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Proceedings of the Research Workshop on the Rapid Estimation of Fish Age Using Fourier Transform Near-Infrared Spectroscopy (FT-NIRS)

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**Proceedings of the Research Workshop on the Rapid Estimation of Fish Age
Using Fourier Transform Near-Infrared Spectroscopy (FT-NIRS)**

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PREFACE

Knowing how long fish live and the number of different age groups in a fish population is essential information to sustainably manage our Nation's valuable fisheries. Age data, in particular, are critical for understanding population dynamics of commercially fished species and for providing management recommendations. Assessing populations for the Nation's biggest fisheries, such as eastern Bering Sea walleye pollock (*Gadus chalcogrammus*), is complex and requires a substantial amount of age data. Traditional ageing methods to estimate the age of each fish require a reader to visually count growth rings on a fish's otolith, or ear stone, using a microscope. Additional time is needed to prepare and process the otolith for ageing; therefore, handling time per otolith is approximately 4-10 minutes plus additional time for quality control readings. With over 60,000 age requests coming in each year to NOAA's Alaska Fisheries Science Center's (AFSC) Age and Growth Program alone, this means a big investment in time, effort, and money. The demand for fish ageing has steadily increased in most if not all regions of the United States, but production capacity has not been able to keep up with this demand due to labor-intensive, traditional ageing methods and limited budgets. Hence, new technology is needed to bridge the gap. National Marine Fisheries Service scientists are evaluating the use of machine-based technology, Fourier Transform Near-Infrared Spectroscopy (FT-NIRS), on otoliths to determine fish age.

Exploratory studies, first by scientists in Australia and then more recently by NMFS scientists, indicate that this technology may provide a more efficient, timely, and cost-effective method for estimating fish age. Efficiency is likely to vary by species depending on

factors such as longevity, preparation method, and clarity of growth zone patterns in the otolith. However, preliminary estimates for walleye pollock from AFSC researchers indicate efficiency could improve by 600%-800%. Additional case studies of other marine fish species (included in these proceedings) suggest this technology may have broader potential applications not only to age other species of fish, but also for measuring important physiological processes such as reproduction and stress. Repeatability (i.e., precision) is also likely to improve as FT-NIRS measures quantitative information from otoliths.

Fourier Transform Near-Infrared Spectroscopy first gained prominence in the pharmaceutical, chemical, and agricultural industries as an efficient and accurate way to measure chemical formulations for product quality and in-process monitoring of factory operations. For example, in the dairy industry, FT-NIRS is used to determine the strictly regulated butterfat content of milk and other dairy products. To determine fish age using FT-NIRS technology, light is focused on the otolith from a special near-infrared source, which absorbs some of this light at characteristic wavelengths or frequencies. The amount of light absorbed is measured and recorded by an instrument known as a spectrometer. This record of the light absorbance by the otolith is called a near-infrared spectrum. The process takes about 30-50 seconds per otolith -- more than 10× faster than traditional methods. Understanding the exact molecular constituents in otoliths that yield the relationship between spectral data measured by FT-NIRS and fish age is paramount. Based on the most informative O-H, N-H and C-H regions in the near-infrared wavelength range, it is plausible that it may be the organic content, including proteins, in otoliths that determine the age of fish. As a fish grows, its otoliths accrete layers of calcium carbonate, including a

protein matrix; hence, the otoliths of a 1-year-old fish will have less protein than those of a 10-year-old fish. If we can measure differences with FT-NIRS, we will have an efficient method to age large numbers of fish.

With the promise of improved precision and efficiency of FT-NIRS age estimation over traditional approaches, the National Marine Fisheries Service funded a 5-year strategic initiative with the goal of operationalizing this technology nationwide. As part of that strategic initiative a workshop entitled, “*Rapid Estimation of Fish Age Using Fourier Transform Near-Infrared Spectroscopy*” was convened at the Alaska Fisheries Science Center in Seattle, WA, April 11th - 12th, 2019. The 2-day workshop served to kick-off the strategic initiative by convening national and international subject matter experts in near-infrared spectroscopy, multivariate predictive modeling, stock assessments, and fish age estimation. The workshop was divided into three sessions. In session one, subject matter experts focused on the theoretical aspects of the technology and the basics of multivariate prediction models for age estimation. In session two, practitioners of FT-NIRS in fisheries and wildlife biology highlighted recent developments, case studies, and experiences of applying FT-NIRS to hard structures and tissues from different species for the estimation of age and animal physiology. The final and third session served as a platform to discuss issues and challenges, prioritization of research needs, standards and best practices, and a pathway to operationalizing FT-NIRS for age estimation.

FT-NIRS for fish ageing is clearly in the research and development stages, but new applications to estimate ages from otoliths of other marine fish species are underway by National Marine Fisheries Service scientists. By the conclusion of the workshop, otolith spectral data from a dozen species over four large marine ecosystems had been collected,

with more than half showing promise to rapidly predict fish age from spectral information. A number of those examples are presented in this document. While basic questions remain and details on the operational aspects of the technology as applied to fish age estimation need to be addressed, the strategic goal is to be able to develop it for use on a large scale and ultimately for use in stock assessments. Once the method is fine-tuned, increased efficiency and reduced costs are only the beginning of the potential benefits of using NIR technology in fisheries research and management.

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INTRODUCTION

Fish ages are a fundamental data element of integrated stock assessments because they provide information on recruitment, growth, maturity, and production (Maunder and Punt 2013, Ono et al. 2015). Estimation of age composition, size at age, maturity at age and longevity of a fish population is critical for assessing the overfishing or overfished status of a stock (Ricker 1975, Hilborn and Walters 1992, Campana and Thorrold 2001, Ono et al. 2015). Fish age has historically been determined by microscopically counting pairs of annual opaque and translucent growth zones in a number of different hard structures including scales, vertebrae, opercula, spines, and most commonly otoliths (Bagenal and Tesch 1978, Chilton and Beamish 1982). In federally managed waters of the United States, over 1.1 M hard structures (minimum estimate) have been examined for age estimates during the period from 2008 to 2015 (T. Helser, AFSC, personal communication) and most management regions have shown a steady increase in the number of otoliths aged per year.

A large investment is made in the age determination and production process of age data to support stock assessments. While the traditional production approach varies in the procedures used to prepare and count the number of growth zones in otoliths depend on species, the hard structures require some degree of handling time in the process of embedding, sectioning, cutting, and burning to enhance growth zones and microscopic examination. Hence, when considering the time from when the otolith is collected at sea to the time a trained expert age reader produces an age estimate, quite an investment has gone into the generation of a single age. Age data have been referred to as one of the most expensive sources of data collected for stock assessments (Campana 1999). Furthermore,

production ageing laboratories employ quality control procedures (to estimate precision and bias) where some percentage (usually between 10 and 20%) of the total specimens are handled and read a second time by another independent expert age reader to estimate age reading precision (Kimura and Anderl 2005).

Historically, machine-based or computerized fish age determination has been attempted with various levels of success using otolith morphometrics (Pilling et al. 2003, Fablet et al. 2009, Mahe et al. 2016), image analysis (Nasreddine et al. 2013) or both (Troadec and Benzinou 2002, Fablet and Le Josse 2005). When a large number of fish need to be aged, such as for stock assessments, the goal may be to produce fish ages more efficiently, while not hindering age accuracy or precision (Fablet and Le Josse 2005). While a number of alternative approaches have been explored for use in age estimation, few if any have assisted experts trained in otolith interpretation and none have been developed as a large-scale production age determination tool.

One of the most exciting new developments in fish ageing is Fourier transform near-infrared spectroscopy (FT-NIRS); a technology widely used in industries including pharmaceutical, chemical, petrochemical, agricultural and food, and feed and dairy manufacturing. Initial efforts to apply this technology to fish ageing were conducted by researchers in Australia to estimate ages from otoliths (Wedding et al. 2014, Robins et al. 2015) and shark vertebra (Rigby et al. 2016). In North America, Helser et al. (2019) conducted the first extensive feasibility study to eastern Bering Sea walleye pollock (*Gadus chalcogrammus*) otoliths and found FT-NIRS had as good or slightly better precision than traditional ageing with almost 10 times more efficiency. With the promise of improved precision and efficiency of FT-NIRS age estimation procedures over traditional approaches,

the National Marine Fisheries Service funded a 5-year strategic initiative with the goal of operationalizing this technology nationwide. As part of that strategic initiative a workshop titled, *“Rapid Estimation of Fish Age Using Fourier Transform Near Infrared Spectroscopy”* was convened at the Alaska Fisheries Science Center in Seattle, WA, 11-12 April 2019. The 2-day workshop served to kick-off the strategic initiative by convening national and international subject matter experts in near- infrared spectroscopy, multivariate predictive modeling, stock assessment and fish age estimation. The workshop was divided into three sessions. In session one, subject matter experts focused on the theoretical aspects of the technology and the basics of multivariate prediction models for age estimation. In session two, practitioners of FT-NIRS in fisheries and wildlife biology highlighted recent developments, case studies, and experiences of applying FT-NIRS to hard structures and tissues of different species for the estimation of age and animal physiology. The final and third session served as a platform to discuss and prioritize research needs, standards and best practices, and a pathway to operationalizing FT-NIRS for fish age estimation.

The workshop was attended by 60 participants representing four countries (Australia, Canada, Republic of South Korea, United States), seven National Marine Fisheries Service ageing laboratories, university researchers, and application scientists from private sector firms (Appendix). While the formal workshop occurred on Thursday, 11 April and Friday, 12 April, members of the FT-NIRS Strategic Initiative Development Team (SIDT) arrived Monday and Tuesday that week to begin training on the use and acquisition of spectral data from otoliths on Bruker MPA II spectrometers. Two Bruker Optics MPA II instruments, purchased by AFSC and SEFSC-Panama City from 2018 ORF funds, were previously optimized upon delivery in Seattle in early January 2019 during

which time Beverly Barnett (SEFSC) began a NOAA Rotational Assignment Program (NRAP).

Otoliths from 10 species including Pacific cod (*Gadus macrocephalus*), haddock (*Melanogrammus aeglefinus*), Acadian redfish (*Sebastes fasciatus*), red snapper (*Lutjanus campechanus*), gag grouper (*Mycteroperca microlepis*), vermillion snapper (*Rhomboplites aurorubens*), gopher rockfish (*Sebastes carnatus*), North Pacific hake (*Merluccius productus*), Pacific sardine (*Sardinops sagax*), and Pacific mackerel (*Scomber japonicus*) were among those chosen for analysis by SIDT members. Predictive models, including calibration and validation steps, were developed as part of a training session by an AFSC chemometric analyst (I. Benson, AFSC) and Bruker Application scientist (J. Erickson, Bruker Optics) on Wednesday; the results of which were presented and discussed by all workshop participants on Friday.

The workshop, which began on Thursday, 11 April, consisted of three sessions focused on 1) background theory and mathematical aspects of the FT-NIRS technology with presentations given by subject matter experts in the field; 2) case studies and other applications from practitioners across the field of fisheries ecology, wildlife biology and biodiversity; and 3) pathway toward operationalization in the National Marine Fisheries Service ageing enterprise and the role of the FT-NIRS SIDT, including fleshing out the activity and budget of a detailed work plan over the next 4-5 years. In session one, for instance, Jason Erickson (Application Scientist from Bruker Optics) presented the physics behind near-infrared (NIR) electromagnetic-matter interaction, molecular vibrational responses, energy absorption profiles, and the unique features of the spectrometer instrumentation that captures it. In session two, FT-NIRS case studies were presented that

dealt with adult and larval fish otoliths, skate vertebrae, and other applications in wildlife physiology studies, including endangered salamanders and frogs (C. Vance, Mississippi State University). These presentations brought to light the broader potential scope of the application of FT-NIRS in aqueous (e.g., blood) biological sample collection for sex determination, reproductive state, physiology response to stress and disease. The third and final session dealt with challenges encountered by the Queensland Department of Agriculture and Fisheries, Australia (DAF) research scientists in the use of FT-NIRS fish age data products in fish stock assessments (B. Wedding and J. Robins, DAF). While proof of concept research showed promising results, the Australian scientists indicated a deeper understanding of the sources of variation in spectra of otoliths is needed, and that no additional operationalization funding is planned. However, the technology was widely favored by the participants as having potential operational success and the Australian scientists were excited to see the National Marine Fisheries Service supporting further advances in NIR spectroscopy.

Each session was followed by a discussion of strengths, weaknesses, and challenges facing the application and operationalization of FT-NIRS to fish ageing. A number of challenges and research questions were identified. These, to mention a few, include the following: better understanding of the molecular constituents of otoliths (and other biological structures) that are informative to FT-NIRS, factors related to sample consistency (preparation, type of medium storage) and presentation to equipment that contribute “noise” to the spectral “signature”, reference data (age) accuracy and precision impacts on predictive models, and spatiotemporal sources of variation that effect predictive model robustness. Richard Methot (NOAA Senior Scientist for Stock

Assessments, Office of Science and Technology), and Jim Ianelli (Senior Assessment Scientist, AFSC) also participated in discussion of potential challenges to integrating FT-NIRS data products into U.S. stock assessments noting that historic “traditional” data in population models may require some sort of “correction” to make it consistent with the new FT-NIR data products. The final wrap up session on Friday afternoon focused on the components of a detailed work plan that identifies specific challenges, research priorities, budget needs, and implementation timelines. The proceedings herein of the workshop is meant to document this important effort toward operationalization, highlight technological applications in wildlife and fisheries ecology through extended abstracts of presenters, and focus our thinking in a coordinated pathway toward operational readiness.

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**Introduction to Near-Infrared Spectroscopy
and Fourier Transform Technology**

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ABSTRACT

Near-Infrared Spectroscopy (NIR) is a widely used technique for the rapid analysis of many types of food products, chemical products, and pharmaceuticals. Recently this technique has been used in the more novel application of fish ageing. NIR is a vibrational spectroscopy technique where the amount of energy absorbed by specific functional groups in a molecule is measured and correlated to sample composition. Fourier Transform (FT) NIR utilizes a specific type of instrument design to measure the energy that is absorbed. The fundamental principles of FT-NIR spectroscopy and Fourier transform technology will be discussed.

Supplementary Notes

Erickson began by presenting the definition of near-infrared spectroscopy (NIRS), which is the measurement of the amount of light/energy that is absorbed by a material. The absorbance is correlated with some property of the material that the user is interested in. Traditional uses for NIRS include pharmaceuticals, chemical, food, animal feed, edible oils, and dairy. Near infrared has wavenumbers in the 12,000 – 4,000 range. Erickson described the process of the interaction of near-infrared light with the sample. He further described the interaction of light with molecules and how different types of molecular vibrations occur at different frequencies that are characteristic of certain molecular combinations. Erickson showed a list of advantages and disadvantages of NIR. There are numerous advantages of NIR, a few of which include: fast method (10-20 seconds is typical scan time), no waste and no pollution, highly precise and accurate, real time monitoring for

process and control. Erickson listed four disadvantages of NIR: 1) “not a primary method”, 2) “perceived as complex”, 3) “chemometrics required due to spectral richness/overlap”, and 4) “many samples may be required for calibration model”. There are three main components for NIR: light source, optics (integrating sphere) and detector (interferometer). NIR uses two main technologies: dispersive and Fourier Transform (FT-NIR). Bruker’s FT-NIRS instrument has a cube-corner mirror, which provides greater stability for the alignment of the instrument over time; therefore, providing repeatability and stability over time. FT-NIR utilizes high resolution for the calibration of the instrument for long-term stability and transferability. Jason described the workflow for using FT-NIR as a secondary method of analysis. He provided a thorough description of the importance of selecting calibration samples, scanning those samples with FT-NIR, and then building the calibration model. This calibration model would be used to predict unknown samples. Models are based on partial least squares (PLS) regression. PLS is very effective in making use of correlated information and discriminating non-useful information; thus allowing for overlapping bands and structures in the spectra to be separated.

**Data Preprocessing for Quantitative and Qualitative Models
Based on Near-Infrared Spectroscopy**

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Supplementary Notes

Wise provided a descriptive overview of chemometric procedures that can be used when creating predictive models. He described the importance of including data preprocessing, which is a step taken prior to modeling the algorithm (e.g., PCA, PLS, etc.). The goal of data preprocessing is to remove the variation that is uninformative (i.e., clutter), which will then allow the analysis to focus on the informative variation or “signal”. He mentioned that clutter (i.e., interferences + uninformative noise) is present in all measurements (both x = spectra and y = prediction). Sources of clutter include the following: systematic background variability, other observed changes in the system, variance due to the physics of the instrument, and non-systematic random noise. Several reasons as to why data should be preprocessed: reduces variance from extraneous sources, makes relevant variance more obvious, statistics will work better, helps with interpretation, and avoids numerical problems. Generally, we are interested in determining how the data varies around the mean so, Wise described “mean centering”, which is a calculation that forms a matrix where each column will have a mean of zero. Scaling is an option that can be used to change variance of variables and therefore changes the weight assigned to them when modeling. Autoscaling is the most commonly used and only takes into account how the variables correlated with each other (i.e., correlation matrix). Wise also described ways to remove low-frequency interferences while keeping higher-frequency features (e.g., detrend, selected-points baselining, weighted least squares baselining, windowed) to remove an offset. He further described methods used to remove variance that results from changing magnitude (e.g., row normalization, standard normal variate, multiplicative signal correction); however, he cautioned that these methods can

also “blow up” low signal noisy samples to have more variance. Savitzky-Golay smoothing and derivatives are useful for removing offsets/slopes. Wise presented a recap on his perspectives for preprocessing. These points included the following: the order for preprocessing matters (1. Background and offset removal; 2. Normalization; 3. Centering; 4. Scaling); keep in mind what each preprocessing step is doing to the data; after data are preprocessed, plot the data and color code the data; and always compare the effect of data preprocessing with model results built on raw data.

**Applications of Near-Infrared Spectroscopy to Questions
in Animal Physiology: Sex Discrimination in a
Monomorphic Amphibian Species with Extension to Juveniles**

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INTRODUCTION

Near-infrared spectroscopy (NIRS) is more frequently being considered as a means to assess animal physiological traits such as species, gender, age, health and reproductive state which contribute to population demographics. In contrast to most assay techniques, which measure a single parameter such as a hormone or nutrient, NIRS captures a suite of chemical information in a single spectrum, and it can be used with a wide variety of samples such as hair, feces, bone, and feather¹. Recent expansion to fundamentally aqueous biological samples including tissues, gametes, embryos, blood, urine, and saliva has given rise to the development of aquaphotomics^{2,3} and other non-traditional means to assess these types of materials. For threatened and endangered species NIR offers a rapid and non-invasive means to acquire physiological information but can also be adapted to *in situ* monitoring if sample collection and exportation are restricted.

This study demonstrates NIRS can be used for sex determination in the Mississippi gopher frog (MGF; *Lithobates sevosa*), North America's most critically endangered amphibian. Estimates suggest only 100 adults remain in the wild. Efforts to combat amphibian extinctions worldwide focus on rescuing and reinvigorating populations using assisted reproductive technologies (ART) and captive breeding programs^{4,5}. Unfortunately, difficulties arise in sexing adult individuals of monomorphic and weakly monomorphic species such as the MGF without characteristic visual or auditory sexual identifiers, and juveniles in general. Human experts often attempt to sex mature adults by examination of the cloaca during the breeding season to pair animals or exchange genetics. Ultrasound is limited to identifying gravid females but not empty females, males,

or juveniles. Validating sex in such amphibians requires either genetic analysis complicated by amphibian XYWZ chromosomal variants, or gamete expression requiring animals to be sexually mature and hormonally induced^{4,6}. As such, a non-invasive means to quickly and accurately identify sex in monomorphic species or juvenile amphibians is needed to support breeding efforts and reintroduction programs. Here we present two experiments that demonstrate calibrations using NIR spectra from MGF can 1) predict the sex of other adult (> 4 years) individuals, and 2) predict the sex of juvenile (< 3 years) animals.

METHODS

Animals

Sex discrimination by NIRS was developed from adult MGF (> 4 years) housed at Mississippi State University (Population 1: 32 males, 27 females) and the Memphis Zoological Society and Florida Zoo (Population 2: 19 males, 28 females, 2 unknown). All animals were identified with passive integrated Transponders (PIT tags). MGFs weighed between 20 and 50 gm each depending on their age and cohort. Validation methods included both ultrasound (U.S.) and exogenous hormone treatments of GnRH (gonadotropin releasing hormone) and hCG (human chorionic gonadotropin) to induce gametogenesis and release of sperm or eggs⁷.

Spectroscopy and Chemometrics

Full Vis-IR spectra (350-2,500 nm) were obtained from live animals using a surface contact probe at the abdomen above the cloaca, and the reflectance captured using a portable ASD FieldSpec®3 NIR Spectrometer. Chemometric analysis used GRAMS®AI/IQ 9.1 software and initial random one-out cross-validations were performed on Population 1 to determine the best parameters for spectral pretreatment; spectral mean centering (MC) and a first derivative GAP function for PCA. Subsequently, Partial Least Squares 2-block analysis was used in discrimination matrices and assign the internal and test spectral sets followed by predicting classification of the external validation sets.

Experimental Design: Calibration, Test and External Validation Sets

Experiment 1. -- An NIR calibration set composed of spectra from 21 male and 21 female adult MGFs and an independent test set of 11 male and 6 female adult MGFs were formed using animals from Population 1, in which sex was validated by hormone expression of gametes. An external validation set of spectra was generated from an unrelated mixed cohort of MGF consisting of 19 males, 28 females, and 2 unknowns, Population 2. These animals were initially sexed by ultrasound which identifies females with developing eggs, but it does not distinguish males and empty females. NIR spectra were collected at the time of the ultrasounds. The sex of these animals was confirmed months later with exogenous hormone treatments of GnRH and hCG.

Experiment 2. -- NIR spectra are collected from 7 adult MGF (> 4 years) of known sex and 14 juveniles (~ 2 years) of unknown sex. Spectra are processed as described above and used for PCA. The juvenile animals were allowed to reach sexual maturity over the next 18-24 months and then treated with exogenous hormones for validation of sex. Sperm is collected from males and egg development in females is monitored.

RESULTS

Average baseline adjusted spectra for adult males and female MGFs (n = 21.21) are shown in Figure 1a, along with scores from the first two PCs (Fig. 1b). In Experiment 1, which examined if sex could be non-invasively discriminated *in vivo*, the PLS-2 block discriminant calibration developed from Population 1 animals gave an $r^2 = 0.83$, standard deviation of 0.14 and SEC = 0.20, Table 1. In the initial cross validation and calibration, all internal (calibration) and test set spectra were correctly classified. For the external validation set generated from Population 2, the NIR calibration identified 18/19 male and 28/28 female gopher frogs from their spectral signatures, with $r^2 = 0.82$, RMSD = 0.23 and SEP = 0.24. Two animals were identified as female by NIR but determination of their sex by hormone treatment and ultrasound took an additional 6 months, and these animals were correctly identified as female. Notably, the NIR spectra were collected on the animals in October, which is not in their breeding season, but the production of gametes to verify these two animals' sex took until April, the spring breeding season for this species.

Experiment 2 investigated as to whether the spectra of adult MGF could be used to identify sex in juvenile MGFs. Figure 2 shows the unadjusted average spectra of adult MGF

for each sex and the PCA scores plots from the first three PCs⁸. Confirmed adults males are indicated in the scores plot and spectra from juvenile males associate with them more strongly than with females. With only three PCs nearly all the variation in the spectra is described and the juvenile animals are correctly classified by sex from the calibrations generated from adult animals.

DISCUSSION AND CONCLUSIONS

NIR can discriminate sex non-invasively in living amphibians, even in monomorphic species, from reflectance spectra obtained from skin. Additionally, NIR spectral calibrations were demonstrated to be transferable across populations, such that a calibration made with one cohort can predict gender of individuals from different cohorts with completely different life histories. Sex can be determined both in and out of the breeding season using NIR, even when ultrasound analysis and hormone induced gamete release may not yield results unless the animals are reproductively active. Of even greater interest, we show that NIRS can predict the sex of juvenile individuals using calibrations made from adult animals. And finally, we also demonstrate that calibrations made in one year for sex discrimination are applicable in future years. Evidence exists that some anurans exhibit sexually dimorphic chemical profiles in their skin composition⁹, such as the Australian splendid tree frog (*Litoria splendida*), where specialized peptides are present only in the skin secretions of the male. Here, we show that NIRS has great potential for non-invasive sex discrimination in live animals, with > 95% reliability. This

project was supported by the Institute of Museum and Library Services Grants MG-30-17-0052-17 and the USDA-ARS Biophotonics Initiative grant #58-6402-3-018.

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Table 1. -- Prediction results and NIR parameters for internal (calibration) test and external validation sets.

Animals	NIR set	Number	NIR parameters	Number correctly predicted	% correctly predicted	Validation Direct Method
Population 1	Calibration	21 M 21 F	$r^2 = 0.83$ SD = 0.14 SEC = 0.20	21/21 M 21/21 F	100% M 100% F	Hormone-induced gametes
Population 1	Test	11 M 6 F	$r^2 = 0.75$	10/10 M 8/8 F	100% M 100% F	Hormone-induced gametes
Population 2 Mixed Cohort Adults, Juveniles	External Sets	19 M 28 F 2 unk	$r^2 = 0.82$ RMSD = 0.23 SEP = 0.24	18/19 M 28/28 F	89% M 100% F	Ultrasound + Hormone-induced gametes

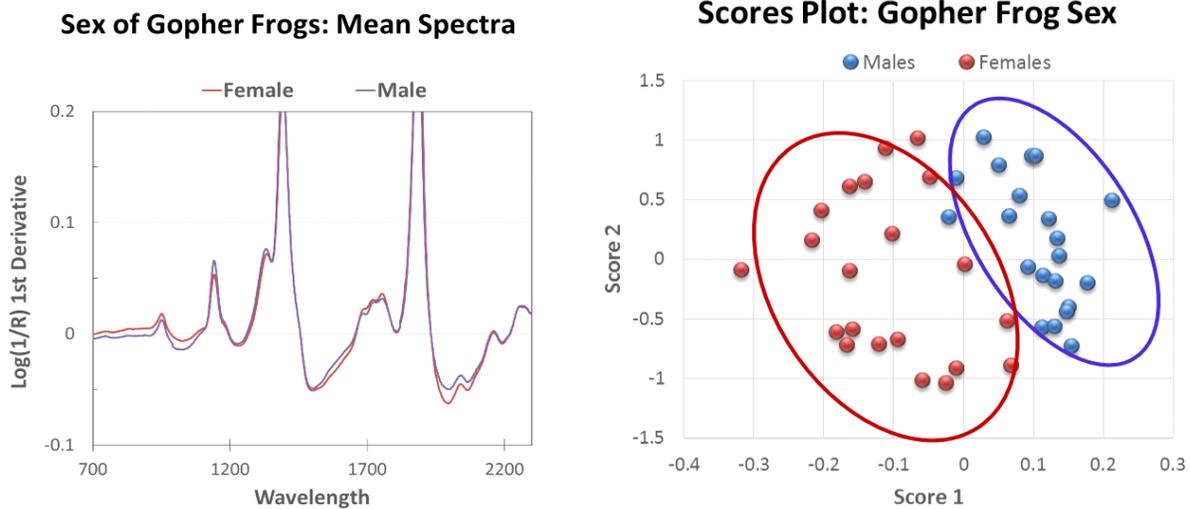


Figure 1. -- Average spectra (700-2,300 nm) generated from adult MGFs (n = 21M, 21F) used in the calibration (A). Scores plots of PC1 and PC2 from the PCA analysis of the external validation set consisting of spectra from mixed animal cohorts different from calibration and test set animals (B).

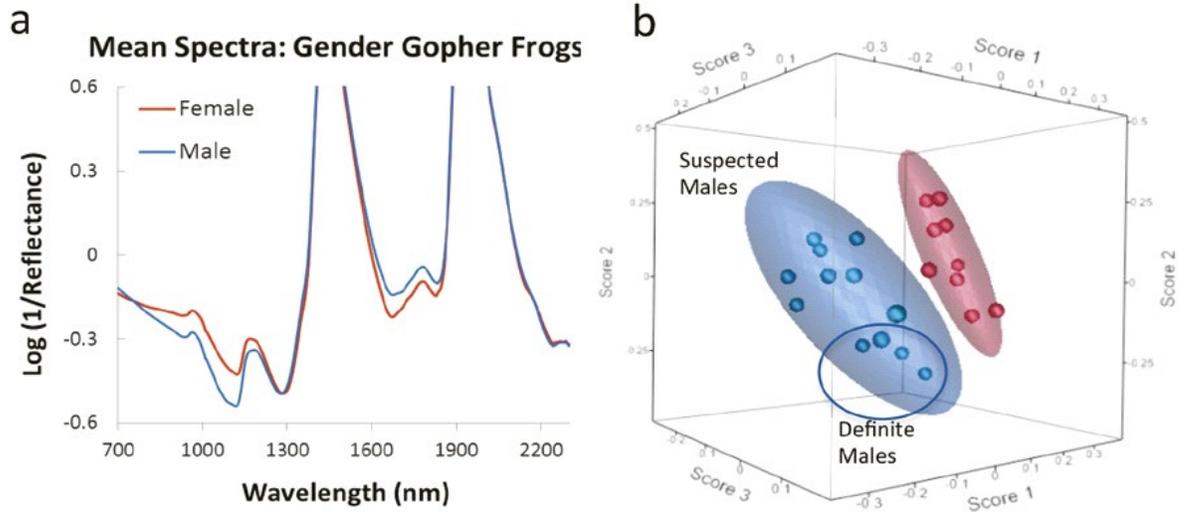


Figure 2. -- Average spectra (700-2,300 nm) generated from adult and juvenile MGFs (n = 12M, 9F) (A). Scores plots of the first three PCs from the PCA analysis (B).

**In Vivo Detection of Induced Pheromone Expression in
Northern Dusky Salamanders (*Desmognathus fuscus*)
Using Near-Infrared Reflectance Spectroscopy**

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ABSTRACT

A hallmark of Plethodontid salamander reproduction is pheromone expression which is triggered by activation of the plasma corticosterone (CORT) cascade in response to gametogenesis and chemical signaling. Our objective was to use Near-Infrared Reflectance (NIR) spectroscopy to detect pheromone expression in male dusky salamanders (*Desmognathus fuscus*) when they were exposed to chemical signals from gravid females. Males of *D. fuscus* species (n = 4) were exposed to Female-Cloacal-Water (FCW), which was generated by soaking ultrasound confirmed gravid and empty (post-gravid) females (n = 4) in 12.5 ml of water for 15 hours. Presence of female pheromones in FCW was determined by aquaphotomics applied to the transmittance NIR spectral signatures. NIR spectra were collected from the males' chemosignaling mental, dorsal tail, and post-cloacal glands both prior to and after exposure to FCW. The presence of pheromones in FCW was evident in the behavioral experiments where the males' responded significantly more ($p < 0.0001$) to FCW from gravid compared to empty (post-gravid) females. Spectral signatures showed chemical changes in the PCA loadings for each chemosignaling gland, indicating pheromone expression in males exposed to FCW of gravid females. In addition, Linear Discriminant Analysis (LDA) predicted 88.8%, 94.1%, and 94.4% of spectra from males exposed to FCW from gravid compared to empty females at the mental, dorsal tail, and the post-cloacal glands, respectively. Overall, we were able to detect and discriminate the glandular chemical changes of male *D. fuscus* in response to female pheromones using NIR spectroscopy, indicating that FCW from gravid

females contains pheromones that stimulate males to release pheromones from their chemosignaling glands.

INTRODUCTION

Northern dusky salamanders (*Desmognathus fuscus*) are a lungless salamander species from the Plethodontidae family. A hallmark of Plethodontid salamander reproduction is pheromone expression, which is triggered by gametogenesis and chemical signaling¹. Initially chemosensory stimuli by pheromone exposure and expression bring males and females together due to activation of the plasma corticosterone (CORT) cascade involving the hypothalamus and the interrenal glands. There is a directly proportional relationship between CORT and the pheromones released from three chemosignaling glands called the mental gland, dorsal tail gland, and post-cloacal gland^{2,3}. Here, we conducted Near-Infrared Reflectance (NIR) Spectroscopy for *in vivo* detection of induced pheromone expression in *D. fuscus* by collecting spectral signatures from 1) Female-Cloacal-Water (FCW) of gravid females, and 2) the males' chemosignaling glands.

METHODS AND MATERIALS

Female-Cloacal-Water (FCW) and Behavioral Experiments

Females were determined to be gravid (contain eggs) or empty by ultrasound (SonoSite® MicroMaxx; 2.7 MHz). In order to induce a chemical and behavioral response in *D. fuscus* males (n=4), Female-Cloacal-Water (FCW) was prepared by placing one female into 12.5 mL of water for 15 hours just prior to behavioral experiments. A total of four trials were performed by pairing each male to each females' FCW. To assess male behavioral response, tanks with platforms containing 1 mL of FCW and 1 mL of control water (CW) were placed. Males were acclimated to the tank for 30 minutes prior to evaluation of six behaviors based on a 1-hour ethogram. Student's T-tests were run to determine if individual behaviors were significantly greater in response to FCW compared to CW using R Core Team (R Studio, R version 3.3.2, Vienna, Austria).

Spectral Signature Collection

Transmittance NIRS spectra were collected for FWC (n = 50) using a portable ASD FieldSpec®3 + Indico®Pro (Malvern Panalytical, ASD Analytical Spectral Devices Inc. Boulder, CO. USA). Samples (300 µl) were analyzed in a 1 mm quartz cuvette mounted in an ASD-fibre optic cuvette adapter. Reflectance NIRS spectra (n = 432) from *D. fuscus* males' chemosignaling glands were collected before and after the behavioral experiments using a small diameter contact probe. Each NIR spectrum was collected across the range 350–2,500 nm (interval = 1 nm; 50 scans; 34 ms integration).

Chemometrics

Aquaphotomic analysis of FCW was performed on the second overtone of water in the vibrational combination band at 900-1,300 nm (Unscrambler®X v.10.5; CAMO Analytics, Oslo, Norway). The mathematical pre-treatments SNV (Standard Normal Variate), Linear detrend, 2nd Derivative, and Savitzky-Golay smoothing were applied to the database. Principal Component Analysis (PCA) on the mean centered matrix was obtained using a full random cross-validation, and algorithm-SVD (Singular Value Decomposition). Water matrix coordinates (WAMACs) and aquagrams were created according to Tsenkova⁴ et al. (2018). Multivariate analysis on males' reflectance signatures included mathematical pre-treatments SNV, Linear detrend, first derivative, and Savitzky-Golay smoothing to the wavelength range 1,005-1,900 nm. Linear discriminant analysis (LDA; Mahalanobis distance method; 9 PCA scores), was applied to difference spectra between the pre- and post-FCW exposure. LDA was reported from the confusion matrix as percent (%) of correctly classified samples. PCA plots are visualized in JMP 14.0 (SAS Institute Inc., NC, USA).

RESULTS

Characteristic transmittance spectral signatures were determined for FCW in comparison with CW. No outliers were found in the influence plots (Hotelling's T^2) for either spectral database. The first PC loadings vectors for spectra collected from CW and FCW are featured in Figure 1a. Twelve dominant peaks were identified in the PC-1 loadings as the water matrix coordinates (WAMACs) and used to generate the aquagram

to compare the FCW from gravid and empty (post-gravid) females following the methods of Tsenkova⁴. Aquagrams of the normalized absorbance based on the WAMACs showed differences in the spectral patterns of each type of sample (Fig. 1b).

The presence of pheromones in FCW was indicated by a significantly greater ($p < 0.0001$) behavior response of *Attracted and Investigated* when males were exposed to FCW from gravid females compared to FCW from empty (post-gravid) (Fig. 2).

Characteristic NIR spectra of the three male chemosignaling glands are featured in Figure 3a. The PCA scores plot distinguishes NIRS signatures at the dorsal tail gland from the mental and post-cloacal glands, which overlap. A total of 97.2% of the spectral variation was described by 3PCs (Fig. 3b). In addition, chemical changes are evident in PCA scores plots at each gland when males were exposed to FCW from gravid versus empty females (Fig. 4).

Classification results from the PCA-LDA of the subtracted reflectance spectra of males exposed to FCW from gravid and empty females yielded 88.9, 94.1 and 94.4 % of samples accurately discriminated at the mental gland, dorsal tail gland and the post-cloacal gland, respectively (Table 1).

CONCLUSIONS

Aquaphotomics analysis showed that NIRS spectral signatures of FCW differ between gravid and empty females. In addition, male behavioral responses to FCW from gravid females were stronger than to FCW from empty females and control water, suggesting that FCW from gravid females contains pheromones that stimulate males to

release pheromones from their chemosignaling glands. Moreover, characteristic chemical signatures of pheromone expression by males' chemosignaling glands can be detected using NIR *in vivo*. Swabs of each gland are being sent for GC-MS analysis of chemical components to determine specific pheromone species. This project was supported by the Institute of Museum and Library Services Grant MG-30-17-0052-17, the USDA-ARS Biophotonics Initiative grant #58-6402-3-018, and the Mississippi State College of Agriculture and Life Sciences Undergraduate Research Scholars Program.

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Table 1. -- Percent (%) accuracy of LDA classification for male's reflectance spectra by chemosignaling glands.

Chemosignaling gland	EXPOSED TO GRAVID FCW (%)	EXPOSED TO EMPTY FCW (%)	Mean (%)
Mental gland	77.8	100.0	88.9
Dorsal tail gland	94.1	94.1	94.1
Post-cloacal gland	88.9	100.0	94.4

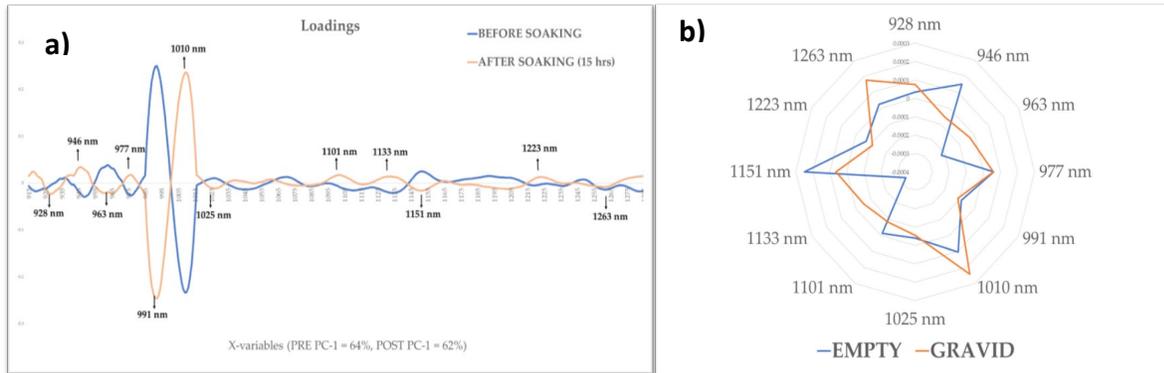


Figure 1. -- Aquaphotomics analysis **a)** PCA-Loadings plots from the transformed spectra of the second overtone of water vibrational-combinations (900-1,300 nm). **b)** Aquagram displaying the normalized absorbance and average for FCW from gravid and empty (post-gravid) females.

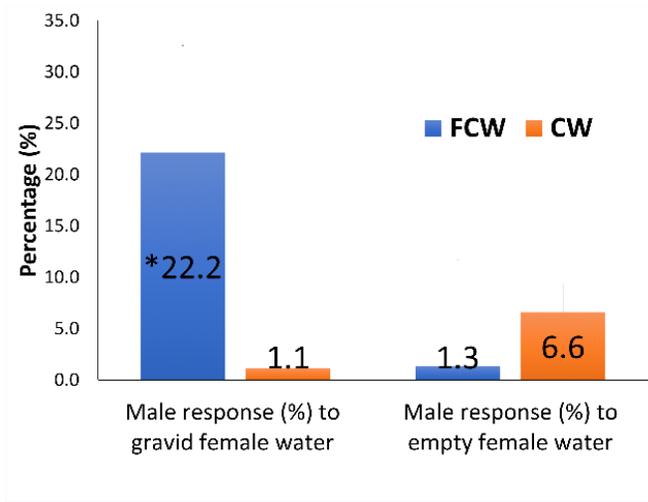


Figure 2. -- Male behavior response to gravid and empty females FWC. * indicates significant difference across FCW treatments, and against control.

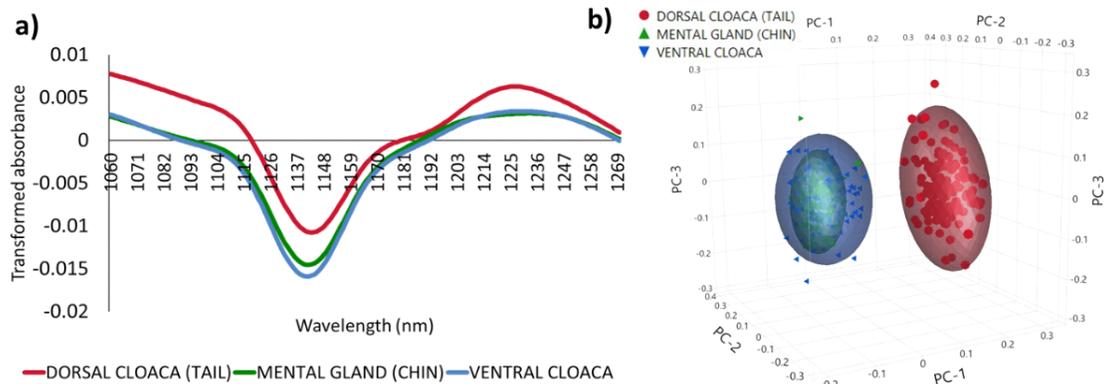


Figure 3. -- PCA results by physiological location **a)** Processed spectral signatures average from male spectra grouped by chemosignaling glands (1,060-1,280 nm). **b)** PCA scores plot (1,005-1,900 nm).

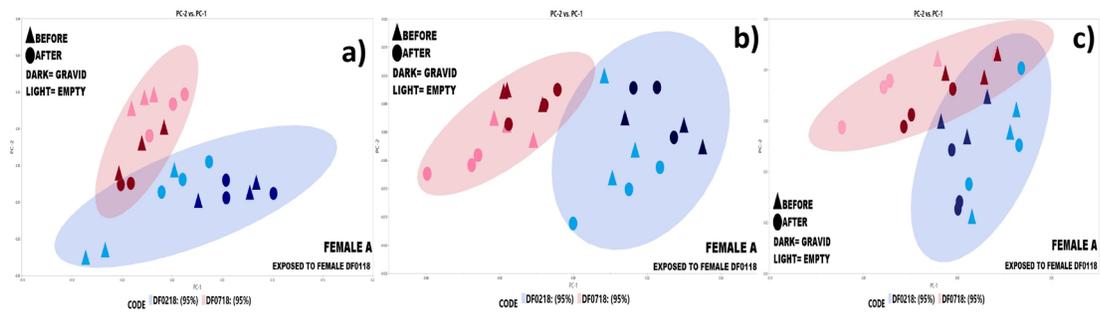


Figure 4. -- PCA results males exposed to FCW from gravid and empty female **a)** Dorsal Tail gland: Two factors explained 85.5% of the database variation. **b)** Mental gland: Two factors explained 78.9% of the database variation. **c)** Post-cloacal gland: Two factors explained 66.7% of the database variation.

Predicting Fish Age at the Speed of Light

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ABSTRACT

Estimating the age structure of fish populations is an important component of sustainable fisheries management. Traditional methods for ageing fish are time-consuming, labor-intensive, and expensive. Previous Australian research has shown that near-infrared spectroscopy (NIRS) has the potential to estimate the age of saddletail snapper (*Lutjanus malabricus*) (Wedding et al. 2014), deepwater sharks (Rigby et al. 2015, Rigby et al. 2014), barramundi (*Lates calcarifer*) and pink snapper (*Pagrus auratus*) (Robins et al. 2015). This current study investigated the effect of seasonal variability for barramundi collected from the Gulf of Carpentaria over four years, 2012-2015. Partial least squares regression (PLS-R) calibration models were developed using samples from successive years with a final model incorporating samples from all four years. The prediction statistics improved as more seasonal variability was introduced into the calibration set. Prediction statistics incorporating seasonal variability from four years with three latent variables, were $r^2 = 0.81$, RMSEP = 7.88 and SDR = 2.3. These results indicate the potential of NIRS to predict the age of barramundi otoliths and the importance of incorporating seasonal variation into a calibration model.

INTRODUCTION

The visual ageing of otoliths is a time-consuming, labor-intensive, and expensive process. Research in Australia have shown that NIRS has great potential to predict the age of saddletail snapper (*Lutjanus malabricus*) (Wedding et al. 2014), deepwater sharks (Rigby et al. 2015, Rigby et al. 2014), barramundi (*Lates calcarifer*) and pink snapper (*Pagrus auratus*) (Robins et al. 2015). Robins et al. (2015) reported a PLS-R model with no seasonal variability for barramundi with prediction statistics of $r^2 = 0.86$, RMSEP = 5.9 and SDR = 2.90. This study extends on the research by Robins et al. (2015) for barramundi by investigating the effect of seasonal variation in barramundi otoliths collected over 2012-2015 from the Gulf of Carpentaria.

METHODS AND MATERIALS

Materials

Whole dry barramundi otoliths from fish caught in the Gulf of Carpentaria fishery in 2012, 2013, 2014, and 2015 were used in this study. Summary statistics for the samples collected are presented in Table 1.

Spectroscopy

All samples in this study were scanned at approximately 12 months after the otoliths were collected. All otoliths were scanned using a Bruker Multi-Purpose Analyser (MPA; Bruker Optics, Ettlingen, Germany; operating software: OPUS™ version 6.5) with an

integrating sphere in diffuse reflectance mode in the 12,500 – 3,597 cm⁻¹ range. In obtaining each sample spectrum, 16 scans at a resolution of 8 cm⁻¹ were collected and averaged. All otoliths were scanned with a concave-up orientation.

Analysis

PLS-R was used to develop calibration models using “The Unscrambler” version 10.5 (Camo, Oslo, Norway). Otoliths included in the calibration set for model development were restricted to otoliths with a reference age of 120 months or less. All spectra were transformed using a 25-point Savitsky-Golay (SG) smooth (2nd order polynomial) and a first derivative transformation (25-point SG smooth, 2nd order polynomial). Calibration models were built up over consecutive years with a base calibration model involving only 2012 samples. This model was used to predict the age of samples collected in 2013. The calibration model was then updated by including 100 samples from 2013 and the process repeated for each consecutive years. The same wavelengths were used in all calibration models and segmented cross validation using 20 segments were used to assess the calibration models.

Outliers included samples in the prediction set that had a deviation associated with the predicted age of greater than 30 months (2.5 years) and samples predicted as >120 months. In reality, predictions with a deviation >30 months are considered unreliable and it would be recommended that these samples be aged using traditional techniques to verify the prediction. The performance of the models were based on the coefficient of determination (r^2) for the calibration and prediction sets; root mean square

error of cross validation (RMSECV) and prediction (RMSEP); bias and the standard deviation ratio (SDR).

RESULTS

Table 2 presents the results for the calibration models and subsequent prediction sets. The base PLS-R calibration models containing only 2012 samples produced poor prediction results for the 2013 samples. For the PLS-R model, 280 of the 404 samples in the prediction set had a deviation >30 months and therefore were considered unreliable and removed.

As the biological variation increased in the calibration set by including samples from different years, the predictive ability of the models improved. The RMSEP decreased to 6.59 months when predicting 2015 samples from a model developed with samples from 2012, 2013 and 2014. The SDR for models predicting samples that are not included in the calibration set also increased as the biological variability in the calibration set increased. Predicting 2013 samples from the 2012 calibration model has an SDR of 1.5. This increases to 1.8 when predicting 2014 samples from a PLS-R calibration with 2012 and 2013 samples, and to 2.4 when predicting 2015 samples from 2012, 2013, and 2014 samples.

CONCLUSION

These results have shown that NIRS can be used to predict the age of barramundi caught in the Gulf of Carpentaria. By introducing greater seasonal variability into the

calibration set, the model robustness clearly increased. The overall prediction statistics improved with increased seasonal variability and fewer samples had a deviation associated with the predicted age >30 months, hence fewer unreliable predictions.

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Table 1. -- Summary of Gulf of Carpentaria otolith samples.

Year	No. otoliths	Age (months)	Mean age (sd*)	Median age
2012	100	28-148	63.0 (20.4)	64
2013	404	17-247	63.1 (24.1)	61
2014	553	28-230	59.3 (25.6)	52
2015	361	28-174	59.9 (17.5)	52

* sd = standard deviation.

Table 2. -- PLS-R model statistics using a generic set of wavelength regions.

Calibration	Prediction	Terms	n	sd	r²	RMSECV	RMSEP	Bias	SDR
			(outliers)						
PLS-R									
2012		3	98	16.5	0.89	5.48		-	3.0
	2013	3	119 (285)	17.3	0.57		11.28	0.06	1.5
2012-2013		3	198	17.9	0.89	6.02		0.01	3.0
	2014	3	543 (10)	20.8	0.70		11.46	-	1.8
								8.24	
2012-2014		4	298	19.0	0.87	6.93		0.01	2.7
	2015	4	357 (4)	15.8	0.83		6.59	-	2.4
								0.24	
2012-2015		3	398	18.9	0.85	7.33		-	2.6
								0.01	
	2013-2015	3	982 (29)	18.1	0.81		7.88	0.05	2.3

Age Prediction of Gulf of Mexico Red Snapper (*Lutjanus campechanus*) Using Near-Infrared Spectroscopy

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INTRODUCTION

In the U.S. Gulf of Mexico (GOM), there is a large demand for more stock assessments to be completed each year, which in turn, requires more otoliths to be processed and aged by scientific staff. While the demand for age data has increased, the capacity to fulfill this need has not increased which has necessitated laboratories involved in production ageing to seek alternative ageing methods that will meet this demand. Fourier transform near-infrared spectroscopy (FT-NIRS) technology, a spectroscopic method that uses the near-infrared region of the electromagnetic spectrum (from 780 nm to 2,500 nm) to measure light absorbance signatures in organic substances, holds the potential to meet this ever-increasing demand. The Southeast Fisheries Science Center's Panama City Laboratory has received approximately 1 million ageing structures representing about 204 species collected from fishery-dependent and fishery-independent sources since the early 1970s. Approximately 20% of these ageing structures have been collected for a single marquee species – red snapper *Lutjanus campechanus*. Red snapper, a long-lived species obtaining ages of 50+ years (Baker et al. 2001, Barnett et al. 2018), supports both commercial and recreational fisheries in the GOM. Age estimates for red snapper have been validated with marginal increment analysis (Allman et al. 2005) and bomb radiocarbon (Baker et al. 2001, Barnett et al. 2018). The current otolith processing method for red snapper includes sectioning otoliths on a Hillquist thin sectioning machine, which is a labor-intensive and time-consuming process. Given the large volume of otoliths received each year for this species and the substantial amount of time it takes to process the otoliths, red snapper was

chosen as the study species for determining whether FT-NIRS could provide a timely and cost-effective method for estimating ages.

METHODS

This study was limited to using fishery-independent samples collected from the eastern (n = 333) and western GOM (n = 453) in 2011 – 2016 since these samples had been previously aged and a second otolith was still available for scanning with FT-NIRS. When possible, 10 otoliths from each age group (ages 1 – 20 years) and all otoliths for ages 20+ years were selected for scanning. Based on visual inspection, only whole clean otoliths were selected from these age groups. An attempt was made to select samples based on the sub-stock structure (eastern and western GOM), which is roughly separated by the Mississippi River (SEDAR 2018). All otoliths were scanned in the concave-up position and covered with a gold-coated reflective stamp. Two independent readers aged the selected otoliths. Thereafter, a consensus age was provided that would be used in the calibration set for model selection.

ANALYSIS

Calibration samples were selected using principal component analysis for data visualization. Optimal wavenumber regions were selected using the optimization algorithm in Bruker OPUS/QUANT2 software and data were preprocessed using a first derivative transformation of the spectra. Calibration and validation models were built with partial

least squares using Bruker OPUS/QUANT2 software. Eastern and western GOM data sets were combined for all models since some age classes were not well represented in each region. The first calibration and validation models were run using all data from all 6 years (2011-2016). The second calibration and validation models were run using year 2012 data to investigate model performance without introducing inter-annual variability that may occur when all years were combined.

RESULTS

Preliminary analysis on GOM red snapper otoliths showed a high correlation when comparing FT-NIRS predicted ages to traditional observed ages (Fig. 1). However, some outliers need to be further investigated to determine if there may have been error in the traditional age estimate, or if the otolith sample was damaged that may have affected the spectral scan. Validation results for model one had an r^2 of 92% (Fig. 1A; Table 1) and model two year 2012 had an r^2 of 95% (Fig. 1B; Table 1); thus, the predictive capability was improved when only one year of data was modeled.

CONCLUSION

This technology once reviewed, tested, calibrated, and implemented would have a major impact on the otolith processing conducted by both state, federal, and academic partners for the fisheries in the Southeast Region. FT-NIRS is a secondary method for age determination; therefore, this method requires the traditional ages used in the calibration-

validation procedure be as accurate as possible. There are two main advantages of using FT-NIRS. First, the rapid speed at which an otolith can be scanned (e.g., approximately 1 minute per otolith) would allow for production ageing to be increased in a timely and cost-effective manner. Second, FT-NIRS provides an objective method rather than a subjective method for generating age estimates; hence, reducing subjectivity that is inherently part of an age reader's interpretation. Overall, FT-NIRS predicted ages were in good agreement with traditional observed ages, thus providing promising results from this preliminary data set of 786 otoliths. Year-specific models with sufficient sample sizes per age class need to be further investigated to determine the effect on model performance. In addition, the effect of fish growth on model predictions also need to be investigated, which would include a spatial component that would be used to produce spatially explicit models.

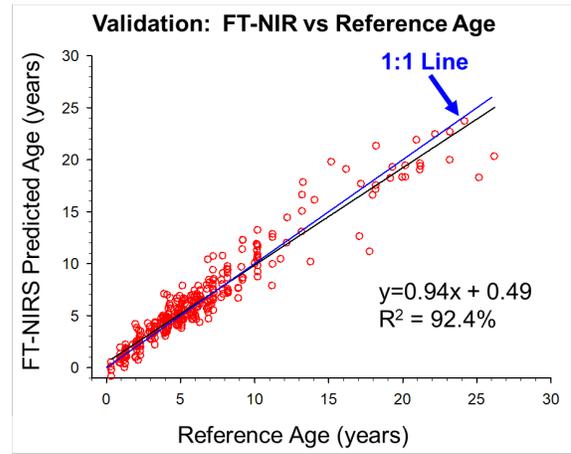
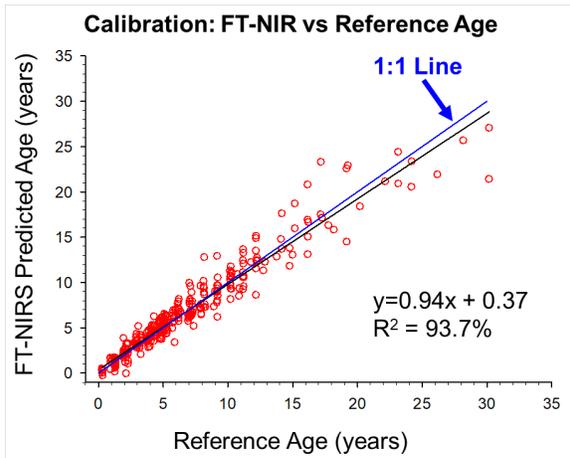
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Table 1. -- Fourier transform near-infrared spectroscopy (FT-NIRS) results from partial least square regressions for red snapper *Lutjanus campechanus* collected from the Gulf of Mexico in years 2011-2016. Metrics are used to evaluate model performance. r^2 = coefficient of determination; RMSECV = root mean square error of cross validation; RPD = residual prediction deviation.

YEARS	Model	n =	r^2	RMSECV	RMSEP	RPD	BIAS
2011-2016	Calibration	333	93.7	1.33	-	3.98	0.0004
2011-2016	Validation	332	92.4	-	1.33	3.63	-0.089
2012	Calibration	84	92.4	1.38	-	3.62	0.0116
2012	Validation	77	94.5	-	0.89	4.5	-0.282

(A)



(B)

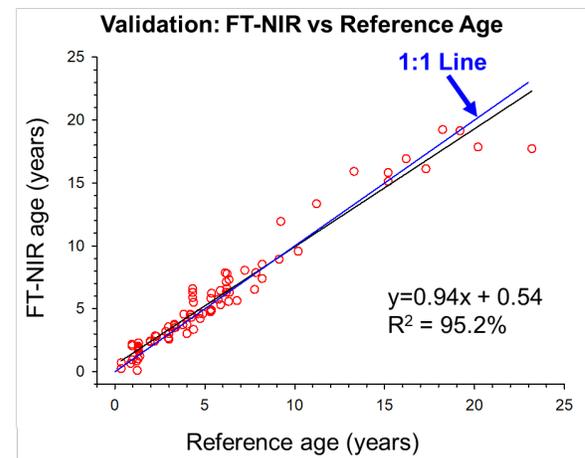
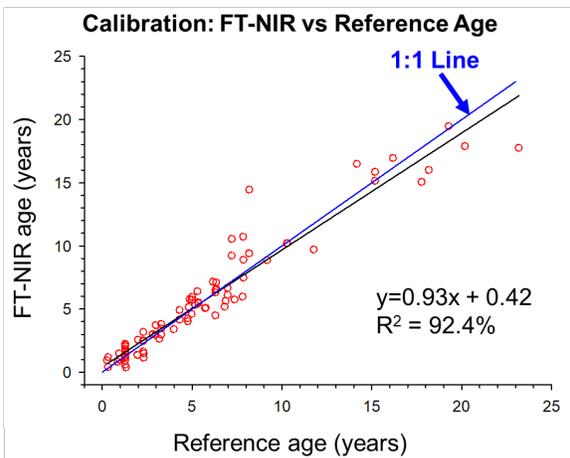


Figure 1. -- Fourier transform near-infrared spectroscopy (FT-NIRS) calibration and validation results from partial least square regressions for red snapper *Lutjanus campechanus* collected from the U.S. Gulf of Mexico in years A) 2011 – 2016 and B) 2012.

**Using Ft-Nirs to Predict Daily Ages in
Juvenile Red Snapper (*Lutjanus campechanus*)**

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ABSTRACT

Fourier transform near-infrared spectroscopy (FT-NIRS) has shown great promise as a rapid and non-destructive method for predicting age from a variety of structures in fish. Its utility for age prediction in otoliths has been demonstrated on an annual scale in multiple studies, but none have evaluated its potential for predicting age on a daily scale. The process of age estimation using daily micro-increments is a time-consuming and difficult task, which requires much skill and technique to yield well-processed otoliths with discernable growth bands. Daily ages are invaluable to the study of early life history dynamics in fisheries, and would thus benefit from an easier and more rapid process to obtain ages. Owing to the promising evidence thus far for the use of FT-NIRS to predict annual age from whole otoliths, we set out to investigate the use of FT-NIRS to estimate daily ages from whole otoliths of juvenile fish. Whole otoliths of juvenile red snapper (*Lutjanus campechanus*) were sourced from archival samples collected from the U.S. Atlantic Ocean, and Gulf of Mexico, whose corresponding paired otolith was aged via traditional methods. Spectral data from whole otoliths (n =153) were collected with a Bruker Matrix-I FT-NIR spectrometer fitted with an external Teflon aperture, which narrowed the light field to 2 mm diameter to maximize signal to noise. Spectral data were preprocessed using a first derivative Savitsky Golay transform (17 smoothing points) and vector normalization and were correlated to traditional daily age estimates via partial least-squares regression to create age prediction models. FT-NIRS models accurately predicted daily age to within about 6 days, on average, relative to traditional age estimates ($r^2 = 91.4$, RMSECV = 6.08 days, bias = -0.04, rank = 5). Our results indicate that FT-NIRS

provides a rapid, non-destructive method to accurately predict age from whole juvenile otoliths, which has broad implications for fisheries applications and management.

Rapid Age Estimation of Longnose Skate (*Raja rhina*) Vertebrae Using Near-Infrared Spectroscopy

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ABSTRACT

Accurate age data are an important component of assessing and managing fish populations, yet traditional age estimation methods are time consuming and expensive. We explored the use of Fourier transform near-infrared spectroscopy (FT-NIRS) to efficiently derive age estimates from the vertebral centra of a batoid species. The longnose skate (*Raja rhina*) is one of just a few species of elasmobranch for which traditional age estimation criteria have been validated. We were therefore able to use robust age estimates to build a predictive model between near infrared spectra and traditional skate age estimates. The model fit the data well with 68% of the traditional ages within 1.41 years of those predicted based on their near infrared spectra (RMSECV = 1.41 years, $R^2 = 0.88$). When externally validated with a separate data set, the model was able to predict the traditionally generated age within 1.45 years 68% of the time (RMSEP = 1.45 years, $R^2 = .87$). The results of this pilot study suggest that the use of FT-NIRS is a promising alternative method for deriving age estimates from longnose skate vertebrae that could reduce cost and improve efficiency.

INTRODUCTION

Biological information regarding the survival, growth, and reproduction of animals is important for predicting the resiliency of a species to exploitation. In fisheries stock assessment, biological information such as age, length, maturity, and reproductive output are used to estimate population productivity in order to estimate sustainable harvest rates (Methot and Wetzel 2013). Specifically, the age when the species matures, the maximum reproductive age, the reproductive output and survival at each age can be used to estimate the rate of population increase in the absence of fishing (Hoenig and Gruber 1990). When combined with a rate of mortality from natural causes, also estimated using age data, a sustainable fisheries harvest rate can be determined. However, information on age is not well known for many species as it is both difficult to attain and time consuming and expensive to process and interpret. This is especially true for elasmobranch species. Elasmobranchs do not possess otoliths which are commonly used for age estimation in teleost fishes. Instead, thin sections of their vertebrae are most commonly used, but this method is especially time consuming (Matta et al. 2017). Additionally, there historically has not been a large research focus on elasmobranch species as they do not support as economically valuable fisheries as teleost fishes (Fowler et al. 2005). This combination of factors has precluded routine age estimation for elasmobranch species. Recently, Fourier-transform near-infrared spectroscopy (FT-NIRS) was applied to rapidly estimate age from the vertebrae of two shark species in Australia (Rigby et al. 2016). This approach may allow for more efficient age estimation of elasmobranchs, enabling more routine acquisition and use of age data in population assessments. However, the application of FT-

NIRS has not yet been explored for estimating ages of a batoid species. Longnose skates (*Raja rhina*) are one species of batoid for which age estimation methods are validated (King et al. 2017). We were therefore able to explore the use of FT-NIRS to estimate ages for this species using robust age estimates to build a predictive model between near infrared spectra and skate age.

METHODS AND MATERIALS

Materials

Samples from longnose skates were collected along the U.S. West Coast on a National Marine Fisheries Service bottom trawl survey in 2011-2012 between the months of May and October. Vertebrae were collected at sea by removing a segment of the vertebral column containing at least five vertebrae. Each specimen was also weighed and total length was recorded by measuring from the tip of the snout to the tip of the tail. Sex was determined by looking for the presence of claspers along the medial edges of the pelvic fins as females lack these structures. Frozen vertebral samples were processed at the Alaska Fisheries Science Center. First, at least four vertebrae per specimen were removed from the frozen vertebral column, cleaned, and stored in a vial with ethanol. For spectroscopic evaluation, one vertebra per specimen was dried for 48 hours before interrogation. Reference age estimates were determined using another vertebra from each specimen by sectioning sagittally through the focus and counting pairs of annual bands corresponding to the age of the skate (Gburski et al. 2007, King et al. 2017).

Spectroscopy

Spectral absorbance data from 648 vertebrae were collected on a Bruker TANGO-R FT-NIR spectrometer. Whole vertebrae were placed on the sampling window in a sagittal orientation and covered with a reflector cap. Absorbance data were acquired at 16 cm^{-1} resolution with 64 co-added scans.

Analysis

Data analysis was conducted in OPUS (version 7.8, Bruker Optics), a chemometric software package. Raw spectral absorbance data, or spectral data, often need to be mathematically transformed to enhance variation between specimens and remove unwanted noise (Robins et al. 2015). All raw spectral data were pre-processed using mean centering, probabilistic quotient normalization, and a 1st derivative (17-point Savitsky-Golay smooth) transformation. Next, a Partial Least Squares (PLS) regression model was fit between reference age and the transformed spectral data. PLS is a type of multivariate regression commonly used for chemometric analysis (Mehmood et al. 2012). The spectral data collected along the full wavenumber range between $11,500$ and $4,000\text{ cm}^{-1}$ contains an abundance of information about the chemistry of the vertebra, not all of which is relevant for estimating age. The model was optimized to identify the least amount of spectral data necessary to differentiate among specimens based on age. We selected a random 50% of specimens that encompassed the full range of spectral variation. This subsample was used to calibrate the model between longnose skate reference age and spectral data using leave-one-out cross validation to maximize the model's predictive ability. In this process, each sample is systematically left out and a PLS model is fit to the remaining samples. The

parameter estimates are then used to estimate the age of the left out sample and the mean error of all predictions versus reference ages can be calculated as the root mean square error of cross validation, or RMSECV. The remaining 324 specimens were used to externally validate the model and a root mean square error of prediction (RMSEP) was calculated as a measure of its predictive ability (Fig. 1). Lastly, to assess the precision of FT-NIRS age estimates relative to traditional methods, we calculated bias between predicted age and reference age and compared it to between human age reader bias.

RESULTS

Two wavenumber ranges were identified to be the most informative for estimating longnose skate age from the spectral absorbance of vertebrae: 5,448 – 6,104 cm^{-1} and 7,496 – 9,400 cm^{-1} . These regions are similar to those identified for otoliths but more constricted (Helser et al. this volume). It is unknown which chemical compounds relate to these regions in skate vertebrae. A calibration model was fit to establish a relationship between light absorbance in these two wavenumber ranges and longnose skate age ($n = 324$). Cross-validation yielded a RMSECV of 1.41 years and a coefficient of determination (R^2) of 0.88. The RMSECV indicates that 68% of the predicted ages from cross-validation fall within 1.41 years of the reference age. The R^2 indicates that over 88% of variation in predicted age is explained by the linear PLS model. When the calibration model was used to predict ages for the remaining 324 samples, it yielded a RMSEP of 1.45 years and an R^2 of 0.87 (Fig. 1). Lastly, the precision between FT-NIRS ages estimates relative to reference ages was compared to the precision between two human age reader estimates. Agreement +/- 0

years between human age readers was 43% compared to 16% for FT-NIRS predictions relative to reference ages. However, agreement +/- 1 year was more comparable between the two methods with 80% between human age readers and 69% between FT-NIR and reference ages. A slight bias was observed in FT-NIRS estimates with a tendency to over-estimate younger ages and under-estimate older ages relative to the reference age (Fig. 1).

DISCUSSION

Prediction of elasmobranch age using FT-NIRS is founded on the concept that there is a relationship between a vertebra's chemical composition and the specimen's age. FT-NIRS measures a light absorbance spectrum from vertebrae that is influenced by that chemical composition. The results of this study suggest that the relationship between chemical composition and age exists for longnose skate vertebrae and that there is potential for FT-NIRS to capture it. Between-reader agreement on longnose skate age estimates is notoriously low given the inherent difficulty in interpreting age visually from their vertebrae. The banding pattern is often diffuse, irregular, and inconsistent between specimens. The process of preparing skate vertebrae for age determination is also incredibly time consuming. On average it takes 40 minutes to prepare and age one vertebra via traditional methods, while it takes just 5 minutes using FT-NIRS once the calibration model is established. Additionally, age estimates generated via spectral absorbance data had similar precision (69%) +/- 1 years to that of between human age reader precision (80%). While a slight bias was observed in FT-NIRS estimates with a tendency to over-estimate age for young skates and under-estimate age for older skates, this may be due to

the unbalanced calibration model that did not contain many old skates. Future work could include using a more balanced dataset to fit the calibration model between spectra and age and may help to resolve this bias. The results of this study demonstrate promise for the use of FT-NIRS to more rapidly and efficiently estimate ages for longnose skates. Longnose skates are commonly caught and retained as bycatch in fisheries along the North American West Coast and their populations are assessed biannually. Age data are a critical component in those assessments, but historically the quantity has been limited by the expertise required and time associated with processing vertebrae for age determination. FT-NIRS to estimate ages for longnose skates could increase the amount of age data available for use in stock assessments. By reducing a source uncertainty in the stock assessment model, we can make more informed management decisions that sustains elasmobranch fisheries and the stocks on which they rely.

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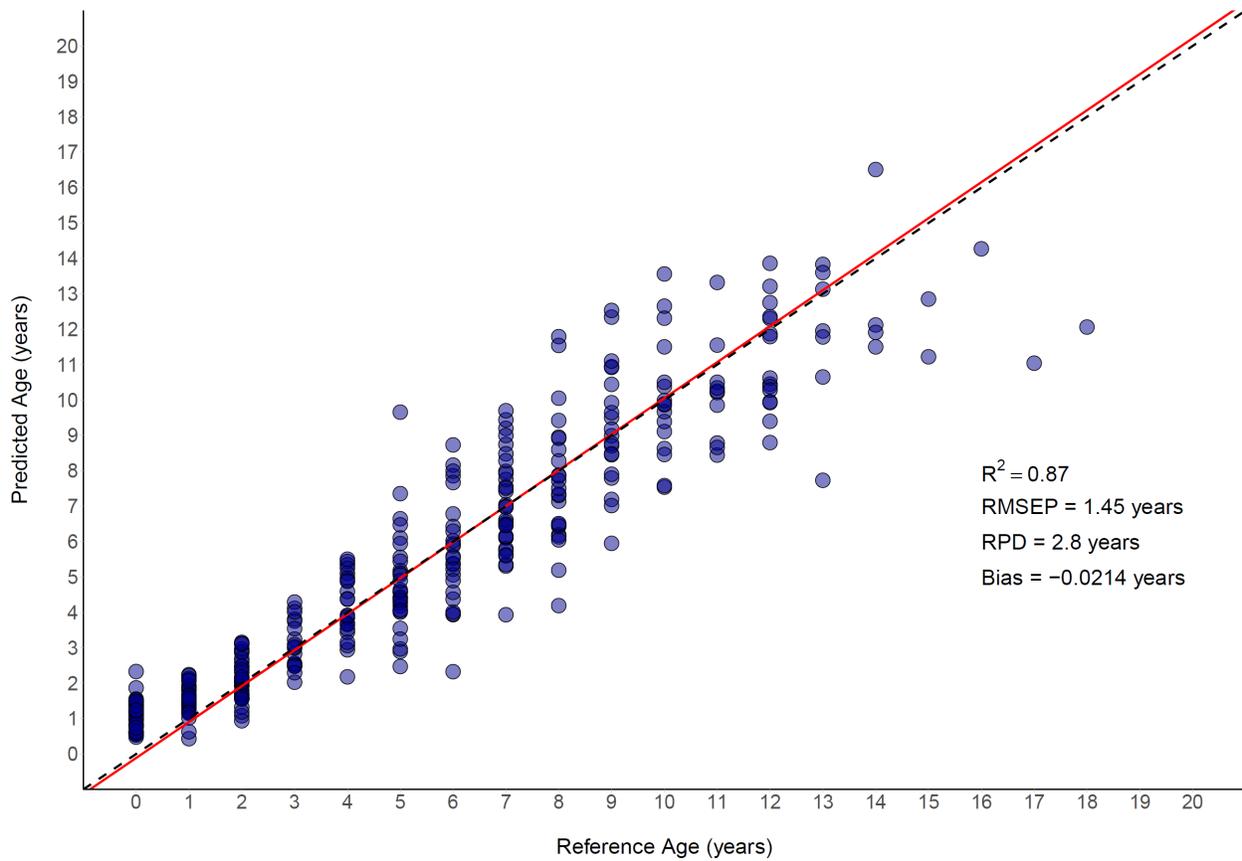


Figure 1. -- Age predictions for the external validation set (n = 324) compared to traditionally estimated reference ages. The dotted black line is the 1:1 line and the red line is the best fit line. Predictions were made based on the calibration model between spectral absorbance data and skate reference age (PLS regression). Sixty-eight percent of FT-NIRS predictions were within 1.45 years of the reference age. Slight bias in FT-NIRS predictions is shown with the tendency to overestimate age for younger skates (< 2 years old) and underestimate age for older skates (> 14 years old).

Case Study of Ft-Nir Spectroscopy Age Estimation for Bering Sea Pacific Cod Stocks

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INTRODUCTION

The Pacific cod (*Gadus macrocephalus*) fishery is the second largest fishery in Alaska bringing in 247,620 (2018) and 223,704 (2017) metric tons of catch biomass (TCB) in the Gulf of Alaska and Bering Sea, respectively. Age-structured stock assessment is used for estimating population growth parameters in *G. macrocephalus* stocks monitored by the Alaska Fisheries Science Center (AFSC) (Grant Thompson, AFSC, 2018). Thus, the acquisition of age data in this species is imperative to its management.

Ages are traditionally estimated by the counting of paired opaque (summer) and translucent (winter) growth bands by trained analysts called age readers. However, this traditional ageing method hosts an array of complications, resulting in time inefficiency and imprecision of ageing fish (Morrongiello and Thresher 2015, Hoie et al. 2009). For example, a fish influenced by atypical seasonal temperature fluctuations at points in its life history can develop anomalous growth bands, called checks, within a single annulus. These checks can be easily confused for true annuli, resulting in difficulty determining a precise age for the fish (Beamish and McFarlane 1995). This challenge is particularly apparent in *G. macrocephalus* under 6 years of age (Roberson et al. 2004). A dominant year class can also introduce challenges. When a dominant year class occurs in a groundfish survey, age readers tend to place samples from adjacent age groups, for which there is uncertainty do to checks and anomalies, into the bin of the dominant year class. This results in difficult to quantify age structure bias in favor of the dominant year class (Beamish and McFarlane 1995, Kimura and Lyons 1990). These are but a couple example of challenges that lead to uncertainty in estimating fish ages, translating to more time spent at the microscope by the

age readers. More time spent at the microscope translates to higher monetary cost in age reading departments.

To reduce cost and increase precision in ageing programs, recent studies have investigated implementation of a new ageing technique using Fourier Transformed Near-Infrared Spectroscopy (FT-NIRs) to model ages from otolith spectra with Partial Least Squares regression (PLSr) analysis (Wedding et al. 2014, Robins et al. 2015, Helser et al. 2019). This pilot study investigates the use of this technique for estimating ages in *G. macrocephalus*, with the purpose of examining its precision when compared against the traditional reader-generated ages.

METHODS

Otolith Collection

Pacific cod otoliths and biological data are procured annually from the AFSC's Bering Sea bottom trawl surveys (BTS). These surveys are conducted using a 20 nautical mile (nmi) fixed grid design at 0-50 m (inner shelf), 50-100 m (middle shelf), and >100 m (outer shelf) depths. The survey area averages 493,000 km² over approximately 350 hauls. Otoliths are collected in a random sample from each haul. Somatic morphometric data (length, mass, sex), diet data, genetic samples, and histology samples are also collected. Otolith samples are transported to the AFSC's Age and Growth Program to be read.

Reference Age Determination

Age readers at the Age and Growth Program estimate ages of fish from the annual growth rings on otoliths, following the guidelines in the “Age and Growth Manual of the Alaska Fisheries Science Center Age and Growth Program” (Matta and Kimura 2012). Of the fish aged by the lab, roughly 20% are aged again by a second reader (also referred to as a tester) to evaluate precision and bias in the age reading process. This between-reader and tester evaluation of precision and bias is used as the standard against which PLSr model performance is compared.

Spectra Collection and Processing

For this study, otoliths were scanned using a Bruker TANGO® spectrophotometer, collecting spectra in 16 cm^{-1} resolution at 8 nm wavenumber intervals from 4,000 to 11,000 wavenumbers, resulting in approximately 875 wavenumber covariates in an $n \times p$ dimensional *X-matrix* of spectral data. An *in situ* Fourier transformation was applied to the spectra to remove excess spectral noise. Spectra were then diagnosed and analyzed in OPUS® chemometric software. A first derivative transformed was applied to further reduce noise and amplify differentiation among samples where separation occurred (Fig. 1.B). A principle components analysis (PCA) was performed on the transformed *X-matrix* of spectra. Eigenvector loadings of the first 2-3 principle components (where > 90% of the variance is explained) were used to select for heavily loaded wavenumber covariates. Wavenumber covariates were removed from the analysis if the amplitude of their eigenvector loadings was close to zero. This reduced the covariates down to only those with heavily loaded covariance (Fig. 1.A). This preprocessing was done iteratively while

looking at PCA plots for obtaining spectra that showed optimized separation among ages in ordination (Fig. 1.C). Broken, crystalized, and otherwise anomalous specimens were removed from analysis. This resulted in $n = 1,526$ and $n = 1,272$ samples that were analyzed from the 2016 and 2017 BTSs, respectively.

Statistical Analysis

Processed spectra were organized in an *X-matrix* of absorbances (X_{np}) with p wavenumber covariates and n samples, which could be fitted to a *Y-vector* of reference ages (Y_n) using a linear model:

$$\begin{array}{c}
 \text{Y-vector of Ages} \\
 \left[\begin{array}{c} Y_1 \\ Y_2 \\ \cdot \\ \cdot \\ \cdot \\ Y_n \end{array} \right] \\
 \text{X-matrix of FT-NIRs absorbances} \\
 \left[\begin{array}{cccccc} 1 & X_{1,1} & X_{1,2} & \cdot & \cdot & X_{1,p-1} \\ 1 & X_{2,1} & X_{2,2} & \cdot & \cdot & X_{2,p-1} \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ 1 & X_{n,1} & X_{n,2} & \cdot & \cdot & X_{n,p-1} \end{array} \right] \\
 \text{Residual Error} \\
 \left[\begin{array}{c} \varepsilon_1 \\ \varepsilon_2 \\ \cdot \\ \cdot \\ \cdot \\ \varepsilon_n \end{array} \right]
 \end{array}
 = \left[\begin{array}{cccccc} 1 & X_{1,1} & X_{1,2} & \cdot & \cdot & X_{1,p-1} \\ 1 & X_{2,1} & X_{2,2} & \cdot & \cdot & X_{2,p-1} \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ 1 & X_{n,1} & X_{n,2} & \cdot & \cdot & X_{n,p-1} \end{array} \right] * \left[\begin{array}{cccccc} B_0 & \cdot & \cdot & \cdot & \cdot & B_{p-1} \end{array} \right] + \left[\begin{array}{c} \varepsilon_1 \\ \varepsilon_2 \\ \cdot \\ \cdot \\ \cdot \\ \varepsilon_n \end{array} \right]$$

Beta estimates for wavenumbers (X)

Spectral data was related to reference ages using a PLSr model. When taken in a univariate (single response) context, as is the case with our model, the PLSr model is similar in concept to the Principle Components Regression (PCR) as it functions by reducing dimensionality of the *X-matrix* to factor loadings and principle component scores, describing the dominant gradients of variation in the spectra across orthogonal principle components. Because of this, PLSr is robust to multicollinearity and a large predictor space

p in the X -matrix. Therefore, the following PLSr model was used to relate spectral data to ages:

$$\begin{aligned} T &= XW \\ Y &= TP^T + E \end{aligned} ,$$

where

$X = n \times p$ matrix of predictors (absorbances)

$T =$ matrix of factor scores from X

$W =$ matrix of factor weights from X

$P =$ matrix of factor loadings for T

$Y =$ vector of responses (ages)

$E =$ model error of T and P fitted to Y .

PLSr models were used for fitting otolith spectra to ages for the years 2016 and 2017 and then validated. For each year, spectral data were partitioned into a testing (70% of n) and training (30% of n) data set for out-of-sample model validation. This validation method was chosen over leave one out (LOO) cross validation as our sample size was large enough and model performance would be tested this way if FT-NIRS were to be operationalized in the future. With future out-of-sample validation, a reader would test a proportion of model-estimated ages. In this case, FT-NIRs would be the primary age reader and a reader would be used as a tester for bias and precision. LOO cross validations were also conducted and yielded similar results to the out-of-sample validation. Validation was used to estimate performance statistics such as r^2 , square root mean squared prediction error ($RMSPE$),

residual predictive deviation (*RPD*), and *Bias*, and most importantly, between reader percent agreement (*%agreement*). These statistics were used to determine how well the models estimated ages when compared against traditionally aged specimens.

RESULTS

Validation of the PLSr models against traditionally derived reference ages yielded high performance ($r^2 = 0.88$ and 0.85) for 2016 and 2017, respectively. *Bias* in model estimated ages was low for both years (*Bias* = -0.0417 and -0.00151) and fit between model and reference ages was nearly 1 to 1. This approach can be expected to predict fish age within ± 0.6 years of the reference age 67% of the time (*RMSEP* = 0.59 and 0.60), in a fraction of the time that traditional ageing is conducted (Fig. 2). Furthermore, results showed that precision among two readers (66 and 64% agreement) was comparable to precision among traditional read ages and PLSr model estimates (64 and 65% agreement), within the two study years (2016 and 2017), with nearly identically skew in not agreed upon estimates (Fig. 3).

DISCUSSION

As the results of this study have illustrated, estimating ages of fish from otoliths with FT-NIRs and PLSr models shows promise in terms of precision and increased efficiency. In closing, however, some substantial considerations need to be made before moving forward. These considerations fall under the auspices of research to 1) develop better modeling

techniques to develop a multiyear global model using FT-NIRs, 2) better understanding the mechanisms of FT-NIRs as a tool for measuring ages, 3) parse out the dominant sources of variability leading to bias and imprecision in age estimation both in the spectra and by the reader, and 4) test how this new age data type might change the way we view and interpret stock assessment models.

One of the goals at the AFSC Age and Growth Program is to implement FT-NIRs as an operational tool for generating age data in a fraction of the current method's time. This would require development of a comprehensive and flexible multiyear age estimation model. The PLSr method, though it has shown promise, assumes a normally distributed *Gaussian* response variable. Age is estimated as an ordinal data type in most agencies and, therefore, lends itself best to modeling under a multinomial distribution. Therefore, it may be worthwhile to consider developing a Multinomial Logistic model which, like PLSr, functions on reducing the spectra to factor loadings and principle components. In addition, a Mixed Effect Model might be worth considering to capture the stochastic nature of modeling across many random year effects. A model of this sort might also allow for greater flexibility in adding biological covariates, unique to each species, which might absorb some of the unexplained variability in the spectra.

Among studies that have sought to investigate new methods for ageing fish, all have involved some proxy for counting annuli. This make FT-NIRs unique in that it tries to age fish without direct analysis of annual growth patterns. This raises question about the legitimacy of the method's functional mechanisms. Is the amplitude of spectral signatures simply a function of otolith mass, or is there a clear chemical mechanism more closely

related to age and development? Understanding this is key to understanding how FT-NIRs should be used in the future.

When readers estimate ages, bias and precision are also estimated. This shows that there is uncertainty (variability) in the ageing process (Kimura and Lyons 1990). A second mode of variability exists in the spectra itself, with at-age spectral signatures varying significantly between years, among regions, and across biological gradients. This means PLSr models have two sources of variability (ϵ_1 and ϵ_2) that are not as of yet fully understood. A study with known- age specimens across multiple species could help us estimate these two sources of variability, which may be unique to each species.

Finally, the resolution and precision of age data is important for reliable growth estimates in a population. The effect that a new data type might have on these estimates, and subsequent stock assessments, needs to be understood. Ages simulated from different distributional properties (different bias and error), as well as traditionally and FT-NIRs derived ages, could be plugged into different growth models. This would allow us to do a sort of sensitivity analysis in growth models before a new data type is operationalized into management.

With these questions answered, FT-NIRs could provide a promising revolution to the future of age estimation in fisheries programs around the world.

ACKNOWLEDGMENTS

The authors would like to thank the team at the AFSC's Age and Growth Program for collaboration and help with this project. We extend a special thank you to Irina Benson and Jason Erickson for hours of help and instruction on using OPUS® and other spectroscopy software packages for data analysis.

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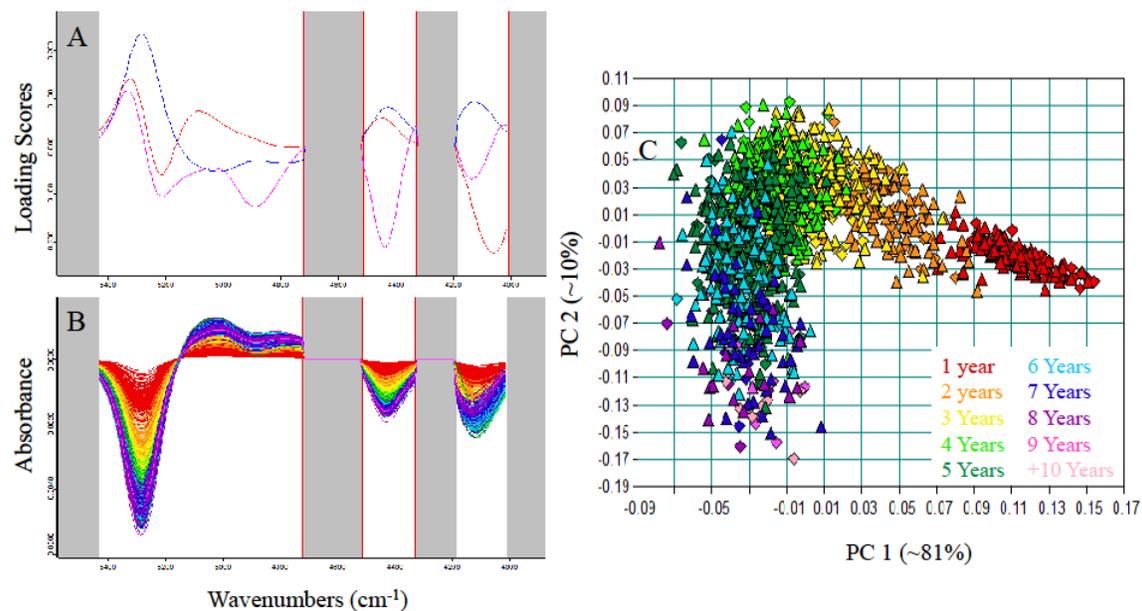


Figure 1. -- Processed spectra was first derivative transformed. Eigenvectors loading scores plotted against wavenumbers in the first three principle components are used to detect heavily loaded wavenumber covariates (A). First derivative transformed spectra are colored by ordinal ages to visualize spectral regions with good separation by age (B). Principle Components Analysis shows the ordination of spectra and the discrimination among ages in *p*-dimensional space (C). Data visualization was done in OPUS® chemometric software.

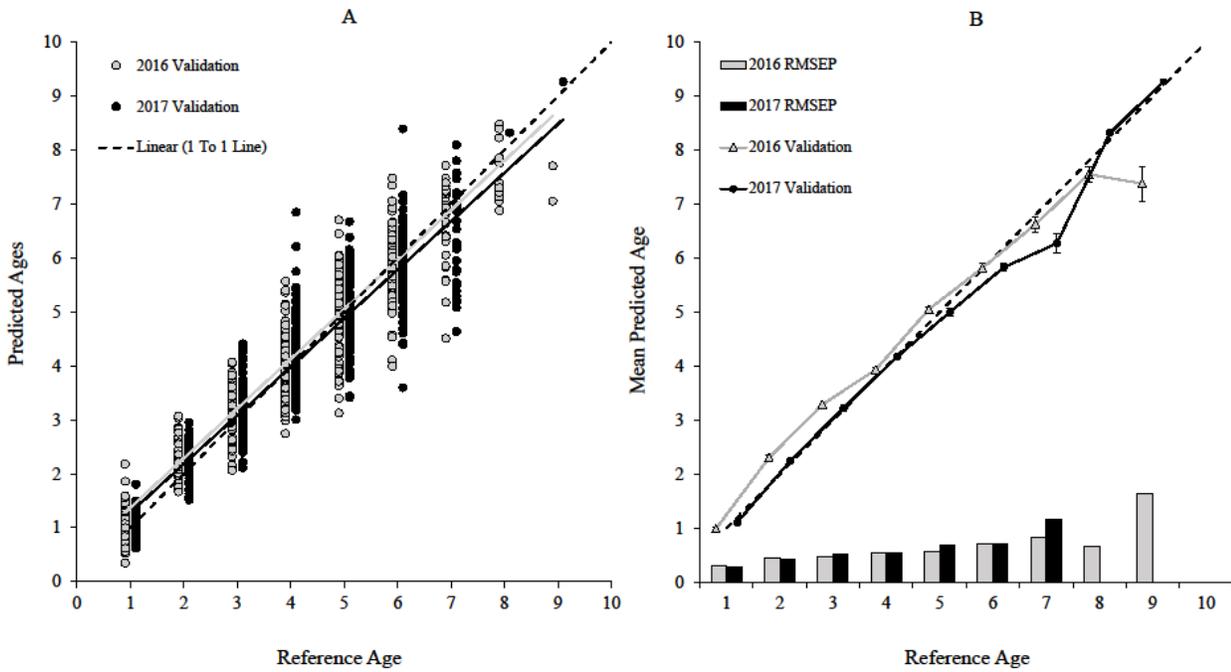


Figure 2. -- PLSr test validation results for 2016 ($R^2 = 0.8819$, $RMSPE = 0.5864$, $RPD = 2.87$, $Bias = -0.0417$) and 2017 ($R^2 = 0.8511$, $RMSPE = 0.6053$, $RPD = 2.57$, $Bias = -0.00151$) Bering Sea BTS data. Figure shows predicted ages (A) and model averages with standard error (B) compared against reference ages. Models were developed and validated on a 50:50 split of the data within each survey year (2016: $n = 1,526$, 2017: $n = 1,272$).

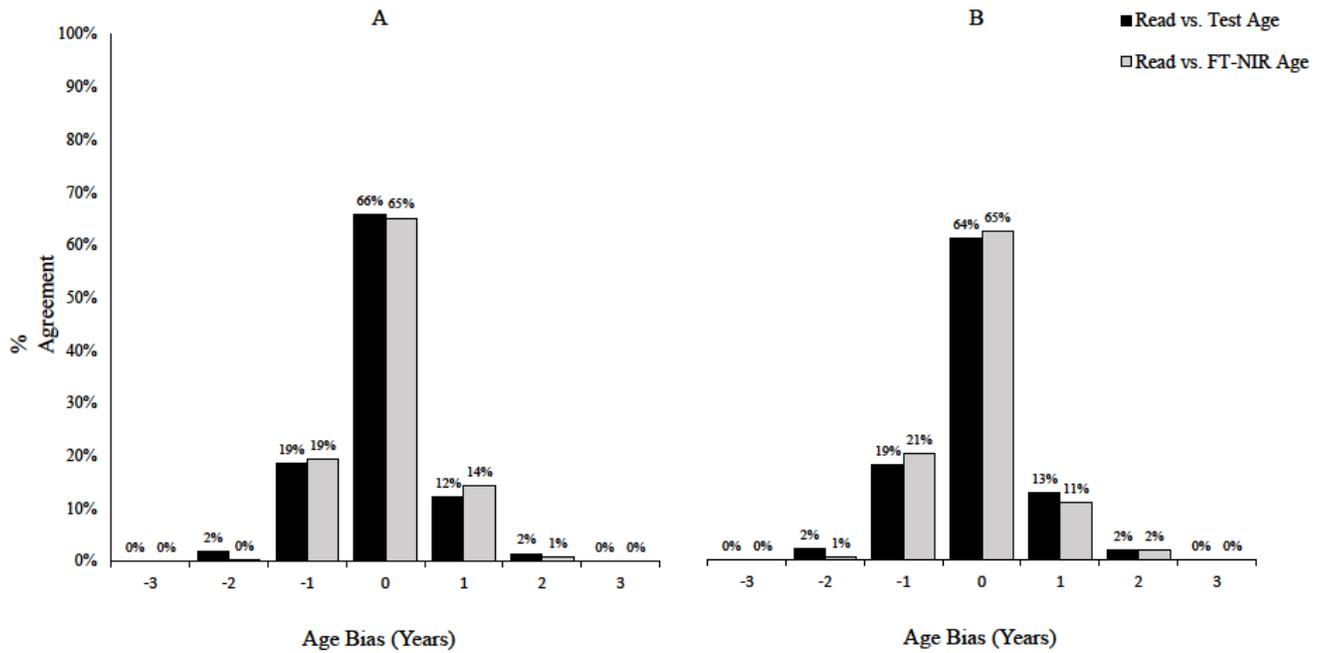


Figure 3. -- Percent agreement and bias between traditional reader and tester ages (black) and between the PLSr models and reader reference ages (textured grey) for 2016 (A) and 2017 (B). These bias agreement plots show how well the FT-NIRs method works compared to the traditional ageing method.

**Ageing Chinook Salmon (*Oncorhynchus tshawytscha*) Otoliths
Using Fourier Transform Near-Infrared Spectroscopy**

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ABSTRACT

Demographic information, such as age, is critical to the conservation and management of ecologically and commercially important fishes. In this study, we investigate the accuracy of otolith ages for Chinook salmon (*Oncorhynchus tshawytscha*) derived using Fourier Transform-Near-Infrared Spectroscopy (FT-NIRS). We focus on Chinook salmon raised at the Priest Rapids Hatchery on the upper Columbia River and released as subyearlings that returned to the hatchery and spawning grounds in 2012 (n = 400) and 2017 (n = 274). We use a combination of known ages based on Coded Wire Tags and Otolith Thermal Marks and partial least squares regression to generate calibration models and evaluate FTNIR otolith ages from test sets. Four separate models and test sets were evaluated: 2012 return year, 2017 return year, combined 2012 and 2017 return year, and 2017 return year with five mixed stocks from the upper Columbia River. For both calibration models and test sets, root mean square error ranged from 0.31 to 0.36, r^2 ranged from 71.06% to 85.26%, and relative percent difference ranged from 1.88 to 2.60. Symmetry tests and bias plots indicated that FT-NIR derived ages for test sets, were not biased except for the mixed stock test set from 2017 (Evans Hoenig Test $p = 0.02$). Percent agreement between known age and FT-NIRS age ranged between 81.53 and 85.11% and agreement was lower for female versus male Chinook salmon (76.40% vs. 90.70%, respectively). Results from this study indicate that ages derived from FT-NIRS are accurate and may be less biased than traditional ages derived from scale analysis of spawning Chinook salmon. Further work investigating spectral differences between male and female Chinook salmon, and different stocks of origin is needed to understand the applicability of

FT-NIRS for determining ages used in run reconstruction and forecasting of Chinook salmon stocks.

INTRODUCTION

Chinook salmon (*Oncorhynchus tshawytscha*) are an anadromous and semelparous fish broadly distributed from Alaska to California (Myers et al. 1998). Chinook salmon generally spend 0-1 years in fresh water followed by several years at sea before returning to spawn in their natal stream (Healey 1991). In Washington State, age composition of Chinook salmon is annually determined for both hatchery and natural populations throughout Puget Sound, coastal Washington, and the Columbia River. Scales are a common structure used for ageing Chinook salmon and age determinations based on scales are cost-effective, but they may vary widely in accuracy (Godfrey et al. 1968, McNicol and MacLellan 2010). For some stocks and age classes, scale resorption during the spawning migration may result in inaccurate age estimates, and in turn, bias run reconstruction and forecasting statistics. For example, ages of spawning lower Columbia River fall Chinook salmon in return year 2015 were highly accurate (97.70%), while accuracy of the upper Columbia River bright summer/fall Chinook salmon was 78.51% (A. Claiborne, WDFW, personal communication). Although corrections to data are made using known ages from physical tags during each assessment, an alternative approach would be to investigate new ageing methods that reduce observational error.

Fourier Transform Near-Infrared Spectroscopy (FT-NIRS) is an established analytical tool used in material testing that has more recently been used to determine fish age of

several species (Wedding et al. 2014, Robins et al. 2015, Rigby et al. 2016, Helser et al. 2019). Briefly, FT-NIRS measures the absorption of near-infrared energy in a sample, which corresponds in part to the quantity of protein in the sample. Otoliths are part of the audio sensory system in fishes and are composed of calcium carbonate, trace elements, and protein molecules that are accreted throughout a fish's life (Campana 1999). As such, FT-NIRS of otoliths may be used to determine age in both freshwater and marine species of fish (Robins et al. 2015, Helser et al. 2019). FT-NIRS may be useful for ageing otoliths of Chinook salmon, but it has not yet been evaluated. In this study, we use a collection of known age fish to evaluate the accuracy, precision, and bias of otolith ages determined using FT-NIRS for a single hatchery stock of Chinook salmon from the Columbia River, USA.

METHODS

Sample Collection

In this study, we focused primarily on the Priest Rapids Hatchery stock, which is released as subyearlings during their first year of life (age-0). Approximately 7 million juveniles are released with otolith thermal marks and 1 million fish are released with coded wire tags (CWT) and otolith thermal marks annually (Regional Mark Processing Center, <https://www.rmpc.org/>), providing a collection of known ages. The Priest Rapids Hatchery stock is part of the federally managed Upper Columbia Summer/Fall Evolutionary Significant Unit and is an important stock in ocean fisheries with significant natural and hatchery production (Myers et al. 1998). Spawning adults from the Priest Rapids Hatchery are sampled on the spawning grounds of the Hanford Reach of the Columbia River and at

the Priest Rapids Hatchery facility. Each return year, based on the approximate run size at lower river dams, the spawning grounds and Priest Rapids hatchery are sampled systematically (e.g., 1 in 10) with the goal of sampling at least 500-1,000 individual fish from various locations. Chinook salmon were sampled for CWT, scales, otoliths, length, adipose fin presence or absence, and sex.

Known Ages

In this study, we define age using the Gilbert-Rich age notation (N_N) for Pacific salmon (*Oncorhynchus* spp.) where N = the year of life of the fish (Gilbert and Rich 1927). For simplicity the subscript N is not shown since all fish were released as subyearlings. In order to create FT-NIRS calibration models and evaluate test sets, we used origin and age information obtained from otolith thermal marks and CWTs from Chinook salmon sampled on the spawning grounds and at Priest Rapids Hatchery. Briefly, otolith thermal marking is a method of mass marking that uses changes in ambient water temperature during the egg and post-hatch stage to apply unique barcodes in otoliths that can be used to identify age and origin with high degree of accuracy (Volk et al. 1994, Volk et al. 1999). After marking and the release of juveniles from the hatchery, otoliths are extracted from adults, ground, and examined to identify the thermal mark (Volk et al. 1999). CWTs are a small magnetic tag inserted into juveniles that are later recovered in fisheries, hatcheries, and on the spawning grounds (Jefferts et al. 1963, Nandor et al. 2010). Each tag corresponds to a batch identifier that indicates the hatchery of origin and age of the fish among other attributes. Similar to the otolith thermal mark, we use CWT age as a known to evaluate the accuracy of

spectral ages. We selected all fish with a positive CWT and/or otolith thermal mark available in 2012 and 2017 for FT-NIRS analysis (2012 n = 400, 2017 n = 274).

Spectroscopy

In this study, one otolith per fish was used to interrogate thermal marks and the second whole otolith was used to collect spectral data from otoliths using FT-NIRS. We did not consistently use the left or right sagittal otolith for spectral data or thermal mark interrogation. Otoliths were presented to the spectrometer clean, dry, distal side up and anterior left in the sample window. Spectral data were collected with the TANGO FT-NIR spectrometer and analyzed using Opus/Quant2 software from Bruker. All spectral analysis was completed at the National Marine Fisheries Service, Alaska Fisheries Science Center's Age and Growth Laboratory in Seattle, Washington. Calibration and test samples were selected using Principal Components Analysis and variable selection was done using Bruker's optimization algorithm and manual selection (Helser et al. 2019). Data was preprocessed by a first derivative transformation and Partial Least Squares Models were built with calibration samples and validated with independent test sets. The FSA package (Ogle et al. 2019) in R (R Core Team 2017) was used to test symmetry of known versus FT-NIRS ages using the Evans-Hoenig Test and to generate bias plots, percent agreement and average percent error index (Beamish and Fournier 1981). We also compare known ages to traditional age determined using scale analysis for run year 2012.

RESULTS

Prediction of test sets indicated that FT-NIRS predicted known age within approximately a third of year 67% of time, indicating a high level of precision and accuracy (Table 1). For both calibration models and test sets, root mean square error ranged from 0.31 to 0.36, r^2 ranged from 71.06% to 85.26%, and relative percent difference ranged from 1.88 to 2.60 (Table 1). Symmetry tests and bias plots indicated that FT-NIR derived ages for test sets were not significantly biased except for the mixed stock test set from 2017 (Fig. 1). Percent agreement between known and FT-NIRS age ranged from 80 to 85% for test sets and APE was consistently under 2.5% (Fig. 1). Overall, percent agreement was lower for females than males (76.40% vs. 90.70%, respectively) indicating some spectral variation related to sex. For run year 2012, traditional age estimates determined using scale analysis had relatively similar precision and accuracy but scale ages were significantly biased (Fig. 1).

CONCLUSION

Results from this study indicate FT-NIRS ages for Chinook salmon otoliths were similar to true age ~85% of time for two run years. We observed that FT-NIRS ages are as accurate as scale ages for this stock and FT-NIRS showed less bias in older age classes compared to scales. We observed significant bias in FT-NIRS ages related to sex where females were under-aged more often than males. Finally, models incorporating multiple

stocks, showed similar accuracy as predictions for just Priest Rapids Hatchery, indicating ages for mixed stocks fisheries and on spawning grounds may be robust.

In this study, we found ages determined using FT-NIRS were as accurate as traditional scale analysis but were less biased than scale estimates for older age classes. Resorption of scales in all species of Pacific salmon occurs during the anadromous migration and is related to sexual maturation and energy used in the upstream migration when fish are primarily fasting (Chilton and Bilton 1986). In Atlantic salmon (*Salmo salar*), resorption of scale minerals (e.g., calcium) occurs through osteoclastic activity (Persson et al. 1998) and is greater in males than females (Kacem et al. 2013). From our observations, the degree of resorption in Chinook salmon varies among spawning populations, run timings, between sexes, and generally increases with increasing freshwater migration distance and time in fresh water. Scale resorption likely influences scale age assignments by reducing the number of winter annuli visible on the scale, and therefore causing the age reader to under-age older age classes in particular. Otolith ages determined using FT-NIRS may be particularly valuable for collections that are known to have significant scale resorption and inaccurate scale ages for older fish.

Results from this study found that age was less accurately predicted for females than males using FT-NIRS, indicating some spectral variation related to sex. Previous studies have found that FT-NIRS calibration models may vary by species, geographic location, season, and year and are likely due to underlying differences in otolith chemistry (Robins et al. 2015). Physiological factors such as growth and reproduction may influence elemental incorporation in otoliths (Campana 1999, Sturrock et al. 2015). For example, trace element otolith and blood plasma chemistry may vary between female and male

fishes during reproduction (Sturrock et al. 2015). Future studies should investigate otolith spectral variation between males and females and the underlying chemical differences.

In conclusion, this study indicates that otolith ages for Chinook salmon derived from FT-NIRS are accurate and may be useful for populations where traditional methods may not be feasible. Further studies incorporating other Pacific salmon species, stocks, natural populations, and a range of freshwater ages (i.e., yearling migrants) will help understand the applicability of FT-NIRS more broadly.

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Table 1. -- Partial Least Squares models statistics for four calibration and four external validation test sets. Also shown is return year, and description of each model including sample size, origin, and age range for each set. Root mean square error (RMSE), r^2 , and residual prediction deviation (RPD) are shown.

Calibration set					Test set			
Return year	Description	RMSE	r^2	RPD	Description	RMSE	r^2	RPD
2012	PRH, n = 138, age-3-5	0.32	85.26	2.60	PRH, n = 249, age-3-5	0.36	80.32	2.26
2012 & 2017	PRH, n = 245, age-2-6	0.36	80.99	2.29	PRH, n = 343, age-2-5	0.37	77.25	2.10
2017	PRH, n = 95, age-2-6	0.31	83.91	2.49	PRH, n = 94, age-2-5	0.34	75.75	2.04
2017	5 stocks, n = 138, age-2-6	0.31	82.13	2.37	4 stocks, n = 131, age-2-6	0.36	71.06	1.88

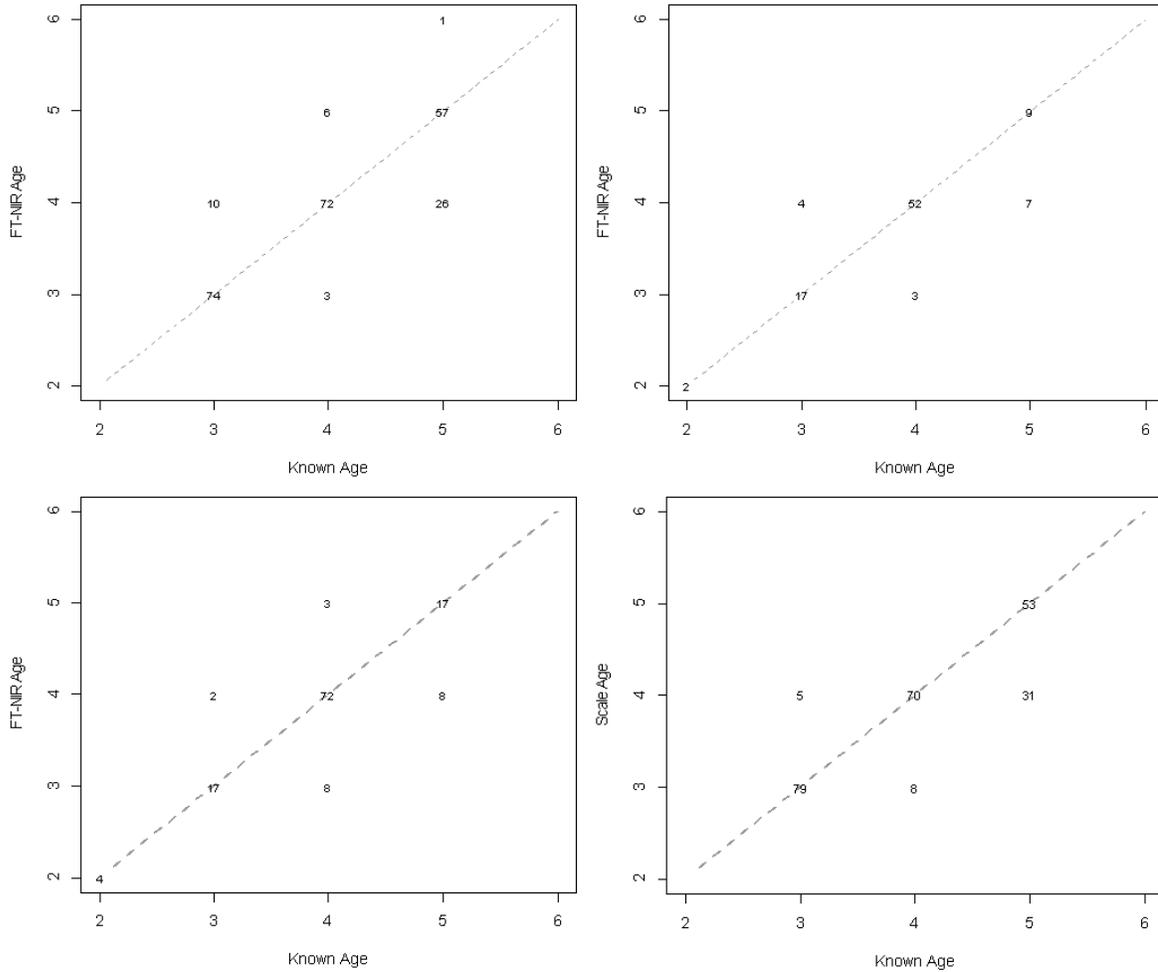


Figure 1. -- Bias plots showing the number of FT-NIRS ages per known age from external validation test sets for return year 2012 (A), return year 2017 (B), and mixed stock of origin (C). Known age versus scale age for return year 2012 is also shown (D). PA = percent agreement, APE = average percent error, and P = the significance of the Evans-Hoenig symmetry test for each comparison.

Fourier Transform Near-Infrared (Ft-Nir) Spectroscopy Ageing of Eastern Bering Sea Walleye Pollock (*Gadus chalcogrammus*) Otoliths

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INTRODUCTION

Age data are critical for understanding population dynamics of commercially fished species and providing management advice. The demand for fish ageing is increasing steadily while production capacity stays the same. To bridge this gap, new technology is needed to derive ages from fish otoliths. The traditional ageing of eastern Bering Sea (EBS) walleye pollock (*Gadus chalcogrammus*) otoliths require handling time in the process of cutting and burning to enhance growth zones and microscopic examination. One advantage of using FT-NIRS over traditional method of ageing is the increase in speed of generating age of each otolith, which can be analyzed whole in about 50 seconds. Recent research in Australia and USA demonstrated that FT-NIRS can be used to age saddletail snapper (Wedding et al. 2014), Australasian snapper and barramundi (Robins et al. 2015) and walleye pollock otoliths (Helser et al. 2019). This study extends on the research by Helser et al. (2019) by investigating geographic variability in EBS walleye pollock otoliths collected in 2016, in addition to analyzing data for 2016-2017 combined. Additionally, von Bertalanffy parameters estimated with FT-NIRS method and traditional ageing method are compared.

METHODS

Otolith Samples

Walleye pollock otoliths were collected during the AFSC's 2016 and 2017 EBS shelf bottom trawl survey (BTS). All otoliths were stored in glycerin-thymol solution. Prior to scanning, otoliths were removed from vials and patted dry with a laboratory tissue to remove glycerin-thymol solution.

Spectroscopy

Diffuse reflectance measurements from all otoliths were obtained using a Bruker Optics TANGO R spectrometer. Each otolith was covered with gold-coated reflector stamp and scanned at 0°, 45°, and 90° orientation with a concave up position. Each final absorbance spectra was acquired from averaging 64 scans at a resolution of 16 cm⁻¹.

Data Analysis

For each year of collection, specimens were divided into north and south EBS groups by the hot spot analysis of fish condition indices based on the calculation of the Getis-Ord G_i^* statistic (ArcGIS Pro 2.3.0). Calibration models were built for each individual group and for all groups combined. Partial least squares regression analysis was used to generate calibration models. All spectra were preprocessed using first derivative with a 17-point Savitzky-Golay smoothing. To assess the performance and robustness of the models, we calculated the coefficients of determination (r^2) of cross validation and external validation, model bias, slope, offset, and the residual prediction deviation (RPD). In addition, the mean

errors of prediction for cross validation and external validation were calculated as the root mean square error of cross validation (RMSECV) and the root mean square error of prediction (RMSEP), respectively. To assess the accuracy between reference and predicted age estimations, we calculated the frequency of the differences as a measure of relative bias. To aid comparison between reference and predicted ages, Von Bertalanffy parameters were estimated. Different scanning orientation of otoliths was analyzed by examining covariance of predicted age on reference age with orientation as a class variable. Data processing and analysis were conducted using the chemometric software OPUS (version 7.8, Bruker Optics) and R statistical computing and graphics software (version 3.2.4).

RESULTS

No statistically significant difference was found for different scanning orientation of otoliths. Calibration and validation results for all data sets are presented in Table 1. The r^2 ranged from 86 to 96 for calibration and 82 to 95 for validation data sets. RMSECV ranged from 0.65 to 0.97 while RMSEP ranged from 0.64 to 1.07. Figure 1 shows calibration models and their validation for 2016 North, 2016 South, and 2016 North and South combined data sets.

A comparison of predicted ages for all specimens combined to the traditional ages indicated that relative bias was minimal for ages 9 years or less in both cases. Age class distribution and von Bertalanffy parameters estimated with reference and predicted ages suggested that both data sets are similar. In 2016 ages a strong 4 year-old year class was

noted by both ageing methods. However, reference-ageing method showed an 8-year-old year class while predicted ages did not form the same dominant age class.

DISCUSSION

FT-NIRS provides rapid non-destructive assessment of fish age with good precision. Subjectivity is reduced and repeatability increased due to the quantitative measurement properties of FT-NIR spectral absorbance in otoliths. However, FT-NIRS is not a primary method and depends on accuracy and precision of the reference method. Here, we demonstrate that walleye pollock age can be reliably predicted within ± 1 year of reference age. Developing protocols for data collection, model maintenance, and stock assessments are the next steps.

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Table 1. -- Prediction results for calibration and validation data sets.

Spectra (n)	Calibration							Validation						
	Data	R ²	RMSECV	RPD	Bias	Slope	Offset	Data	R ²	RMSEP	RPD	Bias	Slope	Offset
651	2016 North Only	96.16	0.646	5.11	0.002	0.961	0.25	2016 North	95	0.64	4.25	-0.082	1.008	0.041
697	2016 South Only	91.87	0.855	3.51	-0.005	0.929	0.436	2016 South	82	0.872	2.23	-0.205	0.831	1.211
1348	2016 North and South	93.26	0.78	3.85	0.002	0.935	0.398	2016 North and South	87	0.882	2.71	-0.195	0.956	0.442
518	2017 North Only	94.68	0.767	4.34	0.0007	0.949	0.331	2017 North	92	1.07	3.21	0.0265	0.891	0.699
513	2017 South Only	86.13	0.863	2.69	-0.004	0.864	0.851	2017 South	86	0.91	2.4	-0.044	0.887	0.718
1031	2017 North and South	88.79	0.966	2.99	-0.003	0.892	0.694	2017 North and South	89	1.04	2.8	-0.004	0.876	0.788
1995	2016 and 2017	90.58	0.826	3.26	-0.001	0.909	0.558	2016 and 2017	89	0.957	2.92	-0.05	0.864	0.867

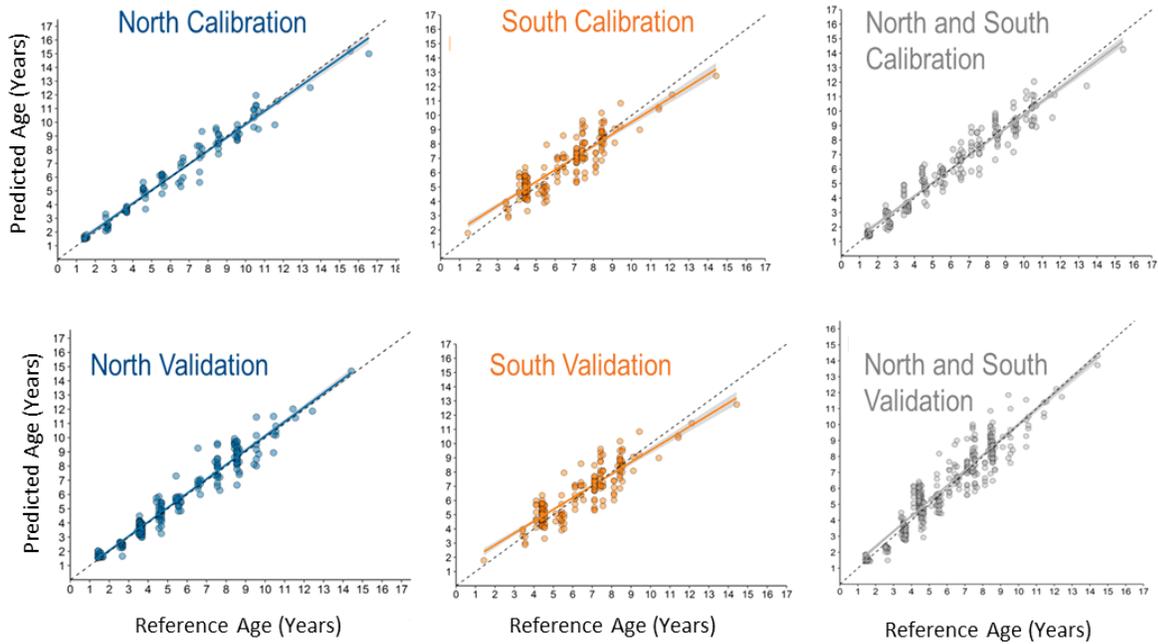


Figure 1. -- Calibration models for 2016 North (A), 2016 South (B), and 2016 North and South combined (C) data sets. Validation of calibration models for 2016 North (D), 2016 South (E), and 2016 North and South combined (F) data sets.

Precision and Accuracy Metrics for Ageing Qa/Qc:

What Is Behind the Numbers?

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ABSTRACT

Error in ageing fish from their hard parts, such as otoliths, is inevitable, requiring our enterprise to measure and reduce it whenever practical. For example, the annuli in scales of older fish become compact to the point they cannot be reliably distinguished even by an expert. This type of ‘process error’ has been demonstrated for many species so that few are still aged using scales. As a different example, inexperienced readers have difficulty estimating the same age from the same individual. This type of ‘observation error’ can be reduced with a training and testing cycle. In this extended abstract, we review the existing framework for evaluating paired age data, and propose how this may be of strategic use for adopting new technology, such as Fourier Transform Near-Infrared Spectroscopy (FT-NIRS).

We begin by distinguishing between quality assurance, quality control, accuracy and precision. Quality assurance (QA) is a matter of testing the reliability of a method, asking if it is both accurate (without bias to the true age) and precise (can produce the same age repeatedly). In a best-case but unusual QA scenario, estimated ages from processed hard parts are compared to known, paired ages for individual fish. Quality control (QC) is a matter of accrediting a set of samples aged using an accepted method. In production ageing operations, a common QC example is to re-estimate a subsample of ages from all the production fish ages, to check for repeatability before forwarding the production ages for use in a stock assessment. In this manner, precision (repeatability) is commonly checked but often for samples of which the true age is unknown.

The idea of evaluating paired data conjures up common statistical techniques, such as a paired t-test or use of the correlation coefficient, but these are not appropriate tests as discussed by Bland and Altman (1986) and McBride (2015). Instead, samples of paired age data are evaluated with three complementary approaches. First, the data are tabulated or graphed to illustrate patterns, second, indices of precision are calculated to evaluate repeatability, and third, tests of symmetry evaluate bias. We illustrate these approaches using a real data set from a small pelagic fish (Atlantic herring, *Clupea harengus*), a species with modest longevity; analyses use software such as Microsoft EXCEL (<http://www.nefsc.noaa.gov/fbi/age-prec>) or R (Ogle 2015; see also <http://derekogle.com/fishR/>).

In this example, 410 herring were aged from a 2017 survey and 85 fish were re-aged to test for repeatability. The production age is first compared to the test age in a 2-way table (left) and summary data are tabulated (right) as: the number of fish examined (n), number of reads for an individual fish otolith (R), the percent agreement of the independent reads (PA), Chang's coefficient of variation of the individual reads (CV), and the probability of asymmetry of mis-matched reads, using the Evans and Hoenig test of symmetry (Prob.) (see McBride [2015], for more details on each test).

Test age	Production age								<u>Parameter</u>	<u>Value</u>
	1	2	3	4	5	6	7	8		
1	4	•	•	•	•	•	•	•	<i>n</i>	85
2	•	6	•	•	•	•	•	•	<i>R</i>	2
3	•	•	17	•	•	•	•	•	PA	94%
4	•	•	•	17	2	•	•	•	CV	0.8
5	•	•	•	•	10	•	•	•	Prob.	0.65 (ns)
6	•	•	•	•	2	15	•	•		
7	•	•	•	•	•	1	7	•		
8	•	•	•	•	•	•	•	4		

Overall, these results are excellent, with 94% of the paired reads agreeing (on the diagonal of the 2-way table), a CV < 5, and no significant (ns) asymmetry of the mismatched reads. However, inspection of the 2-way table depicts uncertainty that appears at age 5 but disappears by age 7. The ageing expert notes that a check appears around age 5, which is difficult to distinguish from the new annulus formed at age 6, but that the check can be recognized at older ages which allows for resolution. With such high agreement and no asymmetry, this sample passes the quality control check; the stock assessment scientist is informed with an ageing error matrix, like the left table above, to use in the assessment model as appropriate.

This general process of QA/QC is relevant to development of FT-NIRS. Development of any new method will require the resulting new ages to be calibrated against independent fish ages from an established method. Confidence in these age estimates depends on the QA/QC results as described above. The use of FT-NIRS technology is also likely to propagate other errors into the final age estimates, from such sources as spectral error and model selection. In the case of a species like herring, FT-NIRS may not achieve gains in optimizing costs and reliability, because of the high repeatability of ageing herring and their relative inexpensive costs of processing (i.e., they can be aged from surface reads of

whole otoliths). Although herring is an extreme example, other species already have validated, highly repeatable age methods as well (i.e., haddock, *Melanogrammus aeglefinus*; Campana 1997, Baumann et al. 2013). However, at the other, less repeatable, extreme are fish like sablefish (*Anoplopoma fimbria*), which can have age-specific values of Chang's CV over 20 (Kimura and Lyons 1991). Here, we propose the use of precision metrics like Chang's CV to rank age repeatability across species, and together with cost estimates for hard part preparation, such a list can prioritize the species for which FT-NIRS is expected to maximize cost-benefits in production ageing.

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**Ageing Outputs in Stock Assessment in Queensland –
Fisheries Concerns Moving the Technology Forward**

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ABSTRACT

The Queensland Department of Agriculture and Fisheries, Australia (DAF) routinely collects length, age, and reproductive information for 15 species (*Lates calcarifer*, *Polydactylus machochir*, *Lutjanus erythropterus*, *L. malabaricus*, *L. carponotatus*, *L. sebae*, *Lethrinus nebulosus*, *Platycephalus fuscus*, *Acanthopagrus australis*, *Sillago ciliata*, *Pagrus auratus*, *Glaucosoma scapulare*, *Mugil cephalus*, *Scomberomorus commerson*, and *S. munroi*), processing in the order of 10,000 otoliths each year. Queensland has a well-established protocol for fish ageing. The exact ageing technique (whole vs. thin section) differs between species, but for all species includes reference collections, annual reader training and qualification prior to expert reading of current samples, centralized data recording and quality control measures (e.g., precision in the form of repeat reads, IAPE, and bias). In 2015, DAF completed a 'proof of concept' study to apply Near Infrared Spectroscopy to age fish (<http://www.frdc.com.au/Archived-Reports/FRDC%20Projects/2012-011-DLD.pdf>). Results were promising and indicated that NIRS could provide cost-efficiencies in routine fish ageing. However, Queensland has not adopted NIRS to routinely age fish due to the moderate cost-efficiency savings and issues with age accuracy and precision. In particular, the fisheries end-users desired to understand what NIRS measures in fish otoliths that is correlated with age and whether the correlation was a function solely of time or was confounded by growth. NIRS is a secondary method of determination and relies on the accuracy of the reference samples, in this case the age based on expert visual interpretation of otoliths. Therefore, age samples used in developing NIRS calibration and validation models should be well understood for their inherent properties as these will influence and

perpetuate through the NIRS predictive models. Inherent properties include age precision, accuracy and bias, as well any physical/chemical changes to the otolith that occur from differences in post-mortem handling (e.g., fresh vs. frozen) which may affect the NIR spectra. Additionally, fisheries biologists/scientists should be included in the spectra acquisition, processing and calibration model development, thus expanding the understanding of fisheries end-users of NIRS age estimates such that these estimates would not be viewed as 'black box' estimates. Predicted age of a fisheries species is not the end-product but is used in numerous subsequent analyses. Age estimates (as age frequencies) are used in catch curve analysis and hindcasting recruitment indices of cohort/year-class strength. 'Old fish' are often poorly estimated for age using NIRS and could be treated as outliers or as relatively unimportant because they occur at low frequency in fishery samples, fishery harvests or biomass estimates. However, such 'outliers' are important as they indicate that the NIRS model doesn't fit as it should and we should strive to understand why the model doesn't fit rather than exclude outliers (without good reason) from the calibration/validation dataset. Old fish (or the lack of them) can be an important indicator of age-truncation in heavily fished species and therefore should be important in NIRS model-fitting considerations. Young fish also may be poorly estimated for age using NIRS. In fisheries with highly variable recruitment, accurately aged young fish can indicate likely pulses in recruitment to the exploitable biomass, which may have implications for setting harvest limits (e.g., quotas). Age estimates are used in growth curves and are fundamental to stock assessment. Age (and ageing error) are just one of multiple data inputs into stock assessment. Precision and accuracy associated with NIRS estimates of fish age should be documented, but it should be kept in perspective that other data inputs to

stock assessments also have error (and assumptions and biases). The Strategic Initiative development team have wisely included fisheries assessment scientists in operationalizing FT-NIRS for age estimation.

**Stock Assessments, Age Data, and New Challenges
in Applying Ft-Nir Data**

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Supplementary Notes

Jim Ianelli is the lead stock assessment author for the walleye pollock resource of the eastern Bering Sea. He began by showing data inputs (abundance, catch, and biological information) for the pollock assessment and how these data sources are integrated in population models to produce numbers of fish at age to estimate recruitment. Due to the large numbers of pollock currently aged by traditional methods, the FT-NIRS approach could potentially convey a large time-savings that could be dedicated to other tasks. However, we first need to recognize the best ways to deal with ageing error. Stock assessments can handle observation error, but first we need to be able to adequately characterize accuracy and precision associated with age estimates. Preliminary studies suggest some differences between FT-NIRS and traditional ages; however, it may be possible to account for this error in the stock assessment model using a conversion matrix.

**Operationalizing Ft-Nirs Ageing Enterprise in the
National Marine Fisheries Service: a Conceptual Pathway Forward**

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Estimating the age of fish is of national relevance since many of the most valuable U.S. fish stocks are managed using age-structured assessment models. Age-structure for stock assessments tend to be data-intensive requiring many years of compositions from both fishery-dependent and -independent data collection efforts. Their net effect can lead to reduced uncertainty in catch projections and key population parameter estimates that support sustainable fishery management. Thus, fish age data play a vital role in identifying appropriate overfishing and annual catch limits. Age data are also essential for estimating growth—and its variability over time and space—and estimating recruitment success, which are key components for ecosystem drivers of population dynamics. Fish age is usually determined by visual microscopic counting of opaque and translucent growth bands from otoliths and other hard structures, which requires a large expense in human capital, equipment and quality control to ensure reliability of the data. The need for age data is exemplified by the fact that roughly 190,000 otoliths per year (5-year average) were processed and read for inclusion in NMFS stock assessments (T. Helser, AFSC, personal communication, 20 May 2017). Despite recent budget cuts, nationwide demand for age data continues to increase, to i) support the development of new assessments, ii) improve existing assessments through addition of historic data, and iii) support models with smaller spatial scale to better reflect genetic differentiation or the definition of management units (e.g., Pacific cod in the EBS and Aleutian Islands). With the demand for age data outstripping our capacity to deliver them using conventional means, new innovative approaches for fish age determination are needed.

Recently, Fourier transform near-infrared spectroscopy (FT-NIRS), which has widespread utility in the pharmaceutical, chemical, and petrochemical industries (Roggo

et al. 2007), has recently been applied to age fish otoliths (Wedding et al. 2014) and shark vertebra (Rigby et al. 2016). Preliminary analyses of saddletail snapper in Australia found that FT-NIRS predictive models could predict the age of fish with a high degree of accuracy leading to reduced subjectivity and costs per sample. Encouraged by their findings, the AFSC conducted a study on the application of FT-NIRS (using TANGO R from Bruker Optics) to age EBS walleye pollock otoliths collected during 2016-2017 trawl surveys. Study results (Helser et al. 2019) found a 96% coefficient of determination (r^2) between the reference ages (best age produced between two readers) and FT-NIRS ages from the spectral information in the otoliths. When comparing approaches, the FT-NIRS method had as good or slightly better precision than the standard method with little or no bias. Considering that a single fish age can be obtained in about 50 seconds using FT-NIRS versus 4-10 minutes using traditional methods, this technology holds the potential for significant nationwide efficiency gains and cost savings.

Fish otoliths are composed of calcium carbonate (CaCO_3) structures on a protein matrix; the quantity of which continually accumulates as fish grow older. FT-NIRS quantitatively measures the absorption of near-infrared energy (800 to 2,500 nm) by material containing covalent bonds such as the protein molecules in the otoliths. When excited by an energy source, the molecules begin to vibrate at characteristic frequencies, during which time the instrument is scanning the object and recording the amount of the energy being absorbed in the form of a near- infrared spectrum. The spectral information most meaningful for otoliths are the regions related to the molecular constituents (or functional groups) in proteins such as carbon-hydrogen (C-H), oxygen-hydrogen (O-H) and nitrogen-hydrogen (N-H) groups. As such, the chemical properties of the otoliths, such as

the quantity of the absorbed energy within those specific regions, are a proxy for fish age. Fish age is predicted using a multivariate calibration method called partial least squares (PLS) regression from the information contained in the spectral data of the otolith and the fish age (either known or estimated by the age reading analyst). Here, reference data represents the collection of ages from otoliths associated with spectra (absorbance at wavenumber). The entire model development process is complete when the predictive calibration model is assessed for performance (robustness) to predict a completely new set of representative unknown samples (*external validation*).

Our continued successful work both at AFSC on walleye pollock, Pacific cod, and yellowfin sole, and with scientists from the SEFSC – Panama City Laboratory on red snapper has also demonstrated that FT-NIRS age estimation has the potential to be widely implemented for ageing other marine species. However, additional research and development is needed before broad-scale operationalization of this technology can be applied to the enterprise of fish age estimation in National Marine Fisheries Service. While FT-NIRS ages can be thought of as a one-to-one substitution for traditional ages, they are fundamentally different, from a statistical sense. Therefore, investigation into three key questions related to the following is required: 1) performance (or robustness) of FT-NIRS calibration models to biological and environmental spatiotemporal variability; 2) integration of new FT-NIRS age data products, such as age compositions, into stock assessment models; and 3) best practices and standards developed for consistent data acquisition, quality control (statistical) metrics, and data products delivered to stock assessment scientists. This paper discusses the conceptual framework and lays out a

pathway for our efforts toward operational readiness of the FT-NIRS age estimation enterprise in National Marine Fisheries Service over the next 4-5 years.

APPROACH AND PRODUCTS

The roadmap to operationalizing the FT-NIRS ageing technology across the NMFS regional science centers is envisioned as three major related tasks that are staged at varying, overlapping time frames. These tasks include: 1) application development, 2) application implementation, and 3) stock assessment integration. Our project or Strategic Initiative Development Team (SIDT), consisting of National Marine Fisheries Service' scientists from AFSC (Seattle, WA), NWFSC (Newport, OR), SEFSC (Panama City, FL; Beaufort, NC), NEFSC (Woods Hole, MA), and SWFSC (La Jolla and Santa Cruz, CA). The ageing laboratories will be split into a west coast (AFSC, NWFSC, SWFSC) and east coast (NEFSC, SEFSC) application development centers. AFSC and SEFSC (Panama City) will serve as the hub for application development since newly purchased Bruker multi-channel MPA II spectrometers are housed at these centers (facilitating savings in travel costs to the project). To make this work relevant to already established age data production processes and age data use in stock assessments, the work will focus on 2 - 3 different managed fish stocks from each region with data covering a 5-year time frame. This will provide an opportunity to evaluate FT-NIRS performance across species with differing life history characteristics (e.g., short-lived vs. long-lived), and the inter-annual stability of calibration model parameters (Table 1).

Application Development

Application Development is already underway at AFSC, which began in 2019 with coordination and optimization of the FT-NIR (MPA II) spectrometer scanning parameters to assure consistency in spectral data acquisition. This coincided with both MPA II spectrometers delivered to AFSC in January of 2019, during which time, Beverly Barnett (SEFSC) began a 3-month NRAP assignment. This process involved assistance from the Bruker Applications Scientist on the project. Moreover, a 2-day workshop was held April 11th and 12th, 2019 in Seattle (these proceedings) which convened subject matter experts and practitioners of FT-NIRS, age reading experts and stock assessment scientists, to explore and discuss the potential application of this technology to the age reading enterprise. This multinational venue of scientists (including 2-3 subject matter experts) and SIDT members from seven of the National Marine Fisheries Service ageing laboratories served as a platform to initiate the project (see introduction of this report).

The general time frame of this project, which includes workflow and activities, is shown in Figure 1. Spectral data from otoliths from the three species from each center will be collected over the first half of the project. Sample presentation to the spectrometers and data acquisition parameters were discussed during the workshop and guidance is outlined in *Discussion Topics and Recommendations*. Simultaneously, ages will be estimated using the standard age determination practices employed by each laboratory. Thus, for each otolith there will exist an otolith spectra and an estimated age by the traditional approach. As typical for each laboratory, age reading precision will be calculated from a random sample of otoliths independently aged by another analyst. Calibration model development and model validation will begin when enough spectra has been collected for a reference

sample data set (traditional age estimates) for a given species. Reference sample sizes and selection approaches were also discussed at the workshop, and while specificity was not provided, guidelines for sample attributes were recommended (*Discussion Topics and Recommendations*). Helser et al. (2019) suggested a two-stage approach for model calibration and validation when first beginning to develop predictive models. They selected reference ages for calibration from a uniform sampling distribution over the age range of a given species, with the actual allocation by age and total sample size dependent on longevity. For the validation data they selected samples, roughly equivalent in number as the calibration, but randomly chosen and hence representative of the population of ages. For instance, the AFSC analysis of walleye pollock used a target of between 200 and 250 samples for calibration/validation representing approximately 10-15% of ages sampled on a typical survey. Other approaches to separating the calibration and validation data sets based on the spectral variation, without regard to age estimates, are often used in the chemometric literature. Helser et al. (2019) also employed this approach to separate calibration/validation samples but found little difference overall in model results for walleye pollock. This does not suggest that one approach is better than the other, and therefore, additional study is needed.

Model development and refinement is expected to continue as more spectra and age data are acquired (during years 1-3), and continue throughout the project, exploring model refinements, testing for predictive model robustness, and building upon innovations and best practices as they apply to the nuances of fish age estimation. For instance, model refinements might include, incorporating unobserved variation in otolith spectra over time, modification of the PLS model for nonlinearity in observational system, and improving

model performance with biologically known ages of fish. Testing model robustness may encompass evaluating predictive model performance in different spatial and temporal scales, particularly under circumstances where changing physiological and environmental conditions can play a role in altering the chemical constituents of the target samples (otoliths, vertebrae, gonad tissue, etc.). Unlike other disciplines where FT-NIRS is used, reference data in fisheries, such as age estimates, are not without errors, and in some cases, are biased. Therefore, innovation will play a role in model refinement where spectra associated with otoliths from fish with unknown (uncertain) and known ages (i.e., from tagging or other validation studies) can be incorporated into calibration models to simultaneously correct for ageing bias and maintain fidelity of prediction error. These topics are highlighted and discussed in more detail in the recommendation section (these proceedings).

Application Implementation

Application Implementation is the delivery of new data generated from the multivariate calibration models, with a focus on fault detection, process control, and preservation of predictive capabilities. The entire system process is dependent upon optimal instrument settings/parameters of otolith spectral data acquisition, appropriate sample sizes and sample distributions for model calibration/validation, a system of predictive-model updating for unusual or degradative performance, model diagnostic measures and intervention, and finally QA/QC reports to assessment authors. The successful application of this system requires a set of standardized procedures and best practices for the enterprise of generating FT-NIRS data products for inclusion in stock

assessments. Best practices are in part achieved from the extent to which that knowledge can be adapted across disciplines, studying success and failure of application in associated fields, and from experience in consistent application of methods of the technology in one's own field of study. Hence, it is crucial that all research and development efforts are coordinated and knowledge rapidly transferred among the seven National Marine Fisheries Service ageing laboratories. The workshop served the purpose as the initial framework for discussions among subject matter experts, practitioners of the technology and expectant innovators to define the goals, identify challenges, priority resources and research, and organizational structure and roles of responsibility. In this regard, AFSC-Seattle will serve as the NOAA-wide FT-NIRS fish ageing innovation center whose mandate is to 1) communicate and disseminate information to the other ageing labs; 2) support/evaluate issues related to data acquisition and model performance; 3) focus/prioritize research efforts among labs, and innovate technological improvements for lab and field-based data acquisition and software development in fisheries research. To facilitate AFSC-Seattle's leadership and communication among the National Marine Fisheries Service ageing laboratories a Virtual-Lab collaboration services webpage has been established (<https://vlab.ncep.noaa.gov/group/advanced-fisheries-research-in-fourier-transform-near-infrared-spectroscopy/welcome>). Additionally, each year the SIDT will meet for a workshop, which will round-robin at each ageing laboratory to further disseminate and transfer technological innovations and accomplishments.

Best practices can also be evaluated though more quantitative approaches using a simulation framework to characterize fault points, uncertainties and weaknesses in assumptions and structural aspects of the complete operational process. For instance, a

'known' operating model can be simulated, using a multivariate normal distribution of the dominant factor loadings of the spectral data, conditioned on each age category paired with a multinomial sampling distribution of traditional age data. New sets of spectra and age data can be generated from this system and the PLS regression (estimating model) will be evaluated with different sample sizes, sampling distributions, ageing precision, response functions (multinomial vs. normal) for different species. The performance measures could consist of a suite of diagnostic measures typically used in chemometric analysis (Wise and Roginski, 2015), and serve as a maintenance roadmap; the goal of which is to preserve or improve models over time under changing conditions with the least amount of cost and effort. For instance, Wedding et al. (2014) found that predictions improved from the base year model which subsequently updated new spectral variation moving forward in time, presumably because biological variability increased in the calibration set by including samples from different years. Discussion points in this regard included how much new data was needed for model updating, how frequently should updating be performed and what criteria should be used to choose samples for updating (i.e., based on spectral and/or age variation).

Stock Assessment Integration

Stock Assessment Integration is the critical end goal of operationalizing FT-NIRS ageing. Although the use of age data in stock assessments varies widely, our focus will be on integrated age-structured stock assessment methods that use either age-length keys to generate marginal age compositions each year or a conditional age-at-length structure for each year in the assessment. In either case, the goal is to conduct a side-by-side

comparison of the FT-NIRS and traditional age data on the assessment model outcomes, documenting the significance of differences in the state variables and benchmarks. Single set of performance measures or benchmarks to compare these data products will not work for all species/stocks, as each assessment is unique in the complement of data types, information content of data, and trade-offs with ages. However, a 5-year time depth of age data, as stated earlier, from both approaches is expected to produce an adequate “effect size” to examine model performance outcomes. In addition, the time-block where the new data are inserted into the time series of the particular assessment will depend on the availability of whole otoliths, historic or contemporary, that are available for analysis. Stock assessments are inherently most variable in state variable estimates near the terminal year, so it may be wise not to incorporate the new data segment in the most recent years if historical whole otoliths are available. As the recommendations state, each SIDT member will coordinate and work closely with assessment scientists at each center to provide both types of data products and in the format tailored specifically to any given assessment model. Members will work closely with assessment authors to provide the most informative measures of FT-NIRS ageing precision and reliability (QA/QC metrics) as these are often incorporated into stock assessments to account for age estimation uncertainty. However, estimation error from traditional ageing methods (between-reader precision) have different properties than prediction errors from FT-NIRS age estimation models and additional work will be needed to harmonize these statistical measures.

FT-NIRS represents a new and transformative technology in fisheries biology to rapidly, reliably and accurately estimate age from otoliths and possibly other hard structures. This report has demonstrated a broad scope of applications in fish ageing for

adult and juvenile life stages over a number of species within several large marine ecosystems. Moreover, it was also demonstrated that FT-NIRS may extend well beyond fish age estimation, to other potential applications of soft tissue for physiological parameters and well as responses of those parameters to stress in relation to changes habitat and climate. While not presented at this workshop, National Marine Fisheries Service scientists at AFSC have already shown preliminary success using FT-NIRS to determine reproductive status from ovary tissue for Pacific cod and northern rockfish, and are in the process of evaluating bioenergetics capacity from lipids in liver tissue. Research and development is solidly underway in a number of fisheries ecology applications. Operational readiness of production age estimation is still a few years out; however, the NIR technology of fish age estimation highlighted at the workshop was widely viewed favorably and could fundamentally change biological sample collection either at-sea or in the laboratory. Its use and comprehensive literature in other fields, new statistical techniques in modern instrumental analysis and number of successful applications for age estimation promise good potential for operational capacity for National Marine Fisheries Service. Exploration of this technology is responsive to the Stock Assessment Improvement Plan in that greater efficiency in generating age data should allow model improvements related to increasing historical time series, developing spatially-/sexually-explicit population models, and evaluating how ecosystem processes affect population dynamics and distribution.

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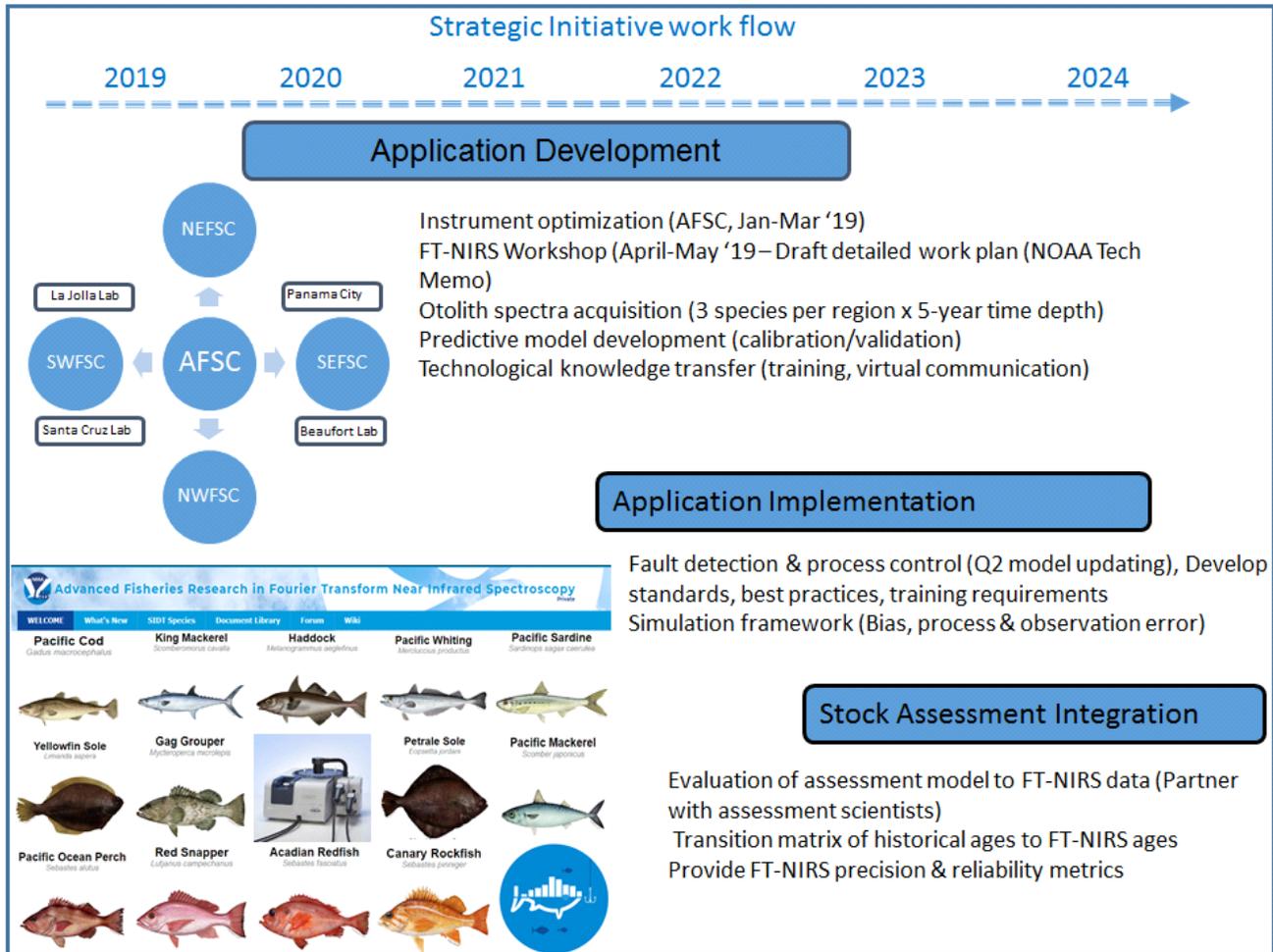


Figure 1. -- Strategic Initiative roadmap to operationalizing FT-NIRS ageing technology across science centers, including workflow, time frame, activities, and target species. The roadmap is envisioned as three major related tasks: Application Development, Application Implementation, and Stock Assessment Integration.

**Presentation of the Week: Ft-Nirs Multispecies Analysis
by the Strategic Initiative Development Team**

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INTRODUCTION

Applications of FT-NIR spectroscopy in fisheries science are currently limited with less than five known studies having focused on biological structures, such as otoliths or vertebrae, of marine fishes. Otoliths are biological structures located in the vestibular system of teleost fishes and are composed primarily of calcium carbonate (aragonite) deposited on a protein matrix (Campana 1999). Otoliths begin forming prior to hatching and continue to grow in three dimensions throughout the life of the fish, even when somatic growth is non-existent. The objective of this study was to examine the use of Principal Component Analysis (PCA) of Fourier transform near-infrared (FT-NIR) spectroscopy as a tool to differentiate between 14 marine fishes representing 13 different species from five large marine ecosystems (Fig. 1). Partial least squares models were also developed for selected species to illustrate that NIR spectra from otoliths can provide informative data for fish age estimation.

METHODS

FT-NIR spectra were acquired from fish otoliths ($n = 3,154$) that were sampled from five large U.S. marine ecosystems (Table 1; Fig. 1). Otoliths were blotted and air-dried prior to scanning. Spectral data from otoliths were collected on Bruker TANGO R or MPA II FT-NIR spectrometers. Diffuse reflectance measurements from all otoliths were obtained using a Bruker Optics TANGO R spectrometer. Each otolith was covered with gold-coated reflector stamp and scanned at 90° orientation with a concave up position. Each final

absorbance spectra was acquired from averaging 64 scans at a resolution of 16 cm⁻¹. Chemometric software Solo v8.7 (Eigenvector Research, Inc.) was used for data preprocessing and evaluation. Selected spectral regions were in the 7,500 to 4,000 cm⁻¹ range. Spectral data were preprocessed with 17-point Savitzky-Golay smoothing (2nd order polynomial), first derivative transformation, and mean-centering. Principal component analysis was used to evaluate the extent of spectral variability across species and ecosystems.

Calibration models were built for each species. Partial least squares regression analysis was used to generate calibration models. To assess the performance and robustness of the models, we calculated the coefficients of determination (r^2) of cross validation and external validation, model bias, slope, offset, and the residual prediction deviation (RPD). In addition, the mean errors of prediction for cross validation and external validation were calculated as the root mean square error of cross validation (RMSECV) and the root mean square error of prediction (RMSEP), respectively. Data processing and analysis were conducted using the chemometric software OPUS (version 7.8, Bruker Optics) and R statistical computing and graphics software (version 3.2.4).

RESULTS

Absorbance spectra of all otoliths were consistent among the different species, as well as with previous studies examining fish otoliths (Wedding et al. 2014, Robins et al. 2015, Helser et al. 2019). All combination of species were analyzed with three principal components (PC) from the PCA. Most variation in the spectral data was described by PC1

and PC2. PC1 (78.80%) described variation in the size of the otoliths separating small coastal pelagic species from flatfish and demersal fishes (Fig. 2). After excluding the small coastal pelagic species Pacific sardine (*Sardinops sagax*) and Pacific mackerel (*Scomber japonicus*), PC1 (69.80%) still described variation in otolith size, while PC2 (22.97%) described the latitudinal variation of the ecosystem from which the species were collected (Fig. 3). The PCAs of spectral data from species collected from either west coast (Fig. 4A) or east coast and Gulf of Mexico (Fig. 4B-C) ecosystems showed the same otolith size (PC1) and latitudinal (PC2) variation as was observed when all species were modeled together (Figs. 2-3). PLS models performed well for Acadian redfish (Fig. 5), haddock (Fig. 6) and Pacific hake (Fig. 7). Calibration of otolith spectra to traditional age estimates had r^2 ranging from 0.92 to 0.96, with equally good performance with external validation samples ($r^2 = 0.91$ to 0.92).

DISCUSSION

Our analyses of otolith spectral data collected from 13 different marine fish species from five marine ecosystems demonstrate the potential applicability of FT-NIRS in fisheries science. The separation of species with different size otoliths, along with the separation of the ecosystems by latitude, may suggest interactions between fish physiology and environmental conditions that can be evaluated using FT-NIRS. The protein matrix in otoliths is composed of amino acids that have been shown to be related to fish diet and trophic food web structure (McMahon et al. 2011) that may have implications for selection of spectral regions, which could improve model performance. It is possible that fish diet in

the different ecosystems may be influencing the latitudinal separation observed in PC2.

Results from this study clearly suggest that further investigations are needed to determine applications of FT-NIRS to otolith chemistry and spatial variability.

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Table 1. -- Ecosystems, fish species, and sample size (n).

ECOSYSTEM	SPECIES	n
Eastern Bering Sea	Pacific cod (<i>Gadus macrocephalus</i>)	297
	walleye pollock (<i>Gadus chalcogrammus</i>)	300
	yellowfin sole (<i>Limanda aspera</i>)	297
North Pacific Ocean	Chinook salmon (<i>Oncorhynchus tshawytscha</i>)	297
	North Pacific hake (<i>Merluccius productus</i>)	294
	Pacific sardine (<i>Sardinops sagax</i>)	76
	Pacific mackerel (<i>Scomber japonicus</i>)	254
U.S. Gulf of Mexico	gopher rockfish (<i>Sebastes carnatus</i>)	113
	red snapper (<i>Lutjanus campechanus</i>)	297
	gag grouper (<i>Mycteroperca microlepis</i>)	357
South Atlantic Ocean	vermilion snapper (<i>Rhomboplites aurorubens</i>)	154
	gag grouper (<i>Mycteroperca microlepis</i>)	116
Northeast Atlantic Ocean	Acadian redfish (<i>Sebastes fasciatus</i>)	143
	haddock (<i>Melanogrammus aeglefinus</i>)	159

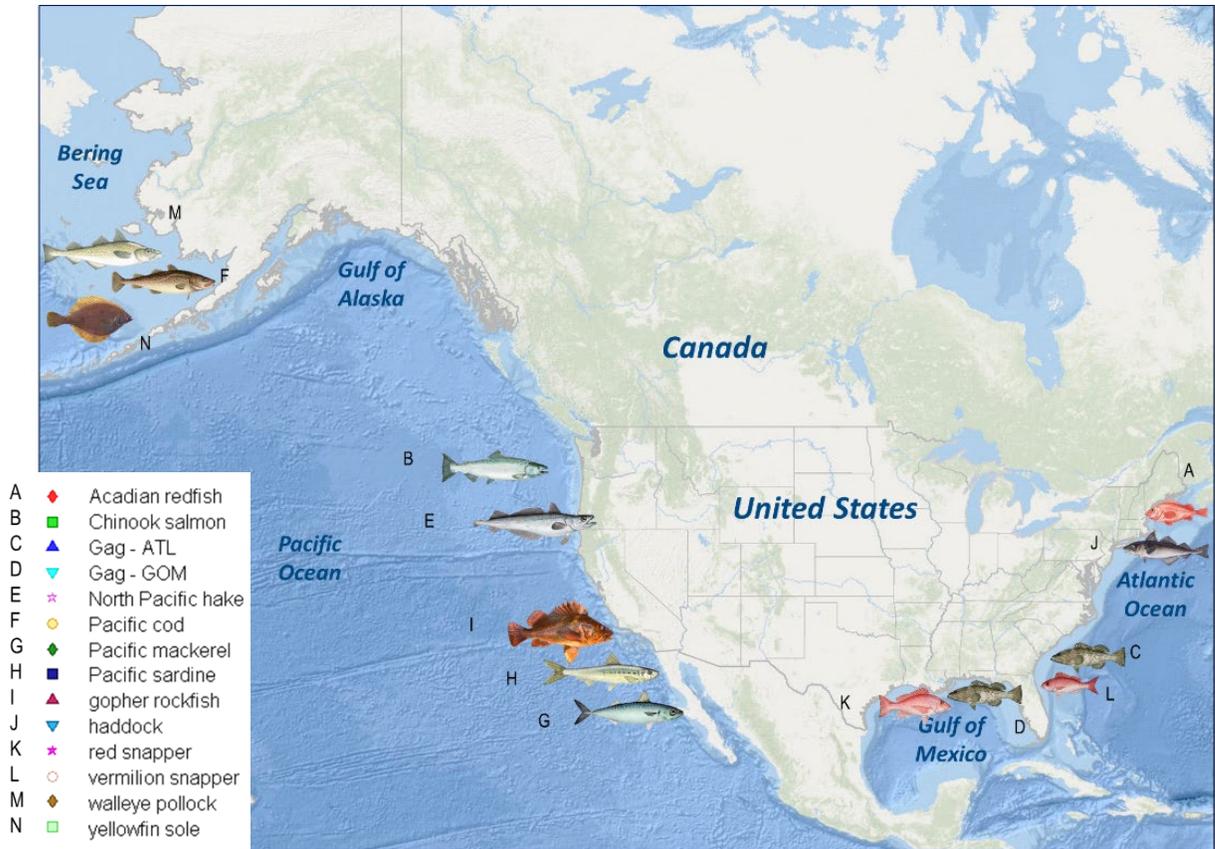


Figure 1. -- Location of fish collection sites and their ecosystems. Letters refer to species shown in the legend.

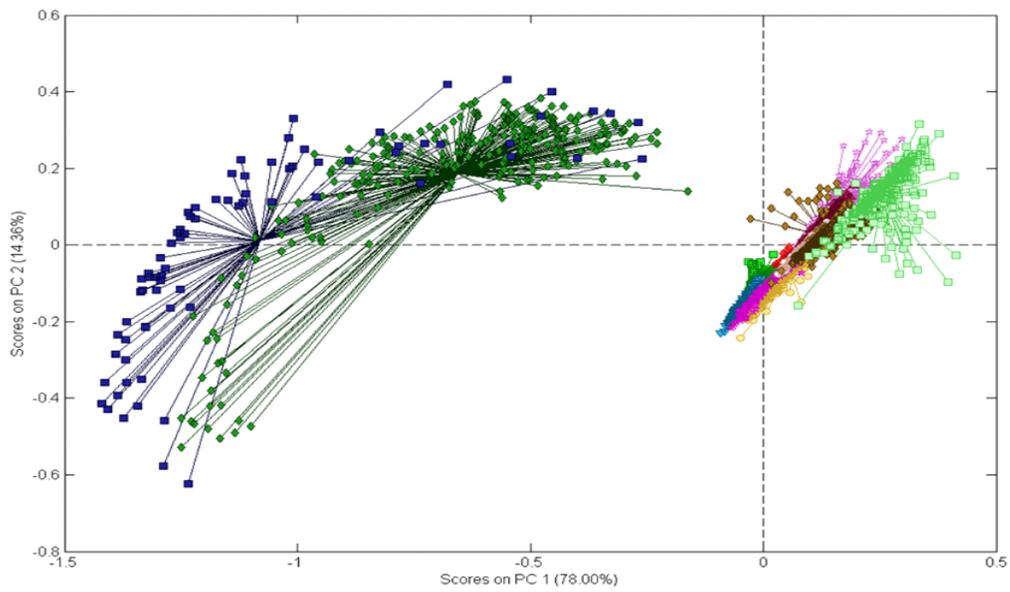


Figure 2. -- PCA score plot of otolith FT-NIR spectra from all 14 marine fishes representing 13 different species.

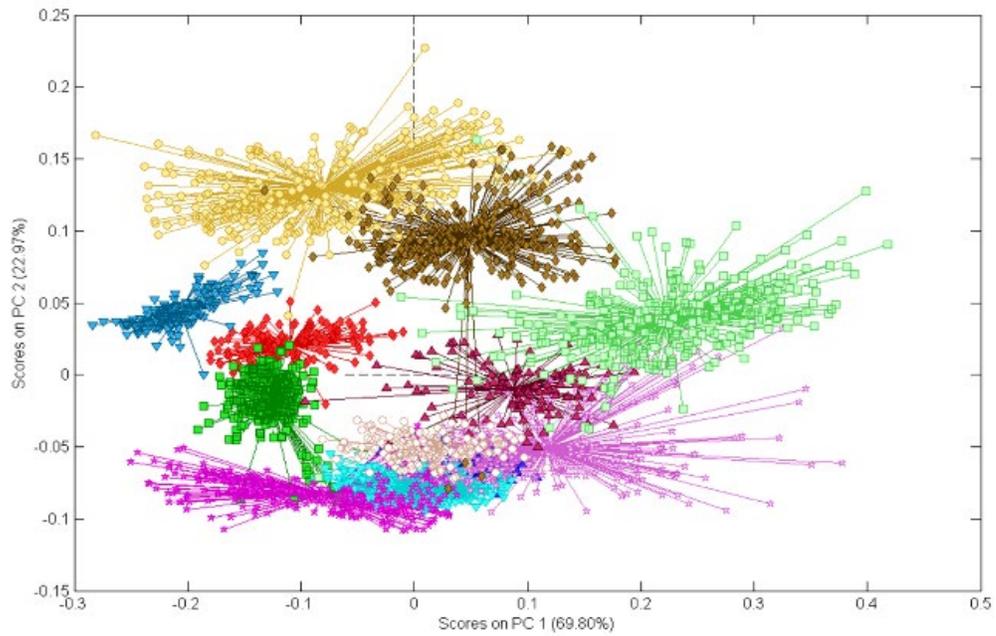


Figure 3. -- PCA score plot of otolith FT-NIR spectra from 12 marine fishes representing 11 species after excluding small coastal pelagic species Pacific sardine (*Sardinops sagax*) and Pacific mackerel (*Scomber japonicus*).

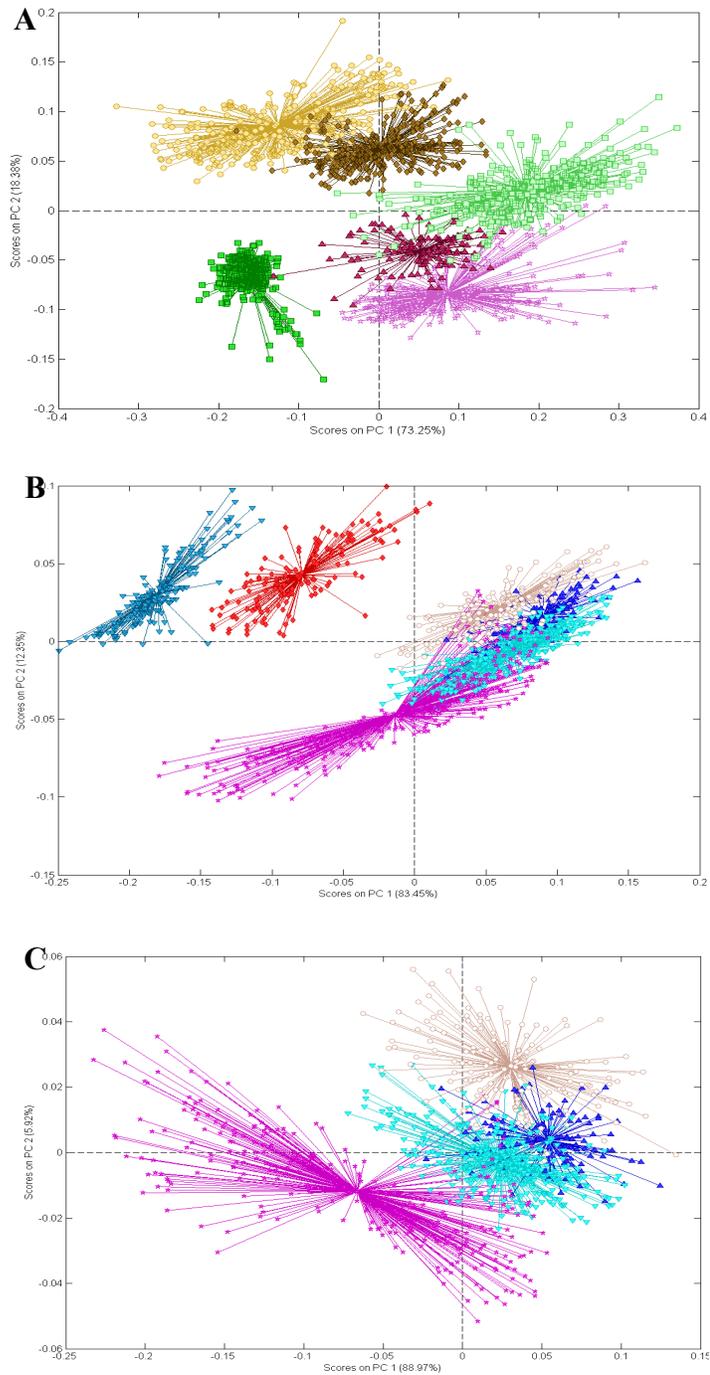


Figure 4. -- PCA score plot of FT-NIR spectra from fish species collected from (A) west coast; (B) east coast and U.S. Gulf of Mexico; and (C) U.S. South Atlantic Ocean and U.S. Gulf of Mexico.

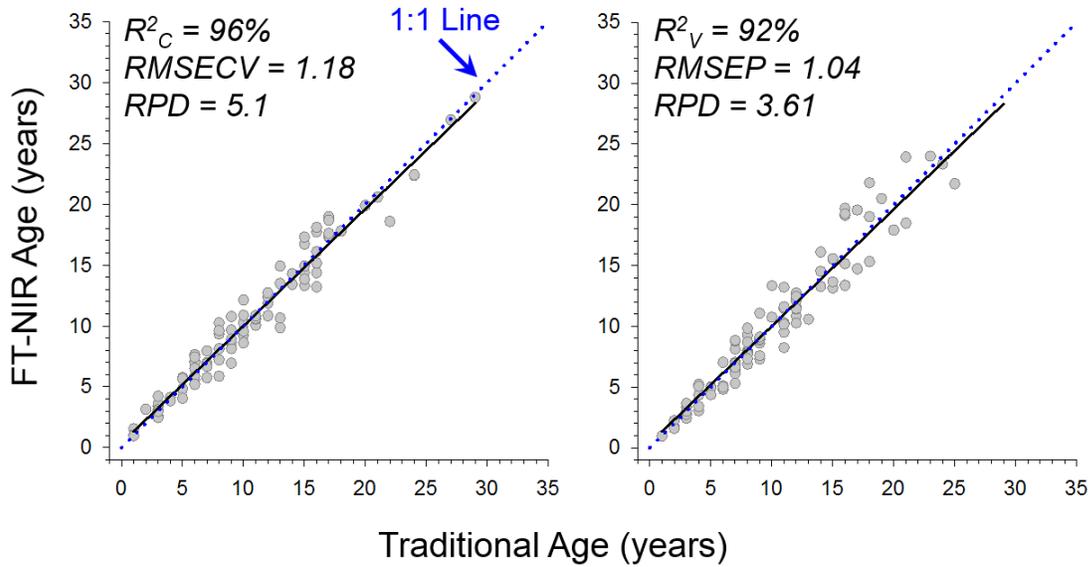
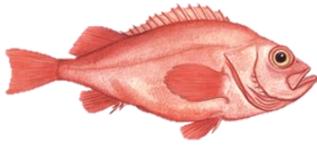


Figure 5. -- PLS calibration (left) and validation (right) models developed for Acadian redfish in the northwest Atlantic Ocean.

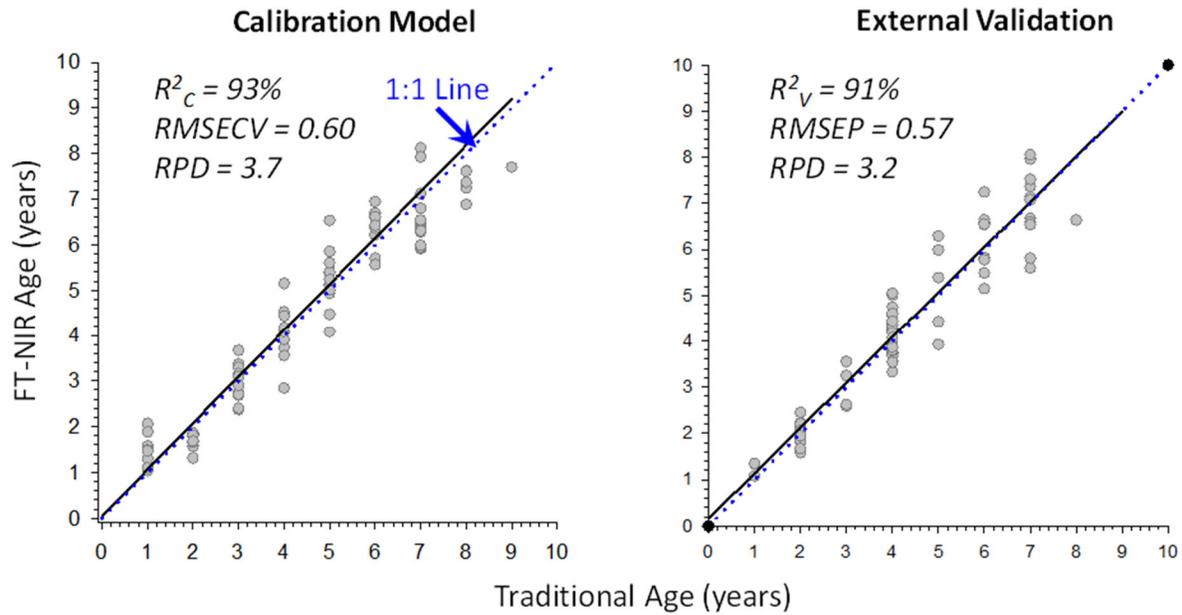


Figure 6. -- PLS calibration (left) and validation (right) models developed for haddock in the northwest Atlantic Ocean.

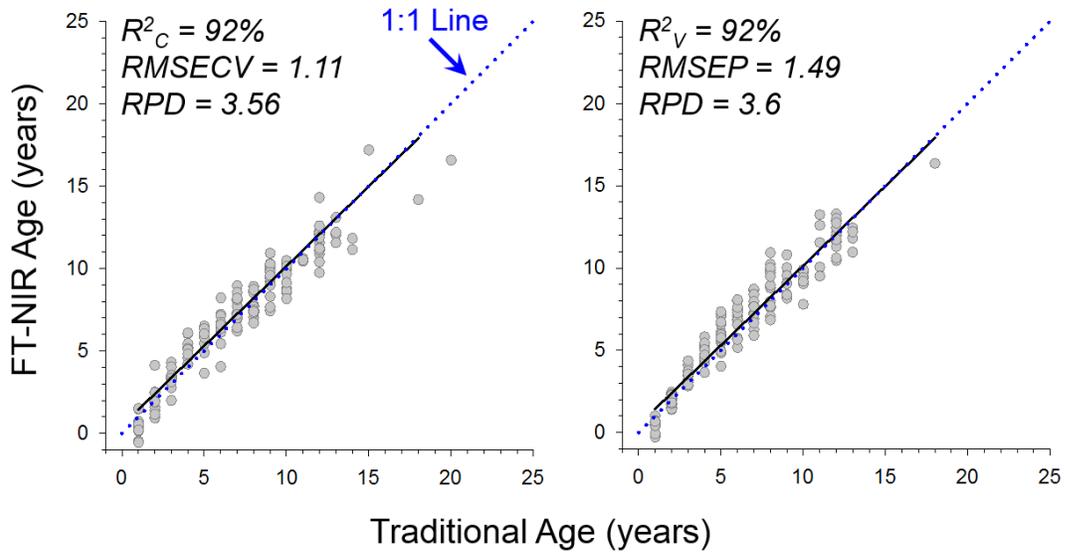


Figure 7. -- PLS calibration (left) and validation (right) models developed for Pacific hake in the northeast Pacific Ocean.

DISCUSSION TOPICS AND RECOMMENDATIONS

The intention of the workshop was not to achieve consensus on all the topics discussed. Rather, the Strategic Initiative Development Team was tasked with fostering discussion in order to identify areas of concern and provide recommendations for research topics. As a result, of the discussion, a few issues were singled out to develop the following recommendations. While the focus of these issues and research priorities are on otoliths for age estimation, the topical areas may be more widely considered to other hard structures such as vertebrae, spines and fin rays, as well as soft tissue such as ovaries and organelles such as eye lenses.

ISSUE # 1: INSTRUMENTS SETTINGS AND OPTIMIZATION

There are currently two Bruker MPA IIs (Seattle, Panama City) and one Bruker TANGO R (Seattle) spectrometers. Based on initial testing of scanning parameters, all instruments are set to 64 scans at resolution 16 cm⁻¹ with 7.5 kHz scanner velocity. All analyses have also been conducted using the MPA II integrating sphere under reflectance mode. Under these settings, the scan time is approximately 50 seconds for each specimen. Scan parameters may be further optimized depending on the otolith attributes (size, thickness, etc.) of different species, which will result in a trade-off between scan time and resolution (signal to noise).

Thus far, analyses have shown that differences in spectra between instruments appears to be inconsequential, although the TANGO R has a base-line shift different from

the MPA IIs due to the fixed preamplifier setting. However, comparative analyses have been limited to a few species (walleye pollock and Pacific cod) which have larger otoliths compared to other species. Between instrument calibration may be necessary when using two or more instruments and for different target species. In addition, instrument drift and shift is possible (i.e., change in response) that may affect the consistency of spectra and therefore calibration transfer.

Scanning of otoliths has also been performed on a 1" diameter scan window using a gold-coated reflective stamp covering the window. While the physical window area is fixed in both reflective and transmittance modes, optional apertures or Teflon focusing disks can be used to reduce the size of the window for smaller otoliths to reduce stray light. During the workshop, it was observed that for species (Pacific cod, red snapper, Pacific cod, etc.) with larger otoliths relative to the scan window area, spectral data were informative (good signal to noise) and produced good calibration models. Species with smaller otoliths (Pacific sardine, Pacific mackerel, or juvenile otoliths) may require a focusing aperture or disc to optimize signal to noise.

Recommendations

Investigate different settings for the instruments, including but not limited to creating experimental xpm files with resolution ranging from 32 to 8 cm^{-1} , number of scans ranging from 64 to 256, and scanner velocity ranging from 7.5 to 15 kHz. The table below shows a matrix of instrument scan parameters and the likely outcomes.

Resolution (cm ⁻¹)	No scans	Scanner velocity (kHz)	Effect
16	64	7.5	Current settings.
8	256	7.5	Potentially more spectral info. Much longer scan time.
16	64	15	Faster measurement. Slightly lower signal to noise.
32	64	7.5	Faster measurement. Potentially less spectral info. Higher signal to noise.
32	64	15	Faster measurement. Potentially less spectral info.

Investigate inter- and intra-instrument calibration of the spectra; spectra should be routinely tested to evaluate consistency of data acquisition. We recommend using a “calibration standard” which consists of a set of otoliths per species over the constituent range (age).

Investigate sample presentation to instrument to focus light for getting more signal relative to the noise. Explore purchasing Bruker’s tablet holder inserts with adjustable aperture or manufacturing inserts to accommodate different size otoliths in the sample wheel. Experiment with Teflon disks for small otoliths. Explore other material that would be more resilient to scratches yet invisible to NIR.

ISSUE # 2: SAMPLE PRESENTATION (PREPARATION AND STORAGE)

As with any analytical instrumentation, consistency with which a sample is presented and interrogated is crucial for robust data acquisition. Whole otoliths have only been used for spectral acquisition thus far in our laboratory. Some otoliths have been observed to

have undergone diagenesis (appearing “chalky”, “cloudy” or “crystalized”) to vaterite or another meta-stable structure and have not yet been compared for spectral differences. It is important to note such differences as otoliths are scanned to provide possible rationale for spectral outliers that may be identified during data analysis. Consistency in orientation may be important depending on otolith attributes, and our efforts thus far have positioned the whole otolith in a concave position with a 90° orientation. Wedding (these proceedings) found no significant difference with operator placement for barramundi as did Helser et al. (2019) for walleye pollock. Orientation effects on spectra, however, may be species-specific and should be evaluated for each species analyzed.

Spectra from otoliths is the result of the interaction between NIR energy and the constituent molecules in the protein matrix-aragonite crystalline structure. As such, changes in the physical or chemical composition of the otolith from exogenous organic material or penetration of aqueous chemicals can alter the spectral signature. Handling, preparation and presentation methods should be consistent as possible. NOAA ageing laboratories may utilize different methods of otolith preservation and storage. For instance, some laboratories collect and store otoliths dry in envelopes, while others preserve otoliths in either ethanol or glycerin thymol. In the former case, poorly cleaned dry stored otoliths may be contaminated with dried organic matter, while in the latter the preservation medium may alter the spectral signatures. In addition, differences in the spectra of historic otolith collections may become an issue if different preservation medium was used through time. It is also plausible that the stability of the internal chemical properties changes through time resulting in spectral changes, which may then affect the NIR calibration model. For instance, the presentation by Wedding (these

proceedings) indicated that NIR spectral data stabilized 6-11 months after dry collection for barramundi and 6 months for pink snapper.

Recommendations

Investigate possible source of spectral variation related to the following:

- Change in sampling or measuring environment (e.g., temperature, humidity)
- Time required for spectral stability for each species and for different preservation media if more than one is used
- Sample handling, preparation, and presentation to instrument (orientation, cleaning, drying, etc.)
- Change in physical or chemical properties (e.g., storage effects)
- Use of focusing disc or aperture for small otoliths

Establish protocol for handling samples that are chipped, or otherwise damaged, and may be acceptable for other ageing methods. Establish a protocol for documenting “abnormal” otoliths that have undergone diagenesis if scanned for spectra as a means of identifying spectral outliers.

Establish protocol for checking and cleaning samples for adhering tissue or other organic matter if the preferred preservation method is dry storage.

Experiment with storing otoliths dry or in the different storage media and comparing resulting calibration models. This may be important for samples that have been stored using different preservation media.

Plan to collect otoliths for a calibration standard to examine all sources of variation identified above. The samples used for the calibration should be representative of the age,

geographic location, and seasonal range. Properly archived samples should be clean, unchipped, and have been stored dry or in appropriate storage media.

ISSUE # 3: CALIBRATION MODELS - STATISTICAL APPROACH TO QUANTITATIVE ANALYSIS

The application of Fourier transform near-infrared spectroscopy to fisheries ecology and for fish age estimation in particular, is quite new. With fewer than a handful of published studies on fish age estimation there is much to learn before this technology can be operationalized. For instance, how does electromagnetic energy interact with a substance (such as an otolith), and what is the exact nature of the specific compounds absorbing NIR light? With nearly a dozen species analyzed so far, we have found a good relationship ($r^2 > 0.88$) between fish age and the spectral data from otoliths. This suggests that the material composition of otoliths seem to be shared across species; however, the exact composition is not yet known. In addition, substantial differences in predictive performance between species suggests that some species will have better predictive performance than others. Moreover, quantitative analyses necessary to build robust predictive models require additional consideration that are particular to the nuances in fisheries science, compared to other fields, because of the complex interaction of biological and environmental systems. For instance, to what extent does variability (systematic and random) in otolith microchemistry and inclusion of that variability (i.e., spectral variability) in the calibration model impact predictive performance? If that variability is systematic, should calibration models be developed for each species separately by region

(e.g., eastern Bering Sea vs. Gulf of Alaska) and time periods (warm vs. cold years)? If random, variability can be accommodated by incorporating sample variability from a number of years, or geographic areas to improve prediction; however, what percentage of new data should be used to update calibration models? Additionally, NIRS is a secondary method of estimation and relies on the accuracy of the reference method (traditional age estimates), and therefore prediction uncertainty will be the sum (or product) of both age estimation error and error associated with the NIRS technique. As such, established analytical techniques such as partial least squares (PLS) regression may require modification to account for both age estimation bias and imprecision.

Recommendations

Conduct analytical or proteomic analysis of otolith chemical constituents to determine the exact compound(s) whose spectral information are functionally related to fish age.

Develop best practices for building calibration models representative of the entire range of variability expected in future prediction, such as age range, stock structure, and environmental variation in space and time.

Establish protocol for periodic updating of the calibration model and validation to incorporate future ecosystem dynamics. Identify the need to add a percent of samples from each collection year and region.

Explore PLS model modification to account for reference age errors (bias and imprecision). Routine age imprecision estimates should be incorporated into model predictions. Age estimation bias should be quantified and incorporated for each species.

For instance, a calibration model might be estimated from reference ages that include age-specific imprecision and a subset of known age fish (e.g., from tagging or microchemical analysis) which are “leveraged” more highly to correct for age estimation bias.

Investigate if the calibration models fit better with whole age (calendar age) or fractional age (biological age), which accounts for birth date and collection date. NIR models predict decimal ages, which must then be rounded or truncated to integer age for comparison to traditional whole age.

Investigate non-linear and hierarchical calibration models as a statistical approach to quantitative analysis to account for nonlinearity between reference age and FT-NIRS predicted age.

Develop best practices to identify spectral outliers. Establish ways to identify and treat spectral errors and or age errors.

Establish best practices for preprocessing for calibration model development and checking of spectral outliers.

ISSUE # 4: COMMUNICATION TO ASSESSMENT REVIEWERS, THE FISHING INDUSTRY, AND POLICY MAKERS

Statistically, data products from traditional age and FT-NIRS age estimation are fundamentally different. The variables or outcomes of the former are strictly estimation errors, which are independent and arise from an assumed moment generating function, such as a normal, log-normal or multinomial distribution. The latter, however, has additional constraints such that the outcomes have expectations that are conditionally

dependent on another random variable that is without or has minimal error and follow some assumed functional response model. While age compositions for assessment models can be generated in much the same way, the statistical properties associated with them are different. As such, reports (QA/QC) and meta-data, such as imprecision matrices, provided to end users must convey the actual properties. Collaboration should be anticipated with assessment biologists to better document the needs, data products, and properties of these age data products. In addition, outreach efforts will be needed to inform working groups, policy makers of the council family, non-governmental organizations, and the fishing industry. Although predicted age estimates with FT-NIRS can land within 10%-20% of the traditional ageing method, some fish are aged with much higher precision; some of which have as high as 100% repeatability. Even for those species where this level of accuracy is acceptable, there appears to be a bias in the FT-NIRS estimation in which older fish will be underestimated in age; therefore, creating a biased view of mortality. Stock assessment biologists will likely need to use age error conversion matrices, which are used widely at some but not all NMFS science centers.

Recommendations

Investigate effects of different data products, such as age compositions and conditional age at length data, on stock assessment model outcomes and estimates. We recommend, if possible, that a 5-year time depth of age data from both traditional ages and FT-NIRS estimated ages be used for this evaluation.

Collaborate closely with stock assessment modelers to identify age data products specifically tailored to a particular stock assessment for use in evaluating model outcomes

as referred above. This includes the time period within the stock assessment time-series for which the FT-NIRS age versus traditional age (fishery, survey, or both) data can be incorporated and compared.

Establish best practices for reporting the age data products, including age estimates, meta-data and QA/QC reports to end users. Characterize precision and accuracy using best possible metrics that are consistent with the statistical properties of the age data product.

Evaluate transition or conversion matrix in the assessment model that harmonizes new FT-NIRS generated ages with historic, traditional estimated ages. Account for all the propagated error (sample presentation, clutter, and preprocessing error) and model selection error (model form and overfitting).

Develop quality control methods to demonstrate the reliability of individual samples needed and to check for bias and precision in FT-NIRS technology.

Develop quality assurance methods to demonstrate reliability of ageing methods because it would have direct return on investment to the ageing enterprise (i.e., more reliable ages in future calibration models and the potential to apply age-based assessment to new species).

ISSUE # 5: QUESTIONS OR CONCERNS ABOUT TECHNOLOGY

FT-NIRS is not a primary method. It depends on accuracy and precision of the reference method (traditional age estimates). In general, application success to a given fish species can be measured on the basis that FT-NIRS ages are as precise, or perhaps even

more precise, in comparison to traditional age estimates, with the caveat that repeatability and efficiency gains of the former outweighs the latter. For some short-lived species where ages are traditionally estimated from otolith surfaces, efficiency gains may not be fully realized, thus, limiting applicability of this technology to these species. Additionally, limitations may exist for species that are inherently difficult to age with high CVs. The threshold of application success, perhaps based on a combination of measured CV and efficiency gains, should nevertheless be decided for each assessed species at the regional level. Ageing bias in the traditional age estimation method does not imply application “failure” of the technology but rather supplemental age validation studies are needed to derive accurate ages for the reference data (traditional age estimates).

In-house expertise in the analytical aspects of this technology will be needed for existing National Marine Fisheries Service staff. Bruker Optics provides the basic training and software operation of OPUS with the purchase of an instrument. Additional training in multivariate statistical analysis and alternative chemometric software packages will provide a greater depth of knowledge and more flexibility in data pre-processing, outlier detection and modeling tools. Traveling to training, buying additional copies of proprietary Bruker software or any other software will require additional funding. Bruker instruments seem reliable with little maintenance involved and have only two consumable parts: light source and desiccant. The number of proprietary software licenses allotted varies by company. Several questions regarding logistics of software and instrument purchases were identified during the workshop. For example, how do we justify purchasing proprietary software such as PLS Toolbox or Unscrambler? Will each lab choose their own software or will all labs use the same software? What are the logistics involved in scanning samples for

ageing labs currently without FT-NIR spectrometers? Will it require purchase or lease with option to purchase instruments from Bruker or traveling to other labs to scan otoliths?

Recommendations

Develop a plan to communicate and coordinate among the National Marine Fisheries Service ageing laboratories scenarios and definitions to evaluate successful application of the technology. The goal is to foster among the team reasonable expectations of situations where the technology will and will not reap benefits.

Information Technology (IT) at each Science Center should be brought on early to review and comment on data storage and management, instrument network connection, and use of proprietary software.

Investigate what kind of instrument maintenance plans Bruker offers and which plan would be more appropriate for which instrument. For instance, these plans differ between the Tango R and MPA II spectrometers.

Investigate potential to hire programmers to customize proprietary software to match its functionality to ageing labs' needs.

Investigate possibility to hire contractors to scan otoliths for each ageing lab to maintain present production ageing rates.

ACKNOWLEDGMENTS

We thank the staff of the AFSC Age & Growth Program for assistance with the many logistics needed to pull together this workshop. Thanks to all the many participants of this workshop for sharing our enthusiasm for use of NIR technology in fish age estimation and for the lively discussions. In particular, we thank all those participants who provided presentations at the workshop. Each was equally fascinating and helped increase our depth of understanding of this technology. We also thank Australian Scientists Brett Wedding and Julie Robbins who traveled so far to participate in the workshop and for openly sharing their R&D experiences of this technology. A very special thanks to Jason Erickson who has helped guide our understanding and application of this technology to fish age estimation. The National Marine Fisheries Service Science Board generously provided funding for this workshop.

APPENDICES

Appendix A. Workshop Agenda



National Marine Fisheries Service, Alaska Fisheries Science Center, Western Regional Center, Building 4, Traynor Room 2076, 7600 Sand Point Way, NE, Seattle WA 98115, April 11th & 12th, 2019

Thursday, April 11, 2019

9:00 Welcome, introductions and workshop purpose (T. Helsler – FT-NIR SIDT Chair)

9:30 Introduction to NIR and FT-technology. Jason Erickson, Applications Scientist, Bruker Optics.

10:00 Data preprocessing for quantitative and qualitative models based on NIR spectroscopy. Barry Wise, President, Eigenvector Research, Inc.

10:30 Applications of near infrared spectroscopy to questions in animal physiology. Carrie Vance, Professor, Mississippi State University.

11:00 Coffee Break

11:20 Near infrared reflectance spectroscopy detection of male northern dusky salamanders (*Desmognathus fuscus*) response to female pheromones. Mariana Santos-Rivera, Mississippi State University.

11:40 Predicting fish age at the speed of light. Brett Wedding, Principle Scientist, Agri-Science Queensland Government, Australia.

12:00 Morning discussion and wrap up

12:30 Lunch and tour of the AFSC Spectroscopy Laboratory

14:00 Age prediction of Gulf of Mexico red snapper using near infrared spectroscopy. Beverly Barnett, Fishery Biologist, Southeast Fisheries Science Center, Panama City Laboratory.

14:20 Using FT-NIR to predict daily ages in juvenile red snapper. Michelle Passerotti, Ph.D. Candidate, University of South Carolina.

14:40 Case study of FT-NIR spectroscopy for Bering Sea Pacific cod stocks. Jordan Healy, M.S. Candidate, University of Washington.

15:00 Application of near FT-NIR spectroscopy for Gulf of Alaska longnose skate vertebrae. Morgan Arrington, M.S. Candidate, University of Washington.

15:20 Anadromous chinook salmon otoliths ageing using near infrared spectroscopy. Andrew Claiborne, Fishery Biologist, Washington Department of Fish and Game.

15:40 Coffee Break

16:00 FT-NIR spectroscopy ageing of Bering Sea walleye pollock: Wavelengths to population parameters. Irina Benson, Research Fishery Biologist, Alaska Fisheries Science Center, Age and Growth Laboratory.

16:20 Discussion and session wrap up.

Workshop Social: TBD

Friday, April 12, 2019

9:00 Precision and accuracy metrics for ageing QA/QC: what is behind the numbers. Richard McBride, Branch Chief, Population Biology, Northeast Fisheries Science Center, Woods Hole Laboratory.

9:30 Ageing outputs in stock assessments in Queensland-focus on fisheries concerns moving the technology forward. Julie Robins, Research Scientist, Department of Fisheries and Agriculture, Queensland, Australia.

10:00 A new paradigm of FT-NIR age estimation and challenges in U.S tock assessments. TBD, Stock Assessment Scientist, Resource Ecology and Ecosystem Modeling, Alaska Fisheries Science Center.

10:30 Operationalizing FT-NIR ageing enterprise in National Marine Fisheries Service: A conceptual pathway forward. Thomas Helser, Supervisory Research Fishery Biologist, Alaska Fisheries Science Center, Age and Growth Laboratory.

11:00 Report of the week's FT-NIRS multispecies analysis by the Strategic Initiative Development Team. Discussion facilitated by T.E. Helser.

12:30 Lunch

14:00 Discussion of detailed strategic initiative work plan and report to National Marine Fisheries Service Science Board.

- 1) Group discussion – likelihood of success for implementing FT-NIRS ageing of fish from otoliths
- 2) Impediments to success - Prioritization and execution of central scientific questions to be answered
- 3) Unique requirements of NIR technology in fisheries science and its scalability
- 4) Implementation time-lines for strategic initiative work plan

Appendix B. Workshop Participants

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