale tests, respectively. These transformed data were used in the MANOVAs, ANOVAs and DFAs for both data sets.

RESULTS

Regional differences in elemental composition

Regional differences in elemental composition of herring otoliths from Sitka, Togiak and NGA were detected when samples were compared using MANOVA ($P < 0.0001$). These regional differences in otolith microchemistry appear to be a function of the relative percentages of Cl and Na (ANOVA, $P < 0.004$) (Table 2). Differences in the percentages of these elements were greatest between Sitka and Togiak. For example, the average percentage of Cl in otoliths collected from Sitka was four times that of otoliths collected in Togiak. In both cases, the percentage of these elements in NGA otoliths was intermediate to the extremes defined by Sitka and Togiak. This can be seen in the plots for the canonical functions. The first of these functions accounted for 83% of the error in the data set ($P < 0 .0001$), while the second did not account for any error ($P = 0.089$). The influence of Na and Cl on both of these functions resulted in the separation between Sitka and Togiak herring along a line drawn diagonally from the upper left to the lower right of the plot (Figure 2). In contrast, the NGA stock appeared to be broadly distributed in the same regions as those described by the Togiak and Sitka observations.

Cross-validation of the functions used to discriminate herring from the three regions further demonstrated that elemental analysis could be used to separate herring from Togiak and Sitka. While the observed error rate for identifying the Togiak samples was 46.7%, the Togiak otoliths were incorrectly identified as Sitka otoliths only 6.7% of the time (Table 3), thus the Togiak samples were identified as "*not-Sitka*" 93.3% of the time. Similarly, Sitka otoliths were only identified as coming from Togiak 10% of the time. However, there appeared to be little discriminating power between Sitka and NGA or Togiak and NGA; the misclassification error rate for the NGA otoliths was 31.3% and these errors were evenly divided between Sitka and Togiak.

Differences in elemental composition among NGA stocks

Despite the poor resolution between Sitka and NGA or Togiak and NGA, MANOVA revealed detectable differences ($P < 0.0001$) in the elemental composition of the otoliths collected from the stocks comprising the NGA group. As with the broader geographic comparison, the univariate ANOVAs indicated these differences were largely the result of differences in the percentages of Na and Cl ($P < 0.0001$) (Table 2). These comparisons suggested the existence of two types of otoliths. For example, Kodiak and PWS-Montague had similar percentages of Cl averaging near 0.16% in contrast to Kamishak and PWS-NE, which both averaged near 0.05%. Similarly, the percentages of Na in the Kodiak and PWS-Montague were more similar to each other than they were to either of those from Kamishak and PWS-NE.

The similarity between Kodiak and PWS-Montague, and their contrast to Kamishak and PWS-NE, was also demonstrated by the DFA. Plots of the canonical function scores indicated a clear separation of the Kodiak and PWS-Montague otoliths from the Kamishak and PWS-NE otoliths (Figure 2). The first function accounted for 97% of the error in the data set ($P < 0.0001$). There was no evidence of further separation of the stocks along the second function, consistent with the observation that this function failed to account for any more error ($P = 0.5938$). Thus, the NGA

stocks could easily be discriminated into two groups based on the percentage of Na and Cl, but these groups did not conform to our *a priori* stock identities.

Cross-validation further demonstrated the robust character of Na and Cl for structuring the data from the NGA stocks (Table 4). Cross-validation identified two distinct groups, one comprising Kamishak and PWS-NE the other Kodiak and PWS-Montague. Errors in assigning Kodiak otoliths to the proper stock occurred 84% of the time, but more than 85% of those errors resulted from their identification as PWS-Montague otoliths. Similarly, the PWS-NE otoliths were only correctly identified 40% of the time, but 83% of the erroneous identifications identified them as Kamishak. Thus, mis-identification of the Kodiak/PWS-Montague type occurred only 9% of the time. Similarly, mis-identification of the Kamishak/PWS-NE type occurred only 10% of the time.

Regional differences in fatty acid composition

Regional differences in the fatty acid composition of herring heart lipids from Sitka, NGA, and Togiak was revealed by MANOVA ($P < 0.0001$). Concentrations of nine of the thirteen fatty acids examined differed among the three groups (ANOVA, $P < 0.003$) (Table 5). The largest F statistics were those associated with C18:2n6-trans, C20:4n6 and C20:1n11 and C16:1n7, with values ranging between 29.17 and 140.80, while the remaining F values were all less than 19.1. Bar charts of the average weight percentage of the four fatty acids with the largest F statistics revealed mean vectors with the region-specific patterns (Figure 4), consistent with the observation that the mean fatty acid compositions differed between regions.

These regional differences in mean fatty acid composition led to a large amount of discriminating power. Plots of the canonical scores for the three regions revealed a high degree of resolution (Figure 5). The first function resolved the Togiak samples from the Sitka and NGA samples and accounted for 70% of the error in the data set ($P < 0.0001$). Examination of the pooled within canonical structure indicated that C18:2n6-trans had the greatest influence in separating these groups (Table 5). This can be seen by noting that the average percentage of C18:2n6-trans in Togiak herring is nearly twice that of the Sitka herring and three times that of the NGA herring. Other fatty acids important in separating Togiak from Sitka and NGA were C16:1n7, C20:1n11, C20:4n3 and C22:6n3. The second function accounted for the remaining error ($P < 0.0001$) and effectively separated the Sitka and NGA herring (Figure 5). The fatty acid with the greatest influence in this separation was C20:4n6. The average percentage of this fatty acid in Togiak and NGA herring was equal, but this value was significantly lower than that of Sitka herring (Table 5).

Cross-validation demonstrated that the discriminating functions were robust. The fatty acid data could be used to correctly identify unknown samples more than 90% of the time. The only errors encountered were with the identification of one of the Sitka samples as NGA and identification of an NGA sample as Sitka (Table 6). In contrast, the Togiak and NGA samples were identified correctly 100% of the time.

Differences in fatty acid composition among NGA stocks

The four NGA stocks- Kodiak, Kamishak, PWS-Montague and PWS-NE, were easily discriminated by their fatty acid compositions. MANOVA results indicated significant

differences among the average fatty acid compositions of the four stocks ($P < 0.0001$). For this analysis, the larger number of herring representing each stock permitted the use of a larger number of fatty acids than was used for the regional comparison. Univariate ANOVAs indicated stock related differences in the percentages of 12 of the 18 fatty acids considered ($P \blacktriangleright 0.003$; Table 7). The largest F statistics were associated with three fatty acids, C20:1n11, C20:1n9 and C20:4n3, ranging between 15.16 and 18.93, while the remaining F statistics were less than 10. Comparison of the average percent weight of the four fatty acids with the largest F statistics in bar charts revealed stock-specific patterns in the relative amounts of these fatty acids (Figure 6).

These differences in fatty acid composition led to a high degree of separation between the stocks along three discriminant functions ($P < 0.0001$). The first function, which accounted for 56% of the error in the data set, separated the two Prince William Sound (PWS) stocks from the Kodiak and Kamishak stocks (Figure 7, upper panel). The fatty acids most associated with this separation were C20:4n3, C20:1n9 and C18:0 (Table 7). Stocks from PWS had significantly different percentages of C20:4n3 and C18:0 than Kodiak or Kamishak. The percentage of C20:1n9 in the heart lipids of Kamishak herring was significantly higher than that of the other three groups (Table 7). The second function, which accounted for another 26% of the error, separated the two Prince William Sound stocks- PWS-NE and PWS-Montague. A large number of fatty acids contributed to this separation, but the two with the largest coefficients were C20:1n9 and C22:5n6. There was little difference in the percentage of C20:1n9 between the two PWS groups, but PWS-NE herring had a significantly greater percentage of C22:5n6 than the PWS-Montague herring. The third function accounted for the remaining error and isolated Kodiak from Kamishak (Figure 7, lower panel) primarily because of the influence of C20:1n11. Note that Kamishak herring have a significantly higher percentage of C20:1n11 in their heart lipids than any of the other stocks (Table 7).

Cross-validation of the discriminating functions indicated that fatty acids could be reliably used to discriminate these four stocks. Erroneous stock identifications occurred less than 10% of the time and stock specific error rates ranged between 8% and 12% (Table 8). Unlike the cross-validation of the elemental data, *a priori* stock identities appeared to best describe the fatty acid data structure. In addition, examination of the mis-identifications revealed evidence of a hierarchical structure to the data, exhibited by the tendency for mis-identified fish to be assigned to the next geographically closest stock. For example, four Kamishak herring were mis-identified, but three of these were identified as Kodiak herring. In turn, the two Kodiak mis-identifications were identified as belonging to the Kamishak group.

DISCUSSION

Discrimination based on elemental analysis

Otolith microchemistry discriminated between herring from Togiak and Sitka (> 1,500 km separation); however, there appeared to be little discriminating power between Togiak and NGA (~500 km separation) or Sitka and NGA (~1,000 km separation). It is possible that our inability to detect stock-specific differences in otolith microchemistry at finer spatial scales resulted from the limited sensitivity of the electron microprobe. For example, Gillanders (2002) used solution-based inductively coupled plasma-mass spectrometry (ICPMS) to discriminate among juvenile

marine fish occupying estuaries less than 30 km apart. While ICPMS provides high resolution data (<0.03 g/l for a standard solution, Houk 1986), it is typically used to measure elemental concentrations in whole, or portions of whole otolith samples (Date 1991) and may be best suited for stocks living the majority of their lives in environments with relatively disparate water chemistries (Campana et al. 1995).

In this study, given our expectation that Kamishak and Kodiak herring resided within their respective natal spawning areas for only a short period, and probably occupied the same over-wintering areas, we needed an elemental analysis technique that allowed us to focus on the microchemistry of the otolith accreted prior to the first annulus. Techniques capable of targeting specific loci, and therefore life history stages, such as EM and Laser-Ablation ICPMS (LA-ICPMS), may be best suited for identifying stocks that spawn in different environments but later reside in similar environments (Coutant and Chen 1993). Laser-ablation ICPMS can be used to analyze trace elements (ppm) at relatively specific loci (30 μm) on the otolith (Gray 1985; Denoyer et al. 1991). Electron microprobes also allow analysis of specific loci (5-7 μm), albeit at a lower sensitivity (100 μg/g of otolith; Gunn et al. 1992).

We chose to use an EM over LA-ICPMS because the equipment and expertise were available in state at competitive rates. Waldman (1999) suggests that when choosing among stock identification techniques, researchers must "…balance costs, sampling needs, repeatability, and likelihood of detecting multiple stocks, and the techniques used should be chosen to fit the particular context and management objectives of the study." For our application, EM appeared to be a more practical tool with which to develop procedures to determine the stock of origin for herring harvested from mixed-stock fisheries or collected during large-scale ecological surveys (e.g., Gulf Ecosystem Monitoring [GEM]).

Because our otolith data were unable to facilitate discrimination between samples from the NGA and adjacent regions, we were surprised to find that samples from our four NGA stocks were easily discriminated into two groups based on their otolith microchemistry. Perhaps more surprising was the fact that these groups did not conform to our *a priori* stock identities. Instead, Kodiak was grouped with PWS-Montague and Kamishak was grouped with PWS-NE. It appears unlikely that these pairings represent *defacto* structuring of NGA herring stocks. Instead, they probably highlight basic similarities in water chemistry between sample locations that more sensitive equipment (e.g., LA-ICPMS) might have been able to tease apart. Montague and Kodiak Islands are both fully exposed to the Gulf of Alaska and are heavily influenced by the Alaska Coastal Current (ACC), whereas Kamishak Bay and PWS-NE reside within large embayments whose water chemistries receive more direct influence from freshwater inputs including glacial silt-laden rivers. However, the possibility that our EA groupings indicate significant gene flow between PWS and Kodiak should not be ignored. When evaluating one distance coefficient (R_{ST}), O'Connell et al. (1998) reported finding that Port Chalmers (Montague Island) and Kodiak herring were more similar to each other than they were to herring from other areas of PWS. However, this Kodiak/Port Chalmers affinity was not apparent when a similar analysis was performed using the $\delta\mu^2$ distance coefficient (O'Connell et al. 1998).

It is difficult to speculate further on what mechanisms led to the similar otolith chemistries of these widely separated spawning aggregates. Dove et al. (1996) cautioned that a comprehensive

understanding of otolith microchemistry results requires knowledge of the relative affinity particular ions have for organic and inorganic components of otoliths. Unfortunately, despite the recent interest and advances in otolith elemental analysis, researchers still do not have a clear understanding of the causal mechanisms involved in determining the concentrations for most elements found in otoliths. Several studies have illustrated that Sr levels in otoliths are strongly influenced by water temperature and salinity (Kalish 1990; Secor and Rooker 2000). Using controlled laboratory studies, Fowler et al. (1995a; 1995b) demonstrated that temperature, salinity, and ontogeny can influence otolith microchemistry. Milton and Chenery (2001) conducted a controlled rearing and diet study and found that "otolith concentrations of some of the commoner trace metals that form divalent ions are related to water concentrations", whereas diet had little effect on otolith composition. We were unable to find any references describing causal mechanisms affecting the uptake of Na and Cl, both of which proved to be the most robust elements for discriminating inter and intra-regional differences among sampled herring stocks in our study.

Aside from the mixed results we had discriminating among samples using otolith microchemistry, several other factors should be considered before further pursuing this herring stock identification tool. Using ICPMS, Gillanders (2002) studied the 'elemental fingerprints' of otoliths from juvenile fish residing in 12-15 estuaries along the Australian coast during each of three consecutive years. Although she found significant differences in otolith chemistry among sample locations and years, she also found that considerable spatial/temporal variation of otolith elemental fingerprints occurred making it possible to confound spatial and temporal differences discovered during single sample event comparisons. Gillanders (2002) suggested that it is difficult to predict whether interannual variability will occur in otolith elemental fingerprints. Rooker et al. (2003) found distinct differences in otolith chemistry between adjacent year classes of Atlantic tuna collected from one area, but no interannual difference between adjacent cohorts from another area. The temporal stability of otolith chemical signatures may be site specific and related to the stability of the areas' water chemistry.

Discrimination based on fatty acids

Heart tissue fatty acid analysis provided a robust basis for discriminating among herring stocks on broad and fine spatial scales. Fatty acid data were used to correctly identify unknown samples more than 90% of the time; the only errors encountered were between individuals sampled from Sitka and NGA. The four NGA stocks- Kodiak, Kamishak, PWS-Montague and PWS-NE, were also reliably (> 90%) discriminated by their fatty acid compositions. Further, the fatty acid data appeared to conform to our *a priori* stock identities. Examination of the mis-identifications revealed evidence of a hierarchical structure to the data. These data indicate that the probability of encountering a herring from a given stock decreases with distance from that stock's spawning site. O'Connell et al. (1998) also observed a hierarchical structure to their genetic data, with Kodiak Island samples always positioned intermediately between the Bering Sea and PWS samples.

This hierarchical structure in our fatty acid data supports the idea that the herring aggregates we sampled were biologically distinct units that experience a limited degree of mixing. This observation is important because multivariate statistical tests often contain sufficient statistical power to reveal differences among groups when the true biological differences are less

meaningful (Waples 1998). This is of particular concern here, because statistically significant differences are more likely to be found when a large number of response variables are used, especially as that number approaches the number of objects that have been measured (Waples 1998). Although we used a large number of response variables relative to the number of herring in a given stock, the results of our univariate analyses indicated a large number of differences in the fatty acid content between stocks. Furthermore, these differences conform to our concept of hierarchical structure. Pair-wise testing of fatty acids found to differ by ANOVA revealed that the NGA values were always intermediate to the extremes defined by Togiak and Sitka. Similarly, the range of values for the fatty acids that differed among the NGA stocks generally conformed to the spatial arrangement of those stocks.

Before fatty acid analysis can proceed as a stock discriminator, it will be important to demonstrate the temporal stability of the differences within and among *a priori* stocks. We targeted heart tissues based on the understanding that heart phospholipids are less subject to environmental influences than other tissues or lipid classes (Grahl-Nielsen and Ulvund 1990, Czesny et al. 2000, McKenzie 2001). This is important because the fatty acid composition of different tissues and lipid classes can be very sensitive indicators of environmental change. For example, numerous studies have shown the effect of diet on the fatty acid composition of the triacylglycerols (e.g., Henderson and Tocher 1987). Thus, tissues whose lipids comprise triacylglycerols will have fatty acid compositions that vary over time. The heart lipids studied here were primarily phosphatidylcholine and phosphatidylethanolamine (data not shown). As a class, these phospholipids are generally less sensitive to diet effects, but dietary influences on the phospholipid content of Atlantic salmon hearts has been demonstrated (Grisdale-Helland et al. 2002). In addition, phospholipids can be influenced by temperature (Hazel 1984; Henderson and Tocher 1987), salinity (Cordier et al. 2002), and developmental stage (Kreps et al. 1969). Therefore, demonstrating that the variation in heart tissue fatty acid composition observed between stocks exceeds that imposed by the environment on a given stock will be a key element in the development of this method (Begg et al. 1999a).

Recently, three laboratory studies have reported evidence of genetic control over some fatty acid concentrations. Joensen et al (2000) found significant differences in the fatty acid profiles of heart tissue extracted from representatives of two cod stocks that had been reared for 44 months under identical conditions. Peng et al. (2003) compared the fatty acid compositions of anadromous and landlocked Atlantic salmon (*Salmo salar*) fry, fed identical diets throughout a 44 d feeding trial, and reported significant differences in their phospholipids. In a companion study, Rollin et al. (2003) concluded that differences in the fatty acid composition of different strains of Atlantic salmon resulted from variation in the rates of desaturation and elongation of linolenic and linoleic acids. This suggests that differences in the activities of enzymes that regulate phospholipid composition might explain the stock differences identified here and in other species examined in field studies (Grahl-Nielsen and Ulvund 1990, Grahl-Nielsen and Mjaavatten 1992).

Other studies have shown that dietary impacts on fatty acid composition are minimized in heart lipids. Viga and Grahl-Nielsen (1990) cultured groups of Atlantic salmon from the same stock for 8 months on diets and found the fatty acid composition of salmon hearts was independent of diet. Grisdale-Helland et al. (2002) found significant differences in the heart phospholipids of

Atlantic salmon fed different diets for approximately three months. However, they identified much greater differences in the composition of heart triacylglycerols. Similarly, studies reviewed by McKenzie (2001) reveal the tendency for heart fatty acid composition to respond to diet but at much lower magnitude than muscle or liver. These data indicate that examination of heart fatty acids should minimize the apparent variation imposed on populations due to diet, ration, and temperature (Grisdale-Helland et al. 2001; Kiessling et al. 2001; Jobling et al. 2002), but the contribution of temporal variation remains unknown.

The concept of genetic control over the composition of heart fatty acids is bolstered by studies demonstrating relationships between cardiac function and fatty acid composition. Bell et al. (1993) reported heart lesions in Atlantic salmon fed diets with high levels of n-6 fatty acids after the fish had been stressed. Agnisola et al. (1996) reported reduced heart rate and cardiac power output in the hearts of sturgeon fed diets high in n-3 fatty acids relative to those fed diets high in n-6 fatty acids. These data demonstrate an influence of heart fatty acid composition on individual fitness, thereby providing selection an opportunity to act on the interaction between the proteins that regulate phospholipid composition, diet and cardiac performance. Alternatively, interactions between phospholipid composition, eicosanoid production and cardiac function have rarely been described for fish (Stenslokken et al. 2002) despite their frequently described impacts on mammalian health (Das 2001). These data may account for the conclusion that C22:6n3 in fish heart phospholipids is not strongly influenced by diet (Thomassen and Røsjø 1989, Caballero et al. 2002, Grisdale-Helland 2002), and in fact may be under strong genetic control (Peng et al. 2003).

CONCLUSIONS

Our pilot study demonstrated that fatty acid analysis of heart tissue could be used to discriminate among Pacific herring spawning aggregates on broad (>1,000 km) and relatively fine ($\leq$100 km) spatial scales. Discrimination of spawning aggregates using otolith microchemistry data was also successful at very broad spatial scales (>1,500 km), but the electron microprobe did not reliably discriminate samples collected 500-1,000 km apart.

Although the fatty acid results are quite promising, more research is necessary in order to verify the utility of fatty acid analysis for discriminating herring stocks. This follow up work should include all biotypes from each population (e.g., all cohorts, sexes, and maturity levels) and aim to satisfy Galvin et al.'s (1995) third criterion for successful application of a mixed stock analysis (MSA) technique by establishing the temporal stability of the stock specific chemical signatures we identified in our pilot study.

Considerable uncertainty exists regarding the ecological significance of herring stock "sub-units" (Stephenson 1999). Thus, the potential value of a tool that can discriminate herring stocks over fine spatial scales cannot be overestimated. In the near term, fatty acid analysis of heart lipids could be used to resolve a number of pressing management questions regarding stock structure in Prince William Sound, Kodiak/Kamishak, Togiak/Dutch Harbor, and in southeastern Alaska. For example, Kamishak Bay contains temporally and spatially separated spawning aggregations, but all are currently considered to comprise a single stock (Otis et al. 1998).

Ultimately, the ability to identify the stock of origin for herring collected away from their natal spawning areas (e.g., during Gulf Ecosystem Monitoring [GEM] studies) would provide a basis for determining larval dispersal patterns, home ranges of individual populations, locations of stock specific over-wintering areas, and perhaps the degree to which Pacific herring home back to their natal spawning areas. With this tool, researchers may also be better able to evaluate cause and effect relationships associated with the population dynamics of NGA herring stocks and thereby improve the management of this commercially and ecologically important resource.

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Table 1. EPMA counting times, standards, detection limits, and analytical errors. Detection limits and analytical errors calculated after Scott et al. 1995.

Element	Counting Time (Peak, sec.)	Counting Time (Background, sec.)*	Standard	Detection Limit (Wt %, 99% Confidence Interval)	Typical Analytical Error (Wt %, 1 sigma)
Na	90	90	Halite (CM Taylor)	0.020	0.016
Mg	90	90	Osumilite (USNM 143967)	0.014	0.007
P	30	30	Apatite (Wilberforce)	0.048	0.035
S	30	30	Gypsum (CM Taylor)	0.032	0.023
Cl	40	40	Halite (CM Taylor)	0.025	0.021
K	30	30	Osumilite (USNM 143967)	0.021	0.012
Ca	34	12	Calcite (NMNH 136321)	N/A	0.201
Fe	190	190	Hematite (CM Taylor)	0.032	0.017
Sr	190	190	Strontianite (Smithsonian R-10065)	0.023	0.012

*Total time, 50% spent on either side of the peak

Table 2. Percentage of various elements in the otoliths of herring. Group identified as NGA is the composite of the PWS-NE, PWS-Montague, Kamishak and Kodiak stocks. Elements marked with an asterisk differed among regions, those with dagger differed among stocks. Percentages superscripted with different letters were found to differ after pairwise testing ($P < 0.005$).

	Region			Stock			
Element	Togiak	Sitka	NGA	PWS-NE	PWS-Montague	Kamishak	Kodiak
Ca	39.14	38.63	38.08	38.62	37.84	38.07	37.74
K	0.09	0.07	0.08	0.09	0.08	0.09	0.07
Cl*†	0.05[a]	0.21[b]	0.10[a]	0.05[a]	0.16[b]	0.04[a]	0.17[b]
P	0.12	0.14	0.10	0.10	0.10	0.10	0.12
S	0.06	0.07	0.06	0.06	0.05	0.06	0.06
Na*†	0.34[a]	0.43[b]	0.38[ab]	0.33[a]	0.44[b]	0.33[a]	0.45[b]
Mg*	0.01	0.17	0.01	0.01	0.01	0.01	0.01
Sr	0.09	0.07	0.08	0.08	0.08	0.09	0.08
Fe	0.01	0.02	0.01	0.01	0.01	0.01	0.01

* Different among regions ($P < 0.0001$)

† Different among NGA stocks ($P < 0.0001$)

Table 3. Results of cross-validating the functions used to discriminate herring from different geographic locations based on the elemental composition of their otoliths. Integers are numbers of samples, below are the percentage of samples assigned to each group.

	Identified as:			
True Identity	Togiak	NGA	Sitka	n
Togiak	8 53.3%	6 40.0%	1 6.7%	15
NGA	20 17.4%	79 68.7%	16 13.9%	115
Sitka	2 10.0%	6 30.0%	12 60.0%	20

Table 4. Results of cross-validating the functions used to discriminate herring from different NGA stocks, based on the elemental composition of their otoliths.

	Identified as:				
True Identity	PWS-NE	PWS-Montague	Kamishak	Kodiak	n
PWS-NE	12 40.0%	0 0.0%	15 50.0%	3 10.0%	30
PWS-Montague	0 0.0%	21 70.0%	1 3.3%	8 26.7%	30
Kamishak	18 60.0%	0 0.0%	9 30.0%	3 10.0%	30
Kodiak	2 8.0%	18 72%	1 4.0%	4 16%	25

Table 5. Percent weight of fatty acids in the heart lipids of herring from different locations. Group identified as NGA is the composite of the PWS-NE, PWS-Montague, Kamishak and Kodiak stocks. Fatty acids marked with an asterisk differed among regions ($P < 0.004$). Percentages superscripted with different letters were found to differ after pairwise testing ($P < 0.005$). Only the fatty acids used in the discriminant model are shown,. Fatty acids were transformed by taking the log ratio of each percentage to that of C16:0 before the analysis.

Fatty Acid	Regions		
	Togiak	Sitka	NGA
C14:0*	1.25	0.08	0.92
C16:0	25.29	22.92	24.33
C16:1n7*	1.97[a]	0.96[b]	1.16[b]
C18:0	3.05	2.93	3.19
C18:1n9*	5.70[a]	4.19[b]	4.60[b]
C18:1n7*	4.86[a]	4.14[b]	4.98[a]
C18:2n6t*	1.70[a]	0.97[b]	0.68[c]
C20:1n11*	0.26[a]	0.98[b]	0.72[b]
C20:4n6*	1.65[a]	2.35[b]	1.60[a]
C20:4n3*	0.57[a]	0.45[b]	0.40[b]
C20:5n3	15.86	16.43	15.94
C22:5n6	0.30	0.29	0.32
C22:5n3	1.49	180	1.65
C22:6n3*	26.11[a]	36.77[b]	35.83[b]

Table 6. Results of cross-validating the functions used to discriminate herring from different geographic locations based on the fatty acid compositions of their heart lipids. Integers are numbers of samples, below are the percentage of samples assigned to each group.

	Identified as:			
True Identity	Togiak	NGA	Sitka	n
Togiak	18 100.0%	0 0.0%	0 0.0%	18
NGA	0 0.0%	131 99.2%	1 0.8%	132
Sitka	0 0.0%	1 5.6%	17 94.4%	18

Table 7. Percent weight of fatty acids in the heart lipids of herring from different NGA stocks. Fatty acids marked with an asterisk differed among stocks ($P < 0.003$). Percentages superscripted with different letters were found to differ after pairwise testing ($P < 0.004$). Only the fatty acids used in the discriminant model are shown. Fatty acids were transformed by taking the log ratio of each percentage to that of C16:0 before analysis.

Fatty Acid	Stocks			
	PWS-NE	PWS-Montague	Kamishak	Kodiak
C14:0*	1.03[ab]	0.83 [ab]	1.09 [ab]	0.76 [b]
C16:0	24.67	24.62	25.06	24.21
C16:1n9	0.07	0.07	0.09	0.08
C16:1n7*	1.38 [a]	1.15 [ab]	1.07[b]	1.03 [b]
C16:1n5*	0.15 [a]	0.19 [b]	0.16 [a]	0.15 [a]
C17:0*	0.14 [a]	0.15 [ab]	0.17 [b]	0.16 [b]
C17:1n7*	0.08 [a]	0.10 [ab]	0.10 [b]	0.11 [b]
C18:0*	3.07 [a]	2.95 [a]	3.55 [b]	3.50 [b]
C18:1n9	4.78	4.41	4.89	4.55
C18:3n6*	0.07[ab]	0.09[a]	0.06 [b]	0.09 [ab]
C18:3n3	0.14	0.15	0.12	0.11
C20:1n11*	0.6 [a]	0.84 [ab]	0.95 [b]	0.49 [a]
C20:1n9*	0.67 [a]	0.47 [a]	1.03 [b]	0.69 [a]
C20:4n6*	1.51 [a]	1.51 [a]	1.72 [b]	1.82 [b]
C20:4n3*	0.44 [a]	0.47 [a]	0.35 [b]	0.34 [b]
C20:5n3	16.19	16.42	15.36	16.74
C22:5n6*	0.35[a]	0.30[b]	0.31 [b]	0.32 [ab]
C22:5n3	1.68	1.63	1.72	1.63
C24:1n9	0.55	0.51	0.61	0.44

Table 8. Results of cross-validating the functions used to discriminate herring from different NGA stocks, based on the fatty acid composition of their heart lipids.

	Identified as:				
True Identity	PWS-NE	PWS-Montague	Kamishak	Kodiak	n
PWS-NE	32 88.9%	2 5.6%	1 2.8%	1 2.8%	36
PWS-Montague	3 7.9%	35 92.1%	0 0.0%	0 0.0%	38
Kamishak	0 0.0%	1 3.0%	29 87.9%	3 9.1%	33
Kodiak	0 0.0%	0 0.0%	2 8.0%	23 92.0%	25

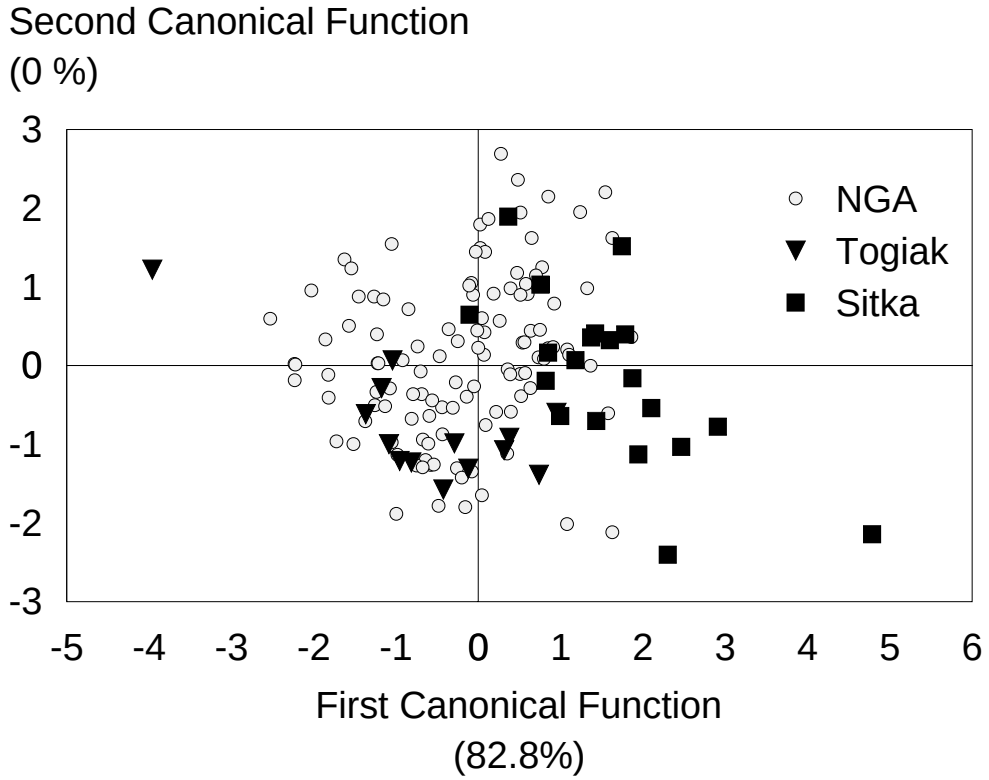

Figure 2. Bi-plot of the variable scores for the first two functions used to discriminate herring from the different regions based on the elemental composition of their otoliths. Note that the dimension of the discriminant space is one ($P < 0.0001$), the second function provides no explanatory value ($P = 0.089$).

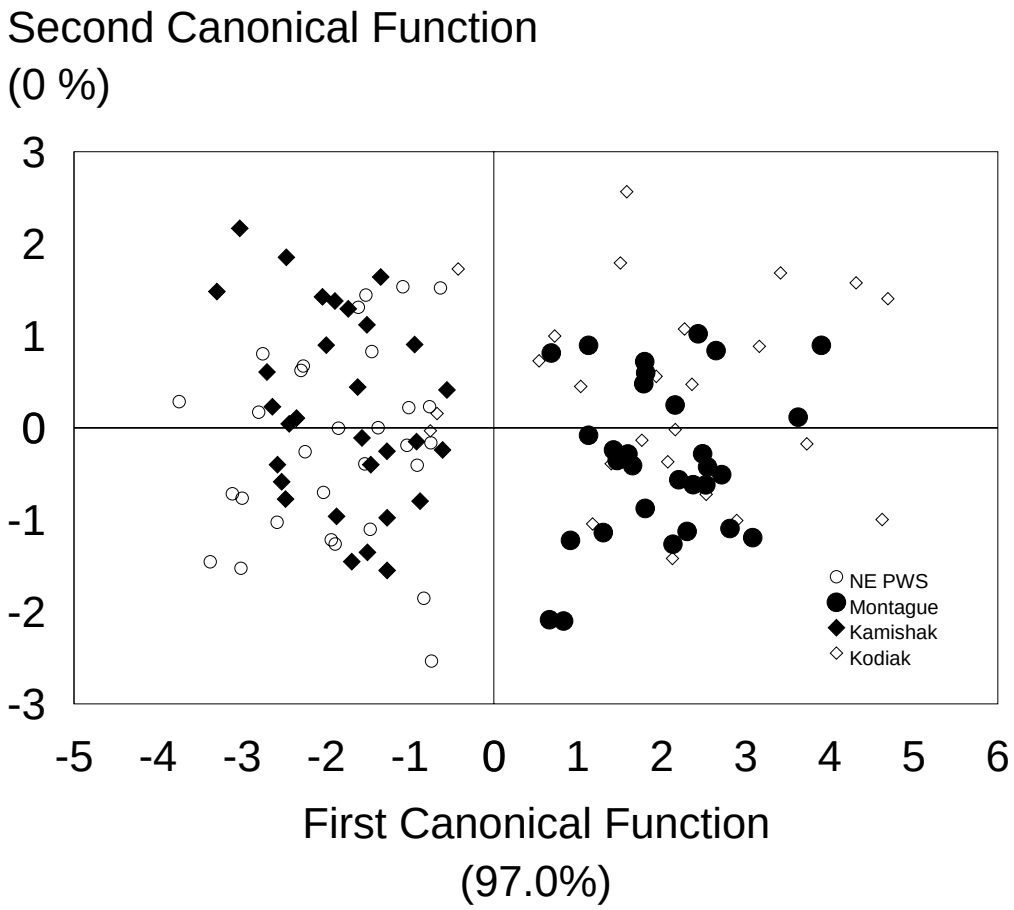

Figure 3. Bi-plot of the variable scores for the first two functions used to discriminate herring from the NGA stocks based on the elemental composition of their otoliths. Note that the dimension of the discriminant space is one ($P < 0.0001$), the second function provides no explanatory value ($P = 0.594$).

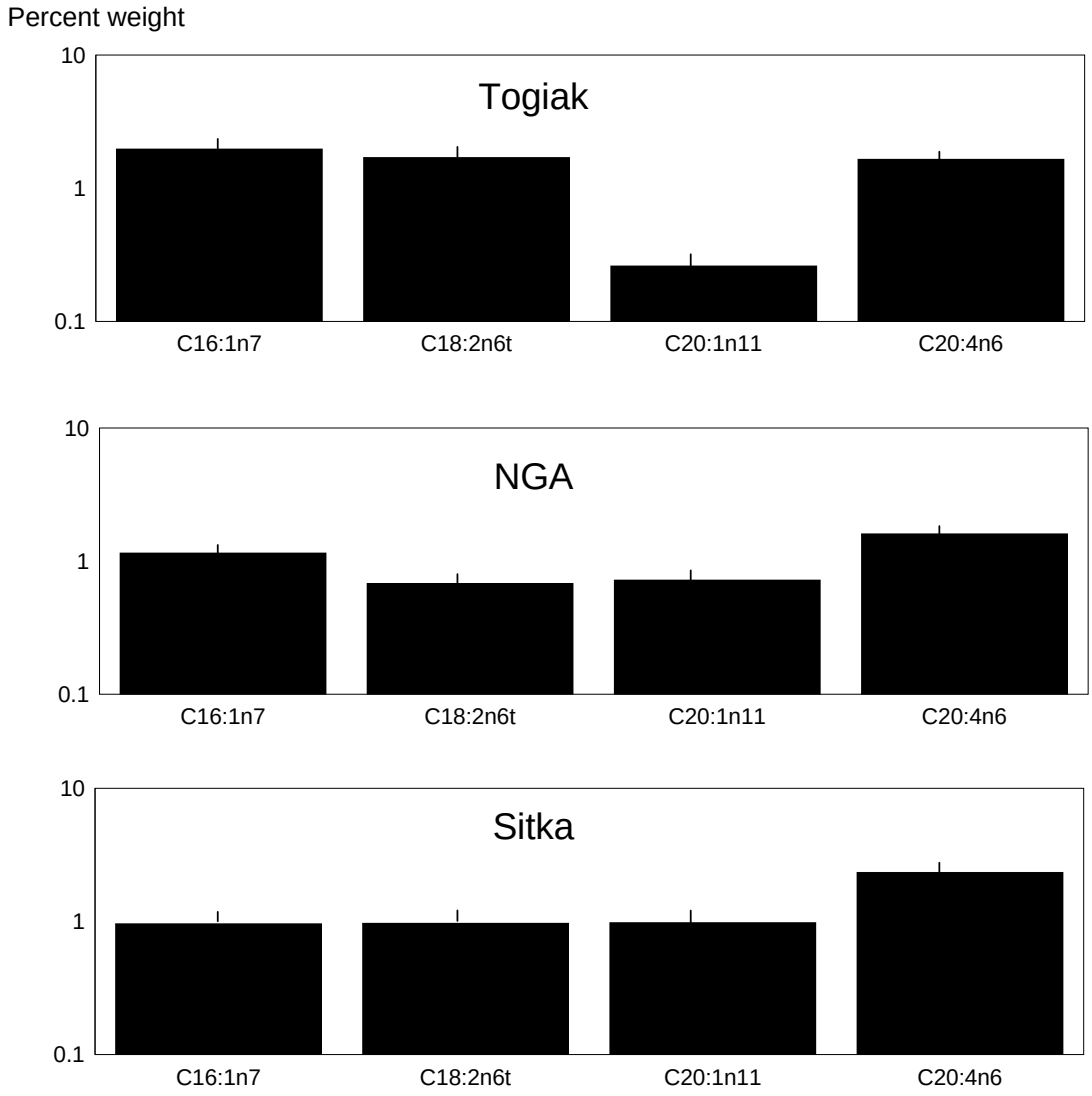

Figure 4. Mean fatty acid vectors for herring from different geographic locations. Vectors shown are limited to fatty acids found to contribute significantly to discriminant functions. Bar heights depict average percent weight (+ 1 s.d.) of fatty acids in herring heart lipids.

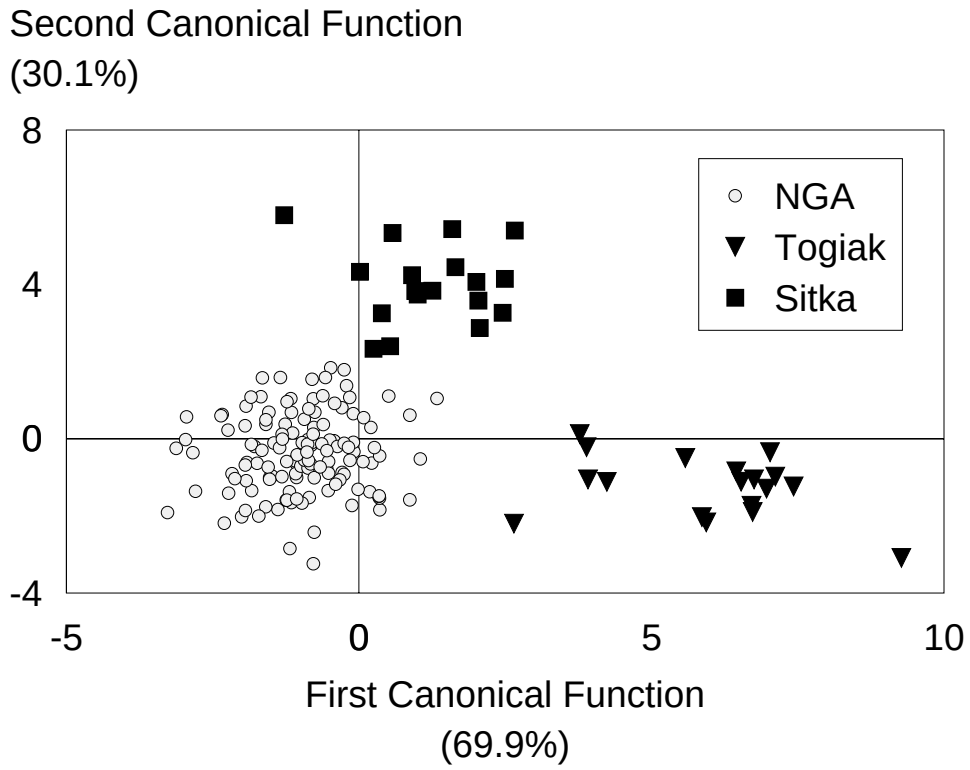

Figure 5. Bi-plots of the scores for the first two functions used to discriminate herring from different locations based on the fatty acid composition of their hearts. Note that the dimension of the discriminant space is two ($P < 0.0001$).

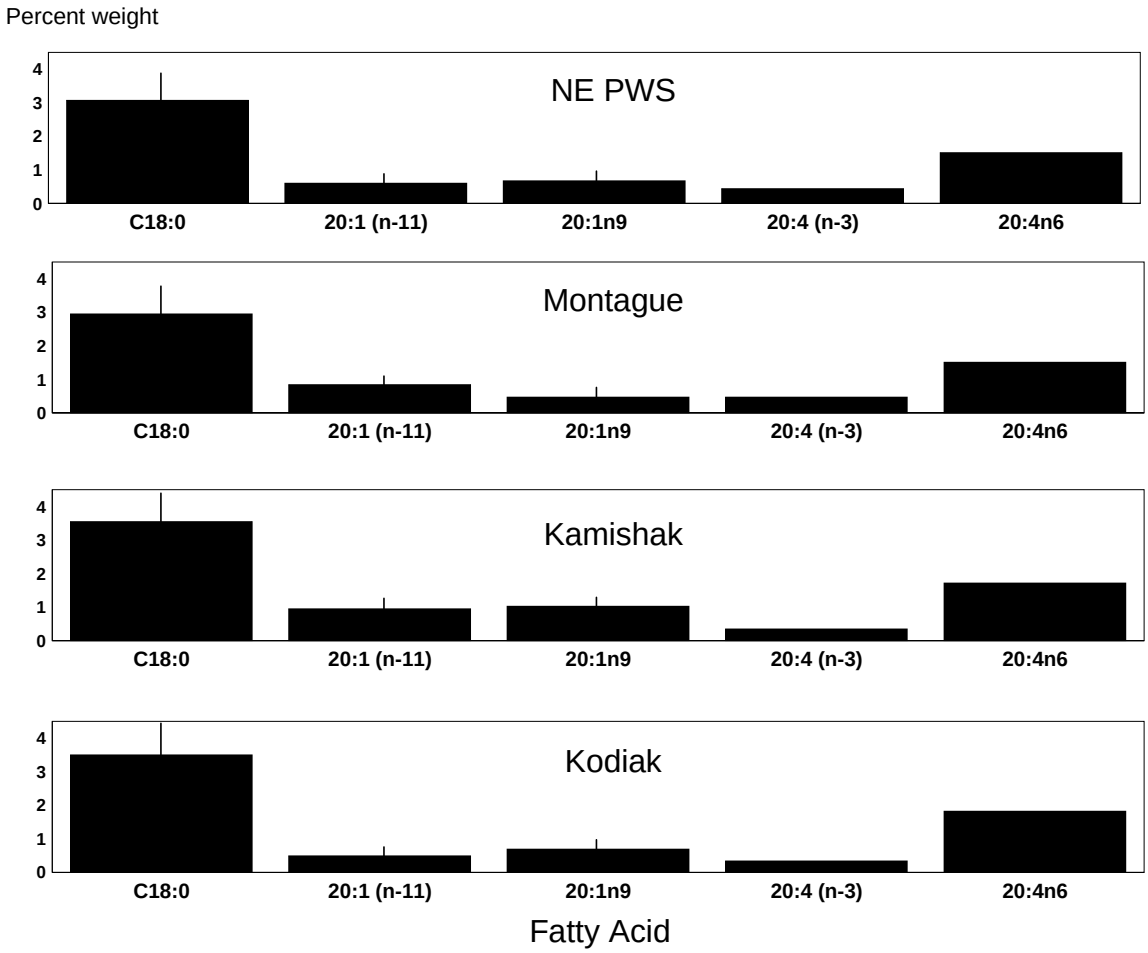

Figure 6. Mean fatty acid vectors for herring from different NGA stocks. Vectors shown are limited to fatty acids found to contribute significantly to discriminant functions. Bar heights depict average percent weight (+ 1 s.d.) of fatty acids in herring heart lipids. Bars without errors indicated have errors too small to be resolved on the figure.

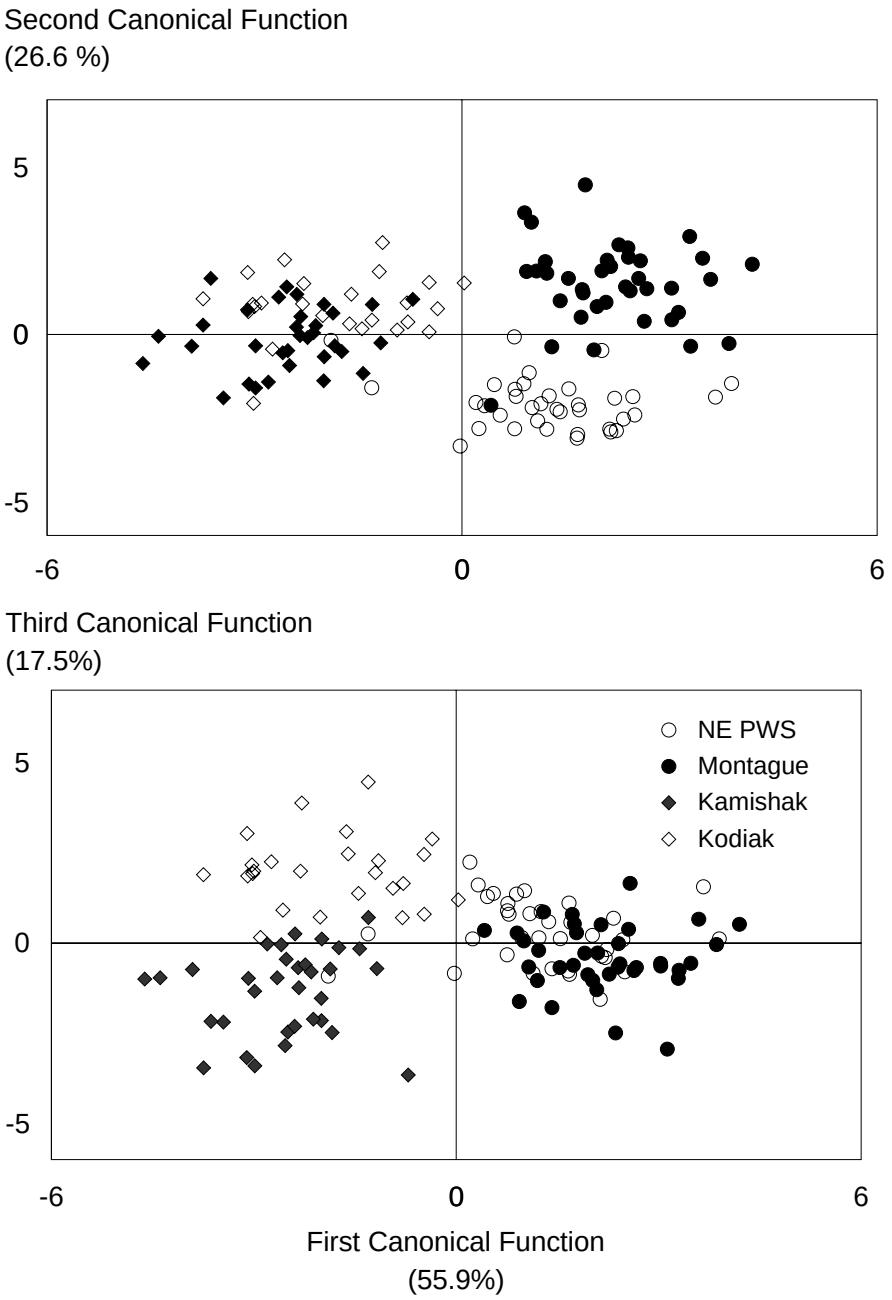

Figure 7. Bi-plots of the variable scores for functions used to discriminate NGA herring stocks based on the fatty acid composition of their hearts. Note that the dimension of the discriminant space is three ($P < 0.0001$).

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