Rapid age estimation of longnose skate (*Raja rhina*) vertebrae using near infrared spectroscopy

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Abstract

There is a paucity of age data for chondrichthyan fishes due, in large part, to limitations in traditional age estimation methods. Fourier transform near infrared (FT-NIR) spectroscopy has shown promise as an alternative, more efficient method for acquiring age data from chondrichthyans. However, studies are limited to sharks in the Southern Hemisphere. We explored FT-NIR spectroscopy to predict age for a batoid species in the Northern Hemisphere. The longnose skate (*Raja rhina)* is one of a small number of batoids for which annual band periodicity in vertebral centra has been validated, allowing for traditional age estimation and making it an ideal candidate for this study. We fit a multivariate partial least squares predictive model between FT-NIR spectra collected from vertebral centra and traditional age estimates, and tested model predictive skill using external validation. Using FT-NIR spectroscopy, we were able to predict age for longnose skates between the ages of 1 and 14 years with near equal precision and bias as traditional methods in less than a quarter of the time. These results support potential for FT-NIR spectroscopy to increase the amount of age data available for assessments used to inform the conservation and management of this sensitive group of species.

1. Introduction

Information on the age of fishes is an essential component of fisheries research and management. Accurate age data allow for a more robust understanding of population dynamics and contribute to our ability to conserve and manage species effectively (Lai and Gunderson 1987; Campana 2001). Fish age data are essential for age-structured methods of stock assessment as they are used to estimate the size of population cohorts as well as important life-history parameters related to growth, mortality, maturity, and longevity. Because these metrics vary over time, monitoring temporal changes in their values is increasingly recognized as an important component of population assessment. Fish growth can be altered by factors such as ecological and environmental conditions (Shelton and Mangel 2012), fishing mortality (Heino and Dieckmann 2008), and density-dependent effects (Lorenzen and Enberg 2001). For example, changes in temperature have been shown to affect fish growth (Matta *et al.* 2010; Pistevos *et al.* 2015; Matta *et al.* 2018), increased mortality can reduce population density leading to increased food availability and faster growth (Heino and Dieckmann 2008), and mortality that is size-selective toward older and larger individuals can favor genotypes that grow faster (Stokes and Law 2000).

Despite its importance, monitoring population parameters that rely on age data is challenging because of the time and expense involved, as well as the difficulties with producing reliable age estimates for some vulnerable species such as chondrichthyans. Historically there has not been a large research focus on chondrichthyan species because they have not supported as many economically valuable fisheries as teleost fishes (Fowler *et al.* 2005; Dulvy *et al.* 2014). Additionally, acquiring information on the age of chondrichthyans is especially difficult because they do not possess otoliths, which are commonly used for age estimation of teleost fishes (Cailliet *et al.* 2006; Matta *et al.* 2017). Growth band patterns visible in thin sections of vertebral centra are often used for ageing chondrichthyans, but this method is time consuming and has not been validated for many species (Matta *et al.* 2017). Furthermore, a growing number of studies have demonstrated that these banding patterns may not form annually throughout life in all species, raising doubts about the accuracy of age estimates generated from vertebral centra (Natanson *et al.* 2018). Validation across species and age groups using independent methods of age determination is important to ensure that the age-estimation protocol yields biologically accurate age estimates. Consequently, age information is generally lacking for chondrichthyan populations (Cailliet *et al.* 2006; Matta *et al.* 2017) even though many species face elevated risk of extinction due to the expansion of fishing, habitat loss, and climate change (Dulvy *et al.* 2014).

The longnose skate *(Raja rhina)* is one of a few species of chondrichthyan for which age estimation methods have been validated (King *et al.* 2017). However, traditional growth-band age estimation is expensive and time consuming for this species, which has precluded routine age estimation. Life history traits such as late age-at-maturity relative to total lifespan and probable low fecundity may make this species more sensitive to exploitation (King and McFarlane 2003), yet a high percentage of bycatch is retained in some areas and there has been interest by industry in developing a target fishery (Farrugia 2017). Thus, more age data would be beneficial to monitor population status with higher spatial and temporal resolution.

Fourier transform near infrared (FT-NIR) spectroscopy is a technology that could be used to improve the efficiency of longnose skate age estimation and enable the enhanced collection of age data for this species. Routinely used in pharmaceutical and agricultural industries to analyze chemical composition (McClure *et al.* 2002; Roggo *et al.* 2007), FT-NIR measures the interaction of near-infrared light with the chemistry of biological materials such that unique molecular structures result in a measure of absorbance across a range of wavenumbers from 12,000 to 4,000 cm^{-1} (Robins *et al.* 2015). It is being increasingly utilized in ecological research including for species identification, physiological status, sex, detection of disease, and diet composition (Vance *et al.* 2016). In the field of fish ageing, FT-NIR is an emerging technology that has been used to more efficiently estimate ages of teleost fish using otoliths and of sharks using vertebrae, fin spines, and fin clips (Rigby *et al.* 2014; Wedding *et al.* 2014; Robins *et al.* 2015; Rigby *et al.* 2016; Helser *et al.* 2018; Passerotti *et al.* 2020a; Passerotti *et al.* 2020b, Wright *et al.* 2021). The ability to predict animal age using FT-NIR spectroscopy is based on the concept that there is a relationship between an ageing structure's chemical composition and the specimen's age. While this technology has shown promise for estimating chondrichthyan ages, it has not yet been applied to vertebrae in the northern hemisphere or from batoids.

Because ages estimated based on annual band periodicity have been validated for longnose skates (King *et al.* 2017), we were presented a unique opportunity to evaluate the use of FT-NIR spectroscopy to estimate ages from the vertebral centra of a batoid species in the northeastern Pacific Ocean. The objective of this study is to evaluate the utility of FT-NIR spectroscopy to estimate longnose skate age and compare results relative to traditional methods.

2. Materials and methods

The longnose skate vertebral centra used in this study were provided by the National Marine Fisheries Service (NMFS) Northwest Fisheries Science Center's (NWFSC) bottom trawl research surveys (Keller *et al.* 2017). A segment of thoracic vertebrae was collected from specimens and frozen at sea following approved NMFS procedures and guidance from the NMFS

Animal Care and Use Policy (Policy Directive 04-112). Collections occurred during 2011-2012 along the U.S. West Coast between May and October. These samples were processed for a separate study on regional variability in the age and growth of longnose skates and more details and sample collection locations are fully described in Arrington (2020). A group of three to four adjacent thoracic vertebrae were dissected out of the frozen vertebral column in the laboratory, neural and hemal arches were removed, and centra were stored in ethanol for preservation (Cailliet and Goldman 2004). One centrum per specimen was used for age estimation and another one was used for spectroscopic evaluation in this study. Age readers estimated ages by counting annually deposited growth bands on vertebral thin sections according to the validated protocol (Gburski *et al.* 2007; King *et al.* 2017). Hereinafter, we refer to reader-generated age estimates as “traditional ages”, while “predicted ages” represent the ages estimated using the calibration model in the FT-NIR spectroscopy approach. The primary reader (Reader 1) aged all specimens. Then, to evaluate ageing error, two additional readers independently aged a random subset of vertebral thin sections. We only evaluated a single vertebra per individual to maximize the number of individuals, but future studies might explore variation in growth across vertebrae within individuals. Traditional age estimates from the full longnose skate dataset ranged from 0 to 19 years (Arrington 2020). For the purpose of the present study, we truncated the dataset at 14 years due to poor representation of the older age classes (fewer than 5 samples per age class), which could have a disproportionately large influence on the predictive model.

To collect FT-NIR spectral absorbance data (hereinafter called spectral data), we placed one whole centrum per specimen under a fume hood and allowed it to air-dry for 48 hours so that ethanol could fully evaporate. We then placed each centrum on the sampling window of a Bruker TANGO-R Fourier transform near infrared spectrometer (Bruker Optics, Ettlingen, Germany) in a standardized orientation with centrum cone side down and covered it with a reflector cap. Spectral data were acquired at 16 cm^{-1} resolution with 64 co-added scans. We collected spectral data from 633 vertebrae (Fig. 1a).

Partial Least Squares (PLS) regression analysis was used to build a predictive model for longnose skate age based on spectral data of whole centra. A PLS regression is a multivariate regression method commonly used for chemometric analysis (Wold *et al.* 1984). In PLS, the independent (here, spectral data) and dependent (here, Reader 1's traditional age estimate) data matrices are decomposed into a set of scores and loadings. Loadings are determined by maximizing the correlation between scores based on least squares. We conducted data analysis in R statistical computing software version 3.6.3 (R Core Team 2020), with chemometric packages *mdatools* (Kucheryavskiy 2020) and *simplerspec* (Baumann 2020).

Raw spectral data often need to be transformed to enhance variation between specimens and remove unwanted noise before modeling (Rinnan *et al.* 2009). First, we estimated the first derivative of the spectral data to enhance separation between spectra while reducing any baseline drift in the data (Brown *et al.* 2000). We calculated the first derivative using a polynomial least squares estimation known as the Savitzky-Golay first derivative (17-point smooth, polynomial order = 1; Savitzky and Golay 1964). The Savitzky-Golay approach is commonly used in spectroscopy due to desirable properties such as reducing unwanted noise in spectral data while preserving the chemical signal of interest. After calculating the first derivative, we mean centered the data (Fig. 1b). We then split the data into a set used to calibrate the model and a set to externally validate it. These sets were selected so that the validation set contained all specimens that each had three independent traditional age estimates. This allowed us to directly compare ageing error between the FT-NIR spectroscopy approach and the traditional method. The calibration set contained 413 samples and the validation set contained 220 samples (Fig. 2). We applied PLS to the pre-processed spectral data from the calibration set and Reader 1's corresponding traditional age estimates using a 10-fold venetian blind cross validation. This is a version of k-fold cross validation in which each fold is constructed from samples in the dataset in the 1 through 10 position until all data have been sorted. Each fold is systematically left out and a PLS regression is applied to the remaining samples. The parameter estimates are then used to estimate the age of the left-out fold and the mean error of all predictions versus Reader 1's estimated ages can be calculated as the root mean square error of cross validation (RMSECV). This is an estimate of the predictive ability of the model on new data. We then applied this model to the validation data set and calculated a root mean square error of prediction (RMSEP) between predicted and Reader 1's traditional age estimates to measure the true predictive ability of the model on an external data set. We also report percentage root mean square error (% RMSE) = (RMSECV/maximum age × 100) to estimate the average predictive ability of the PLS model within the range of the age data (Couture *et al.* 2016; Passerotti *et al.* 2020a). This metric also provides a standardized measure of error that can be compared across studies (Passerotti *et al.* 2020a).

Lastly, we assessed age estimation error in the FT-NIR spectroscopy approach compared to the traditional method. There are two types of error associated with traditional age and growth studies: process error and observation error. Process error occurs when growth zones in the ageing structure do not reflect true age. We did not address process error in this study. However, King *et al.* (2017) suggest annual band periodicity in the vertebrae of longnose skates. Observation error is due to interpretation of the ageing structure and can be evaluated by comparing age estimates among multiple age readers. In the FT-NIR approach, we considered observation error to be error between FT-NIR predicted age and Reader 1's traditional age estimates.. This definition of observation error is based on the simplifying assumption that there is no process error in FT-NIR measurements that would result in different age estimates for the same specimens, though this has yet to be evaluated or quantified for this species. To evaluate the precision of each method, we compared the coefficient of variation (CV) between Reader 1's traditional age estimates and the FT-NIR predicted ages to the CV among the three age reader estimates (Chang 1982). McBride (2015) found that CV tracks precision better than other commonly used measures, such as average percent agreement (APE). We evaluated relative bias among traditional methods and the FT-NIR approach visually using age bias plots and Bowker's test of symmetry (Hoenig *et al.* 1995; McBride 2015). Since the PLS regression returns fractional age estimates, we first had to round predictions to the nearest whole number (i.e. 2.45 years = 2 years) so that precision and relative bias could be compared between the two methods. We evaluated current levels of observation error based on precision and relative bias among traditional age estimates to compare to the performance of the FT-NIR approach and to evaluate the utility of this approach for generating age estimates for longnose skates.

3. Results

The FT-NIR spectra of longnose skate vertebral centra correlated with Reader 1's traditionally estimated age with a coefficient of determination (R^2) of 0.86, a RMSECV of 1.38 years, and a %RMSE of 9.87% (Fig. 3). The RMSECV of 1.38 years indicates that 67% of the predicted ages from cross-validation fell within 1.38 years of the traditionally estimated age. When the model was applied to the external validation set and predictions compared to Reader 1's traditional age estimates, RMSEP was 1.32 years (Fig. 3).

The FT-NIR approach predicted longnose skate ages between 1 and 14 years in the external validation set with as much precision as the traditional method but with a slight increase in bias for individuals at either end of the age range. Specimens with Reader 1's traditional age estimate of 0 years were imprecisely estimated by the PLS regression (Fig. 3). When age-0 specimens were included, the traditional method had a CV of 22.6% among the three age readers and no systematic disagreement between Reader 1 and 2 (Bowker's χ^2 = 32.10, d.f. = 29 , P = 0.315) or Reader 1 and 3 (Bowker's χ^2 = 46.91, d.f. = 40, P = 0.210). Precision was lower between the FT-NIR predictions and the Reader 1's traditional age estimates with a CV of 34.2% and the null hypothesis of no systematic disagreement between methods was rejected (Bowker's χ^2 = 61.06, d.f. = 37, P = 0.008). When specimens with Reader 1's traditional age estimate of 0 were removed, the precision between FT-NIR predictions and Reader 1's traditional age estimates (CV of 19.6%) was comparable to that among readers (CV of 19.1%) and Bowker's test of symmetry indicated no systematic disagreement between methods (Bowker's χ^2 = 42.86, d.f. = 35, P = 0.170). We visually observed a slight bias in FT-NIR predictions, with a tendency to over-estimate the age of younger skates (< 2 years) and under-estimate the age of older skates (> 13 years) relative to the three age reader estimates (Fig. 4). When the magnitude of discrepancies in age estimates was compared between traditional and FT-NIR spectroscopy methods, we found that the percentage of samples with complete agreement (discrepancy of 0 years) was slightly higher among age readers using traditional methods (41.5–45.1% agreement) than between Reader 1's estimates and FT-NIR predictions (33.0% agreement), but otherwise had a similar distribution (Fig. 5).

4. Discussion

The results of this study suggest that FT-NIR spectra of longnose skate centra can be used to estimate skate ages, similar to results published for other chondrichthyan species (Rigby *et al.* 2014; Rigby *et al.* 2016). When applied to an external validation set, the FT-NIR spectroscopy method produced age predictions with comparable precision to traditional methods for longnose skates between the ages of 1 and 14 years (the full age range in this study was 0-14). Since the traditional method has been shown to produce biologically accurate age estimates (King *et al.* 2017), these findings support the validity of using FT-NIR predicted ages in age-based assessments of longnose skates. Additionally, this method provides considerable efficiency gains -- a scan takes just one minute and requires minimal preparation of the sample. Traditional methods for longnose skate age determination require extensive preparation and take between 15 and 30 minutes per sample. Estimates can also be biased among age readers, especially among different agencies (King *et al.* 2017). FT-NIR spectroscopy may allow age estimation to be standardized among age readers and agencies due to its reproducibility. This technology has the potential to improve the frequency, quantity, and reproducibility of longnose skate age data for use in stock assessments and management.

This study marks the first known application of this technology to a batoid and results were comparable to those found by Rigby *et al.* (2014, 2016) for shark vertebrae. For *Sphyrna mokarran*, *Carcharhinus sorrah*, and *Squalus megalops*, Rigby *et al.* (2014, 2016) developed calibration models that yielded R^2 values between 0.78 and 0.89, RMSECV values of 1.23 to 2.48 years, and uncertainty between 7 and 9% (%RMSE of 7.40 to 8.97). The FT-NIR spectroscopy analysis of longnose skate vertebrae in this study produced comparable calibration models with an R^2 of 0.86, RMSECV of 1.38 years, and uncertainty of 9.87% (Fig. 3).

The biased age predictions we observed for age-0 longnose skates in the external validation relative to traditional methods could be due to the exceedingly small size of age-0 vertebrae (1.5 mm). Passerotti *et al.* (2020b) hypothesized that excess stray light from the FT-NIR spectrometer might confound their results for otoliths of juvenile red snapper that were between 1.5 and 7.0 mm. They found better calibration models when using a Teflon aperture to constrain the field of light. Future work for longnose skates could include using an aperture that may improve the predictive ability of the FT-NIR spectroscopy approach for younger skates.

Except for age 0 skates, the FT-NIR spectroscopy approach yielded age predictions for the external validation set that had comparable precision to the traditional method currently used for production ageing. However, neither method was as precise as is typical for teleost age determination. Poor precision in age estimation is common for chondrichthyan species due to the difficulty of interpreting their ageing structures. Published CVs for chondrichthyan vertebrae are generally much higher (>10%) than those for teleost otoliths (~5%) (Campana 2001). The current study reports slightly poorer precision in the traditional method (CV = 19.1%) than prior ageing studies for longnose skates by Thompson (2006), Gburski *et al.* (2017), and King *et al.* (2017) that found CVs between 11.9% and 15.2%. However, this is likely due to the large number of young skates included in the current study. It is difficult to distinguish growth increments on longnose skate vertebrae under the age of 2 years and any disagreement within this age range is weighed more heavily in the calculation of the overall CV. For instance, when one reader assigns an age of 0 and another assigns an age of 1 to a given individual, the disagreement dramatically inflates the CV.

The low precision of traditional age estimates in this study relative to teleosts is likely due to observation error. However, the equivalently low precision of FT-NIR predictions could be due to several factors. First, any observation error associated with the traditional age estimates used to calibrate the model could reduce the precision of age predictions. Second, some process error is likely present in vertebrae which could cause a difference in the visual versus spectral interpretation of the structure. Third, any natural variation in chemical composition of vertebrae may affect the spectral data collected using FT-NIR spectroscopy. This may be amplified because we were unable to standardize the exact position of the thoracic vertebral samples used for spectroscopy. Size of vertebrae is variable along the vertebral column, and it is possible that larger thoracic vertebrae could impart different FT-NIR spectra than smaller thoracic vertebrae within an individual. Finally, the calibration model did not contain as many older skates as young skates, which could affect its predicative capability in that age range. Ideally, calibration samples would have even representation from all age classes. Rigby *et al.* (2014) found that age predictions markedly improved when older age classes were better represented in the calibration model.

An increasing number of studies show that vertebral banding patterns may not form annually throughout life in all species (Natanson *et al.* 2018). This finding raises doubts about the accuracy of age estimates from vertebral centra in general and suggests that process error may be common. However, Rigby *et al.* (2014) found FT-NIR to be a promising alternative method for estimating age from vertebrae even when no visible banding pattern is present. Rigby *et al.* (2016) also found that using known-age samples to build predictive models based on FT-NIR spectra had improved precision relative to using traditionally estimated ages with RMSECV of +/- less than a year (0.87 and 0.88 years). This demonstrates the potential for FT-NIR spectroscopy to estimate age despite process error if accurate ages are used to calibrate the model. These findings indicate that as the accuracy and representation of age estimates used to calibrate the model improve, we may be able to improve the precision of age predictions. Future work for longnose skates could include using a more balanced dataset to fit the calibration model between spectra and age estimates, utilizing known-age specimens, and standardizing the position of centra along the vertebral column. This may help to resolve bias in younger and older ages and improve precision while also improving the accuracy of age estimates.

The results of this study as well as those of Rigby *et al.* (2014, 2016) indicate that there is a correlation between age and the chemical composition of vertebrae. It is unknown what specific chemical compound(s) correlate with age in chondrichthyans, but it could be related to the quantity of calcified phosphate mineral, hydroxyapatite, in their cartilaginous vertebral centra, as mineralization occurs incrementally with age in many chondrichthyans (Cailliet 1990; Kerr and Campana 2014). Calcium hydroxyapatite is detectable in the NIR spectrum due to stretching and bending of OH bonds in surface hydroxyl groups when exposed to near infrared light (Elkabouss *et al.* 2004). Relative quantities of the unmineralized components of vertebrae may also contribute to the observed relationship between NIR spectra and age. The unmineralized components of chondrichthyan vertebrae include water, proteoglycan, and collagen fibers, which are also detectable in the NIR region between 5,400 and 3,800 cm^{-1} (Baykal *et al.* 2010). Future work is needed to determine the specific chemistry of chondrichthyan vertebrae related to age to better understand the mechanism driving the observed correlation between age and FT-NIR spectra.

The results of this study show promise for the use of FT-NIR spectroscopy to more rapidly and efficiently estimate ages for longnose skates. The FT-NIR approach provides considerable efficiencies over the labor-intensive traditional process of preparing vertebrae for age determination. This is important because it may allow for a larger quantity and higher frequency of age estimates to be generated for use in stock assessments to monitor the status of this species. Additionally, modern stock assessment software allows the inclusion of ageing error in population models and the results of this study provide the basis for quantifying this error. The most recent assessment for longnose skate off the US West Coast included age-reading error matrices estimated using the approach of Punt et al. (2008) based on various models of the relationship between the CV of age-reading error and true age (Gertseva *et al.* 2019). This study also adds to a growing body of literature demonstrating the successful application of this technology to estimate age for chondrichthyan species. Fourier transform near infrared spectroscopy may provide a way to estimate age for other members of this sensitive group of fishes that previously had little to no age data available.

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Research (https://fisheries.org/docs/policy_useoffishes.pdf; Chapter V). Permits acquired for sample collection including NOAA, NMFS, WCR (West Coast Region) Scientific Research Permit 2011: SRP-06-2011 and 2012: SRP-06-2012, Oregon Scientific Taking Permit for Fish and Marine and Freshwater Invertebrates: 2011: Permit 16328 and 2012: Permit 17200, CA Scientific Collecting Permit from the California Department of Fish and Game for 2011-2013: SC-11678, National Marine Sanctuary Permit for 2010 -2012: Multi-2010-004. The authors thank Aimee Keller for providing permit information. This research was supported by a grant from the NOAA Fisheries Office of Science and Technology's Improve a Stock Assessment program and a graduate fellowship from the University of Washington's School of Aquatic and Fishery Sciences. The scientific results and conclusions, as well as any views or opinions are those of the author(s) and do not necessarily reflect those of NOAA or the Department of Commerce. Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support this study will be shared upon reasonable request to the corresponding author. More information available at https://www.fisheries.noaa.gov/resource/data/age-and-growth-otolith-collections-ageing-methods.

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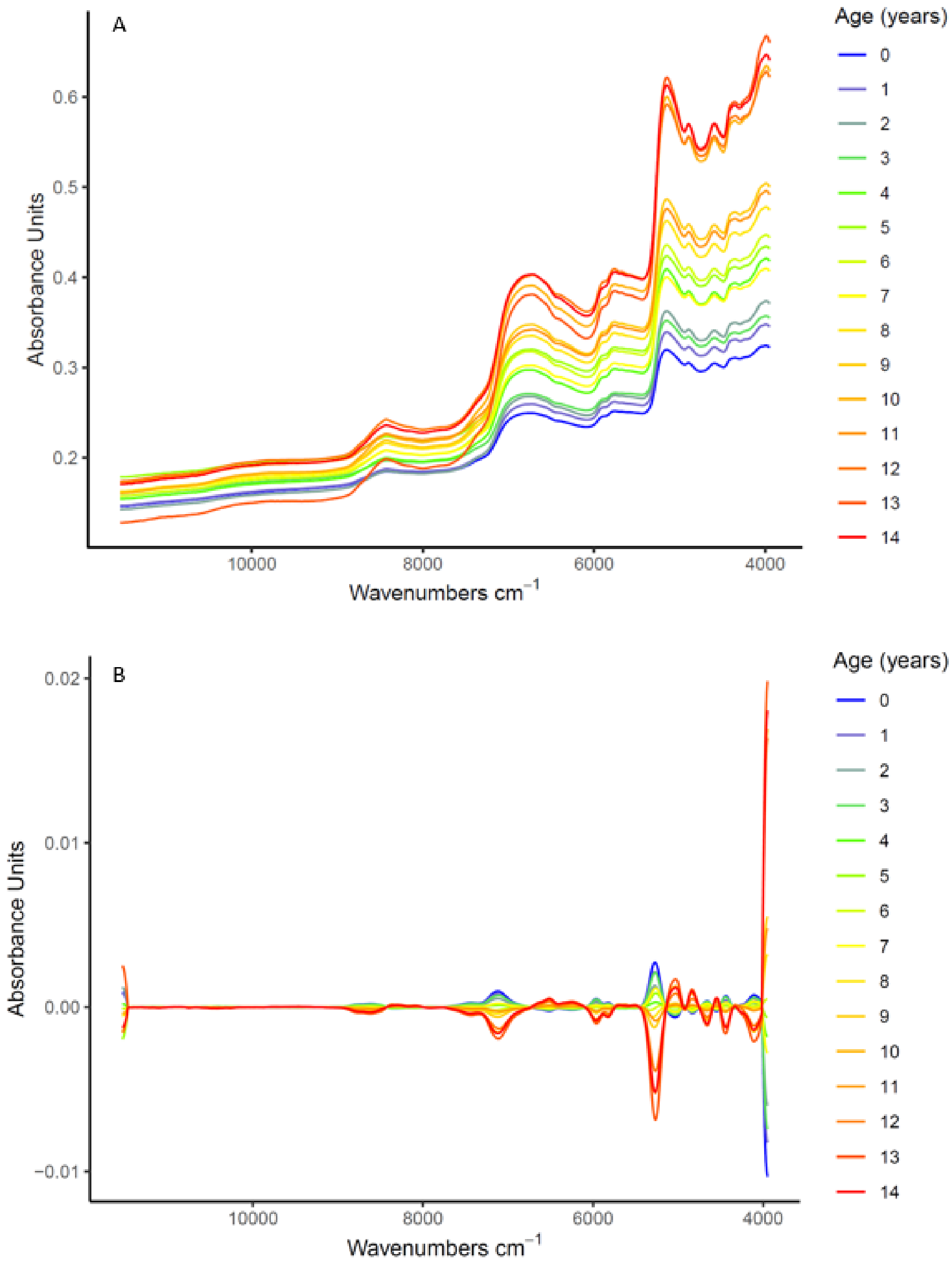

Figure 1. Fourier transform near infrared spectral absorbance data of all Longnose Skate vertebrae. Spectra were averaged by traditionally estimated age, represented in colors: (A) raw absorbance data and (B) pre-processed spectral data using a 1st derivative Savitzky-Golay transform (17-point smooth, polynomial order = 1) and mean centering.

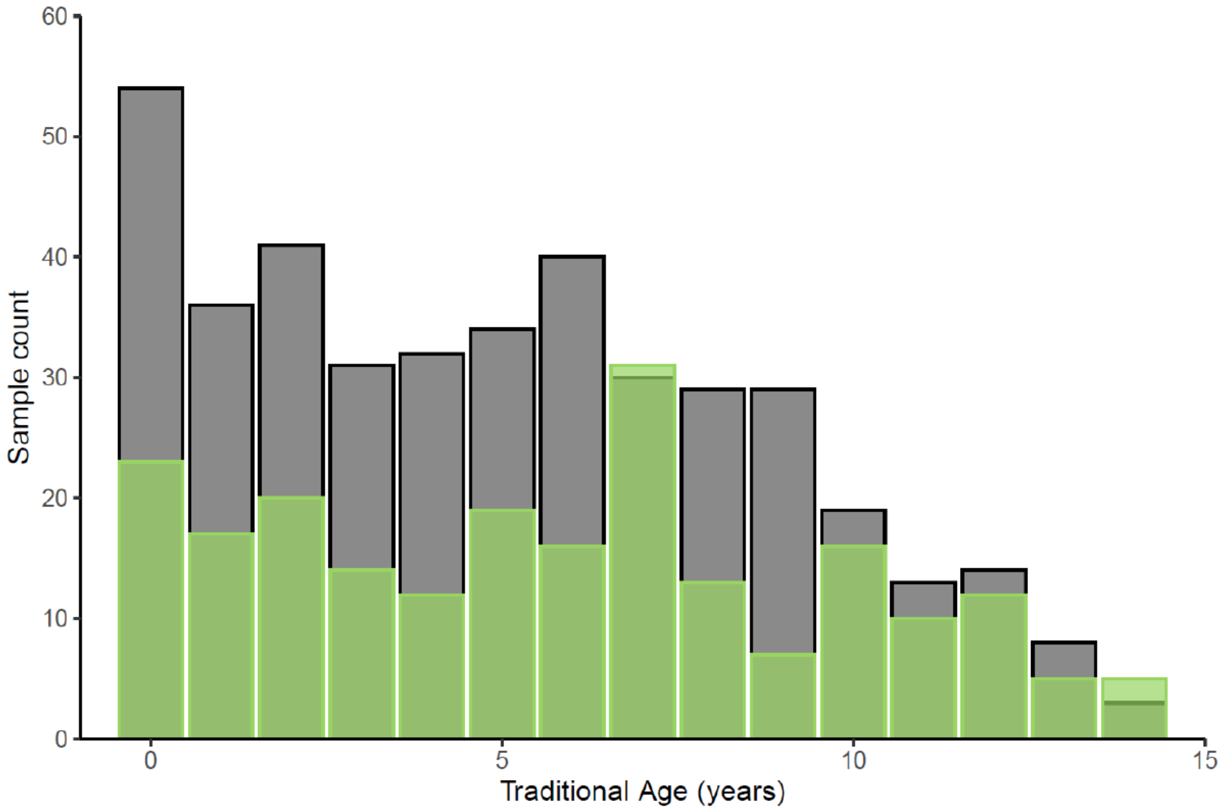

Figure 2. Age-specific sample sizes for the calibration set (grey) overlapped by external validation set (green) used in the age estimation of longnose skate by Fourier transform near infrared (FT-NIR) spectroscopy.

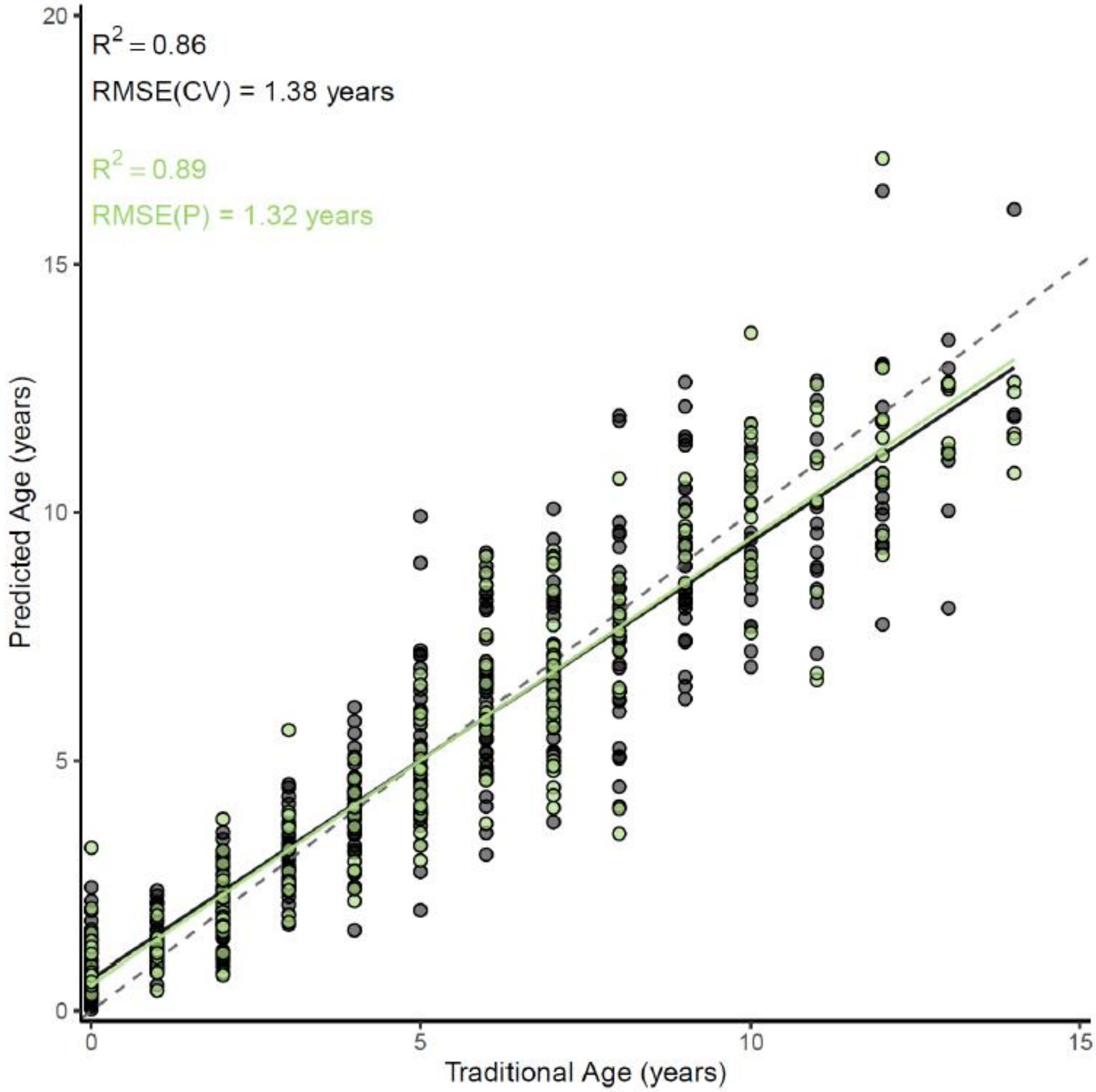

Figure 3. Results of a partial least squares model fit between reader-estimated age (traditional age) and Fourier transform near infrared spectra (predicted age) of Longnose Skate vertebral centra. Age predictions relative to traditionally estimated ages shown for the cross-validation (black) and for the external validation set (green) for each specimen. The solid lines are the regression lines for the cross-validation (black) and external validation (green). The dashed line represents one-to-one agreement between prediction and traditional estimate. Transparency in point shading shows overlapping data points.

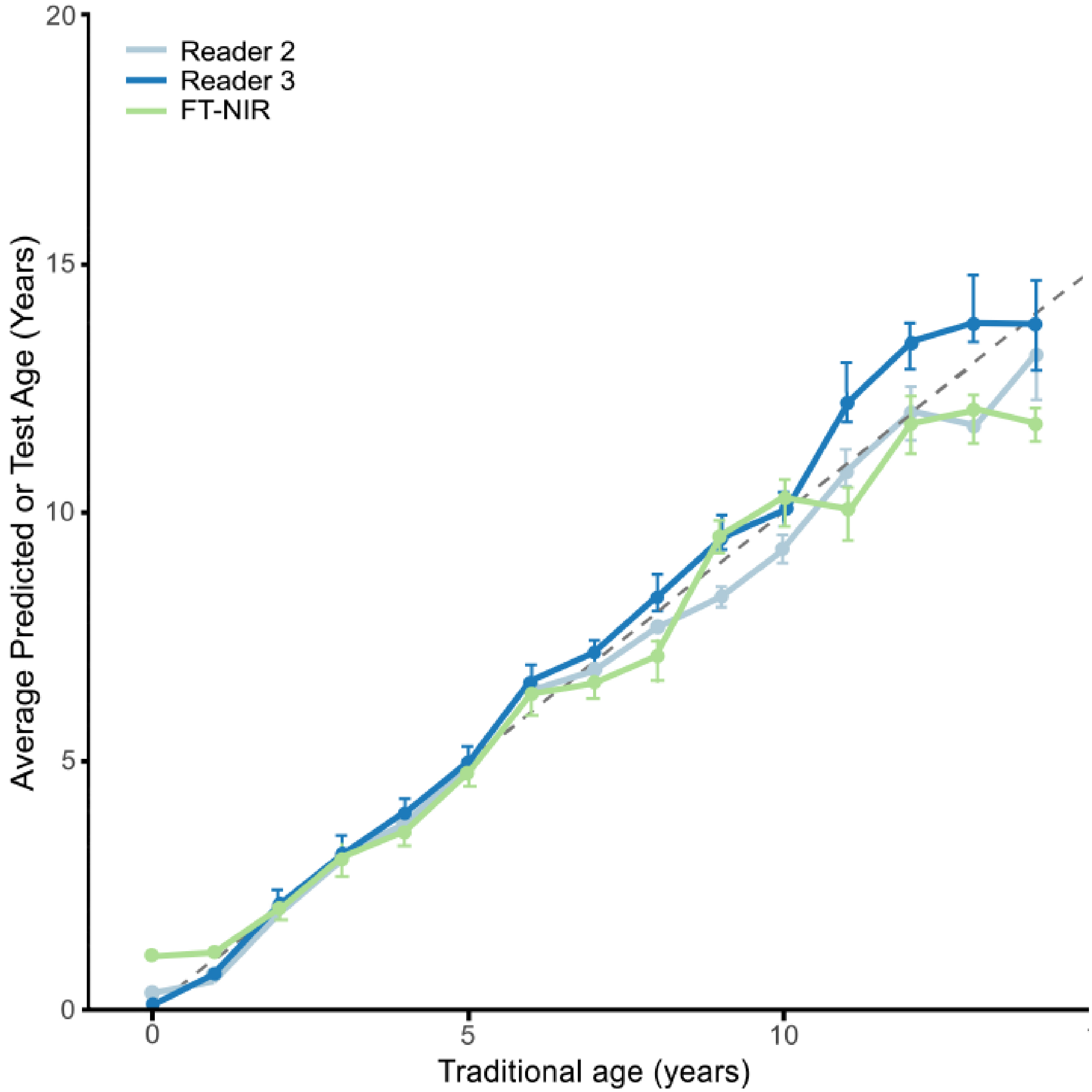

Figure 4. Bias plot comparing Reader 1's traditional age estimates to: average age estimates of Reader 2 (light blue), Reader 3 (in dark blue) and Fourier transform near infrared (FT-NIR) predictions (green) for longnose skates. Standard error bars shown for age groups with multiple samples. Dashed line represents 1:1 agreement. Sample count for each age category are represented by grey bars.

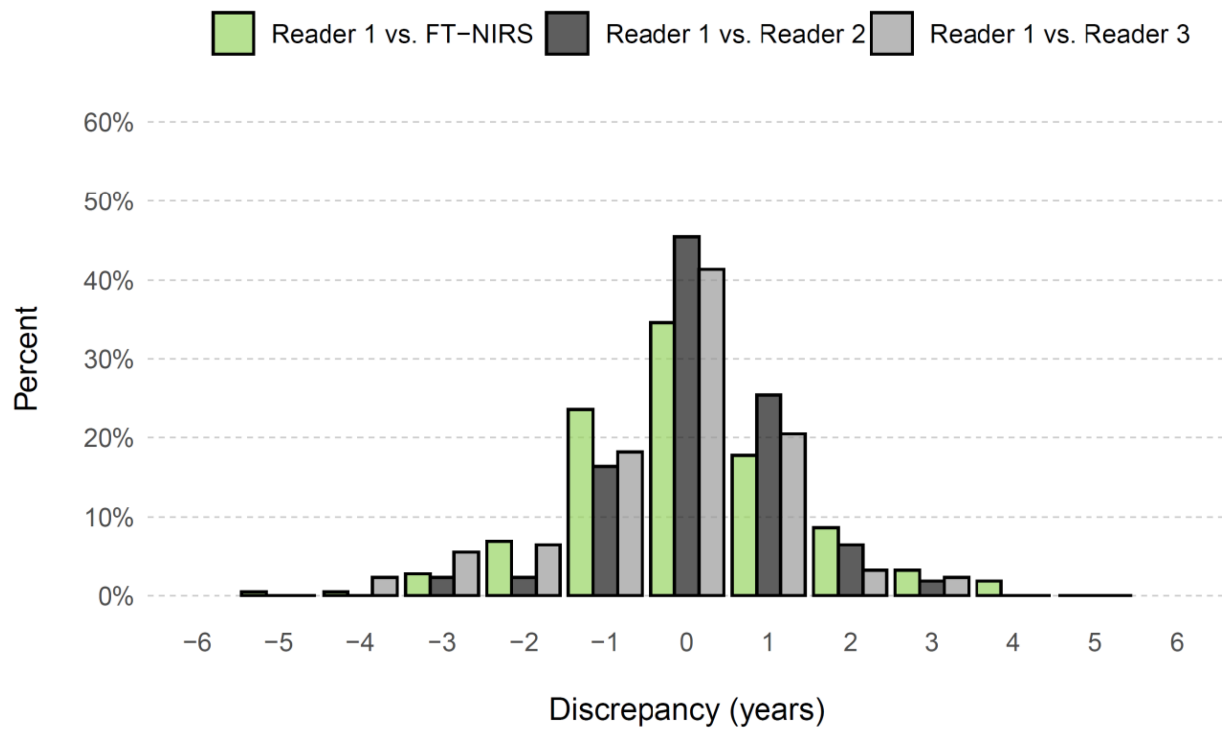

Figure 5. Comparing discrepancies in longnose skate (Raja rhina) age estimates for the validation set from Fourier transform near infrared spectroscopy (FT-NIRS) and three age readers using traditional methods. Discrepancies are shown as percentage of specimens with age estimate differences of 0 to 6 years. In grey: traditional age estimated by Reader 1 – 2 and Reader 1 - 3, and in green: Reader 1 – FT-NIR prediction.