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Are Pacific spiny dogfish lying about their age? A comparison of ageing structures for *Squalus suckleyi*

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Abstract. Historically, Pacific spiny dogfish (*Squalus suckleyi*) have been aged using dorsal fin spines, a method that was validated through bomb radiocarbon analysis and oxytetracycline tagging. However, ages generated using this method generally have poor precision and require estimation of missing growth bands in eroded spines, prompting a search for improved age determination methods. In the present study, spiny dogfish were aged using the historical spine method and a new method involving stained thin sections of vertebral centra. Results of an inter-laboratory exchange demonstrated the need for readers to calibrate ageing criteria with a reference collection before reading structures, a practice that yielded significant improvements in between-reader precision of spine band pair counts. After calibration, the primary readers examined the full sample set. The two structures yielded similar age estimates for younger animals, but centrum estimates were consistently younger than spine estimates after age-10. Although further work is necessary to fully explore potential reasons for the observed bias, such as centrum size and location within the vertebral column, at the present time centra are not a suitable alternative to dorsal fin spines for age determination of Pacific spiny dogfish >10 years of age.

Additional keywords: age calibration, elasmobranch, reader agreement.

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Introduction

Accurate age data are critical to understanding fish population dynamics and to promote effective fish stock assessment and fishery management. Choosing the most accurate age determination method is critical to fishery management because age determines growth and mortality estimates from which biological reference points are derived to prescribe target harvest rates and to prevent overfishing (Campana 2001). Traditional fish age determination methods rely on periodic growth patterns within hard structures of the body that grow continuously throughout the life of the animal (Campana and Thorrold 2001). Pairs of alternating concentric growth bands, frequently referred to as 'opaque' and 'translucent', are counted to arrive at an estimate of age (Goldman *et al.* 2012). Although these band pairs are often presumed to be deposited annually, temporal periodicity of formation must be verified (Morales-Nin and Panfili 2002; Cailliet and Goldman 2004; Cailliet *et al.* 2006). There are several steps in undertaking fish age determination: (1) an appropriate structure is chosen; (2) the best preparation method for viewing growth patterns is selected; (3) the temporal periodicity of band pair formation is determined; (4) the repeatability or precision of age interpretations is evaluated; and, finally and most importantly, (5) the validity or accuracy of age estimates is established (Morales-Nin and Panfili 2002). However, fish age determination can be fraught with difficulty, both in selecting an appropriate hard structure and in interpreting growth patterns and validating their frequency of formation. Otoliths or scales are often used to age teleost species; however, sharks and other elasmobranchs, which are cartilaginous, do not possess otoliths or scales that can be aged (Cailliet 2015). Numerous alternative structures have been examined for elasmobranchs, ranging from vertebral centra (the central bodies of vertebrae, hereafter simply termed 'centra') to caudal thorns, but not all structures are appropriate for all species (Goldman *et al.* 2012). Furthermore, it should be noted that, in some species, hard structures may not grow continuously over time, but may instead be a function of fish size (e.g. Francis *et al.* 2007; Natanson *et al.* 2008).

The Pacific spiny dogfish (*Squalus suckleyi*) is a small shark species common to the coastal waters of the eastern North Pacific Ocean. This species is so named because it possesses a large spine anterior to each of its two dorsal fins. The two spines extend from the animal's body into the environment and are thus subject to wear and possible breakage. *S. suckleyi* is a close relative of the North Atlantic spiny dogfish (*Squalus acanthias*) and, until recently, they were considered the same species (Ebert *et al.* 2010). Much of the literature on the North Pacific Ocean spiny dogfish published before 2010 refers to the species as '*S. acanthias*'; based on current nomenclature, those cases actually refer to *S. suckleyi* (Bonham *et al.* 1949; Ketchen 1975; McFarlane *et al.* 1987; Saunders and McFarlane 1993; Tribuzio *et al.* 2010).

A method for ageing *S. suckleyi* was first defined in the 1930s from a study that examined centra, teeth, fin rays and fin spines (Kaganovskaia 1933). Ultimately, Kaganovskaia (1933) determined that the only appropriate ageing structure was the posterior dorsal fin spine and only selected specimens with 'ideal' or intact and unworn spines for ageing. In particular, Kaganovskaia (1933) rejected centra because that study only examined unstained centrum sections, in which band pairs are hard to discern. This work was followed by that of Bonham *et al.* (1949), who also only included ideal spines but made a point of acknowledging that such selection could bias the results. The posterior dorsal fin spine was deemed superior to the anterior spine because it is generally subject to less wear (Kaganovskaia 1933; Ketchen 1975). Subsequently, Ketchen (1975) developed an approach that accounted for lost band pairs on broken or worn spines. Numerous studies have used the methods of Ketchen (1975) to generate age estimates for Pacific spiny dogfish (McFarlane *et al.* 1987; Saunders and McFarlane 1993; McFarlane and King 2009; Tribuzio *et al.* 2010). The use of the posterior dorsal fin spine as an ageing structure was validated using bomb radiocarbon dating (Campana *et al.* 2006) and recapture of tagged fish with known periods of liberty that had been previously injected with oxytetracycline to verify annual periodicity of growth increments on spines (Beamish and McFarlane 1985; McFarlane *et al.* 1987; McFarlane and King 2009).

The approach put forth by Ketchen (1975) uses simple exponential regression to estimate the number of band pairs lost due to spine erosion. This approach assumes that the variability in age data is due to the size of the spine and that band pairs are accreted in a predictable pattern. An alternative approach has been proposed using non-linear mixed-effects modelling in which the number of lost band pairs is treated as a random effects variable (Cheng 2012). These two approaches result in different age estimates for older fish, with estimates using the method of Cheng (2012) being substantially older (Taylor *et al.* 2013). Taylor *et al.* (2013) critiqued both methods as problematic in stock assessments and called for investigations into new ageing structures that would eliminate the need for estimating lost band pairs. Furthermore, age imprecision (CV = 19%) and systematic bias among laboratories have been documented for *S. suckleyi* (Rice *et al.* 2009), further highlighting the need for alternative methods of age determination.

Centra have been proposed as an alternative to fin spines for ageing *S. suckleyi* because this method eliminates the need to estimate the number of missing band pairs associated with worn spines. Indeed, recent research suggests that it is an appropriate structure for the congener *S. acanthias* (Bubley *et al.* 2012). Gross sagittal sections of centra prepared using staining procedures adapted from Natanson *et al.* (2007) resulted in more precise and accurate age estimates for *S. acanthias* than the traditional fin spine method (Bubley *et al.* 2012). Thus, the objectives of the present study were to: (1) adapt the centrum ageing methods used in Bubley *et al.* (2012) to centra of *S. suckleyi*; (2) examine the suitability of the centrum method for age determination of *S. suckleyi* through age estimate precision and edge analysis (i.e. edge type and marginal increment); (3) compare age estimates derived from the historical fin spine method with those from the centrum method; and (4) conduct an inter-laboratory exchange to determine whether there are significant differences between laboratories in band pair counts, and to examine whether those differences are affected by readers who calibrate with a reference collection.

Materials and methods

Sample collection and preparation

Samples were collected opportunistically from commercial fisheries bycatch and routine assessment surveys. Fishery-independent collections occurred on the National Marine Fisheries Service (NMFS) Alaska Fisheries Science Center's (AFSC) biennial bottom trawl (2011) and annual longline surveys (2010, 2011 and 2013) in the Gulf of Alaska (Fig. 1). Specimens were also collected in 2012 by the Seattle Aquarium and during an *S. suckleyi* tagging survey operated by the NMFS Northwest Fisheries Science Center in Puget Sound (WA, USA; Fig. 1). Personnel at the Alaska Department of Fish and Game provided samples from *S. suckleyi* caught incidentally in commercial longline fisheries in both south-east Alaska and Prince William Sound, and Oregon Department of Fish and Wildlife personnel collected samples from commercial trawl bycatch along the Oregon coast (Fig. 1). All samples were collected opportunistically, and thus a sampling design was not applied; however, efforts were made to collect samples from each month of the year. Sex and precaudal length (PCL) were recorded for each fish, and the posterior fin spine and attached section of vertebral column removed for ageing (Tribuzio *et al.* 2017). Samples were frozen and processed at the AFSC Age and Growth Program for age determination.

In preparation for the present study, a pilot project was conducted to determine whether banding patterns were visible in centra using the methods of Bubley *et al.* (2012). As part of this unpublished pilot study, both anterior and posterior centra were examined to assess whether position in the vertebral column could affect band pair counts. The pilot project was based on six fish (three males, all 68 cm PCL, and three females ranging from 64 to 81 cm PCL) from which both anterior and posterior centra were sampled. A two-tailed paired *t*-test (H_0: $\mu_d = 0$) showed no significant difference between anterior and posterior centra in band pair counts ($P = 0.322$; $t_{0.05(2),5} = 2.571$; mean paired difference ($\bar{d}$) = 1.083; 95% confidence interval (CI) −1.453, 3.620). Thus, predominantly only posterior centra were collected for the analyses in the present study because of the ease with which they could be collected simultaneously with

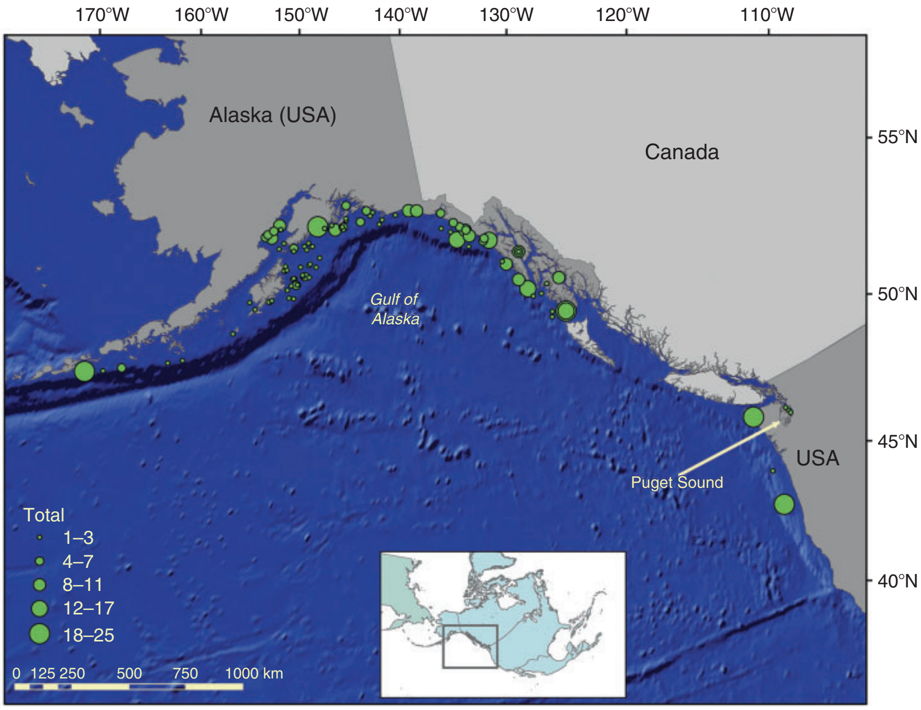

Fig. 1. Map showing sampling locations of *Squalus suckleyi*; the size of the symbol is proportional to the number of fish collected at each location. Owing to confidentiality restrictions, samples from commercial fishing operations are shown, but the locations have been shifted to the centroids of non-confidential (400 km^2) grid squares.

the posterior dorsal fin spine. In some animals, sections of centra ventral to each dorsal fin were collected to compare age estimates from anterior and posterior structures to supplement the analysis conducted in the pilot study.

Fin spines (hereafter termed 'spines') and centra were processed separately. A detailed description of the methods to prepare both structures is available in Tribuzio *et al.* (2017). Spine preparation was relatively simple: all soft tissue was removed and the spines were allowed to air dry and then stored in paper envelopes. Centrum processing was more intensive. First, centra were gently cleaned of soft tissue and stored in ethanol until further processing. Multiple centra were mounted in resin blocks and sectioned along the sagittal plane at ~0.4 mm. Sections were then decalcified, stained with haematoxylin, destained, fixed in glycerol and then mounted on slides.

Reference collection and ageing criteria development

An integral part of ageing studies is to ensure consistent age determination criteria among all readers. Therefore, a set of reference samples (both spines and centrum sections) was created and examined collectively by several experienced age readers to reach a consensus age for each structure. Included in the reference collection was a set of high-resolution images of spines in which the number of band pairs and absolute age were validated using bomb radiocarbon, provided by the Bedford Institute of Oceanography (Campana *et al.* 2006). An age determination manual was developed with a standard set of ageing criteria for both spines and centrum sections (Tribuzio *et al.* 2017). This reference collection and manual were used to train all readers who participated in the present study to ensure consistency among readers. The reference collection is available upon request from the AFSC Age and Growth Program.

Spine and centrum age determination

All samples in the present study were read once by two to four experienced readers at the AFSC (the 'primary' readers), with the lead reader examining each structure at least twice. Spines were examined using reflected light and low-power magnification (approximately 10–20× magnification), whereas centrum sections were examined using transmitted light at up to 40× magnification (Tribuzio *et al.* 2017). The terms 'opaque' and 'translucent' are often used to describe pairs of growth bands on centra. However, because spines do not have opaque and translucent bands, we use the terms 'dark' and 'light' to refer to growth bands in both structures for the sake of consistency. For both structures, a pair of growth bands (one dark and one light) was assumed to represent 1 year of growth, although it should be noted that periodicity of band pair formation in centra has not been validated (Tribuzio *et al.* 2017). On spines, all band pairs were counted between the base of the spine and the last readable point; on centra, all band pairs were counted distal to the birth mark, which represents age-0 (Fig. 2; Tribuzio *et al.* 2017). It is important to distinguish between age and band pair counts. The number of band pairs counted on a centrum thin section is equivalent to the age estimate; however, as discussed earlier, spines sometimes require estimation of missing band pairs if wear has occurred and, as such, the band pair count may only represent a portion of the total age of the fish.

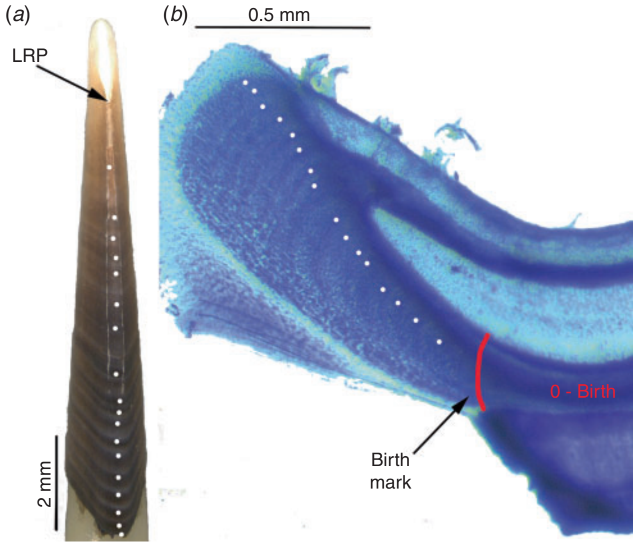

Fig. 2. The two structures used to age *Squalus suckleyi*: (*a*) a posterior spine from a male (70-cm precaudal length) with the last readable point (LRP) indicated by the arrow and band pairs marked by white dots; and (*b*) a stained thin section of a posterior centrum from the same animal with the birth mark indicated by the arrow and band pairs indicated by white dots.

The readability of each structure was assessed by the reader on a scale of 1–3 as follows: 1, the structure is clear, the reader is confident in the band pair count and could duplicate the count in another reading; 2, the reader can generate a single band pair count, but with variable level of confidence; and 3, the reader has little confidence in duplicating the band pair count and is unable to generate a single count, but can assign a range (Tribuzio *et al.* 2017). The enamel base diameter (EBD), diameter at the last readable point (LRD) and band pair count were recorded for spines (Tribuzio *et al.* 2017). For centra, each reader recorded a band pair count and, when possible, edge type, characterised as being either 'dark' (slow growth) or 'light' (fast growth; Tribuzio *et al.* 2017). All structures were examined and photographed using a Leica (Wetzlar, Germany) S6D stereomicroscope with an attached Leica DFC295 camera; however, images were not used to count band pairs. For centrum specimens with clear increment boundaries, ImageJ image analysis software (https://imagej.nih.gov/ij/, accessed 25 August 2013) was used to measure centrum radius, edge width (EW) and last band pair width (LAW) from photographs for marginal increment analysis (Conrath *et al.* 2002; Tribuzio *et al.* 2017), as described in further detail below.

Testing suitability of centra for age determination

The centrum method is a new approach for *S. suckleyi* and it is thus important to examine the feasibility of using centra as ageing structures. This was accomplished in four ways. First, to verify the formation of one dark and one light growth band per year, the proportion of each edge type was calculated for each month based on posterior centra. Second, marginal increment analysis (MIA) was conducted on a subset of posterior centrum samples to further examine the annual pattern of band deposition following the methods of Conrath *et al.*(2002), where EW was divided by LAW to yield the marginal increment ratio (MIR), which was then plotted by month of capture. MIA was performed on centrum thin sections that were from animals <60 cm PCL and that had easily definable banding patterns, in which the reader had high confidence in the EW and LAW measurements. Third, the posterior centrum radius was compared with PCL to determine whether centra grow continuously, based on a significant positive relationship between centrum size and animal size. Centrum radius and PCL were log transformed and fit with a linear regression to determine whether data from the sexes should be fit separately. Then, the untransformed radius and PCL data were fit with various models (e.g. linear, quadratic, exponential) by least-squares regression. *F*-tests based on analysis of variance (ANOVA) values were used to determine whether the regressions between centrum radius and PCL were significant, and the best fitting model was chosen based on the R^2 value. Finally, a paired *t*-test was conducted to determine whether anterior and posterior centra had significantly different band pair counts. A linear model was applied to the difference between the anterior and posterior band pair counts to estimate bias.

Age estimate precision and bias among readers and structures

Inter-reader precision was examined by calculating the average percentage error (APE), CV and percentage agreement (%A) between readers after calibration (Beamish and Fournier 1981; Chang 1982; Campana 2001). Ageing studies generally examine %A within one or two band pairs to gauge reader agreement (Cailliet and Goldman 2004). In the case of *S. suckleyi*, a long-lived species, such a narrow range of agreement may not be appropriate; thus, %A within a 10% range was calculated (Tribuzio *et al.* 2010). For example, if Reader 1 counted 10 band pairs on a structure, Reader 2 would be considered in agreement if they counted nine to 11 band pairs. If Reader 1 counted 30 band pairs, Reader 2 would be considered in agreement if they counted 27–33 band pairs. Age–bias plots (Campana *et al.* 1995) were also created to examine differences between the three primary readers who aged the greatest number of samples.

Band pair counts from centrum thin sections were then compared with band pair counts from spines. Only unworn spines were used because worn, or eroded, spines may have lost band pairs; in such cases the band pair count underestimates age and would not be comparable to centrum band pair counts. Unworn spines were those in which the LRD was less than the assumed size of the spine after 2 years of growth (LRD ≤ 2.45 mm; Ketchen 1975; McFarlane and King 2009). Mean CVs of band pair counts for each structure were estimated to compare the precision of spine and centrum band pair counts, and an ANOVA was conducted to determine whether there was a relationship between band pair count and CV. A paired *t*-test was conducted to compare the sample CVs of the spines and centra. Median band pair count was calculated across all four readers (three readers for spines because one of the readers conducted their reads before calibration, and those were removed from the analysis) for each structure for the following analyses. Two paired *t*-tests were conducted to compare band pair counts from centrum with band pair counts from unworn spines: one between only anterior centrum sections and unworn spines and the other between posterior centrum sections and unworn spines.

Inter-laboratory exchange of samples and effects of calibration

To address concerns about consistency among age determination laboratories, we included an inter-laboratory exchange of samples. A randomly selected set of 25 spines and 25 centrum sections (not from the same animals) was sent to six laboratories with varying experience in ageing *S. suckleyi*. Readers from each laboratory were instructed to examine the structures as they normally would. Then, the readers were asked to read through the ageing manual and examine the reference collection before reading the exchange structures a second time (allowing at least 1 day between reads). All readers in the inter-laboratory exchange were asked to classify their experience level with each structure as follows: 1, new age reader; 2, experienced age reader with limited experience ageing elasmobranchs; 3, experienced elasmobranch age reader but not with this structure; 4, experienced elasmobranch age reader with this structure, but not *S. suckleyi*; 5, experienced *S. suckleyi* age reader with this structure.

Analysis of the inter-laboratory exchange data served two purposes: (1) to determine the value of having readers examine a reference collection (hereafter termed 'calibration') before age estimation; and (2) to determine whether there were significant differences in band pair counts among laboratories. Analyses of the inter-laboratory exchange were conducted at the level of the individual reader, because only one reader participated from most of the laboratories. Cases where the reader classified the readability of the spine or centrum as '3' were not included in the inter-laboratory data analysis. The interquartile range (IQR) was used to estimate a 95% CIs around the median for each specimen's reads before and after calibration using the formula 1.58 $\mathrm{IQR}/\sqrt{n}$ (Zar 1999). A paired *t*-test was conducted on spine and centrum thin section band pair counts and CVs before and after calibration for each reader. The effect of band pair count and calibration on CV was estimated with linear regression and ANOVA (Zar 1999). Finally, a paired Wilcoxon signed-rank test was conducted for each reader to determine whether the band pair counts (before and after calibration tested separately) were significantly different from the median of all readers. All statistical tests were conducted at the $\alpha = 0.05$ level of significance using R, ver. 3.1.0, statistical computing software (https://cran.r-project.org/, accessed 15 June 2014).

Results

In all, 389 spines (162 males, 221 females, six of unknown sex) and 497 centra (211 males, 281 females, five of unknown sex (i.e. where sex was not recorded)) were collected. Fish lengths ranged from 29 to 80 cm PCL for males, from 36 to 102 cm PCL for females and from 60 to 87 cm PCL for unknown sex (Fig. 3). In total, 59 anterior centra (29 from males and 30 from females) and 438 posterior centra (182 from males, 251 from females, and five from individuals of unknown sex) were collected. A linear model of the band pair count against the PCL by sex indicated no significant interaction between the sex term and the model; thus, the data for both sexes were combined.

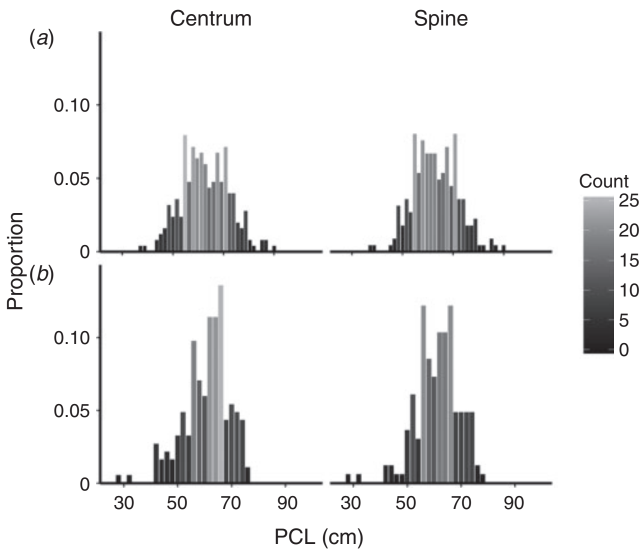

Fig. 3. Proportional size (precaudal length, PCL) distribution of *Squalus suckleyi* spine and centrum samples in (*a*) females and (*b*) males. The degree of shading of the columns represents the sample size for each size bin.

Testing suitability of centra for age determination

The feasibility of the centrum as an ageing structure was supported several ways. The edge type showed a clear annual pattern. For specimens collected in February and March, nearly 100% of centrum samples in which an edge type was discernable were the 'dark' edge type (Fig. 4*a*). The proportion of samples with the dark edge type decreased through the summer, reaching 0% in September, and then increased back to 100% in December. Similarly, the trend in MIR suggested an annual pattern of deposition (Fig. 4*a*); however, sample sizes of centrum sections that were clear enough to allow increment measurement were small, and no suitable samples were available in the months of April, June or December. Thus, our assessment of MIR remains qualitative.

There was a significant interaction between sex and log-transformed centrum radius when compared with PCL (ANOVA, $P < 0.001$); thus, the radius-at-fish-length models were fit for the sexes separately. A linear model provided the best fit of centrum radius to PCL and was significant for both sexes (Fig. 4*b*; females: $\mathrm{PCL} = 52.102 + 160.394 \times \mathrm{radius}$, $R^2 = 0.970$; males: $\mathrm{PCL} = 186.410 + 119.440 \times \mathrm{radius}$, $R^2 = 0.870$; ANOVA, $P < 0.001$ for both models), demonstrating that centrum radius increases consistently with fish length.

Band pair counts from anterior centra were significantly greater than band pair counts of posterior centra ($P < 0.001$; $t_{0.05(2),54} = 2.005$; $\bar{d} = 0.836$; 95% CI 0.372, 1.301). However, bias plots (Fig. 4*c*) and linear regression of the residuals (slope = 0.027 (95% CI −0.099, 0.153; $P = 0.665$); intercept = 0.519 (95% CI −1.016, 2.054; $P = 0.501$)) showed no systematic bias between band pair counts of centra from the anterior and posterior regions of the column.

Age estimate precision and bias among readers and structures

Inter-reader bias plots showed no systematic bias between readers (Fig. 5). Across all readers, the APE and CV were

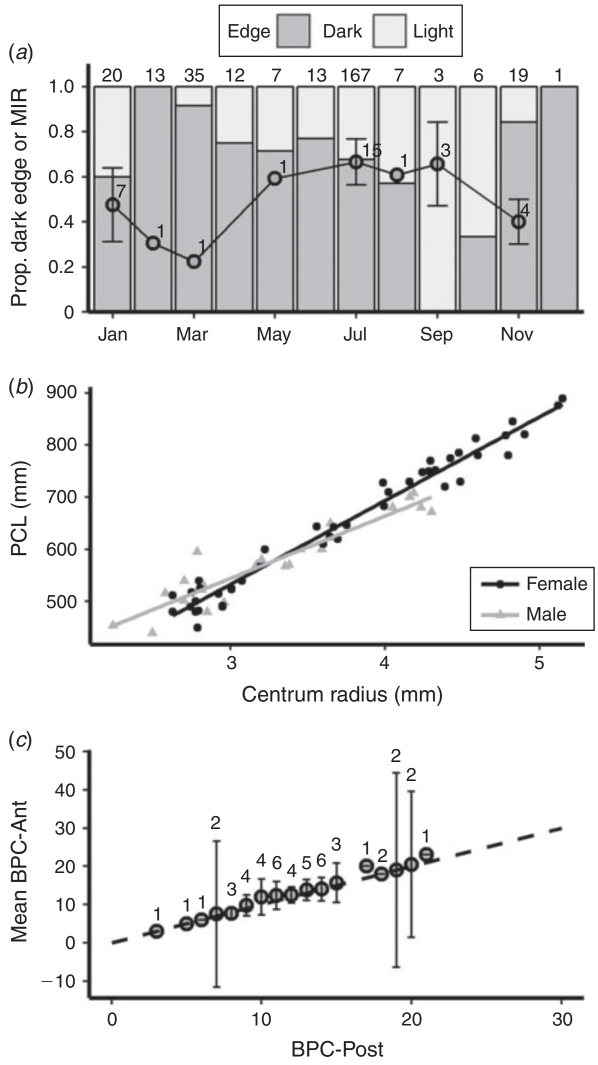

Fig. 4. Testing the suitability of centra as an ageing structure for *Squalus suckleyi* using (*a*) the proportion (Prop.) of samples with the dark edge type by month (shaded bars; numbers above the bars are the sample size for each month) and marginal increment ratio (MIR) of the edge width to the last band pair width (symbols with error bars indicating 95% confidence intervals; numbers are sample size), (*b*) precaudal length (PCL) plotted against the centrum radius for males and females with least-squares regression fits and (*c*) agreement plot of the mean band pair count for anterior centra (BPC-Ant) *v.* the band pair count of posterior centra (BPC-Post), with error bars indicating 95% confidence intervals, numbers showing the sample size and the dashed line indicating 1 : 1 equivalence.

generally lower (and %A was greater) for spines than for centra (Table 1). %A (±10% band pair counts) between readers was highly variable for both structures, ranging from 0 to 100%, and the CV between readers was also variable, ranging from 0 to 48.7%. The paired *t*-test of the spine and centrum CVs showed a significant difference between the two structures ($P < 0.001$; $t_{0.05(2),166} = 1.974$; $\bar{d} = 2.969$; 95% CI 1.393, 4.545). The mean CV at band pair count was greater for centra until 11 years, after which the spine CV was consistently greater. Linear regression of the CV on band pair count showed a significant relationship for both spines and centra (Fig. 6), with the slope for spines increasing and that for centra decreasing with increasing band pair count (ANOVA, $P < 0.001$ for both).

There was no significant difference between band pair counts of unworn spines and anterior centra (Fig. 7*a*; $P = 0.299$; $t_{0.05(2),6} = 2.447$; $\bar{d} = 1.944$; 95% CI −2.066, 5.954); however, sample size was small ($n = 7$). There was a significant difference between band pair counts of unworn spines and posterior centra (Fig. 7*b*; $P < 0.001$; $t_{0.05(2),199} = 1.972$; $\bar{d} = 6.571$; 95% CI 6.07, 7.070). Centrum band pair counts were systematically lower than spine band pair counts for fish older than 10 years (Fig. 7).

Inter-laboratory exchange of samples

Results of the inter-laboratory exchange suggested that reader calibration led to a significant change in band pair counts. The range of band pair counts was generally narrower for spines after calibration, often with a significant decrease in the median (Fig. 8). The paired *t*-test of reads before and after calibration showed a significant change in band pair counts for spines from five of the readers, as well as when the data from all readers were combined (Table 2). Similarly, for centra, calibration resulted in a significant change in band pair counts for all but one reader, as well as when the data from all readers were combined. One reader classified all the centra with a readability code of 3 both before and after calibration (Reader 1) and another reader classified them all as 3 after calibration (Reader 2); thus, those readings were not included in further analysis. However, the failure of Reader 2 to produce any single-point age estimates after calibration because of the change of the readability code should be considered a significant result.

The CV was significantly different before and after calibration for spines ($P < 0.001$; $t_{0.05(2),24} = 2.064$; $\bar{d} = -9.173$; 95% CI −13.398, 4.948), but not for centra ($P = 0.052$; $t_{0.05(2),24} = 2.064$; $\bar{d} = 5.150$; 95% CI −0.0404, 10.341). Calibration (ANOVA, $P < 0.001$; Fig. 9*a*) and band pair count (ANOVA, $P = 0.007$; Fig. 9*b*) were significant factors in the relationship between CV and band pair count. The number of readers whose band pair counts were significantly different from the median band pair count decreased after calibration for both spines and centra (Table 3).

Discussion

Stained centrum thin sections initially showed promise as an alternative ageing structure to dorsal fin spines for *S. suckleyi*. Growth band pairs were visible using the preparation methods of Bubley *et al.* (2012). Centrum radius increased linearly with fish length, suggesting that centra grow continuously as the animal grows. Furthermore, edge analysis and MIA both suggested that one band pair is deposited per year, verifying our age determination criteria for centra, although it should be noted that these analyses were performed predominantly on young (3–14 years) fish because they tended to have the clearest growth increment boundaries. Despite these findings and the successful use of stained centrum thin sections as an age determination method for *S. suckleyi*'s shorter-lived Atlantic counterpart *S. acanthias* (Bubley *et al.* 2012), *S. suckleyi* posterior centrum band pair counts did not agree with unworn spine band pair counts in fish older than 10 years (~60 cm PCL). In these older fish, a

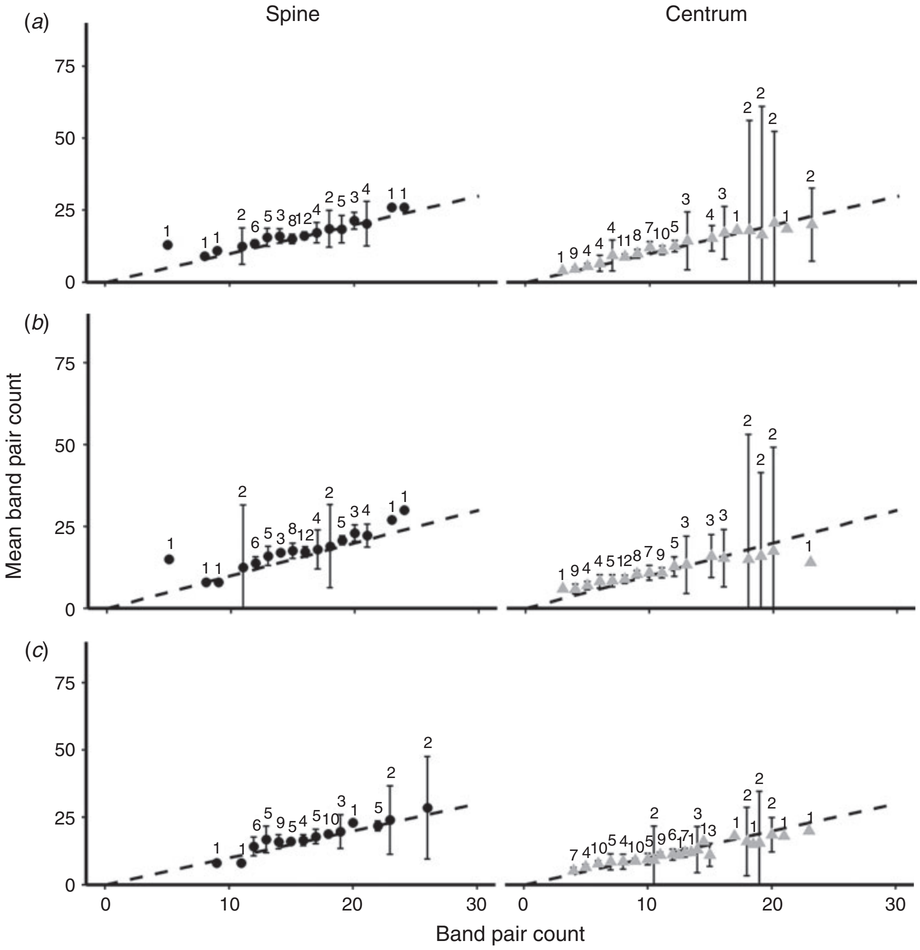

Fig. 5. Agreement plots comparing band pair counts made by three primary readers for *Squalus suckleyi* spines and centra. (*a*) Reader 2 *v*. Reader 1; (*b*) Reader 3 *v*. Reader 1; (*c*) Reader 3 *v*. Reader 2. Error bars are 95% confidence intervals around the mean band pair count. Numbers are the sample size for each band pair count. Dashed lines indicate 1 : 1 equivalence.

Table 1. Average percentage error (APE), CV and percentage agreement (%A) between the three primary readers

	Source	Number of specimens	APE	CV	%A
Reader 1 *v*. Reader 2	Spine	59	5.9	8.3	74.6
Reader 1 *v*. Reader 3	Spine	59	7.9	11.2	49.2
Reader 2 *v*. Reader 3	Spine	59	6.8	9.6	72.9
Reader 1 *v*. Reader 2	Centrum	88	8.7	12.3	51.1
Reader 1 *v*. Reader 3	Centrum	80	10.3	14.6	52.5
Reader 2 *v*. Reader 3	Centrum	83	7.9	11.2	51.8

systematic bias was detected in which centrum band pair counts were consistently younger than spine band pair counts. Given that the spine method of age determination has been validated (Campana *et al.* 2006; McFarlane and King 2009), posterior centra do not provide an improved alternative to spines, at least for fish over 10 years of age. Further investigation is needed to

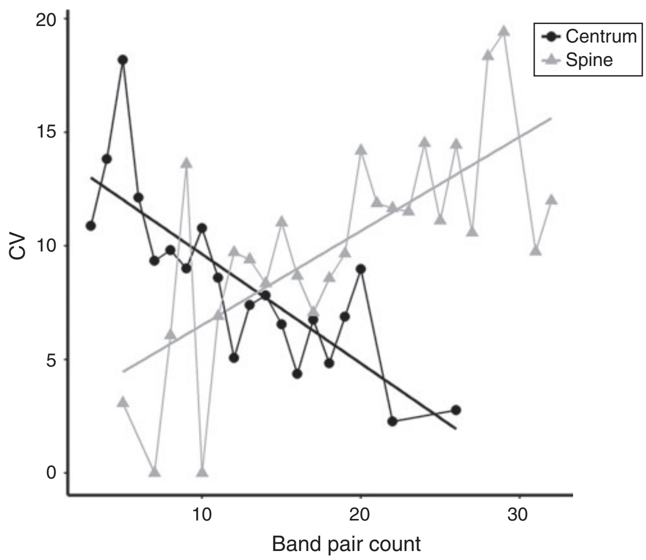

Fig. 6. Mean CV based on band pair counts for *Squalus suckleyi* for spines and centra. Linear regression models were fitted to show trend lines.

resolve potential reasons for the observed differences between the two methods.

The location within the vertebral column of samples selected for age determination could have affected age estimates for older fish. Owing to sample size limitations, the present study was not able to sufficiently determine whether band pair counts from anterior centra agree with band pair counts from unworn spines. Generally within a given individual, the largest centra are easier to age because their band pairs are less compressed (closely spaced), or because growth bands can sometimes be lacking in the smaller caudal centra (Cailliet *et al.* 1983; Cailliet and Goldman 2004; Goldman *et al.* 2012). For *S. suckleyi*, anterior centra tend to be larger in diameter than posterior centra. Thus, it is quite possible that posterior centrum band pair counts were lower than anterior centrum band pair counts because the band pairs are either absent or more difficult to discern. The results of the present study do not agree with the results of the pilot study comparing band pair counts from different regions of the vertebral column. The sample size for the pilot study ($n = 6$) was likely too small to detect the difference between anterior and posterior band pair counts that was revealed by the present study. The majority of centrum samples used in the present study were collected from the section of the vertebral column ventral to the posterior dorsal fin. Most of the field collections were conducted during routine ongoing surveys, in which field personnel have other duties and collecting samples for this project was a secondary responsibility; thus, the sampling plan was simplified to prioritise collection of posterior structures. The present study showed a significant difference between band pair counts of anterior and posterior centra, but the agreement plots suggested a substantial degree of variability and no systematic bias (Fig. 4*b*). This result could be a product of a small sample size ($n = 48$), difficulty of interpreting band pairs near the outer edges in posterior centra or variability in centrum section quality.

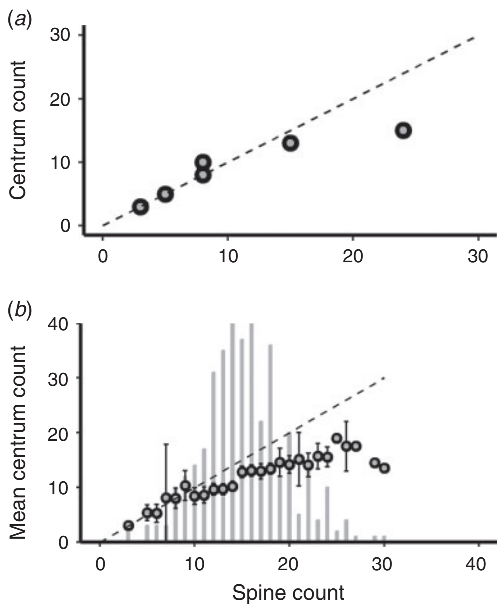

Fig. 7. Comparisons of spine and centrum band pair counts for *Squalus suckleyi* (*a*) anterior centra *v*. spines ($n = 7$), showing all samples, and (*b*) agreement plot of mean posterior centrum band pair count *v*. spine band pair count with 95% confidence intervals (grey columns represent sample size). Dashed lines indicate 1 : 1 equivalence.

Several factors could have affected the quality of each centrum section. Although the methods used to process each centrum were standardised, section appearance could vary among specimens. Centrum size, section thickness and freshness of the haematoxylin stain can affect how quickly and

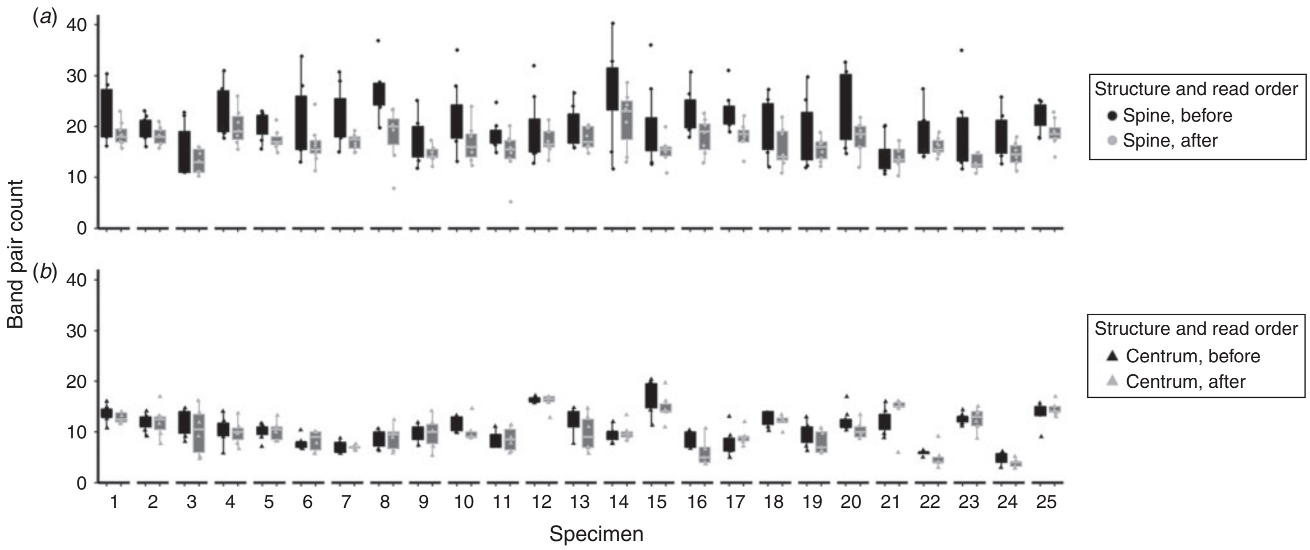

Fig. 8. Band pair count data for (*a*) spines and (*b*) centra from each laboratory's reads of *Squalus suckleyi* specimens in the inter-laboratory exchange. Points are jiggered to show overlapping points. Reads where the reader categorised the structure with a readability of '3' (see text for details) were not included. Specimen number does not represent a pair of structures (i.e. spine Specimen #1 is not from the same animal as centrum Specimen #1) for two reasons: (1) specimens for the exchange were selected at random from within a structure type; and (2) specimen order was randomised.

uniformly the centrum section absorbs stain, and can consequently affect interpretation of band pairs. Replacing specimens of low quality with new preparations would have been ideal but impractical given the time-consuming nature of sectioning and staining. Finally, as described earlier, band pairs become compressed at the distal edges of centra in older animals and are thus more difficult to identify and count. This is possibly exacerbated by the staining procedure, because sectioned and stained centra appear grainy under high magnification, making it difficult to delineate between individual band pairs. Section thickness could also have affected age interpretations. Future work could include testing the effect of different thicknesses on the appearance and quantification of band pairs near centrum section distal edges. In the present study, the section thickness was standardised to 0.4 mm; however, some centra may have absorbed stain more effectively than others, possibly as a function of centrum size or the degree of mineralisation, and thus it could be more appropriate to adapt section thickness to achieve the best staining results.

Although the overall sample size for the present study was sufficiently large, there was only a small number of samples from larger, older *S. suckleyi* (i.e. females >90 cm PCL and males >75 cm PCL, corresponding to animals >35 years of age). This species is known to segregate by sex and size; previous work in the Gulf of Alaska found that the greatest catch rates of the largest animals were often found in nearshore environments (Tribuzio and Kruse 2012), which were not possible to sample during the present study. *S. suckleyi* is reported to live to at least 80 years (Saunders and McFarlane 1993), so it is necessary to examine the full age range to determine whether an ageing structure is appropriate. Spines have been used for ageing *S. suckleyi* for many decades, so historical collections are quite large, including many samples from large, old fish. This preponderance of samples has allowed researchers to validate age estimates from spines using bomb radiocarbon (Campana *et al.* 2006). Furthermore, a separate

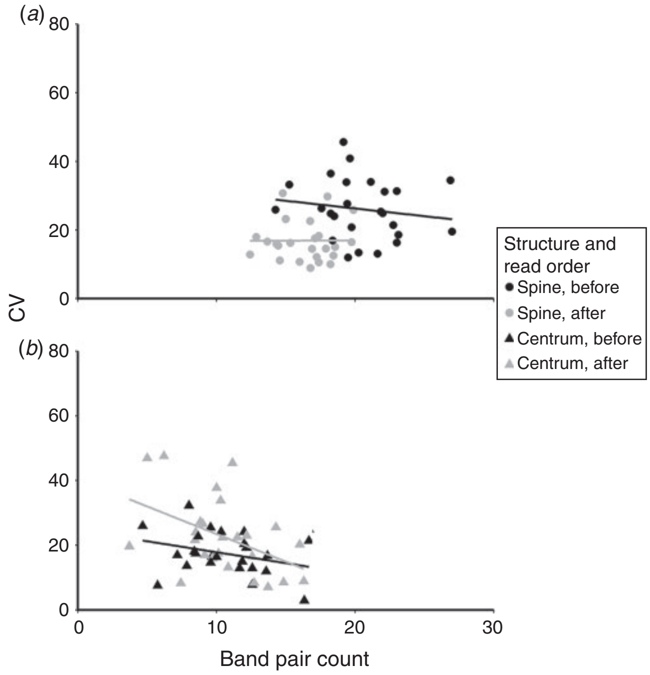

Fig. 9. Inter-laboratory CV for band pair counts of *Squalus suckleyi* (*a*) spines and (*b*) centra before and after calibration with linear regressions.

Table 2. Paired *t*-test results between band pair counts for *Squalus suckleyi* before and after calibration in the inter-laboratory exchange
Reader 1 scored all centra, both before and after calibration as readability code '3' (see text for details), and Reader 2 classified all centra as readability code '3' after calibration; thus, these reads were not included in this analysis. Probabilities are statistically significant at $P < 0.05$. $\bar{d}$, mean difference between estimates; CI, confidence interval; NA, not applicable

Source	Laboratory	d.f.	$t_{0.05(2),\text{d.f.}}$	*P*-value	$\bar{d}$	95% CI	
						Lower	Upper
All	Spine	181	1.973	<0.001	−3.648	−4.452	−2.845
All	Centrum	130	1.978	0.001	−0.657	−1.033	−0.280
Reader 1	Centrum	NA	NA	NA	NA	NA	NA
Reader 2	Centrum	NA	NA	NA	NA	NA	NA
Reader 3	Centrum	14	2.145	0.038	1.000	0.064	1.936
Reader 4	Centrum	24	2.064	0.001	−1.680	−2.616	−0.744
Reader 5	Centrum	20	2.086	0.605	0.286	−0.849	1.420
Reader 6	Centrum	24	2.064	0.001	−1.400	−2.154	−0.646
Reader 7	Centrum	19	2.093	0.036	0.750	0.056	1.444
Reader 8	Centrum	24	2.064	<0.001	−1.800	−2.291	−1.309
Reader 1	Spine	23	2.069	0.029	−0.917	−1.732	−0.101
Reader 2	Spine	20	2.086	0.308	−0.619	−1.853	0.615
Reader 3	Spine	16	2.120	0.850	−0.118	−1.414	1.179
Reader 4	Spine	24	2.064	<0.001	−2.640	−3.607	−1.673
Reader 5	Spine	24	2.064	<0.001	−13.760	−16.090	−11.430
Reader 6	Spine	24	2.064	<0.001	−7.120	−8.571	−5.669
Reader 7	Spine	20	2.086	0.002	−1.238	−1.956	−0.520
Reader 8	Spine	23	2.069	0.142	−0.542	−1.278	0.195

Table 3. Results of a paired Wilcoxon signed-rank test comparing the *Squalus suckleyi* sample band pair counts for each reader with the sample median band pair counts of all readers in the inter-laboratory exchange, both before and after calibration

Reader 1 scored all centra, both before and after calibration as readability code '3' (see text for details), and Reader 2 classified all centra as readability code '3' after calibration; thus, these reads were not included in this analysis. Probabilities are statistically significant at $P < 0.05$

Source	Reader	*P*-value	
		Before calibration	After calibration
Centrum	Reader 1	NA	NA
Centrum	Reader 2	0.081	NA
Centrum	Reader 3	0.115	0.474
Centrum	Reader 4	0.002*	0.001*
Centrum	Reader 5	0.003*	0.670
Centrum	Reader 6	0.621	<0.001*
Centrum	Reader 7	<0.001*	<0.001*
Centrum	Reader 8	<0.001*	0.487
Spine	Reader 1	0.001*	0.087
Spine	Reader 2	0.329	<0.001*
Spine	Reader 3	0.600	0.002*
Spine	Reader 4	<0.001*	0.017*
Spine	Reader 5	<0.001*	0.107
Spine	Reader 6	<0.001*	<0.001*
Spine	Reader 7	<0.001*	<0.001*
Spine	Reader 8	<0.001*	0.983

series of studies was performed that examined the band pair counts of fish that had been injected with oxytetracycline, tagged and released, and then recovered after up to 20 years at liberty (McFarlane *et al.* 1987; McFarlane and King 2009). There are no historical collections of *S. suckleyi* centra, and few large, old animals were sampled in the present study; thus, validation of this structure via bomb radiocarbon is not possible at this time. Samples collected during the present study do not appear to be from fish old enough to have been alive during the 1960s when oceanic bomb radiocarbon peaked; however, it is possible that heavy isotopes of certain elements released by the Fukushima disaster (e.g. strontium and caesium) could be used as alternative age validation chronometers in the future. A further concern regarding centra as a valid ageing structure is that centrum growth has been shown to cease in certain species, such that discernable band pairs do not form on the outer portions of centra in older individuals (Francis *et al.* 2007; Natanson *et al.* 2008). The results of our edge analysis and MIA suggested that deposition of visible band pairs is likely annual. However, these analyses were restricted to relatively young fish. Given the fact that spine ages did not match centrum ages in fish over 10 years, it is possible that band pairs either stop forming or become too difficult to discern in *S. suckleyi* centra. More work is needed to determine whether similar results would be found with larger anterior centra.

In addition to examining centra as a potential ageing structure, the present study incorporated an inter-laboratory exchange to investigate potential biases between laboratories. The result of this effort underscores the importance for readers to calibrate with a known reference collection (i.e. to standardise their ageing criteria). Reader calibration significantly changed the band pair counts and reduced the CV in spines (Fig. 9). Many readers' age estimates were significantly different from the median, some before and after calibration, highlighting the variability and difficulty associated with age determination of this species.

Although the centrum thin section method is appealing in that centrum band pair counts should represent a direct estimate of fish age with no erosion of band pairs and higher inter-reader precision, a bias was detected between spine and centrum band pair counts after approximately age-10. At this point in time, centra do not provide an alternative to spine-based ageing methods for *S. suckleyi* over 60 cm PCL until the discrepancy can be investigated further. Specifically, the effects of variability in thin section thickness on the staining process and readability of band pairs should be examined, anterior centra should be further evaluated and the spine and centrum methods should be compared for a larger sample of large, older fish to fully investigate the source of the observed bias.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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