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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, 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.

Keywords: age validation, beaks, cephalopods, fisheries, increment analysis, methods, octopus ageing, stylets.

Introduction

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

A range of methods have been explored to estimate octopus age, including direct enumeration of growth increments in hard structures such as stylets (Doubleday *et al.* 2006) and beaks (Perales-Raya and Hernández-González 1998; Perales-Raya *et al.* 2010), and indirect methods that act as an age proxy, such as eye lens diameter or weight (Baqueiro-Cárdenas *et al.* 2011), stylet weight (Leporati and Hart 2015), and lipofuscin quantification (Doubleday and Semmens 2011). Stylet and beak increment analyses remain the most effective and broadly used octopus ageing methods and have been validated across different life stages for multiple species (Doubleday *et al.* 2006; Hermosilla *et al.* 2010; Rodríguez-Domínguez *et al.* 2013; Bárcenas *et al.* 2014; Perales-Raya *et al.* 2014*a*). However, because of species-specific variations in beak and stylet microstructure, not all

preparation techniques can be applied to all species. Therefore, a period of method development that includes increment visualisation and validation of increment periodicity is usually required when ageing a species for the first time.

The following guide outlines common and successfully applied methods for stylet and beak preparation, increment analyses, and age validation, as well as guidance on selecting the most suitable method for different octopus species. Although we acknowledge that 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* (Rodríguez-Domínguez *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.

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).

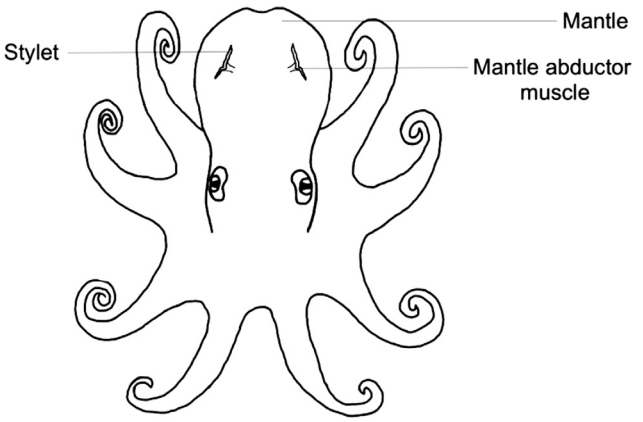

Fig. 1. Diagram of an octopus from a dorsal viewpoint, indicating stylet location within the mantle.

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 4–6 to retrieve the stylet on the opposite side.

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 utilised in multiple octopus ageing studies (Barratt and Allcock 2010; Leporati and Hart 2015; Durante *et al.* 2023). 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 ~1-mm 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 h. Solutions can be reused up to three 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

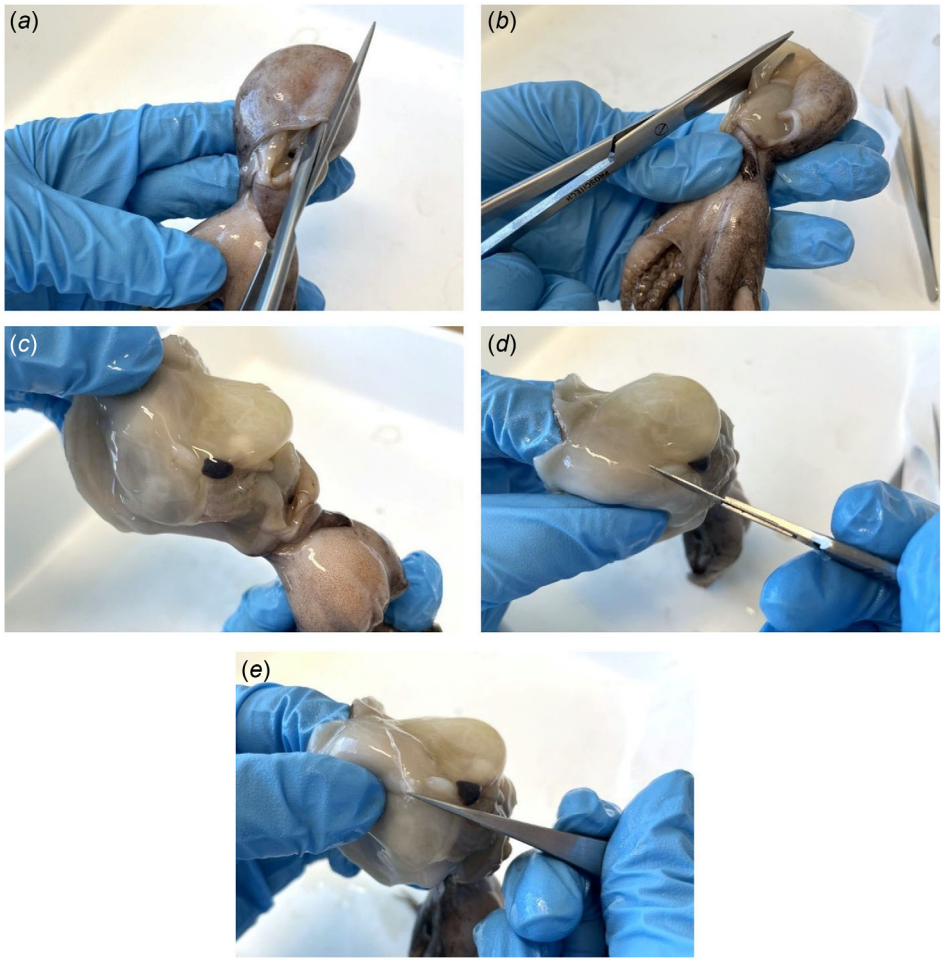

Fig. 2. Stylet dissection process involving (*a*) a vertical incision from the base (anterior) to the top (posterior) of the mantle, (*b*) a horizontal incision through the muscular septum, (*c*) stylet location, (*d*) an incision to separate the stylet and mantle abductor muscle, and (*e*) removal of the stylet from the mantle.

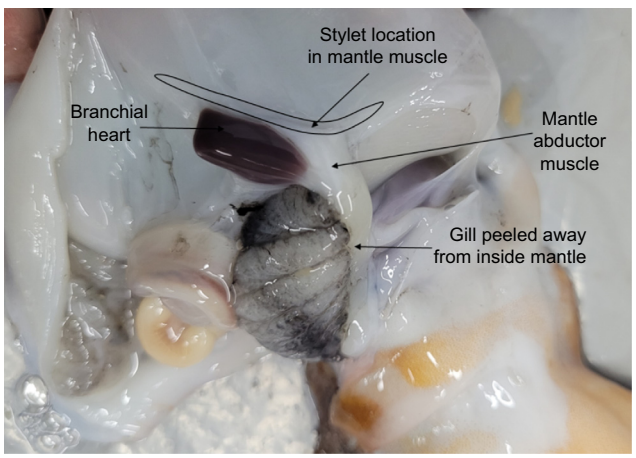

Fig. 3. Close-up image of the stylet location within the mantle muscle at the base of the abductor muscle and branchial heart, after the gill has been peeled away from the inside of the mantle.

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 of 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 h.
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

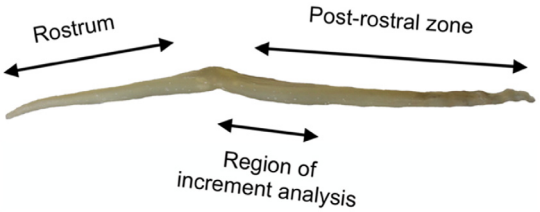

Fig. 4. Image showing the region of increment analysis on the post-rostral zone of an *Octopus pallidus* stylet.

Table 1. Table outlining the preparation sequence for stylets to be preserved through resin infiltration.

Solution	Duration (h)
90% ethanol	1
100% ethanol	1
LR white resin (medium grade)	24

The LR white resin solution was catalysed prior to adding accelerator (hardener).

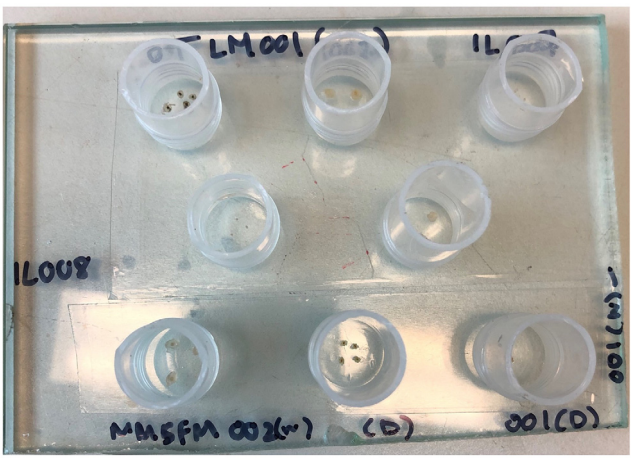

Fig. 5. Image displaying resin infiltration moulds used for stylet preparation including glass base, double-sided tape and plastic tubing.

polishing. For stubborn residue, surface-safe adhesive removers may be useful.

10. Using wet 1000 grit sandpaper, followed by 15-, 6- and 3-μm 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 motorised 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 h (Fig. 6*a*). 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.

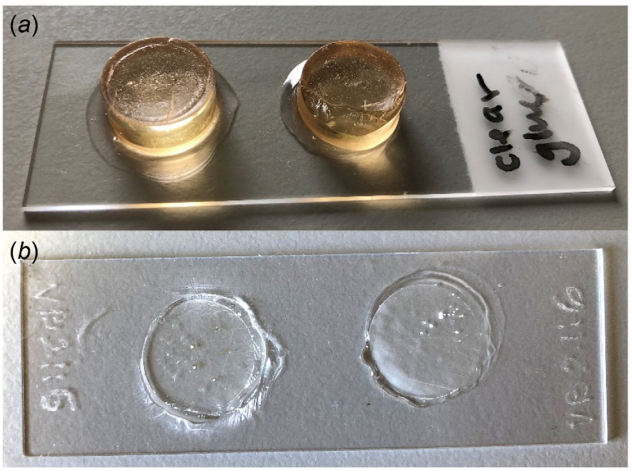

Fig. 6. Images of resin blocks with stylet sections when (*a*) glued to slide and (*b*) cut and polished.

Alternatively, a motorised 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-μm lapping film until a thin section of the stylet is visible (Fig. 6*b*). Extra scratches can then be buffed out with 0.5 μm of aluminium oxide powder and a carwash chamois or any smooth, soft cloth.

Preparing stylets from hatchlings and juveniles

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

Visualising and counting growth increments

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

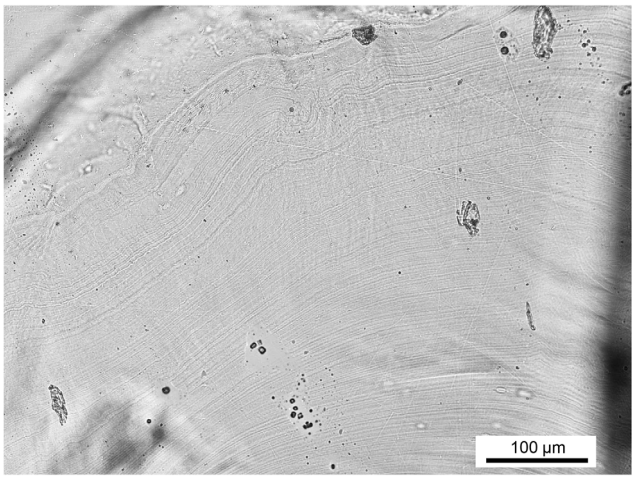

Fig. 7. Micrograph of *Macroctopus maorum* stylet section showing growth increments at 100× magnification.

were best viewed between 400 and 1000× (oil immersion) magnification (Donlon *et al.* 2019).

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

Determining age using stylet weight

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

Part 2: beak increment analysis

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

We provide a detailed outline of the steps involved for beak increment analyses using the LWS. For methods using the RSS and LRS, refer to Perales-Raya *et al.* (2010, 2014*a*, 2018) and Franco-Santos *et al.* (2016).

Dissection and storage

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

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

After the majority of tissue is cleaned, beaks can be preserved indefinitely in 70% ethanol until ready for use

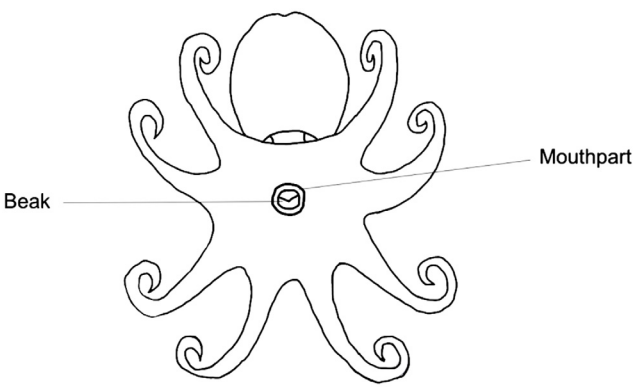

Fig. 8. Diagram of an octopus from a ventral viewpoint, indicating the beak embedded within the buccal mass.

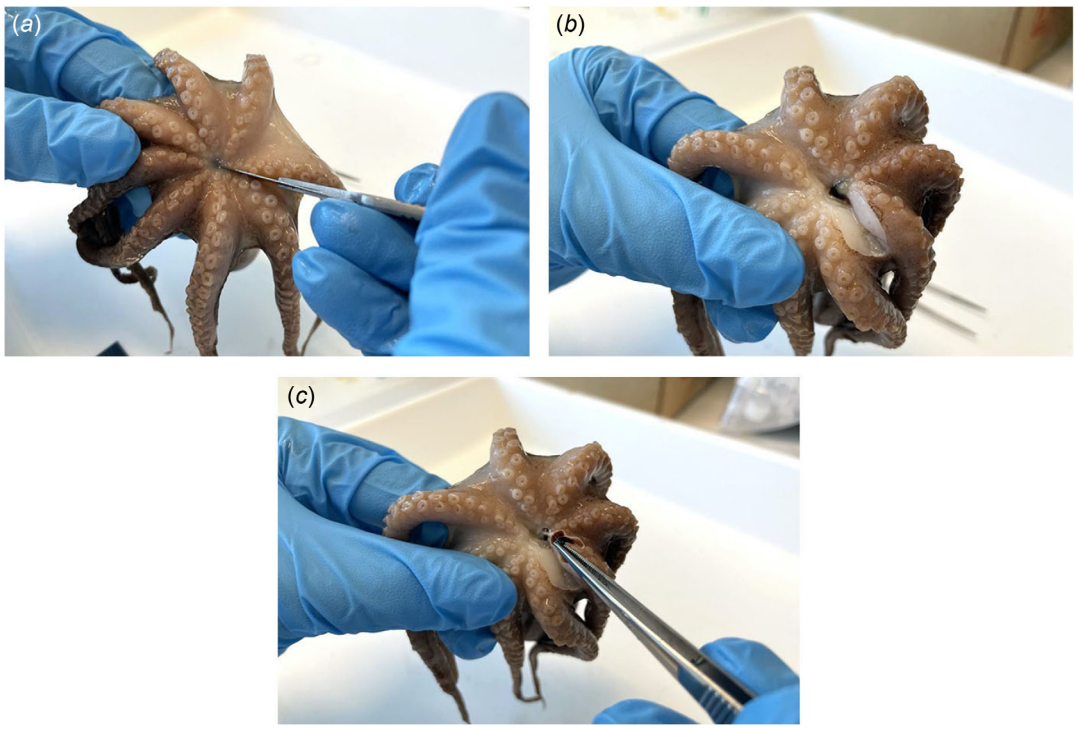

Fig. 9. Beak dissection process involving (*a*) an incision to one side of the mouth musculature to expose the beak, (*b*) another incision to the other side, and (*c*) removal of the beak from the buccal mass.

or, if analysis occurs shortly after, they can be preserved in distilled water at 4°C. The latter preservation method has been found to better preserve the microstructure, but trials should always be done for each species to determine whether ethanol significantly degrades the microstructure or not.

Preparing the LWS of beaks from adults and large individuals

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

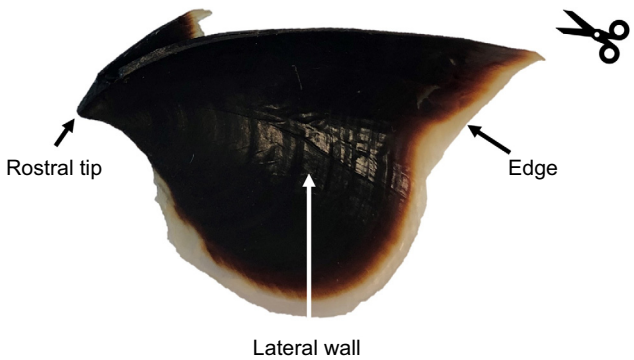

Fig. 10. Image of a *Macroctopus maorum* beak indicating the rostral tip, lateral wall and beak edge according to Clarke (1986). The counting line indicates the direction for counting of growth increments (from edge to rostral tip), and the scissors indicate where to section if using the lateral wall surface.

Preparing the LWS of beaks from hatchling and juveniles

Extra small and thin beaks, such as those in hatchlings, are carefully dissected, cleaned with water and a plastic

Fig. 11. Flattening beak half onto a slide with warm crystalbond adhesive.

pipette, butterflied with the inside facing up, and mounted to a slide in warmed glycerol gelatin and a coverslip. Slightly larger hatchling beaks are cut in half sagittally, as in adult octopus, and mounted face up on a slide with glycerol gelatin and a coverslip. The beak should be completely covered by the gelatin before placing the cover slip, and overheating of the gelatin should be avoided to prevent air bubbles from forming.

Visualising and counting growth increments

Beak growth increments can be visualised through microscopy (Fig. 12). Increments on thicker, larger beaks are more visible using reflective light, and increments on thinner, smaller beaks are more visible with transmitted light, but this varies with each species, and both and a combination of both should be trialled.

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

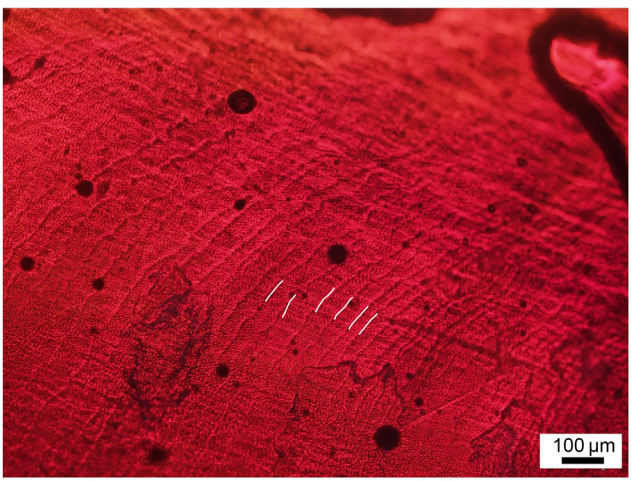

Fig. 12. Micrograph of *Octopus tetricus* beak section (lateral wall surface) showing growth increments at 100× magnification. White lines highlight a few growth increments that can be seen.

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.* (2014*a*). 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 (Xavier *et al.* 2022; Durante *et al.* 2023). 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, 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; Doubleday *et al.* 2011; 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 *Octopus maya* (Rodriguez-Domínguez *et al.* 2013), *Octopus vulgaris* (Perales-Raya *et al.* 2014*a*), *Octopus insularis* (Batista *et al.* 2021) and *Octopus berrima* (Durante *et al.* 2023). In addition, stylet increment periodicity has been validated in *Octopus vulgaris* (Hermosilla *et al.* 2010), *Octopus djinda* (previously *Octopus* cf. *tetricus*) (Leporati and Hart 2015), *Robsonella huttoni* (previously *Octopus huttoni*) (Donlon *et al.* 2019), *Octopus pallidus* (Doubleday *et al.* 2006) and *Octopus australis* (Nuttall 2009).

Validation assumptions

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

Known-age method

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

Marking method (chemical staining and stress marking)

There are two well-known methods of marking hard parts: chemical staining (Hermosilla *et al.* 2010; Canali *et al.* 2011; Perales-Raya *et al.* 2014*a*; Leporati and Hart 2015; Batista *et al.* 2021) and stress marking (Perales-Raya *et al.* 2014*a*, 2014*b*). Stress marking can be done by either the stress of handling and capture (Perales-Raya *et al.* 2014*b*) or by thermal stress (Canali *et al.* 2011). The chemical staining method uses fluorescent stains to mark growing hard structures in individuals where hatch date is unknown. After staining, individuals are held for a known amount of time prior to euthanasia. Alternatively, marked animals could be released into the wild and recaptured after a designated time period, but this would be logistically challenging and has yet to be achieved for octopus. To determine increment periodicity, the total number of growth increments deposited after marking is compared with the total number of days held or at liberty (Perales-Raya *et al.* 2014*a*).

This method relies on the method effectively marking the hard part to the extent that a mark can be visualised through microscopy. With chemical staining, often the mark is fluorescent and requires a microscope with light of an appropriate wavelength. It is also essential that the stain is not toxic to the octopus.

Several stains have been successfully used to mark stylets and beaks, whereas others have been unsuccessful (Table 2). However, success is not always consistent among species or structures. For example, we found that Calcofluor white, a fluorescent stain that binds to cellulose and chitin in cell walls, effectively stained the stylets, but surprisingly, not the beaks of *O. berrima* (Durante *et al.* 2023), whereas Perales-Raya *et al.* (2014*a*) reported that it successfully marked *O. vulgaris* beaks. Tetracycline hydrochloride is a commonly used stain but can cause adverse effects on

Table 2. Summary of chemical stains used to validate periodicity in octopus stylets and beaks and their effectiveness.

Chemical stain	Structure	Species	Reference
Tetracycline hydrochloride	Stylet	*Octopus berrima*: clear stain mark, but adverse effect on octopus health	Durante *et al.* (2023)
		Octopus huttoni: clear stain mark	Donlon *et al.* (2019)
		Octopus vulgaris: clear stain mark	Hermosilla *et al.* (2010)
		Octopus australis: diffused stain mark	Nuttall (2009)
	Beak	*Octopus berrima*: diffused stain mark	Durante *et al.* (2023):
Calcofluor White	Stylet	*Octopus berrima*: clear stain mark	Durante *et al.* (2023)
	Beak	*Octopus vulgaris*: clear stain mark	Perales-Raya *et al.* (2014*a*)
		Octopus berrima: diffused stain mark	Durante *et al.* (2023)
Congo red	Beak	*Octopus vulgaris*: diffused stain mark	Perales-Raya *et al.* (2014*a*)
Alizarin Complexone	Stylet	*Octopus australis*: clear stain mark	Nuttall (2009)
Alizarin Red S	Stylet	*Octopus australis*: clear stain mark	Nuttall (2009)
Calcine	Stylet	*Octopus dijinda* (*Octopus* cf. *tetricus*): clear stain mark	Leporati and Hart (2015)

octopus health in some species (e.g. injection in adults can trigger arm autophagy) (Durante *et al.* 2023; Karina Hall, pers. comm.). Therefore, we do not recommend tetracycline as a stain for new species due to potential adverse effects. In this guide, we will describe how to chemically mark octopus using Calcofluor white.

Injection is the most widely practised and recommended method of chemical stains for octopus. Submersion in a seawater bath containing the chemical stain has also been explored. However, adequate levels of chemical may not be absorbed and there is also a risk of the chemical becoming oxidised and losing its fluorescent ability (Donlon *et al.* 2019). Euthanased octopus that have undergone chemical staining should be stored and dissected in the dark. Similarly, stylet and beak samples must be stored, prepared and embedded in a darkened room to prevent stain oxidation.

Stock solution preparation

A stock solution of Calcofluor can be prepared following the methods outlined in Perales-Raya *et al.* (2014*a*). This solution is concentrated to 50 mg mL^{-1} to minimise injection volume. However, the concentration can be altered as required for different sized octopus.

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

Sedation or anesthesia

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

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 (y, mL) following recommended injection concentration as per Perales-Raya *et al.* (2014*a*) and formula below:

$$y = (\text{total weight} \times \text{stain concentration}) \div 50$$

where total weight is the mass in grams, stain concentration is the mass in milligrams, and concentration of stock solution is 50 mg mL^{-1}.

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 is currently 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 colouration returns, octopus respond to stimuli and all arms are functioning. When recovered, they can be returned back to their original housing.

Analysing stained samples

To analyse stained stylets and beaks, follow the same procedures as outlined 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 ultraviolet filter or other light source of an appropriate wavelength (~380–475 nm).

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

Identifying first post-hatch increment in stylets and stylet core

To estimate the position of the first post-hatch increment or size of the stylet core, as well as determine if stylets are present immediately post-hatching, whole hatchlings can be sectioned using histological methods outlined in Lourenço *et al.* (2015) and summarised below (Fig. 14):

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

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

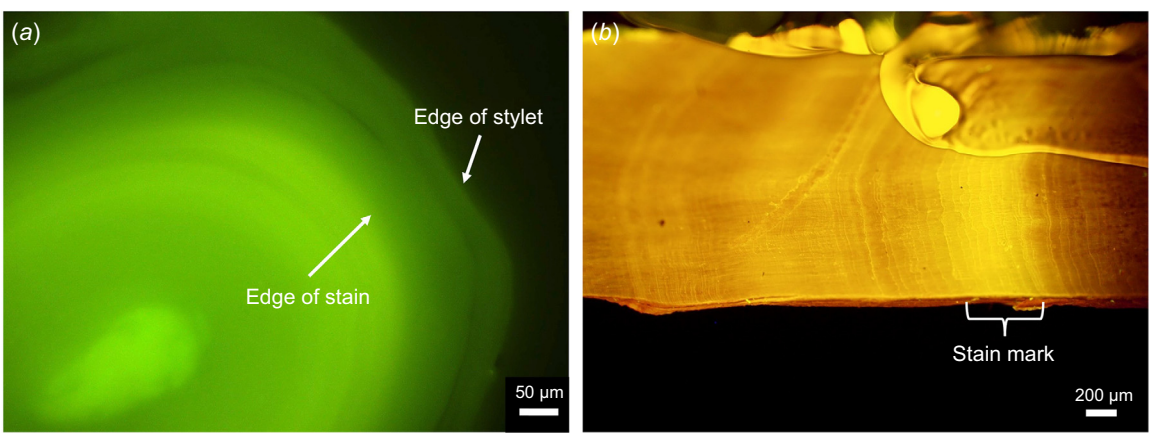

Fig. 13. (*a*) Micrograph of a Calcofluor-stained *Octopus berrima* stylet section showing the edge of the stain mark and the edge of the stylet and (*b*) micrograph of the lateral wall of a *Octopus tetricus* upper beak that has been stained with tetracycline. Brackets indicate the section in which the fluorescent mark was formed from the tetracycline.

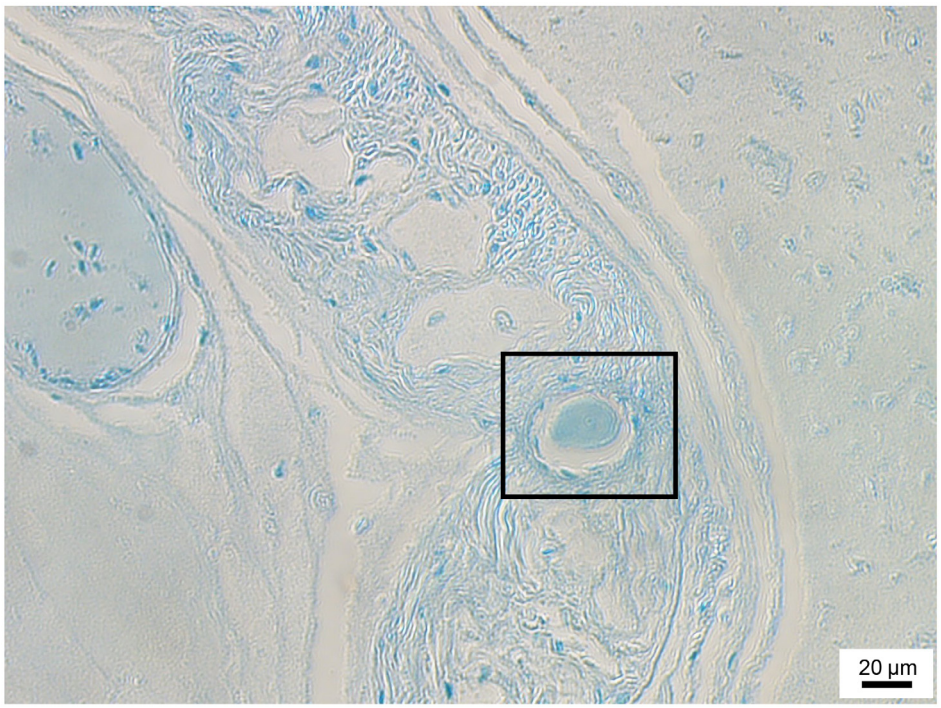

Fig. 14. Micrograph of a 3-day-old *Octopus berrima* hatchling cross section at 20× magnification. Stylet section is indicated within the box.

Table 3. Paraffin embedding sequence for octopus hatchlings (<100 days old).

Solution	Duration (min)
95% ethanol	60
100% ethanol	60
100% ethanol	60
Xylene	40
Xylene	40
Paraffin	40
Paraffin	40
Paraffin	40

Table 4. Dewaxing and staining sequence for octopus hatchlings (<100 days old).

Solution	Duration (min)
Xylene	2
Xylene	2
100% ethanol	2
100% ethanol	2
70% ethanol	2
30% ethanol	2
~3% (2.7%) methyl blue in water	5
Rinse in water	Until water is clear
70% ethanol	2
100% ethanol	2
100% ethanol	2
Xylene	2
Xylene	2

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.

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-Cárdenas *et al.* 2011; Rodriguez-Domínguez *et al.* 2013) or embedded in synthetic resin to produce thin slides (Baqueiro-Cárdenas *et al.* 2011). Baqueiro-Cárdenas *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-Domínguez *et al.* 2013).

Lipofuscin quantification

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

Part 5: choosing the best method

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

For some octopus species, stylet and beak increments have been detected but periodicity is yet to be validated, and for a handful of others, increment periodicity has been validated, and ageing methods successfully applied (Table 5). These past successes provide a valuable starting point for future ageing studies. However, for many octopus species, stylet and beak growth increments are yet to be visualised; therefore, an initial period of method development is required. To assist with the ageing process, we provide a flow chart indicating the main steps and decision points (Fig. 15).

Table 5. Recommended ageing methods for holobenthic and merobenthic octopus species whereby full methods have been already developed and published.

Species	Increments visualised	Periodicity validated	Recommended ageing method(s)	Reference
Holobenthic				
Octopus berrima	Yes	Yes (non-daily)	Further validation required on different life history stages	Durante *et al.* (2023)
Octopus maya	Yes	Yes	Beak increment analysis	Rodriguez-Domínguez *et al.* (2013) Bárcenas *et al.* (2014)
Octopus pallidus	Yes	Yes	Stylet increment analysis	Doubleday *et al.* (2006) Leporati *et al.* (2008)
Merobenthic				
Eledone cirhhosa	Yes	No		Barratt and Allcock (2010)
Octopus djinda (*Octopus* cf. *tetricus*)	Yes	Yes	Stylet increment analysis Stylet weight	Leporati and Hart (2015)
Octopus huttoni	Yes	Yes	Further validation required on different life history stages	Donlon *et al.* (2019)
Octopus vulgaris	Yes	Yes	Stylet increment analysis Beak increment analysis	Barratt and Allcock (2010) Hermosilla *et al.* (2010) Hernández-López *et al.* (2001) Perales-Raya *et al.* (2014*a*)

We also list species that have readable increments, but validation is still required.

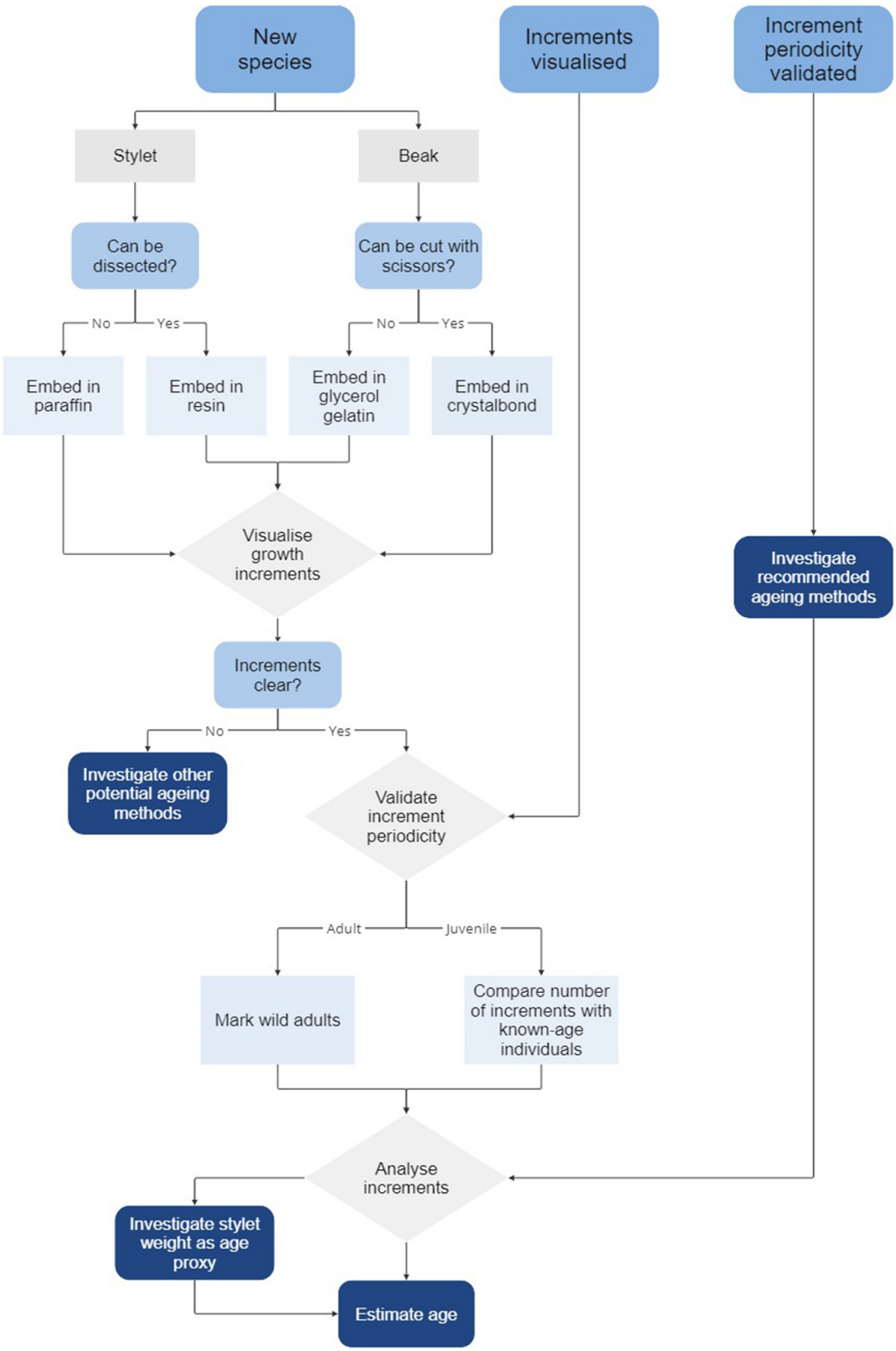

Fig. 15. Flow chart indicating the main steps and decision points involved in ageing octopus.

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Data availability. There are no data associated with this study.

Conflicts of interest. The authors declare that they have no conflicts of interest.

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