Original Article

Ageing bias and precision for deep-water snappers: evaluating nascent otolith preparation methods using novel multivariate comparisons among readers and growth parameter estimates

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Tropical deep-water snappers (Etelinae) support valuable fisheries across the Indo-Pacific, with stock assessments reliant on age-based information in the absence of reliable catch and effort statistics. These long-lived species have been considered notoriously difficult to age. However, nascent developments in ageing protocols, particularly thinner transverse sections of otoliths (∼180–200 μm), are providing improvements in growth zone clarity, interpretation and repeatability of annuli counts. At a recent international workshop, thin sectioned otoliths from three deep-water snappers were read under reflected light by eight fisheries scientists from across the Indo-Pacific, with various levels of fish-ageing experience. Precision and bias were assessed using traditional ageing precision metrics (index of average percent error, IAPE; and coefficient of variation, CV), and a novel approach using multivariate analyses (metric multidimensional scaling, mMDS) based on Euclidean dissimilarity among readers' counts and subsequent von Bertalanffy (vB) growth parameter estimates. Annuli counts between the primary reader and all other readers were within two for 80% of estimates, with uniform variation across a wide age range for *Etelis carbunculus* (intraclass correlation coefficient (ICC) = 0.924, n = 20, 3–25 annuli) and *Etelis* sp. (ICC = 0.933, n = 15, 2–27 annuli). In contrast, annuli counts for *Pristipomoides filamentosus* (n = 14, 4–49) were less precise (i.e. ICC = 0.835, 66% of counts within two of primary reader) with a bias toward greater variation in younger, pre-maturational life stages (≤5 annuli). Traditionally accepted ageing precision (IAPE ≤ 5.5%, CV ≤ 7.6%) was achieved for each species, but was commensurate with reader experience. The multivariate mMDS ordination was more informative in identifying both distance (i.e. dissimilarity) and direction (i.e. form) of variations in annuli counts and vB growth parameter estimates among readers. The acceptable level of ageing precision and bias achieved among most readers indicated that deep-water snappers can be aged precisely when appropriate otolith preparation methods are used. This study contributes towards ageing protocols that can be used

to (i) interrogate bias and precision of age data for stock assessments, and (ii) standardise comparisons of age-based life history characteristics across a species range.

Introduction

Transverse sections of sagittal otoliths are one of the most reliable means for obtaining age estimates of teleosts based on counts of annually formed translucent and opaque growth zones (herein referred to as annuli, Beamish and McFarlane, 1987; Secor *et al.*, 1995; Campana, 2001; Maceina *et al.*, 2007). Although the methods for otolith section preparation may vary between species and/or laboratories, they generally involve similar principles of transverse division through the primordium to a predetermined thickness to achieve acceptable growth zone clarity and consistent counts among reads and/or readers. Variations in repeated annuli counts are used to determine the extent of ageing precision and bias, which refers to differences that are either random or systemic, respectively (see Punt *et al.*, 2008). Ageing precision and bias are traditionally measured using metrics of error (Beamish and Fournier, 1981; Chang, 1982), symmetry (McNemar, 1947; Bowker, 1948; Evans and Hoenig, 1998; McBride, 2015), or visually assessed based on univariate comparisons in age-bias scatter plots or tables (Campana *et al.*, 1995). Studies that investigate ageing precision metrics for fish typically gauge estimates against acceptable reference levels of $\leq$ 5.5% for the index of average percent error (IAPE) and/or $\leq$ 7.6% for the CV (Campana, 2001; Matta and Kimura, 2012). It is unclear how appropriate these reference levels are for species with otolith sections that are relatively difficult to interpret, and thus likely to produce greater variations in annuli counts among reads and/or readers. Additionally, the influence of ageing error on estimates of age-based life history parameters, such as growth, is not well understood.

Although traditional ageing precision metrics (i.e. IAPE and CV) provide a suitable method to assess variability among annuli counts, they provide limited information on the direction and form of variation among counts. Multivariate analysis provides an alternate approach for determining ageing bias and precision by comparing high-dimensional relationships among multiple annuli reads through nonparametric dissimilarity measures calculated between every pairwise comparison. Using this approach, variations in annuli counts can be tested or visually assessed based on the distances and directions of their separation reflecting their level of dissimilarity within a data cloud that is projected into either a two or three dimensional ordination. The dissimilarity values among pairwise comparisons of annuli reads can be preserved, or scaled, to the relative distances between data points within these ordinations through the use of metric scaling. Furthermore, the forms of variation in annuli counts can be diagnosed by overlaying vectors for important variables (e.g. vB growth parameters) based on correlation coefficients to show the strength and direction of their relationships (i.e. trend in higher to lower values) within the data cloud of reads and/or readers within an ordination.

Age-based studies of deep-water tropical Etelinae snappers have demonstrated varied success in interpreting counts of annual growth increments from otolith sections (Pilling *et al.*, 2000; Newman and Dunk, 2003; Andrews *et al.*, 2012), due mostly to variations in methods for the preparation and/or microscopy of

otolith sections. For example, simply using higher magnification (i.e. a compound microscope) to view extremely narrow annuli near the outer margin of otolith sections of *Salvelinus namaycush* resolved discrepancies between annuli counts and bomb radiocarbon validated ages (see Campana, 2005). Similarly, recent developments in otolith section preparation and interpretation have improved growth zone clarity and repeatability of annuli counts for many deep-water teleosts (Peres and Haimovici, 2004; Stransky *et al.*, 2005; Wakefield *et al.*, 2010; Williams *et al.*, 2015). These developments include thinner otolith sections ($\sim$180-200 μm, but may be species-specific), quality control for consistent cutting orientation (e.g. transverse sections through the primordium perpendicular to the sulcus acusticus), calibration of sectioning equipment (e.g. micrometer on low-speed saws), rinsing of otolith sections in an acidic solution (Gauldie *et al.*, 1990) and interpreting growth zones in otoliths relative to life history stages (see Newman *et al.*, 2015). Given these recent advances in otolith preparation and interpretation, there is a need to standardise methods for estimating ages for these species to facilitate comparisons of life history parameters across their typically wide geographic range and to reduce uncertainty in age-based stock assessments.

Deep-water Etelinae snappers account for a large proportion of the catches from deep-water (i.e. $\sim$250–500 m) commercial, recreational, artisanal and subsistence fisheries across the tropical and subtropical waters of the Indo-Pacific region. In most locations, catch and effort statistics from these fisheries are limited, unreliable or absent. Consequently, there have been few quantitative stock assessments for single species, with those that have been assessed relying on good quality age-based data (see Newman *et al.*, 2015). In general, deep-water fishes are characterised as more inherently vulnerable to exploitation compared with more productive shallow-water species, due to life history traits that suggest lower production potential and resilience (e.g. extended longevity, slow growth, delayed maturity, Koslow *et al.*, 2000; Wakefield *et al.*, 2013a; Williams *et al.*, 2013; Newman *et al.*, 2016). Such life history traits suggest sustainable harvest levels are likely to be relatively low, which emphasises the need for robust age-based monitoring and assessment programs to inform sustainable management and maintain adequate spawning biomass for these stocks. Unfortunately, the lower productivity of deep-water teleost fisheries tends to be correlated with inherently lower economic value, which typically limits the resources available for collecting age-based data. This may be further compounded when these fisheries occur in less-developed countries (e.g. Pacific Island countries) with limited access to fish ageing laboratories.

The use of age data to derive parameter estimates (e.g. mortality, growth, selectivity, age-at-maturity, recruitment) that underpin age-based stock assessment models relies on, among other things, measures of the level of ageing precision and unbiased age data. This study aims to (i) apply a novel technique using a multivariate metric-ordination to annuli counts and growth parameter estimates as an alternate criterion for diagnosing the forms of variation in ageing among multiple readers, and (ii) determine

whether acceptable levels of ageing precision and bias in annuli counts among multiple readers using traditional and novel methods for comparisons is achievable for deep-water snapper otoliths that are difficult to interpret. Precision and bias in annuli counts were explored for three species of deep-water snappers, i.e. *Etelis carbunculus*, Etelis sp. and *Pristipomoides filamentosus*, and examined relative to the different levels of experience among readers in interpreting growth zones in thin sectioned otoliths. The otolith growth zone interpretations and ageing precision protocols for deep-water snappers outlined in this study are not just important for these species, but can provide specific criteria by which age-based analyses can be standardised to facilitate useful comparisons of life histories and fishing mortality estimates from age structures to better inform stock assessments for sustainable fishery management.

Methods

Reader training, otolith preparation and annuli counts

In May 2015, an international workshop was held to develop standardised methods for estimating life-history parameters for deep-water tropical snappers (see Newman *et al.*, 2015). This workshop brought together fisheries scientists from across the Indo-Pacific region who have responsibilities for monitoring, assessment and management of deep-water tropical fisheries. A primary reader who had extensive experience was identified. Training and discussions on ageing techniques and annuli interpretations of several Etelinae snapper otolith sections ensued among the group for ~2 h. The participants then independently counted growth zones in thin otolith sections (~180–200 μm) without knowledge of the fishes length or date of capture, for three Etelinae snapper species, *E. carbunculus* (n = 20, Cuvier 1828), Etelis sp. (n = 15, see Andrews *et al.*, 2016) and *P. filamentosus* (n = 14, Valenciennes 1830). Note that recent taxonomic revision has demonstrated that *E. carbunculus* is the correct scientific name for the species previously referred to as *E. marshi*, and that Etelis sp. is used to refer to the species previously referred to as *E. carbunculus* (e.g. previous nomenclature used in Wakefield *et al.*, 2014; current nomenclature used in Andrews *et al.*, 2016). Fish samples were collected during scientific surveys or from commercial catches between 1997 and 2013 from either the western central Pacific or eastern Indian Oceans. For each fish, the standard fork length (FL) was measured to the nearest 1 mm and the two sagittal otoliths were removed and stored dry in plastic vials or paper envelopes prior to sectioning. The gonads of each fish were macroscopically identified as either ovaries, testes, or indeterminate (i.e. juveniles).

The collections of thin transverse otolith sections for each species were selected across a wide range of annuli counts and readabilities based on preliminary otolith readings. Otolith sections had been prepared at two separate laboratories following the same methods (i.e. Department of Fisheries, Western Australia and the Pacific Community, New Caledonia), which were consistent with those used in recent age-based studies of deep-water species that produced thin sections to improve annual growth zone clarity and produced an acceptable index of ageing precision (e.g. Peres and Haimovici, 2004; Wakefield *et al.*, 2013a,b). The sagittae of each fish were embedded in epoxy resin and sectioned transversely through the primordium in a direction perpendicular to the sulcus acusticus. Prior to sectioning, the micrometer on each low speed saw was calibrated with each diamond tipped

blade to improve the consistency in the thickness of otolith sections. Otolith sections were cut thinly at about ~180–200 μm, rinsed in an acidic solution for ~30 s (2% hydrochloric acid, see Gauldie *et al.*, 1990), and mounted on a glass slide with a cover slip using clear casting resin.

Separate microscope stations were set up for each of the three Etelinae snapper species. At each station, images of otolith sections with opaque zones (presumed annuli) denoted were available as a reference guide for each species (Figure 1). Otolith sections were viewed at ~40–50× magnification under reflected light, with the external light sources placed at a very low angle essentially creating a dark-field appearance. Annuli were counted along four reading axes including one each from the primordium to each crista (i.e. inferior and superior) adjacent to the posterior and anterior margins of the sulcus; and from the primordium to both the anterior and posterior outer margins in close proximity to the subcupular meshwork fibre zones. Otolith terminology follows Kailish *et al.* (1995) and Tuset *et al.* (2008). Annuli counts were cross-referenced among multiple reading axes, with only the most confident annuli count from each reader for each fish used in the subsequent analyses to determine ageing precision and bias among readers and species.

Determining ageing precision and bias

Ageing precision was considered to be the repeatability of annuli counts and ageing bias was considered to be any systemic variation in annuli counts among the eight readers for each species. The precision and bias in these counts were assessed visually using univariate age-bias plots and a multivariate mMDS ordination. The intraclass correlation coefficient (ICC) based on a one-way single measure model was used to determine the consistency of annuli counts among the eight readers for each species, where values range between 0-1 and a value of one indicates precise agreement (Shrout and Fleiss, 1979). Ageing precision was also assessed empirically using a modified form of the IAPE (Beamish and Fournier, 1981), whereby the error among annuli counts for each fish was expressed as a fraction of the median, rather than a mean, of counts among readers. By comparing individual annuli counts against the group median for each fish, the distribution of IAPEs was maintained and better represented, which improved comparisons of variations in counts among the most and least accurate readers. In addition, the relative IAPEs for each of the eight readers were determined separately for each species and all fish combined by using a restructured form of the equation from Beamish and Fournier (1981). This involved changing the summation unit from reader (R), which provides the relative error contribution for each fish, to fish aged (N), which provides the relative error contribution for each reader. Thus, the equation used was

$$\text{IAPE}_{\text{reader}} = \frac{1}{N}\sum_{j=1}^{N}\frac{|x_{ij} - \tilde{x}_j|}{\tilde{x}_j};\qquad(1)$$

where N was the number of fish aged, x_{ij} was the ith annuli count of the jth fish, and $\tilde{x}_j$ was the median annuli count for the jth fish.

The overall IAPEs for each species and all fish combined were compared among, (i) all readers (n = 8), (ii) the five readers with the lowest IAPE, and (iii) the two readers with the lowest IAPE. This approach facilitated comparisons of consistency in precision indices among readers for each species based on their inclusion, or
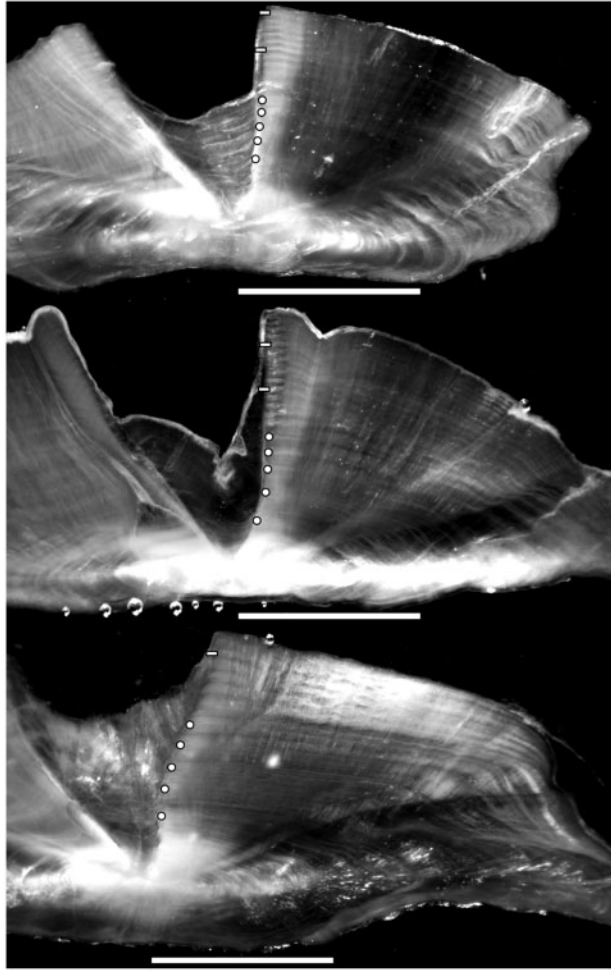
Figure 1. Images of thin transverse sections (∼180-200 μm) of a sagittal otolith of (a) *E. carbunculus* of 305 mm FL with 15 opaque zones (top), (b) *Etelis* sp. of 805 mm FL with 19 opaque zones (middle), and (c) *P. filamentosus* of 447 mm FL with 11 opaque zones (bottom). White circles show the location of the first five opaque zones and white bars the location of every fifth opaque zone thereafter. Scale bar 1 mm (bottom of each image).

exclusion, from higher precision groupings and thus avoided the need to assign subjective ratings for each reader's level of fish-ageing experience. Overall IAPEs were estimated using the equation,

$$\mathrm{IAPE_{overall}} = \frac{1}{N} \sum_{j=1}^{N} \left[\frac{1}{R} \sum_{i=1}^{R} \frac{|x_{ij} - \tilde{x}_j|}{\tilde{x}_j} \right]; \quad (2)$$

where R was the number of times each fish was aged (i.e. $R = 8$ readers). These IAPE$_{\mathrm{overall}}$ results were compared with the generally accepted reference level of $< 5.5\%$ (Campana, 2001).

Variations in annuli counts among readers for all fish combined were also assessed using a multivariate analysis with dissimilarities based on the distances and directions of separation among data points within an mMDS ordination. These analyses were performed using the software PRIMER (version 7.0.4, Clarke *et al.*, 2014). A resemblance matrix based on Euclidean dissimilarity was produced from annuli counts for all fish and

used to derive the mMDS ordination. The scale of relative distance between data points within the ordination was preserved from the input resemblance matrix by fitting a linear regression (through the origin) to their relationship with the corresponding Euclidean dissimilarity among all pairwise combinations of readers ($n = 28$). This linear correlation was evaluated using a Shepard diagram and stress coefficient. The performance of each reader was also determined as their average Euclidean dissimilarity with all other readers. The relationship between traditional ageing precision metrics (i.e. IAPE and CV) and Euclidean dissimilarity were determined using linear regression analysis of comparisons of annuli counts among all pairwise combinations of readers for all fish combined.

Variations in growth parameter estimates among readers

Variations in estimated growth parameters among readers were only assessed for *Etelis* sp. because (i) despite a small sample size, they exhibited a length at age relationship that conformed to asymptotic growth; and (ii) they were sampled from the same location (eastern Indian Ocean), which was not the case for the other two species (see Supplementary Material). A vB growth curve was fitted to the length and annuli count data for each reader using the equation,

$$\mathrm{FL}_t = L_\infty \left(1 - e^{-k(t - t_0)} \right); \quad (3)$$

where FL_t = the predicted FL (mm) of fish of age t, L_∞ = the asymptotic FL (mm), k = the growth coefficient (year^{-1}, a constant that determines the rate at which FL_t approaches L_∞), t = annuli count (years) and t_0 = the hypothetical annuli count (years) at which fish would have zero length. Variations in vB growth parameters among readers were assessed using the aforementioned multivariate mMDS ordination. The vB growth parameters were square root transformed prior to analyses to stabilise the variance among these variables, based on the gradient of the linear relationship between the logarithms of the mean and standard deviations for each parameter. Relationships between each vB growth parameter and relative IAPE$_{\mathrm{reader}}$ values were visually assessed using Draftsman pairwise scatter plots, to determine if the transformed data conformed with approximate (multivariate) normality based on roughly symmetrical distributions across their range and, if relationships existed, then they were linear (rather than curvilinear).

Considering the vB parameters are not on comparable scales, the square root transformed values were standardised prior to analysis, i.e.

$$Z_{(x)} = \frac{x - \mu}{\sigma}; \quad (4)$$

where $Z_{(x)}$ is the Z-score for each vB growth parameter estimate x, μ = mean and σ = SD of parameter estimates. The univariate relationship between the Z-scores for L_∞ and t_0 was used to determine how the vB growth parameters varied between growth trajectories derived from each reader's annuli counts. In addition, a dissimilarity matrix based on Euclidean dissimilarity between readers was produced from the Z-scores for all three vB parameters and used to derive an mMDS ordination. The scale of relative distance between data points within the ordination was
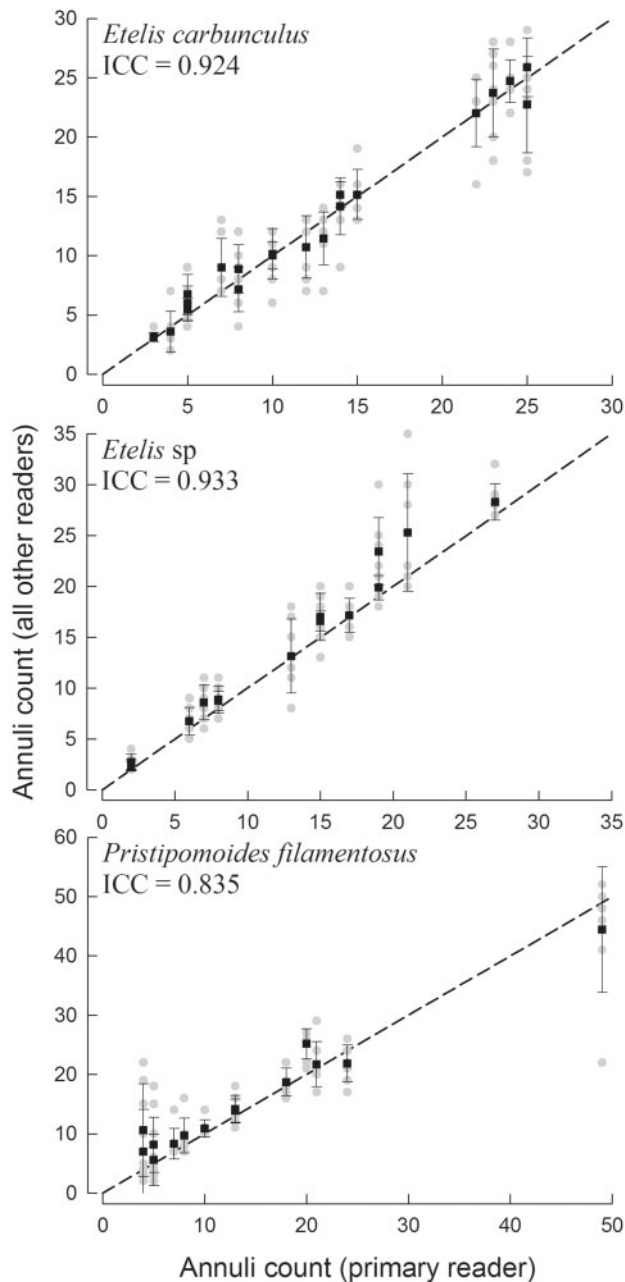
Figure 2. Age-bias plots comparing annuli counts between the primary reader and all other readers (grey circles; black squares denote mean ± 1 SD of counts from all other readers) for *E. carbunculus* (top), *Etelis* sp. (middle) and *P. filamentosus* (bottom). Equivalent annuli counts (dashed line) and ICCs are shown.

preserved from the input resemblance matrix by fitting a linear regression (through the origin) to their relationship with the corresponding Euclidean dissimilarity among all pairwise combinations of readers (n = 28). This linear correlation was evaluated using a Shepard diagram and stress coefficient. Vectors were overlaid onto the mMDS based on Pearson correlation coefficients to identify the strength and direction of the relationships between each vB growth parameter and the data cloud of eight readers within the mMDS ordination. The location of each readers' data

point relative to these growth parameter vectors was used to discern the forms of variation in ageing precision and bias among readers.

Results

Annuli counts and age-bias plots

A wide range of annuli counts were represented for all three deep-water snapper species, i.e. 3–25 annuli for *E. carbunculus* (n = 20), 2-27 annuli for *Etelis* sp. (n = 15) and 4–49 annuli for *P. filamentosus* (n = 14), as estimated by the primary reader. Variations in annuli counts between the primary reader and all other readers were within two for 80% of estimates for *E. carbunculus* and *Etelis* sp. In addition, high values of ICC for *E. carbunculus* (ICC = 0.924, 0.869–0.964 95% CI) and *Etelis* sp. (ICC = 0.933, 0.874–0.973 95% CI) indicated significant concordance of annuli counts among readers ($p < 0.001$, Figure 2). The magnitude of variation in annuli counts also appeared uniform across the wide age range, with standard deviations in annuli counts averaging 2.1 (σ ranging from 0.4 to 4.1) for *E. carbunculus* and 1.9 (σ ranging from 0.5 to 5.8) for *Etelis* sp. (Figure 2). The individual *Etelis* sp. with the highest variation in annuli counts was estimated by the primary reader to have 21 annuli (20–35 annuli all readers, $\sigma = 5.8$, Figure 2).

In contrast to the two *Etelis* species, the trends among annuli counts in the age-bias plot for *P. filamentosus* exhibited a significant bias, with less precision among younger individuals. For example, SD in annuli counts ranged from 4.4 to 7.8 for fish with ≤ 5 annuli compared with 1.5–3.8 for those with 7–24 annuli. Nonetheless, variations in annuli counts between the primary reader and all other readers were within two for 66% of estimates and concordance among readers was significant (ICC = 0.835, 0.708–0.932 95% CI, $p < 0.001$). The variation in annuli counts for the oldest and largest *P. filamentosus* (775 mm FL) ranged between 46 and 52 for six of the eight readers, with the other two readers counting 41 (reader 7) and 22 (reader 8) annuli. Reader 8 also counted 22 annuli for the two smallest *P. filamentosus* (i.e. 381 and 390 mm FL) for which the majority of other readers' counts ranged between two and six. This suggested that the ageing precision of some relatively inexperienced readers varied markedly from the majority of the group, but was species-specific.

Ageing precision indices and multivariate reader comparisons

The ageing precision for individual readers (IAPE$_{reader}$) relative to the rest of the group exhibited two general trends that were either consistently precise or imprecise, or inconsistent with respect to fish genus. For example, readers 1 and 2 consistently exhibited some of the lowest ageing error indices among readers for all species; conversely readers 7 and 8 consistently had some of the highest ageing error indices (Figure 3). Reader 3 had relatively lower ageing error for *P. filamentosus* than for the two *Etelis* species; conversely reader 6 exhibited relatively lower ageing error for the two *Etelis* species than for *P. filamentosus* (Figure 3). Variations in ageing bias and precision relative to genus was not surprising as growth zone interpretations in otolith sections were very similar for the two *Etelis* species, but varied markedly for *P. filamentosus* (see Figure 1), which corresponded with the trends in concordance and standard deviation in annuli counts observed in the age-bias plots. For *P. filamentosus* otolith sections, there
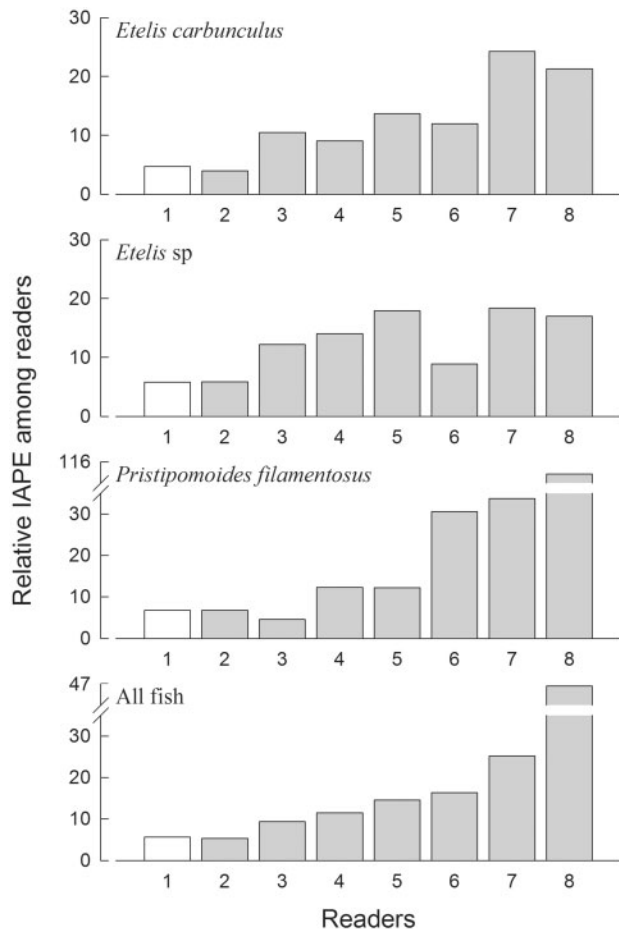
Figure 3. Relative IAPE (IAPE$_{reader}$) among eight readers (primary reader 1, white bars, and seven other readers, grey bars) for *E. carbunculus*, *Etelis* sp., *P. filamentosus* and all fish combined (reader numbers correspond with subsequent figures).
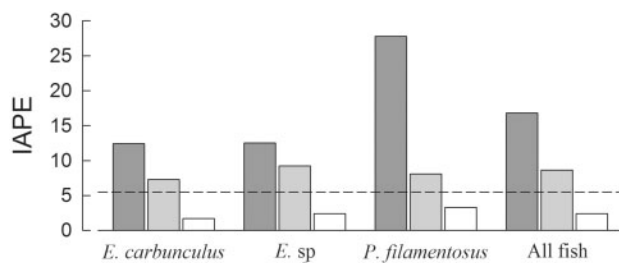

Figure 4. Overall IAPE (IAPE$_{overall}$) for all readers (dark grey bars), top five readers (light grey bars) and top two readers (white bars) for *E. carbunculus*, *Etelis* sp., *P. filamentosus* and all fish combined. Dashed line denotes the reference level for generally accepted IAPE values of $\leq 5.5\%$ (Campana, 2001).

appeared to be a greater separation in ageing precision between inexperienced readers (i.e. readers 6–8) relative to more experienced readers (readers 1–5, Figure 3).

The overall ageing error among readers for each species and all fish combined declined precipitously from values derived for all readers (IAPE$_{overall}$ = 12.4–27.8), to those for the five most precise readers (IAPE$_{overall}$ = 7.3–9.2), to those for the two most precise readers (IAPE$_{overall}$ = 1.7–3.3, Figure 4). The three readers

excluded from the five most precise were consistently readers 7 and 8 for each species and all fish combined, with reader 5 excluded for the two *Etelis* species and reader 6 excluded for *P. filamentosus* and all fish combined. In addition, readers 1 and 2 were always included as the two most precise readers, with their IAPE$_{overall}$ values consistently lower than the generally acceptable level of 5.5% (Figure 4).

The relationship between traditional ageing precision metrics (IAPE and CV) and Euclidean dissimilarity for pairwise comparisons among all readers was strongly correlated ($r^2 = 0.82$, Figure 5). In addition, the sequential rank in ageing performance among readers for all fish combined was very similar between these univariate (IAPE$_{reader}$, Figure 3) and multivariate metrics (average Euclidean dissimilarity, Figure 6a), with only readers 4 and 5 switching order between methods. The use of Euclidean dissimilarity among readers facilitated a multi-dimensional assessment of variations in ageing within an mMDS ordination based on their relative distances and directions among data points, with Euclidean dissimilarity well preserved within the ordination (stress = 0.15, Figure 6b and c). Readers with the lowest ageing precision indices (i.e. readers 1 and 2) also had the smallest distance between data points and were distributed centrally within the data cloud of readers in this ordination (Figure 6c). Although the relative distances between data points for readers 5 and 6 were at similar distances from readers 1 and 2, they were located at opposing directions and thus their variations in annuli counts were not consistent. The trends exhibited in this ordination also concur with the univariate analysis, with reader 3 being the closest in ageing precision to readers 1 and 2, and readers 7 and 8 being markedly dissimilar to all other readers based on their relative distance and direction (Figure 6c).

Evaluating forms of ageing precision from growth parameter estimates

The data points within the Draftsman pairwise scatter plots between a readers' ageing precision index (IAPE$_{reader}$) and each vB growth parameter were roughly symmetrically distributed, and the only conspicuous relationship was a strong linear correlation between vB parameters L_∞ and k ($k = -0.004L_\infty + 3.188$, $r^2 = 0.95$, see Supplementary Materials). Thus, the data used in the multivariate analysis was assumed to conform to approximate (multivariate) normality and the square root transformation was deemed suitable for stabilising the variance among variables. Visual assessment of the variations in growth trajectories among readers appeared subtle, particularly in older ages where the curves reach an asymptote (Figure 7a). However, the univariate relationship between standardised (i.e. Z-scores) L_∞ and t_0 parameters demonstrated that the two readers with the lowest ageing error indices (readers 1 and 2) also exhibited the least variation in these two subsequent growth parameter estimates and these readers were distributed centrally within the data cloud of eight readers (Fig. 7b). This trend in the distribution of data points was very similar to that derived for multivariate mMDS of growth parameters among readers, with readers 1 and 2 again distributed centrally within the data cloud (Figure 7d). However, the mMDS incorporated vectors depicting the form of variation for all three growth parameters relative to the distribution of data points, while preserving almost exact Euclidean dissimilarity between points within the ordination (stress = 0.01, Figure 7c). The relative distribution of data points within the mMDS relative to the strength
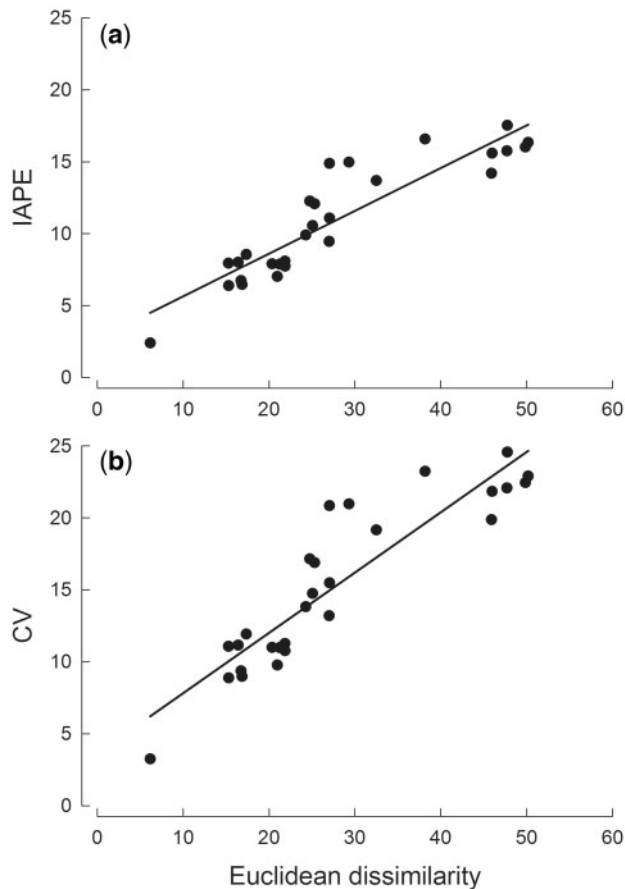
Figure 5. Relationships between traditional ageing precision metrics (a) IAPE and (b) CV and Euclidean dissimilarity for annuli counts of all fish among pairwise comparisons of the eight readers ($n = 28$, a, IAPE $= (0.2972*Euc.\ dist.) + 2.6773$, $r^2 = 0.82$; b, CV $= (0.4191*Euc.\ dist.) + 3.6251$, $r^2 = 0.82$).

and direction of growth parameter vectors demonstrated that readers 1, 2, 3, 5 and 6 were strongly influenced by the correlated estimates of L_∞ and k, with their estimates of t_0 being relatively consistent. In contrast, readers 4, 7 and 8 had higher estimates of t_0 than the other readers, with readers 4 and 8 also having higher estimates of k and reader 7 a higher estimate of L_∞ (Figure 7d).

Discussion

Despite deep-water snapper otolith sections being considered notoriously difficult to age (Ralston and Miyamoto, 1983; Pilling *et al.*, 2000; Newman and Dunk, 2003; Andrews *et al.*, 2012), traditionally accepted levels of ageing precision among readers were achieved (i.e. IAPE < 5.5%, Campana, 2001) using nascent methods in otolith preparation (particularly thinner sections ~180–200 μm) and interpretation. These acceptable levels of ageing precision occurred consistently between only two of the eight readers, who had the most experience in deriving annuli counts from thinner otolith sections of these species using reflected light. Nonetheless, based on very limited training (~4 h, see Newman *et al.*, 2015), age readers from across the Indo-Pacific with relatively less experience in interpreting thin otolith sections achieved significant concordance in annuli counts (ICC $= 0.835–0.933$, $P < 0.001$); their counts were within two of the primary reader
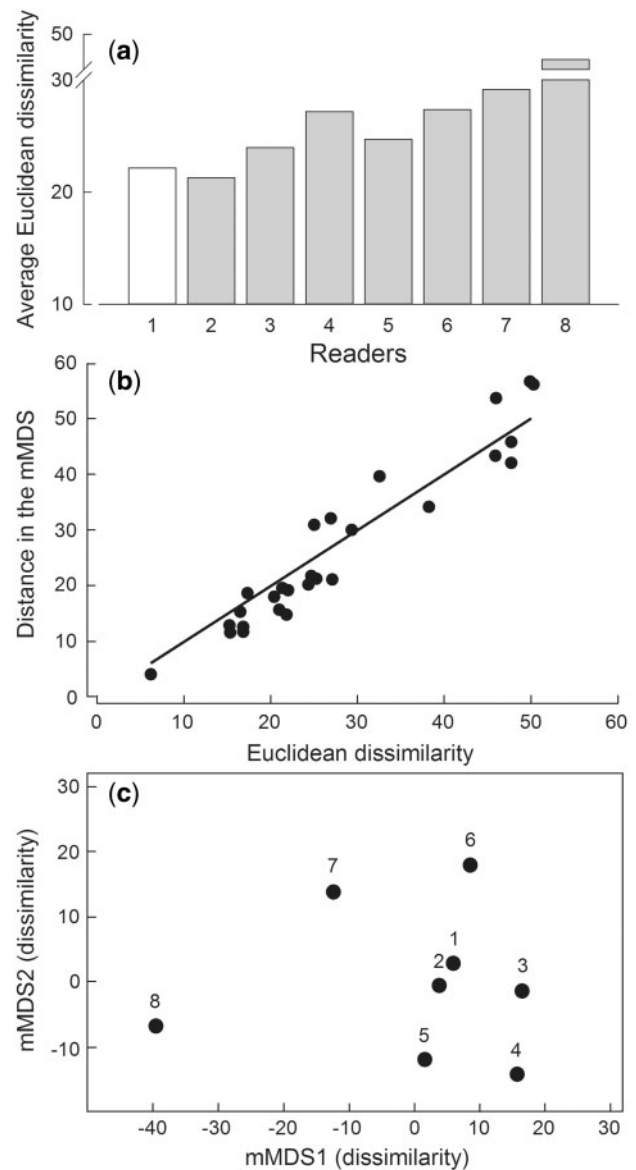

Figure 6. (a) Average Euclidean dissimilarity in variations for opaque zone counts among readers (primary reader 1, white bar) for all fish combined, derived from thin transverse otolith sections. (b) Shepard Diagram showing the relationship between the Euclidean dissimilarities among pairwise comparisons of all eight readers relative to those distances in the metric multi-dimensional scaling (mMDS) ordination (line represents equivalent distances, $n = 28$, stress $= 0.15$). (c) mMDS ordination derived from the Euclidean dissimilarities among annuli counts for all fish combined for the eight readers (*denotes primary reader, stress $= 0.15$).

for 80% of estimates for the two *Etelis* species and 66% for *P. filamentosus*; and they achieved ageing precision indices close to acceptable levels (IAPE$_{overall}$ $= 7.3–9.2$ for the top five most precise readers). Importantly, the otolith sections used in this study were more than half as thin as previous evaluations of ageing precision that used "thin" sections (i.e. 500 μm, Stransky *et al.*, 2005), but consistent with other studies of deep-water teleosts (Peres and Haimovici, 2004; Wakefield *et al.*, 2010, 2013b, 2015).

Based on the level of concordance in annuli counts and ageing precision indices among readers, the growth zones in these
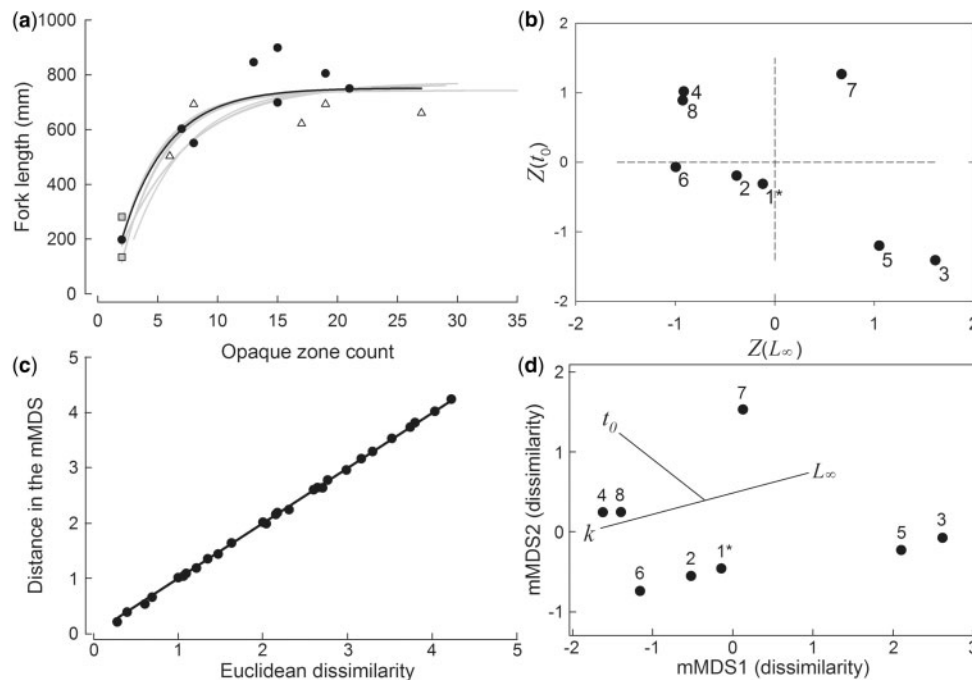
Figure 7. (a) Relationship between FL and annuli counts from thin transverse sections for female (black circles), male (white triangles), and juvenile (grey squares) *Etelis* sp. otoliths (n = 15) by the primary reader, with vB growth curves fitted to annuli counts for the primary reader (black line), and the other seven readers (grey lines). (b) Univariate relationship between Z-scores for L_∞ and t_0 parameters of the vB growth equation for each reader (numbered, * denotes primary reader). (c) Shepard Diagram showing the relationship between the pairwise Euclidean dissimilarities among combinations of eight readers Z-scores for vB parameters relative to those distances in the mMDS ordination (line represents equivalent distances, n = 28, stress = 0.01). (d) mMDS ordination (stress = 0.01) of Z-scores for the vB parameters for each reader, with vectors showing the strength and direction of the variations for each vB parameter among readers (lines).

thinner otolith sections were easier to interpret for the two *Etelis* species than for *P. filamentosus*. The greatest variations in counts among readers were recorded for the younger *P. filamentosus* with ≤ ∼5 annuli, which corresponds with the pre-maturational period for this species (Ralston and Miyamoto, 1983; Mees, 1993). This ageing bias for younger *P. filamentosus* arises from the appearance of their early growth zones being much broader and more diffuse (i.e. greater potential for counting false annuli), with the contrast between these wide opaque and thin translucent zones being less conspicuous. When greater numbers of annuli were present, the first ∼5 zones appeared easier to interpret based on (i) the relative incremental widths typically reducing with increasing age; and (ii) the delineation between opaque and translucent zones being more conspicuous in the recently formed zones (i.e. > 5 annuli), which in turn improved the relative perspective for younger growth zonation (see Figure 1c). This interpretation was supported by the markedly lower variation in annuli counts between readers for older *P. filamentosus*. As a result, the ageing precision for some readers varied markedly with respect to genus, suggesting age estimates of *P. filamentosus*, and potentially similar congeners, may require readers to possess a higher level of ageing experience. This also highlights the need for the calibration of growth zone interpretations prior to commencing an ageing project to ensure consistency between reads. This is generally achieved and measured through the periodic rereading of a reference collection of otolith sections and should be incorporated into ageing protocols (see Campana *et al.*, 1995).

This study used a novel application of multivariate analysis based on Euclidean dissimilarity to compare annuli counts among all eight readers. The Euclidean dissimilarity measure was strongly correlated with traditional ageing precision metrics (i.e. IAPE and CV), which meant trends in ageing precision among readers were consistent, and that acceptable levels of dissimilarity could be established similar to those of ≤ 5.5% for IAPE and/or ≤ 7.6 for CV (Campana, 2001; Matta and Kimura, 2012). The primary advantage in using the mMDS ordination to compare annuli estimates among readers is that it provides both the relative distance and direction of variations relative to the proximity of data points within the two-dimensional ordination plot, in comparison to only a one-dimensional distance measure for IAPE and CV metrics. For example, readers 5 and 6 had similar IAPE and Euclidean dissimilarity measures for annuli counts from all fish, but the mMDS ordination indicated that the variation in their counts was in opposing directions from the primary reader. Further diagnosis of variations among readers could be performed by overlaying vectors in the mMDS for fish with annuli counts that differed significantly based on a correlation coefficient (e.g. Pearsons). Multivariate mMDS can also provide an effective comparison for displaying lower numbers of reads and/ or readers than that performed in this study (i.e. n ≥ 2). The use of multivariate analysis complemented the standard ageing precision protocol for deep-water Etelinae snappers, and could easily be incorporated into age-based studies of fish species more generally.

The novel application of a multivariate mMDS ordination of vB growth parameter estimates provided a useful assessment for diagnosing the forms of variations in annuli counts among the eight readers. The position of each reader's data point relative to the direction and strength of each growth parameter vector within the mMDS ordination could be used to discern bias. For example, if estimates of t_0 varied significantly among readers (based on Pearson correlation), then the vector for this parameter could identify those readers with higher t_0 values, associated with overestimates of age for younger fish. In contrast, if estimates of the correlated parameters L_∞ and k varied among readers, but t_0 was consistent, then the bias would have been greater for mid to older aged fish. The two readers with the most precise ageing indices (i.e. readers 1 and 2) also exhibited the lowest level of variation in growth parameters among the eight readers as determined by their closest proximity to each other (lowest Euclidean dissimilarity), close proximity to the junction of the vB parameter vectors, and their central distribution within the data cloud. Thus, not surprisingly, ageing precision had direct implications for growth estimates, and would likely extend to other age-based life history attributes that utilise the same age data (e.g. mortality, selectivity, age-at-maturity, recruitment).

The annuli counts derived using nascent methods in otolith preparation and interpretation from this study provide comparable estimates of longevity to those derived from radiochemical dating of deep-water snapper otoliths (e.g. Andrews *et al.*, 2011, 2012). The annuli counts for the oldest *P. filamentosus* for the six readers with the most precise ageing indices ranged from 46 to 52 years, which is slightly older than previous longevity estimates for this species based on radiochemical dating (ca 46 years, Andrews *et al.*, 2012). Recent improvements in the preparation and optical resolution (e.g. microscopy and image enhancement) of thin otolith sections have resulted in greater accuracy and precision in interpreting annual growth zones (Campana, 2001; Choat *et al.*, 2009; Campana *et al.*, 2016). Studies that establish ageing protocols from sectioned otoliths for a group of species can facilitate age estimates from annuli counts using the same interpretations for the same species in different locations or, potentially, congeneric species. The dissemination of these ageing protocols provides an opportunity to increase the capacity for fishery assessments by partitioning the already limited resources toward the collection of sufficient and representative age-based data (Choat *et al.*, 2009). This would increase fish ageing capabilities for deep-water teleost fisheries that are inherently lower valued and resourced (i.e. less productive) and occur in many less-developed countries (e.g. Pacific Island countries), which would inevitably result in more cost-effective monitoring and assessment programs that better inform sustainable management. As such, the necessity for age validation should be considered on a species and location basis, with caution exercised if age-based studies opt to follow already established ageing protocols by placing a larger emphasis on (i) quality control during otolith preparation to ensure growth zone clarity is maximised in transverse sections, and (ii) adhering to stringent interrogation of ageing bias and precision among annuli counts.

There are other potentially useful methods for diagnosing ageing bias and precision that have not been included in this study due to data limitations (e.g. low sample sizes). These include, among others, (i) consistencies in strong and weak year class frequencies between periodic age-structures (e.g. Casselman and Gunn, 1992); (ii) tests of symmetry (McBride, 2015); and (iii) empirical relationships among age-based life history invariants

(Charnov, 1993; Jensen, 1996). Here, we provide a protocol that incorporates traditional and novel methods of estimating and examining ageing bias and precision that can be applied to all fish species, including those that are relatively data-poor and long lived. This study indicates that deep-water snappers can be aged precisely, when appropriate otolith preparation methods are applied. It also identified some genera and/or species of Etelinae that may require a greater level of fish-ageing experience, and emphasises the need to calibrate readings prior to ageing by using an otolith reference collection (Newman *et al.*, 2015). The ageing protocols established in this study provide methods that can contribute toward (i) accounting for bias and precision within age-based stock assessments, and (ii) standardised comparisons of life history characteristics across a species range.

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

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