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Novel method for shark age estimation using near infrared spectroscopy

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Abstract. Accurate age determination is an important component of assessing and managing fish populations, yet traditional ageing using growth bands is time-consuming and has limitations. In the present study, an alternative approach to shark age estimation using near infrared spectroscopy (NIRS) was investigated using two species. The ages of *Sphyrna mokarran* and *Carcharhinus sorrah* vertebrae that had been traditionally aged and validated were successfully predicted up to 10 years of age using NIRS. The correlations between the known ages of the vertebrae and their near infrared spectra were strong, with R^2 values of 0.89 and 0.84 for *S. mokarran* and *C. sorrah* respectively. The major advantage of the NIRS ageing approach was the rapid speed of age estimation, which could enable large numbers of sharks to be aged quickly. This would offer the fisheries management benefit of improving the reliability of age information for stock and risk assessments.

Additional keywords: *Carcharhinus sorrah*, *Sphyrna mokarran*, vertebrae.

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Introduction

Reliable age information is essential to accurately assess the status of shark populations and predict their ability to sustain exploitation (Goldman *et al.* 2012). Age estimates are important for the development of conservation and sustainable management strategies because they are the basis of many of the key biological parameters used in demographic and fisheries assessments, such as growth rate, mortality rate and productivity (Campana 2001; Cailliet *et al.* 2006). The traditional approach to estimating age in sharks is the enumeration of periodic growth bands in their hard structures, mostly vertebrae or dorsal fin spines (Cailliet *et al.* 1983; Cailliet and Goldman 2004). As the animals age, calcified material accumulates in these structures and can produce visible band pairs that, once periodicity has been validated, enable determination of age (Cailliet *et al.* 1986, 2006; Cailliet and Goldman 2004).

The traditional age reading approach of band pair counts requires practice and experience to achieve accurate results and can require time-consuming sample preparation, such as sectioning and enhancement techniques that are often necessary on shark ageing structures to improve band resolution (Cailliet *et al.* 1983; Campana 2001; Irvine *et al.* 2006). Band counts are highly subjective, with quality control measures required to maintain consistency and precision to reduce the risk of reader bias (Goldman *et al.* 2012). The traditional approach also has its limitations, because many sharks do not have dorsal fin spines and may have vertebrae that are either too poorly calcified to provide age information, such as numerous deep-water species, or have reduced band resolution that creates difficulties in age interpretation (Cailliet 1990; Gallagher *et al.* 2006; Barnett *et al.* 2009). Consequently, there is a need to investigate alternative approaches to the ageing of sharks that may be more cost-effective, and that have the potential to address shark age estimation limitations.

Near infrared spectroscopy (NIRS) is a technology that may provide an alternative approach to traditional age reading methods. It is a non-destructive technique (i.e. the original sample remains intact after ageing) that uses light data from the near infrared (NIR) region of the electromagnetic spectrum (12 820–4000 cm^{-1}) in combination with chemometric methods (multivariate statistics) to analyse the chemical composition of a material (Murray and Williams 1987; Wold and Sjöström 1998; Williams 2008). All organic matter consists of atoms, mainly carbon, oxygen, hydrogen, nitrogen, phosphorus and sulfur, with a minor amount of other elements. These atoms combine to form molecules that are constantly in motion and that vibrate (bend or stretch) at specific frequencies, referred to as wavelengths in NIRS terminology (Murray and Williams 1987). When an energy source such as NIR light is focussed onto a sample, it causes the molecules within the whole sample to increase their vibrational energy by absorbing specific wavelengths to a greater or lesser degree, depending on the atomic

masses, bond strengths and molecular geometry of the material within the sample. The original NIR light energy is thus modified and reflected, and can be detected and analysed by a NIRS instrument to produce a graph of the different amount of absorbance at each wavelength. This graph is an undulating pattern of peaks and troughs known as the spectrum and is characteristic of all the absorbing molecules in the sample; that is, it represents the molecular composition of the sample. Chemometric procedures are applied to analyse these spectra into the qualitative (identity) or quantitative chemical constituents of the sample (Murray and Williams 1987; Siesler *et al.* 2002; Reich 2005).

NIRS is widely used in agriculture, pharmaceutical and other industries, and was recently investigated as a method for teleost age estimation (McClure *et al.* 2002; Solberg *et al.* 2003; Wedding *et al.* 2014). The NIRS approach was demonstrated to rapidly and accurately estimate the age of saddletail snapper (*Lutjanus malabaricus*) using otoliths, which are the most commonly used structures to age fish (Campana and Thorrold 2001). NIRS is generally used as a secondary method of determination, which means the spectra must be calibrated against a primary reference method to develop a calibration model (Murray and Williams 1987). For instance, in the Wedding *et al.* (2014) otolith study, 100 whole otoliths were scanned using NIRS to produce 100 NIRS spectra and were then aged by traditional sectioning and increment counts. The 100 spectra were related to the age estimates from the increment counts (the primary reference method) using partial least-squares regression to develop a calibration model. That calibration model could then be used to predict the age of a saddletail snapper otolith scanned by NIRS and not aged by traditional increment counts. In that study, the calibration model was shown to have a strong capacity to accurately predict the age of such an otolith (Wedding *et al.* 2014). This process required a sufficient number of samples to cover the variability in age among fish of similar length, which in this instance was only 100 otoliths. The scanning and analysis of each whole otolith took ~25 s; hence, once the initial 100 otoliths aged by traditional means were scanned and a calibration model developed, the NIRS method had the capacity to conduct up to 150–200 otolith age estimates per hour. This would provide substantial time and cost savings not only in preparation time, because no sectioning was required, but also in age reading time because the NIRS scans were rapid (Dub *et al.* 2013; Wedding *et al.* 2014). This cost-efficiency and the non-destructive nature of the analyses are the major benefits of NIRS (Roggo *et al.* 2007; Williams 2008). The technique is considered non-destructive because whole otoliths can be scanned using NIRS once the calibration model is developed; they do not need to be destroyed by sectioning. Following on from the otolith study, a more detailed assessment of using NIRS to age fish was undertaken (Robins *et al.* 2015).

The success of NIRS age estimation in teleost otoliths provided the impetus to investigate the potential ability of the NIRS method for age estimation in sharks. Although the chemistry of the otoliths differs from shark vertebrae and dorsal fin spines (Walker *et al.* 1995; Campana 1999; Hamlett 1999), visible band pair counts have been determined as useful for age estimation in both teleost and shark structures (Campana and Thorrold 2001; Goldman *et al.* 2012), so it is a feasible proposition that the NIRS approach may be viable with sharks. A current limitation of the NIRS approach is that it must be used in combination with traditional age reading, although only for potentially 100 structures (e.g. vertebrae), to develop the calibration model. The calibration model incorporates errors associated with the traditional age reading, so the more accurate and precise the growth band counts, the more accurate and precise the NIRS calibration model (Williams 2008; Wedding *et al.* 2014). NIRS could be used to scan the whole vertebrae and, after the 100 structures have been traditionally aged, the calibration model applied to estimate age. Because the NIRS age estimate can be performed on whole structures, it would reduce the amount of time needed to prepare structures for age reading. The NIRS process is mechanised, rapid and precise (Siesler *et al.* 2002) and offers the potential of considerable cost savings associated with the conventional age reading. It also would remove age reading subjectivity and risk of reader bias for all vertebral ages predicted using NIRS after the calibration model has been developed (Williams and Norris 1987; Wedding *et al.* 2014). Because NIRS is a chemical assay approach, if the method is viable for shark age estimation it has the potential to be used to address other limitations of shark ageing, such as poor calcification and reduced band resolution.

The purpose of the present study was to determine the feasibility of the use of NIRS for age estimation in sharks. Whole vertebrae from age-validated and -verified coastal and pelagic sharks were scanned. Their NIRS spectra were related to their age estimates derived from the traditional method of band pair counts to examine whether robust calibration models capable of predicting age could be developed. Finally, the NIRS estimated ages were used to fit growth models and these were compared with growth curves from vertebral ageing to determine whether there were differences.

Materials and methods

Study species

Samples of whole vertebral centra from great hammerhead *Sphyrna mokarran* (Rüppell, 1837) and spot-tail shark *Carcharhinus sorrah* (Müller & Henle, 1839) were provided from another research project on the life history of tropical sharks (Harry *et al.* 2011, 2013). Using these two species provided the opportunity to trial NIRS across habitats, body size and contrasting life histories. *Sphyrna mokarran* is a large, long-lived (up to 39 years), slow-growing, coastal-pelagic and semi-oceanic shark (von Bertalanffy (VB) growth constant $k = 0.079$ year^{-1}; Harry *et al.* 2011). *Carcharhinus sorrah* is a medium-sized, faster-growing, coastal shark (k female $= 0.34$ year^{-1}), with a shorter life span (of up to 14 years; Harry *et al.* 2013). The 80 *S. mokarran* (44 females, 32 males, 4 sex unknown) were collected from waters down to 110 m depth along the east coast of Australia from northern to southern Queensland between 2005 and 2010 (Harry *et al.* 2011). The 102 *C. sorrah* (all females) were collected from inshore waters less than 25 m depth in the Townsville region of Queensland between 2007 and 2012 (Harry *et al.* 2013).

In the tropical shark study, both species were aged by growth band counts of sectioned vertebral centra. A segment of five

vertebrae was taken from each individual, the neural and haemal arches removed, the remaining centra bleached briefly, rinsed thoroughly and dried in an oven for 24 h (Harry *et al.* 2011, 2013). One of the five vertebral centra was sectioned for age estimation by band counts and another was provided whole to the present NIRS study. The band counts of each individual were adjusted to partial ages in the tropical shark study using a mean population birth date for each species based on their seasonal reproductive cycle (Harry *et al.* 2011, 2013). Those partial ages were used in the present study to represent the estimated age of each individual based on band counts and were called 'vert-age', with the term 'NIRS-age' used to represent age estimated by the NIRS calibration model.

The 80 *S. mokarran* ranged in size from 801 to 4391 mm stretched total length (L_{ST}) and in vert-age from 0.3 to 39.1 years. Ages were validated up to 9.5 vert-age years through calcein mark, tag and recapture. Five of the thirty-eight calcein-marked animals were recaptured, with vertebrae from two animals (7.1 and 9.5 vert-age years) used to validate annual band pair deposition (Harry *et al.* 2011). The 102 *C. sorrah* ranged in size from 634 to 1301 mm L_{ST} and in vert-age from 0.5 to 13.7 years. Age validation through calcein mark and recapture was unsuccessful because of low recapture rates and poor calcein uptake, although periodicity of annual growth band deposition had been verified previously in *C. sorrah* up to 2 years old (Davenport and Stevens 1988). It was suggested by Harry *et al.* (2013) that this same periodicity may occur throughout life, because there was a recapture in a large northern Australian tagging program of an animal at liberty for 9.9 years estimated to be at least 12 years old (Stevens *et al.* 2000; Harry *et al.* 2013). For clarity, in referring to ages that have been validated and verified, for both species known vert-age represents animals up to 10 years old and unknown vert-age represents those >10 years old. In addition, although 'vert-age' refers to the age of an individual, 'vert-ages' refers to all individuals (i.e. the entire age range).

Spectral acquisition and data analysis

To obtain a NIRS spectrum from each vertebral centrum, each centrum was scanned by a Bruker multipurpose analyser (MPA), Fourier transform (FT) NIR spectrophotometer (Bruker Optics, Ettlingen, Germany; operating software: OPUS version 6.5, Bruker Optics) in the 12 500–3600 cm^{-1} wavenumber range (corresponding to wavelengths of 800–2778 nm; wavenumbers are the inverse of wavelengths). Each individual centrum was hand positioned, with the centrum placed face down onto the sample window where the NIR light irradiates the centrum, and the diffusely reflected NIR light was detected and analysed. For each centrum, 16 individual scans at every 8 cm^{-1} of NIR wavenumber were taken and averaged (by the software) to produce a raw NIRS spectrum, which took ~16 s per centrum (Fig. 1).

For each species, the NIRS spectra of the centra and the vert-ages of the centra (reference age estimation by band counts) were compared to determine whether a robust calibration model could be developed. This was done by chemometric analyses methods, which included partial least-squares (PLS) regression that attempted to build a calibration model (i.e. establish a regression relationship between all the NIRS spectra and all the vert-ages) that was represented by a single plot. The plot defined the weights given to the different wavelengths in the linear PLS regression equation, with regression coefficients on the *y*-axis and wavelengths (expressed as wavenumbers) on the *x*-axis. If a calibration model could be developed, it was analysed further to identify the specific NIRS wavelengths within the plot that represented the molecular compounds in the centra most strongly related to the vert-ages. The final calibration model was presented as: (1) a calibration model plot; (2) calibration model statistics; and (3) a calibration model regression graph of the predicted NIRS-ages against vert-ages (Murray and Williams 1987; Foley *et al.* 1998; Wedding 2007). This chemometric process is explained in further detail below. All data analyses were undertaken using the multivariate software package The Unscrambler (version 9.8; Camo, Oslo, Norway).

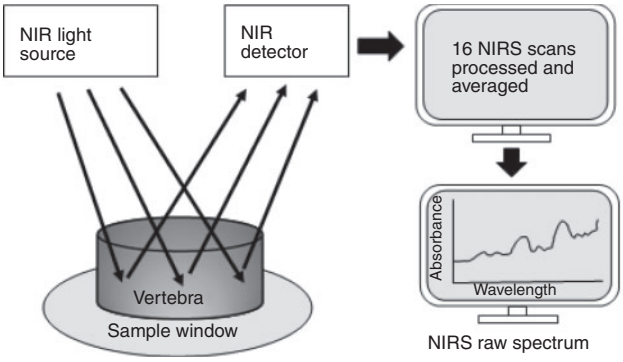

Fig. 1. Schematic of the near infrared (NIR) spectroscopy (NIRS) scanning process.

As a first step in the analyses, before the PLS, raw NIRS spectral data often need to be mathematically transformed to both remove noise in the raw spectra (mostly caused by instrument effects) and to enhance the visual resolution of spectral peaks (Reich 2005; Wedding 2007). The raw NIRS spectra for all the scanned centra of *S. mokarran* and *C. sorrah* are shown in Fig. 2. Several pretreatments were investigated and, for all calibration models, all raw spectral data were transformed before model development using a 25-point Savitsky–Golay spectral smoothing and a first derivative transformation. During the PLS analysis, validation of the calibration model was undertaken to ensure the model accurately predicted the vert-ages. This was done by a process of segmented cross-validation, as recommended for small sample sets of less than 120 (Williams 2013). In the cross-validation process, a group of samples was withdrawn from the total set and the calibration model developed using the remaining samples. The resulting model was then used to predict the ages of the samples that had been withdrawn. This process was repeated using successive groups of randomly withdrawn samples until all the sample ages had been predicted, with none of them having been used in the development of the models used to predict them (Williams 2013).

The PLS analysis initially used the full spectrum of the NIRS wavelengths of the scanned centra to develop the calibration

model (Foley *et al.* 1998). This full spectrum contained an extensive amount of information on the chemical composition of the centra (Williams 2008). The next step was to develop an optimal calibration model. This included a process to identify the minimum subset of wavelengths in the initial calibration model plot that most closely related to the vert-ages. Spectral areas of the plot were serially removed and the PLS analysis run each time. If the removal of a spectral area improved the calibration model, that spectral area was considered as not closely correlated with age and was permanently removed; conversely, if the removal of a spectral area worsened the calibration model, that spectral area was considered as more strongly correlated with age and was retained. This was repeated until the optimal calibration model was obtained. The optimal calibration model was the model that best predicted age (NIRS-age), and was defined by a combination of several statistical criteria, namely the combination of the highest possible coefficient of determination (R^2), the lowest root mean square error of cross-validation (RMSECV), the least number of terms, or factors, and the lowest bias (Wedding 2007; Williams 2008). Bias was the average difference between the vert-ages and the predicted ages (NIRS-ages; Williams 2008). The standard deviation ratio (SDR = standard deviation of the vert-ages ÷ RMSECV) was calculated to enable comparisons of model performance between the two species; the higher the SDR, the greater the relative predictive power of the model (Golic and Walsh 2006; Wedding 2007).

To provide further assessment of the accuracy of the predicted NIRS-ages, the predicted NIRS-ages for both species were used to fit a three-parameter version of the von Bertalanffy growth models (von Bertalanffy 1938) given by:

$$L_t = L_0 + (L_\infty - L_0)(1 - e^{(-kt)})$$

where L_t is length at age t, L_0 is length at birth, L_∞ is asymptotic length and k is the Brody growth coefficient. These models were compared with the von Bertalanffy growth models of the known vert-ages using likelihood ratio tests (Kimura 1980), as implemented by Haddon (2001).

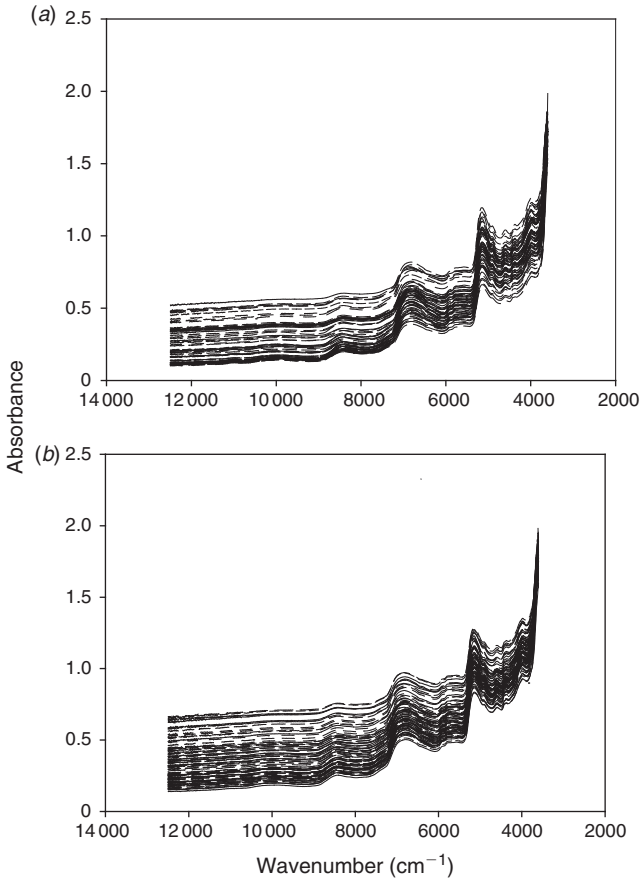

Fig. 2. Raw near infrared spectroscopy (NIRS) spectra of centra of (*a*) 80 *Sphyrna mokarran* and (*b*) 102 *Carcharhinus sorrah.*

Results

There was a relationship between the vert-ages of both *S. mokarran* and *C. sorrah* and the spectral output of their vertebrae, evidenced by calibration models with R^2 values of 0.83 and 0.78 respectively (Table 1). The older unknown vert-age animals were identified as outliers during the initial PLS regressions. In NIRS, an outlier was a sample that did not conform to the rest of the population and unduly influenced the calibration (Wedding 2007). Technically, it was a sample that differed from the rest of the population by three or more Mahalanobis distances (Williams 2008). The raw spectral data (Fig. 2) did not indicate the spectra of these older vertebrae were anomalies; that is, there had been no uncharacteristic interferences, such as power supply fluctuations, which would require rescanning of the samples (Williams 2008). Most of the *S. mokarran* data (76 samples) were of animals up to 10 years of

Table 1. Partial least-squares regression calibration model statistics for *Sphyrna mokarran* and *Carcharhinus sorrah*

The coefficient of determination (R^2), the root mean square error of cross validation (RMSECV), standard deviation ratio (SDR) and standard deviation (s.d.) are given for vert-ages and known vert-ages, where the ages are based on band counts and the term 'vert-ages' represents the estimated ages of all individuals and 'known vert-ages' represents the ages that have been validated and verified

Structure	Samples (*n*)	Age range (years)	s.d.	Terms	R^2	RMSECV (years)	Bias (years)	SDR
S. mokarran vert-ages	80	0.3–39.1	6.05	9	0.83	2.48	−0.146	2.44
S. mokarran known vert-ages	76	0.3–10.2	2.55	5	0.89	0.87	0.012	2.93
C. sorrah vert-ages	102	0.3–13.7	2.60	9	0.78	1.23	−0.007	2.11
C. sorrah known vert-ages	99	0.5–9.8	2.19	5	0.84	0.88	−0.005	2.50

known vert-age with just four older animals of 14, 19, 35 and 39 unknown vert-age years. For the *C. sorrah*, most of the data (99 samples) were of animals up to 10 years of known vert-age with only three older animals of 11, 12 and 14 unknown vert-age years.

Because no additional vertebrae from older unknown vert-age animals of these two species were available to increase the sample size for these older size classes, these older animals were removed from the dataset and new calibration models developed. This improved the overall calibration model statistics for both species, with an improvement in R^2 values from 0.83 to 0.89 for *S. mokarran* and from 0.78 to 0.84 for *C. sorrah*, and a reduction in the error (RMSECV) for *S. mokarran* from 2.48 to 0.87 years and for *C. sorrah* from 1.23 to 0.88 years (Table 1; Fig. 3). For the known vert-age models, *S. mokarran* had a marginally stronger predictive power, with a SDR of 2.93, compared with a SDR of 2.50 for *C. sorrah* (Table 1). In summary, the NIRS spectra correlated well with vert-ages; however, this correlation was stronger when only the vertebrae of validated ages were used. The final NIRS calibration models could be used reliably to predict the ages of *S. mokarran and C. sorrah* from Queensland, up to the validated and verified ages of 10 years.

The VB NIRS-age growth curves were not significantly different to the VB known vert-age growth curves for either species (Fig. 4). Likelihood ratio tests indicated that, for both species, there was no significant difference between the two curves, with *S. mokarran* coincident curve $\chi^2 = 7.66$, $P = 0.0536$, and *C. sorrah* coincident curve $\chi^2 = 6.95$, $P = 0.0734$.

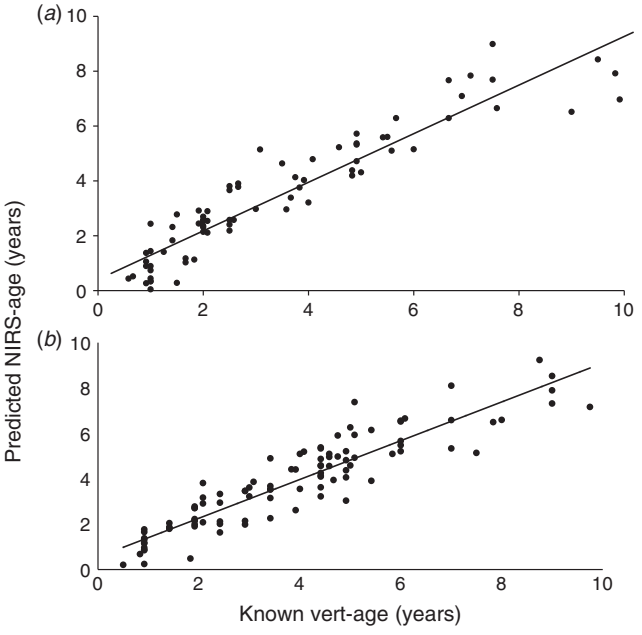

Fig. 3. Partial least-squares regression calibration model of predicted near infrared spectroscopy (NIRS)-ages plotted against known vert-ages (based on validated and verified band counts) for (*a*) *Sphyrna mokarran* ($R^2 = 0.89$) and (*b*) *Carcharhinus sorrah* ($R^2 = 0.84$).

The spectral areas that correlated most strongly with the known vert-ages of the vertebral centra were very similar for both *S. mokarran* and *C. sorrah* (Fig. 5). This suggested that similar molecular compounds correlated with known vert-age

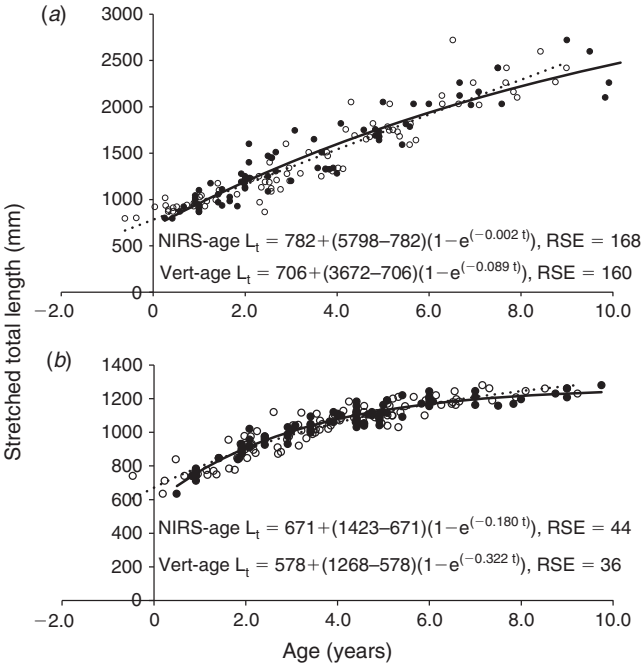

Fig. 4. Length-at-age data for known vert-age (based on validated and verified band counts; solid circles) and near infrared spectroscopy (NIRS)-age (open circles) with the fitted von Bertalanffy growth models and residual standard error (RSE) for (*a*) *Sphyrna mokarran* and (*b*) *Carcharhinus sorrah* known vert-age (solid line) and predicted NIRS-age (dotted line). L_t, length at age t.

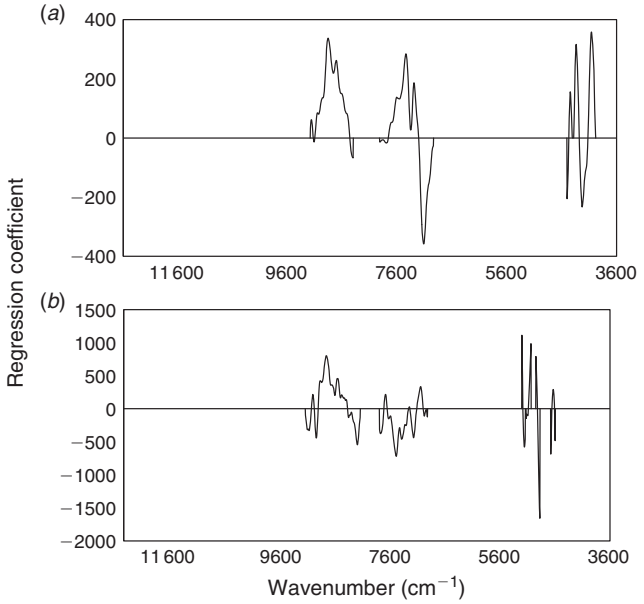

Fig. 5. Spectral areas of the calibration model plot for (*a*) *Sphyrna mokarran* and (*b*) *Carcharhinus sorrah.*

were being detected by the NIRS in the vertebrae of both species. There were three main spectral areas identified between 9200 and 4000 cm^{-1} (Fig. 5). Without further NIRS research on the chemistry of the vertebrae, it is not possible to relate these spectral areas to a specific ageing chemical compound, such as calcium phosphate. At this stage, these main spectral areas can only be described in terms of the main molecular compounds to which they correspond, based on a table of group frequencies. This is a table of groups of wavelengths (frequencies) characteristic of molecules that are prominent absorbers in the NIR region, which is commonly found in NIRS textbooks (Workman and Weyer 2008). The three main spectral areas corresponded to: Carbon–Carbon alkene, CH (aromatic), $–CH_3$ methyl, $–CH_2$ methylene all combination and second overtones; CH second overtone; $–NH_2$ primary amines combination and first overtone; $–CONH_2$ primary amides combination, first and second overtones; and –CONH secondary amides combination and second overtone.

Discussion

The results of the present study demonstrate that NIRS can be used to estimate the age of sharks. For both *S. mokarran* and *C. sorrah*, the NIRS method produced robust calibration models with a good ability to accurately predict the ages of vertebrae from these species up to the validated and verified known vert-ages of 10 years. These predicted NIRS-ages also produced comparable VB growth curves to those of the traditional ageing method. We showed that a robust age calibration model can be developed with 80–100 animals aged by the traditional method of growth band counts (Cailliet *et al.* 1986). Once the NIRS calibration model has been developed, further ageing of individuals of these species up to the validated and verified ages of 10 years included in the calibration model can be done rapidly and objectively. Their whole, dried vertebrae can be scanned with the NIRS instrument and the NIRS calibration model applied to predict their ages. Some random checks to monitor the age predictions would be required by occasionally using traditional ageing to verify the predicted age (Siesler *et al.* 2002). Although the NIRS method needs to be used in conjunction with traditional age reading methods and is not a validation method in itself, it does provide verification of the band counts and, most importantly, it can provide rapid age estimations of vertebrae that have not been traditionally aged up to the maximum age included in the calibration model.

The predicted age estimations can be done by NIRS very rapidly, which is one of the major benefits of the NIRS approach to ageing (Murray and Williams 1987; Roggo *et al.* 2007). Once the calibration model for a species has been developed, the predicted NIRS-age can be determined from a 16-s scan of a vertebra, without the need for sectioning or enhancement. An additional benefit of this NIRS approach to ageing is that because this predicted NIRS-age is mechanised, it is also objective and would maintain the precision of the age estimate of the calibration model and avoid further age reading subjectivity that would occur if all vertebrae needed to be aged by traditional visual band count estimates (Williams 2008). One aspect of age reading that cannot be avoided by NIRS is any errors associated with the traditional age reading of the reference samples. These errors would be incorporated into the calibration model and thus NIRS is reliant on accurate traditional age estimates to develop a calibration model that will be useful for prediction. NIRS instruments are widely available in industrial and research organisations, because NIRS is regularly used in the commercial and research sectors of agriculture, food, pharmaceuticals and medicine (Siesler *et al.* 2002; Ferrari *et al.* 2004). The availability of portable hand-held units is also increasing because of improvements in their precision and accuracy in recent years, which has raised demand (Herberholz *et al.* 2010; Alcalà *et al.* 2013).

The NIRS ageing method offers the potential for major savings in time and cost over the traditional shark age estimation process. It would be particularly beneficial where large numbers of vertebrae need to be aged, such as for inclusion in stock and risk assessments for commercially captured sharks (Kritzer *et al.* 2001; Thorson and Simpfendorfer 2009). Ages could be reliably predicted up to the maximum validated age used in the calibration model, and the resources saved by negating the need to traditionally age all vertebrae could be invested to increase the number of sharks aged. This would improve the reliability of age information for stock and risk assessments.

A limitation of the present study is that older age-validated material from the two species was not available and consequently it is yet to be determined whether the NIRS method would be viable for older animals of these two species. In the present NIRS investigation, the vertebral scans from individuals of both species >10 years of age were removed from the models because they were identified as outliers during calibration model development. This may have been because their ages were not well represented in the dataset, with very few older individuals among a much larger population of younger animals. Ideally, the calibration samples would consist of an even representation of all age classes (Reich 2005; Williams 2008), although with sharks this can be logistically difficult. Alternatively, age underestimation of the older individuals that had not been age validated could have caused poor correlations between the vertebral ages and their corresponding spectra that led to the identification of these older animals as outliers. Age underestimation has been identified for older individuals of several species (Campana *et al.* 2002; Francis *et al.* 2007; Andrews *et al.* 2011). In the tropical shark study, a low recapture rate of larger *S. mokarran* precluded validation of animals >10 years of age and for *C. sorrah*, although some calcein-marked larger animals were recaptured, the calcein failed to mark their vertebrae and it was suggested that somatic growth and calcification of their vertebrae had either slowed or ceased (Harry *et al.* 2011, 2013).

The issue of NIRS age predictions for older animals could not be resolved in the present study because no additional older individuals were available; however, it was addressed in subsequent work that extended the NIRS shark ageing research to deep-water shark species (Rigby *et al.* 2014). Two dogfish species (*Squalus megalops* and *S. montalbani*) were traditionally aged up to 25 and 31 years respectively using band counts on their dorsal fin spines (DFS). Vertebrae and fin clips were also removed from the same *S. megalops* animals aged by DFS because DFS annual band pair deposition had been verified for *S. megalops* (Braccini *et al.* 2007). Scans of the three structures

from *S. megalops* (DFS, vertebrae and fin clips) and the DFS from *S. montalbani* all produced NIRS calibration models with a good ability to predict ages up to the maximum ages of 25 and 31 years (Rigby *et al.* 2014). In that study, the vertebrae themselves could not be aged by traditional age reading because growth bands were not visible, yet the DFS proxy ages from the same animals clearly indicated that chemical changes occurred in the vertebrae correlated with age up to the maximum ages in the study. The older age classes were much better represented for *S. megalops* and *S. montalbani* than for *S. mokarran* and *C. sorrah*, which suggested that the older *S. mokarran* and *C. sorrah* were outliers in the NIRS models because of a lack of representation in the dataset. However, possible age underestimation of the older animals by traditional age reading cannot be discounted, but was outside the capacity of the present study to resolve.

The NIR spectra of the vertebrae contained large amounts of information about the chemistry of the vertebrae, yet the spectral areas that correlated most strongly with age were very similar for both *S. mokarran* and *C. sorrah*. This suggested that chemical changes in the vertebrae associated with age were detected by the NIRS method. These chemical changes could have been related to hydroxyapatite $3(Ca_3PO_4)_2 \cdot Ca(OH)_2$, the primary growth mineral in the calcified cartilage of vertebrae (Walker *et al.* 1995; Hamlett 1999). The deposition of hydroxyapatite within an organic matrix creates the growth bands used for vertebral ageing, with different ratios of the mineral and organic matrix producing the two optically distinct opaque and translucent bands (Casselman 1974; Kerr and Campana 2014). However, the specific chemical compound in the vertebrae that correlated with the NIRS spectra is unknown at this stage. A limitation of NIRS is that the spectra are very complex (Murray and Williams 1987) and to determine the identity of the chemical compounds that could be related to the vertebral ages would require considerably more research into NIRS and the chemistry of shark ageing structures, which was beyond the scope of the present study.

Future work to investigate what the NIRS is detecting in the shark vertebrae could include NIRS scans of pure hydroxyapatite to create a reference spectrum for comparison with the spectra of vertebrae from age-validated animals. This could also enable a quantitative measure of the ageing compounds and the potential to measure chemical changes in the vertebrae, which may help to address the issue of sharks that cannot be aged because of poorly calcified vertebrae. Unlike previous chemical assay approaches that were very time consuming and costly (Jones and Geen 1977; Cailliet and Radtke 1987), the NIRS technique is cost-effective, simple and rapid, and consequently offers a realistic ability to investigate vertebral chemistry. Validation of the quantitative chemical changes with age would be required, and bomb radiocarbon validation may be the most promising approach because it has proven effective with shark vertebrae (Campana *et al.* 2002; Francis *et al.* 2007; Andrews *et al.* 2011).

Regional variability in the intraspecific age and growth of sharks is well documented (Cailliet and Goldman 2004; Cope 2006; Rigby and Simpfendorfer 2015) and should be considered in the development of future NIRS calibration models for age estimation. The models from the present study could be used to reliably predict ages up to 10 years for *S. mokarran* and *C. sorrah* from Queensland. To increase the regional scope of the model, additional samples from a wider geographic area could be added to the existing calibration model as they became available, providing they were from animals with the same age and growth parameters as the Queensland populations (Foley *et al.* 1998; Williams 2008). This would also then include the potentially different water bodies that can affect the microchemistry of the age structure and the corresponding spectral characteristics (Tillett *et al.* 2011; Kerr and Campana 2014; Wedding *et al.* 2014). For NIRS in general, it is recommended to include the entire range of biological and geographic variability in the NIRS calibration model to ensure acceptable accuracy in the predicted parameter (Siesler *et al.* 2002; Bobelyn *et al.* 2010). In the teleost otolith study (Wedding *et al.* 2014), seasonal rather than regional differences were detected and separate calibration models developed. However, the accuracy of prediction improved when both seasons were included in one calibration model because of the inclusion of greater biological variability. The shark NIRS age prediction models could possibly be improved by the development of separate models for males and females where sufficient data are available, which is done for traditional age and growth studies because sexually dimorphic growth is a general feature of shark populations (Cortés 2000; Cailliet and Goldman 2004). The present study was inconclusive on this matter because the NIRS model for *S. mokarran* was based on combined sexes and was stronger than that of *C. sorrah*, which used just females.

The present investigation of NIRS for ageing sharks clearly demonstrated it is feasible for age estimation of sharks. Although the NIRS approach needs to be first used in concert with accurate traditional age reading and validated ages to develop a robust calibration model, it then has the ability to rapidly estimate ages of vertebrae that have not been traditionally aged up to the maximum ages used in the calibration model. This offers the potential to cost-effectively age large numbers of sharks, which could improve the reliability of age information for stock and risk assessments and provide benefits to fisheries management. We consider the NIRS approach to ageing could be applicable to a wide range of shark taxa, because the two study species were of different habitats, body sizes and life histories.

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