A step by step guide to ageing octopus

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Abstract

Global octopus catch has doubled over the past four decades and is likely to grow in importance as many key fisheries continue to decline. Lack of age data is a critical limitation in assessing the resource status of octopus. Over the past 30 years or so, studies have investigated various methods to age octopus, with some methods better suited to certain species than others. However, only a small number of researchers have the hands-on knowledge to execute these methods in the laboratory. Here we present the first step-by-step guide to ageing octopus, as well as a decision tool, which should enable readers to carry out the ageing process and make an informed decision on the most suitable method for their species. We provide guidance on age validation, increment analysis of both beaks and stylets, materials needed, as well as avenues for further research. We hope this guide will provide a starting point for researchers new to octopus ageing, and for those working with octopus species that have never been aged before. We also encourage researchers to use this guide as a forum for open discussion to support the ongoing development of effective octopus ageing methods.

Additional keywords: Octopus ageing, age validation, stylets, beaks, fisheries

30 **Introduction**

31 Fisheries are fundamental to the maintenance of global food security and contribute to the
32 livelihoods of an estimated 600 million people (FAO 2022). However, decades of overfishing
33 have resulted in the depletion of some finfish stocks (FAO 2022). Simultaneously, some
34 cephalopod populations have proliferated, and octopus fisheries have expanded, which may
35 be in part, due to diminished finfish supply (Balguerías *et al.* 2000; Caddy and Rodhouse
36 1998; Doubleday *et al.* 2016; Sauer *et al.* 2019). Octopus fisheries are expected to expand
37 further as humanity strives to effectively meet the nutritional demands of a rising global
38 population (Rodhouse *et al.* 2014; Sauer *et al.* 2019). However, many commercially harvested
39 octopus species remain critically understudied and the potential impact of fishing on these
40 populations is poorly understood (Martino *et al.* 2021; Sauer *et al.* 2019). Long-term
41 maintenance of these fisheries will rely on sustainable management practices supported by a
42 robust understanding of life history and population dynamics, such as maturation, mortality
43 and recruitment, to which age and growth data are essential (Rodhouse *et al.* 2014).
44

45 A range of methods have been explored to estimate octopus age, including direct enumeration
46 of growth increments in hard structures such as stylets (Doubleday *et al.* 2006) and beaks
47 (Perales-Raya *et al.* 2010; Raya and Hernández-González 2010), and indirect methods that act
48 as an age proxy, such as eye lens diameter or weight (Cardenas *et al.* 2011), stylet weight
49 (Leporati *et al.* 2015), and lipofuscin quantification (Doubleday and Semmens 2011). Stylet
50 and beak increment analyses remain the most effective and broadly used octopus ageing
51 methods and have been validated across different life stages for multiple species (Doubleday
52 *et al.* 2006; Hermosilla *et al.* 2010; Rodríguez-Domínguez *et al.* 2013; Bárcenas *et al.* 2014;
53 Perales-Raya *et al.* 2014a). However, due to species-specific variations in beak and stylet
54 microstructure, not all preparation techniques can be applied to all species. Therefore, a
55 period of method development that includes increment visualisation and validation of
56 increment periodicity is usually required when ageing a species for the first time.
57

58 The following guide outlines common and successfully applied methods for stylet and beak
59 preparation, increment analyses, and age validation, as well as guidance on selecting the most
60 suitable method for different octopus species. While we acknowledge ageing methods will

continue to evolve, we hope this guide will provide a starting point for researchers new to octopus ageing, and for those working with octopus species that have never been aged before.

Part 1: Stylet increment analysis

Stylets are cartilaginous vestigial internal shells consisting of a pair of thin rod-like structures embedded within the muscle behind the two brachial hearts on either side of the mantle (Bizikov 2004). As octopus grow, the stylet is formed in layers and increments are periodically deposited, thus facilitating age estimation through increment analysis. Stylet increments were first discovered by Sousa-Reis and Fernandes (2002) and then validated as an ageing method by Doubleday *et al.* (2006) in which transverse sections were taken, embedded in Crystalbond™ 509, and polished. However, as stylets are sensitive to heat and drying out, Barratt and Allcock (2010) created a method for permanent stylet preparation using a low viscosity resin. In both methods, growth increments are visualised under microscopy and counted through micrographs taken of the section. Thus far, these methods have been used for a variety of species including, but not limited to, *Octopus pallidus* (Doubleday *et al.* 2006), *Octopus vulgaris* (Hermosilla *et al.* 2010), *Octopus maya* (Rodriguez-Dominquez *et al.* 2013), and *Octopus huttoni* (Donlon *et al.* 2019). However, stylet shape, consistency, and increment readability vary between species. Thus, stylet increment analysis may not be suitable for all species.

1.1 Dissection and storage

Stylets are embedded within the mantle musculature where the mantle abductor muscles attach to the mantle (Fig. 1), and can be dissected through the following method (Fig. 2):

1. Begin from the ventral side of the octopus.
2. Adjacent to the muscular septum, make a vertical incision from the base (anterior) to the top (posterior) of the mantle.
3. Make a horizontal incision through the muscular septum.

4. Peel back the ventral mantle wall to separate from the visceral sac and gill, and on one side, locate the stylet at the base of the abductor muscle and branchial heart (Fig. 3).
5. Make an incision into the mantle muscle where the mantle abductor muscle and stylet adjoin as close to the stylet elbow as possible.
6. Carefully remove the stylet from the mantle and preserve in 70% ethanol until ready for use.
7. Repeat steps 4 to 6 to retrieve the stylet on the opposite side.

1.2 Preparing stylets from adults and large individuals

A permanent stylet preservation method developed by Barratt and Allcock (2010) using a low viscosity resin has been utilized in multiple octopus ageing studies (Barratt and Allcock 2010; Durante *et al*., 2023; Leporati and Hart 2015). In these studies, LR White resin was chosen as it can be cold cured to prevent the exothermic reaction that often damages stylet sections. The resin infiltration process using LR White resin can be undertaken through the following method:

1. Using a single-sided razor blade, transversely section the post-rostral zone of the stylet (region of increment analysis in Fig. 4) into ~ 1mm lengths, preparing up to three lengths for each stylet.
2. Prepare three tubes (with lids) per sample following the solutions outlined in Table 1.
3. Dehydrate and impregnant the stylets lengths following Table 1 making sure to blot excess solution from each length using a tissue before placing in the next solution. It is especially important to ensure all excess ethanol is removed before placing in the resin for 24 hours. Solutions can be reused up to 3 times, although ideally, they should be changed after each sample as solutions can be diluted over time as ethanol evaporates and some may mix in the resin solution.
4. Mount stylet lengths vertically (cut side down) onto a glass base with double sided tape. Any double-sided tape is suitable as long as it has enough stick.
5. Place cylindrical moulds over the top of each group of lengths on the tape (Fig. 5). Here, we have cut the bottoms from 5 mL plastic sample tubes and used the tops cut side up. However, any shape mould is suitable. In our experience, silicone moulds do

not work, and hard plastic (polyethylene) moulds are best. Be sure to clean tubes with ethanol and wipe down after each use to ensure they adhere to the tape.

6. Mix a new aliquot of catalysed resin with accelerator (5 mL resin per 1 drop of accelerator) in a disposable cup or jar and mix well by pipetting up and down with a disposable pipette. Prepare enough to cover all stylet pieces.
7. Carefully pipette resin mixture into the mould until stylet lengths are covered. Transfer to a fridge and leave to set for at least 2 hours.
8. Remove the resin block from its mould and wipe away excess resin with paper towel.
9. Remove any sticky residue from the tape by carefully scraping with a razor blade, ensuring not to cut any resin. The idea is to form a smooth, flat surface for polishing. For stubborn residue, surface-safe adhesive removers may be useful.
10. Using wet 1000 grit sandpaper, followed by 15-, 6- and 3-micron lapping film, sand and buff the bottom of the block until the stylet end is visible. Regular checks under a microscope will help visualise progress. The surface should be as flat as possible. Using a slab of glass as the working surface under the sandpaper and lapping film is best, but a motorized turntable would also work.
11. Using clear Gorilla glue, affix the block polished side down to a clean microscope slide and leave to fully dry for 24 hours (Fig. 6A). In our experience, superglue is not adequate as it is not waterproof and degrades during polishing, therefore water-resistant glue is best.
12. Using a cutting device such as a diamond saw, remove excess resin to make 100–200 µm thick sections. Alternatively, a motorized turntable or rotary tool (e.g., Dremel) with sandpaper may be useful. It is important to make the surface as evenly flat as possible which is more difficult with a handheld Dremel.
13. Grind and polish the remaining resin block using wet 1000-grit sandpaper followed by 15-, 6- and 3-micron lapping film until a thin section of the stylet is visible (Fig. 6B). Extra scratches can then be buffed out with 0.5 micron aluminium oxide powder and a car wash chamois or any smooth, soft cloth.

148 **1.3 Preparing stylets from hatchlings and juveniles**

149 If stylets can be readily dissected and removed from a juvenile or hatchling, they can be
150 prepared as described above, but it should be noted that often, increments in small stylets are
151 difficult to read due to the loss of resolution at high magnifications. If stylets cannot be
152 removed from very young hatchlings, they may be identified using histological methods (see
153 section 3.3), but again increment visualisation may be impossible.

154

155 **1.4 Visualising and counting growth increments**

156 Stylet growth increments can be visualised using transmitted brightfield microscopy and
157 either counted directly through the eyepiece while under the microscope, from an enlarged
158 digital image on an attached computer screen, or from a single or series of saved digital
159 images using an image analysis software application such as ImageJ (Fig. 7). The best
160 viewing magnification will vary for each octopus species, although resolution is often lost at
161 higher magnifications. For example, for *Octopus berrima*, stylet increments were best viewed
162 between 200 and 400x magnification (Durante et al. 2023) and for *Robsonella huttoni*
163 (*Octopus huttoni*), increments were best viewed between 400 and 1000x (oil immersion)
164 magnification (Donlon et al. 2019).

165 Ideally, increments, from the core to the edge, should be counted at least twice, non-
166 consecutively, by one or more trained readers, with the average of multiple counts used to
167 define age (if increment periodicity is known). Aging precision is typically measured by
168 taking the percent difference between counts. Then, if the counts differ by more than a set
169 percentage for a single stylet section (i.e., more than 10% is a typical standard), the section is
170 discarded (Barratt and Allcock 2010; Leporarti and Hart 2015; Perales-Raya *et al.* 2010). We
171 refrain here from recommending a set number of consecutive counts, number of readers, and
172 percent cut off for precision, because these may need to vary based on species, number of
173 samples available, and application. However, we suggest that practitioners refer to published
174 methods, particularly if their species has been aged before.

175

176 **1.5 Determining age using stylet weight**

177 Once increment periodicity is validated and stylet increment analysis undertaken, there is
178 potential to take the ageing method further by determining if stylet weight (or another
179 morphometric measure) can be used as a proxy for age. For example, Leporati *et al.* (2015)
180 found that there was a strong relationship between age and stylet weight in *Octopus djinda*
181 (formally *Octopus* cf. *tetricus*), suggesting that stylet weight can be used as a rapid, cost-
182 effective, and reliable ageing method.

183

184

185 **Part 2: Beak increment analysis**

186 Beaks are composed of a mixture of chitin and protein and embedded within the buccal mass
187 (mouth musculature) located at the centre of the arms on the ventral side of the octopus
188 (Bizikov 2004). As octopus grow, beak increments are periodically deposited on the edge of
189 the rostrum and lateral wall, thus facilitating age determination through increment analysis.
190 Beak increment analysis was first explored in octopus by Raya and Hernández-González
191 (1998) and can be prepared through a range of methodologies such as the rostrum sagittal
192 section (RSS), lateral wall surface (LWS), or lateral rostrum surface (LRS) (Arkhipkin *et al.*
193 2018). Of these methods, the LWS appears to be a more accurate age indicator than the RSS
194 (Perales-Raya *et al.* 2014a), but the most recent suggestion is to analyse both LWS and RSS
195 of upper and lower beaks of new species to determine the best reading location (Xavier *et al*.
196 2022). The LRS is typically only used on hatchling, paralarvae, or translucent adult beaks in
197 which increments are only visible in this area (Arkhipkin *et al*. 2018; Franco Santos *et al*.
198 2016; Perales-Raya *et al.* 2014a; Perales-Raya *et al*. 2017).

199 We provide a detailed outline of the steps involved for beak increment analyses via the LWS.
200 For methods using the RSS and LRS refer to Perales-Raya *et al.* (2010), Perales-Raya *et al.*
201 (2014a), Perales-Raya *et al*. (2017), and Franco Santos *et al*. (2016).

202

203 **2.1 Dissection and storage**

204 Octopus beaks are embedded within the buccal mass on the ventral side of the octopus (Fig
205 8). Dissection can be undertaken through the following method (Fig. 9) and is best dissected
206 after the octopus or entire buccal mass is previously frozen:

207 1. Begin on the ventral side of the octopus between the arms.
208 2. Make an incision to both sides of the mouth musculature to expose the beak.
209 3. Using tweezers, carefully remove the upper and lower beak.

210 After the majority of tissue is cleaned, beaks can be preserved indefinitely in 70% ethanol
211 until ready for use or, if analysis occurs shortly after, they can be preserved in distilled water
212 at 4 °C. The later preservation method has been found to better preserve the microstructure,
213 but trials should always be done for each species to determine whether ethanol significantly
214 degrades the microstructure or not.

215

216 **2.2 Preparing the LWS of beaks from adults and large individuals**

217 1. Using scissors, cut the upper beak in half to obtain two sagittal sections (Fig 10).
218 Select the flattest half for sample preparation.
219 2. Remove any remaining tissue from the beak using distilled water and scrub gently
220 with the tip of a plastic pipette. For stubborn tissue, place beak halves in a tube with
221 5% hydrogen peroxide in an ultrasonic cleaner for ~ 5 minutes and scrub again with
222 pipette tip. Rinse with water.
223 3. If the beak drying out is a concern, they can be stored in water at 4 °C and then placed
224 under the microscope when counting. To keep the beak flat, we suggest placing the
225 beak between two pieces of glass secured with an adhesive tape during counting.
226 4. If it is determined that increments are not compromised with the beak is dry, we
227 suggest using an appropriate adhesive to fix your beak section to a microscope slide,
228 flattening the section as much as possible with a wide, flat scalpel or knife (Fig. 11).
229 Our preferred adhesive is Crystalbond™ 509 because it can be reheated to reshape

230 mounts and cures quickly as it cools. The slide can then be easily referred to when
231 needed.

232

233 **2.3 Preparing the LWS of beaks from hatchling and juveniles**

234 Extra small and thin beaks such as those in hatchlings are carefully dissected, cleaned with
235 water and a plastic pipette, butterflied with the inside facing up and mounted to a slide in
236 warmed glycerol gelatin and a coverslip. Slightly larger hatchling beaks are cut in half
237 sagittally as in adult octopus and mounted face up on a slide with glycerol gelatin and a
238 coverslip. The beak should be completely covered by the gelatin before placing the cover slip
239 and overheating of the gelatin should be avoided to prevent air bubbles from forming.

240

241 **2.4 Visualising and counting growth increments**

242 Beak growth increments can be visualised through microscopy. Increments on thicker, larger
243 beaks are more visible using reflective light and increments on thinner, smaller beaks are
244 more visible with transmitted light, but this varies with each species, and both and a
245 combination of both should be trialed.

246 If good micrographs can be taken, increments can be successfully counted from a series of
247 digital images that are individually focused and later stitched together (Fig. 11). These images
248 can then be easily referred back to and measurements such as increments width can be taken.
249 In our experience, it is sometimes easier to count increments on beaks directly through the
250 eyepiece while LWS sections are under the microscope because the three-dimensional surface
251 profile of the increments require careful adjustment of the field of view across the section.
252 Often, the edge of the beak needs to be scanned to find the area in which more increments are
253 visible to find a starting point. As other studies have pointed out (Perales-Raya *et al.* 2010;
254 Perales-Raya *et al.* 2014a), there are many scratches near the rostral tip due to feeding on hard
255 shelled crustaceans, making it difficult to read this area. Similarly, with stylets, we
256 recommend multiple non-consecutive counts per trained reader, with data treated as described
257 above (section 1.4).

258

2.5 Determining age using beak morphometrics

As with stylets, beak morphometrics such as weight and various measurements can also be used as a proxy of age but increment periodicity first needs to be validated to determine the relationship between age and beak morphometrics. This methodology has been applied to *Octopus vulgaris* in which Perales-Raya *et al.* (2010) found well fitted power relationships (R^2=0.76) between the number of beak increments and beak mass as well as hood length. Although periodicity was not validated in this study, it was later validated as daily by Perales-Raya *et al.* (2014a). These data suggest that beak morphometrics have the potential to be effective proxies of age.

Part 3: Validating periodicity of growth increments

Stylet and beak increment analysis are undertaken through counting growth increments, each of which often represent a single day of life (Donlon *et al.* 2019). However, increment deposition may be influenced by various abiotic and biotic factors, and non-daily periodicity has been observed in *Octopus berrima* stylets and beaks, with periodicity varying between the two structures (Durante *et al.* 2023; Xavier *et al.* 2022). Thus, daily growth ring deposition cannot be assumed. Consequently, validation of growth increment is a crucial first step in the ageing process for each species and each ageing structure. Age validation can be achieved through the analysis of known-age individuals, or chemical staining or stress marking of the hard structures to mark time at liberty or in captivity when hatch date is unknown (e.g., for wild caught octopus). Determining the age and location of the first increment is also crucial for validation to determine if any increments are formed before hatching or there is a delay in which the first increment in formed (e.g. at 3-days old instead of at hatching) (Campana, 2001; Lourenço *et al.,* 2015). Only after both periodicity and the identification of the first increment have been described, can precise age estimates be made (if validation assumptions, discussed below, hold true).

At present, beak increment periodicity has been validated in a variety of species, including *O. maya* (Rodriguez *et al.* 2013), *O. vulgaris* (Perales-Raya *et al.* 2014a), *Octopus insularis*

288 (Batista *et al*. 2021), and *Octopus berrima* (Durante *et al*. 2023). In addition, stylet increment
289 periodicity has been validated in *O. vulgaris* (Hermosilla *et al.,* 2010), *Octopus djinda*
290 (previously *Octopus* cf. *tetricus*) (Leporati and Hart, 2015), *Robsonella huttoni* (previously
291 *Octopus huttoni*) (Donlon *et al.,* 2019), *O. pallidus* (Doubleday *et al.,* 2006), and *Octopus*
292 *australis* (Nuttall, 2009).

293

294 **3.1 Validation assumptions**

295 Validation that involves laboratory reared animals obviously assumes that captivity does not
296 influence increment periodicity, as such, age calculated from wild caught individuals should
297 always be regarded as an estimate. It has also been observed in one species of octopus
298 (Octopus berrima) that periodicity may vary with factors such as temperature and rearing
299 density (Durante *et al*. 2023). While, ideally, periodicity should be validated throughout the
300 life cycle of an individual, this is rarely feasible, and validation methods also generally
301 assume that increment periodicity remains constant throughout an individual's life. However,
302 periodicity can be validated in juvenile stages using known age methods and adult stages
303 using chemical marking methods (Durante *et al.* 2023).

304

305 **3.2 Known-age method**

306 The known-age method can be used for ageing octopus with a known hatch date (e.g., for
307 octopus raised in captivity). Age in days is compared with the number of growth increments
308 counted on a structure (e.g., stylet or beak) to validate the periodicity of increment deposition
309 (Barratt and Allcock 2010; Doubleday *et al.* 2006; Hernández López *et al*. 2001; Villegas-
310 Bárcenas *et al.* 2014). To validate periodicity in known-age individuals, sample preparation
311 and visualisation methods follow those described in Parts 1 and 2. A disadvantage of this
312 method may be that somatic growth rates and increment deposition in individuals held in
313 captivity may differ from individuals collected from the wild (Campana 2001). But because
314 the best way to have known-age octopus is to raise them in captivity, conditions should be as
315 natural as possible including seawater quality, temperature, and ambient light.

316

317 **3.3 Marking method (chemical staining and stress marking)**
318 There are two well-known methods of marking hard parts; chemical staining (Batista *et al*.,
319 2021; Hermosilla *et al*., 2010; Perales-Raya *et al*., 2014a; Leporati and Hart, 2015) and stress
320 marking (Perales-Raya *et al.,* 2014a; Perales-Raya *et al.,* 2014a; Canali *et al*., 2011). Stress
321 marking can be done by either the stress of handling and capture (Perales-Raya *et al.,* 2014b)
322 or by thermal stress (Canali *et al*., 2011). The chemical staining method uses fluorescent
323 stains to mark growing hard structures in individuals where hatch date is unknown. After
324 staining, individuals are held for a known amount of time prior to euthanasia. Alternatively,
325 marked animals could be released into the wild and recaptured after a designated time period
326 but this would be logistically challenging and has yet to be achieved for octopus. To
327 determine increment periodicity, the total number of growth increments deposited after
328 marking is compared with the total number of days held or at liberty (Perales-Raya *et al.*
329 2014a).
330
331 This method relies on the method effectively marking the hard part to the extent that a mark
332 can be visualised through microscopy. With chemicals staining, often the mark is fluorescent
333 and requires a microscope with light of an appropriate wavelength. It is also essential that the
334 stain is not toxic to the octopus.
335
336 Several stains have been successfully used to mark stylets and beaks, while others have been
337 unsuccessful (Table 2). However, success is not always consistent among species or
338 structures. For example, we found that Calcofluor white, a fluorescent stain that binds to
339 cellulose and chitin in cell walls, effectively stained the stylets, but surprisingly, not the beaks
340 of *O. berrima* (Durante *et al*. 2023); whereas, Perales-Raya *et al.* (2014a), reported it
341 successfully marked *O. vulgaris* beaks. Tetracycline hydrochloride is a commonly used stain
342 but can cause adverse effects on octopus health in some species (e.g., injection in adults can
343 trigger arm autophagy) (Durante *et al*. 2023; Karina Hall pers comm). Therefore, we do not
344 recommend tetracycline as a stain for new species due to potential adverse impacts. In this
345 guide, we will describe how to chemically mark octopus using Calcofluor white.
346

347 Injection is the most widely practiced and recommended method of chemical stains for
348 octopus (section 3.2.2). Submersion in a seawater bath containing the chemical stain has also
349 been explored. However, not enough of the chemical was absorbed and there is also a risk of
350 the chemical becoming oxidized and losing its fluorescent ability (Donlon *et al.* 2019).
351 Euthanised octopus which have undergone chemical staining should be stored and dissected
352 in the dark. Similarly, stylet and beak samples must be stored, prepared, and embedded in a
353 darkened room to prevent stain oxidation.
354

355 ***3.3.1 Stock solution preparation***

356 A stock solution of Calcofluor can be prepared following the methods outlined in Perales-
357 Raya *et al.* (2014a). This solution is concentrated to 50 mg/mL to minimise injection volume.
358 However, the concentration can be altered as required for different sized octopus.

359 1. Add 750 mg of Calcofluor White to 15 mL of autoclaved seawater, place on a
360 magnetic stir plate with a stir bar and heat to 30 °C.
361 2. Add 15 drops of potassium hydroxide to increase solubility and 3.75 mL of 0.2 M
362 phosphate buffer solution (pH 6.8).
363 *3.* Wrap solution in tin foil, allow to cool to room temperature, and store in the dark at
364 4°C until use.

365

366 ***3.3.2 Sedation or anesthesia***

367 For chemical staining, octopus have been sedated through cold water immersion (Donlon *et*
368 *al.* 2019, Perales-Raya *et al.* 2014a) or anesthetised through chemical solution immersion
369 prior to the injection process (Fiorito *et al.* 2015). In our experience, octopus sedated with
370 cold water are stiff, making it difficult to inject staining solution into the muscle. In
371 comparison, octopus anaethetised with magnesium chloride have relaxed muscles, which may
372 make it easier for injections (Erica Durante pers comm.). Magnesium chloride is also one the
373 most widely used sedatives for octopus. However, we recommend referring to the following
374 guides for comprehensive information on the care and welfare of cephalopods in the
375 laboratory, including sedation: Andrews *et al*. (2013), Fiorito *et al.* (2015) and Doubleday *et*
376 *al.* (2022). We also highly recommend that researchers review the latest best-practice

procedures for chemical staining and sedation of octopus in the literature, as well as through their local animal ethics committees.

3.3.3 Stain injection

1. Once sedated, place octopus on tared scale and record weight. This does not need to be exact as it is just to calculate the quantity of stain to inject.
2. Return octopus to water and calculate injection volume required following recommended injection concentration as per Perales-Raya *et al.* (2014a) and formula below.

$$\text{Calcofluor injection concentration: 0.1 mg/g of body weight}$$

$$\text{Total weight (g)}\ \ 0.1\ \text{(mg)} = \text{y (concentration required)}$$

$$\frac{\text{y mg (calcofluor concentration required)}}{\text{50 mg (solution required)}} = \text{injection volume (ml)}$$

3. Inject solution intramuscularly at the base of the thickest arm (usually a ventral arm). Some researchers suggest injecting in the mantle, but the site of injection had not been investigated thoroughly and at the moment, is based off of what worked for previous studies.
4. Return octopus to a solitary container and flush fresh seawater into the mantle and over the gills until octopus movement recovers. Octopus are considered fully recovered when breathing returns to a normal rate, skin coloration returns, octopus respond to stimuli and all arms are functioning. When recovered, they can be returned back to their original housing.

3.3.4 Analysing stained samples

To analyse stained stylets and beaks follow the same procedures as outline in parts 1 and 2. However, all work must be carried out in the dark to prevent oxidisation of the stain. Visualisation of the fluorescent mark also requires a microscope fitted with an UV filter or other light source of an appropriate wavelength (~380-475 nm).

404 1. Take an image of the stained section under a fluorescent microscope to locate the
405 mark (Fig. 13).
406 2. Take another image in the same position under white light to visualise increments.
407 3. Aligning the two images, count the number of growth increments in the second image
408 from the edge of the chemical stain to the edge of the section.
409 4. Repeat to produce at least two, non-consecutive counts as with unstained sections.
410 5. Average the counts and compare with the number of days from staining to euthanasia
411 to validate growth increment periodicity.

412

413 **3.4 Identifying first post-hatch increment in stylets and stylet core**

414 To estimate the position of the first post-hatch increment or size of the stylet core, as well as
415 determine if stylets are present immediately post hatching, whole hatchlings can be sectioned
416 using histological methods outlined in Lourenco *et al.* (2015) and summarized below:

417 1. Fix whole hatchlings in a mixture of formalin acetic acid calcium chloride (FAACC)
418 for 48 hours then transfer to 70% ethanol and store for at least 24 hours before
419 processing.
420 FAACC is comprised of:
421 • 400mL, 10% neutral buffered formalin
422 • 13g calcium chloride (0.117M)
423 • 50mL glacial acetic acid
424 • 550 mL distilled water
425 2. Process samples following the paraffin embedding sequence outlined in Table 3.
426 3. Trim paraffin blocks until a cross section of the mantle is seen and cut 5 μm sections.
427 Additional trimming may be required if the stylet is not visible post staining and
428 mounting.
429 4. Using a warm water bath, place sections on a slide, flatten under filter paper soaked
430 with 20% ethanol and a roller, and leave to dry for a few hours or overnight.
431 5. Dewax and stain samples following the sequence outlined in Table 4. Alternative
432 stains can also be used, e.g., Lourenco et al. (2015) used Masson's trichrome stain, but
433 we found methyl blue to be sufficient.

6. Cover slip with slide mounting medium DPX.
7. Using a microscope, observe sections and measure the diameter of the stylet cross section and any visible increments.

3.5 Identifying first post- hatch increment in beaks

As with stylets, it is important to know when the first beak increment was formed and how many, if any, they hatch with. This is done by using the methods for small beaks described above to closely observe freshly hatched hatchlings or paralarvae to determine if any increments are present. Everyday thereafter, beaks of individuals raised in captivity should be observed to determine at what age the first increment forms.

Part 4: Potential ageing methods: avenues for further research

In some instances, increment analysis of stylets and beaks may not be a suitable ageing method due to poor increment readability or variable increment periodicity. Further research is needed to develop ageing methods for application in such instances. We present two additional potential avenues below.

4.1 Eye lens analysis

Analysing growth increments in eye lenses has been explored as an ageing method when traditional ageing methods have yielded unsatisfactory readings. Lenses can be fixed in neutral formalin before being dehydrated, and either embedded in paraffin to produce histological slides (Luna 1968; Baqueiro-Cardenas *et al.* 2011; Rodriguez *et al.* 2013) or embedded in synthetic resin to produce thin slides (Baqueiro-Cardenas *et al.* (2011). Baqueiro-Cardenas *et al.* (2011) found a correlation between the number of eye lens growth increments and age in *Enteroctopus megalocyathus.* However, subsequent validation of this method using *O. maya* indicated no relationship between number of eye lens increments and age (Rodriguez *et al.* 2013).

462

463 **4.2 Lipofuscin quantification**

464 Lipofuscin quantification involves quantification of age pigment lipofuscin using histological
465 methods (Arkhipkin *et al.* 2018). Lipofuscin is generated during normal metabolism and
466 accumulates within nervous tissue over time; thus, it may be used as a proxy for age
467 (Doubleday and Semmens 2011). Lipofuscin quantification is currently the primary method
468 used for ageing in crustaceans, having been successfully applied to a range of marine species
469 (Kodama *et al.* 2006; Puckett *et al.* 2008; Matthews *et al.* 2009; Harvey 2010). Lipofuscin
470 quantification has been explored as an alternative ageing method in *O. pallidus* (Doubleday
471 and Semmens 2011) and *O. huttoni* (Donlon *et al.* 2019), with mixed results. However, more
472 research is needed on more individuals, across different life stages, and species.

473

474

475 **Part 5: Choosing the best method**

476 Given that periodicity validation experiments are usually costly and challenging to complete,
477 the first step in developing an ageing method for a new octopus species is to ascertain whether
478 any clear growth increments can be visualised in the hard structures. Initial trials to establish
479 preparation methods can usually be achieved with a small number of specimens and at
480 minimal expense using the steps outlined in this guide. Once an approach for increment
481 visualisation and analysis has been established, it is essential to follow with some form of age
482 validation to determine the periodicity of increment formation. Validation should be
483 preferably done for different life stages (Campana 2001; Doubleday *et al.* 2006), as well as
484 different ageing structures if multiple ageing structures are used (Durante et al. 2023). Only
485 then can increment counts from hard structures be converted into accurate age estimates.

486

487 For some octopus species, stylet and beak increments have been detected but periodicity is yet
488 to be validated, and for a handful of others, increment periodicity has been validated, and
489 ageing methods successfully applied (Table 5). These past successes provide a valuable

490 starting point for future ageing studies. However, for many octopus species, stylet and beak
491 growth increments are yet to be visualised, therefore an initial period of method development
492 is required. To assist with the ageing process, we provide a flow chart indicating the main
493 steps and decision points (Fig. 15).

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499

500 **Conflict of Interest Statement**

501 The authors declare no conflicts of interest.

502

503 **Data Availability Statement**

504 There are no data associated with this study.

505

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510 Fishermen's Co-operative, Clarence River Fishermen's Co-Operative Ltd and several NSW
511 commercial fishers for assistance in collecting and processing octopus samples.

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