The hardest part of being a cephalopod:
Unravelling ecological and environmental histories of southern Australian cephalopods

by

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Summary

The hard parts of marine animals contain a wealth of information about biological and environmental histories. For cephalopods, the term "hard part" commonly refers to calcified or chitinous structures, which typically exhibit incremental or accretionary growth. Hard parts offer two avenues for studying cephalopod ecology: analysing 1) morphology including growth increments and 2) biochemical markers within the hard part. In this thesis, I employ diverse methods to extract data from cephalopod hard parts and subsequently use these methods to unravel the ecological and environmental histories of cephalopod species from southern Australia.

Growth increments can be used to collect age and growth data, which are important for fisheries and conservation management. I have estimated the age of a commercially harvested octopus species, *Octopus berrima*, by validating the periodicity of growth increments in the beaks and stylets (vestigial shells) (Chapter 2). Previous studies have either validated or assumed daily periodicity, but here, I confirm that increments are non-daily and that periodicity also varies between structures (0.75/day for stylets and 0.53/day for beaks). Based on this evidence, this study highlights the importance of age validation studies for different octopus species and different structures, and that daily periodicity should not be readily assumed. Aligned to this study, I have also created a step-by-step octopus ageing guide, as part of a collaborative project (see Chapter 3). This guide provides hands-on guidance to help researchers through the ageing process, particularly for species that have never been aged before.

Biochemical markers, such as stable isotopes are powerful tools for unravelling the life histories of animals, but they are used to a limited extent in cephalopod hard parts. I have explored the relationships between $\delta^{13}C$ and $\delta^{18}O$ values in octopus statoliths as proxies of metabolic rate and experienced temperature, respectively, by rearing *O. berrima* hatchlings in aquaria at different temperatures (Chapter 4). As a result, a strong, inverse relationship between $\delta^{18}O_{statolith}$ values and temperature was evident. I use this relationship to develop an oxygen-thermometry equation for octopus, which could be applied to reconstruct temperatures in the field. In addition, $\delta^{13}C_{statolith}$ values were positively correlated with body mass, suggesting it is a useful proxy for metabolic rate. In Chapter 5, I collate historic *Sepia apama* cuttlebones from museum collections to create a timeseries spanning 60 years, which I use to investigate spatial and temporal trends on $\delta^{13}C$ and $\delta^{18}O$ values across southern Australia. When the dataset was analysed as a whole, there were no detectable temporal trends, as these were likely

overwhelmed by strong spatial effects driven by local oceanography and temperature. However, temporal trends were detected when a smaller subset of the data representing a single oceanographic region were analysed. Carbon isotope values were also influenced by body mass (cuttlebone size), suggesting that historic cuttlebones may be used to help reconstruct metabolic rate through time. However, further research is needed.

Hormones are a novel biochemical marker that has the potential to unravel reproductive histories of cephalopods. I have developed a new method to extract and analyse steroid reproductive hormones from the beaks of two commercial species of octopus, *Macroctopus maorum* and *Octopus pallidus* (Chapter 6). By sub sampling along the beak, I successfully extracted progesterone and estradiol from each sub-sample, which were then analysed on a liquid chromatography-mass spectrometer (LC/MS/MS). Because beaks have incremental growth, peaks in hormone concentrations could be related back to "age", and physiological maturity for both males and females could be identified. The advantage of this new method is that the reproductive histories of cephalopods can be reconstructed without the use of whole specimens, thus providing valuable data on rare species or species living in inaccessible environments, such as the deep sea (i.e., where beaks are available in the stomach contents of predators).

Collectively, these case studies showcase the variety of information that can be extracted from cephalopod hard parts such as age, metabolism, thermal histories, and reproductive histories. My thesis also highlights the challenges and potential of using opportunistically collected samples and historical specimens. This thesis contributes to the knowledge of cephalopod ecology, demonstrates the exciting potential of integrating new and established methods, and lays the foundation for future research utilising cephalopod hard parts.

Declaration

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.

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[redacted]

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General Introduction

Cephalopods in global ocean ecosystems and fisheries

Cephalopods, a class of invertebrates within the phylum Mollusca, exhibit distinct characteristics that set them apart from their mollusc relatives. Unlike other molluscs such as bivalves and gastropods, cephalopods have evolved for enhanced mobility since the Cambrian period (500-550 million years ago) (Norman and Reid, 2000; Bizikov, 2004). Some, like the nautilus, achieve this mobility by modifying the shape of their external shells, while others, such as squid, cuttlefish, and octopus, have entirely shed their external shells to become less cumbersome and quickly avoid predators (Kröger *et al.*, 2011). Squid, cuttlefish, and octopus are part of the Coleoidae or soft-bodied cephalopods in which modern coleoids exhibit a 'live fast, die young' life history strategy (Semmens *et al.*, 2004). Most coleoids, such as octopus, are semelparous in which they only live approximately 1-2 years (5 years in the case of the giant Pacific octopus, *Enteroctopus dofleini*) and die shortly after reproducing (Semmens *et al.*, 2004). These coleoids are phenotypically plastic and highly responsive to environmental change (O'Dor and Webber, 1986; Rodhouse *et al.*, 2014). There is evidence that some cephalopod populations are even increasing, potentially driven by a changing climate and declines in fish stocks (Rodhouse *et al.*, 2014; Doubleday *et al.*, 2016).

Coleoid cephalopods are found throughout the world's oceans, inhabiting diverse environments ranging from coastal intertidal zones to the deep sea, and play fundamental roles in marine ecosystems (Hofmeister, 2018). They are voracious predators of crustaceans, small fish, and various invertebrates, as well as important sources of prey for many other species, including marine mammals (Clarke, 1996; Klages, 1996), seabirds (Croxall and Prince, 1996), fish (Smale, 1996), and humans. Cephalopods are a staple food source in many cultures around the world, and as the global demand for sustainable seafood increases and traditional finfish stocks decline or remain stagnant, cephalopod fisheries have continued to grow (Fig. 1.1) (Rodhouse *et al.*, 2014; Arkhipkin *et al.*, 2015; Sauer *et al.*, 2021).

Although cephalopods are important in maintaining the balance of marine ecosystems, and their demand as a seafood is increasing, knowledge about their ecology and biology is lacking for many species. This paucity of knowledge can hamper management decisions underpinning sustainable fisheries and conservation (Rodhouse *et al.*, 2014). For example, sustainable fisheries require knowledge of life-span and spawning periods to set regulations on catch limits and fishing seasons (Gebremedhin *et al.*, 2021).

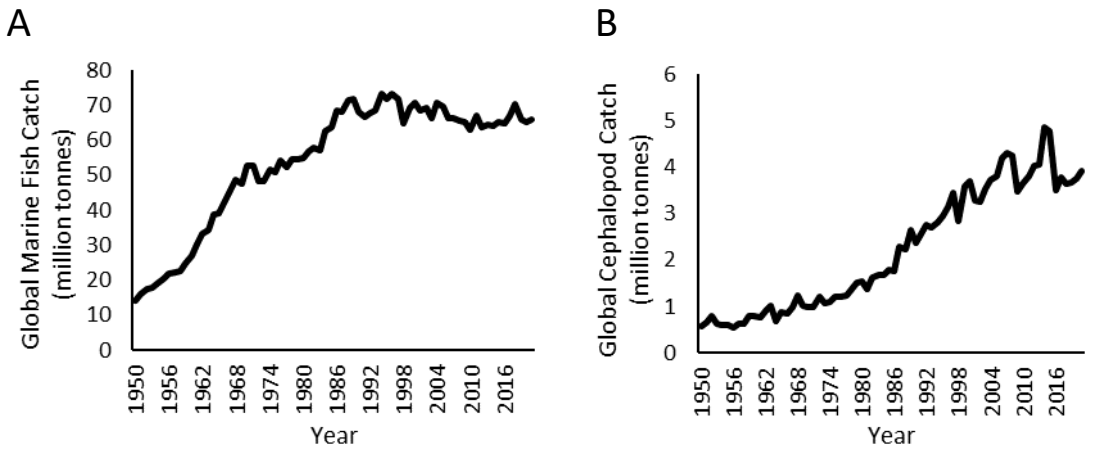


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Using hard parts to understand the biology and ecology of cephalopods

The hard parts or hard tissues of marine animals have been used to reconstruct the environmental and life histories of marine animals for decades. For example, fish otoliths have been used to reconstruct the age and movement histories of thousands of species; these data are crucial for fisheries and conservation management (Smith *et al.*, 1995; Campana, 2001; Jackson, 2007; Zoë A. Doubleday *et al.*, 2022). Although cephalopods are invertebrates, they possess various calcified or chitinous structures (i.e., hard parts) that can be used to understand their ecological and environmental histories (Table 1.1). Like other molluscs, some cephalopod such as nautilus have external shells whereas coleoid cephalopods have internalised this shell into a backbone like structure such as cuttlebones, squid gladii and octopus stylets (Kröger *et al.*, 2011). All cephalopods also have beaks (mouth part) and a pair of statoliths. Statoliths are calcium carbonate ($CaCO_3$) structures that aid in orientation and stability (Stephens and Young, 1978), and are analogous to fish otoliths. (Table 1.1). Many cephalopod hard parts grow incrementally, and thus contain temporally explicit information that represents different periods of an individual's life. Two primary approaches for utilising cephalopod hard parts include growth increment analysis, which provides data on age and somatic growth rates (Doubleday *et al.*, 2006; Donlon *et al.*, 2019), and chemical analyses (e.g., trace elements, stable isotopes, and hormones), which can provide data on an animal's environmental and biological histories (Jackson *et al.*, 2007; Fang *et al.*, 2019; Queirós *et al.*, 2020).

Using hard parts to understand the ecology and biology of cephalopods can be particularly cost effective, as alternative monitoring methods such as tagging, field observations, and lab-based

studies of live animals, can be expensive and time consuming (Semmens *et al.*, 2007). Furthermore, archived hard parts can be often sourced from museums and research agencies, allowing large datasets to be extracted from pre-existing samples at relatively little cost (Izzo *et al.*, 2016). In the following sections, I describe in more detail how the ecology of cephalopods can be interpreted from their various hard parts.

Table 0.1: Summary of the hard parts found in cephalopods and their current and potential applications for unravelling the ecological and environmental histories of cephalopods.

Hard part	**Taxa**	**Composition**	**Function**	**Applications**	**Example studies**
Beak	All cephalopods	Chitin-protein complex	Mandible for eating	Ageing, trophic ecology	Cherel and Hobson (2005) Perales-Raya *et al*. (2014)
Statolith	All cephalopods	Crystalized aragonite	Balance and orientation	Ageing, population structure, connectivity, provenance	Arkhipkin (2005) Chiang *et al.* (2020)
Stylet	Incirrate octopus	Chitin-protein complex	Vestigial	Ageing, population structure	Doubleday *et al*. (2006) Donlon *et al.* (2019)
Cuttlebone	Cuttlefish	Three layers of calcium carbonate (aragonite), chitin and proteins	Buoyancy	Growth, movement, metabolism	Bettencourt and Guerra (2001) Dance *et al*. (2014) Chung *et al*. (2020)
Gladius	Squid and cirrate octopus	Three layers of chitin and protein	Support for fins	Ageing, growth, movement, connectivity	Perez *et al*. (2006) Ruiz-Cooley *et al.* (2010)
External Shell	Nautilus	Calcium carbonate (aragonite)	Protection	Movement, trophic ecology	Oba *et al.* (1992) Auclair *et al.* (2004)

					Kashiyama *et al.* (2010)
Brooding shell	Argonaut	Calcium carbonate (calcite)	Brooding eggs	Movement	Stevens *et al.* (2015)
Spirula shell (ram's horn)	*Spirula spirula* squid	Calcium carbonate (aragonite)	Buoyancy	Movement, trophic ecology	Lukeneder *et al.*, (2008) Price *et al*. (2009) Ohkouchi *et al.* (2013)

Estimating age

One of the most important pieces of information for fisheries management is age data. Many hard parts have incremental growth "rings" called increments and by counting these increments with the knowledge of how often then are deposited or periodicity, individual age can be estimated. Ageing fish using increments in otoliths (fish equivalent of statoliths) first began in the late 1800s (Isely and Grabowski, 2007) and has since been applied to many species of fish across the world. This same idea of estimating age using increments in hard parts has since been applied to cephalopods since the 1970s (Lipinski, 1978; Spratt, 1979). Squid statoliths have clear daily growth increments and have been used widely to estimate age of squid (Arkhipkin, 2005). In contrast, octopus statoliths have no incremental growth bands (Clarke, 1978b). As such, increments on the beaks and stylets are used to estimate age in octopus (Doubleday *et al.*, 2006; Donlon *et al.*, 2019).

However, simply counting increments within these hard parts is not enough to estimate age. The periodicity in which these increments are formed must also be known. As cephalopods are short lived, increments are often deposited on a daily basis and have been validated in statoliths (Lipinski, 1986; Bettencourt and Guerra, 2001; Arkhipkin and Shcherbich, 2012), beaks (Hernández-López *et al.*, 2001; Perales-Raya, Almansa, *et al.*, 2014; Liu *et al.*, 2015), and octopus stylets (Doubleday *et al.*, 2006; Donlon *et al.*, 2019). Although many cephalopod species deposit increments daily, this cannot be assumed for all species or hard structures due to different life histories and ecologies (i.e., shallow water vs deep water species). Furthermore, for octopuses, daily increments have only been validated in a small number of species (Hermosilla *et al.*, 2010; Arkhipkin *et al.*, 2018; Donlon *et al.*, 2019). If periodicity is assumed without validation, inaccurate age estimates can be made (Doubleday *et al.*, 2006). Therefore, proper age validation is an important step for estimating ages of commercially important species and aid in understanding the role of cephalopods within marine food web systems (i.e., species and age of individuals being consumed).

Reconstructing histories

Chemical elements and isotopic ratios within the hard parts can reflect an animal's changing environment and biological processes (Campana and Neilson, 1985; Grossman and Ku, 1986;

Campana, 1999). A range of trace elements such as strontium and barium and isotopic makers such as $\delta^{13}C$, $\delta^{18}O$, $\delta^{15}N$ have been used in various marine animals including but not limited to fish (Thorrold *et al.*, 1997; Bath *et al.*, 2000), marine mammals (Newsome *et al.*, 2007; Borrell *et al.*, 2018; Vighi *et al.*, 2019), and bivalves (Pfister *et al.*, 2011; Schöne *et al.*, 2011; Wanamaker and Gillikin, 2019). For cephalopods, there has been a particular focus on $\delta^{13}C$ and $\delta^{15}N$ in beaks (Cherel *et al.*, 2009; Xavier *et al.*, 2022) as well as trace elements in statoliths (Zumholz *et al.*, 2007; Gillanders *et al.*, 2013; Daryanani *et al.*, 2021). $\delta^{13}C$ and $\delta^{18}O$ in cephalopod carbonate structure have been used to a much lesser extent, especially in octopus, and are the focus on my PhD research. $\delta^{13}C$ and $\delta^{18}O$ in carbonates are useful for tracking environmental changes such as temperature and salinity as well as biological changes such as metabolic rate and are valuable tools for marine ecology.

Environmental histories

$\delta^{18}O$ values in marine carbonates are in close equilibrium with $\delta^{18}O$ values in seawater making carbonates excellent $\delta^{18}O$ recorders (Grossman and Ku, 1986; McConnaughey, 1989a, 1989b; Kalish, 1991b). $\delta^{18}O$ values in the ocean differ with different oceanographic processes such as evaporation, freshwater runoff (Fry, 2006), and the movement of ocean currents (Trueman and St John Glew, 2019), but are generally recorded as having an inverse relationship with temperature (Grossman and Ku, 1986; Kalish, 1991b). This is why $\delta^{18}O$ values in carbonates have been used to geolocate individuals back to their geographic origin (Daryanani *et al.*, 2021; Martino, Trueman, *et al.*, 2022; Duarte *et al.*, 2023) as well as reconstruct experienced temperatures (Martino, Chung, *et al.*, 2022).

If historical samples are available, changes over a long temporal scale can also be observed and can reveal information about habitat preference and climate change. For example, previous studies have observed an increase in atmospheric CO_2 reflected in the carbon isotopic values in hard parts such as fish otoliths (Schloesser *et al.*, 2009; Fraile *et al.*, 2016), fur seal teeth (Newsome *et al.*, 2007), and mussel shells (Pfister *et al.*, 2011) over time.

Biological histories

In addition to observing environmental changes, hard parts can help reconstruct the biological histories of organisms such as metabolic rate. Metabolic rate is known to change with body size and temperature (Brown *et al.*, 2004) and recent studies have shown the use of carbon isotopic values ($\delta^{13}C$) in hard parts to be an effective proxy of metabolic rate. Carbon in carbonates originates from two sources; the environment (dissolved inorganic carbon; DIC) and diet (Solomon *et al.*, 2006; McConnaughey and Gillikin, 2008). The proportional contribution from these two sources can then be used as a proxy of metabolism (C_{resp}) with the idea that higher contribution from respiratory sources (environment) equals a higher metabolic rate (Solomon *et al.*, 2006; Chung, Trueman, Godiksen and Grønkjær, 2019; Martino *et al.*, 2020; Chung *et al.*, 2021). The study of metabolism is another important aspect of ecological studies, as the rate an animal uses energy in response to various conditions (i.e., rising temperatures) is important for understanding how that animal will respond and survive in the future. Metabolic rate is difficult to measure in the field and laboratory measurements are often unnatural, which is why the use of C_{resp} is useful (Treberg *et al.*, 2016; Chung, Trueman, Godiksen and Grønkjær, 2019). Currently, C_{resp} has only been calculated for a few species of fish (Solomon *et al.*, 2006; Chung, Trueman, Godiksen, Holmstrup, *et al.*, 2019) and one species of cuttlefish (Chung *et al.*, 2021), with potential to reveal unique insights into cephalopod biology.

Reproductive histories can also be investigated using hard parts. Traditionally, reproductive stages in cephalopods are determined by observing gonads, which requires the dissection of deceased animals (Jackson, 1990; Jackson and Forsythe, 2002), but this method allows for only a snapshot of that individual's maturity stage at that time. Recently, a novel method for extracting hormones from hard parts, such as pinniped whiskers and fish opercula, was developed (Trumble *et al.*, 2013, 2018; Kathleen E Hunt *et al.*, 2014; Keogh *et al.*, 2020, 2021; Charapata *et al.*, 2022). This method suggests that a sharp increase in steroid reproductive hormones such as cortisol, oestradiol, progesterone, and testosterone can indicate physiological age of maturity as well as spawning events (Charapata *et al.*, 2018, 2022). The peak can then be matched to the growth increments or age of the hard part. Hormone analysis in cephalopod hard parts, like beaks, has the potential to offer powerful insights into the reproductive biology of many species, and could be particularly valuable for species living in inaccessible environments like the deep-sea, where the knowledge of their life histories remains limited, and whole specimens are rare. Moreover, hormone analysis within hard parts

offers more detailed insight into an individual's entire life history, with a fine temporal resolution down to the scale of days, as opposed to the limited view provided by gonad analysis, which only reveals the current reproductive status.

Study species

My thesis focusses on a several southern Australian species including the southern keeled octopus (*Octopus berrima*), the giant Australian cuttlefish (*Sepia apama*), the pale octopus (*Octopus pallidus*), and the Māori octopus (*Macroctopus maorum*). All are found in temperate, coastal waters of Australia in a range of habitats (Fig. 1.2). *O. berrima* forms an important developing fishery in in South Australia in which it comprises 94% of the catch (Martino *et al.*, 2021). *O. pallidus* and *M. maorum* constitute bycatch and target fisheries in Tasmania, Victoria, South Australia, and New South Wales (Moore *et al.*, 2018; Fraser *et al.*, 2022). *S. apama* inhabits the entire southern Australian coast but is renowned for its breeding aggregation in Spencer Gulf, South Australia, whereby thousands of cuttlefish come inshore to reproduce during the winter months (Hall and Hanlon, 2002). All four of these cephalopod species are important parts of the ecosystem of the southern Australian coast. The information resulting from this thesis will advance cephalopod research, as well as aid in the management of these local South Australian species.

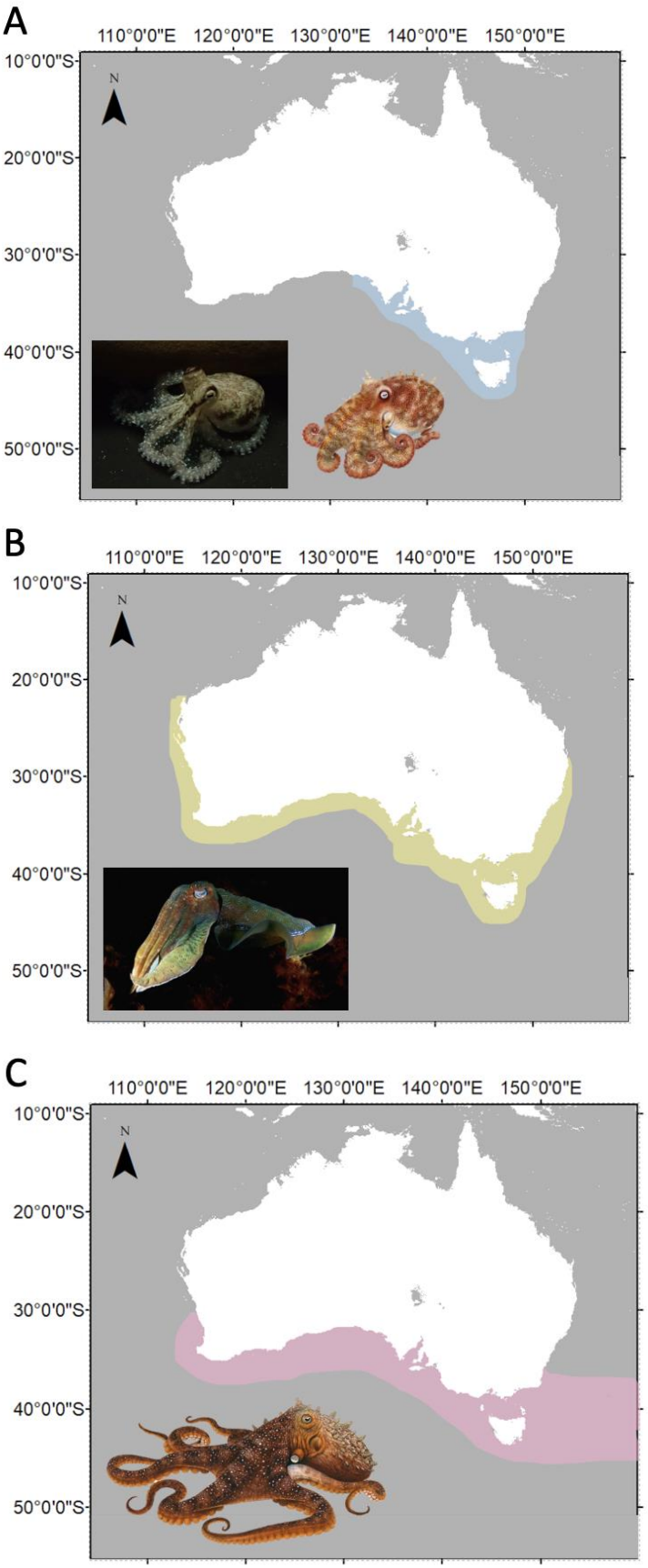


Figure 0.2: Distributions of study species (A) *Octopus berrima*, *Octopus pallidus*, (B) *Sepia apama*, (C) and *Macroctopus maorum* across southern Australia.

Thesis aims and structure

The overarching aim of this thesis is to develop and carry out innovative techniques using increment and chemical analysis of cephalopod hard parts to study their biology and ecology and inform their sustainable management. The thesis is organised into five data chapters (Chapters 2 to 6) that are written as stand-alone papers accepted or prepared for publication. I provide a brief overview of the chapters below.

Chapter 1: Introduction

This chapter provides a brief background and review of relevant previous studies.

Chapter 2: Non-daily growth increments in the commercial species, *Octopus berrima,* and the importance of age validation

Aim: To develop the first age data for the commercially harvested octopus species.

Age data is crucial for the proper management of commercially harvested species, and until now, the commercial species of octopus, *Octopus berrima,* has never been aged. In this chapter, I have validated increment periodicity in both the stylet and beak using a chemical staining method with adults and a known-age method with juveniles. With the resulting periodicities, I estimated age for wild-caught octopus and from the maximum age caught from the samples, estimated a life span of approximately 11.6 months for *O. berrima*. For the first time, non-daily periodicity have been validated in an octopus species with differing periodicities between the stylet and beak, strengthening the need for proper validation not only for each species, but for each structure as well.

This chapter has been published in ICES Journal of Marine Science as a Quo Vadimus article.

Chapter 3: A definitive guide to ageing octopus

Aim: To create an ageing guide for researchers to refer to when ageing octopus.

This chapter represents a practical, step-by-step guide on ageing octopus, with a review of the relevant literature. This was created due to a lack of such information in the literature and accompanied a collaborative project with the New South Wales, Department of Primary Industries, whereby I trained their researchers to age octopus.

This chapter has been published in Marine and Freshwater Research.

Chapter 4: Developing isotopic proxies to reconstruct the metabolic rates and thermal histories of octopus

Aim: To determine if carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotopic values in octopus statoliths can be used as proxies of metabolic rate and experienced temperature.

Oxygen isotopic values in biogenic carbonates are a known proxy of water temperature, and carbon isotopic values have been linked to metabolic rates in fish and cuttlefish. Before $\delta^{18}O$ and $\delta^{13}C$ values can be used for reconstructing temperature and calculating metabolic rate of wild animals, the relationship between these variables much first be determined with controlled laboratory rearing. In this chapter, I raised *O. berrima* hatchlings from hatching to 110 days old at three different temperatures to determine if $\delta^{13}C$ and $\delta^{18}O$ values in octopus statoliths can be used as metabolic and temperature proxies, respectively. An increase in metabolic rate with body mass was observed, supporting the hypothesis that octopus juveniles have an initial, fast-growing stage. In addition, $\delta^{18}O$ values had a significant inverse relationship with temperature, supporting the use as proxy of temperature. With these data, I created an oxygen thermometer equation that can be used to estimate experienced temperatures of wild octopus.

This chapter has been published in Marine Environmental Research.

Chapter 5: Isotopic trends in an historic cuttlebone archive spanning six decades

Aim: To determine temporal and spatial trends in $\delta^{13}C$ and $\delta^{18}O$ values in historic cuttlebones.

I collected historic *S. apama* cuttlebones spanning a 60-year time period from multiple museums in Australia. $\delta^{13}C$ and $\delta^{18}O$ values were then analysed in the cuttlebones and a series of generalised additive models and generalised linear models were used. As a result, I demonstrated how historic archives can be utilised to investigate $\delta^{13}C$ and $\delta^{18}O$ values across spatial and temporal scales. I also discuss the implications of using opportunistically collected samples.

This chapter is being prepared for publication in Estuarine, Coastal and Shelf Science.

Chapter 6: Reproductive histories revealed with steroid hormones in octopus beaks

Aim: To develop a method to extract and analyse steroid hormones from growth increments in octopus beaks.

The common way of assessing maturity in cephalopods is by observing the gonads post-dissection, which only determines the maturity stage of that individual at that time and also requires the use of whole specimens. I have developed a new method to extract and analyse hormones along the growth axis of cephalopod beaks. This novel method can then be applied to other species of cephalopods, especially rare species for which gonad samples are difficult to come by, including deep sea and Antarctic species.

This chapter has been submitted for publication for a special issue in Limnology and Oceanography Letters and is under review.

Chapter 7: Discussion

This chapter discusses key findings from this thesis and future directions and suggestions for future studies.

Non-daily growth increments in the commercial species, *Octopus berrima*, and the importance of age validation

This research is published as:

Durante, E.D., Grammer, G.L., Martino, J.C., Payne, J.L. and Doubleday, Z.A. (2023) 'Nondaily growth increments in the commercial species, Octopus berrima, and the importance of age validation', ICES Journal of Marine Science, 0(0), pp. 1–14.

Abstract

Age data are crucial for understanding and managing target species in commercial and recreational fisheries. Octopus catches are increasing worldwide, yet ageing methods are still in their infancy. Octopus age is estimated by counting growth increments in stylets and beaks. These increments have been validated as daily in some species or assumed to be daily in other species. Here I conducted an age validation study on both the stylets and beaks of a commercial octopus species (*Octopus berrima*). Subsequently, I compared and assessed the readability of growth increments in beaks and stylets of wild-caught *O. berrima*. I found that increment periodicity was non-daily for both structures, with increment periodicity also varying between structures (0.75 increments per day for stylets and 0.53 increments per day for beak). This study highlights the importance of validating increment periodicity for different octopus species and for both stylets and beaks to obtain accurate age and growth data. Without accurate age data, a species may be incorrectly managed with the potential for negative effects on the sustainability of wild populations.

Introduction

Cephalopods, such as squid and octopus, are a staple food source in many cultures around the world. However, as the global demand for sustainable seafood increases and some traditional finfish stocks decline, cephalopod fisheries are expanding (Jereb *et al.*, 2014; Rodhouse *et al.*, 2014; Arkhipkin *et al.*, 2015; Sauer *et al.*, 2021). Cephalopods are an important link in marine ecosystems being both predators and prey for many animals, yet the understanding of basic biology in many cephalopod species is limited.

Determining the sustainable harvest of a fishery requires data on growth, maturation, mortality, and recruitment, all of which are underpinned by age. For example, in Australia, ageing methods and age data (Doubleday *et al.*, 2006; Leporati *et al.*, 2009; Semmens *et al.*, 2011; Leporati and Hart, 2015; Hart *et al.*, 2016, 2019) have helped develop stock assessments for *Octopus pallidus* Hoyle, 1885 (Fraser *et al.*, 2022) and *Octopus* cf*. tetricus*, now known as *Octopus djinda* Amor, 2021 (Hart *et al.*, 2018), in the Tasmanian and Western Australian octopus fisheries respectively. In the South Australian fishery, the majority of catch is composed of the southern keeled octopus, *Octopus berrima* Stranks & Norman, 1992 (94% of catch), but age or growth data have

not yet been recorded for the species (Martino *et al.*, 2021). A lack of age data is one of the most critical limitations in assessing the resource status of octopus (Sauer *et al.*, 2021). Without age data, it is difficult to assess the sustainability of a given stock.

Despite techniques being well-developed for many other marine animals, such as fish, methods to age octopus are still in their infancy. The discovery of concentric rings in cephalopod tissues dates back to 1893 by Appellöf, followed by Naef (1928) in which they described increments in cuttlebones (in German). Yasuda (1951) and Yagi (1960) then followed suit in Japanese, leading to the first account of cephalopod increments in English by Choe (1963) and then Clarke (1965) who first discovered 'microrings' within squid beaks. Since then, numeration of growth increments in various hard parts have been used to age cephalopods (Arkhipkin *et al.*, 2018). Statoliths are the most widely used structures for ageing squid as they are structurally similar to calcium carbonate otoliths of fish and have been found to have daily growth increments (Lipinski, 1978; Spratt, 1979; Rodhouse and Hatfield, 1990; Jackson and Forsythe, 2002; Hunt *et al.*, 2011). In comparison, octopus statoliths form irregularly and lack the concentric growth increments seen in squid (Clarke, 1978a). This prompted Raya and Hernández-González (1998) to estimate age from *Octopus vulgaris* Cuvier, 1797 beaks due to the discovery of growth increments within the Rostral Sagittal Section (RSS), followed by Hernández-López *et al.* (2001) who used the Lateral Wall Surface (LWS). Sousa Reis and Fernandes (2002) then observed growth increments in stylets, a pair of cartilaginous-like vestigial shell embedded in octopus mantle tissue, which were then validated as daily by Doubleday *et al.* (2006). From then on, there have been several studies estimating age from both octopus stylets and beaks (Table 1).

Before increments can be used for ageing, the rate, or periodicity, at which they are deposited must first be validated. This validation can be conducted through 1) rearing known-aged animals in captivity (Doubleday *et al.*, 2006), 2) chemically staining the hard part either by immersion or injection (Hermosilla *et al.*, 2010; Perales-Raya, Almansa, *et al.*, 2014) or 3) evaluating stress mark through stressful events such as exposing the animal to a temperature at their thermal limit or from handling (Perales-Raya, Almansa, *et al.*, 2014; Perales-Raya, Jurado-Ruzafa, *et al.*, 2014) (Table 2.1). In fish, fluorescent and calcium-binding chemicals such as calcein and tetracycline are often used to mark otoliths. The fish are then either released and recaptured or kept in captivity for a known amount of time and their otoliths examined under a microscope with UV light

(Campana, 1992). Many fish species are longer lived than cephalopods and generally also have annual or seasonal growth increments visible in otoliths, on top of daily increments (Campana, 1992). Using the above validation methods, research to date has found that the deposition of increments in cephalopod beaks and stylets are typically daily and because this is common, studies on different species are known to assume daily periodicity without validation. Although, validation is a complex process and can rarely provide absolute assurance of increment periodicity throughout the life cycle of an octopus, it is a critical step to improve the accuracy of age estimates.

Here I conducted an age validation study on an important commercial octopus species, the southern keeled octopus, *O. berrima. O. berrima* is a small bodied octopus that can weigh up to approximately 380 g (Martino *et al.*, 2021) and is holobenthic, which means it produces crawl-away demersal juveniles instead of planktonic paralarvae (merobenthic species) (Stranks and Norman, 1992; Sweeney *et al.*, 1992). *O. berrima* are targeted by Marine Scalefish Fishers in South Australia, and approximately 10 tonnes are caught per annum, with interest to expand the fishery (Martino *et al.*, 2021). To collect the first age data on this species, age validation was performed on both beak and stylets of *O. berrima* using chemical staining and known-age methods. Both beaks and stylets were used as often one structure is optimal over the other, depending on the species. I also examined increment patterns of wild-caught octopus to investigate increment readability.

Table 0.1: Octopus species and populations that have been aged using stylets and beaks and the extent of age validation. LWS indicated the lateral wall surface of the beak, RSS indicates the rostrum sagittal section of the beak, and RS indicates the rostrum surface.

* Indicates a commercial harvested species, † indicates a recreational harvested species, and § indicates a species often caught as bycatch. Although *O. vulgaris* is a complex and has a number of species, it is believed that all of the studies included in this table are of the same species/ type as they all originate from the north-east Atlantic (Amor *et al.*, 2019; Avendaño *et al.*, 2020).

Species	**Structure**	**Periodicity validated for species/ structure**	**Validation method**	**References**
MEROBENTHIC				
*Octopus vulgaris**† (north-east Atlantic)	Stylet	No	None	Sousa Reis and Fernandes (2002)
		Yes, daily in adults	Tetracycline injection	Hermosilla *et al.* (2010)
		Assumed daily	None	Barratt and Allcock (2010) Nafkha *et al.* (2019)
	Beak	No, proposed daily (RSS)	None	Raya and Hernández-González (1998)
		Yes, daily up to 26 days old (LWS)	Known-aged	Hernández-López *et al.* (2001)
		Assumed daily (LWS, RSS)	None	Perales-Raya *et al.* (2010)
		Yes, daily in adults, 70-370 days old (LWS)	Thermal marking	Canali *et al.* (2011)
		Assumed daily (LWS)	None	Cuccu *et al.* (2013)

		Assumed daily (LWS, RSS)	Stress marking and wild senescent individuals	Perales-Raya, Jurado-Ruzafa, *et al.* (2014)
		Yes, daily in adults, 200-734 days old (LWS, RSS) and early stages up to 98 days old (RS)	Calcofluor injection, stress marking, and known-aged	Perales-Raya, Almansa, *et al.* (2014)
		Yes, daily up to 15 days old (RS)	Known-aged	Franco-Santos *et al.* (2016)
		Yes, daily up to 30 days, but <1 increment day^{-1} at cold temperatures (~14°C) (RS)	Known aged	Perales-Raya *et al.* (2018)
		No, proposed daily (RS)	Observation of embryo and paralarvae beak formation	Armelloni *et al.* (2020)
*Eledone cirrhosa**	Stylet	Assumed daily	None	Barratt and Allcock (2010) Regueira *et al.* (2015)
Macroctopus maorum§†	Stylet	No	None	Doubleday *et al.* (2011)
Octopus cyanea†*	Stylet	Assumed daily	None	Herwig *et al.* (2012)
*Octopus tetricus**†§	Stylet	Assumed daily	None	Ramos *et al.* (2014)
*Octopus djinda**† *(Octopus* cf. *tetricus)*	Stylet and stylet weight	Yes, daily in adults	Calcine injection	Leporati and Hart (2015)
Robsonella huttoni § *(Octopus huttoni)*	Stylet	Yes, daily in adults	Tetracycline injection	Donlon *et al.* (2019)
	Beak (LWS)	No	None	

Octopus insularis	Beak (LWS)	Yes, daily in adults	Tetracycline injection	Batista *et al.* (2021)
HOLOBENTHIC				
*Octopus pallidus**†	Stylet	Yes, daily up to 8 months old	Known-aged	Doubleday *et al.* (2006)
		Assumed daily	None	Leporati *et al.* (2008)
Octopus australis§	Stylet	Yes, daily in adults	Immersion and injection of chemical stains (oxytetracycline, Alizarian Complexone, Alizarian Red S, and Methylene Blue)	Nuttall (2009)
Bathypolypus sponsalis	Stylet	No	None	Barratt and Allcock (2010)
Megaleledone setebos	Stylet	No	None	Barratt and Allcock (2010)
	Beak (LWS)	Assumed daily	None	Schwarz *et al.* (2019)
*Octopus maya**†	Stylet	Yes, daily 124-233 days old octopus	Known-aged	Rodríguez-Domínguez *et al.* (2013)
	Beak (LWS)	Yes, daily, but not properly validated in all age groups. 120-160 days old octopus	Known-aged	Rodríguez-Domínguez *et al.* (2013)

		Periodicity=0.75, low precision due to poor visibility in older age groups		
	Beak (RSS)	Yes, daily up to 122 days old	Known-aged	Villegas-Bárcenas *et al.* (2014)
Pareledone aequipapillae, Pareledone charcoti, Muusoctopus rigbyae, Adelieledone polymorpha, Pareledone aurata, Pareledone felix, Pareledone turqueti,	Beak (LWS)	Assumed daily, but proposed non-daily	Maintained *P. charcoti* in captivity for >12 months and increments were <365. Suggesting a periodicity less than daily.	Schwarz *et al.* (2019)
Japetella diaphna, Vampyroteuthis infernalis	Beak (LWS)	No	None	Schwarz *et al.* (2020)
*Octopus berrima**	Beak (LWS)	Yes, **not daily** **Periodicity=0.46** up to 110 days old	Known-aged	Durante *et al.* (this study)
	Stylet	Yes, **not daily** **Periodicity=0.75** Adults 84-348 days old	Calcofluor white injection	

Methods

Collection

To first validate the periodicity of growth increments in stylets and beaks of *O. berrima,* 24 live octopus were collected from a commercial octopus fisher in Venus Bay, South Australia (~2 m depth) (Fig. 2.1) in 2021 using 45- and 50-mm diameter pots attached to lines and a buoy. Octopus were then transported in 10 L buckets by car to the South Australian Research and Development Institute (SARDI) Aquatic Sciences facilities at West Beach, South Australia. Buckets were fitted with battery operated aerators and PVC "dens" fitted to the size of the bucket to offer refuge and minimise stress. Upon arrival at SARDI, octopus were acclimated to tank conditions overnight by running a small tube into their transportation bucket with local seawater. The next morning, octopus were transferred to their own individual "octo-pod" which floated in a 5,000 L tank with their PVC den and fed *ad libitum* with live shore crab. These octopus ranged from 20.5 to 190 grams in weight in which 54% were male and 46% were female.

In addition, stylets and beaks from another 445 octopus, that were collected from five sites around the Eyre Peninsula, South Australia, in 2020 as part of a previous study (Martino *et al.*, 2021) (Fig. 2.1), were used to investigate increment readability and age of wild *O. berrima.* These octopus were collected using the same method mentioned above and ranged from 18 to 363 grams in weight in which 27% were male and 73% were female.

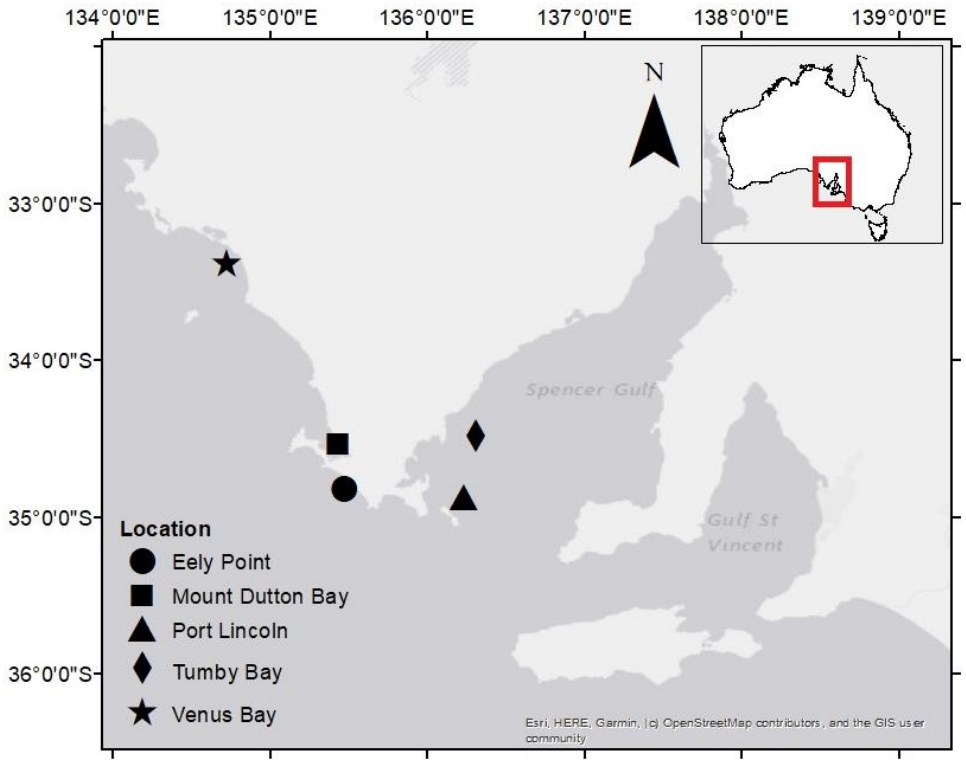


Figure 0.1: Map of sample sites for *Octopus berrima*. Live octopus were collected from Venus Bay which is indicated by the star (this study). Wild-caught octopus for age estimation were collected from all five sites from a previous project and information on sampling sites such as depth can be found in (Martino *et al.*, 2021).

Validating growth increment periodicity

To validate periodicity, two methods were used: chemical staining and known-age animals.

The chemical staining method used here, followed the methods developed by Hermosilla et al. (2010) and Perales-Raya et al. (2014) and involved staining stylets and beaks with tetracycline hydrochloride and Calcofluor White (Sigma Aldrich). To summarise, 22 live octopus were anesthetised in cold water (2 ±1°C) and injected with one of the two solutions (tetracycline (n=11): 0.12 mg per gram of body weight, Calcofluor (n=11): 0.10 mg per gram of body weight) at the base of one of the ventral arms, maintained in captivity for a known number of days, then euthanised in a magnesium chloride bath (6%) (Fig. 2.2). Octopus who were stained with tetracycline appeared distressed, with some individuals removing their injected arm via autophagy. These individuals were euthanised promptly in line with animal ethics code of best

practice. This adverse reaction suggests that the results from tetracycline-stained individuals may not be viable. On the other hand, Calcofluor did not cause any adverse reaction or cause any apparent stress and individuals were euthanised 5 (n=3), 15 (n=3), 30 (n=3), and 40 (n=1) days post-staining, with one octopus laying eggs post stain and was therefore left to care for her eggs until natural death.

Neither the tetracycline nor the Calcofluor left a visible fluorescent mark on the beaks, therefore only the stylets could be validated with this staining method.

For known-age validation, hatchlings were sourced from one of the females that laid eggs during the acclimation period and one female that laid post Calcofluor staining (Fig. 2.2). Hatchlings hatched after an average incubation period of 80 days of incubation and were then raised up to 110 days old in flow-through tanks. One clutch was reared under three controlled temperature treatments (20, 22, 24°C) and one clutch under ambient temperature conditions (ambient; ~22°C). All hatchlings were fed wild-caught shrimp daily (*Palaemon serenus*). The temperature-controlled treatments had 10 octopus per 30 L tank (1 octopus/3 L or 100 octopus/m^2) with either 4 or 6 replicate tanks depending on the number of octopus available. The ambient temperature treatment had ~50 octopus per 55 L tank (~1 octopus/L or 250 octopus/m^2) with 3 replicate tanks. The temperature in each tank was recorded every 10 minutes with an Aquatronics system. Octopus (n= 2-3 per tank) were sampled at various ages throughout the 110 days by euthanising in a 6% magnesium chloride bath. All octopus were frozen prior to dissection.

All procedures followed the rules and regulations for animal ethics and care of the Department of Primary Industries and Regions South Australia Animal Ethics Committee (PIRSA AEC: 08/21).

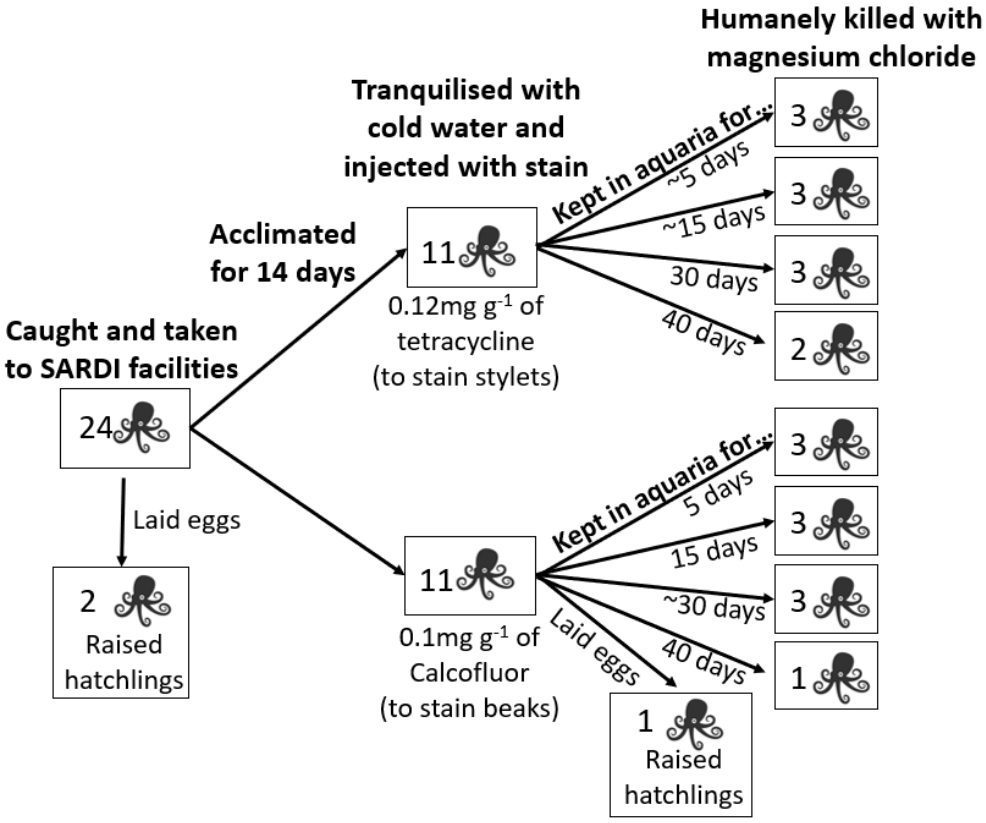


Figure 0.2: Flow chart of experimental design for age validation experiment.

Preparing structures

Stylets and beaks from each octopus used in age validation were dissected from frozen octopus and stored in Eppendorf tubes with 70% ethanol. Stylets and beaks collected from Martino et al. 2021 were already dissected and stored in in 70% ethanol. While preservation in distilled water at 4°C can be preferred over ethanol, which can sometimes dehydrate the beaks, ethanol is more suitable for longer-term storage (Arkhipkin *et al.*, 2018). Furthermore, to determine potential effects of ethanol storage, a pilot study was conducted whereby one half of a beak was preserved in water and another half in 70% ethanol for 3 months. After which, no visible differences in beak microstructure were seen for this species across the entire beak. To prevent oxidation of the stain, structures from stained individuals were wrapped with tin foil and processing occurred in a darkened room.

Increments in stylets were revealed by thinly sectioning at the post-rostral zone following Sousa Reis and Fernandes (2002) (Fig. 2.3) and embedding following the method described by Barratt and Allcock (2010) which uses a low viscosity resin (LR White medium grade, Agar Scientific Ltd.,

UK). Sections were then photographed under a phase contrast microscope (Olympus BX53) and stained stylets were photographed under a blue filter (peak at 470nm) as well. I were unable to visualise increments for the known-age juvenile stylets, because growth increments were too small and compact to visualise (see table 2.2 for a summary of validation methods used on different structures).

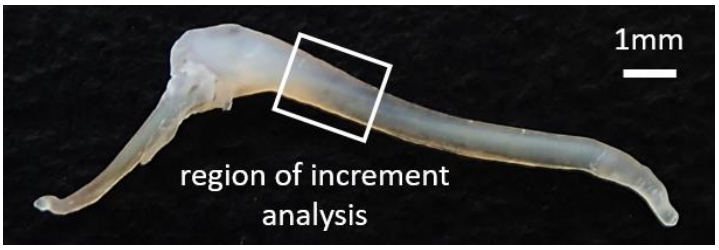


Figure 0.3: Photo of an *Octopus berrima* stylet showing the region of increment analysis indicated by the white rectangle.

Table 0.2: Increment periodicity validation methods that either worked (✓) or did not work (×) for *Octopus berrima* beaks and stylets.

Structure	**Chemical staining**		**Known-age octopus**
	Tetracycline	Calcofluor	
Beak	×	×	✓
Stylet	✓ (stained but results were inconclusive and had adverse impacts on octopus health)	✓	×

To observe increments in beaks, I analysed the internal LWS on the upper beak following the advice of Hernández-López *et al.* (2001). Beaks were cleaned gently with water and a plastic pipette tip. If tissue proved difficult to remove, beaks were placed in a sonic cleaner with 5% hydrogen peroxide for 5 minutes before scrubbing again. Adult beaks were cut longitudinally with scissors and mounted to a slide with the inside facing up using the thermoplastic cement, Crystalbond™ 509 (Fig. 2.4). Juvenile beaks, if the beak was large (>5mm) the same longitudinal cut was done on the upper beak and one half was mounted on a side with glycerol gelatine and a

coverslip (Fig. 2.4). If the beak was too small to cut with scissors (<5mm), the whole upper beak was butterflied with the inside facing up and mounted the same way with glycerol gelatine.

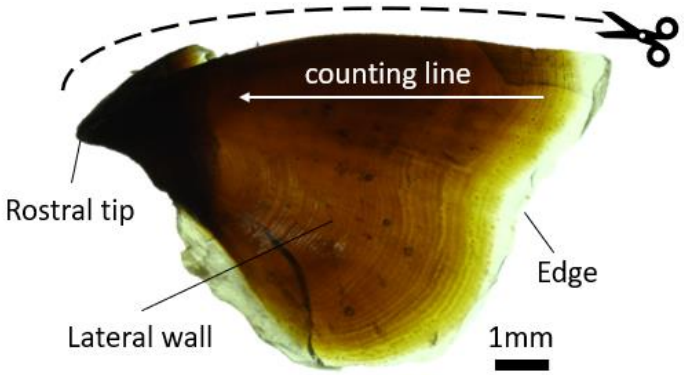


Figure 0.4: Diagram of an *Octopus berrima* upper beak with cutting line indicated by the dotted line and visualization of the growth bands on the lateral wall.

Counting increments

Increments in stylets were counted from microphotographs using the image analysis program FIJI (ImageJ) from the first visible increment in the core to the last visible increment on the edge. To validate the number of days post staining in stained stylets, the number of increments from the edge of the stain to the edge of the stylet (Fig. 2.5) was counted.

Increments in beaks were observed under a phase contrast microscope from the first visible increment on the edge of the lateral wall surface to the last visible increment near the rostral tip (Fig. 2.6).

For beaks, two non-consecutive increment counts were taken by a single, experienced reader. If the counts differed by more than 10%, that sample was discarded. For stylets, increments were fainter and more difficult to read, therefore, three non-consecutive increment counts were taken by a single, experienced reader. If a count differed by more than 10%, that sample was discarded.

The average of the counts was then compared to the expected number of days (age of the known-age octopus or number of days post stain) using a Pearson's correlation.

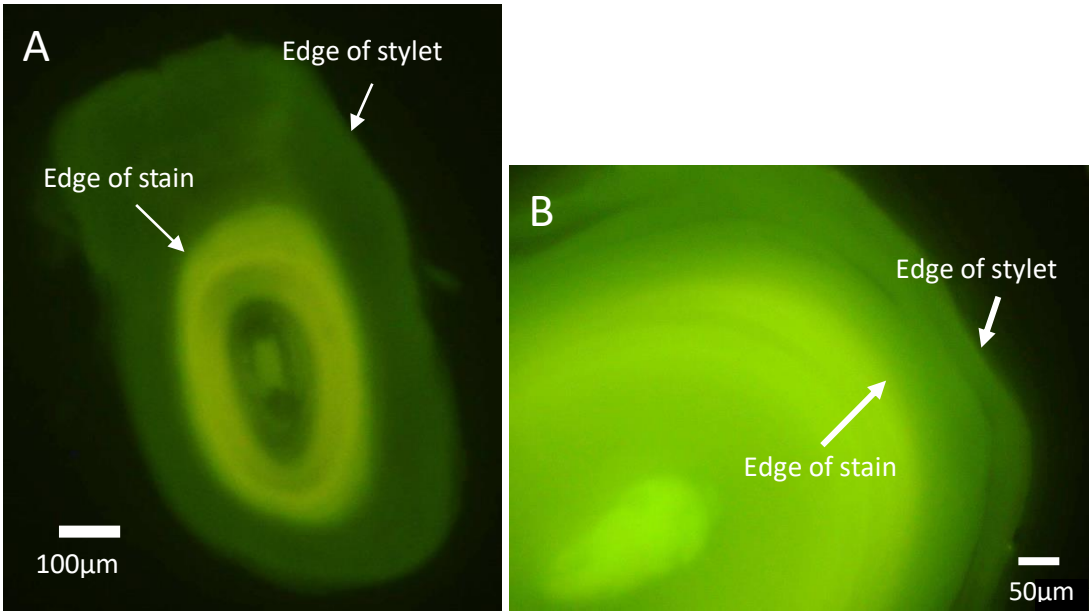


Figure 0.5: Microphotograph of a (A) tetracycline-stained and (B) Calcofluor stylet section indicating the edge of the stain marks and the edge of the stylets with white arrows.

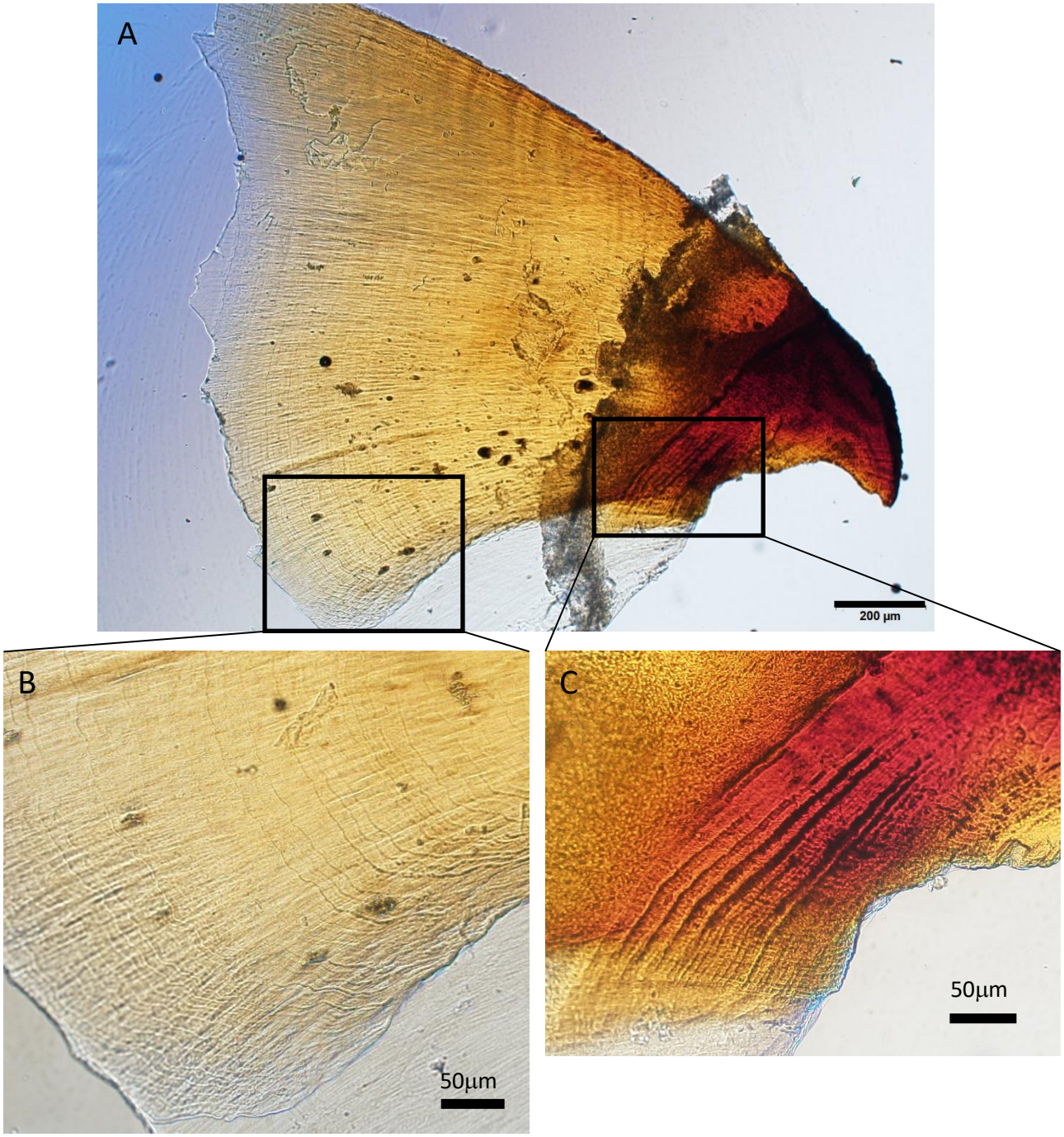


Figure 0.6: Microphotograph of the upper beak of a 91-day old juvenile *Octopus berrima* mounted on a microscope slide with glycerol gelatine showing a close up of the increments on the lateral wall (B) and the bottom edge of the rostrum (C).

First-hatch increment

Finally, to determine when the first-hatch increment was deposited, the beaks and stylets of young hatchlings (1-10 days old) were observed. Beaks were present in hatchlings from day 0 and were prepared using the above method of butterflying and mounting with glycerol gelatine.

Because stylets are so small and difficult to dissect out at this young age, whole known-age hatchlings were prepared following modified methods of Lourenço *et al.* (2015) which involved embedding in paraffin wax, cutting 5µm sections and staining with methyl blue (0.27%). These sections were then photographed, and the size of the stylet core measured.

Investigating growth increment patterns in wild-caught octopus

Growth increments can visibly differ between lab-raised and wild-caught animals (Doubleday *et al.*, 2006). Therefore, increments were compared between these two types of octopus. Each sample was given a readability score from 1 to 5 (Fig. 2.7 & 2.8), with 3 considered the minimum needed to be counted with confidence. Increments were counted as described above with 3 non-consecuative counts by the same experienced reader. Age could then be estimated by using the periodicity validated by the above methods.

All statistical analyses were carried out in the R Statistical Environment (R Core Team, 2022).

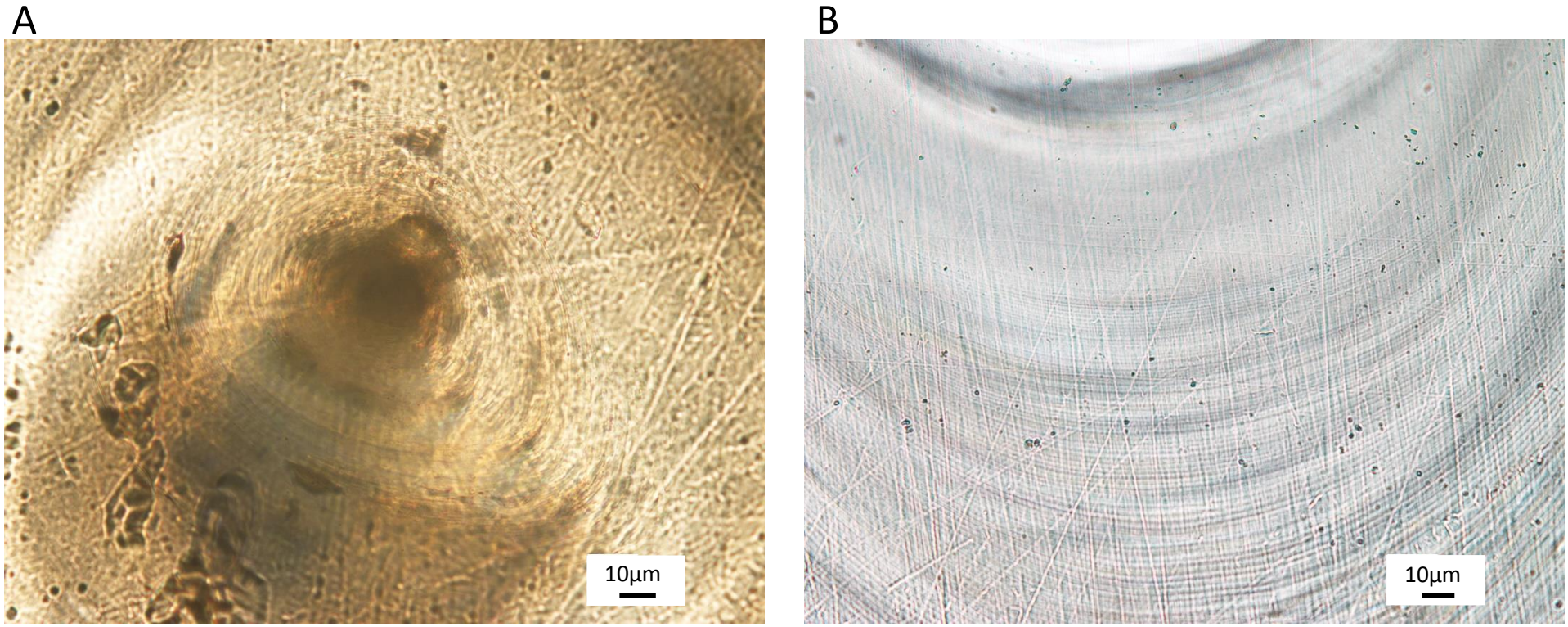


Figure 0.7: Examples of stylets sections with readability scores of (A) 1 (worst) and (B) 4 (best)

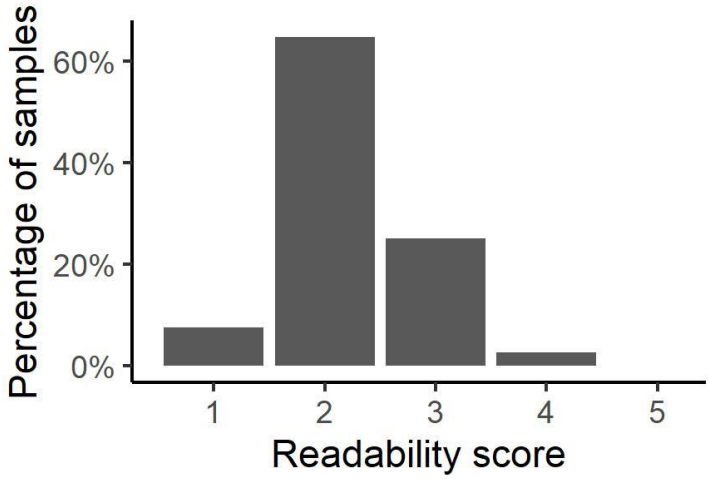


Figure 0.8: Readability scores given to wild-caught *Octopus berrima* stylets. Score of 1=most difficult to read, score of 5=perfect section.

Results

Validating growth increment periodicity

Calcofluor clearly stained the stylets and indicated an average increment periodicity of 0.75 increments per day in adult octopus (Fig. 2.9a) (Linear regression, df=6, F=8.023, R^2=0.539, *p-value*=0.037). However, periodicity was more variable as time passed (i.e., number of days post stain) (Fig. 2.9a). Tetracycline also stained the stylets, but there was no clear pattern in increment periodicity and had an adverse impact on octopus health (Fig. 2.9b) (Linear regression, df=9, F=1.46, R^2=0.44, *p-value*=0.258).

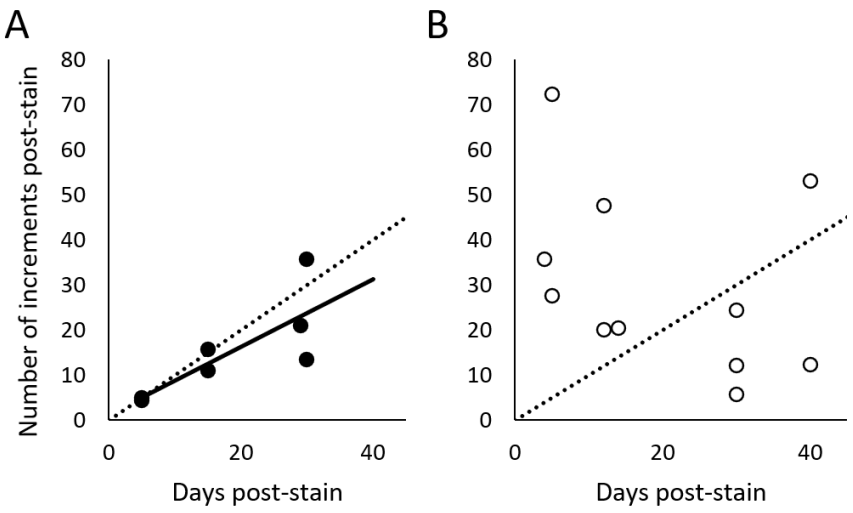


Figure 0.9: Comparison between the number of counted increments post-stain and number of days passed for (A) Calcofluor (n=7)- and (B) tetracycline (n=11)-stained stylets of wild-caught octopus. The dotted line displays a 1:1 line that is representative of hypothetical daily deposition of increments. The solid line is the calculated linear trend for Calcofluor stained stylets ($y=0.749x+1.335$, $R^2=0.616$).

Using known-age validation for the beaks, the number of increments on hatchling beaks increased with individual age at an average rate of 0.53 increments per day (Linear regression, df=25, F=35.02, $R^2=0.57$, *p-value* <0.001) with rearing temperature and stocking density potentially affecting deposition periodicity (Table 2.3). Beak increment periodicity varied with rearing temperature with those reared in cooler (20°C) water depositing 0.60 increments per day and those reared in warmer temperatures (24°C) depositing 0.42 increments per day.

Day-old hatchlings had fully formed beaks and visible, uniform growth increments (Fig. 2.6). I observed varying beak periodicity depending on age in which 0-5 days old hatchlings had sub-daily periodicity, 5 to 10 day old hatchlings had daily increments, and older hatchlings and juveniles had supra-daily periodicity (Supp Table 1). With this in mind, the average periodicity for juveniles from 0 to 110 days old was still supra-daily at 0.53 increments per day. However, stylets did not form until the hatchlings were 2 days old, whereby the stylet cores measured approximately 20 µm in diameter (Fig. 2.10). Stylets in hatchlings from 0-1 days old were absent.

Table 0.3: Variation in increment periodicity on *Octopus berrima* beaks with rearing temperature and stocking density

Rearing temperature	**Stocking density (# of octopus/ m^2)**	**Periodicity**	**Sample size**	**Equation**	**R^2**
20°C	100/m^2	0.60	7	y=4.28+0.598x	0.61
22°C	100/m^2	0.57	8	y=2.92+0.565x	0.66
24°C	100/m^2	0.42	14	y=0.498+0.424x	0.55
Average	-	0.53	-	y=0.701+0.534x	0.58
Ambient (21.69 ±1.32°C)	250/m^2	0.29	19	y=4.56+0.287x	0.71

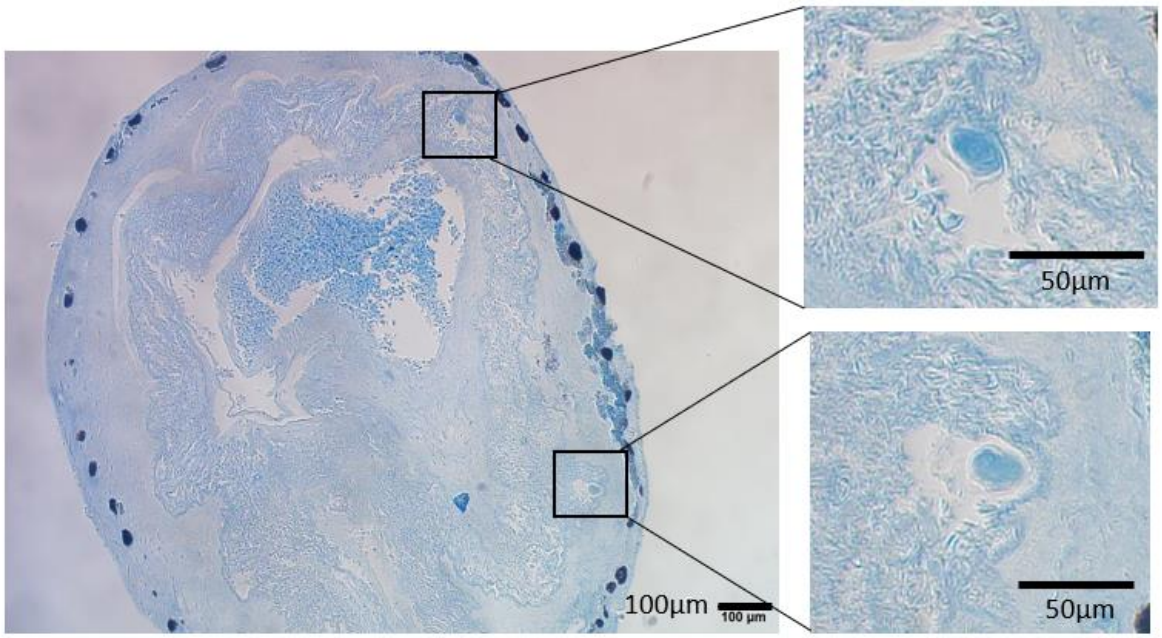


Figure 0.10: Microphotograph of a transversely sectioned 2-day old *Octopus berrima* hatchling to show the first presence of stylets (stained dark blue within the marked squares) in hatchlings.

Growth increment patterns in wild-caught octopus

Only 29.7% of stylets from wild-caught octopus (n =135) were considered readable (i.e., readability score ≥ 3) (Fig. 2.8) and no samples were graded with the highest score of 5. Of these samples (n=135) 76 were discarded due to varying counts, with 59 stylet counts used for further analysis.

Two types of increments were observed on the wild-caught *O. berrima* beaks from all collection sites: small/narrow increments (~20-30 µm) and large/wide increments (~100 µm) (Fig. 2.11). There appeared to be no consistency on where each type of increment was located or between individuals. For example, some beaks only contained large increments, some only had small increments, but the majority of beaks contained a mix of both. Those raised in captivity did not have this variation between increment type, which is most likely due to the consistent growth seen in laboratory reared octopus.

Although varying in width, all visible increments were counted on beaks. A total of 54 beaks were counted and 27 samples were discarded due to varying counts, with 27 beak counts used for analysis.

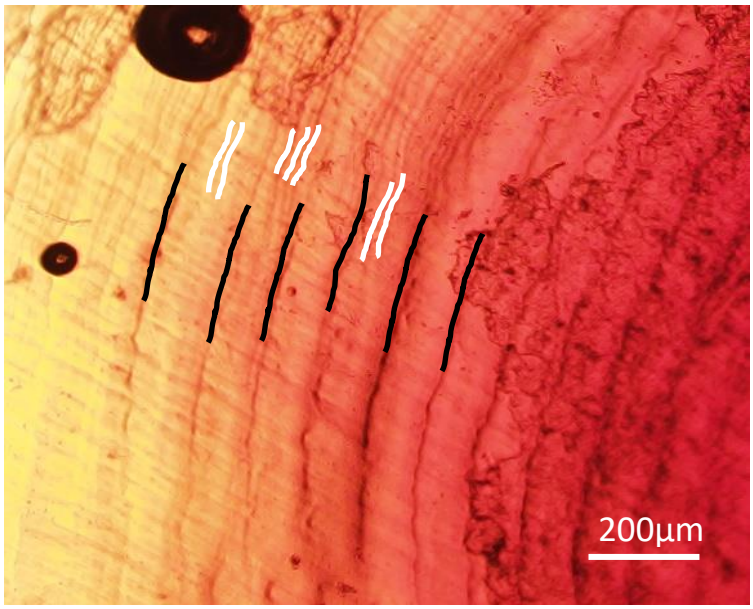


Figure 0.11: Microphotograph of the lateral wall of an adult wild-caught *Octopus berrima* beak showing the difference between large (wide) increments indicated using black lines and small (narrow) increments indicated using white lines.

Estimating age of wild-caught octopus

Number of increments in stylets ranged from 63 to 261 from octopus that ranged from 34 to 268 g in weight. Number of increments in beaks ranged from 44 to 160 from octopus that ranged from 28 to 258 g in weight.

Assuming that the validated increment periodicity remains unchanged throughout an individuals' entire lifespan, the maximum lifespan recorded was 348 days (11.6 months) based on stylets (0.75 periodicity) and 302 days (10 months) based on beaks (0.53 periodicity). The average estimated age using stylets was 131 days old and the average estimated age using beaks was 94 days old noting different animals were represented in the different datasets.

With these age estimates from the stylets of wild-caught octopus, I can then investigate the efficacy of morphometrics as indirect ageing methods. As a result, stylet weight increased with age (linear regression model; *df*=56, *p*=0.0001), but beak morphometrics (upper and lower beak weight, upper beak crest length and upper beak hood length) did not increase significantly with age (linear regression models; *df*=25, *p*>0.15) (Supplementary Fig. 1).

Discussion

Here I present one of the first examples of validated non-daily increment periodicity in adult octopus stylets and juvenile octopus beaks, with periodicity varying between structures. With this new information, I emphasise the importance of validating increment periodicity for each octopus species and age structure, rather relying on the assumption of daily increment periodicity.

Delayed formation of stylets

Counts of stylet increments always start at the core or the first increment visible, but for many species, it is unknown at what age the core represents (Doubleday *et al.*, 2011; Lourenço *et al.*, 2015). In *O. berrima,* stylets were not formed at 2 days old, suggesting that counts should start at 2 as that is the age the core is formed. This is the first evidence of octopus hatching without developed stylets. While Lourenço *et al.* (2015) did observe a stylet nucleus in 3-day-old *O. vulgaris* paralarvae, they did not examine younger individuals. Therefore, it is possible that this delay in stylet formation is apparent in other species as well.

Non-daily increment periodicity

Non-daily increment periodicity was observed in stylets of wild-caught adult *O. berrima* using chemical marking methods, with 0.75 increments deposited per day on average. Non-daily periodicity was also observed in the beaks of lab-raised known age juveniles, with 0.53 increments deposited per day on average, or every other day up to 110 days old. Perales-Raya *et al.* (2018) also found periodicity to be <1 increment per day in the beaks of *O. vulgaris* paralarvae reared at colder temperatures (14°C) and attributed this slower deposition to slower growth at this temperature. Another study observed a validated periodicity of 0.75 in *Octopus maya* G. L. Voss &Solís, 1966 beaks (Rodríguez-Domínguez *et al.*, 2013). However, because the slope (0.75)

did not statistically differ from 1, there was a significant bias between readers, and an increase in erosion in older octopus beaks, Rodríguez-Domínguez *et al.* (2013) concluded that increments were deposited daily and that it was possible that not all increments were visible.

In this study, temperature and stocking density appeared to influence increment periodicity, with individuals reared at a higher temperature (24°C) having slower periodicity than those reared at moderate temperatures (20 and 22°C). Thermal stress is known to negatively affect cephalopod growth (Gowland *et al.*, 2002; Higgins *et al.*, 2012) and it is likely that 24°C is near the thermal limit of the *O. berrima* based on temperature records from Venus Bay (the collection location of the hatchling's parents) (Martino *et al.*, 2021). Octopus reared at ambient temperature (21.69 ±1.32°C) had the slowest increment deposition periodicity (0.29), likely because they were raised in a different tank set up with a higher density of octopus per tank. These variations in beak increment periodicity suggest how rearing temperature and living conditions (e.g., tank stocking density) may influence increment deposition and further stresses the importance of validating periodicity accurately.

Age validation: moving forwards

Some studies assume daily increment deposition based on studies on other species and only validate from a portion of their life (i.e., only adults or only juveniles) (Table 2.1). However, I suggest that increment periodicity should be validated for each species, age structure, and potentially genetically distinct populations. This suggestion also applies to species that are not yet officially described. For example, there are multiple "species" within the *O. vulgaris* complex such as type I and type II in the West Atlantic, and type III in the Indian Ocean around South Africa (Jereb *et al.*, 2014; Amor *et al.*, 2019; Van Nieuwenhove *et al.*, 2019; Avendaño *et al.*, 2020). Until recently, all species within the *O. vulgaris* complex have been treated as *O. vulgaris*, but it has since been discovered that these similar species are genetically different (Amor *et al.*, 2019; Avendaño *et al.*, 2020). The taxonomic status of octopods is poor in general, and there are likely many undescribed and cryptic species yet to be discovered. For example, during an assessment of the diversity, connectivity and genetic structure of *O. vulgaris* and *Octopus cyanea* Grey, 1849 in the western Indian Ocean, Van Nieuwenhove *et al.* (2019) discovered a range expansion of *Octopus oliveri* S. S. Berry, 1914 and *Callistoctopus luteus* Sasaki, 1929, which until then, had only been recorded in the western Pacific Ocean. Even if they were all the same species, increment

periodicity can change with different abiotic and biotic influence, as observed here, and could therefore differ between octopus populations exposed to different environmental conditions. Thereby, strengthening the suggestion that daily increment deposition periodicity should not be assumed across species, structure, or locations.

Ideally, periodicity would be validated throughout the life cycle using the same method, but this is rarely feasible. Instead, periodicity can be validated in juvenile stages (through known age methods) and larger adult stages through chemical marking. The only caveat with these validation methods is that it is unknown if periodicity is a function of captivity, as it is impossible to replicate the natural conditions as well as the variety of live food available in the laboratory. Therefore, the assumption is that the periodicity observed in captivity also occurs in the wild and any age calculated for wild caught individuals should be regarded as an estimate and not absolute age.

Chemical staining methods had varying results. While Calcofluor produced correlated results, tetracycline results were inconsistent, and octopus had an adverse effect in which they participated in autophagy. Often when octopus have a damaged or sick arm, they have been observed to bite it off at the base (autotomy) or eat it (autophagy) (Budelmann, 1998; Anderson *et al.*, 2001). This can either be due to stress, bacterial or viral infections affecting the nervous system (Budelmann, 1998). I concluded that the cause of autophagy was due to the tetracycline and not the injection procedure as those injected with Calcofluor had normal behaviour. Although tetracycline injections have been successful in other species of octopus (Hermosilla *et al.*, 2010; Donlon *et al.*, 2019), *O. berrima* in this study and *Macroctopus maorum* (Karina Hall, pers comm.) from a separate study had a negative reaction to it. In the future, I recommend Calcofluor over tetracycline as a chemical stain to validate growth increment periodicity in new octopus species, due to the adverse effects seen here.

However, despite these potentially confounding factors, as well as non-daily increment periodicity recorded in this species, the lifespan estimate of 10/11.6 months for *O. berrima* is reasonable as other small-bodied octopus species range from 7.4 months for *Octopus digueti* Perrier & Rochebrune, 1984 (Derusha *et al.*, 1987), to 8.3 months for *Robsonella huttoni* Benham, 1943 (Donlon *et al.*, 2019) to 11 months for *Octopus joubini* G. C. Robson, 1929 (Forsythe, 1984). Sympatric octopus species in southern Australia have a large range from 11 months for *O. tetricus*

(Ramos *et al.*, 2014), to 18 months for *O. pallidus* (Leporati, Semmens, *et al.*, 2008), to 22 months for *O. djinda* (Leporati and Hart, 2015), however, these species are significantly larger than *O. berrima*.

Improving readability of growth increments

Clear visualisation of growth increments is also a critical part of ageing octopus. Others have found that poor visualisation of fish otoliths (Campana, 1992) and squid statoliths (Lipinski and Durholtz, 1994; Arkhipkin and Shcherbich, 2012) is often due to inadequate sample preparation methods and microscopy resolution, and I have found the same for octopus stylets and beaks.

Visibility of increments in octopus structures varies with individual, cleanliness (beak), and preparation of the structure. When Doubleday *et al.* (2006) first investigated the stylet as an ageing method for *O. pallidus*, the stylets were prepared similarly to squid statoliths; in warmed crystalbond, polished and photographed quickly as they would degrade (darken and crack) after a few minutes. Barratt and Allcock (2010) then developed a permanent method to preserve stylet sections, which was used in the present study, and yielded 60% viable, readable samples. Repeating this method on other species yielded 40% (*O. tetricus*, Ramos *et al.*, 2014) and 30% viable samples (this study). The primary reason for discarding most samples is due to poor visibility of growth increments.

One key issue with the method described by Barratt and Allcock (2010) is that when the stylet is small, it is difficult to orient correctly when embedding. I were able to decrease this occurrence by using a dissecting microscope during the embedding process, but poor increment visibility was still high in the study.

Microscope optical resolution can also be problematic. There may be hundreds of growth increments in the stylet of a small octopus that are packed tightly on a tiny structure less than 1 mm in diameter, therefore the resolution and magnification needed can go beyond standard laboratory microscopes. This problem has arisen with octopus stylets (Doubleday *et al.*, 2011; Lourenço *et al.*, 2015), squid statoliths (Arkhipkin and Shcherbich, 2012), as well as fish otoliths (Campana, 1992).

Growth increments on wild-caught *O. berrima* beaks were fainter and more difficult to visualise than those of other octopus species that I have observed (*R. huttoni*) (Donlon *et al.*, 2019), and it was the first time the authors had seen this variation in increment width across an individual beak. There was a 70-80 µm difference in increment width and no obvious pattern, which could be related to various abiotic and biotic factors that affect metabolism such as food availability, stress, and growth rate, as this is what has been concluded for other species including *O. vulgaris* (Campana, 1992; Perales-Raya *et al.*, 2010; Perales-Raya, Almansa, *et al.*, 2014).

Beaks tend to have less complications during sample preparation, but if organic tissue is not removed with this process, increments are not easy to discern. Previous studies (Hernández-López *et al.*, 2001; Perales-Raya *et al.*, 2010; Perales-Raya, Almansa, *et al.*, 2014) on beaks have cleaned them by hand with water to remove mucus and only one other study has used hydrogen peroxide to thoroughly clean the beak (Perales-Raya and Hernández-González, 1998). The method of cleaning the beaks with 5% hydrogen peroxide in an ultrasonic cleaner before scrubbing with a soft plastic pipette took suggestions from both methods to improve visualisation and, in turn, readability of beak increments. The curvature of the beak also adds a layer of difficulty. I have found that it is easier and more accurate to count from the LWS under the microscope rather than from an image, so the focus can be adjusted for each section. However, manually counting increments under a microscope is often more taxing on the researcher compared to counting increments within a captured image.

Xavier *et al.* (2022) now recommends the analysis of both LWS and RSS as well as both upper and lower beak to determine the best method for each species, but at the time of this study, the LWS on the upper beak was thought to be the best location for ageing (Perales-Raya, Almansa, *et al.*, 2014; Arkhipkin *et al.*, 2018). For this reason, only the upper beak LWS was used for the present study.

Alternative ageing methods

Moving forward, more studies investigating other ageing methods should continue to be done. The age pigment, lipofuscin, has been trialled as proxy of age in octopus (Doubleday and

Semmens, 2011; Donlon *et al.*, 2019) and has shown some promising results, but this method is still a work in progress and is more of a proxy for age (% aged cells) rather than a direct method that produces a numerical result (age in days). Other indirect ageing methods include using morphometric measurements of beaks and stylets to estimate age; however, validated ages are required first. For example, the *O.* cf. *tetricus* (now *O. dijinda*) fishery in Western Australia now uses stylet weight as a proxy of age, as it was found to be highly correlated with the age of the animal (R^2=0.81-0.88) (Leporati and Hart, 2015). In the present study, I also found that estimated age and stylet weight were significantly correlated, but the relationship was weaker (R2 = 0.24). As such, I suggest that stylet weight could be cautiously used as a broad proxy of age in *O. berrima*, but further analysis would be useful.

Conclusions

Age is one of the most important pieces of life history information used in fisheries management. This is the first study to show non-daily growth increments in both the stylets and beaks of octopus using two different validation methods (known age and chemical staining), whereas other octopus ageing studies either validate daily increments or assume daily periodicity. Here, I highlight the importance of age validation for both species and age structure, as increment periodicity is not always daily and likely influenced by a range of abiotic and biotic factors.

Data availability

The data underlying this article are available in the supplementary material and The Open Science Framework, at:
https://osf.io/5pejb/?view_only=4e274ecd393f49de988cf0ee78f8511b

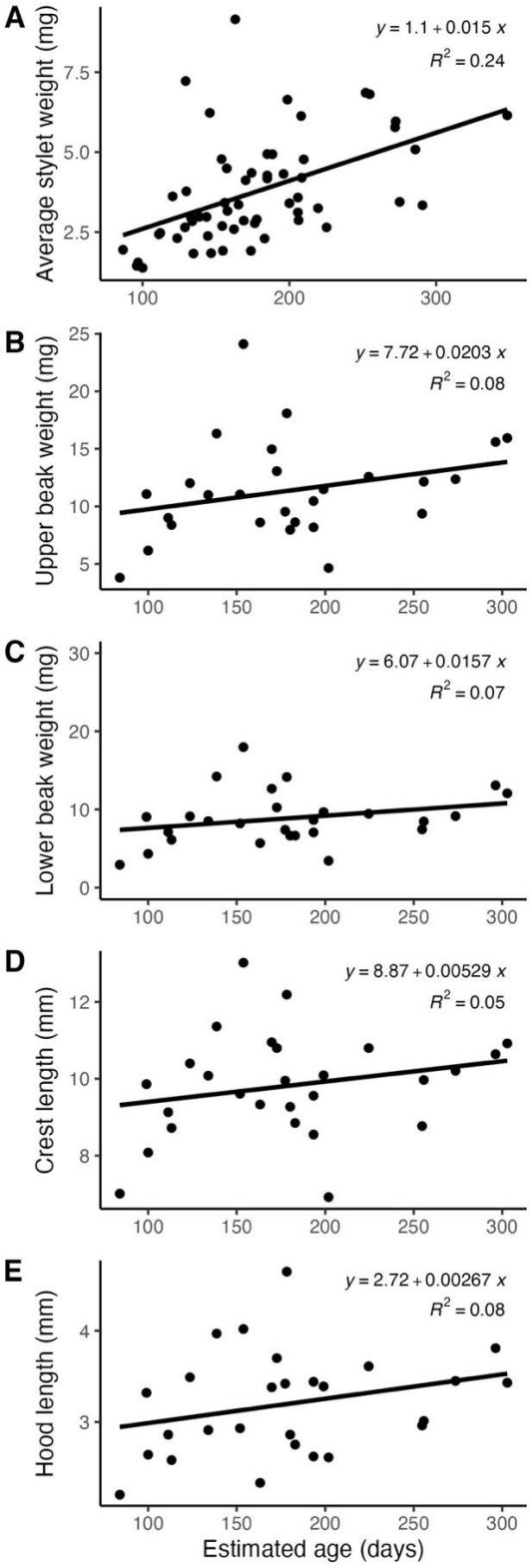
Supplementary Material

Supplementary Table 0.1: Raw data of juvenile beak increment counts from the upper beak lateral wall surface with rearing temperature and age (days).

Temperature	Age (days)	Average number of increments on upper beak LWS
22	2	4

Ambient	3	5
Ambient	3	9
Ambient	3	6
20	4	7
Ambient	4	8
24	5	12
Ambient	7	7
Ambient	7	8
Ambient	7	7
24	8	6
24	8	6
24	8	5
Ambient	9	9
Ambient	9	8
Ambient	9	8
Ambient	10	9
Ambient	10	10
Ambient	60	13
Ambient	60	6
Ambient	60	12
Ambient	60	31
Ambient	60	23
Ambient	60	24
24	72	10
24	72	15
24	72	11
20	90	57
22	90	62
22	90	72

22	90	31.5
20	91	55
20	91	34
22	91	62
22	91	52
22	91	44
24	91	38
24	91	30
24	91	65
20	92	80
20	92	48
20	92	77
24	99	30
24	99	63
24	99	59
Ambient	110	21
Ambient	110	51
Ambient	110	40
Ambient	110	42



Supplementary Figure 0.1: (A) Average stylet weight (mg), (B) upper beak weight (mg), (C) lower beak weight (mg), (D) upper beak crest length (mm), and (E) upper beak hood length in relation to estimated age using counts of increments on (A) stylets with the validated periodicity of 0.75 and (B-E) beaks and the validated periodicity of 0.53.

A definitive guide to ageing octopus

This research is under review with Marine and Freshwater Research

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 I 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. I provide guidance on age validation, increment analysis of both beaks and stylets, materials needed, as well as avenues for further research. I 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. I also encourage researchers to use this guide as a forum for open discussion to support the ongoing development of effective octopus ageing methods.

Introduction

Fisheries are fundamental to the maintenance of global food security and contribute to the livelihoods of an estimated 600 million people (FAO, 2022). However, decades of overfishing have resulted in the depletion of some finfish stocks (FAO, 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; 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, Almansa, *et al.*, 2014), and indirect methods that act as an age proxy, such as eye lens diameter or weight (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; Perales-Raya, Almansa, *et al.*, 2014; Villegas-Bárcenas *et al.*, 2014). However, due to 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 periodicity validation 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. While I acknowledge ageing methods will continue to evolve, I 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, whilst acting as a forum for discussion to support the development of optimal ageing methods.

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. 3.1), and can be dissected through the following method (Fig. 3.2):

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. 3.3).
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.

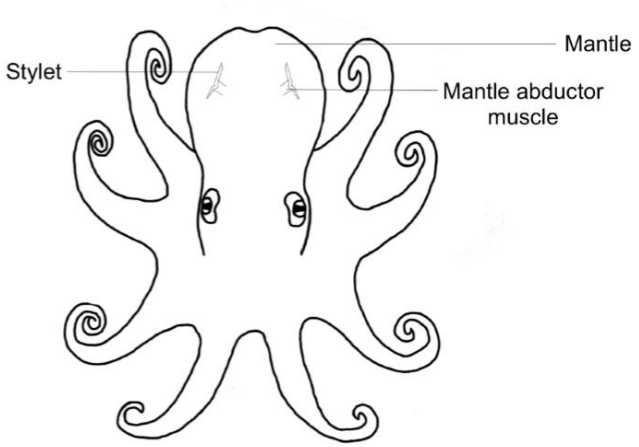


Figure 0.1: Diagram of an octopus from a dorsal viewpoint, indicating stylet location within the mantle

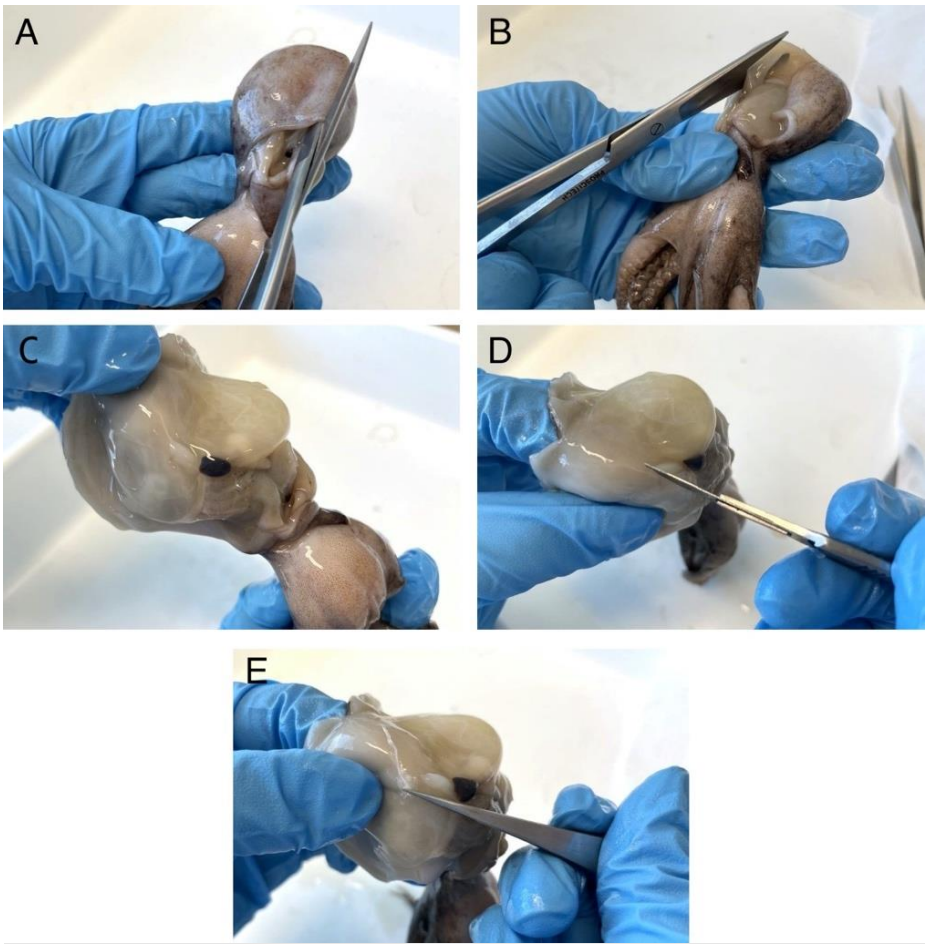


Figure 0.2: Stylet dissection process involving (A) a vertical incision from the base (anterior) to the top (posterior) of the mantle, (B) a horizontal incision through the muscular septum, (C) stylet location, (D) an incision to separate the stylet and mantle abductor muscle, and (E) removal of the stylet from the mantle.

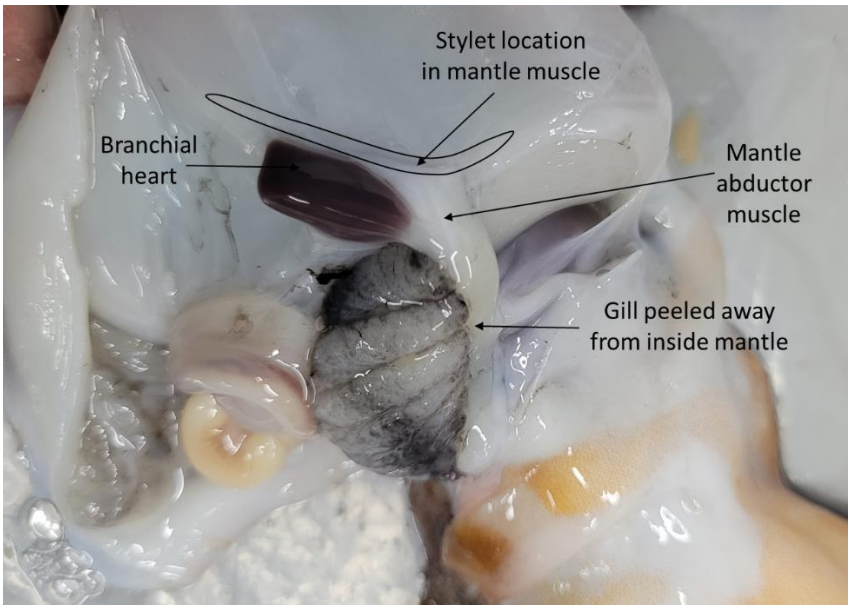


Figure 0.3: Close-up image of the stylet location within the mantle muscle at the base of the abductor muscle and branchial heart, after the gill has been peeled away from the inside of the mantle.

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; Leporati and Hart, 2015). The resin infiltration process can be undertaken through the following method:

1. Using a single-sided razor blade, transversely section the post-rostral zone of the stylet into ~ 1mm lengths (Fig. 3.4), preparing up to three lengths for each stylet.

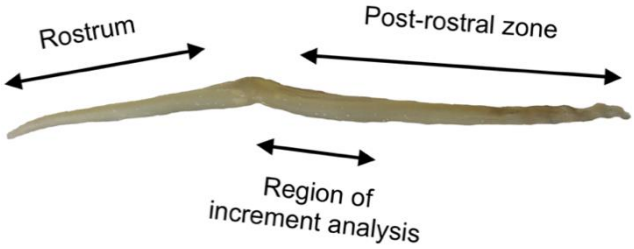


Figure 0.4: Image showing the region of increment analysis on the post-rostral zone of an *Octopus pallidus* stylet.

2. Place the lengths in solution-filled, numbered tubes (one for each stylet) following the sequence outlined in Table 3.1. Make sure to blot excess solution from each length before placing in the next solution. It is especially important to ensure all 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.

Table 0.1: Table outlining the preparation sequence for stylets to be preserved through resin infiltration.

Solution	Duration
90% ethanol	1 hour
100% ethanol	1 hour
LR white resin (medium grade) *	24 hours

*catalysed resin solution, prior to adding accelerator (hardener)

3. 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.
4. Place cylindrical moulds over the top of each group of lengths on the tape (Fig. 3.5). Here, I have cut the bottoms from 5 mL plastic sample tubes and used the tops cut side up. However, any shape mould is suitable. In my 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.

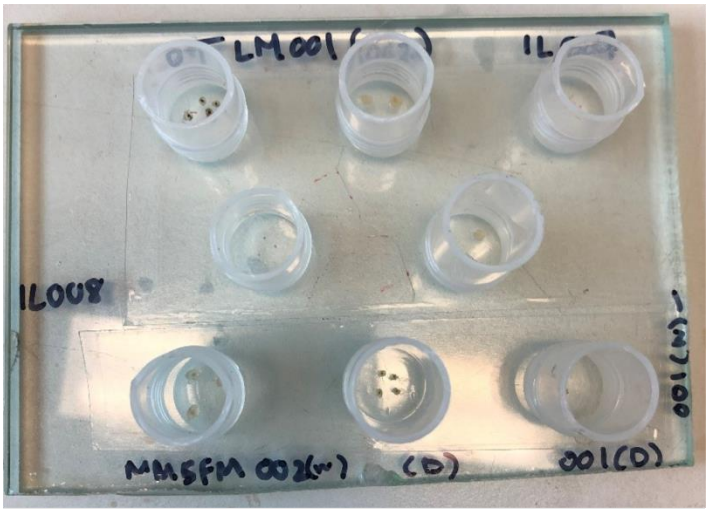


Figure 0.5: Image displaying resin infiltration moulds used for stylet preparation including glass base, double-sided tape, and plastic tubing.

5. 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.
6. 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.
7. Remove the resin block from its mould and wipe away excess resin with paper towel.
8. 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.
9. 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.
10. Using clear Gorilla glue, affix the block polished side down to a clean microscope slide and leave to fully dry for 24 hours (Fig. 3.6A). In my experience, superglue is not adequate as it is not waterproof and degrades during polishing, therefore water-resistant glue is best.
11. 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.

12. 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. 3.6B). Extra scratches can then be buffed out with 0.5 micron aluminium oxide powder and a car wash chamois or any smooth, soft cloth.

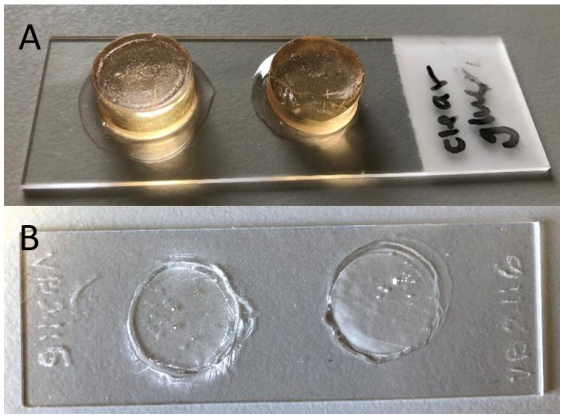


Figure 0.6: Images of resin blocks with stylet sections when (A) glued to slide and (B) cut and polished.

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 due to 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 visiualisation maybe impossible.

Visualising and counting growth increments

Stylet growth increments can be visualised using 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. 3.7). The best viewing magnification will vary for each octopus species, although resolution is often lost at higher magnifications.

Ideally, increments, from the core to the edge, should be counted at least twice, non-consecutively, by one or more trained readers. Multiple approaches to defining reader precision and octopus age can be taken, however, a common approach is to take the average of multiple counts to define age (if increment periodicity is known). Then if the counts differ by more than 10% for a single stylet section, the section is discarded (Barratt and Allcock, 2010; Perales-Raya *et al.*, 2010; Leporati and Hart, 2015).

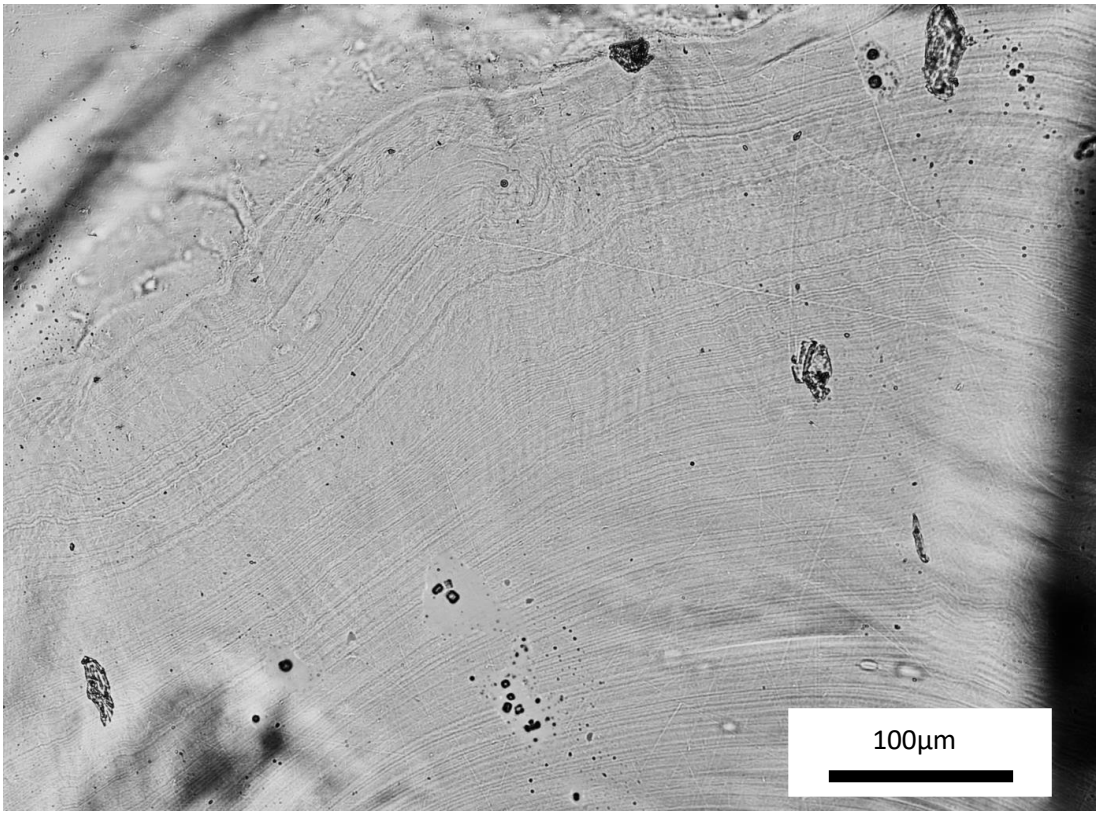


Figure 0.7: Micrograph of *Macroctopus maorum* stylet section showing growth increments at 100x magnification.

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 *et al.* (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 lateral rostrum and wall, thus facilitating age determination through increment analysis. Beak increment analysis was first explored in octopus by 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 and is applicable to a wider range of species than the LRS (Perales-Raya, Almansa, *et al.*, 2014). At present, beak increment periodicity has been validated in a variety of species, including *O. maya* (Rodríguez-Domínguez *et al.*, 2013), *O. vulgaris* (Perales-Raya, Almansa, *et al.*, 2014), *Octopus insularis* (Batista *et al.*, 2021), and *Octopus berrima* (Durante *et al.*, 2023).

I provide a detailed outline of the steps involved for beak increment analyses via the LWS. For methods using the RSS and RS refer to Pearles-Raya *et al.* (2010) and Pearles-Raya *et al.* (2014).

Dissection and storage

Octopus beaks are embedded within the buccal mass on the ventral side of the octopus (Fig 3.8). Dissection can be undertaken through the following method (Fig. 3.9) and is best dissected after the octopus or entire buccal mass is 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.
4. After the majority of tissue is cleaned, beaks can be preserved indefinitely in 70% ethanol until ready for use or, if analysis occurs shortly after, they can be preserved in distilled water at 4°C. The later preservation method has been found to better preserve the microstructure, but trials should always be done for each species to determine whether or not ethanol significantly degrades the microstructure or not.

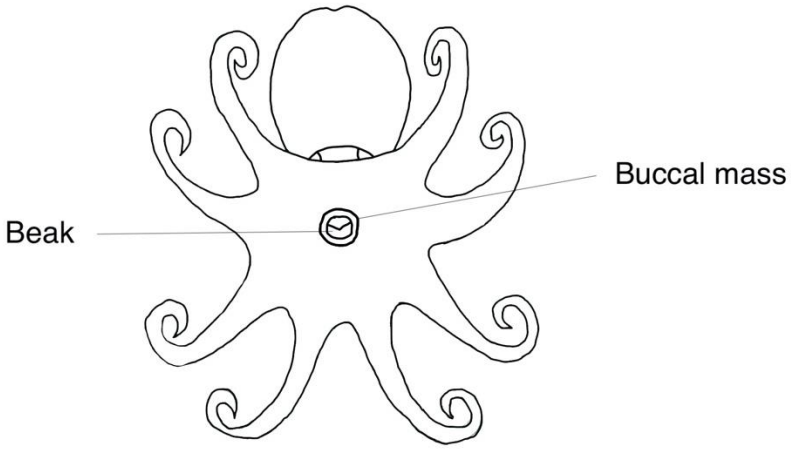


Figure 0.8: Diagram of an octopus from a ventral viewpoint, indicating the beak embedded within the buccal mass.

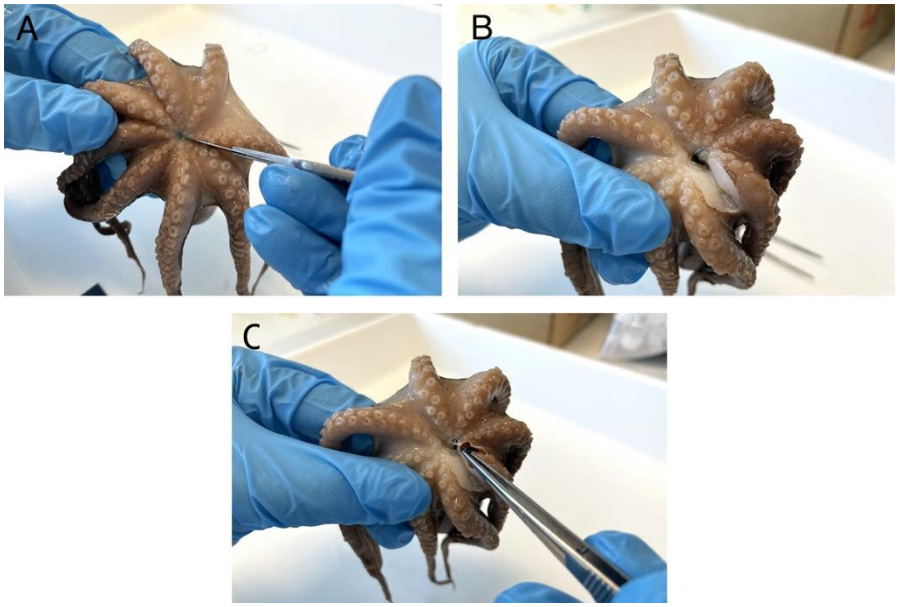


Figure 0.9: Beak dissection process involving (A) an incision to one side of the mouth musculature to expose the beak (B) another incision to the other side, (C) and removal of the beak from the buccal mass.

Preparing beaks from adults and large individuals

1. Using scissors, cut the upper beak in half to obtain two sagittal sections (Fig 3.10). Select the flattest half for sample preparation.

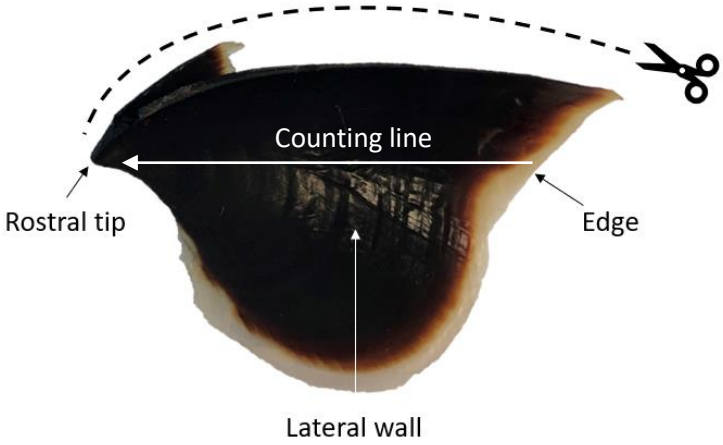


Figure 0.10: Image of a *Macroctopus maorum* beak indicating the rostral tip, lateral wall, and beak edge. The counting line indicates the direction for counting of growth increments (from edge to rostral tip), and the scissors indicate where to section if using the lateral wall surface (LWS).

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 minutes and scrub again with pipette tip. Rinse with water.
3. Using an appropriate adhesive, fix your beak section to a microscope slide, flattening the section as much as possible with a wide, flat scalpel or knife (Fig. 3.11). The preferred adhesive is Crystalbond™ 509 because it can be reheated to reshape mounts and cures quickly as it cools.



Figure 0.11: Flattening beak half onto a slide with warm crystalbond adhesive.

Preparing beaks from hatchling and juveniles

Extra small and thin beaks such as those in hatchlings are carefully dissected, cleaned with water and a plastic 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. Increments on thicker, larger beaks are more visible using a combination of transmitted and reflective light and increments on thinner, smaller beaks are more visible with transmitted light, but this varies with each species, and both should be trialled.

In my experience, it is easiest to count increments on beaks directly through the eyepiece while sections are under the microscope because of the three-dimensional surface profile of the increments that require careful adjustment of the field of view across the section. However, it is possible to successfully count increments from a good micrograph or series of digital images that are individually focused and later stitched together (Fig. 3.11). Increments should be counted from the edge of the beak to the rostrum (Fig. 3.10). 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, Almansa, *et al.*, 2014), 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, I recommend multiple non-consecutive counts per trained reader, with data treated as described above.

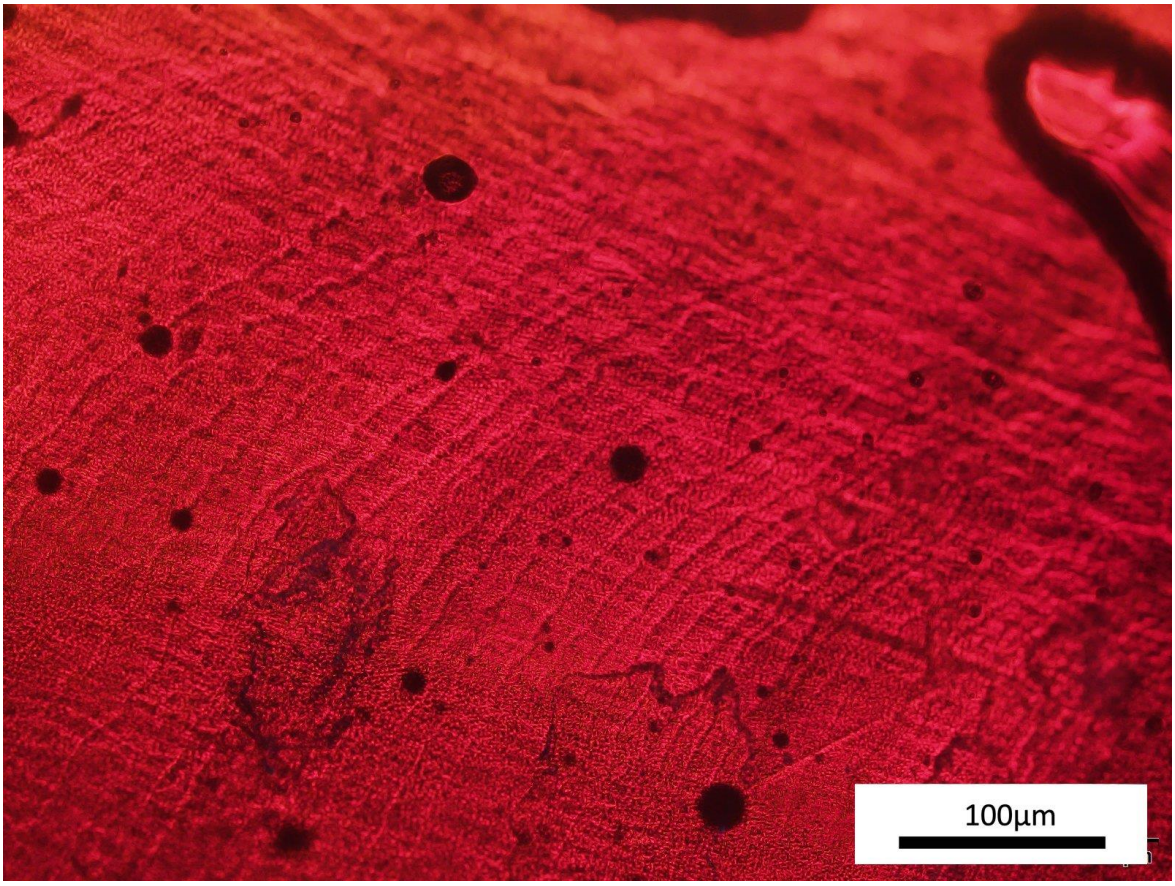


Figure 0.12: Micrograph of *Octopus tetricus* beak section (lateral wall surface) showing growth increments at 100x magnification.

Part 3: Periodicity validation

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 (Xavier *et al.*, 2022; Durante *et al.*, 2023). Thus, daily growth ring deposition cannot be assumed. Consequently, validation of growth ring periodicity is a crucial first step in the ageing process for each species. Age validation can be achieved through the analysis of known-age individuals, or chemical staining of the hard structures to mark time at liberty or in captivity when hatch date is unknown (e.g., for wild caught octopus).

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 or marked and recaptured from the wild.) 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 (Doubleday *et al.*, 2006; Barratt and Allcock, 2010). 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). But 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.

Chemical staining method

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 staining is compared with the total number of days held or at liberty (Perales-Raya, Almansa, *et al.*, 2014).

This method relies on the chemical effectively staining the hard part to the extent that a fluorescent mark can be visualised through microscopy 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, while others have been unsuccessful (Table 3.2). However, success is not always consistent among species or structures. For example, I 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, Almansa, *et al.* (2014), reported it successfully marked *O. vulgaris* beaks. Tetracycline hydrochloride is a commonly used stain but can cause adverse effects on octopus health in some species (e.g., injection in adults can trigger arm autophagy) (Durante *et al.*, 2023; Karina Hall pers comm). Therefore, I do not recommend tetracycline as a stain for new species due to potential adverse impacts.

Table 0.2: 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 impact to 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, Almansa, *et al.* (2014)
		Octopus berrima: **Diffused stain mark**	Durante *et al.* (2023)
Congo red	*Beak*	*Octopus vulgaris:* **Diffused stain mark**	Perales-Raya, Almansa, *et al.* (2014)
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)

Injection is the most widely practiced and recommended method of chemical stains for octopus. Submersion in a seawater bath containing the chemical stain has also been explored. However, not enough of the chemical was absorbed and there is also a risk of the chemical becoming oxidized and losing its fluorescent ability (Donlon *et al.*, 2019). Euthanised octopus which 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, Almansa, *et al.* (2014). This solution is concentrated to 50 mg/mL 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, Almansa, *et al.*, 2014; Donlon *et al.*, 2019; Durante *et al.*, 2023) or anesthetised through chemical solution immersion prior to the injection process (Fiorito *et al.*, 2015). In my 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 (Erica Durante pers comm.). Magnesium chloride is also one the most widely used sedatives for octopus. However, I 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). I 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 following the formula below.

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

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

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

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 original container and flush fresh seawater into the mantle and over the gills until octopus movement recovers.
5. Octopus are considered fully recovered when breathing returns to a normal rate, skin coloration returns, octopus respond to stimuli and all arms are functioning.

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.

1. Take an image of the stained section under a fluorescent microscope to locate the mark (Fig. 3.13).

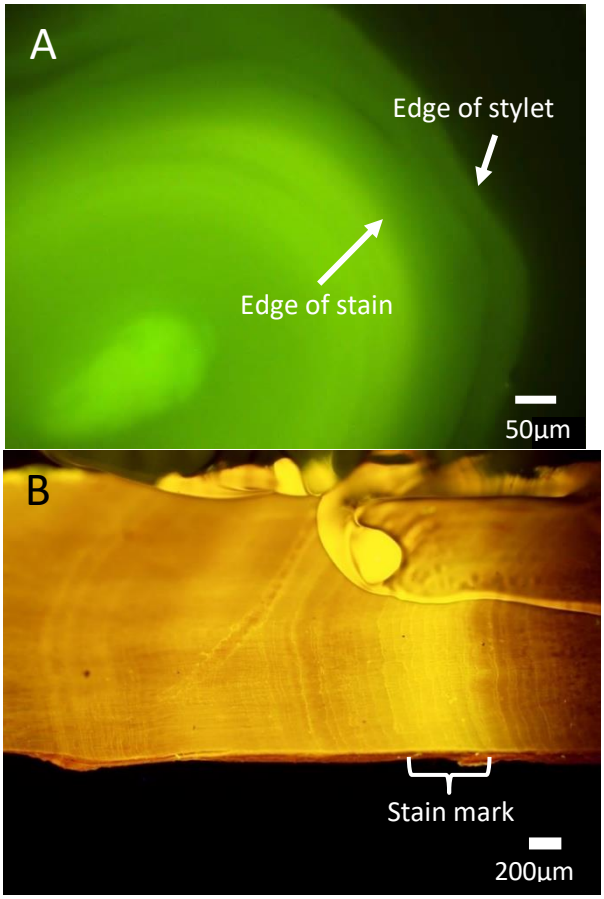


Figure 0.13: (A) Micrograph of a Calcofluor-stained *Octopus berrima* stylet section showing the edge of the stain mark and the edge of the stylet and (B) micrograph of the lateral wall of a *Octopus tetricus* upper beak that has been stained with tetracycline. Brackets indicate the section in which the fluorescent mark was formed from the tetracycline.

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 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 summarized below:

1. Fix whole hatchlings in a mixture of formalin acetic acid calcium chloride (FAACC) for 48 hours then transfer to 70% ethanol and store for at least 24 hours before processing. FAACC is comprised of:
    - 400mL, 10% neutral buffered formalin
    - 13g calcium chloride (0.117M)
    - 50mL glacial acetic acid
    - 550 mL distilled water
2. Process samples following the paraffin embedding sequence outlined in Table 3.3.

Table 0.3: Paraffin embedding sequence for octopus hatchlings (<100 days old).

Solution	Duration
95% ethanol	1 hour
100% ethanol	1 hour
100% ethanol	1 hour
Xylene	40 minutes
Xylene	40 minutes
Paraffin	40 minutes
Paraffin	40 minutes
Paraffin	40 minutes

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 Table 3.4. Alternative stains can also be used, e.g., Lourenço *et al.* (2015) used Masson's trichrome stain, but I found methyl blue to be sufficient.

Table 0.4: Dewaxing and staining sequence for octopus hatchlings (<100 days old).

Solution	Duration
Xylene	2 minutes
Xylene	2 minutes
100% Ethanol	2 minutes
100% Ethanol	2 minutes
70% Ethanol	2 minutes
30% Ethanol	2 minutes
~3% (2.7%) Methyl blue in water	5 minutes
Rinse in water	Until water is clear
70% Ethanol	2 minutes
100% Ethanol	2 minutes
100% Ethanol	2 minutes
Xylene	2 minutes
Xylene	2 minutes

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.

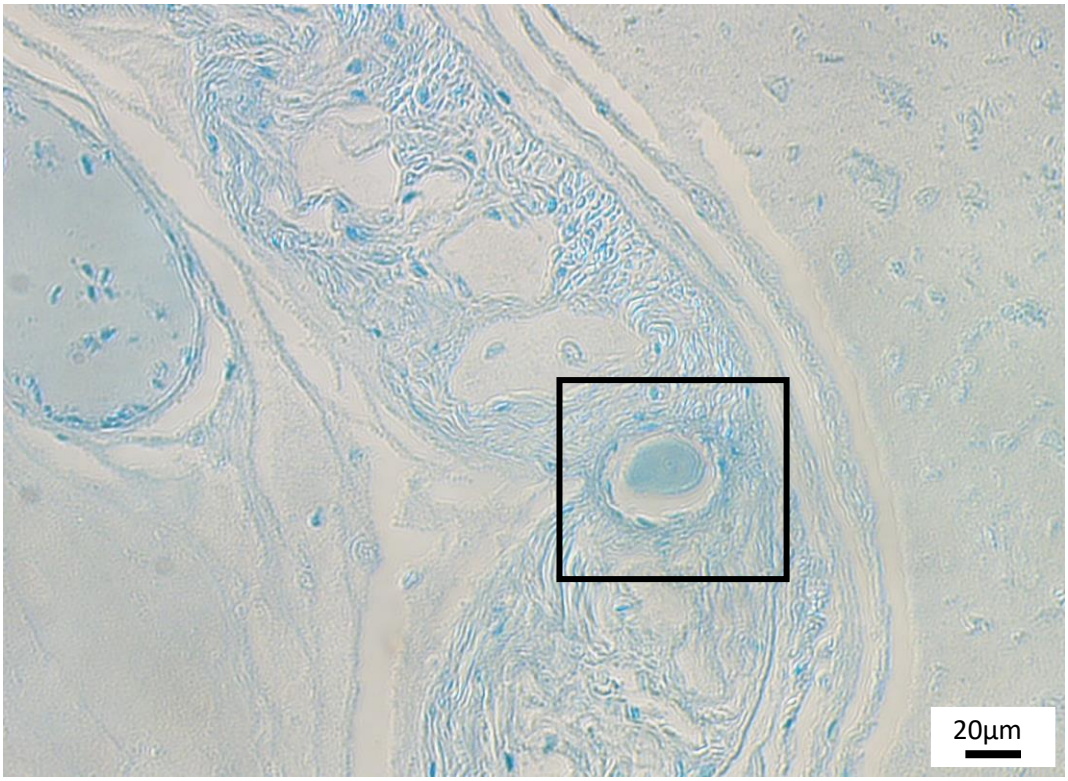


Figure 0.14: Micrograph of a 3-day old *Octopus berrima* hatchling cross section at 20x magnification. Stylet section is indicated within the box.

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. I 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 (Cárdenas *et al.*, 2011; Rodríguez-Domínguez *et al.*, 2013) or embedded in synthetic resin to produce thin slides (Cárdenas *et al.*, 2011). 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 (Rodríguez-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, and preferably over different life stages (Campana and Thorrold, 2001; Doubleday *et al.*, 2006). 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 3.5). 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, I provide a flow chart indicating the main steps and decision points (Fig. 3.15).

Table 0.5: Recommended ageing methods for holobenthic and merobenthic octopus species whereby full methods have been already developed and published. I also list species that have readable increments, but validation is still required.

Species	Increments Visualised	Periodicity Validated	Recommended Ageing Method/s	Literature
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	Rodríguez-Domínguez *et al.* (2013) Villegas-Bárcenas *et al.* (2014b)
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	Hernández-López *et al.* (2001) Barratt and Allcock (2010) Hermosilla *et al.* (2010) Perales-Raya, Almansa, *et al.* (2014)

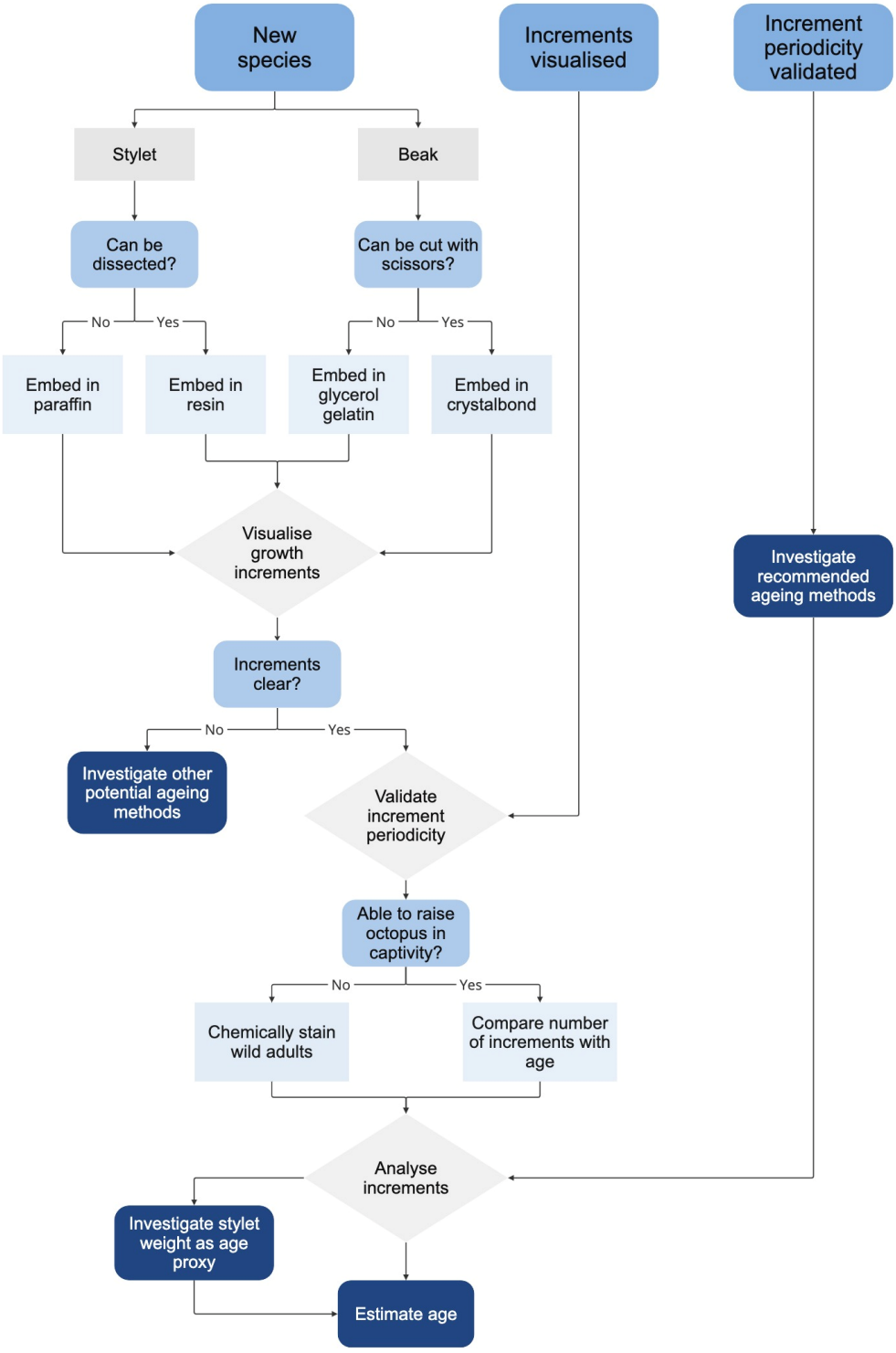


Figure 0.15: Flow chart indicating the main steps and decision points involved in ageing

Developing isotopic proxies to reconstruct the metabolic rates and thermal histories of octopus

This research is under review with Marine Environmental Research

Abstract

Understanding an animal's metabolic rate and thermal history is pivotal for ecological research. Recent studies have proposed the use of stable carbon and oxygen isotopes ($\delta^{13}C$ and $\delta^{18}O$) in biogenic carbonates as proxies of metabolic rate and experienced temperature, respectively, to overcome the challenges of gathering these data from the field. This study represents the first experimental investigation to develop $\delta^{13}C$ and $\delta^{18}O$ proxies in octopus. *Octopus berrima* hatchlings were raised in captivity, at varying water temperatures, for up to 110 days. *O. berrima* statoliths were then subsequently analysed for $\delta^{13}C$ and $\delta^{18}O$ values. The proportion of metabolically derived carbon or respired carbon (C_{resp}) increased as the octopus grew (slope = 0.076, R^2 = 0.72), indicating a significant influence of somatic growth rate and body mass on $\delta^{13}C$ values. Additionally, I identified a strong inverse correlation between $\delta^{18}O$ values and environmental temperature (slope = -0.163, R^2 = 0.91), which was subsequently used to develop a thermal reconstruction model. This experiment aids in understanding how stable isotopic signatures in biogenic carbonates can be used as proxies in future field-based studies. Such proxies have the potential to enhance the understanding of these ecologically and commercially significant animals, contributing to their conservation and effective management.

Introduction

Energy is the sustenance of life and understanding the energy demands of a species is important for conservation and management. Energy is best quantified through metabolic rate which indicates the rate at which energy is required and expended. Metabolism can be defined as basal metabolic rate (BMR) for an endotherm or standard metabolic rate (SMR) for an ectotherm, which indicates the minimal energy needed to survive. In contrast, field metabolic rate (FMR) indicates the amount of energy needed to thrive and includes functions such as growth, movement and reproduction (Treberg *et al.*, 2016). Metabolic rates vary with feeding strategies (i.e., carnivore vs herbivore), body size, and environmental temperature within the species' natural temperature range (Gillooly *et al.*, 2001; Brown *et al.*, 2004; Nagy, 2005). For ectothermic animals, temperature drives metabolic rates and metabolic rate increases with temperature until the thermal maximum for a species is reached. After this point, increasing temperature becomes detrimental and metabolic rate and growth rapidly declines (André *et al.*, 2009; Meza-Buendía *et al.*, 2021; Borges *et al.*, 2023). Of the marine invertebrates, coastal cephalopods have the highest metabolic rates (O'Dor and Webber, 1986) and have a "live fast, die young" life history strategy (Semmens *et al.*, 2004). With increased ocean

temperatures, the metabolic rates of marine ectotherms are predicted to increase (Pimentel *et al.*, 2012; Borges *et al.*, 2023) incurring a larger need for oxygen and food to maintain energy requirements. Increasing physical demands are likely to be particularly detrimental to cephalopods at high temperatures outside their natural range. For example, at higher temperatures, squid paralarvae have been found to hatch prematurely and thereby have a lower chance of survival (Pecl and Jackson, 2008; Pimentel *et al.*, 2012). Octopus have also been found to avoid water temperatures above their thermal limit and when held at these high temperatures, octopus exhibit spasms, rigor, and death (Higgins *et al.*, 2012).

As the oceans continue to warm, it is increasingly important to investigate how the metabolic rates of marine animals respond to thermal change. Metabolic rate is typically determined by measuring respiration rate of the animal in rest in a respiration chamber (Treberg *et al.*, 2016), but there are also various other methods to measure metabolic rate. For example, in cephalopods, telemetered jet pressure (O'Dor and Webber, 1986; O'Dor *et al.*, 1995; Aitken *et al.*, 2005) or accelerometry (Payne *et al.*, 2011) and analysis of enzyme activities (Seibel and Childress, 2000) have been used to monitor metabolism. Analysis telemetered jet pressure and accelerometry requires the attachment of transmitters to the animal in which it is then placed in a chamber to swim (O'Dor and Webber, 1986; O'Dor *et al.*, 1995; Aitken *et al.*, 2005; Payne *et al.*, 2011) which is an expensive and time-consuming process and not suitable for all species. Analysis of enzyme activities requires a sample of soft tissue, such as a piece of arm or mantle, and thus only represents a snapshot of an individual metabolism at that time (Seibel and Childress, 2000). In contrast, biogenic carbonates, like statoliths, provide a permanent record of an individual's metabolic history, and can be a cost-effective and time-efficient method to obtain metabolic data retrospectively.

Carbon isotope values ($\delta^{13}C$) in carbonates represent carbon from two sources; 1) dissolved inorganic carbon (DIC) in the ambient seawater and 2) carbon from diet. The ratio of incorporation of carbon from these two sources is dependent on the metabolic rate of the animal (Chung, Trueman, Godiksen and Grønkjær, 2019). Higher metabolic rates increase the proportional contribution from the diet, which has significantly depleted $\delta^{13}C$ compared to the DIC in water, resulting in lower measured $\delta^{13}C$ values in carbonates (Chung, Trueman, Godiksen and Grønkjær, 2019; Martino *et al.*, 2020; Chung *et al.*, 2021). By using a two-component mixing model (Schwarcz *et al.*, 1998; Solomon *et al.*, 2006; McConnaughey and Gillikin, 2008) and $\delta^{13}C$ values from biogenic carbonates, the proportion of carbon from metabolic sources (C_{resp}) can then be calculated and used as a proxy of metabolic rate.

This new method has been examined in controlled experiments on various marine organisms including, fish otoliths (Høie *et al.*, 2003; Shirai *et al.*, 2018; Martino *et al.*, 2019), cuttlefish statoliths (the cephalopod equivalent to otoliths) and cuttlebones (Chung, Chen, *et al.*, 2020), and mollusc shells (Owen *et al.*, 2008; Nishida *et al.*, 2015). For example, Høie *et al.* (2003) and Martino *et al.* (2019) found that 28-32% of carbon in Atlantic cod otoliths and 21-28% of carbon in snapper otoliths were metabolically derived (i.e., from the diet), respectively. These percentages of metabolically derived carbon increased with temperature as predicted for metabolic rates. Studies such as these can aid in understanding the thermal sensitivity of metabolism occurring between individuals.

As temperature is a significant driver of metabolic rate, it is useful to know the thermal history of the animal (i.e., range of temperatures the animal experienced). Oxygen isotope values ($\delta^{18}O$) in biogenic carbonates are a useful tool for estimating experienced temperatures as there is a well recorded inverse relationship between oxygen isotopic values and temperature (Kalish, 1991a; Trueman and St John Glew, 2019). Historical temperatures have been reconstructed using a variety of biogenic carbonates such as, fish otoliths (Surge and Walker, 2005; Darnaude *et al.*, 2014), corals (Sun *et al.*, 2005; Han *et al.*, 2020), and mollusc shells (Schöne *et al.*, 2005; Bayer *et al.*, 2020; Samin *et al.*, 2023). The relationships between $\delta^{18}O$ values and temperature are described using oxygen thermometry equations, which predict experienced temperature using $\delta^{18}O$ values and have been developed for fish otoliths (Høie *et al.*, 2003, 2004; Kitagawa *et al.*, 2013; Sakamoto *et al.*, 2017; Shirai *et al.*, 2018), mollusc shells (Grossman and Ku, 1986; Owen *et al.*, 2008; Nishida *et al.*, 2015), and more recently, cuttlefish statoliths, and cuttlebones (Chung, Chen, *et al.*, 2020). Although the general trend is the same across all recorded marine carbonates, the slope and intercept of this relationship varies and should therefore be individually modelled for species and carbonate type. For example, Martino *et al.* (2022) reconstructed experienced temperatures for an octopus, a cuttlefish, and a squid species using thermometry equations from Grossman and Ku (1986) for aragonitic structures and Chung *et al.* (2020) for *Sepia pharaonis*, and found that overall, the equations accurately predicted experienced temperatures, but predictions became less accurate for octopus living at higher temperatures. These results suggest that species specific thermometry equations are required to confidently predict experienced temperatures (Martino, Chung, *et al.*, 2022).

Here, I reared octopus hatchlings under controlled temperature conditions to investigate the potential of $\delta^{18}O$ and $\delta^{13}C$ values in statoliths to track the metabolic rate and thermal histories of octopus. Specifically, I analysed isotopes in statoliths, seawater and diet and calculated the proportion of respired carbon or carbon from metabolic sources (C_{resp}) and then evaluated the relationships between isotopic values, C_{resp}, body mass and water temperature. Finally, I developed a

thermal reconstruction model using the $\delta^{18}O$ data to enable the retrospective reconstruction of water temperature using $\delta^{18}O$ statolith values.

Methods

Study species and hatchling rearing

This study focused on the southern keeled octopus, *Octopus berrima,* which is a commercially important species in South Australia and found in shallow coastal habitats throughout southern Australia (Martino *et al.*, 2021). Mature *O. berrima* females were collected from octopus fishery shelter pots from around the Eyre Peninsula, South Australia, and maintained in tanks at the South Australian Research and Development Institute (SARDI), West Beach. Octopus were fed a diet of live shore crabs (Grapsidae) sourced from the wild until the females laid eggs. After eggs were laid, no food was provided so the female could brood her eggs undisturbed as females do not eat during brooding and the presence of live crabs in the tank would add unnecessary stress. Monitoring checks continued every second day to ensure the healthy development of the brood.

Hatchlings from one female (clutch 1) were raised at ambient conditions (21.69 ±1.32 °C) for 110 days and hatchlings from a second female (clutch 2) were raised under controlled temperature conditions at 20 (19.8 ±0.8 °C), 22 (21.96 ±0.48 °C), and 24 °C (24.19 ±0.53 °C) for 90 days.

All tanks were continuous flow-through systems in which water was filtered multiple times before it was pumped into the tanks. Each tank had an air stone to ensure enough dissolved oxygen. Hatchlings reared in ambient seawater conditions were housed in 3, 55 L black, plastic flow through tanks (bottom area: 645 mm x 313 mm) in a covered outdoor facility (Fig. 4.1A) at a stocking density of ~1 octopus/L or 250 octopus/m^2 for about 55 octopus per tank. In these tanks, temperature and salinity was recorded with Onset HOBO (Massachusetts, USA) data loggers every 10 minutes.

Hatchlings reared at controlled temperatures, were housed in clear, glass 30 L (bottom area: 490 mm x 190 mm) flow-through tanks in an indoor controlled environment room (CER) at a stocking density of 1 octopus/3 L or 100 octopus/ m^2. Each tank housed 10 octopus and each temperature treatment had six replicate tanks except for the 24 °C treatment. This treatment had four tanks because animals in two tanks died due to an unforeseen system malfunction. Water temperature of each tank was recorded every 10 minutes with an Aquatronics system (Idaho, USA), and salinity was measured every second day with a refractometer.

Each tank had a clear lid and 350 µm mesh covering the inflow and outflow pipes to prevent escapes and evaporation. Octopus dens made of 15 mm diameter PVC tubes (Fig. 4.1B) were available in each tank, and octopus were offered one live rock pool shrimp (*Palaemon serenus*) per day. The shrimp were stunned by cold water prior to feeding. The size of the shrimp varied with octopus size but was typically ~50-100% of the size of the octopus.

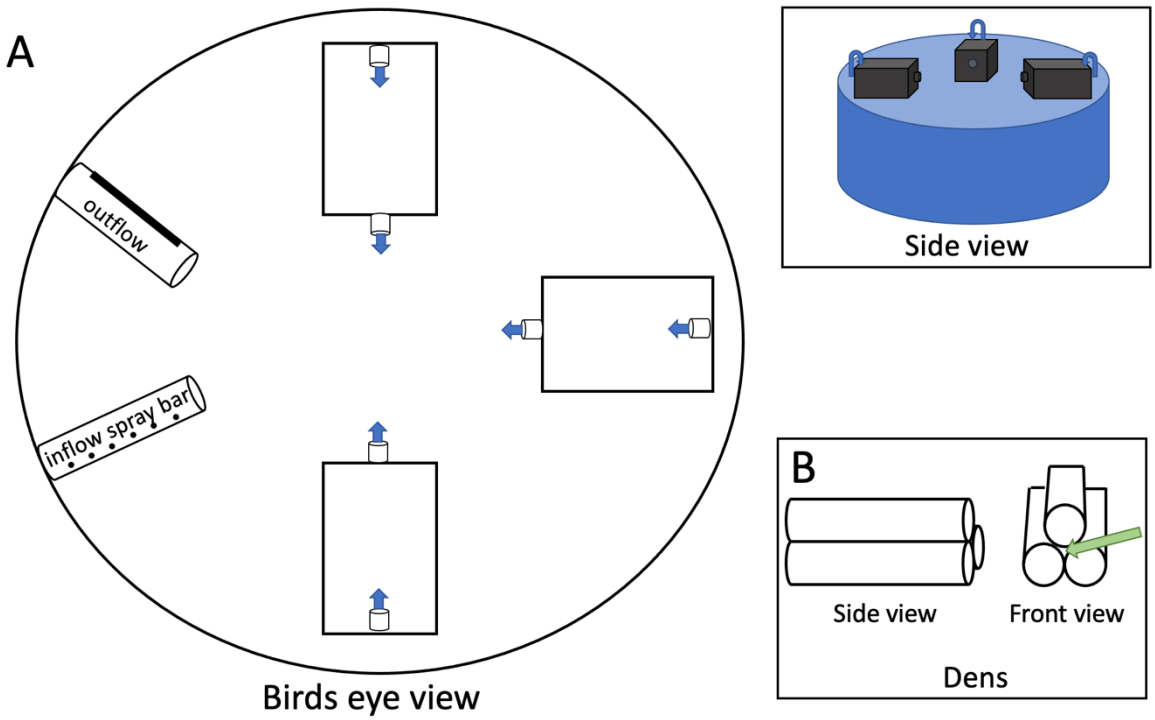


Figure 0.1: (A) Diagram of tank set up for octopus reared at ambient temperature where the large cylindrical tank is 5000 L and each smaller rectangular tank is 55 L. Blue arrows indicate direction of flow. (B) Diagram of an octopus den constructed of 15 mm diameter PVC tubes; the green arrow indicates the preferred den of the hatchlings when small.

Three octopus were randomly sampled from each tank at the beginning and end of the experiment, and any octopus that died during the experiment were also sampled. The sampled octopus were humanely euthanised with a lethal dose of magnesium chloride (3.5-4%), weighed, measured, and frozen for later dissection. The magnesium chloride bath comprised of 25-30 g of $MgCl_2$ dissolved in 200 mL of distilled water and then added to 500mL of seawater to maintain a normal salinity level. All procedures were approved by the Department of Primary Industries and Regions of South Australia Animal Ethics Committee (AEC#22/21).

Isotopic analyses

Statoliths and a section of muscle tissue from the mantle were extracted from each of the sampled octopus. Muscle tissue was sampled so that $\delta^{13}C$ values between muscle and diet could be compared, and aid future field studies where diet values are difficult to obtain. Muscle tissues were immediately frozen until further processing and statoliths were rinsed in Milli-Q water, airdried, and stored until cleaning. Organic material was cleaned from statoliths using 5% hydrogen peroxide in an ultrasonic cleaner for 5 minutes. The statoliths were then washed for five minutes in ultrapure water in an ultrasonic cleaner three times and dried overnight at 50 °C. Cleaned statoliths were analysed for $d^{18}O$ and $d^{13}C$ using a Nu Instruments NuCarb device coupled with a Nu Perspective Isotope Ratio Mass Spectrometer (IRMS) (UK) equipped with a dual inlet at the Mawson Analytical Spectrometry Services, University of Adelaide, South Australia. Approximately 80 μg of the sample was weighed, placed into glass vials, and evacuated to a high vacuum. Each sample was then reacted with 105% phosphoric acid at 70 °C, and the resultant carbon dioxide was transferred to the dual inlet and measured. The measured isotopic compositions were monitored and normalised to IAEA-CO-1 and internal calcite standards ANU P3 and UAC-1. External precision was calculated from repeated measurement of standards and was better than ±0.05 per mil (1 standard deviation (1sd)). Due to the miniscule size of the statoliths from the day 0 hatchlings, these were pooled, which resulted in only one sample per clutch for the day 0 age group (pooled weight: ~20 μg). The total statolith samples analysed were: 14 ambient temperature samples, one day-0 temperature treatment sample, nine 20 °C samples, nine 22 °C samples, and nine 24 °C samples.

Muscle tissues from 110-day old hatchlings were freeze dried and then powdered by hand with a mortar and pestle. Although octopus muscle tissue is low in lipids (Lee, 1994), lipids were extracted following the methods of Kojadinovic *et al.* (2008) to be consistent with other studies. Briefly, the methods were as follows: 2 mL cyclohexane was added per 0.1 g of powdered tissue, vortexed 2 times for 15 seconds, centrifuged for two minutes at 804 *g*. The supernatant was then pipetted off and discarded and the pellet was oven dried at 60 °C overnight. Approximately 2 mg of dry powdered muscle tissue was weighed, packed into tin capsules, and analysed for $\delta^{13}C$ values using an Elementar VarioIsotope cube coupled to an Isoprime isotope ratio mass spectrometer (IRMS) at the Flinders Analytical Lab in South Australia. The measured isotopic compositions were monitored and normalised to USGS40 and USGS41a standards. Precision was calculated from repeated measurement of standards and was better than ±0.07 per mil (1sd). Octopus diet (whole shrimp) was also analysed for $\delta^{13}C$ using the same methods.

The carbon and oxygen isotopic values of carbonates and muscle tissues are reported in the delta notation (δ) in per mil (‰), relative to the international isotope reference standard of Vienna Pee Dee Belemnite (VPDB).

$$\delta^{13}C = \left(\frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{standard}} - 1 \right) \times 1000\ ‰$$

$$\delta^{18}O = \left(\frac{\left(\frac{^{18}O}{^{16}O}\right)_{sample}}{\left(\frac{^{18}O}{^{16}O}\right)_{standard}} - 1 \right) \times 1000\ ‰$$

Water samples were taken from the inflow at the beginning, middle, and end of the experiment for subsequent analysis of $\delta^{18}O$ values and $\delta^{13}C_{DIC}$ values at the West Australian Biogeochemistry Centre (WABC), The University of Western Australia. $\delta^{18}O$ values were analysed using an Isotopic Liquid Water and Continuous Water Vapour Analyser Picarro 2130i (Picarro, Santa Clara, California, USA). Each sample was analysed six times with the first four results discarded to minimize any possible instrument memory effect. The raw $^{18}O/^{16}O$ values of samples were normalized to the VSMOW (Vienna Standard Mean Ocean Water) scale, based on three laboratory standards, each replicated twice and reported in per mil (‰) following the principles of the three-point normalization (Skrzypek, 2013). The laboratory standards provided by the international Atomic Energy Agency; VSMOW2, SLAP2 and GISP (Coplen, 1996), were calibrated against international reference materials that determine the VSMOW2-SLAP2 scale (Coplen, 1996) (VSMOW2 and $\delta^{18}O$ equal to 0 ‰ and SLAP equal to $\delta^{18}O$ = -55.50 ‰). The long-term analytical uncertainty (one standard deviation (1σ)) was determined as <0.05 ‰ for $\delta^{18}O$ (Skrzypek and Ford, 2014).

$\delta^{13}C_{DIC}$ values were analysed using a GasBench II coupled with Delta XL Mass Spectrometer (Thermo-Fisher Scientific) also at WABC. Prior to water sample injection, 12 mL vials with 0.1 mL of 100 % phosphoric acid were flushed with ultra-high purity helium (99.999 %), and then reacted at 25 °C over 24 hour (Paul and Skrzypek, 2006). The water volume used for each sample was adjusted

accordingly to match the peak heights of reference materials and avoid potential non-linearity effects. All results were expressed using the standard δ-notation ($\delta^{13}C$) and were reported in per mil (‰) after normalization to Vienna Pee Dee Belemnite isotope scale [VPDB]. The multi-point normalization was based on three international standards NBS18, NBS19 and L-SVEC for $\delta^{13}C$, each replicated twice (Skrzypek, 2013). The analytical uncertainty was ≤ 0.10 ‰ (1σ).

Isotopic mixing model

To calculate the proportional contribution from carbon sources to statoliths, the following isotopic mixing model was used (Schwarcz *et al.*, 1998; Solomon *et al.*, 2006; McConnaughey and Gillikin, 2008; Chung, Trueman, Godiksen and Grønkjær, 2019):

$$\delta^{13}C_{stato} = C_{resp} \times \delta^{13}C_{diet} + \left(1 - C_{resp}\right) \times \delta^{13}C_{DIC} + \varepsilon_{total}$$

or

$$C_{resp} = (\delta^{13}C_{DIC} - \delta^{13}C_{stato} \times \varepsilon_{total})/(\delta^{13}C_{DIC} - \delta^{13}C_{diet})$$

Where C_{resp} is the proportion of respired carbon or carbon from metabolic sources (diet), $\delta^{13}C_{stato}$ is the $\delta^{13}C$ value from the statolith, $\delta^{13}C_{diet}$ is the $\delta^{13}C$ value from diet, $\delta^{13}C_{DIC}$ is the value from dissolved inorganic carbon (DIC) in ambient seawater, and ε_{total} is the total net isotopic fractionation during carbon exchange between the DIC, blood, and fluid in which the statolith is formed. Because there is no validated value for cephalopod statoliths, the value of 2.7 ‰ is used for ε_{total} which was estimated from an experiment on inorganic aragonite formation (Romanek *et al.*, 1992; Chung *et al.*, 2021).

Statistical Analyses

Growth rates of ambient and temperature controlled reared octopus were explored to better understand how two different tank set ups affect growth. The best method to display growth rate was determined by fitting different regression curves such as linear, polynomial, power, and exponential, to weight with age data, and the best fit was chosen by selecting the highest r^2.

To determine if octopus muscle tissue can be used as a proxy of diet, $\delta^{13}C_{diet}$ was subtracted by $\delta^{13}C_{muscle}$ to calculate the trophic discrimination factor (TDF), and a t-test was done to test if $\delta^{13}C_{diet}$ and $\delta^{13}C_{muscle}$ were significantly different.

To assess whether there were differences in $\delta^{13}C$ values and $\delta^{18}O$ values between replicate tanks within temperature treatments, PERMANOVAs were conducted with replicates (tanks) included as fixed factors. No significant tank effects were present for $\delta^{13}C$ values, $\delta^{18}O$ values, or C_{resp} ($\delta^{13}C$ and C_{resp}: p=0.82; $\delta^{18}O$: p=0.08). Therefore the PERMANOVAs were re-run with tanks pooled within each treament. Pair-wise Wilcoxon tests were then conducted to determine differences among treatments.

Linear regression models were used to determine the relationships between body mass and $\delta^{13}C$ values, C_{resp}, and $\delta^{18}O$ values for each clutch. There was no clear linear relationship with $\delta^{18}O$ therefore, a Kendall's Tau rank test was used test for correlation.

A collated dataset of C_{resp}, and $\delta^{18}O$ values from cephalopod carbonates from Chung *et al.* (2021) were used as a comparison to understand the isotopic incorporation of *O. berrima*.

Analyses and figures were created in the statistical program, R (R Core Team, 2022).

Thermal reconstruction model

To further develop $\delta^{18}O$ values as a tool to reconstruct the thermal histories of octopus, I then used the $\delta^{18}O$ and temperature data to create a simple thermal reconstruction model or thermometry equation in which:

$$\delta^{18}O_{\text{statolith, VPDB}} - \delta^{18}O_{\text{water, VSMOW}} = X * temperature\ (°C) + b$$

or

$$Estimated\ temperature\ (°C) = X * (\delta^{18}O_{\text{statolith}} - \delta^{18}O_{water}) + b \quad \text{(Eq. 1)}$$

While the above equations are in the form more familiar to a biologist or ecologist, to enable comparisons with palaeogeological studies, I also present the model in different units (noting the slope and intercept remain the same). Specifically, temperature (°C) was converted to 1000/ temperature (Kelvins) and $\delta^{18}O_{statolith,\ VPDB} - \delta^{18}O_{water,\ VSMOW}$ was converted to 1000 times the natural log (ln) of the fractionation factor, which was defined as α where:

$$\alpha_{carbonate-water} = \frac{\delta^{18}O_{carbonate,VPDB} + 1000}{\delta^{18}O_{water,VPDB} + 1000}$$

$\delta^{18}O_{water,\ VSMOW}$ was converted to $\delta^{18}O_{water,\ VPDB}$ by using the equation of Friedman and O'Neil (1977):

$$\delta^{18}O_{water,VPDB} = \delta^{18}O_{water,VSMOW} \times 0.97006 - 29.94$$

To calculate uncertainties associated with the thermometry equation, Isoplot in Microsoft excel and a Monte Carlo approach in the program R (R Core Team, 2022) were used.

Equation 1 was then used to "reconstruct" the average experienced tempeatures of six individuals that were raised at ambient conditions (3 60-day olds, and 3 110-day olds). These individuals were raised in different tanks and were from a different clutch from those reared at controlled temperatures. Reconstructed temperatures were then plotted against recorded temperatures to determine the efficacy of the model.

Results

Octopus rearing

Water temperature in aquarium tanks were relatively constant throughout the experiment (Table 4.1), alongside salinity which remained at 40 ‰. $\delta^{18}O$ and $\delta^{13}C_{DIC}$ values from analysed tank water were also fairly constant throughout the experiment ($\delta^{18}O$= 0.97 ±0.04 ‰ VSMOW; $\delta^{13}C_{DIC}$= 0.60 ±0.16 ‰).

Octopus in the 24 °C treatment had the lowest body weight and survivability (Table 4.1). Hatchlings from both clutches exhibited exponential growth with those raised in the controlled temperature experiment having the fastest growth (Fig. 4.2).

Table 0.1: *Octopus berrima* rearing conditions, average weight (g) at 90 days old, and survivability to 90 days of age.

Water temperature	Actual recorded	Average body weight (g)	Survivability

treatment (°C)	temperature (1SD, °C)		(%)
20	19.8 ±0.8	3.05 ±0.57	83
22	21.96 ±0.48	3.17 ±0.97	82
24	24.19 ±0.53	1.67 ±0.55	25

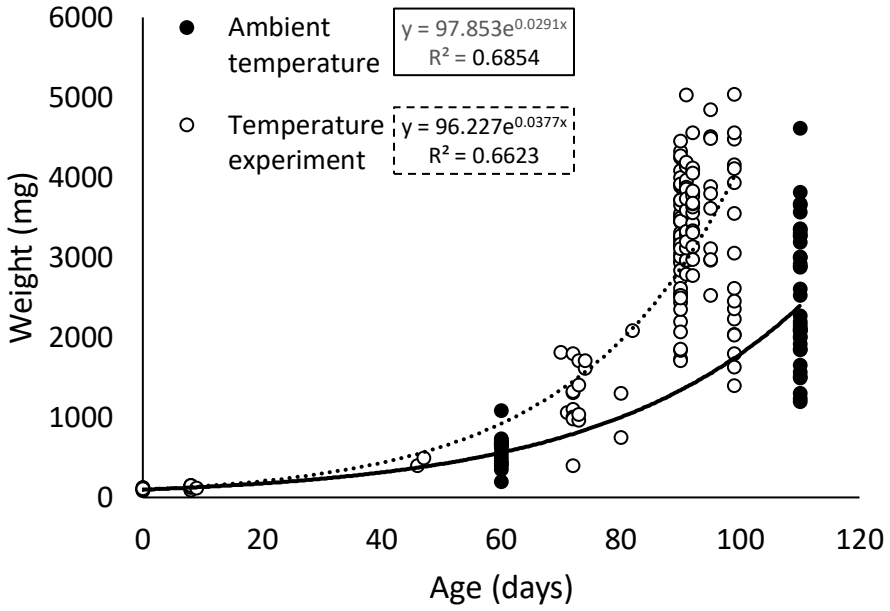


Figure 0.2: Growth rates of *Octopus berrima* hatchlings. Black circles represent clutch 1 raised at ambient conditions (21.69 ±1.32 °C) and white circles represent clutch 2 reared at three controlled temperature treatments (19.8 ±0.8 °C, 21.96 ±0.48 °C, 24.19 ±0.53 °C). Data are fit with exponential curves.

Isotopic results

The $\delta^{13}C$ values in muscle tissue (mean: -12.88 ±0.06 ‰) and diet (mean: -14.84 ±0.30 ‰) were significantly different (Welch two sample t-test, *p*= 0.0007) and yield a trophic discrimination factor (TDF: $\delta^{13}C$ or $\Delta^{13}C$) of ~2 ‰.

For octopus reared at ambient conditions, there was a significant relationship between octopus body mass and $\delta^{13}C$ values in statoliths and C_{resp} (Fig. 4.3A & 4.B) (linear regression models: $\delta^{13}C$ and C_{resp}, *df*= 5, R^2= 0.72, *p* =0.016). $\delta^{13}C$ values in statoliths were closer to $\delta^{13}C_{DIC}$ values in the seawater (0.60 ±0.16 ‰) for younger and smaller octopus and closer to $\delta^{13}C_{diet}$ values (-14.84 ±0.30 ‰) for older and larger octopus. The opposite trend was observed for C_{resp}, whereby C_{resp} increased with increasing body mass. There was no correlation between $\delta^{18}O$ values and octopus body mass (Kendall's Tau: *T*=9, *p*=0.77, *tau*=-0.14) (Fig. 4.3C).

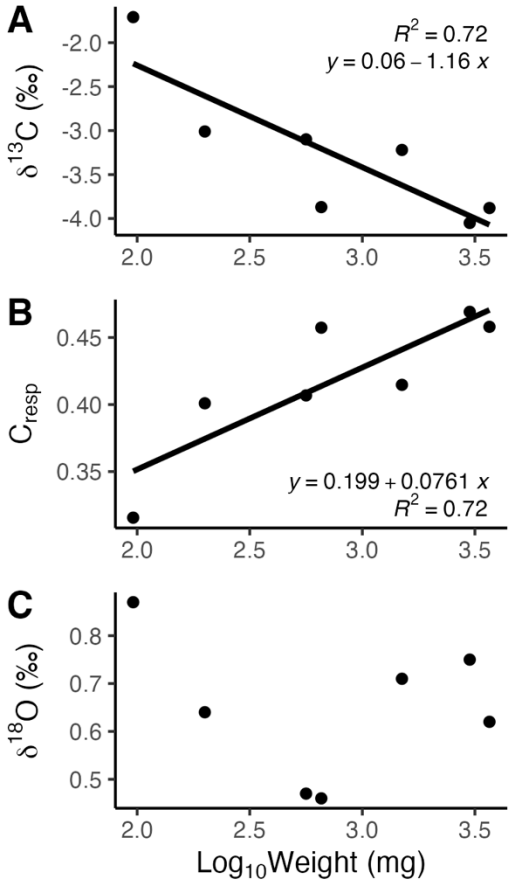


Figure 0.3: Change in (A) $\delta^{13}C$ values (slope= -0.076), (B) proportion of metabolically derived carbon (C_{resp}) (slope= 0.076), and (C) $\delta^{18}O$ values in *Octopus berrima* statoliths with octopus body mass (Log_{10} weight, mg) when reared at ambient temperature (clutch 1).

For octopus reared in controlled temperatures (clutch 2), $\delta^{13}C$ values and C_{resp} were weakly correlated with octopus body mass (Fig. 4.4A and C) (linear regression: *df*= 25, R^2= 0.16, *F*= 5.94, *p*=0.02).

PERMANOVAs demonstrated that $\delta^{13}C$ values and subsequently C_{resp} were not significantly different with different rearing temperatures (*df*=2, *p*=0.64) (Fig. 4.4B and 4.4D). Conversely, $\delta^{18}O$ values were significantly different between octopus raised at 20, 22, and 24°C (*df*= 2, *p*=0.001) (Fig. 4.4F).

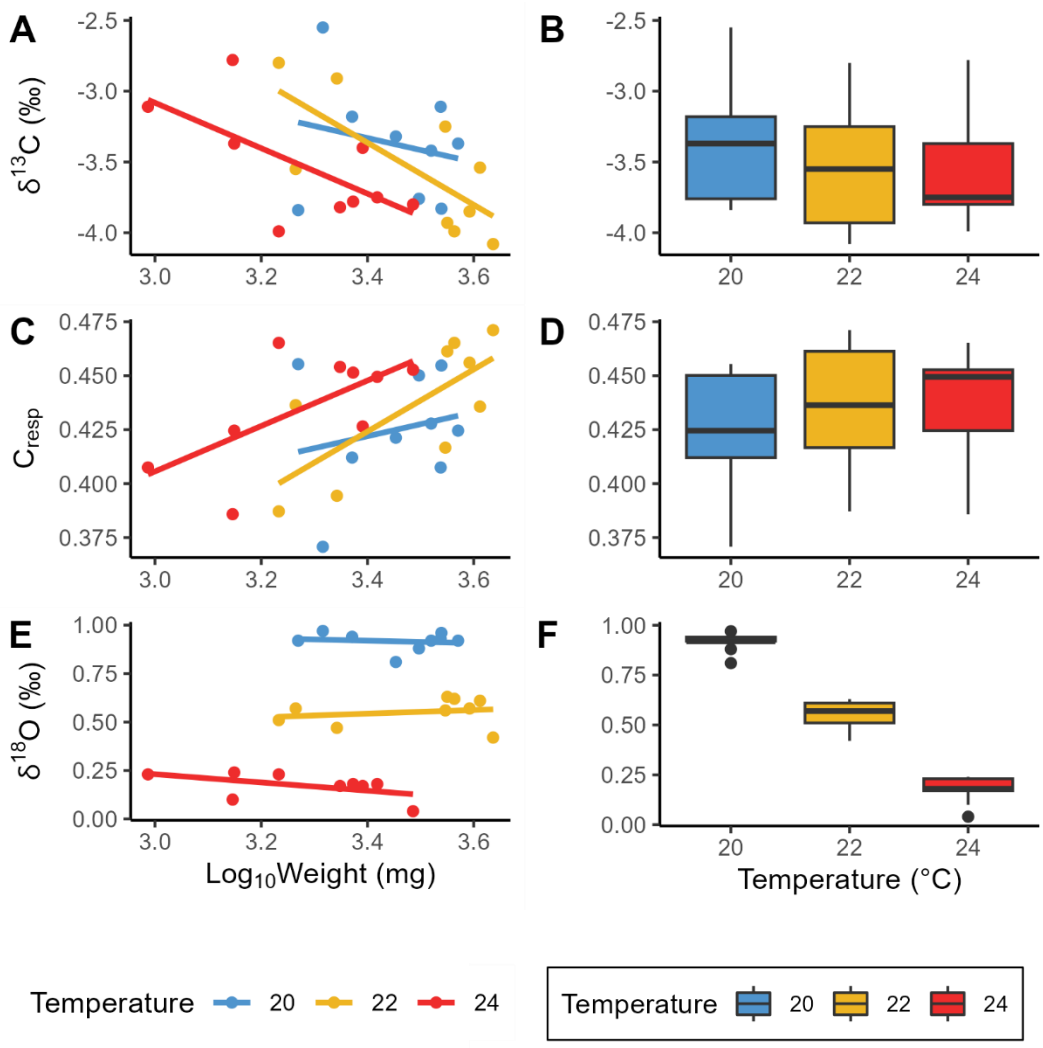


Figure 0.4: Change in $\delta^{13}C$ values (A-B), proportion of metabolically derived carbon (C_{resp}) (C-D), and $\delta^{18}O$ values (E-F) in *Octopus berrima* statoliths with body mass (weight, mg) and rearing temperature (20, 22, 24 °C) for clutch 2 (73-99 days old).

Table 0.2: Relationships between proportional metabolic carbon (C_{resp}) and $\delta^{18}O$ values (‰) with temperature (°C) recorded from the field and in laboratory experiments from different cephalopod species collected from Chung *et al.* (2021) including *Octopus berrima* from this current study. A visual representation including $\delta^{13}C$ values (‰) is included in the supplementary material as supplementary figure 4.1.

Cephalopod species	Habitat type	C_{resp} range	Relationship between C_{resp} and temperature		Relationship between $\delta^{18}O_{statolith}$ values and temperature		Study ([W] for wild caught and [C] for captive raised)
			Slope	Intercept	Slope	Intercept	
Architeuthis dux	Deep sea	0.23-0.25	0.009	0.134	-0.209	4.396	Landman *et al.* (2004)[W]
Argonauta argo	Pelagic	0.19-0.23	0.010	0.078	-0.217	2.638	Stevens *et al.* (2015)[W]
Dosidicus gigas	Deep sea	0.42-0.48	0.014	0.168	-0.200	3.360	Trasviña-Carrillo *et al.* (2018)[W]
Illex illecebrosus	Open pelagic	0.46-0.50	-0.008	0.572	-0.072	1.397	Radtke (1983)[W]
Nautilus macromphalus	Open pelagic Vertical migrations	0.16	0.000	0.160	0.290	-4.169	Taylor and Ward (1983)[W] Auclair *et al.* (2004)[W]
Nautilus pompilius	Open pelagic Vertical migrations	0.08-0.20	-0.013	0.348	-0.039	1.651	Taylor and Ward (1983)[W] Zakharov *et al.* (2006)[W] Kruta *et al.* (2014)[W]
Octopus berrima	Coastal benthic	0.36-0.47	0.002	0.383	-0.163	4.114	Current study[C]
Sepia officinalis	Coastal semi-benthic	0.27-0.35	0.002	0.276	-0.147	3.451	Bettencourt and Guerra (1999)[W] Rexfort and Mutterlose (2006)[CW] Dance *et al.* (2014)[W]

Sepia pharaonis	Coastal semi-benthic	0.40-0.50	0.011	0.161	-0.113	1.817	Chung *et al.* (2020, 2021)[C]
Spirula spirula	Deep sea Vertical migrations	0.20-0.24	0.018	-0.043	-0.228	4.896	Price *et al.* (2009)[W]
Average	all	0.35	0.017	0.009	-0.102	2.500	Compiled data from all above studies

Reconstructing temperature using $\delta^{18}O$ values

A linear regression model showed a significant decrease in $\delta^{18}O$ values of statoliths with increases in temperature (*df*= 40, R^2= 0.91, *F*= 382.8, *p* <0.0001) (Fig. 4.5A). The resulting linear equation or thermal reconstruction model could be described as:

$$Estimated\ temperature\ (°C,\ \pm 1.77) = -6.29\ \pm 0.44 * (\delta^{18}O_{statolith} - \delta^{18}O_{water}) + 19.15 \pm 0.48 \quad \text{(Eq. 1.1)}$$

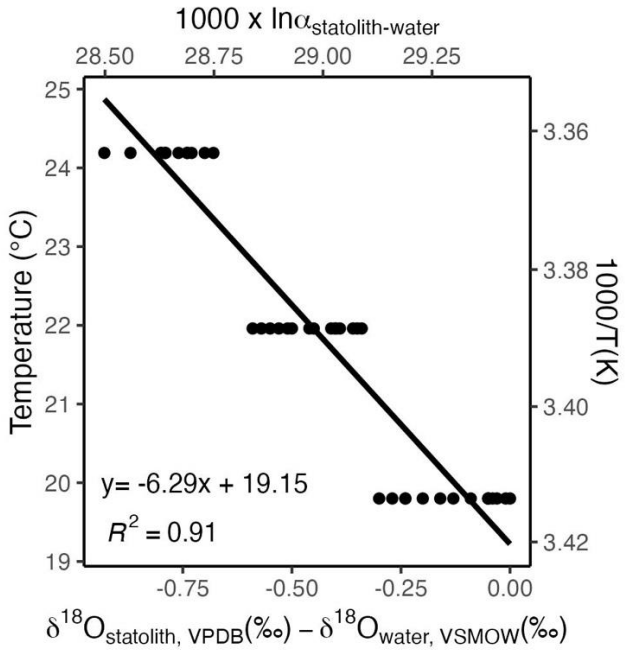


Figure 0.5: The relationship between temperature (°C) and $\delta^{18}O$ values from *Octopus berrima* statoliths (VPDB) - $\delta^{18}O_{seawater}$ values (VSMOW). The data is presented using units traditionally used in ecology (left and bottom axes) and geology (right and top axes).

The resulting uncertainty for the model's reconstructed temperature was ±1.77 °C. When testing the accuracy of the thermometry model, temperatures reconstructed from statolith $\delta^{18}O$ values were slightly lower than recorded temperatures for Clutch 1 (raised at ambient temperatures) but well within the margins of uncertainty (±1.77 °C) (Fig. 4.6).

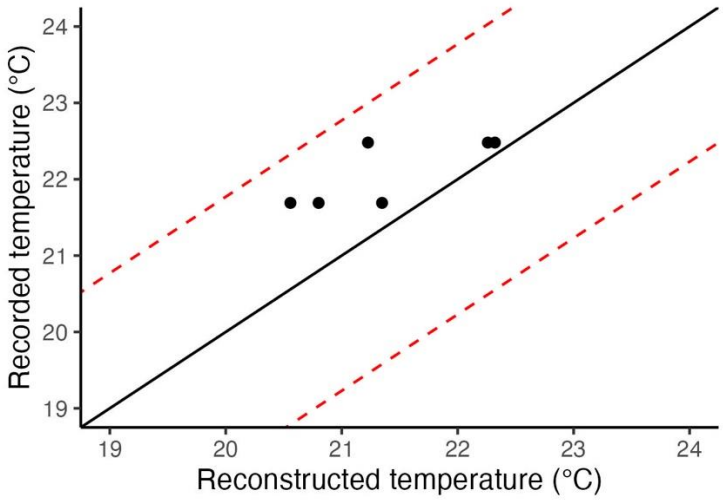


Figure 0.6: Relationship between reconstructed temperature (derived using equation 1.2 and $\delta^{18}O$ values) and experienced temperatures (derived from the average recorded tank temperature) of six *Octopus berrima* individuals raised in ambient conditions. The black line represents the mean estimated temperature and the red dotted lines represent the standard error (±1.77 °C).

Discussion

$\delta^{13}C$ values as a metabolic proxy

I show experimental evidence of a strong link between $\delta^{13}C$ values and body mass, and therefore metabolic rate (C_{resp}). I found that there was a negative relationship between $\delta^{13}C$ values and body mass, which suggests that as octopus grow, they assimilate more carbon from metabolic sources, such as diet. This relationship contrasts with relationships observed in fish where, typically, body size and $\delta^{13}C$ values are positively correlated and C_{resp} negatively correlated (Mulcahy *et al.*, 1979; Schwarcz *et al.*, 1998; Brown *et al.*, 2004). The difference in the result may be due to different growth patterns in octopus compared to fish. Octopus have a "live fast, die young" strategy and grow rapidly throughout their lives, with an initial fast, exponential phase and a slower power phase (O'Dor and Webber, 1986; Zielinski and Portner, 2000; Semmens *et al.*, 2004). Another reason why these results may differ to those of fish is because studies on fish (Schwarcz *et al.*, 1998; Jamieson *et al.*, 2004) and other longer lived species (Kleiber, 1932, 1947) often observe older individuals (i.e., >1 year old), whereas this study focused on octopus under 110 days old when they are growing exponentially. Some longer lived species, such as cod (Schwarcz *et al.*, 1998), also often have ontogenetic shifts to consume higher trophic level food with higher $\delta^{13}C$ values which influences the calculation of metabolic rate. There were no diet changes in the present study, suggesting a more direct link to metabolic rate.

In this study, C_{resp} ranged from 0.3 to 0.47 meaning 30-47 % of the carbon in the statoliths was derived from their diet. In fish, C_{resp} ranges between 0.1 and 0.3 meaning they only get 10-30 % of their carbon from their diet and 70-90 % from DIC through processes such as respiration (Kalish, 1991a; Solomon *et al.*, 2006; Chung, Trueman, Godiksen and Grønkjær, 2019; Martino *et al.*, 2019; Alewijnse *et al.*, 2021). The high C_{resp} in octopus could be attributed to their rapid growth rates at this age as more food is needed to grow so more carbon is being absorbed from their diet.

Among the controlled temperature treatments, I expected to see a statically significant increase in C_{resp} with temperature, and only observed a slight positive trend. A similar trend had previously been found for *Sepia officialis* (Table 2, Supp. Fig. 1) in which no correlation was found between $\delta^{13}C$ values (and therefore C_{resp}) and environmental temperature (Bettencourt and Guerra, 1999). In contrast, Chung *et al.* (2021) observed a significant change in $\delta^{13}C$ statolith values and C_{resp} with increasing temperature, in the pharaoh cuttlefish*, Sepia pharaonis.* However, they had a wider temperature range among treatments (9 °C) compared to this study (4 °C). Chung *et al.* (2021) also had a larger difference between DIC and $\delta^{13}C_{diet}$ values (26 ‰) than in this study (14 ‰), which can amplify these effects.

When calculating proxies such as C_{resp}, a $\delta^{13}C_{diet}$ value is required, but if the diet is unknown or not easily accessible, $\delta^{13}C$ from muscle tissue is often used as a proxy. Studies on fish have justified doing so because the trophic discrimination factor (TDF) is often around 1 ‰ which can be considered minimal (DeNiro and Epstein, 1978; Deniro and Epstein, 1981; Post, 2002; Stephens *et al.*, 2022), but data on the TDF in cephalopods is lacking. Here, I showed a TDF of 2 ‰ for *O. berrima* which is double of the average TDF of fish (Stephens *et al.*, 2022). Because of the relatively high TDF for *O. berrima* and limited data to date, I suggest that the TDF should be validated for individual cephalopod species, but if this is not feasible a TDF of 2 ‰ should be used to adjust the $\delta^{13}C_{muscle}$ value to predict a more accurate $\delta^{13}C_{diet}$ value.

$\delta^{18}O$ values as an environmental temperature proxy

Our results suggest that $\delta^{18}O$ values in octopus statoliths can be used as a temperature proxy to estimate past thermal histories, with clear differences in $\delta^{18}O$ values among temperature treatments. Using $\delta^{18}O$ values as a thermal proxy has been tested and applied in other cephalopods with a slope ranging from -0.23 to -0.14 (Chung *et al*., 2021, supplementary material; Martino *et al.,* 2022). The similarity in slopes between cephalopod species suggest that they assimilate $\delta^{18}O$ values into their carbonates at a similar rate. The relationship from this present study (-0.16) is most like that of the

cuttlefish, *Sepia officinalis* (-0.15) (Bettencourt and Guerra, 1999; Rexfort and Mutterlose, 2006; Dance *et al.*, 2014), as well as the Japanese eel, *Anguilla japonica* (-0.15) (Shirai *et al.*, 2018). With similar slopes, but different y-intercepts, these species are a good example of a similar fractionation rates with different species, and with different thermal ranges (Morissette *et al.*, 2023). Although species might have similar fractionation rates, the relationship between temperature and $\delta^{18}O$ values needs to first be determined for each species to accurately reconstruct temperatures from carbonate $\delta^{18}O$ values. Since $\delta^{18}O$ values did not change with weight in the current study, it indicates that size does not influence $\delta^{18}O$ values and that $\delta^{18}O$ temperature reconstruction models can likely be applied to all life stages.

When I tested the thermometry model on *O. berrima* individuals raised in ambient tank conditions, reconstructed temperatures were within the margins of model uncertainty (±1.77 °C). However, to reduce the uncertainty and improve the model's accuracy, more data would be needed at a wider range of temperatures. Furthermore, application of this equation to other samples will require propagation or modelling of uncertainties using the relevant measurement uncertainties.

Comparison with other cephalopods

I found that the isotope-temperature relationships in *O. berrima* were similar to cuttlefish (Sepiidae) and quite different from pelagic squid (Ommastrephidae) and nautilus (Nautilidae). This is most likely due to the range of life history strategies: nautilus are taxonomically distinct from all other cephalopods and are long-lived, slow-growing, and undergo vertical migrations, whereas pelagic squids are highly mobile and spend their entire life in the water column migrating between different water masses with varying temperatures. Due to their highly migratory behaviour, the use of isotope-temperature relationships for pelagic squid and nautilus is much more complex than nonmigratory species such as cuttlefish and octopus.

While cuttlefish and octopus are fast growing and short-lived like squid, they are largely demersal or benthic and have limited lifetime movement as adults (Seibel and Childress, 2000; Seibel and Drazen, 2007; Seibel, 2016). *O. berrima* also have large, benthic, hatchlings similar to cuttlefish. Due to their similar lifestyles, it is logical that octopus and cuttlefish are similar in the way they incorporate $\delta^{13}C$ and $\delta^{18}O$ than more mobile species.

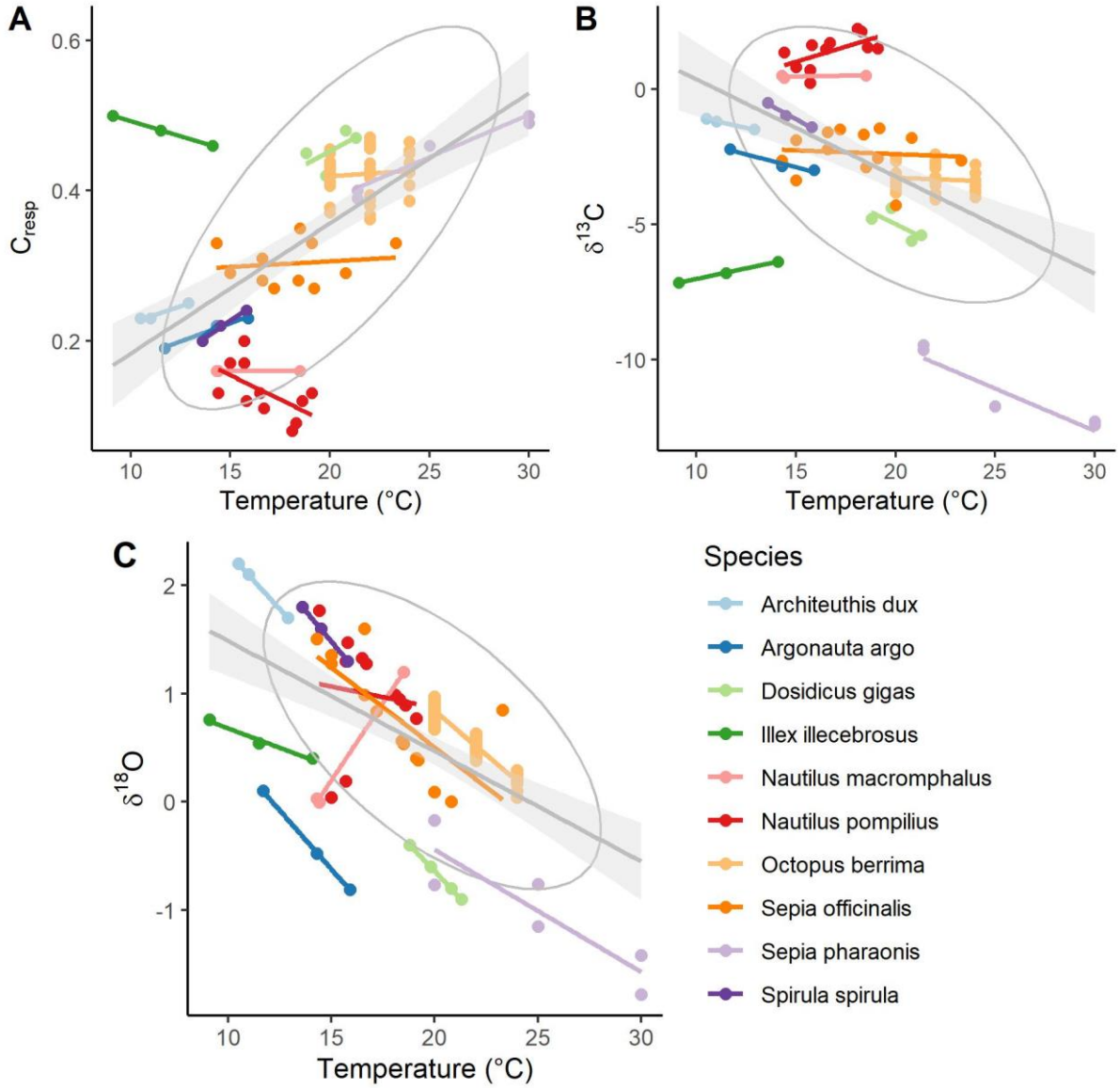
Conclusion

In conclusion, I present evidence that $\delta^{13}C$ values in juvenile octopus statoliths serve as an effective proxy for metabolic rate. By knowing this relationship between metabolic rate and body weight as well as temperature, future research can predict how the metabolic demands for octopus might change with ocean warming. Furthermore, $\delta^{18}O$ values in juvenile octopus statoliths were accurate recorders of experienced temperatures. Knowing these relationships between temperature and $\delta^{18}O$ carbonate values can also provide insight into how cephalopods are responding and adapting to ocean warming. Overall, this research underscores the value of using stable isotopes in biogenic carbonates to reconstruct the biological and environmental histories of marine animals, particularly cephalopod species, further expanding the ability to understand their ecology in a rapidly changing world.

Data Availability

The data underlying this article are available in Open Science Framework at: https://osf.io/ge9fn/?view_only=f00e02daa92043cdb0534bf0ad6c4617

Supplementary Material



Supplementary Figure 0.1: Change in (A) proportional metabolically derived carbon (C_{resp}), (B) $\delta^{18}O$ values (‰), and (C) $\delta^{13}C$ values (‰) with temperature (°C) for different cephalopod species (compiled by Chung *et al.*, 2021) including *Octopus berrima* from this current study (details included in Table 2). The grey line and ellipse indicate an average for all species and the shaded portion is the 95% CI.

Isotopic trends in an historical cuttlebone archive spanning six decades

This research is in preparation to be submitted to Estuarine, Coastal, and Shelf Science

Abstract

Isotopic markers ($\delta^{13}C$ and $\delta^{18}O$) in marine animals can unearth long lasting records of biological and environmental change, including climate change, but have rarely been studied in cephalopod hard parts. To understand the challenges and limitations of isotopic markers in cuttlebones and reconstruct long-term histories, I collated the first multi-decadal archive and isotopic record of cuttlebones. A collection of Australian giant cuttlefish (*Sepia apama*) cuttlebones was sourced from museums, research repositories and private donations and represented multiple sites around southern Australia. Stable carbon isotopes ($\delta^{13}C$) and oxygen isotopes ($\delta^{18}O$) in the cuttlebones were analysed. Then, generalised additive models (GAM) and linear regression models were used to assess temporal and spatial trends, and the influence of cuttlebone size on $\delta^{13}C$ and $\delta^{18}O$ cuttlebone values. I found that spatial trends overrode temporal trends, and that $\delta^{13}C$ and $\delta^{18}O$ values in carbonate were strong geographical predictors. However, I observed temporal changes in $\delta^{13}C$ and $\delta^{18}O$ values in cuttlebones collected from a single oceanographic region. This is likely driven by a changing climate, and specifically, ocean warming and the increase in atmospheric carbon, otherwise known as the Suess effect. This study highlights both the challenges and benefits of using novel carbonate archives to reconstruct biological and environmental change in the marine environment.

Introduction

Marine hard parts composed of calcium carbonate ($CaCO_3$), such as shells, ear stones, and coral skeletons, are valuable tools for studying past environmental conditions (McConnaughey, 1989a; McConnaughey and Gillikin, 2008). Hard parts provide a permanent record of chemical data, preserve well, and are often abundant in museums and research agencies. Although working with historical archives can be challenging because their opportunistic collection leads to intermittent records, they offer a cost-effective alternative to direct monitoring and generating multi-decadal data, which is sorely lacking in the marine environment (McClenachan *et al.*, 2012). As such, hard tissues offer a unique opportunity to unveil the environmental and biological histories of marine animals, which would otherwise be difficult or impossible to collect.

Stable oxygen and carbon isotopes are useful for reconstructing environmental and biological change, particularly in biogenic carbonates. Oxygen isotopic ratios (herein referred to as $\delta^{18}O$) in marine carbonates are in close isotopic equilibrium with the seawater (Grossman and Ku, 1986; McConnaughey, 1989a, 1989b; Kalish, 1991b) and inversely correlated with temperature, whereby $\delta^{18}O$ values are lower at higher temperatures and higher in cooler temperatures (Grossman and Ku, 1986). The negative correlation between $\delta^{18}O$ in carbonates and temperature has been demonstrated in a variety of marine animals including fish otoliths (Høie *et al.*, 2003, 2004; Kitagawa *et al.*, 2013; Sakamoto *et al.*, 2017; Shirai *et al.*, 2018), cuttlefish statoliths and cuttlebones (Chung, Chen, *et al.*, 2020), and mollusc shells (Grossman and Ku, 1986; Owen *et al.*, 2008; Nishida *et al.*, 2015). Carbon isotopic ratios ($\delta^{13}C$) are also commonly studied in biogenic carbonates. Carbon in carbonates are derived from two sources: dissolved inorganic carbon (DIC), such as CO_2 in seawater, and diet (Solomon *et al.*, 2006; McConnaughey and Gillikin, 2008), in which relative proportions are dependent on biological factors such as metabolic rate (Chung, Trueman, Godiksen and Grønkjær, 2019; Martino *et al.*, 2019). Therefore, $\delta^{13}C$ values can provide information on both environmental carbon and metabolic rate of the animal.

As a proportion of the carbon in biogenic carbonates originates from the environment, $\delta^{13}C$ values can reveal the environment that the animal was exposed to. For example, climate change, particularly the increase in environmental carbon dioxide (CO_2) through the burning of fossil fuels coupled with mass deforestation and the general decline of photosynthetic organisms can be observed through a phenomenon called the Suess effect (Keeling, 1979; Körtzinger *et al.*, 2003). The Suess effect is the dilution of rare, heavy isotopes (^{13}C and ^{14}C) by the addition of the dominant, light

isotope (^{12}C) in the atmosphere, changing the isotopic ratio of carbon in the atmosphere and oceans (Keeling, 1979; Körtzinger *et al.*, 2003). The Suess effect can be identified by the decrease in $\delta^{13}C$ values over time, and as the ocean's isotopic composition is reflected in tissue isotopes, the Suess effect can be observed in marine tissues including fish otoliths (Schloesser *et al.*, 2009; Fraile *et al.*, 2016), whale skin (Borrell *et al.*, 2018), seal teeth (Newsome *et al.*, 2007), and mussel shells (Pfister *et al.*, 2011). The magnitude of the Suess effect can vary by region, since different oceans have different CO_2 absorption rates, thereby enhancing or diluting the effect (Eide *et al.*, 2017). Archived collections are useful because they can be used to reconstruct past conditions and specifically to observe changes in atmospheric CO_2.

The Australian giant cuttlefish (*Sepia apama*) is a demersal species and has limited lifetime movement as juveniles and adults (Gillanders *et al.*, 2016) As such, the species is particularly useful for recording long-term environmental change. Here, I utilised museum and past research collections by first collating historical and modern cuttlebone samples of *Sepia apama*, and then analysing samples for $\delta^{13}C$ and $\delta^{18}O$ values to reconstruct environmental and biological change in the marine environment. The resulting cuttlebone collection spanned several decades but were sporadically distributed along the southern coastline of Australia. Therefore, specifically, I aimed to investigate and disentangle temporal and spatial trends in isotopic values, as well as investigate the influence of cuttlebone size (body size) on spatial and temporal values.

Methods

Study species

The Australian giant cuttlefish (*Sepia apama*) is a neritic demersal species found in shallow waters up to 100m in depth and is distributed across southern Australia from Moreton Bay, Queensland (27° 25'S, 153° 20'E) to Point Cloates, Western Australia (22° 43'S, 113° 40'E) (Roper *et al.*, 1984; Voss and Sweeney, 1998) (Fig. 5.1). *Sepia apama* is the largest species of cuttlefish in the world and forms a well-known breeding aggregation in the upper Spencer Gulf in South Australia (Hall and Hanlon, 2002) (Fig. 5.1).

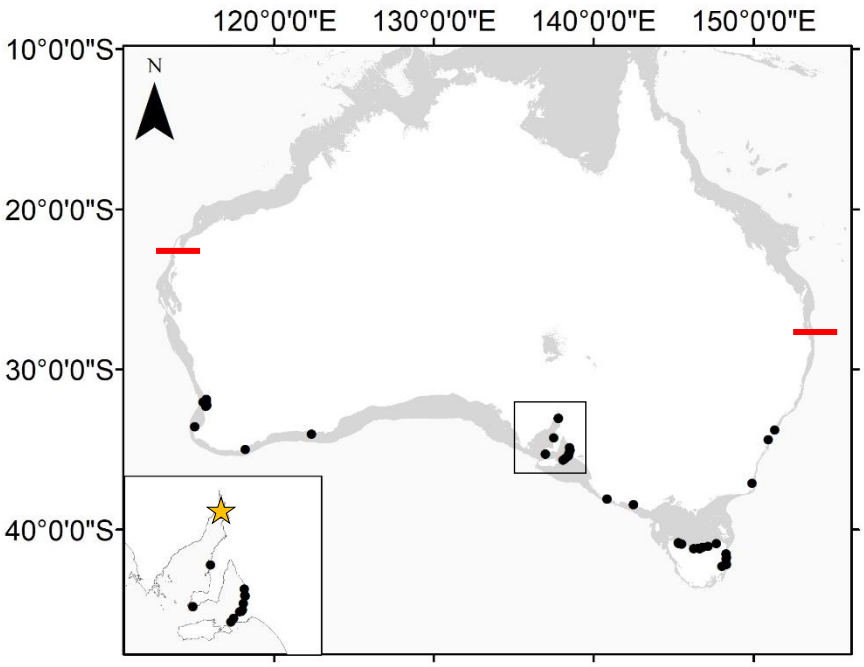


Figure 0.1: Map of *Sepia apama* sites where cuttlebones were collected (black dots) and the location of the breeding aggregation in which cuttlefish were collected (yellow star). Between one and three cuttlebones were collected from each site except for the aggregation where 10 cuttlebones were collected for a total of 71 samples. Note that some sites appear to overlap due to the resolution of the map. The grey shaded portion represents the 100-meter depth contour and the northern limits of the species distribution is indicated by red lines.

Cuttlebone collection

Modern and historical *S. apama* cuttlebone samples from coastal southern Australia were sourced from museum archives (South Australian Museum, the Tasmania Museum and Art Gallery, the Western Australian Museum), scientific surveys (Kassahn *et al.*, 2003), and donations from beachcombers. Cuttlebones were identified as *S. apama* by the presence of a large callus (Fig. 5.2). A total of 71 cuttlebones were obtained spanning from 1861 to 2022. Some cuttlebones were obtained whole and other samples were available as small cuttlebone biopsies (1 mm^3), which had been extracted from each cuttlebone 50 mm from the posterior tip of the callus (Fig. 5.2), representing a mid-life stage. Whole cuttlebones were biopsied in a similar manner. As *S. apama* typically have a one- two-year lifespan (Hall *et al.*, 2007), I assumed the carbonate biopsy sample represented the year the cuttlebone was collected. As only 5 historical samples were available from 1861 to 1959, these were excluded from the statistical analyses. The length of each cuttlebone was also measured as it is a strong predictor of cuttlefish size (Almonacid-Rioseco *et al.*, 2009; Chung, Huang, *et al.*, 2020).



Figure 0.2: Photo of a *Sepia apama* cuttlebone demonstrating the site of biopsy (red circle), 50 mm from the callus (indicated by the arrow), and along the ventral midline. Scale bar equals 5 cm.

Isotopic analyses of cuttlebones

Cuttlebone biopsies were cleaned following the methods in (Chung, Chen, *et al.*, 2020), where the biopsies were soaked in hydrogen peroxide in an ultrasonic cleaner for 5 minutes followed by 3 rinses of Milli-Q water in the ultrasonic cleaner for 5 minutes each. Biopsies were then dried overnight at 50 °C and powdered. Samples were then analysed for $\delta^{18}O$ and $\delta^{13}C$ values using a Nu Instruments NuCarb device coupled with a Nu Perspective Isotope Ratio Mass Spectrometer (IRMS) equipped with a dual inlet at the Mawson Analytical Spectrometry Services, University of Adelaide. Approximately 90 μg of sample was weighed into glass vials and evacuated to high vacuum. Each sample was then reacted with 105 % phosphoric acid at 70 °C and the resultant carbon dioxide was transferred to the dual inlet and measured. The measured isotopic compositions were monitored and normalised to IAEA-CO-1 and internal standards ANU P3 and UAC-1. Precision was calculated from repeated measurement of standards and less than ±0.05 per mil (sd).

All isotopic results are in the per mil (‰) delta (δ) notation using the Vienna Peedee Belemnite (VPDB) isotope reference standard.

Statistical analyses

To investigate trends in carbon and oxygen isotopes over space and time, I used a series of generalised additive models (GAM) and linear regression models using the software program R (R Core Team, 2022). Covariates included were latitude, longitude, year of collection, and cuttlebone length. Cuttlebone length was included in the analysis, as it is a strong predictor of cuttlefish size, and thus metabolic rate, which, in turn, can influence isotopic values (Chung, Trueman, Godiksen and

Grønkjær, 2019; Chung *et al.*, 2021). First, I examined the presence of outliers or extreme values in the data using Cleveland dot plots, and collinearity using scatterplots and Pearson correlation coefficients (Zuur *et al.*, 2010). There was evidence of collinearity between latitude and longitude (0.76), latitude and cuttlebone length (0.5), and longitude and cuttlebone length (0.6). However, latitude and longitude were treated as one spatial term and are hereafter collectively referred to as "location".

The response variables, $\delta^{13}C$ and $\delta^{18}O$ values, were plotted against all covariates and time to determine whether the relationship was linear or non-linear. Clear non-linear relationships were found. Therefore, I applied GAMs with Gaussian distributions and restricted maximum likelihood (REML) estimations to estimate the smoothing parameters to undertake the initial analysis of the data (Wood, 2017). For visualization of all models used and their results, see Tables 5.1 and 5.2.

For analyses on $\delta^{13}C$ values in cuttlebones, a GAM (C1) was first used in which all covariates were taken as smoothing functions to allow for non-linear effects using the *gam* function from the package *mgcv* (Wood, 2011). This model showed that time (year) was effectively removed from the model (edf ≈ 0) and that the relationship with cuttlebone length was essentially linear (edf ≈ 1) (Zuur, 2012). I then proceeded with a GAM (C2) with cuttlebone length as a linear variable and location as a (tensor) smoother. Additional models, including a linear regression model (C3) with only cuttlebone length and another GAM (C4) with only location in a smoother, were also carried out to avoid collinear effects. For the GAM (C5) with the single location covariate, a soap film smoother was used to identify geographical boundaries to prevent any predictions for areas in which cuttlefish do not inhabit (i.e., on land) (Wood *et al.*, 2008). Data were visualised using the function *ggplot*.

After fitting the models, model validation was applied using the package *DHARMa* (Hartig, 2020). Scaled quantile residuals were plotted against the fitted values and each covariate used in the model. Spatial dependency in these residuals was then checked using a Moran's I test.

For analyses on $\delta^{18}O$ values in cuttlebones, a GAM (O1) was first used that included all covariates as smoothing functions. This model showed that year and cuttlebone length had no effect and were effectively removed from the model (edf ≈ 0). To determine if location was overriding year and cuttlebone length, due to the slight collinearity between location and cuttlebone length, I used another GAM (O2) with only year and cuttlebone length as smoothers. Here, cuttlebone length again had no effect and was removed from the model, but year was significant. When location and time (year) were included in the same model (O4), year was effectively removed from the model (edf ≈ 0) suggesting that the effect from location overrode any effect from time, making the covariates

difficult to disentangle. Therefore, location and time were separated into different models to further observe the influence of time and space (O3 and O5). In the final model used for location (O6), a soap film smoother was used and plotted similarly as with $d^{13}C$. Residuals and spatial dependency were also checked in the same manner as for $\delta^{13}C$.

Finally, because the influence of space and time were difficult to separate, I also analysed trends from samples collected from a single region, the South Australian gulfs, and ran the same analyses as above. Samples from the South Australian gulfs were used because the samples spanned multiple decades, and the gulfs represent a distinct oceanographic region. There was a single sample from 1960-1980 that was removed from further analysis. Two GAMs (C6 and O7), both with year as the smoother, were used to analyse $\delta^{13}C$ values over time and $\delta^{18}O$ values over time. Results were plotted and residuals and spatial dependency were also checked in the same manner as above.

All statistical analyses were carried out in the software program R (R Core Team, 2022) using packages *ggplot2* (Wickham, 2011)*, mgcv* (Wood, 2011)*, gratia* (Simpson, 2023)*,* and *DHARMa* (Hartig, 2020).

Table 0.1: Step by step of models used to analyse $d^{13}C$ values in *Sepia apama* cuttlebones with the variables entered, the result (edf and p-value) and a summary of the result.

Model name	Model	Covariate	edf	p-value	Summary of result
All samples (1963-2022)					
C1	$d^{13}C$ = Intercept + s(year) + s(cuttlebone length) + s(latitude, longitude)	Year	<0.0001	0.94	Year had no effect and was effectively removed from the model (edf ≈ 0). Cuttlebone length was linear (edf ≈ 1).
		Cuttlebone length	1.11	0.11	
		Latitude, Longitude	1.57	0.003	
C2	$d^{13}C$ = Intercept + cuttlebone length+ s(latitude, longitude)	Cuttlebone length	-	0.11	Cuttlebone length was not significant, but latitude and longitude were.
		Latitude, Longitude	1.35	0.034	
C3	$d^{13}C$ = Intercept + slope(cuttlebone length) *Linear model*	Cuttlebone length	-	0.002	Cuttlebone length was only significant on its own. However, the spatial effect could be overriding the effect of cuttlebone length due to weak collinearity between latitude and cuttlebone length (0.5) as well as longitude and cuttlebone length (0.6).
C4	$d^{13}C$ = Intercept + s(latitude, longitude)	Latitude, Longitude	1.62	0.0004	Location on its own was more significant than location and cuttlebone length. This model makes predictions over land where cuttlefish do not inhabit, so in next model I use a normal smoother with boundaries instead of a tensor smoother.
C5	$d^{13}C$ = Intercept + s(latitude, longitude) *with set geographic boundaries using soap film smoother*	Latitude, Longitude	1.81	<0.0001	
South Australia gulf samples (1984-2022)					

C6	$d^{13}C$ = Intercept + s(year)	Year	0.89	0.006	Relationship is linear

Table 0.2: Step by step of models used to analyse $d^{18}O$ values in *Sepia apama* cuttlebones with the variables entered, the result (edf and p-value) and a summary of the result.

Model name	Model	Covariate	edf	p-value	Summary of result
All samples (1963-2022)					
O1	$d^{18}O$ = Intercept + s(collection year) + s(cuttlebone length) + s(latitude, longitude)	Year	<0.0001	0.68	Year and cuttlebone length were effectively removed from the model (edf ≈ 0), but latitude and longitude were significant. I then determined if location was overriding year and cuttlebone length.
		Cuttlebone length	<0.0001	0.90	
		Latitude, Longitude	9.47	<0.0001	
O2	$d^{18}O$ = Intercept + s(collection year) + s(cuttlebone length)	Year	1.90	0.0005	Cuttlebone length edf was close to 0 meaning it has effectively been removed from the model, but year was significant without location in the model.
		Cuttlebone length	0.11	0.32	
O3	$d^{18}O$ = Intercept + s(collection year)	Year	1.92	0.0005	Year was significant on its own.
O4	$d^{18}O$= Intercept + s(collection year) + s(latitude, longitude)	Year	<0.0001	0.68	When included with location, year was effectively removed from the model (edf ≈ 0), indicating that location was overriding time effect.
		Latitude, Longitude	9.47	<0.0001	
O5	$d^{18}O$ = Intercept + s(latitude, longitude)	Latitude, Longitude	5.06	<0.0001	This model makes predictions over land where cuttlefish do not inhabit, so in O6 a normal smoother was used with boundaries instead of a tensor smoother.
O6	$d^{18}O$= Intercept + s(latitude, longitude) *with set geographic boundaries using soap film smoother*	Latitude, Longitude	6.79	<0.0001	
South Australia gulf samples (1984-2022)					
O7	$d^{18}O$ = Intercept + slope(year)	Year	-	0.134	

	Gulf St. Vincent				
O8	$d^{18}O$ = Intercept + slope(year) *Spencer Gulf*	Year	-	0.037	

Results

$\delta^{13}C$ values

When all cuttlebones across southern Australia were analysed, $\delta^{13}C$ values did not exhibit a significant trend over time (Fig. 5.3A, Table 5.1), but linear models showed significant positive relationships between $\delta^{13}C$ and cuttlebone length (*df*=59, *F-statistic*= 10.46, *p-value*=0.002) (Fig. 5.4, Table 5.1) with no evidence of spatial dependency (Moran's I test; *sd*=0.108, *p-value*=0.77).

When only South Australian gulf samples were analysed, there was a significant linear decrease in $\delta^{13}C$ values over time (Fig. 5.3B, Table 5.1) with no evidence of spatial dependency (Moran's I test; *sd*=0.118, *p-value*=0.69).

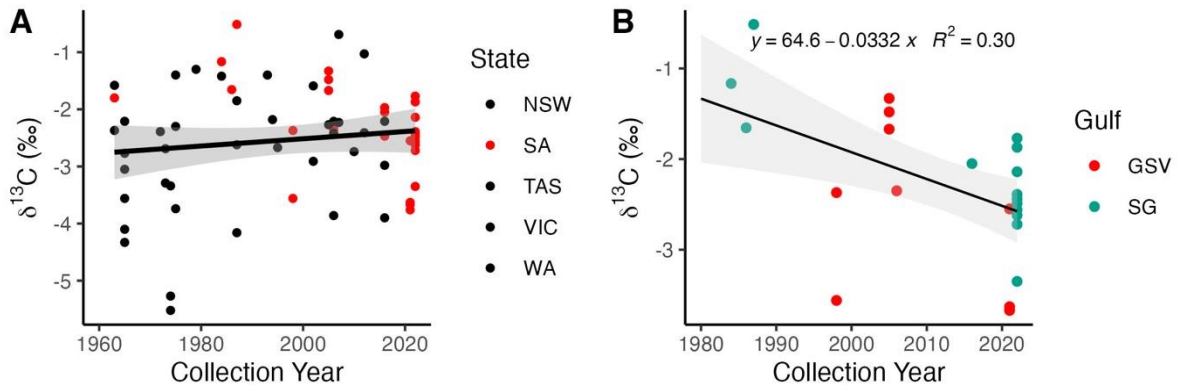


Figure 0.3: Temporal change in $\delta^{13}C$ values from *Sepia apama* cuttlebones with (A) data across southern Australia fit with a linear regression and 95% confidence intervals (shaded portion) and (B) data from the South Australian gulfs (GSV: Gulf St. Vincent, SG: Spencer Gulf) fit with generalised additive model C6 (Table 1) and 95% confidence intervals (shaded portion).

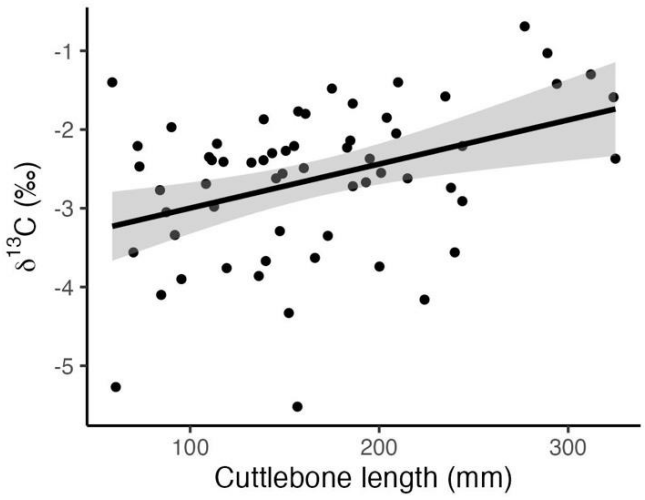


Figure 0.4: Relationship between $\delta^{13}C$ values from *Sepia apama* cuttlebones collected from across southern Australia and cuttlebone length (mm) fit with a linear regression and 95% confidence intervals (shaded portion).

A GAM also showed a significant relationship between $\delta^{13}C$ and location (*edf*= 1.62, *p-value*=0.0004), but when cuttlebone length and location are included in the GAM, cuttlebone length no longer had a significant relationship with $\delta^{13}C$ (*t-value*=1.64, *p-value*= 0.11) (Table 5.1). This suggests that location has a larger effect on $\delta^{13}C$ values than cuttlebone length and when $\delta^{13}C$ values are mapped (Fig. 5.5), there is a clear gradient from lower values in the west to higher values in the east.

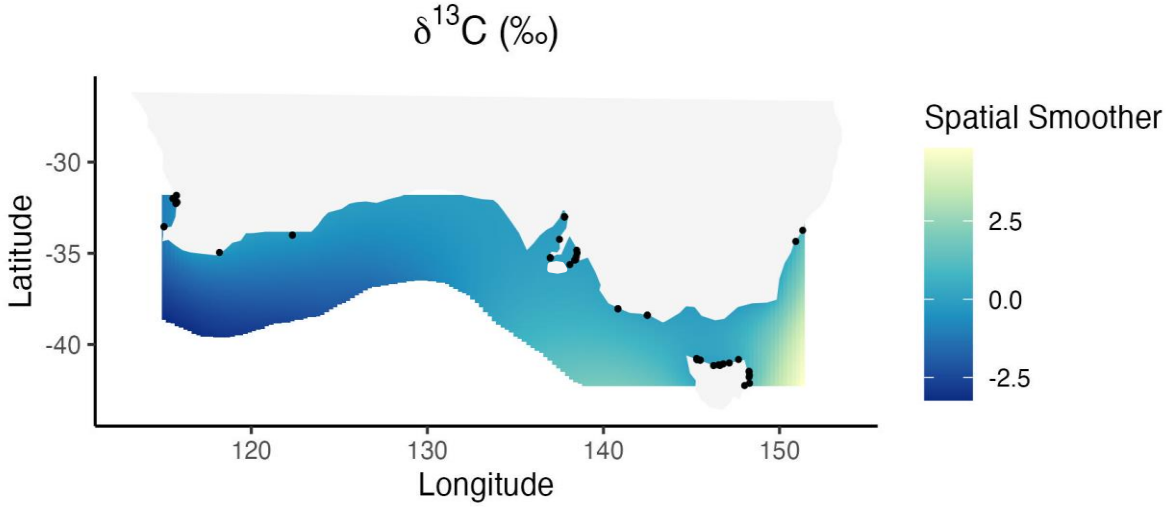


Figure 0.5: Geographical variation of $\delta^{13}C$ values from *Sepia apama* cuttlebones across southern Australia. Created with generalised additive model C5 (Table 1) in which $d^{13}C$ = Intercept + s(latitude, longitude) with geographic boundaries set using soap film smoother. Black dots indicate sampling locations.

$\delta^{18}O$ values

When all samples from across southern Australia were analysed, there was a strong effect of location (GAM; *edf*=6.79, *p-value*<0.0001; Fig. 5.6) and a smaller effect of time (GAM; *edf*=1.92, *p-value*=0.0005; Fig. 5.7A) on $\delta^{18}O$ values (Table 5.2). By mapping the location effect, higher $\delta^{18}O$ values were present around the two gulfs in South Australia, as indicated by the yellow spot in the centre of the map, and lower $\delta^{18}O$ values were present along the western and eastern edges of southern Australia (Fig. 5.6).

For South Australian gulf samples, there was a significant decrease in $\delta^{18}O$ values over time for Spencer Gulf (linear regression; *df*= 12, *p-value*= 0.037), but not for Gulf St. Vincent (linear regression; *df*= 7, *p-value*= 0.134) (Fig. 5.7B, Table 5.2). There was also no evidence of spatial dependency (Moran's I test; sd=0.118, p-value=0.91).

In contrast, cuttlebone length did not have a significant effect on $\delta^{18}O$ values (GAM; *p-value*=0.32; Table 5.2).

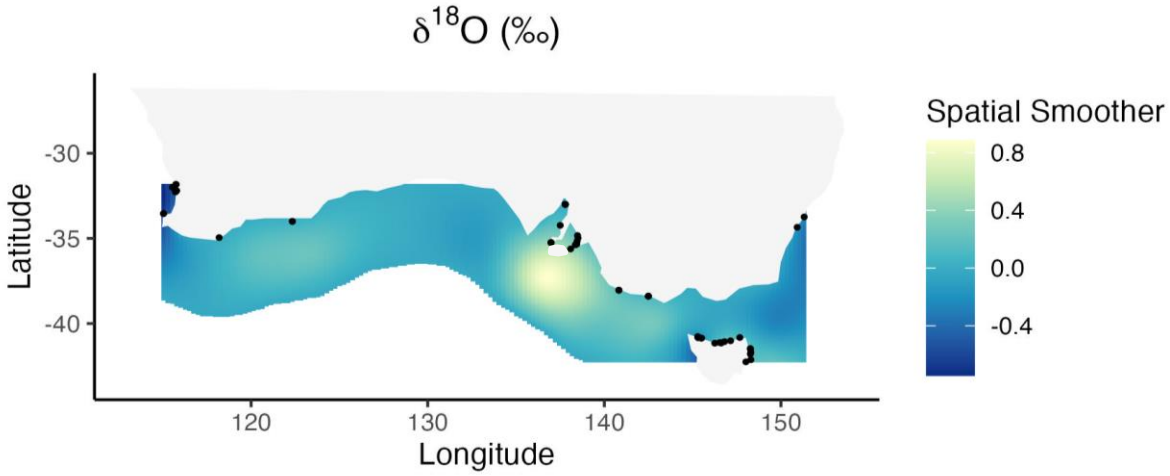


Figure 0.6: Geographical variation of $d^{18}O$ values from *Sepia apama* cuttlebones across southern Australia. Created with generalised additive model O6 (Table 2) in which $d^{18}O$= Intercept + s(latitude, longitude) with geographic boundaries set using soap film smoother. Black dots indicate sampling locations.

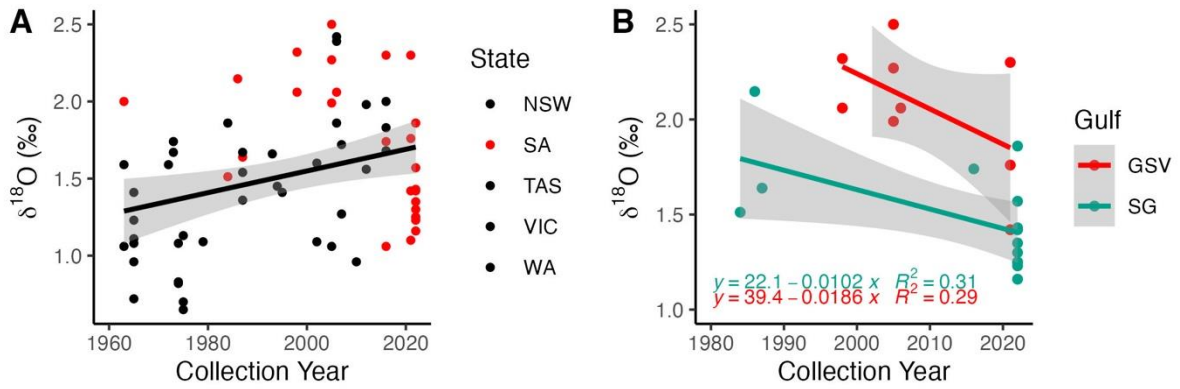


Figure 0.7: Temporal change in d^{18}O values from *Sepia apama* cuttlebones collected from (A) across southern Australia fit with a linear regression and (B) the South Australia gulfs (GSV: Gulf St. Vincent, SG: Spencer Gulf) fit with linear models for each site. Shaded portions indicate the 95% confidence intervals.

Discussion

In this study, I assembled the first multi-decadal archive of cuttlebones, which spanned six decades. I found both $\delta^{13}C$ values and $\delta^{18}O$ values from cuttlebones are strong predictors of geographical origin, regardless of long-term temporal effects. However, analysis of a single oceanographic region revealed temporal trends in $\delta^{13}C$ and $\delta^{18}O$ values, which could reflect global climate change (i.e. global warming and increases in atmospheric CO_2).

Carbon and oxygen isotopes as predictors of geographic origin

Although the dataset spanned several decades, I found that spatial trends overwhelmed any effects seen from cuttlebone length or time. Spatial trends are likely to be strongly influenced by local oceanography.

There are various currents flowing along the 5500 km southern Australian coastline and while the oceanography is complex, the general flow is from west to east during the winter and east to west during summer (Ridgway and Condie, 2004; Middleton and Bye, 2007). Briefly, the main coastal currents include the Leeuwin Current which runs between North West Cape in Western Australia and the Great Australian Bight, the South Australian Coastal Current which runs between eastern Great Australian Bight and western Bass Strait, and the Zeehan Current which runs off western Tasmania (Ridgway and Condie, 2004). Within these currents, there are two distinct water masses; tropical,

warm, low-salinity water from the west spread by the Leeuwin Current and warm, high-salinity water from Spencer gulf in South Australia spread by the South Australian Current (Ridgway and Condie, 2004).

Carbon isotopic values in marine carbonates are largely driven by temperature, whereby lower $\delta^{13}C$ values are present in areas with higher temperatures (Grossman and Ku, 1986; Thorrold *et al.*, 1997). This is seen on a global scale in phytoplankton (Magozzi *et al.*, 2017) as well as in experimental rearing of different marine ectotherms including cuttlefish (Martino *et al.*, 2020; Chung *et al.*, 2021). On the southern coast of Australia, the warmer waters in the west and cooler waters in the east are represented by the results with $\delta^{13}C$ values ranging from more negative values on the west to more positive values on the east. Although the oceanography is complex and strength and direction of the coastal currents off southern Australia changes with season (Ridgway and Condie, 2004; Middleton and Bye, 2007), the presence of warmer water in the west and cooler water in the east remains the same. It should also be noted that the model in this study is based on sparce, random sampling across a large area. Resulting in a coarse scale model with many spatial gaps which cannot be over interpreted.

Cuttlebone $\delta^{18}O$ values had a more complex spatial distribution as it is a function of salinity and temperature. In seawater, $\delta^{18}O$ values vary with salinity as a function of freshwater inputs and evaporation (LeGrande and Schmidt, 2006). In biogenic carbonates of ectotherms, $\delta^{18}O$ values also vary with temperature as higher temperatures increase fractionation into the carbonate (Trueman and St John Glew, 2019; Zoë A. Doubleday *et al.*, 2022). The model displayed a large cluster of positive $\delta^{18}O$ values off South Australia, which is influenced by samples collected from within the two gulfs. The gulfs are large, inverse estuaries with high levels of evaporation and low levels of precipitation and are thus hypersaline relative to the open ocean (Nunes and Lennon, 1986). As such, the gulfs have elevated $\delta^{18}O$ seawater values (1.62 ‰; Durante, unpublished data from Spencer Gulf) relative to the open ocean (~0.5 ‰; Schmidt *et al.*, 1999). Hence the elevated levels of $\delta^{18}O$ in the cuttlebones from the gulfs is expected. Higher $\delta^{18}O$ values can also indicate cooler waters and lower $\delta^{18}O$ values can indicate warmers waters, which is possibly why I see lower $\delta^{18}O$ values on the western and eastern edges of the country where warm tropical water is mixing. Because $\delta^{18}O$ values have this inverse relationship with temperature, they also vary with latitude similarly to $\delta^{13}C$ values (Schmidt *et al.*, 1999; Martino, Trueman, *et al.*, 2022; Zoë A. Doubleday *et al.*, 2022). Although both $\delta^{13}C$ and $\delta^{18}O$ values in cuttlebones have shown to be useful spatial predictors, $\delta^{13}C$ values have shown to be the best at a longitudinal scale, while $\delta^{18}O$ values provides us with a smaller, more specific scale for the study area.

Carbon and oxygen isotopes as indicators of environmental change

Although the sample size was small, temporal trends in $\delta^{13}C$ values from the South Australian Gulfs (i.e., removing the spatial effect) likely reflect increases in atmospheric CO_2 (i.e., the Suess effect). In this study, $\delta^{13}C$ values declined at a rate of 0.033 ‰ per year over 38 years. This is slightly lower than the rate observed in Atlantic bluefin tuna otoliths (0.05 ‰) in the Mediterranean Sea (Fraile et al. (2016) and is even less than what was found in California mussels shells (0.07 ‰) (Pfister *et al.*, 2011), which spanned 20 and 50 years respectively. On the contrary, these results are slightly higher than those that Schloesser et al. (2009) observed in Atlantic bluefin tuna otoliths (0.025 ‰) from the west Atlantic and that (Newsome *et al.*, 2007) observed in northern fur seal teeth (0.021 ‰) in the northern Pacific Ocean.

These rates of change are consistent with what has been found for the Suess effect, which varies slightly between different oceanic locations (Keeling, 2024). Unfortunately, there are no additional studies on marine biogenic carbonates in the South Pacific for comparison, but atmospheric $\delta^{13}C$ values from sampling stations such as Mauna Loa, Hawai'i and Baring Head, New Zealand suggest that the rate of Suess effect in the southern Pacific is around 0.025 ‰ per year (Keeling *et al.*, 2005).

In addition to the Suess effect, $\delta^{13}C$ values in cephalopod carbonates have also been linked to diet, metabolic rate and temperature in which δ13C values decrease with increased temperature (Martino *et al.*, 2020; Chung *et al.*, 2021). Therefore, it becomes difficult to determine if this change in $\delta^{13}C$ values over time is due to direct effects of climate change (i.e., the Suess effect) or indirect effects of climate change (i.e., diet preference, metabolic rate).

I also observed a significant decrease in $\delta^{18}O$ values in *S. apama* cuttlebones from the 1980s onwards from Spencer Gulf. Because $\delta^{18}O$ values increase with salinity and decrease with temperature (Friedman and O'Neil, 1977; Grossman and Ku, 1986), this decrease can either be attributed to global ocean warming or a decrease in salinity over time. However, because inverse estuaries are such a highly dynamic environment, it is likely that there are a variety of factors contributing to this change in $\delta^{18}O$ values over time such as increased freshwater runoff and temperatures changes, which then becomes difficult to pinpoint the exact cause.

The influence of body size on isotopic trends

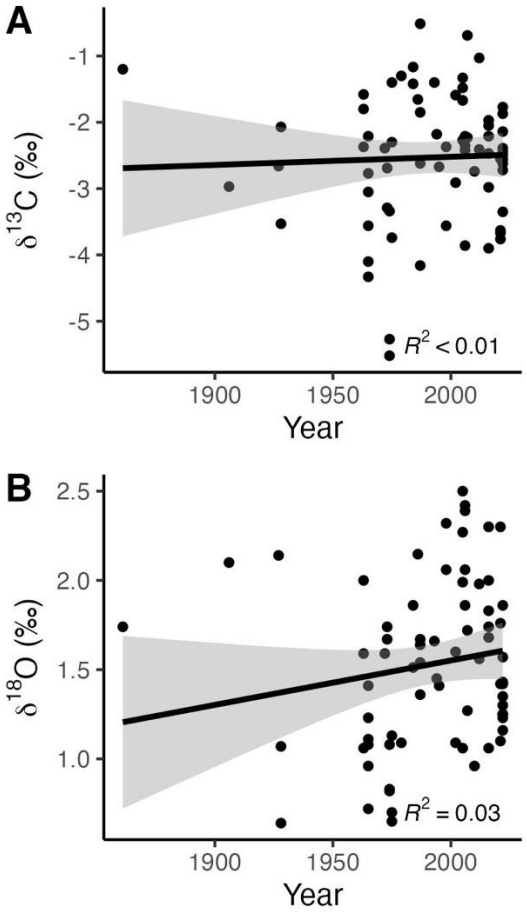
Here, I observed an increase in $\delta^{13}C$ values with cuttlefish size. As metabolic rate is often inversely related to $\delta^{13}C$ values (Chung, Trueman, Godiksen and Grønkjær, 2019; Chung *et al.*, 2021), I could hypothesise that this suggests a decrease in metabolic rate with size, which is a trend that has also been recorded in fish (Brown *et al.*, 2004; Chung, Trueman, Godiksen and Grønkjær, 2019) and one species of cephalopods, *Sepia officinalis,* (Bettencourt and Guerra, 1999; Chung *et al.*, 2021). But because I did not have all of the information to calculate metabolic rate, such as $\delta^{13}C_{diet}$ and $\delta^{13}C_{DIC}$ values for all samples, I cannot be sure.

There is also the question of why cuttlebone $\delta^{13}C$ values vary with cuttlefish size as all biopsies were taken from the same location (50 mm from callus). Cuttlebones are comprised of growth lamellae or layers and instead of being correlated with age, they are instead correlated with growth rate (Ré and Narciso, 1994; Bettencourt and Guerra, 2001; Chung and Wang, 2013). I also sampled on the ventral side which remains unchanged after deposition as the cuttlebone grows from the dorsal side (Bandel and von Boletzky, 1979). Therefore, I can suggest that although sampled at the same length, individuals were not the same age, but rather at the same size. If an individual is younger at that size, it would suggest that they have a faster growth and metabolic rate and would reach larger sizes quicker than others which is the opposite to the data. This suggests that there may be some kind of metabolic rate change with size, but to properly determine how, samples should be taken from the cuttlebone's anterior tip to obtain a signature representative of its current size.

Conclusion

I collated a cuttlebone archive and isotopic record spanning six decades and the entire southern Australian coast. While spatial trends largely overrode temporal trends, the result suggests that $\delta^{13}C$ and $\delta^{18}O$ values in cuttlebones are strong indicators of geographic origins and can therefore be used to geolocate or track animals back to region. Despite limited sample size, I also found temporal changes in $\delta^{13}C$ and $\delta^{18}O$ values from cuttlebones from the South Australian gulfs suggesting that cuttlebone archives can be useful recorders of global change (i.e., atmospheric CO2 and temperature). While challenges remain and further studies should limit spatial effects where possible, historical hard tissue archives can provide a valuable insight into past environments, which would be otherwise difficult or impossible to collect.

Supplementary Material



Supplementary Figure 0.1: (A) $\delta^{13}C$ and (B) $\delta^{18}O$ values from *Sepia apama* cuttlebones correlated with collection year including the 5 samples from 1861 to 1959 that were excluded from the final analysis. Fit with a linear regression with 96% confidence intervals and R^2 indicated on each graph.

Reconstructing life-time reproductive histories using steroid hormones in octopus beaks

This research is in preparation to be submitted to a special sclerochronology issue in Limnology and Oceanography Letters with the proposal already approved for review.

Abstract

Understanding the reproductive attributes of marine animals, such as size- or age-at-maturity, is vital for both fisheries and conservation management. For cephalopods, reproductive information is usually obtained by analyzing the gonads, which only provides reproductive data on an individual at a given time (i.e., maturity status), as well as requires the use of whole specimens. Here, I present a novel method for extracting reproductive hormones, such as testosterone, progesterone, and estradiol, from multiple regions within octopus beaks, effectively providing a life time history of their reproduction. Consecutive subsections were taken from individual beaks, digested with hydrogen peroxide and chitinase, after which lipids were extracted with methanol. Concentrations of progesterone, estradiol, and testosterone were quantified from extracts using liquid chromatography with tandem mass spectrometry (LC/MS/MS). By utilising the beak, which accretes material periodically in discernible increments, I were able to obtain a record of hormonal trends for individual male and female Māori Octopus (*Macroctopus maorum*) and Pale octopus (*Octopus pallidus*) throughout their entire life. The steroid hormones estradiol and progesterone were successfully detected whereas testosterone fell below detection limits in both species and sexes of octopus. Clear peaks in hormone levels were observed indicating timing of sexual maturity. The hope is that this novel technique may be further developed and applied to support the understanding of reproductive biology in cephalopods and improve their management and conservation.

Introduction

Understanding the reproductive history of an animal provides fundamental data on age of maturity, spawning seasonality, and past spawning events, which is essential for understanding a species' life history and for their management and conservation (Adams, 1980; Morgan, 2008). Most cephalopods are semelparous, meaning they reproduce only once and, typically, maturity stage (i.e., immature, mature, post-spawning) is assessed by analyzing the gonads post dissection (Arkhipkin, 1992; D'Aniello *et al.*, 1996; Di Cosmo *et al.*, 2001; Di Cristo *et al.*, 2004). The disadvantage of determining maturity stage via dissection is that only that individual at that specific time can be staged and many individuals of different ages are required to establish reproductive stages throughout the entire life cycle. Whole, well preserved, specimens are also required.

Steroid hormone analysis offers an alternative to analysing gonads. Just like vertebrates, steroid hormones such as progesterone, estradiol, and testosterone are present in some invertebrates, such as cephalopods, and are involved in their reproduction. In cephalopods, instead of the pituitary

gland, the optic glands are the endocrine structures involved in maturation of the gonads (Wells and Wells, 1959; D'Aniello *et al.*, 1996). Sexual maturity can be inferred from steroid hormone analysis, as it is usually associated with a surge in progesterone, estradiol, and/or testosterone (Di Cosmo *et al.*, 2001). In octopus, steroid hormones have been recorded in ovaries, oviducts, and oviductal glands in females, and testes, vas deferens, seminal vesicle, prostate, and Needham's sac in males (D'Aniello *et al.*, 1996; Di Cosmo *et al.*, 2001). Small amounts have also been recorded in the brain, liver and haemolymph of male *Octopus vulgaris* (D'Aniello *et al.*, 1996). However, as with the visual analysis of gonads, hormone analyses of reproductive soft tissues can only provide a snapshot of the reproductive status of that individual at that specific time. Hormones have also been analysed in mucus swabs taken from the surface of the skin (Chancellor *et al.*, 2023) and from octopus fecal matter (Larson and Anderson, 2010), which allows for multiple non-invasive sample collection throughout the individual's life; however, this requires maintenance of the octopus in aquaria which is time consuming and expensive and, in many case, not feasible.

Hard, accretionary tissues grow throughout an individual's life and are not resorbed, providing a permanent record of their biotic and abiotic growth conditions. Analysing steroid hormones in these hard, accretionary tissues is a novel approach to reveal the life-time reproductive histories of an individual, and has been recently applied to vertebrate structures, including pinniped whiskers (Keogh *et al.*, 2020, 2021), walrus bones (Charapata *et al.*, 2018), fish opercula (Charapata *et al.*, 2022), whale baleen (Fernández Ajó *et al.*, 2022), and whale earplugs (Trumble *et al.*, 2013, 2018). By analysing hormones along the growth axis, the whole reproductive cycle or history of an individual can be reconstructed. Many accretionary tissues also have periodic growth increments or rings that can be used to determine age, and thus, the age of reproductive events or maturity stages. Recently, Charapata *et al.* (2022) successfully identified age-at-maturity of female yelloweye rockfish by quantifying the hormone profile with age.

Cephalopod beaks also have incremental growth which can be used to estimate age if increment periodicity has been validated (Doubleday *et al.*, 2006; Durante *et al.*, 2023). Another advantage of beaks is that they remain intact in the digestive system of their predators such as marine mammals and seabirds (Clarke and Kristensen, 1980; Clarke and Macleod, 1982; Clarke and Young, 1998) and can be stored in museum collections indefinitely. Thus, beak collections obtained either directly from cephalopods, or via predators, could allow for the collection of reproductive data across large spatial and temporal scales. This is especially useful for the study of rare or inaccessible species, such as those that inhabit the deep-sea or Southern Ocean, as whole animals are difficult to obtain.

In this study, I aimed to develop a method to extract steroid hormones from subsections of octopus beaks, and thus visualise maturation throughout an individual's lifetime. Cephalopod beaks are comprised of a chitin-protein complex (Hunt and Nixon, 1981); therefore, I developed a method to digest and extract lipids from a chitinous structure. I then applied liquid chromatography with tandem mass spectrometry (LC/MS/MS) to quantify the concentrations of steroid sex hormones from the lipid extracts. The advantage of LC/MS/MS is that it requires less sample material, making it more suitable for sub-sampling structures with lower lipid content. This is the first time that this method has been applied to a chitinous structure from an invertebrate species, and further application of this novel technique may deepen the understanding of reproduction in cephalopods and other taxonomic groups with chitinous structures.

Methods

Study species

I opportunistically sourced octopus beaks from previous or ongoing projects, which also avoided the need to euthanise additional animals. I focused on two common coastal octopus species from southern Australia, the Māori octopus, *Macroctopus maorum*, and the pale octopus, *Octopus pallidus,* which have clear growth increments along their beaks. *Macroctopus maorum* is merobenthic, producing many small eggs and small hatchlings that are initially planktonic whereas *O. pallidus* is holobenthic with fewer, larger eggs and much larger demersal hatchlings (Norman and Reid, 2000). The *M. maorum* specimens were collected from southern New South Wales, Australia as part of a separate, ongoing study for the Fisheries Research and Development Cooperation and were categorised as either juvenile, maturing, mature, or spent following a simplified version of the reproductive stages outlined in Arkhipkin (1992). Specimens of *O. palldius* were sourced from eastern Victoria, Australia, as part of a previous study (Hua *et al.*, 2023) and reproductive stages were categorised following De Lima *et al.* (2014) and Kivengea *et al.* (2014). Samples were collected in accordance with the relevant animal ethics requirements.

While both species have incremental growth on their beaks, periodicity has not been validated for these species. Therefore, hormone levels are assessed relative to number of increments rather than absolute age.

Sub-sampling the beaks

Beaks from two mature male and two mature female octopus for each species were analysed for reproductive hormones by sub-sectioning along the beak. All beaks had been preserved in 70% ethanol; therefore the beaks were first rehydrated in distilled water at 4°C for at least one day. They were then cut in half longitudinally with scissors and then sectioned into 3-5 mm wide segments (Fig. 6.1). Growth increments from each segment were counted under a stereo microscope. Segments were then dried at 40°C for 24 hours, weighed to the nearest 0.01 mg, and then cut into smaller, more easily digested pieces.



Figure 0.1: Diagram of beak sectioning with (A) white lines indicating where the beak was sectioned and (B) the beak segments after sectioning.

Extracting hormones

I developed a method to extract hormones from chitinous structures, building on the methods described by Dillon *et al.* (2021) and Kallenbach *et al.* (2021), which described how to digest chitin and extract lipids from keratinous structure respectively.

To digest the chitin, 1 mL of hydrogen peroxide per 20 mg of beak tissue was added to a plastic tube and capped. This solution was incubated at 50°C on an orbital shaker at 150 rpm for 24 hours. A chitinase solution was then added at 1 mL per 10 mg of beak tissue and incubated at 37°C on an orbital shaker at 150 rpm for 3 to 5 days until beak pieces were clear (Fig. 6.2).

The chitinase solution was made following the protocol from (Kallenbach *et al.*, 2021) in which 50 units of chitinase from *Streptomyces griseus* (Sigma-Aldrich) was dissolved into 50 mL of glycerol and

then 450 mL of a NaOAc buffer was added to reach an activity level of 100 U L^{-1}. The NaOAc buffer was made of 400 mL ultra-pure Milli-q water and 41.015 g of sodium acetate. After the addition of the buffer, glacial acetic acid was added to the chitinase solution until it reached a pH of 5.

After the samples were fully digested, the solution was dehydrated under nitrogen gas at ~40°C. Lipids were then extracted by adding 3 mL of absolute methanol and put on an orbital shaker at 175 rpm for 24 hours at room temperature. Samples were then dehydrated again under nitrogen gas at ~40°C and stored at -80°C until analysis (Fig. 6.2).

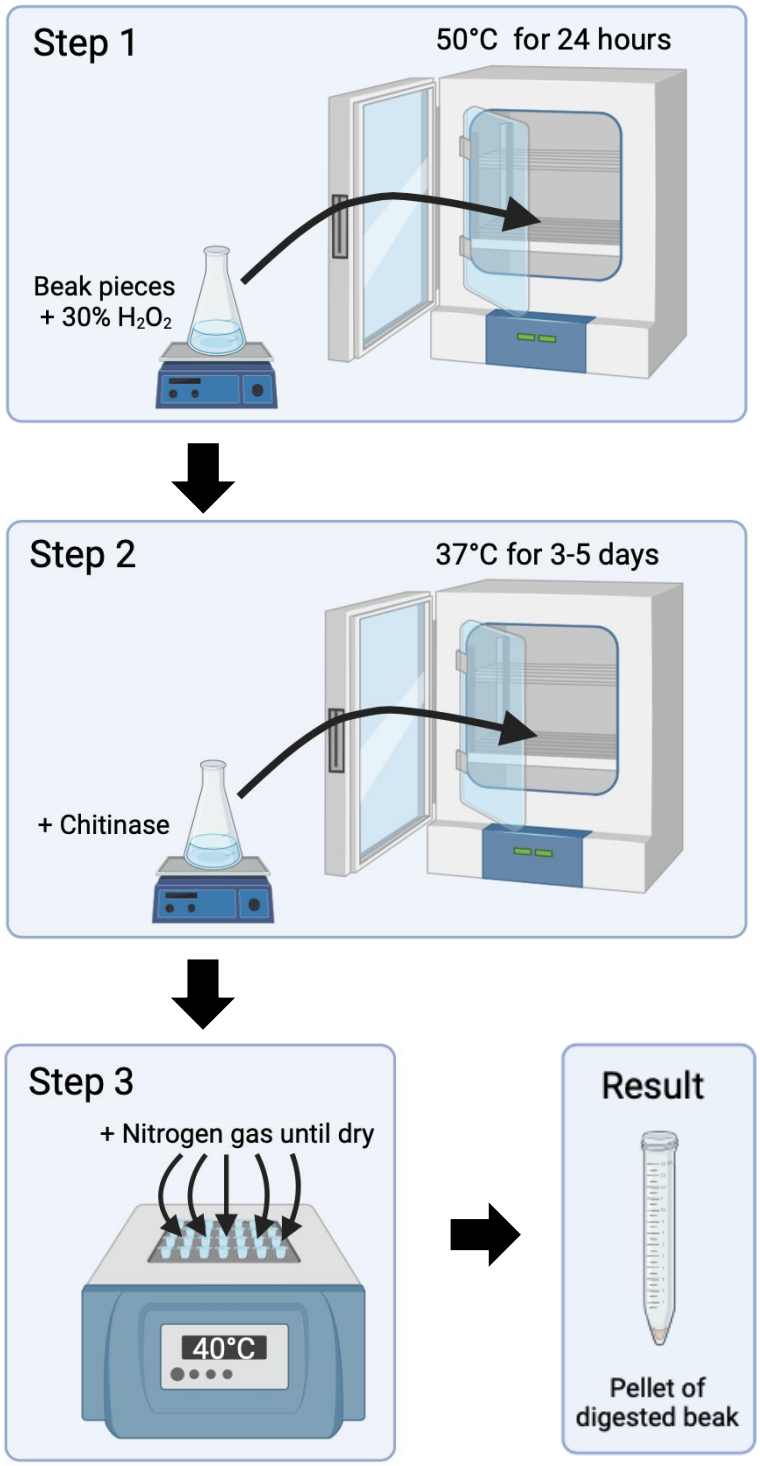


Figure 0.2: Diagram of the chitin digesting process. Step 1: Add 1 mL of hydrogen peroxide (H_2O_2) per 20 mg of beak tissue to a vial and incubate on an orbital shaker at 150 rpm at 50°C for 24 hours. Step 2: Add 1 mL of chitinase solution per 10 mg of beak tissue to peroxide solution and return to orbital shaker at 150 rpm and incubate at 37°C for another 3-5 days until beak tissue is digested. Step 3: Remove vials from incubator and dry on a heat block at 40°C with nitrogen gas until dry. Figure created with BioRender.com

Analysing hormones

Progesterone, testosterone, and estradiol content were analysed from each sample using liquid chromatography (LC; Shimadzu Nexera XR, Shimadzu, Japan) coupled to a SCIEX 6500 Triple-Quad system (MS/MS; SCIEX, US) at the University of South Australia following the methods from Häkkinen *et al.* (2018). In previous studies on pinniped whiskers and fish opercula (Keogh *et al.*, 2020, 2021; Charapata *et al.*, 2022), enzyme-linked immunosorbent assay (ELISA) kits are often used as a more affordable option. The advantages of using an LC/MS/MS method rather than an assay kit are that: 1) more than one hormone can be measured at a time; 2) the targeted hormone can be measured specifically rather than the hormone plus its metabolites; and 3) it is more sensitive, and thus suitable for small sample sizes with low concentrations (i.e. when taking sub-samples along an accretionary hard tissue).

To prepare the extracts for the LC/MS/MS, they were reconstituted in 1 mL absolute methanol containing 100ng/ml of cortisol-9,11,12,12-d4 (Toronto Research Chemicals, Toronto, Canada) and evaporated to dryness using GeneVac EZ-2 Plus Evaporating System (GeneVac, UK). Samples were reconstituted in phosphate buffer saline and then subjected to liquid-liquid extraction as previously described (Dimasi *et al.*, 2023). In brief, 300 µl acetonitrile was added to each sample, vortexed for 1 min and then centrifuged at 12,000 *g* for 10 min. Supernatant was transferred to a fresh Eppendorf tube and the remaining sample pellet was resuspended in 300 µl ethyl acetate, vortexed for 1 min and then centrifuged for 12,000 *g* for 10 min. Supernatant was added to the acetonitrile, mixed by inversion, and then evaporated to dryness using the GeneVac EZ-2 Plus Evaporating System (GeneVac, UK). Dried samples were reconstituted in 50% methanol and then injected onto an ACQUITY UPLC BEH C18 Column 130Å, 1.7 µm, 2.1 mm X 100 mm (Waters Corp, US). Mobile phases were 0.2% Formic acid in water (A) and 0.2% Formic acid in acetonitrile (B). Flow rate was 0.3 ml/min and mobile phase B was initially 10% and increased linearly to 90% over 10 min and then held at 90% for 2 min, after which it was returned to 10% over 3 min prior to injection of the next sample. Hormone concentrations were calculated via integration with a standard curve that ranged from 0.05 to 100 ng/ml and then normalised to tissue weight.

Hormone concentration (ng per g) was calculated by dividing the quantity of each hormone (progesterone and estradiol) by the weight of each beak subsample (g).

All figures were created in the software program R (R Core Team, 2022).

Results

Estradiol and progesterone were detected in all samples, providing a history of maturity for each individual's lifetime. Testosterone was below the limit of detection and was therefore not analysed further.

Estradiol and progesterone were detected in male beaks in similar concentrations to female beaks for both *M. maorum* and *O. pallidus*. Progesterone was present in higher concentrations than estradiol in all except one male *M. maorum* (Fig. 6.3A).

In *M. maorum*, the maximum concentration of progesterone was around 800 ng/g whereas the maximum concentration of estradiol varied between individuals (Fig. 6.4). In the beaks of female *M. maorum*, there was an obvious peak in progesterone around 100 increments followed shortly by a peak in estradiol and a rapid decline thereafter. In the beak of one male *M. maorum*, three sub samples were excluded from the results due to large standard error, but a progesterone peak could still be identified (Fig. 6.3B).

In *O. pallidus*, the maximum concentration of progesterone was around 2000 ng/g except for one individual (Fig. 6.4). Like with *M. maorum*, estradiol varied between individuals (Fig. 6.4). Both males and females of *O. pallidus* show an initial increase in hormones levels at around 20-30 increments and then a distinct peak at around 80 increments for both estradiol and progesterone, except in one of the females (Fig. 6.3G). The beak increments for individuals depicted in Fig. 6.3D and 6.3G were more difficult to differentiate, so it possible that all increments were not counted.

In all but one individual (6.3F), the distinct, large peak in hormonal levels was followed by a decline at the growing edge of the beak just prior to capture, and yet all individuals were classified as mature via gonadal staging. For this reason, I believe that although the individual depicted in Fig. 6.3F was mature, it had not yet reached peak maturity.

The maximum recorded hormone concentrations observed here surpassed those found in the gonads and feces of other octopus species (Table 6.1). However, baleen plates in whales analysed with enzyme immunoassays had comparable concentrations of progesterone as octopus beaks from the present study (Table 6.2).

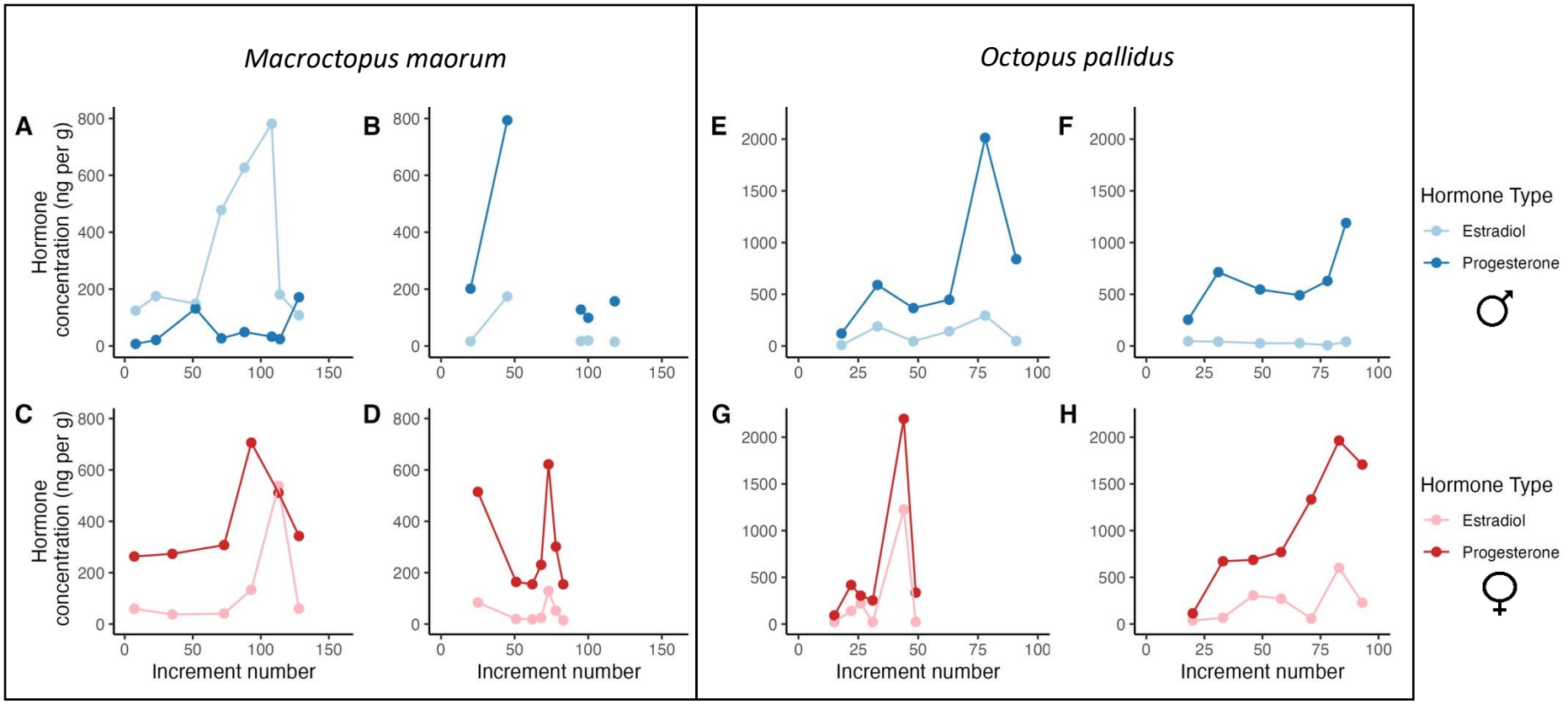


Figure 0.3: Estradiol and progesterone concentrations (ng g^{-1}) in beaks of *Macroctopus maorum* (A-D) and *Octopus pallidus* (E-H) males (top row; blue) and females (bottom row; red). Hormone samples were taken at various points along the beak as indicated by the increment number on the x-axis.

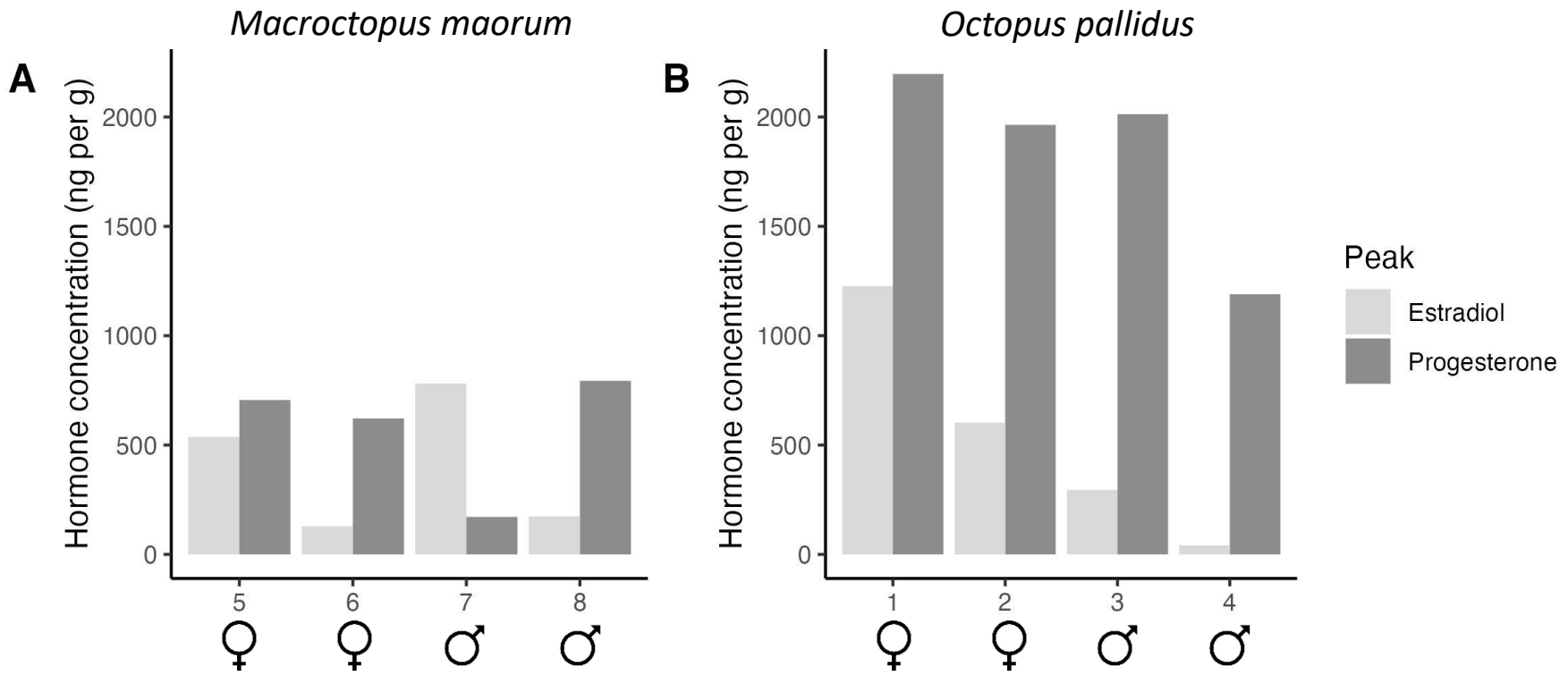


Figure 0.4: Peak estradiol and progesterone concentrations (ng/g) in beaks of individual female (♀) and male (♂) (A) *Macroctopus maorum*, a merobenthic species, and (B) *Octopus pallidus*, a holobenthic species.

Table 0.1: Review of maximum recorded hormone (estradiol, progesterone, and testosterone) concentrations in octopus tissues and methods used. [M] next to the species name indicates that the species has merobenthic reproductive strategy and [H] indicates a holobenthic reproductive strategy.

Species	**Method**	**Tissue**	**Sex**	**Max hormone concentrations (ng/g)**			**Reference**
				Estradiol	Progesterone	Testosterone	
Octopus vulgaris[M]	Radioimmunoassay	Gonads	F	~0.175	~0.2	-	Di Cosmo *et al.* (2001)
	Radioimmunoassay, Immunoenzymatic, High performance liquid chromatography	Gonads and other soft tissue	M	0.92	4.8	5.2	D'Aniello *et al.* (1996)
Octopus maya[H]	Radioimmunoassay	Gonads	F	-	10.11	0.56	Avila-Poveda *et al.* (2013)
			M	-	6.57	0.67	
	Radioimmunoassay	Gonads	F	-	0.61	0.08	Avila-Poveda *et al.* (2015)
			M	-	0.44	1.15	
Enterocotpus dofleini[M]	Enzyme immunoassay	Feces	F	99.4	232	-	Larson and Anderson (2010)
			M	-	-	18.2	
Octopus bimaculoides[H]	Enzyme immunoassay	Dermal swab	F	4619 ng/mL ethanol	1262 ng/mL ethanol	873 ng/mL ethanol	Chancellor *et al.* (2023)
			M	-	-	876 ng/mL ethanol	
Macroctopus maorum[M]	LC/MS/MS	Beak	F	537	706	below detection	Present study

			M	781	794	below detection	
Octopus pallidus[H]	LC/MS/MS	Beak	F	1226	2197	below detection	
			M	294	2013	below detection	

Table 0.2: Review of maximum recorded hormone (estradiol, progesterone, and testosterone) concentrations in the hard tissues of marine animals and methods used.

Species	**Method**	**Tissue**	**Sex**	**Max hormone concentrations (ng/g)**			**Reference**
				Estradiol	Progesterone	Testosterone	
Bowhead whale (*Balaena mysticetus*)	Enzyme immunoassay	Baleen	F	-	2,143.3	-	Hunt *et al.* (2014)
			F	~13	~1,100	-	Lysiak *et al.* (2023)
			M	-	9	-	Hunt *et al.* (2014)
			M	-	-	11	Hunt *et al.* (2018)
North Atlantic right whale (*Eubalaena glacialis*)	Enzyme immunoassay	Baleen	F	-	~3,000	-	Hunt *et al.* (2016)
			M	-	-	8.5	Hunt *et al.* (2018)
Blue whale	Enzyme	Baleen	M	-	-	40	Hunt *et al.* (2018)

(*Balaenoptera musculus*)	immunoassay						
Humpback whale (*Megatera novaeangliae*)	Enzyme immunoassay	Baleen	F	-	966.5	-	Lol *et al.* (2021)
			F	11.84	-	22.45	Lol *et al.* (2022)
Pacific walrus (*Odobenus rosmarus divergens*)	LC/MS/MS	Bone	M	-	-	1,334 ng/ g lipid	Charapata *et al.* (2018)
			F	9,461 ng/ g lipid	30,330 ng/ g lipid	7,622 ng/ g lipid	
	LC/MS/MS	Bone	Both	9,754 ng/ g lipid	276,408 ng/ g lipid	14,393 ng/ g lipid	Charapata *et al.* (2021)
Harbor seal (*Phoca vitulina*)	Enzyme immunoassay	Whiskers	F	87	2.1	-	Keogh *et al.* (2020)
Ringed seal (*Pusa hispida*)	Enzyme immunoassay	Whiskers	F	54	3.5	-	Keogh *et al.* (2020)
Northern fur seal (*Callorhinus ursinus*)	Enzyme immunoassay	Whiskers	F + 1 unknown	5.5	~490	21	Keogh *et al.* (2021)
Steller sea lion (*Eumetopias jubatus*)	Enzyme immunoassay	Whiskers	F	0.9	~55	31	Keogh *et al.* (2021)
Yelloweye rockfish (*Sebastes ruberrimus*)	Enzyme immunoassay	Opercula	F	~200 ng/ g lipid	~700 ng/ g lipid	-	Charapata *et al.* (2022)

Discussion

Here, I developed a new method to extract sex hormones from an accretionary hard tissue of a marine invertebrate. By employing LC/MS/MS, I successfully detected progesterone and estradiol in small sub-samples (as small as 2 mg) taken along the beak growth axis.

Our findings revealed a consistent pattern with progesterone, which was consistently higher than estradiol; a trend also recorded in the soft tissues of octopus (D'Aniello *et al.*, 1996; Di Cosmo *et al.*, 2001; Larson and Anderson, 2010). The role of progesterone in maturation and reproduction in octopus is well documented, contributing to the proliferation of follicle cells, and therefore the synthesis of egg yolk (Di Cristo *et al.*, 2004; Di Cristo, Paolucci, *et al.*, 2010), and activation of stored spermatozoa to facilitate fertilization (Tosti *et al.*, 2001). While more research has focused on progesterone, the similar patterns observed with estradiol suggests that estradiol also plays a part in reproductive strategies.

In *O. pallidus* individuals, both hormones initially rose, possibly at the onset of gonadal development, followed by a larger peak likely coinciding with peak vitellogenesis. Whereas for *M. maorum*, only a single large peak was present. While the reason for the small peak is unknown, I hypothesise that it may coincide with the holobenthic reproductive strategy of *O. pallidus*. Regardless of the smaller peak, I hypothesise that the time leading up to the large peak indicates the pre/ early vitellogenic period in which the cells in the ovaries are maturing, the large peak indicates full vitellogenesis in which the individual becomes sexually mature, and any time thereafter indicates the late vitellogenic period (Di Cosmo *et al.*, 2001). In longer lived, live bearing species like yelloweye rockfish (*Sebastes ruberrimus*) and seals (Phocidae), peaks can indicate significant events such as the birthing of offspring (Keogh *et al.*, 2020; Charapata *et al.*, 2022), but since octopus only lay eggs at the end of life and all individuals samples were classified as mature (rather than spent), I do not expect that the peaks are associated with egg laying.

Although I cannot relate the increment counts to absolute age, I can utilise these peaks in progesterone and estradiol to estimate that the physiological maturity of males occurs around 80-125 increments in both species, and females at around 75-100 increments. When periodicity is validated in the beaks for these species, number of increments can then be correlated to age in days.

In the present study, *O. pallidus* individuals exhibited more than twice the hormone concentrations of *M. maorum*, possibly reflecting differences in reproductive strategies as *O. pallidus* have fewer, large eggs and demersal hatchlings (holobenthic) and *M. maorum* have many, smaller eggs and planktonic paralarvae (merobenthic) (Leporati, Pecl, *et al.*, 2008; Carrasco, 2014). I hypothesise that holobenthic species such as *O. pallidus* may require higher hormone levels for the development and incubation of large eggs. However, merobenthic species tend to have a higher gonadosomatic index (GSI) than holobenthic species, suggesting that higher hormone levels are not related to bigger gonads. For example, the maximum GSI (i.e., GSI at maturity) for merobenthic species such as *M. maorum* (12%, Grubert and Wadley, 2000), *O. vulgaris* (6%, Smale and Buchan, 1981; 10%, Di Cosmo *et al.*, 2001), *Octopus dofleni* (15%, Hartwick, 1983) and *Eledone cirrhosa* (19%, Boyle *et al.*, 1988) are higher than what is recorded for *O. pallidus* (<4.2%, Leporati *et al.*, 2015).

The difference between the maximum hormone concentrations I observed and those from the gonads and feces of other octopus species could either be due to the difference in tissue type or analysis method as these previous studies used assays instead of LC/MS/MS. Of the few studies that have used LC/MS/MS, comparisons are difficult, as they present hormone concentration relative to amount of extracted lipid rather than relative to the amount of tissue (Charapata *et al.*, 2018, 2022). To determine if higher hormone concentrations are due to tissue type or analysis method, a future study comparing different tissue types within the same species while using the LC/MS/MS method is needed.

The method developed here could be particularly useful for the study of rare or rarely encountered species since cephalopod beaks are often found in stomachs of predators such as seabirds and marine mammals (Clarke and Kristensen, 1980; Clarke and Macleod, 1982; Clarke and Young, 1998). For example, the extent of the knowledge of deep sea squid has primarily come from juvenile specimens that have been found dead, caught in nets, or from beaks found in predator's stomachs, (Clarke, 1965; Rosa *et al.*, 2017; Laptikhovsky *et al.*, 2019). Therefore, information such as reproductive maturity can only be inferred from observing the gonads of the few intact whole specimens (Laptikhovsky *et al.*, 2007, 2019). In contrast, the novel method of analysing hormones in the beak provides information on the maturity stages across the individual's life span, and only the beak is required. Importantly, this method may also be applied to other chitinous structures, such as squid gladii, which are also comprised of chitin (Cherel *et al.*, 2009) and exhibit incremental growth (Arkhipkin *et al.*, 2018).

Conclusion

In conclusion, I have shown that LC/MS/MS is an effective method for the quantitation of estradiol and progesterone in a chitinous structure, which allows identification of sexual maturity in octopus. The differences in concentrations between the two species also suggests that reproductive strategy may influence the production and use of the two hormones. This novel method may be applied to a variety of cephalopod beaks and other structures, such as squid gladii, to further the knowledge of a species' lifetime maturation so that critical information such as age at maturity can be inferred without the need for whole specimens.

General discussion

Although invertebrates, cephalopods have a large range of hard parts that can be used for ecological studies. Hard parts have been widely used in vertebrates such as fish, but only to a limited extent in cephalopods. The advantage of utilising hard parts as an ecological tool is that they allow a wide range of data to be obtained including life history and environmental data that may otherwise be too expensive or too difficult to collect. They are also small and preserve well once dry allowing for easy storage in historical archives. In this thesis, I utilised both modern and historical samples to demonstrated a number of ways in which cephalopod hard parts are currently used (e.g., ageing) as well as developed new methods which have the potential to greatly advance cephalopod research in the future (e.g., hormone analysis). This research also aids in the future management of these commercial and culturally important species. For example, the age data produced here will aid fisheries managers to understand more about the current sustainability of *O. berrima* and if further conservation measures need to be put into place. In addition, the methods from the hormone study (Chapter 6) opens doors for further research to easily assess age of maturity of commercial species across different populations to determine if the populations need different rules and regulations.

Hard parts for ageing octopus

To properly regulate a fishery, life history information, including age data, of the target species is required (Campana, 2001; Arkhipkin *et al.*, 2018). Ageing commercial marine species typically involves counting growth increments, as well as validating the periodicity of those increments. For octopus, 19 species have been aged, but only 8 species have been aged using validated methods (see Table 1, Chapter 2) (Doubleday *et al.*, 2006; Hermosilla *et al.*, 2010; Perales-Raya *et al.*, 2017; Donlon *et al.*, 2019; Batista *et al.*, 2021). Growth increments in beaks and stylets are often assumed to be daily, based on validation studies done on other species. However, as seen in Chapter 2, I found evidence of nondaily periodicity in both beaks and stylets, and that increment periodicity varied between ageing structures and was also influenced by temperature. As such, I recommend that age validation is undertaken for species that are aged for the first time, and potentially for each distinct sub-species or sub-population (i.e., *Octopus vulgaris* in the Mediterranean vs west Atlantic), as well as for each ageing structure (i.e., beak or stylet). Validation can be done with known age octopus that have been raised in captivity or with chemical staining. While both validation methods should be done under conditions as close to natural as possible, a key limitation of validation experiments is that it is unknown how much captivity affects increment periodicity.

Optimising sample preparation for new species is also crucial to successfully ageing octopus and for researchers that have never aged octopus before, it is difficult to know where to start. Therefore, to ensure the continued advancement and uptake of correct octopus ageing methods, I also collaborated with colleagues to create a step-by-step ageing guide (Chapter 3) as a starting point for researchers looking to age octopus. Advancements made in Chapters 2 and 3 will improve the science of octopus ageing around the world.

Hard parts for reconstructing experienced temperatures

The use of stable isotopes in marine calcified structures has long been used as a tool for paleoenvironmental studies, as well as modern-day studies, in which oxygen and carbon isotopic ratios (δ^{18}O and δ^{13}C) are used to reconstruct past environmental conditions (Fraile *et al.*, 2016; Wu *et al.*, 2021). The δ^{18}O values in marine carbonates are widely used to reconstruct temperatures as they are in near isotopic equilibrium with seawater and there is a well-known inverse relationship between temperature and δ^{18}O values in seawater (Grossman and Ku, 1986; Kalish, 1991a, 1991b). Although this trend is similar across different taxa, the degree of this relationship, such as slope and intercept, differs between species (Grossman and Ku, 1986; Chung, Chen, *et al.*, 2020). Thus, before thermal histories can be reconstructed accurately, the relevant oxygen-thermal relationship first needs to be described, which can be achieved through controlled experimental rearing.

There are no known oxygen thermometry equations for octopus, with little research undertaken on octopus statoliths (carbonate structures analogous to fish otoliths) (but see Daryanani *et al.*, 2021; Martino, Chung, *et al.*, 2022). As such, for Chapter 4, I reared octopus from hatchling to 110 days old under three different temperatures to determine the relationship between statolith δ^{18}O values and temperature. Results confirmed that octopus (*Octopus berrima*) reared at warmer temperatures had a depletion in statolith δ^{18}O values much like previous studies on other marine species (Grossman and Ku, 1986; Kalish, 1991b; Chung, Chen, *et al.*, 2020). I was then able to create a thermal reconstruction equation with the relationship in which the rate of change for *O. berrima* (-0.16) is similar to that of the cuttlefish, *Sepia officinalis* (-0.15) (Bettencourt and Guerra, 1999; Rexfort and Mutterlose, 2006; Dance *et al.*, 2014) and Japanese eel, *Anguilla japonica* (-0.15) (Shirai *et al.*, 2018). With this equation established for *O. berrima*, future studies can reconstruct temperatures

experienced by wild-caught octopus to determine habitat use, study a population, and determine seafood provenance.

In using statoliths in temperature reconstructions, there are some important caveats to note. While cephalopod statoliths are valuable tools for thermal reconstructions, the statoliths of *O. berrima* are very small, and the entire statolith needs to be analysed to measure the $\delta^{18}O$ values of an individual. Analysing the entire statolith means that the reconstructed temperature represents the lifetime average. Furthermore, octopus statoliths grow irregularly (Young, 1960), and it is not well known what, if any, part of the statolith represents the birth or death of the animal (Daryanani *et al.*, 2021). As such, questions relating specifically to natal origins or capture conditions are more difficult to isolate in statoliths compared to clearly incremental structures such as stylets or otoliths. A second caveat is that although octopus are not highly mobile, they spend most of their time in a den or shelter which might be thermally different than the open seafloor. Octopus may also seek thermal refugia if conditions are not optimal, which is common in ectotherms (Higgins *et al.*, 2012; Abram *et al.*, 2017). Salinity also plays a part in the incorporation of $\delta^{18}O$ values from seawater into carbonate and these oxygen-thermal equations do not include the salinity effect (Chung, Chen, *et al.*, 2020; Martino, Chung, *et al.*, 2022). Therefore, accounting for natural salinity deviation through incorporating uncertainty margins within oxygen-thermometry models can enable more accurate temperature reconstructions.

Hard part archives to record environmental changes across time and space

Because hard parts preserve so well naturally, they have a large presence in historical archives, which allows access to samples over a long timeframe that otherwise would have been impossible to obtain. Since hard parts are excellent recorders of their environment, historical archives have the potential to unravel past environmental conditions. Historical samples are often used for geological studies, but rarely for marine biological studies (McClenachan *et al.*, 2012). A recent example of the use of historical hard parts is the use of California mussel shells from middens in Washington, USA showed the decline in $\delta^{13}C$ over the past 1350 years (Pfister *et al.*, 2011). Newsome *et al.* (2007) also observe the decline in $\delta^{13}C$ over the past 50 years by analysing fur seal teeth that were originally collected to monitor the population age structure.

When working with opportunistic samples, there is not much control on when and where samples were collected. For example, in Chapter 5, I found that although the data spanned a

large spatial and temporal scale, this hindered analyses as it became difficult to disentangle effects from space and time. Using beach collected cuttlebones can also be problematic because of their buoyant nature, and it is possible that cuttlebones drifted significant distances before washing up on shore at the final site of collection (Reid, 2016). Therefore, to remove spatial effects and potential effects of drifting, I analysed cuttlebones from specific oceanographic regions (two South Australian gulfs) where large-scale drifting is likely minimal. In Spencer Gulf, I was able to detect a temporal change in cuttlebone $\delta^{13}C$ and $\delta^{18}O$ values over time possibly due to the increase in atmospheric CO_2 (i.e., Suess effect) and global warming. However, sample size and temporal span was greatly reduced. Despite this, the robust nature of the spatial effects, suggest that $\delta^{13}C$ and $\delta^{18}O$ values in biogenic carbonates are strong geographic predictors, which is useful for population studies and seafood provenance. For future studies interested in using hard parts to detect change over time, I recommend using only samples from a single area (e.g., a gulf) to remove the confusion of space and time effects.

Hard parts for reconstructing biological histories

Reconstructing metabolic rate

Stable isotopes such as carbon ($\delta^{13}C$) can also be used to study different biological changes, such as metabolic rate. $\delta^{13}C$ values can be used as a proxy of metabolic rate by using a two-component mixing model to calculate C_{resp} (i.e., metabolic rate) (Schwarcz *et al.*, 1998; Solomon *et al.*, 2006; Chung, Trueman, Godiksen and Grønkjær, 2019; Chung *et al.*, 2021). Typically, because the way the model is formatted, lower $\delta^{13}C$ values indicates higher metabolic rate. In fish, a decrease in metabolic rate is often observed with age (Mulcahy *et al.*, 1979; Schwarcz *et al.*, 1998; Brown *et al.*, 2004), but for the short-lived octopus, I found that metabolic rate increases with body mass (up to 110 days old). This negative relationship with body mass was likely due to an observed shift from $\delta^{13}C$ values similar to those of dissolved inorganic carbon (DIC) in the water to $\delta^{13}C$ values similar to those of their diet (Chapter 4), which is why as the octopus grow, they are assimilating more $\delta^{13}C$ from their diet than from the seawater. This is represented by the increase in metabolic rate. This is an inverse metabolic relationship compared to many longer-lived fish species, because coastal species of octopus only live for about one year, and the majority of their life is spent in a fast growing, exponential phase (Semmens *et al.*, 2004).

Temperature also influences metabolic rate, with higher metabolic rates at higher temperatures until the optimal thermal maximum is reached (Brown *et al.*, 2004; Martino *et al.*, 2020; Chung *et al.*, 2021). While there was a slight trend for *O. berrima*, I was unable to show that temperature had a statistically significant effect on metabolic rate. This lack of relationship could be due to the relatively narrow temperature ranges that I focused on (4 °C) as opposed to other studies using a larger temperature range that did find a significant effect (e.g., 10 °C range, Chung *et al.* 2021; 8 °C range, Martino *et al.* (2020)). Evidence also suggests that 24 °C is reaching the thermal maximum of this species due to the poor growth and survivability (Chapter 4) and maximum recorded temperatures from their habitat (Martino *et al.*, 2021).

Reconstructing reproductive histories and maturity status

Hard parts can also be used to reconstruct reproductive histories to reveal information such as size- and age-at-maturity. Typically for cephalopods, reproductive stages and age-at-maturity are determined by analysing the gonadal structure of many individuals (Arkhipkin, 1992; de Lima *et al.*, 2014; Kivengea *et al.*, 2014). Hormonal concentrations from the gonads (D'Aniello *et al.*, 1996; Di Cristo, Di Donato, *et al.*, 2010), dermal swabs (Chancellor *et al.*, 2023) and faeces (Larson and Anderson, 2010) have also been used to identify maturity stages. But both gonad observation and soft tissue hormone analysis only allow a snapshot of the individual at that particular time.

In Chapter 6, I developed a method to reconstruct the entire reproductive history of male and female individuals of two species of octopus. I extracted steroid hormones, such as progesterone and oestradiol, from two species of octopus beaks and then analysed the extracts by LC/MS/MS to observe changes in hormone concentration along the beak axis. Because beaks have incremental growth, this produced a history of hormone concentrations over the individual's entire life. This method of using hormones to construct reproductive histories has been done in a other marine animals including fish (Charapata *et al.*, 2022), seals and sea lions (Keogh *et al.*, 2020, 2021), walruses (Charapata *et al.*, 2018), and whales (Trumble *et al.*, 2013, 2018; Hunt *et al.*, 2018; Crain *et al.*, 2020), but never in an invertebrate like octopus. The advantage of this method that I developed is that it can be applied to any cephalopod beak and is particularly useful for species that are difficult to obtain, such as those inhabiting the deep sea or Antarctic waters. Since cephalopod beaks are often discovered

intact in the stomachs of their predators, the beaks are easier to obtain than whole specimens. The development of this technique will enable further research on the reproductive histories on other species of cephalopods to further the knowledge of cephalopod biology around the world.

Future directions

In this thesis, I showed how cephalopod hard parts can be used for a range of ecological and fisheries focussed questions. However, there are a range of challenges and exciting possibilities that could be addressed in future research.

Firstly, octopus statoliths represent the potential to reveal more about octopus biology and ecology. However, little is known about how octopus statoliths grow. Currently, whole statoliths are used for chemical analyses, but different parts of the statolith could be targeted for unique chemical markers, allowing researchers to pinpoint different life history stages. Octopus statoliths are conical in shape and have no growth increments (Young, 1960; Clarke, 1978a). Interestingly, sea lamprey have statoliths, unlike other teleost fish, which are also conical in shape and have been shown to grow in length and width with age (Potts *et al.*, 2015). Therefore, future studies could focus more on how octopus statoliths grow by chemically marking individuals in the laboratory at known intervals and determining growth patterns. If I understood the direction of growth in octopus statoliths, then I could subsample along the growth axis or at least target the natal and adult portions.

Second, with the development of hormone analysis in beaks (Chapter 6), there is a huge opportunity for future research to investigate reproductive histories for a variety of cephalopod species and over long time periods. For example, with the use of historical beak archives, it may be possible to determine how size- or age-at-maturity is changing in cephalopod populations over many years. Alternatively, maturity could be identified for iconic species like the giant and colossal squids, since it is often only the juveniles that are found. However, more research on different species is needed to link hormone levels to reproductive events and life history strategies. Furthermore, if hormonal data can be linked to validated growth increments in beaks, then hormonal peaks can be related back to age. Ideally the octopus will have been raised in captivity, and hormonal peaks can be related back to major life-time events, such as egg laying.

Lastly, the idea of using carbonate $\delta^{13}C$ values as a proxy of metabolic rate is relatively new and has potential to investigate metabolic changes over long periods of time with the use of historical archives. In addition to carbonate $\delta^{13}C$ values, $\delta^{13}C_{diet}$ and $\delta^{13}C_{DIC}$ values are also needed to calculate metabolic rate, but if unknown, $\delta^{13}C_{DIC}$ values can also be predicted from Kroopnick (1985). It is difficult to obtain a $\delta^{13}C_{diet}$ value, since this can change between individuals. Therefore, studies should be done to investigate how to extract $\delta^{13}C_{diet}$ from the already available carbonate. If possible, future studies will have the tools to investigate changes in diet and metabolic rates over decades.

Conclusions

Cephalopods, compared to other commercially exploited species, are understudied. Here, I have shown that hard parts of cephalopods are great tools to utilise as cephalopod research accelerates. These hard parts are prevalent in historical archives because they are metabolically inert and naturally preserve well. Hard parts also hold a wealth of information within their small structures and are a useful tool for studying environmental and biological changes across space and time. In this thesis, I have demonstrated how cephalopod hard parts can be used for ageing, reconstructing temperatures, metabolic histories, reproductive histories, trends in environmental CO_2 and possible geolocation. The research within this thesis contributes to the knowledge of cephalopods and their environment and aids in the future conservation and management of cephalopod species around the world.

References

Abram, P.K., Boivin, G., Moiroux, J. and Brodeur, J. (2017) 'Behavioural effects of temperature on ectothermic animals: unifying thermal physiology and behavioural plasticity', *Biological Reviews*, 92(4), pp. 1859–1876. Available at: https://doi.org/10.1111/brv.12312.

Adams, P.B. (1980) 'Life-history patterns in marine fishes and their consequences for fisheries management', *Fishery Bulletin*, 78(1), pp. 1–12.

Aitken, J.P., O'Dor, R.K. and Jackson, G.D. (2005) 'The secret life of the giant Australian cuttlefish *Sepia apama* (Cephalopoda): Behaviour and energetics in nature revealed through radio acoustic positioning and telemetry (RAPT)', *Journal of Experimental Marine Biology and Ecology*, 320(1), pp. 77–91. Available at: https://doi.org/10.1016/j.jembe.2004.12.040.

Alewijnse, S., Stowasser, G., Saunders, R., Belcher, A., Crimmen, O., Cooper, N. and Trueman, C. (2021) 'Otolith-derived field metabolic rates of myctophids (Family Myctophidae) from the Scotia Sea (Southern Ocean)', *Marine Ecology Progress Series*, 675, pp. 113–131. Available at: https://doi.org/10.3354/meps13827.

Almonacid-Rioseco, E., Hernández-García, V., Solari, A.P., Del Pino, Á.S. and Castro, J.J. (2009) 'Sex identification and biomass reconstruction from the cuttlebone of *Sepia officinalis* ', *Marine Biodiversity Records*, 2(May 2014). Available at: https://doi.org/10.1017/s1755267208000079.

Amor, M.D., Doyle, S.R., Norman, M.D., Roura, A., Hall, N.E., Robinson, A.J., Leite, T.S. and Strugnell, J.M. (2019) 'Genome-wide sequencing uncovers cryptic diversity and mito-nuclear discordance in the *Octopus vulgaris* species complex', *bioRxiv*, p. 573493. Available at: https://doi.org/10.1101/573493.

Anderson, R., Wood, J.B. and Bryne, R.A. (2001) 'Octopus senescence: The beginning of the end', *Journal of Applied Animal Welfare Science*, 5(4), pp. 37–41. Available at: https://doi.org/10.1207/S15327604JAWS0504.

André, J., Grist, E.P.M., Semmens, J.M., Pecl, G.T. and Segawa, S. (2009) 'Effects of temperature on energetics and the growth pattern of benthic octopuses', *Marine Ecology Progress Series*, 374, pp. 167–179. Available at: https://doi.org/10.3354/meps07736.

Andrews, P.L.R., Darmaillacq, A.S., Dennison, N., Gleadall, I.G., Hawkins, P., Messenger, J.B., Osorio, D., Smith, V.J. and Smith, J.A. (2013) 'The identification and management of pain, suffering and distress in cephalopods, including anaesthesia, analgesia and humane killing',

Journal of Experimental Marine Biology and Ecology, 447, pp. 460–64. Available at: https://doi.org/10.1016/j.jembe.2013.02.010.

Appellöf, J.J.A. (1893) *Die schalen von Sepia, Spirula, und Nautilus: Studien über den bau und das wachsthum*, *PA Norsted & söner*.

Arkhipkin, A.I. (1992) 'Reproductive system structure, development and function in cephalopods with a new general scale for maturity stages', *Journal of Northwest Atlantic Fishery Science*, 12, pp. 63–74. Available at: https://doi.org/10.2960/j.v12.a7.

Arkhipkin, A.I. (2005) 'Statoliths as "black boxes" (life recorders) in squid', *Marine and Freshwater Research*, 56(5), pp. 573–583. Available at: https://doi.org/10.1071/MF04158.

Arkhipkin, A.I., Bizikov, V.A., Doubleday, Z.A., Laptikhovsky, V. V., Lishchenko, F. V., Perales-Raya, C. and Hollyman, P.R. (2018) 'Techniques for estimating the age and growth of molluscs: Cephalopoda', *Journal of Shellfish Research*, 37(3), pp. 1–10. Available at: https://doi.org/10.2983/035.037.0300.

Arkhipkin, A.I., Rodhouse, P.G.K., Pierce, G.J., Sauer, W., Sakai, M., Allcock, L., Arguelles, J., Bower, J.R., Castillo, G., Ceriola, L., Chen, C.S., Chen, X., Diaz-Santana, M., Downey, N., González, A.F., Granados Amores, J., Green, C.P., Guerra, A., Hendrickson, L.C., Ibáñez, C., Ito, K., Jereb, P., Kato, Y., Katugin, O.N., Kawano, M., Kidokoro, H., Kulik, V. V., Laptikhovsky, V. V., Lipinski, M.R., Liu, B., Mariátegui, L., Marin, W., Medina, A., Miki, K., Miyahara, K., Moltschaniwskyj, N., Moustahfid, H., Nabhitabhata, J., Nanjo, N., Nigmatullin, C.M., Ohtani, T., Pecl, G., Perez, J.A.A., Piatkowski, U., Saikliang, P., Salinas-Zavala, C.A., Steer, M., Tian, Y., Ueta, Y., Vijai, D., Wakabayashi, T., Yamaguchi, T., Yamashiro, C., Yamashita, N. and Zeidberg, L.D. (2015) 'World squid fisheries', *Reviews in Fisheries Science and Aquaculture*, 23(2), pp. 92–252. Available at: https://doi.org/10.1080/23308249.2015.1026226.

Arkhipkin, A.I. and Shcherbich, Z.N. (2012) 'Thirty years' progress in age determination of squid using statoliths', *Journal of the Marine Biological Association of the United Kingdom*, 92(6), pp. 1389–1398. Available at: https://doi.org/10.1017/S0025315411001585.

Armelloni, E.N., Lago-Rouco, M.J., Bartolomé, A., Felipe, B.C., Almansa, E. and Perales-Raya, C. (2020) 'Exploring the embryonic development of upper beak in *Octopus vulgaris* Cuvier, 1797: New findings and implications for age estimation', *Fisheries Research*, 221(August 2019), p. 105375. Available at: https://doi.org/10.1016/j.fishres.2019.105375.

Auclair, A.C., Lecuyer, C., Bucher, H. and Sheppard, S.M.F. (2004) 'Carbon and oxygen isotope

composition of *Nautilus macromphalus*: A record of thermocline waters off New Caledonia', *Chemical Geology*, 207(1–2), pp. 91–100. Available at: https://doi.org/10.1016/j.chemgeo.2004.02.006.

Avendaño, O., Roura, Á., Cedillo-Robles, C.E., González, Á.F., Rodríguez-Canul, R., Velázquez-Abunader, I. and Guerra, Á. (2020) '*Octopus americanus*: a cryptic species of the *O. vulgaris* species complex redescribed from the Caribbean', *Aquatic Ecology*, 54(4), pp. 909–925. Available at: https://doi.org/10.1007/s10452-020-09778-6.

Avila-Poveda, O.H., Montes-Pérez, R.C., Benitez-Villalobos, F. and Rosas, C. (2013) 'Development and validation of a solid-phase radioimmunoassay for measuring progesterone and testosterone in octopus gonad extracts', *Malacologia*, 56(1–2), pp. 121–134. Available at: https://doi.org/10.4002/040.056.0209.

Avila-Poveda, O.H., Montes-Pérez, R.C., Koueta, N., Benítez-Villalobos, F., Ramírez-Pérez, J.S., Jimenez-Gutierrez, L.R. and Rosas, C. (2015) 'Seasonal changes of progesterone and testosterone concentrations throughout gonad maturation stages of the Mexican octopus, *Octopus maya* (Octopodidae: Octopus)', *Molluscan Research*, 35(3), pp. 161–172. Available at: https://doi.org/10.1080/13235818.2015.1045055.

Bandel, K. and von Boletzky, S. (1979) 'A comparative study of the structure development and morphological relationships of chambered cephalopod shells', *The Veliger*, 21(3), pp. 313–354.

Barratt, I.M. and Allcock, A.L. (2010) 'Ageing octopods from stylets: Development of a technique for permanent preparations', *ICES Journal of Marine Science*, 67(7), pp. 1452–1457. Available at: https://doi.org/10.1093/icesjms/fsq047.

Bath, G.E., Thorrold, S.R., Jones, C.M., Campana, S.E., McLaren, J.W. and Lam, J.W.H. (2000) 'Strontium and barium uptake in aragonitic otoliths of marine fish', *Geochimica et Cosmochimica Acta*, 64(10), pp. 1705–1714. Available at: https://doi.org/10.1016/S0016-7037(99)00419-6.

Batista, B.B., Matthews-Cascon, H., Marinho, R.A., Kikuchi, E. and Haimovici, M. (2021) 'The growth and population dynamics of *Octopus insularis* targeted by a pot longline fishery in north-eastern Brazil', *Journal of the Marine Biological Association of the United Kingdom*, 101(6), pp. 935–946. Available at: https://doi.org/10.1017/S0025315421000898.

Bayer, S., Beierlein, L., Morán, G.A., Doldán, M.S., Morsan, E.M., Brey, T., Mackensen, A., Farias, L., García, G. and Gordillo, S. (2020) 'Late Quaternary climatic variability in northern Patagonia,

Argentina, based on $\delta^{18}O$ of modern and fossil shells of Amiantis purpurata (Bivalvia, Veneridae)', *Palaeogeography, Palaeoclimatology, Palaeoecology*, 560, p. 110012. Available at: https://doi.org/10.1016/j.palaeo.2020.110012.

Bettencourt, V. and Guerra, A. (1999) 'Carbon- and oxygen-isotope composition of the cuttlebone of *Sepia officinalis*: A tool for predicting ecological information?', *Marine Biology*, 133(4), pp. 651–657. Available at: https://doi.org/10.1007/s002270050505.

Bettencourt, V. and Guerra, A. (2001) 'Age studies based on daily growth increments in statoliths and growth lamellae in cuttlebone of cultured *Sepia officinalis*', *Marine Biology*, 139(2), pp. 327–334. Available at: https://doi.org/10.1007/s002270100582.

Bizikov, V. (2004) 'The shell in Vampyropoda (Cephalopoda): Morphology, funtional role and evolution', *Ruthenica*, Supplement.

Borges, F.O., Sampaio, E., Santos, C.P. and Rosa, R. (2023) 'Climate-Change Impacts on Cephalopods: A Meta-Analysis', *Integrative And Comparative Biology*, pp. 1–26. Available at: https://doi.org/10.1093/icb/icad102.

Borrell, A., Saiz, L., Víkingsson, G.A., Gaufier, P., López Fernández, A. and Aguilar, A. (2018) 'Fin whales as bioindicators of multi-decadal change in carbon and oxygen stable isotope shifts in the North Atlantic', *Marine Environmental Research*, 138(March), pp. 129–134. Available at: https://doi.org/10.1016/j.marenvres.2018.04.014.

Boyle, P.R., Mangold, K. and Ngoile, M. (1988) 'Biological variation in *Eledone cirrhosa* (Cephalopoda: Octopoda): simultaneous comparison of North Sea and Mediterranean populations.', *Malacologia*, 29(1), pp. 77–87.

Brown, J.H., Gillooly, J.F., Allen, A.P., Savage, V.M. and West, G.B. (2004) 'Toward a metabolic theory of ecology', *Ecology*, 85(7), pp. 1771–1789.

Budelmann, B.U. (1998) 'Autophagy in *Octopus*', *South African Journal of Marine Science*, (20), pp. 101–108. Available at: https://doi.org/10.2989/025776198784126502.

Caddy, J.F. and Rodhouse, P.G. (1998) 'Cephalopod and groundfish landings: Evidence for ecological change in global fisheries?', *Reviews in Fish Biology and Fisheries*, 8(4), pp. 431–444. Available at: https://doi.org/10.1023/A:1008807129366.

Campana, S.E. (1992) 'Measurement and Inteipretation of the Microstructure of Fish Otoliths', in D.K. Stevenson and Steven E. Campana (eds) *Otolith Microstructure Examination and*

Analysis. Ottawa: Canadian Special Publication of Fisheries and Aquatic Sciences, pp. 59–71. Available at: https://doi.org/10.1006/jmsc.1993.1053.

Campana, S.E. (1999) 'Chemistry and composition of fish otoliths: Pathways, mechanisms and applications', *Marine Ecology Progress Series*, 188, pp. 263–297. Available at: https://doi.org/10.3354/meps188263.

Campana, S.E. (2001) 'Accuracy , precision and quality control in age determination , including a review of the use and abuse of age validation methods', *Journal of Fish Biology*, 59, pp. 197–242. Available at: https://doi.org/10.1006/jfbi.2001.1668.

Campana, S.E. and Neilson, J.D. (1985) 'Microstructure of fish otoliths', *Canadian Journal of Fisheries and Aquatic Sciences*, 42, pp. 1014–1032.

Campana, S.E. and Thorrold, S.R. (2001) 'Otoliths, increments, and elements: keys to a comprehensive understanding of fish populations?', *Canadian Journal of Fisheries and Aquatic Sciences*, 58(1), pp. 30–38. Available at: https://doi.org/10.1139/cjfas-58-1-30.

Canali, E., Ponte, G., Belcari, P., Rocha, F. and Fiorito, G. (2011) 'Evaluating age in *Octopus vulgaris*: Estimation, validation and seasonal differences', *Marine Ecology Progress Series*, 441, pp. 141–149. Available at: https://doi.org/10.3354/meps09399.

Cárdenas, E.R.B., Correa, S.M., Guzman, R.C., Barahona, N., Briceño, F., Villegas, M.J. and Paredes, R. (2011) 'Eye lens structure of the octopus *Enteroctopus megalocyathus*: Evidence of growth', *Journal of Shellfish Research*, 30(2), pp. 199–204. Available at: https://doi.org/10.2983/035.030.0201.

Carrasco, S.A. (2014) 'The early life history of two sympatric New Zealand octopuses : eggs and paralarvae of *Octopus huttoni* and *Pinnoctopus cordiformis*', *New Zealand Journal of Zoology*, 41(1), pp. 32–45. Available at: https://doi.org/10.1080/03014223.2013.827126.

Chancellor, S., Grasse, B., Sakmar, T., Scheel, D., Brown, J.S. and Santymire, R.M. (2023) 'Exploring the effect of age on the reproductive and stress physiology of *Octopus bimaculoides* using dermal hormones', *Animals*, 13(3115), pp. 1–21. Available at: https://doi.org/10.21203/rs.3.rs-655419/v1.

Charapata, P., Horstmann, L., Jannasch, A. and Misarti, N. (2018) 'A novel method to measure steroid hormone concentrations in walrus bone from archaeological, historical, and modern time periods using liquid chromatography/tandem mass spectrometry', *Rapid Communications in Mass Spectrometry*, 32(23), pp. 1999–2023. Available at: https://doi.org/10.1002/rcm.8272.

Charapata, P., Horstmann, L. and Misarti, N. (2021) 'Steroid hormones in Pacific walrus bones collected over three millennia indicate physiological responses to changes in estimated population size and the environment', *Conservation Physiology*, 9(1), pp. 1–20. Available at: https://doi.org/10.1093/conphys/coaa135.

Charapata, P., Oxman, D., McNeel, K., Keith, A., Mansouri, F. and Trumble, S. (2022) 'Lifetime hormone profiles for a long-lived teleost: opercula reveal novel estimates of age-specific reproductive parameters and stress trends in yelloweye rockfish (*Sebastes ruberrimus*)', *Canadian Journal of Fisheries and Aquatic Sciences*, 17, pp. 1–17. Available at: https://doi.org/10.1139/cjfas-2022-0048.

Cherel, Y., Fontaine, C., Jackson, G.D., Jackson, C.H. and Richard, P. (2009) 'Tissue, ontogenic and sex-related differences in δ13C and δ15N values of the oceanic squid *Todarodes filippovae* (Cephalopoda: Ommastrephidae)', *Marine Biology*, 156(4), pp. 699–708. Available at: https://doi.org/10.1007/s00227-008-1121-x.

Cherel, Y. and Hobson, K.A. (2005) 'Stable isotopes, beaks and predators: A new tool to study the trophic ecology of cephalopods, including giant and colossal squids', *Proceedings of the Royal Society B: Biological Sciences*, 272(1572), pp. 1601–1607. Available at: https://doi.org/10.1098/rspb.2005.3115.

Chiang, C.I., Chung, M.T., Shiao, J.C., Wang, P.L., Chan, T.Y., Yamaguchi, A. and Wang, C.H. (2020) 'Seasonal Movement Patterns of the Bigfin Reef Squid *Sepioteuthis lessoniana* Predicted Using Statolith $\delta^{18}O$ Values', *Frontiers in Marine Science*, 7(April). Available at: https://doi.org/10.3389/fmars.2020.00249.

Choe, S. (1963) 'Daily age markings on the shell of cuttlefishes', *Nature*, 197(4864), pp. 306–307. Available at: https://doi.org/10.1038/197306b0.

Chung, M., Chen, C., Shiao, J., Shirai, K. and Wang, C. (2021) 'Metabolic proxy for cephalopods: Stable carbon isotope values recorded in different biogenic carbonates', *Methods in Ecology and Evolution* [Preprint], (May). Available at: https://doi.org/10.1111/2041-210x.13630.

Chung, M.T., Chen, C.Y., Shiao, J.C., Lin, S. and Wang, C.H. (2020) 'Temperature-dependent fractionation of stable oxygen isotopes differs between cuttlefish statoliths and cuttlebones', *Ecological Indicators*, 115(2), p. 106457. Available at: https://doi.org/10.1016/j.ecolind.2020.106457.

Chung, M.T., Huang, K.F., You, C.F., Chiao, C.C. and Wang, C.H. (2020) 'Elemental Ratios in

Cuttlebone Indicate Growth Rates in the Cuttlefish *Sepia pharaonis '*, *Frontiers in Marine Science*, 6(January), pp. 1–12. Available at: https://doi.org/10.3389/fmars.2019.00796.

Chung, M.T., Trueman, C.N., Godiksen, J.A. and Grønkjær, P. (2019) 'Otolith $\delta^{13}C$ values as a metabolic proxy: Approaches and mechanical underpinnings', *Marine and Freshwater Research*, 70(12), pp. 1747–1756. Available at: https://doi.org/10.1071/MF18317.

Chung, M.T., Trueman, C.N., Godiksen, J.A., Holmstrup, M.E. and Grønkjær, P. (2019) 'Field metabolic rates of teleost fishes are recorded in otolith carbonate', *Communications Biology*, 2(1), pp. 1–10. Available at: https://doi.org/10.1038/s42003-018-0266-5.

Chung, M.T. and Wang, C.H. (2013) 'Age validation of the growth lamellae in the cuttlebone from cultured *Sepia pharaonis* at different stages', *Journal of Experimental Marine Biology and Ecology*, 447, pp. 132–137. Available at: https://doi.org/10.1016/j.jembe.2013.02.020.

Clarke, M. (1965) '"Growth rings" in the beaks of the squid *Moroteuthis ingens* (Oegopsida: Onychoteuthidae)', *Malacologia*, 3(2), pp. 287–307.

Clarke, M. and Young, R. (1998) 'Description and analysis of cephalopod beaks from stomachs of six species of odontocete cetaceans stranded on Hawaiian shores', *Journal of the Marine Biological Association of the United Kingdom*, 78(2), pp. 623–641. Available at: https://doi.org/10.1017/S0025315400041667.

Clarke, M.R. (1978a) 'The Cephalopod Statolith- An Introduction to its Form', *Journal of Marine Biol. Ass. U.K.*, 58, pp. 701–712.

Clarke, M.R. (1978b) 'The Cephalopod statoltih- An introduction to its form', *Journal of Marine Biology*, (May), pp. 701–712.

Clarke, M.R. (1996) 'Cephalopods as prey. III. Cetaceans', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 351(1343), pp. 1053–1065. Available at: https://doi.org/10.1098/rstb.1996.0093.

Clarke, M.R. and Kristensen, T.K. (1980) 'Cephalopod beaks from the stomachs of two northern bottlenosed whales (*Hyperoodon ampullatus*)', *Journal of the Marine Biological Association of the United Kingdom*, 60(1), pp. 151–156. Available at: https://doi.org/10.1017/S002531540002419X.

Clarke, M.R. and Macleod, N. (1982) 'Cephalopod remains in the stomachs of eight Weddell seals', *British Antarctic Survey Bulletin*, 57, pp. 33–40.

Coplen, T.B. (1996) 'New guidelines for reporting stable hydrogen, carbon, and oxygen isotope-ratio data', *Geochimica et Cosmochimica Acta*, 60(17), pp. 3359–3360. Available at: https://doi.org/10.1016/0016-7037(96)00263-3.

Di Cosmo, A., Di Cristo, C. and Paolucci, M. (2001) 'Sex steroid hormone fluctuations and morphological changes of the reproductive system of the female of *Octopus vulgaris* throughout the annual cycle', *Journal of Experimental Zoology*, 289(1), pp. 33–47. Available at: https://doi.org/10.1002/1097-010X(20010101/31)289:1<33::AID-JEZ4>3.0.CO;2-A.

Crain, D.D., Thomas, A., Mansouri, F., Potter, C.W., Usenko, S. and Trumble, S.J. (2020) 'Hormone comparison between right and left baleen whale earplugs', *Conservation Physiology*, 8(1), pp. 1–9. Available at: https://doi.org/10.1093/conphys/coaa055.

Di Cristo, C., Di Donato, P., Palumbo, A., D'Ischia, M., Paolucci, M. and Di Cosmo, A. (2010) 'Steroidogenesis in the brain of *Sepia officinalis* and *Octopus vulgaris*', *Frontiers in Bioscience - Elite*, 2 E(2), pp. 673–683. Available at: https://doi.org/10.2741/e127.

Di Cristo, C., Paolucci, M. and Cosmo, A. Di (2004) 'Progesterone and 17ß-estradiol involvement in the yolk protein synthesis in the ovary of *Octopus vulgaris*', *Journal of Experimental Biology*, pp. 1–4.

Di Cristo, C., Paolucci, M. and Di Cosmo, A. (2010) 'Progesterone affects vitellogenesis in *Octopus vulgaris*', *The Open Zoology Journal*, 1(1), pp. 29–36.

Croxall, J.P. and Prince, P.A. (1996) 'Cephalopods as prey. I. Seabirds', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 351(1343), pp. 1023–1043. Available at: https://doi.org/10.1098/rstb.1996.0091.

D'Aniello, A., Di Cosmo, A., Di Cristo, C., Assisi, L., Botte, V. and Di Fiore, M.M. (1996) 'Occurrence of sex steroid hormones and their binding proteins in *Octopus vulgaris* Lam', *Biochemical and Biophysical Research Communications*, 227(3), pp. 782–788. Available at: https://doi.org/10.1006/bbrc.1996.1585.

Dance, M.A., Bello, G., Furey, N.B. and Rooker, J.R. (2014) 'Species-specific variation in cuttlebone $\delta^{13}C$ and $\delta^{18}O$ for three species of Mediterranean cuttlefish', *Marine Biology*, 161(2), pp. 489–494. Available at: https://doi.org/10.1007/s00227-013-2346-x.

Darnaude, A.M., Sturrock, A., Trueman, C.N., Mouillot, D., Craven, J.A., Campana, S.E. and Hunter, E. (2014) 'Listening in on the past: What can otolith $\delta^{18}O$ values really tell us about the environmental history of fishes?', *PLoS ONE*, 9(10). Available at:

https://doi.org/10.1371/journal.pone.0108539.

Daryanani, D.S., Martino, J.C. and Doubleday, Z.A. (2021) 'Statolith chemistry: a new tool to understand the ecology and provenance of octopus', *Reviews in Fish Biology and Fisheries*, 31(4), pp. 923–934. Available at: https://doi.org/10.1007/s11160-021-09671-x.

Deniro, M.J. and Epstein, S. (1981) 'Influence of diet on the distribution of nitrogen isotopes in animals', *Geochimica et Cosmochimica Acta*, 45(3), pp. 341–351. Available at: https://doi.org/10.1016/0016-7037(81)90244-1.

DeNiro, M.J. and Epstein, S. (1978) 'Influence of diet on the distribution of carbon isotopes in animals', *Geochimica et Cosmochimica Acta*, 42, pp. 495–506. Available at: https://doi.org/10.1002/mop.25285.

Derusha, R.H., Forsythe, J.W. and Hanlon, R.T. (1987) 'Laboratory growth reproduction and life span of the Pacific pygmy octopus *Octopus digueti*', *Pacific Science*, 41(1–4), pp. 104–121.

Dillon, D., Fernández Ajó, A., Hunt, K.E. and Buck, C.L. (2021) 'Investigation of keratinase digestion to improve steroid hormone extraction from diverse keratinous tissues', *General and Comparative Endocrinology*, 309(April). Available at: https://doi.org/10.1016/j.ygcen.2021.113795.

Dimasi, C.G., Darby, J.R.T., Cho, S.K.S., Saini, B.S., Holman, S.L., Meakin, A.S., Wiese, M.D., Macgowan, C.K., Seed, M. and Morrison, J.L. (2023) 'Reduced in utero substrate supply decreases mitochondrial abundance and alters the expression of metabolic signalling molecules in the fetal sheep heart', *Journal of Physiology*, 0, pp. 1–22. Available at: https://doi.org/10.1113/JP285572.

Donlon, E.M., Lamare, M.D., Damsteegt, E.L., Mckinnon, J. and Higgins, F.A. (2019) 'Growth and age of the midget octopus, *Octopus huttoni* ', *Aquatic Ecology*, 53, pp. 689–706. Available at: https://doi.org/https://doi.org/10.1007/s10452-019-09719-y.

Doubleday, Z., Semmens, J.M., Pecl, G. and Jackson, G. (2006) 'Assessing the validity of stylets as ageing tools in *Octopus pallidus*', *Journal of Experimental Marine Biology and Ecology*, 338(1), pp. 35–42. Available at: https://doi.org/10.1016/j.jembe.2006.06.027.

Doubleday, Zoë A, Martino, J.C., Semmens, J.M. and Boultby, E. (2022) 'Cephalopods', in *Wildlife Research in Australia: Practical and Applied Methods*, pp. 513–518.

Doubleday, Zoë A., Martino, J.C. and Trueman, C. (2022) 'Harnessing universal chemical

markers to trace the provenance of marine animals', *Ecological Indicators*, 144(August). Available at: https://doi.org/10.1016/j.ecolind.2022.109481.

Doubleday, Z.A., Prowse, T.A.A., Arkhipkin, A., Pierce, G.J., Semmens, J., Steer, M., Leporati, S.C., Lourenço, S., Quetglas, A., Sauer, W. and Gillanders, B.M. (2016) 'Global proliferation of cephalopods', *Current Biology*, 26(10), pp. R406–R407. Available at: https://doi.org/10.1016/j.cub.2016.04.002.

Doubleday, Z.A. and Semmens, J.M. (2011) 'Quantification of the age-pigment lipofuscin in known-age octopus (*Octopus pallidus*): A potential tool for age determination', *Journal of Experimental Marine Biology and Ecology*, 397(1), pp. 8–12. Available at: https://doi.org/10.1016/j.jembe.2010.11.010.

Doubleday, Z.A., White, J., Pecl, G.T. and Semmens, J.M. (2011) 'Age determination in merobenthic octopuses using stylet increment analysis: Assessing future challenges using *Macroctopus maorum* as a model', *ICES Journal of Marine Science*, 68(10), pp. 2059–2063. Available at: https://doi.org/10.1093/icesjms/fsr135.

Duarte, B., Mamede, R., Duarte, I.A., Caçador, I., Reis-Santos, P., Vasconcelos, R.P., Gameiro, C., Rosa, R., Tanner, S.E. and Fonseca, V.F. (2023) 'Elemental and spectral chemometric analyses of *Octopus vulgaris* beaks as reliable markers of capture location', *Journal of Food Science*, (January), pp. 1349–1364. Available at: https://doi.org/10.1111/1750-3841.16492.

Durante, E.D., Grammer, G.L., Martino, J.C., Payne, J.L. and Doubleday, Z.A. (2023) 'Nondaily growth increments in the commercial species, *Octopus berrima*, and the importance of age validation', *ICES Journal of Marine Science*, 0(0), pp. 1–14.

Eide, M., Olsen, A., Ninnemann, U.S. and Eldevik, T. (2017) 'A global estimate of the full oceanic $\delta^{13}C$ Suess effect since the preindustrial', *Global Biogeochemical Cycles*, 31(3), pp. 492–514. Available at: https://doi.org/10.1002/2016GB005472.

Fang, Z., Liu, B., Chen, X. and Chen, Y. (2019) 'Ontogenetic difference of beak elemental concentration and its possible application in migration reconstruction for *Ommastrephes bartramii* in the North Pacific Ocean', *Acta Oceanologica Sinica*, 38(10), pp. 43–52. Available at: https://doi.org/10.1007/s13131-019-1431-5.

FAO (2022) *The state of world fisheries and aquaculture*, *Towards Blue Transformation*. Rome. Available at: https://doi.org/10.4060/cc0463en.

Fernández Ajó, A., Hunt, K.E., Dillon, D., Uhart, M., Sironi, M., Rowntree, V. and Loren Buck, C.

(2022) ‘Optimizing hormone extraction protocols for whale baleen: Tackling questions of solvent:sample ratio and variation’, *General and Comparative Endocrinology*, 315(May 2021). Available at: https://doi.org/10.1016/j.ygcen.2021.113828.

Fiorito, G., Affuso, A., Basil, J., Cole, A., de Girolamo, P., D’angelo, L., Dickel, L., Gestal, C., Grasso, F., Kuba, M., Mark, F., Melillo, D., Osorio, D., Perkins, K., Ponte, G., Shashar, N., Smith, D., Smith, J. and Andrews, P. lr (2015) ‘Guidelines for the Care and Welfare of Cephalopods in Research –A consensus based on an initiative by CephRes, FELASA and the Boyd Group’, *Laboratory Animals*, 49, pp. 1–90. Available at: https://doi.org/10.1177/0023677215580006.

Forsythe, J.W. (1984) ‘*Octopus joubini* (Mollusca : Cephalopoda): a detailed study of growth through the full life cycle in a closed seawater system’, *The Zoological Society of London*, 202, pp. 393–417.

Fraile, I., Arrizabalaga, H., Groeneveld, J., Kölling, M., Santos, M.N., Macías, D., Addis, P., Dettman, D.L., Karakulak, S., Deguara, S. and Rooker, J.R. (2016) ‘The imprint of anthropogenic CO_2 emissions on Atlantic bluefin tuna otoliths’, *Journal of Marine Systems*, 158, pp. 26–33. Available at: https://doi.org/10.1016/j.jmarsys.2015.12.012.

Franco-Santos, R.M., Perales-Raya, C., Almansa, E., De Troch, M. and Garrido, D. (2016) ‘Beak microstructure analysis as a tool to identify potential rearing stress for *Octopus vulgaris* paralarvae’, *Aquaculture Research*, 47(9), pp. 3001–3015. Available at: https://doi.org/10.1111/are.12753.

Fraser, K., Hartmann, K. and Krueck, N. (2022) *Tasmanian Octopus Assessment 2020/2021*.

Friedman, I. and O’Neil, J.R. (1977) *Compilation of Stable Isotope Fractionation Factors of Geohemical Intrest*, *Data of Geochemistry*.

Fry, B. (2006) *Stable Isotope Ecology*. Springer Science+Business Media, LLC.

Gebremedhin, S., Bruneel, S., Getahun, A., Anteneh, W. and Goethals, P. (2021) ‘Scientific methods to understand fish population dynamics and support sustainable fisheries management’, *Water*, 13(574), pp. 1–20. Available at: https://doi.org/10.3390/w13040574.

Gillanders, B.M., Donnellan, S.C., Prowse, T.A.A.C., Fordham, D., Izzo, C., Myers, S., Rowling, K., Steer, M. and Woodcock, S.H. (2016) *Giant Australian cuttlefish in South Australian waters*.

Gillanders, B.M., Wilkinson, L.M., Munro, A.R. and de Vries, M.C. (2013) ‘Statolith chemistry of two life history stages of cuttlefish: Effects of temperature and seawater trace element

concentration', *Geochimica et Cosmochimica Acta*, 101, pp. 12–23. Available at: https://doi.org/10.1016/j.gca.2012.10.005.

Gillooly, J.F., Brown, J.H., West, G.B., Savage, V.M. and Charnov, E.L. (2001) 'Effects of size and temperature on metabolic rate', *Science*, 293(5538), pp. 2248–2251. Available at: https://doi.org/10.1126/science.1061967.

Gowland, F.C., Boyle, P.R. and Noble, L.R. (2002) 'Morphological variation provides a method of estimating thermal niche in hatchlings of the squid *Loligo forbesi* (Mollusca: Cephalopoda)', *Journal of Zoology*, 258(4), pp. 505–513. Available at: https://doi.org/10.1017/S0952836902001668.

Grossman, E.L. and Ku, T.L. (1986) 'Carbon and oxygen isotopic fractionation in biogenic aragonite-temp effects', *Chemical Geology*, 59(April), pp. 59–74. Available at: https://www.researchgate.net/publication/291049277.

Grubert, M.A. and Wadley, V.A. (2000) 'Sexual maturity and fecundity of *Octopus maorum* in southeast Tasmania', *Bulletin of Marine Science*, 66(1), pp. 131–142.

Häkkinen, M.R., Heinosalo, T., Saarinen, N., Linnanen, T., Voutilainen, R., Lakka, T., Jääskeläinen, J., Poutanen, M. and Auriola, S. (2018) 'Analysis by LC–MS/MS of endogenous steroids from human serum, plasma, endometrium and endometriotic tissue', *Journal of Pharmaceutical and Biomedical Analysis*, 152, pp. 165–172. Available at: https://doi.org/10.1016/j.jpba.2018.01.034.

Hall, K.C., Fowler, A.J. and Geddes, M.C. (2007) 'Evidence for multiple year classes of the giant Australian cuttlefish *Sepia apama* in northern Spencer Gulf, South Australia', *Reviews in Fish Biology and Fisheries*, 17(2–3), pp. 367–384. Available at: https://doi.org/10.1007/s11160-007-9045-y.

Hall, K.C. and Hanlon, R.T. (2002) 'Principal features of the mating system of a large spawning aggregation of the giant Australian cuttlefish *Sepia apama* (Mollusca: Cephalopoda)', *Marine Biology*, 140(3), pp. 533–545. Available at: https://doi.org/10.1007/s00227-001-0718-0.

Han, T., Yu, K., Yan, Hong, Jiang, W., Yan, Hongqiang and Tao, S. (2020) 'Coral δ18O-based reconstruction of El Niño-Southern Oscillation from the northern south China sea since 1851 AD', *Quaternary International*, 550(100), pp. 159–168. Available at: https://doi.org/10.1016/j.quaint.2020.04.032.

Hart, A.M., Leporati, S.C., Marriott, R.J. and Murphy, D. (2016) *Innovative development of the*

Octopus (cf) tetricus *fishery in Western Australia FRDC Project No 2010/200*.

Hart, A.M., Murphy, D., Hesp, S.A., Leporati, S. and Arkhipkin, A. (2019) 'Biomass estimates and harvest strategies for the Western Australian *Octopus aff. tetricus* fishery', *ICES Journal of Marine Science*, 76(7), pp. 2205–2217. Available at: https://doi.org/10.1093/icesjms/fsz146.

Hart, A.M., Murphy, D.M., Harry, A.. and Fisher, E.A. (2018) *Resource Assessment Report Western Australian Octopus Resource*. Western Australia.

Hartig, F. (2020) 'DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models'. R package version 0.4.3.

Hartwick, E.B. (1983) '*Octopus dofleini*', in *Cephalopod Life Cycles Vol. 1*, pp. 277–291.

Harvey, H.R., Ju, S.J., Son, S.K., Feinberg, L.R., Shaw, C.T. and Peterson, W.T. (2010) 'The biochemical estimation of age in Euphausiids: Laboratory calibration and field comparisons', *Deep-Sea Research Part II: Topical Studies in Oceanography*, 57(7–8), pp. 663–671. Available at: https://doi.org/10.1016/j.dsr2.2009.10.015.

Hermosilla, C.A., Rocha, F., Fiorito, G., González, Á.F. and Guerra, Á. (2010) 'Age validation in common octopus *Octopus vulgaris* using stylet increment analysis', *ICES Journal of Marine Science*, 67(7), pp. 1458–1463. Available at: https://doi.org/10.1093/icesjms/fsq054.

Hernández-López, J.L., Castro-Hernández, J.J. and Hernández-García, V. (2001) 'Age determined from the daily deposition of concentric rings on common octopus (*Octopus vulgaris*) beaks', *Fishery Bulletin*, 684(April), pp. 679–684.

Herwig, J.N., Depczynski, M., Roberts, J.D., Semmens, J.M., Gagliano, M. and Heyward, A.J. (2012) 'Using age-based life history data to investigate the life cycle and vulnerability of *Octopus cyanea*', *PLoS ONE*, 7(8), pp. 1–6. Available at: https://doi.org/10.1371/journal.pone.0043679.

Higgins, F.A., Bates, A.E. and Lamare, M.D. (2012) 'Heat tolerance, behavioural temperature selection and temperature-dependent respiration in larval *Octopus huttoni*', *Journal of Thermal Biology*, 37(1), pp. 83–88. Available at: https://doi.org/10.1016/j.jtherbio.2011.11.004.

Hofmeister, J.K.K. (2018) 'Variation in *Octopus bimaculatus* Verrill, 1883 Diet as Revealed through $\delta^{13}C$ and $\delta^{15}N$ Stable Isotope Analysis: Potential Indirect Effects of Marine Protected Areas', *American Malacological Bulletin*, 36(1), pp. 96–108. Available at: https://doi.org/10.4003/006.036.0103.

Høie, H., Folkvord, A. and Otterlei, E. (2003) 'Effect of somatic and otolith growth rate on stable isotopic composition of early juvenile cod (*Gadus morhua* L) otoliths', *Journal of Experimental Marine Biology and Ecology*, 289(1), pp. 41–58. Available at: https://doi.org/10.1016/S0022-0981(03)00034-0.

Høie, H., Otterlei, E. and Folkvord, A. (2004) 'Temperature-dependent fractionation of stable oxygen isotopes in otoliths of juvenile cod (*Gadus morhua* L.)', *ICES Journal of Marine Science*, 61(2), pp. 243–251. Available at: https://doi.org/10.1016/j.icesjms.2003.11.006.

Hua, Q.Q.H., Thomson, V.A., Strugnell, J.M., Martino, J.C., Gillanders, B.M. and Doubleday, Z.A. (2023) 'Using genomics and morphometrics to monitor data-poor and commercially exploited octopod populations', *Marine Biology*, 170(10), pp. 1–21. Available at: https://doi.org/10.1007/s00227-023-04270-9.

Hunt, K. V., Steer, M.A. and Gillanders, B.M. (2011) 'Validating age in southern calamary (*Sepioteuthis australis*) over seasonal and life history extremes', *Journal of the Marine Biological Association of the United Kingdom*, 91(4), pp. 857–863. Available at: https://doi.org/10.1017/S002531541000113X.

Hunt, K.E., Lysiak, N.S., Moore, M.J. and Rolland, R.M. (2016) 'Longitudinal progesterone profiles in baleen from female North Atlantic right whales (*Eubalaena glacialis*) match known calving history', *Conservation Physiology*, 4(1), pp. 1–9. Available at: https://doi.org/10.1093/conphys/cow014.

Hunt, K.E., Lysiak, N.S.J., Matthews, C.J.D., Lowe, C., Ajó, A.F., Dillon, D., Willing, C., Heide-Jørgensen, M.P., Ferguson, S.H., Moore, M.J. and Loren Buck, C. (2018) 'Multi-year patterns in testosterone, cortisol and corticosterone in baleen from adult males of three whale species', *Conservation Physiology*, 6(1), pp. 1–16. Available at: https://doi.org/10.1093/conphys/coy049.

Hunt, Kathleen E., Stimmelmayr, R., George, C., Hanns, C., Suydam, R., Brower, H. and Rolland, R.M. (2014) 'Baleen hormones: A novel tool for retrospective assessment of stress and reproduction in bowhead whales (*Balaena mysticetus*)', *Conservation Physiology*, 2(1), pp. 1–12. Available at: https://doi.org/10.1093/conphys/cou030.

Hunt, Kathleen E, Stimmelmayr, R., George, C., Hanns, C., Suydam, R., Jr, H.B. and Rolland, R.M. (2014) 'Themed Issue Article : Stress in Vertebrates Baleen hormones : a novel tool for retrospective assessment of stress and reproduction in bowhead whales (*Balaena mysticetus*)', 2(April), pp. 1–12. Available at: https://doi.org/10.1093/conphys/cou030.

Hunt, S. and Nixon, M. (1981) 'A comparative study of protein composition in the chitin-protein complexes of the beak, pen, sucker disc, radula and oesophageal cuticle of cephalopods', *Comparative Biochemistry and Physiology -- Part B: Biochemistry and*, 68(4), pp. 535–546. Available at: https://doi.org/10.1016/0305-0491(81)90071-7.

Isely, J.J. and Grabowski, Ti.B. (2007) 'Age and growth', in *Analysis and Interpretation of Freshwater Fisheries Data*, pp. 187–228. Available at: https://doi.org/10.1201/9781315119861.

Izzo, C., Doubleday, Z.A., Grammer, G.L., Gilmore, K.L., Alleway, H.K., Barnes, T.C., Disspain, M.C.F., Giraldo, A.J., Mazloumi, N. and Gillanders, B.M. (2016) 'Fish as proxies of ecological and environmental change', *Reviews in Fish Biology and Fisheries*, 26(3), pp. 265–286. Available at: https://doi.org/10.1007/s11160-016-9424-3.

Jackson, G.D. (1990) 'Age and growth of the tropical nearshore loliginid squid *Sepioteuthis lessoniana* determined from statolith growth-ring analysis', *Fishery Bulletin*, 88(1), pp. 113–118.

Jackson, G.D., Bustamante, P., Cherel, Y., Fulton, E.A., Grist, E.P.M., Jackson, C.H., Nichols, P.D., Pethybridge, H., Phillips, K., Ward, R.D. and Xavier, J.C. (2007) 'Applying new tools to cephalopod trophic dynamics and ecology: Perspectives from the Southern Ocean Cephalopod Workshop, February 2-3, 2006', *Reviews in Fish Biology and Fisheries*, 17(2–3), pp. 79–99. Available at: https://doi.org/10.1007/s11160-007-9055-9.

Jackson, G.D. and Forsythe, J.W. (2002) 'Statolith age validation and growth of *Loligo plei* (Cephalopoda: Loliginidae) in the north-west Gulf of Mexico during spring/summer', *Journal of the Marine Biological Association of the United Kingdom*, 82(4), pp. 677–678. Available at: https://doi.org/10.1017/S0025315402006069.

Jackson, J.R. (2007) 'Earliest references to age determination of fishes and their early application to the study of fisheries', *Fisheries*, 32(6), pp. 270–277. Available at: https://doi.org/10.1577/1548-8446(2007)32.

Jamieson, R.E., Schwarcz, H.P. and Brattey, J. (2004) 'Carbon isotopic records from the otoliths of Atlantic cod (*Gadus morhua*) from eastern Newfoundland, Canada', *Fisheries Research*, 68(1–3), pp. 83–97. Available at: https://doi.org/10.1016/j.fishres.2004.02.009.

Jereb, P., Roper, C.F.E., Norman, M.D. and Finn, J.K. (2014) *Cephalopods of the world: An annotated and illustrated catalogue of species known to date. Volume 3. Octopods and Vampire Squids.*, *FAO Species Catalogue for Fishery Purposes*.

Kalish, J.M. (1991a) 'Oxygen and carbon stable isotopes in the otoliths of wild and laboratory-

reared Australian salmon (*Arripis trutta*)', *Marine Biology*, 110, pp. 37–47.

Kalish, J.M. (1991b) 'δ^{13}C and δ^{18}O isotopic disequilibria in fish otoliths: metabolic and kinetic effects', *Marine Ecology Progress Series*, 75(2–3), pp. 191–203. Available at: https://doi.org/10.3354/meps075191.

Kallenbach, E.M.F., Hurley, R.R., Lusher, A. and Friberg, N. (2021) 'Chitinase digestion for the analysis of microplastics in chitinaceous organisms using the terrestrial isopod Oniscus asellus L. as a model organism', *Science of the Total Environment*, 786, p. 147455. Available at: https://doi.org/10.1016/j.scitotenv.2021.147455.

Kashiyama, Y., Ogawa, N.O., Chikaraishi, Y., Kashiyama, N., Sakai, S., Tanabe, K. and Ohkouchi, N. (2010) 'Reconstructing the life history of modern and fossil nautiloids based on the nitrogen isotopic composition of shell organic matter and amino acids', *Cephalopods: present and past*, pp. 67–75.

Kassahn, K.S., Donnellan, S.C., Fowler, A.J., Hall, K.C., Adams, M. and Shaw, P.W. (2003) 'Molecular and morphological analyses of the cuttlefish *Sepia apama* indicate a complex population structure', *Marine Biology*, 143(5), pp. 947–962. Available at: https://doi.org/10.1007/s00227-003-1141-5.

Keeling, C.D. (1979) 'The Suess effect: ^{13}Carbon-^{14}Carbon interrelations', *Environment International*, 2(4–6), pp. 229–300. Available at: https://doi.org/10.1016/0160-4120(79)90005-9.

Keeling, C.D., Piper, S.C., Bacastow, R.B., Wahlen, M., Whorf, T.P., Heimann, M. and Meijer, H.A. (2005) 'Atmospheric CO_2 and $^{13}CO_2$ Exchange with the Terrestrial Biosphere and Oceans from 1978 to 2000: Observations and Carbon Cycle Implications', *A History of Atmospheric CO2 and Its Effects on Plants, Animals, and Ecosystems*, pp. 83–113. Available at: https://doi.org/10.1007/0-387-27048-5_5.

Keeling, R. (2024) *All global stations isotopic ^{13}C trends*, *Scripps CO_2 Program*. Available at: https://scrippsco2.ucsd.edu/graphics_gallery/isotopic_data/all_global_stations_isotopic_c13_trends.html.

Keogh, M.J., Charapata, P., Fadely, B.S., Zeppelin, T., Rea, L., Waite, J.N., Burkanov, V., Marshall, C., Jones, A., Sprowls, C. and Wooller, M.J. (2021) 'Whiskers as a novel tissue for tracking reproductive and stress-related hormones in North Pacific otariid pinnipeds', *Conservation Physiology*, 9(1), pp. 1–19. Available at: https://doi.org/10.1093/conphys/coaa134.

Keogh, M.J., Charapata, P., Karpovich, S., Jones, A., Sprowls, C. and Marshall, C.D. (2020) 'Reproductive and stress-related hormones in whiskers from two North Pacific phocids: Harbor and ringed seals', *Marine Mammal Science*, 36(4), pp. 1322–1333. Available at: https://doi.org/10.1111/mms.12709.

Kitagawa, T., Ishimura, T., Uozato, R., Shirai, K., Amano, Y., Shinoda, A., Otake, T., Tsunogai, U. and Kimura, S. (2013) 'Otolith $\delta^{18}O$ of Pacific bluefin tuna Thunnus orientalis as an indicator of ambient water temperature', *Marine Ecology Progress Series*, 481(Anon 2010), pp. 199–209. Available at: https://doi.org/10.3354/meps10202.

Kivengea, G.M., Ntiba, M.J., Sigana, D.O. and Muthumbi, A.W. (2014) 'Reproductive biology of the common octopus (*Octopus vulgaris* Cuvier, 1797) in South Kenya', *Western Indian Ocean J. Mar. Sci*, 13(1), pp. 47–56.

Klages, N.T.W. (1996) 'Cephalopods as prey. II. Seals', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 351(1343), pp. 1045–1052. Available at: https://doi.org/10.1098/rstb.1996.0092.

Kleiber, M. (1932) 'Body Size and Metabolism', *Hilgardia Journal of Agricultural Science*, 6(11), pp. 315–353.

Kleiber, M. (1947) 'Body size and metabolic rate', *Physiological Reviews*, 27(9), pp. 511–541.

Kodama, K., Shiraishi, H., Morita, M. and Horiguchi, T. (2006) 'Verification of lipofuscin-based crustacean ageing: Seasonality of lipofuscin accumulation in the stomatopod *Oratosquilla oratoria* in relation to water temperature', *Marine Biology*, 150(1), pp. 131–140. Available at: https://doi.org/10.1007/s00227-006-0337-x.

Körtzinger, A., Quay, P.D. and Sonnerup, R.E. (2003) 'Relationship between anthropogenic CO_2 and the ^{13}C Suess effect in the North Atlantic Ocean', *Global Biogeochemical Cycles*, 17(1). Available at: https://doi.org/10.1029/2001gb001427.

Kröger, B., Vinther, J. and Fuchs, D. (2011) 'Cephalopod origin and evolution: A congruent picture emerging from fossils, development and molecules: Extant cephalopods are younger than previously realised and were under major selection to become agile, shell-less predators', *BioEssays*, 33(8), pp. 602–613. Available at: https://doi.org/10.1002/bies.201100001.

Kroopnick, P.M. (1985) 'The distribution of ^{13}C of ΣCO_2 in the world oceans', *Deep Sea Research Part A, Oceanographic Research Papers*, 32(1), pp. 57–84. Available at: https://doi.org/10.1016/0198-0149(85)90017-2.

Kruta, I., Landman, N.H. and Cochran, J.K. (2014) 'A new approach for the determination of ammonite and nautilid habitats', *PLoS ONE*, 9(1), pp. 1–7. Available at: https://doi.org/10.1371/journal.pone.0087479.

Landman, N.H., Cochran, J.K., Cerrato, R., Mak, J., Roper, C.F.E. and Lu, C.C. (2004) 'Habitat and age of the giant squid (*Architeuthis sanctipauli*) inferred from isotopic analyses', *Marine Biology*, 144(4), pp. 685–691. Available at: https://doi.org/10.1007/s00227-003-1245-y.

Laptikhovsky, V. V., Arkhipkin, A.I. and Hoving, H.J.T. (2007) 'Reproductive biology in two species of deep-sea squids', *Marine Biology*, 152(4), pp. 981–990. Available at: https://doi.org/10.1007/s00227-007-0749-2.

Laptikhovsky, V. V., Fock, H., Piatkowski, U., Schwarz, R. and Hoving, H.J.T. (2019) 'Reproductive strategies of deep-sea squid (Mastigoteuthidae, Chiroteuthidae, Batoteuthidae and Cranchiidae)', *Marine Biology*, 166(7), pp. 1–10. Available at: https://doi.org/10.1007/s00227-019-3532-2.

Larson, S.E. and Anderson, R.C. (2010) 'Fecal hormones measured within giant pacific octopuses *Enteroctopus dofleini*', *Journal of Aquatic Animal Health*, 22(3), pp. 152–157. Available at: https://doi.org/10.1577/H09-027.1.

Lee, P.G. (1994) 'Nutrition of Cephalopods: Fueling the System', in *Physiology of Cephalopod Molluscs*, pp. 35–51.

LeGrande, A.N. and Schmidt, G.A. (2006) 'Global gridded data set of the oxygen isotopic composition in seawater', *Geophysical Research Letters*, 33(12), pp. 1–5. Available at: https://doi.org/10.1029/2006GL026011.

Leporati, S.C. and Hart, A.M. (2015) 'Stylet weight as a proxy for age in a merobenthic octopus population', *Fisheries Research*, 161, pp. 235–243. Available at: https://doi.org/10.1016/j.fishres.2014.08.001.

Leporati, S.C., Hart, A.M., Larsen, R., Franken, L.E. and De Graaf, M. (2015) 'Octopus life history relative to age, in a multi-geared developmental fishery', *Fisheries Research*, 165, pp. 28–41. Available at: https://doi.org/10.1016/j.fishres.2014.12.017.

Leporati, S.C., Pecl, G.T. and Semmens, J.M. (2008) 'Reproductive status of *Octopus pallidus*, and its relationship to age and size', *Marine Biology*, 155(4), pp. 375–385. Available at: https://doi.org/10.1007/s00227-008-1033-9.

Leporati, S.C., Semmens, J.M. and Pecl, G.T. (2008) 'Determining the age and growth of wild octopus using stylet increment analysis', *Marine Ecology Progress Series*, 367(2), pp. 213–222. Available at: https://doi.org/10.3354/meps07558.

Leporati, S.C., Ziegler, P.E. and Semmens, J.M. (2009) 'Assessing the stock status of holobenthic octopus fisheries: Is catch per unit effort sufficient?', *ICES Journal of Marine Science*, 66(3), pp. 478–487. Available at: https://doi.org/10.1093/icesjms/fsn224.

de Lima, F.D., Leite, T.S., Haimovici, M. and Lins Oliveira, J.E. (2014) 'Gonadal development and reproductive strategies of the tropical octopus (*Octopus insularis*) in northeast Brazil', *Hydrobiologia*, 725(1), pp. 7–21. Available at: https://doi.org/10.1007/s10750-013-1718-z.

Lipinski, M. (1986) 'Methods for the validation of squid age from statoliths', *Journal of Marine Biology*, 66, pp. 505–526.

Lipinski, M.R. (1978) 'The age of squids, *Illex illecebrosus* (LeSueur, 1821), from their statoliths', *International Comission for the Northwest Atlantic Fisheries Research Documents*, 78/11/15(5167).

Lipinski, M.R. and Durholtz, M.D. (1994) 'Problems associated with ageing squid from their statoliths: Towards a more structured approach', *Antarctic Science*, 6(2), pp. 215–222. Available at: https://doi.org/10.1017/S0954102094000337.

Liu, B.L., Chen, X.J., Chen, Y. and Hu, G.Y. (2015) 'Determination of squid age using upper beak rostrum sections: technique improvement and comparison with the statolith', *Marine Biology*, 162(8), pp. 1685–1693. Available at: https://doi.org/10.1007/s00227-015-2702-0.

Lourenço, S., Moreno, A., Narciso, L., Pereira, J., Rosa, R. and González, Á.F. (2015) 'Stylet (vestigial shell) size in *Octopus vulgaris* (Cephalopoda) hatchlings used to determine stylet nucleus in adults', *Journal of the Marine Biological Association of the United Kingdom*, 95(06), pp. 1237–1243. Available at: https://doi.org/10.1017/S0025315415000478.

Lowe, C.L., Hunt, K.E., Neilson, J.L., Gabriele, C.M., Teerlink, S.S. and Buck, C.L. (2022) 'Reproductive Steroid Hormone Patterns in Baleen of Two Pregnant Humpback Whales (*Megaptera novaeangliae*)', *Integrative and Comparative Biology*, 62(2), pp. 152–163. Available at: https://doi.org/10.1093/icb/icac070.

Lowe, C.L., Hunt, K.E., Rogers, M.C., Neilson, J.L., Robbins, J., Gabriele, C.M., Teerlink, S.S., Seton, R. and Buck, C.L. (2021) 'Multi-year progesterone profiles during pregnancy in baleen of humpback whales (*Megaptera novaeangliae*)', *Conservation Physiology*, 9(1), pp. 1–14.

Available at: https://doi.org/10.1093/conphys/coab059.

Lukeneder, A., Harzhauser, M., Müllegger, S. and Piller, W.E. (2008) 'Stable isotopes ($\delta^{18}O$ and $\delta^{13}C$) in *Spirula spirula* shells from three major oceans indicate developmental changes paralleling depth distributions', *Marine Biology*, 154(1), pp. 175–182. Available at: https://doi.org/10.1007/s00227-008-0911-5.

Lysiak, N.S.J., Ferguson, S.H., Hornby, C.A., Heide-Jørgensen, M.P. and Matthews, C.J.D. (2023) 'Prolonged baleen hormone cycles suggest atypical reproductive endocrinology of female bowhead whales', *Royal Society Open Science*, 10(7). Available at: https://doi.org/10.1098/rsos.230365.

Magozzi, S., Yool, A., Vander Zanden, H.B., Wunder, M.B. and Trueman, C.N. (2017) 'Using ocean models to predict spatial and temporal variation in marine carbon isotopes', *Ecosphere*, 8(5). Available at: https://doi.org/10.1002/ecs2.1763.

Martino, J.C., Chung, M.-T., Chiang, C.-I., Wang, C.-H., Shirai, K. and Doubleday, Z.A. (2022) 'Systematic evaluation of oxygen isotopes in cephalopod statoliths as thermal proxies', *ICES Journal of Marine Science*, 79(6), pp. 1719–1729. Available at: https://doi.org/10.1093/icesjms/fsac102.

Martino, J.C., Doubleday, Z.A., Chung, M.T. and Gillanders, B.M. (2020) 'Experimental support towards a metabolic proxy in fish using otolith carbon isotopes', *Journal of Experimental Biology*, 223(6). Available at: https://doi.org/10.1242/jeb.217091.

Martino, J.C., Doubleday, Z.A. and Gillanders, B.M. (2019) 'Metabolic effects on carbon isotope biomarkers in fish', *Ecological Indicators*, 97(October 2018), pp. 10–16. Available at: https://doi.org/10.1016/j.ecolind.2018.10.010.

Martino, J.C., Steer, M. and Doubleday, A. (2021) 'Supporting the sustainable development of Australia's octopus industry : First assessment of an artisanal fishery', *Fisheries Research*, 241(May). Available at: https://doi.org/10.1016/j.fishres.2021.105999.

Martino, J.C., Trueman, C.N., Mazumder, D., Crawford, J. and Doubleday, Z.A. (2022) 'The universal imprint of oxygen isotopes can track the origins of seafood', *Fish and Fisheries*, 23(6), pp. 1455–1468. Available at: https://doi.org/10.1111/faf.12703.

Matthews, T.R., Maxwell, K.E., Bertelsen, R.D. and Derby, C.D. (2009) 'Use of neurolipofuscin to determine age structure and growth rates of Caribbean spiny lobster *Panulirus argus* in Florida, United States', *New Zealand Journal of Marine and Freshwater Research*, 43(1), pp. 125–137.

Available at: https://doi.org/10.1080/00288330909509987.

McClenachan, L., Ferretti, F. and Baum, J.K. (2012) 'From archives to conservation: Why historical data are needed to set baselines for marine animals and ecosystems', *Conservation Letters*, 5(5), pp. 349–359. Available at: https://doi.org/10.1111/j.1755-263X.2012.00253.x.

McConnaughey, T.A. (1989a) '^{13}C and ^{18}O Isotopic disequilibrium in biological carbonates: 1. Patterns', *Geochimica et Cosmochimica Acta*, 53(1), pp. 151–162.

McConnaughey, T.A. (1989b) '^{13}C and ^{18}O isotopic disequilibrium in biological carbonates: II. In vitro simulation of kinetic isotope effects', *Geochimica et Cosmochimica Acta*, 53(1), pp. 163–171. Available at: https://doi.org/10.1016/0016-7037(89)90282-2.

McConnaughey, T.A. and Gillikin, D.P. (2008) 'Carbon isotopes in mollusk shell carbonates', *Geo-Marine Letters*, 28(5–6), pp. 287–299. Available at: https://doi.org/10.1007/s00367-008-0116-4.

Meza-Buendía, A.K., Trejo-Escamilla, I., Piu, M., Caamal-Monsreal, C., Rodríguez-Fuentes, G., Diaz, F., Re, D., Galindo-Sánchez, C.E. and Rosas, C. (2021) 'Why high temperatures limit reproduction in cephalopods? The case of *Octopus maya*', *Aquaculture Research*, 52(11), pp. 5111–5123. Available at: https://doi.org/10.1111/are.15387.

Middleton, J.F. and Bye, J.A.T. (2007) 'A review of the shelf-slope circulation along Australia's southern shelves: Cape Leeuwin to Portland', *Progress in Oceanography*, 75(1), pp. 1–41. Available at: https://doi.org/10.1016/j.pocean.2007.07.001.

Moore, B., Steer, M., Green, C., Hall, K. and Hartmann, K. (2018) *Status of Australian Fish Stocks Report: Pale Octopus*.

Morgan, M.J. (2008) 'Integrating reproductive biology into scientific advice for fisheries management', *Journal of Northwest Atlantic Fishery Science*, 41(2003), pp. 37–51. Available at: https://doi.org/10.2960/J.v41.m615.

Morissette, O., Trueman, C.N., Sturrock, A.M., Geffen, A.J. and Shirai, K. (2023) 'Limited evidence for species-specific sensitivity of temperature-dependent fractionation of oxygen stable isotope in biominerals: A meta-analysis', *Methods in Ecology and Evolution*, 14(7), pp. 1719–1731. Available at: https://doi.org/10.1111/2041-210X.14122.

Mulcahy, S., Killingley, J., Phleger, C.F. and Berger, W.H. (1979) 'Isotopic composition of otoliths from a benthopelagic fish, *Coryphaenoides acrolepis*, Macrouridae,: Gadiformes', *Oceanologica*

Acta, 2(4), pp. 423–427. Available at: http://archimer.ifremer.fr/doc/00122/23358/21185.pdf.

Naef, A. (1928) 'Cephalopoda Embryology', *Fauna and Flora of the Bay of Naples*, 1(2).

Nafkha, B., Rocha, F., Ben Abdallah-Ben, O., Hamida, H., Ben, N., Chemmam, B., Velasco, C., Ezzeddine-Najai, S. and Jarboui, O. (2019) 'How long can *Octopus vulgaris* live? An extended life cycle in southern Tunisian coasts from age estimation using stylet analysis', *Cah. Biol. Mar*, 60(March 2021), pp. 243–252. Available at: https://doi.org/10.21411/CBM.A.DE35B895.

Nagy, K.A. (2005) 'Field metabolic rate and body size', *Journal of Experimental Biology*, 208(9), pp. 1621–1625. Available at: https://doi.org/10.1242/jeb.01553.

Newsome, S.D., Etnier, M.A., Kurle, C.M., Waldbauer, J.R., Chamberlain, C.P. and Koch, P.L. (2007) 'Historic decline in primary productivity in western Gulf of Alaska and eastern Bering Sea: Isotopic analysis of northern fur seal teeth', *Marine Ecology Progress Series*, 332(Gentry 1998), pp. 211–224. Available at: https://doi.org/10.3354/meps332211.

Van Nieuwenhove, A.H.M., Ratsimbazafy, H.A. and Kochzius, M. (2019) 'Cryptic diversity and limited connectivity in octopuses: Recommendations for fisheries management', *PLoS ONE*, 14(5), pp. 1–20. Available at: https://doi.org/10.1371/journal.pone.0214748.

Nishida, K., Suzuki, A., Isono, R., Hayashi, M., Watanabe, Y., Yamamoto, Y., Irie, T., Nojiri, Y., Mori, C., Sato, M., Sato, K. and Sasaki, T. (2015) 'Thermal dependency of shell growth, microstructure, and stable isotopes in laboratory-reared *Scapharca broughtonii* (Mollusca: Bivalvia)', *Geochemistry Geophysics Geosystems*, 16(1), pp. 2395–2408. Available at: https://doi.org/10.1002/2014GC005634.Received.

Norman, M. and Reid, A. (2000) *A guide to squid, cuttlefish and octopuses of Australasia*.

Nunes, R.A. and Lennon, G.W. (1986) 'Physical Property Distribution and Seasonal Trends in Spencer Gulf South Australia: an Inverse Estuary', *Australian Journal of Marine and Freshwater Research*, 37, pp. 39–53.

Nuttall, A.M. (2009) *Determining the Age and Growth of* Octopus australis.

O'Dor, R.K., Hoar, J.A., Webber, D.M., Carey, F.G., Tanaka, S., Martins, H.R. and Porteiro, F.M. (1995) 'Squid (*Loligo Forbesi*) Performance And Metabolic Rates In Nature', *Marine and Freshwater Behaviour and Physiology*, 25(1–3), pp. 163–177. Available at: https://doi.org/10.1080/10236249409378915.

O'Dor, R.K. and Webber, D.M. (1986) 'The constraints on cephalopods: why squid aren't fish',

Canadian Journal of Zoology, 64(8), pp. 1591–1605. Available at: https://doi.org/10.1139/z86-241.

Oba, T., Kai, M. and Tanabe, K. (1992) 'Early life history and habitat of *Nautilus pompilius* inferred from oxygen isotope examinations', *Marine Biology*, 217, pp. 211–217.

Ohkouchi, N., Tsuda, R., Chikaraishi, Y. and Tanabe, K. (2013) 'A preliminary estimate of the trophic position of the deep-water ram's horn squid *Spirula spirula* based on the nitrogen isotopic composition of amino acids', *Marine Biology*, 160(4), pp. 773–779. Available at: https://doi.org/10.1007/s00227-012-2132-1.

Owen, E.F., Wanamaker, A.D., Feindel, S.C., Schöne, B.R. and Rawson, P.D. (2008) 'Stable carbon and oxygen isotope fractionation in bivalve (*Placopecten magellanicus*) larval aragonite', *Geochimica et Cosmochimica Acta*, 72(19), pp. 4687–4698. Available at: https://doi.org/10.1016/j.gca.2008.06.029.

Paul, D. and Skrzypek, G. (2006) 'Flushing time and storage effects on the accuracy and precision of carbon and oxygen isotope ratios of sample using the Gasbench II technique', *Rapid Communications in Mass Spectrometry*, 20, pp. 2033–2040. Available at: https://doi.org/10.1002/rcm.

Payne, N.L., Gillanders, B.M., Seymour, R.S., Webber, D.M., Snelling, E.P. and Semmens, J.M. (2011) 'Accelerometry estimates field metabolic rate in giant Australian cuttlefish *Sepia apama* during breeding', *Journal of Animal Ecology*, 80(2), pp. 422–430. Available at: https://doi.org/10.1111/j.1365-2656.2010.01758.x.

Pecl, G.T. and Jackson, G.D. (2008) 'The potential impacts of climate change on inshore squid: Biology, ecology and fisheries', *Reviews in Fish Biology and Fisheries*, 18(4), pp. 373–385. Available at: https://doi.org/10.1007/s11160-007-9077-3.

Perales-Raya, C., Almansa, E., Bartolomé, A., Felipe, B.C., Iglesias, J., Sánchez, F.J., Carrasco, J.F. and Rodríguez, C. (2014) 'Age validation in *Octopus vulgaris* beaks across the full ontogenetic range: Beaks as recorders of life events in octopuses', *Journal of Shellfish Research*, 33(2), pp. 481–493. Available at: https://doi.org/10.2983/035.033.0217.

Perales-Raya, C., Bartolomé, A., García-Santamaría, M.T., Pascual-Alayón, P. and Almansa, E. (2010) 'Age estimation obtained from analysis of octopus (*Octopus vulgaris* Cuvier, 1797) beaks: Improvements and comparisons', *Fisheries Research*, 106(2), pp. 171–176. Available at: https://doi.org/10.1016/j.fishres.2010.05.003.

Perales-Raya, C. and Hernández-González, C.L. (1998) 'Growth lines within the beak microstructure of the octopus Octopus vulgaris Cuvier , 1797', *South African Journal of Marine Science*, 20(1), pp. 135–142. Available at: https://doi.org/10.2989/025776198784126368.

Perales-Raya, C., Jurado-Ruzafa, A., Bartolomé, A., Duque, V., Carrasco, M.N. and Fraile-Nuez, E. (2014) 'Age of spent *Octopus vulgaris* and stress mark analysis using beaks of wild individuals', *Hydrobiologia*, 725(1), pp. 105–114. Available at: https://doi.org/10.1007/s10750-013-1602-x.

Perales-Raya, C., Nande, M., Roura, A., Bartolomé, A., Gestal, C., Otero, J.J., García-Fernández, P. and Almansa, E. (2017) 'Comparative study of age estimation in wild and cultured Octopus vulgaris paralarvae: effect of temperature and diet', *Marine Ecology Progress Series*, 598, pp. 1–13. Available at: https://doi.org/10.3354/meps12218.

Perez, J.A.A., de Aguiar, D.C. and dos Santos, J.A.T. (2006) 'Gladius and Statolith as Tools for Age and Growth Studies of the Squid *Loligo plei* (Teuthida : Loliginidae) off Southern Brazil', *Brazilian Archives of Biology and Technology*, 49(September), pp. 747–755.

Pfister, C.A., McCoy, S.J., Wootton, J.T., Martin, P.A., Colman, A.S. and Archer, D. (2011) 'Rapid environmental change over the past decade revealed by isotopic analysis of the california mussel in the northeast pacific', *PLoS ONE*, 6(10), pp. 4–11. Available at: https://doi.org/10.1371/journal.pone.0025766.

Pimentel, M.S., Trübenbach, K., Faleiro, F., Boavida-Portugal, J., Repolho, T. and Rosa, R. (2012) 'Impact of ocean warming on the early ontogeny of cephalopods: A metabolic approach', *Marine Biology*, 159(9), pp. 2051–2059. Available at: https://doi.org/10.1007/s00227-012-1991-9.

Post, D.M. (2002) 'Using stable isotopes to estimate trophic position: Models, methods, and assumptions', *Ecology*, 83(3), pp. 703–718. Available at: https://doi.org/10.1890/0012-9658(2002)083[0703:USITET]2.0.CO;2.

Potts, D.D., Dawson, H.A. and Jones, M.L. (2015) 'Validation of a relationship between statolith size and age of larval Great Lakes sea lamprey (*Petromyzon marinus*)', *Environmental Biology of Fishes*, 98(8), pp. 1859–1869. Available at: https://doi.org/10.1007/s10641-015-0403-7.

Price, G.D., Twitchett, R.J., Smale, C. and Marks, V. (2009) 'Isotopic analysis of the life history of the enigmatic squid *Spirula spirula,* with implications for studies of fossil cephalopods', *Palaios*, 24(5–6), pp. 273–279. Available at: https://doi.org/10.2110/palo.2008.p08-067r.

Puckett, B.J., Secor, D.H. and Ju, S. (2008) 'Validation and application of lipofuscin-based age

determination for Chesapeake Bay blue crabs *Callinectes sapidus*', *Transactions of the American Fisheries Society*, 137(6), pp. 1637–1649. Available at: https://doi.org/10.1577/t07-278.1.

Queirós, J.P., Fenwick, M., Stevens, D.W., Cherel, Y., Ramos, J.A. and Xavier, J.C. (2020) 'Ontogenetic changes in habitat and trophic ecology of the giant Antarctic octopus *Megaleledone setebos* inferred from stable isotope analyses in beaks', *Marine Biology*, 167(5), pp. 1–9. Available at: https://doi.org/10.1007/s00227-020-3666-2.

R Core Team (2022) 'R: A language and environment for statistical computing. R Foundation for Statistical Computing'. Vienna, Austria. Available at: https://www.r-project.org/.

Radtke, R.L. (1983) 'Chemical and structural characteristics of statoliths from the short-finned squid *Illex illecebrosus*', *Marine Biology*, 76(1), pp. 47–54. Available at: https://doi.org/10.1007/BF00393054.

Ramos, J.E., Pecl, G.T., Moltschaniwskyj, N.A., Strugnell, J.M., León, R.I. and Semmens, J.M. (2014) 'Body size, growth and life span: Implications for the polewards range shift of *Octopus tetricus* in South-Eastern Australia', *PLoS ONE*, 9(8), pp. 1–9. Available at: https://doi.org/10.1371/journal.pone.0103480.

Ré, P. and Narciso, L. (1994) 'Growth and cuttlebone microstructure of juvenile cuttlefish, *Sepia offcinalis* L., under controlled conditions', *Journal of Experimental Marine Biology and Ecology*, 177(1), pp. 73–78.

Regueira, M., Gonzalez, A.F. and Guerra, A. (2015) 'Determination of age and growth of the horned octopus *Eledone cirrhosa* (Cephalopoda: Octopoda) using stylet increment analysis', *Scientia Marina*, 79(1), pp. 71–79.

Reid, A. (2016) 'Post-mortem drift in Australian cuttlefish sepions: its effect on the interpretation of species ranges', *Molluscan Research*, 36(1), pp. 9–21. Available at: https://doi.org/10.1080/13235818.2015.1064366.

Rexfort, A. and Mutterlose, J. (2006) 'Stable isotope records from *Sepia officinalis*-a key to understanding the ecology of belemnites?', *Earth and Planetary Science Letters*, 247(3–4), pp. 212–221. Available at: https://doi.org/10.1016/j.epsl.2006.04.025.

Ridgway, K.R. and Condie, S.A. (2004) 'The 5500-km-long boundary flow off western and southern Australia', *Journal of Geophysical Research: Oceans*, 109(4), pp. 1–18. Available at: https://doi.org/10.1029/2003JC001921.

Rodhouse, P.G. and Hatfield, E.M.C. (1990) 'Age determination in squid using statolith growth increments', *Fisheries Research*, 8, pp. 323–334. Available at: https://doi.org/10.1016/0165-7836(90)90002-D.

Rodhouse, P.G.K., Pierce, G.J., Nichols, O.C., Sauer, W.H.H., Arkhipkin, A.I., Laptikhovsky, V. V., Lipiński, M.R., Ramos, J.E., Gras, M., Kidokoro, H., Sadayasu, K., Pereira, J., Lefkaditou, E., Pita, C., Gasalla, M., Haimovici, M., Sakai, M. and Downey, N. (2014) 'Environmental effects on cephalopod population dynamics: Implications for management of fisheries', *Advances in Marine Biology*, 67, pp. 99–233. Available at: https://doi.org/10.1016/B978-0-12-800287-2.00002-0.

Rodríguez-Domínguez, A., Rosas, C., Méndez-Loeza, I. and Markaida, U. (2013) 'Validation of growth increments in stylets, beaks and lenses as ageing tools in *Octopus maya*', *Journal of Experimental Marine Biology and Ecology*, 449, pp. 194–199. Available at: https://doi.org/10.1016/j.jembe.2013.10.001.

Romanek, C.S., Grossman, E.L. and Morse, J.W. (1992) 'Carbon isotopic fractionation in synthetic aragonite and calcite: Effects of temperature and precipitation rate', *Geochimica et Cosmochimica Acta*, 56, pp. 419–430.

Roper, C.F.E., Sweeney, M.J. and Nauen, C.E. (1984) *FAO Species catalogue: Cephalopods of the world (Nototodarus sloanii)*.

Rosa, R., Lopes, V.M., Guerreiro, M., Bolstad, K. and Xavier, J.C. (2017) 'Biology and ecology of the world's largest invertebrate, the colossal squid (*Mesonychoteuthis hamiltoni*): a short review', *Polar Biology*, 40(9), pp. 1871–1883. Available at: https://doi.org/10.1007/s00300-017-2104-5.

Ruiz-Cooley, R.I., Villa, E.C. and Gould, W.R. (2010) 'Ontogenetic variation of $\delta^{13}C$ and $\delta^{15}N$ recorded in the gladius of the jumbo squid *Dosidicus gigas*: Geographic differences', *Marine Ecology Progress Series*, 399, pp. 187–198. Available at: https://doi.org/10.3354/meps08383.

Sakamoto, T., Komatsu, K., Yoneda, M., Ishimura, T., Higuchi, T., Shirai, K., Kamimura, Y., Watanabe, C. and Kawabata, A. (2017) 'Temperature dependence of $\delta^{18}O$ in otolith of juvenile Japanese sardine: Laboratory rearing experiment with micro-scale analysis', *Fisheries Research*, 194(May), pp. 55–59. Available at: https://doi.org/10.1016/j.fishres.2017.05.004.

Samin, E., Malaiz, B., Dassi, P., Charlier, K., Genty, D., Richard, P., Vieira, J. and Baudrimont, M. (2023) 'Reconstruction of annual and seasonal variations in water temperature in the Haute-

Dronne River of southwest France based on $\delta^{18}O$ records of freshwater pearl mussel shells (*M . margaritifera*), and its palaeoenvironmental implications', *Evolving Earch*, 1(September). Available at: https://doi.org/10.1016/j.eve.2023.100012.

Sauer, W.H., Gleadall, I.G., Downey-Breedt, N., Doubleday, Z., Gillespie, G., Haimovici, M., Ibáñez, C.M., Katugin, O.N., Leporati, S., Lipinski, M.R., Markaida, U., Ramos, J.E., Rosa, R., Villanueva, R., Arguelles, J., Briceño, F.A., Carrasco, S.A., Che, L.J., Chen, C.S., Cisneros, R., Conners, E., Crespi-Abril, A.C., Kulik, V. V., Drobyazin, E.N., Emery, T., Fernández-Álvarez, F.A., Furuya, H., González, L.W., Gough, C., Krishnan, P., Kumar, B., Leite, T., Lu, C.C., Mohamed, K.S., Nabhitabhata, J., Noro, K., Petchkamnerd, J., Putra, D., Rocliffe, S., Sajikumar, K.K., Sakaguchi, H., Samuel, D., Sasikumar, G., Wada, T., Zheng, X., Tian, Y., Pang, Y. and Yamrungrueng, A. (2021) 'World Octopus Fisheries', *Reviews in Fisheries Science and Aquaculture*, 29(3), pp. 1–151. Available at: https://doi.org/10.1080/23308249.2019.1680603.

Schloesser, R.W., Rooker, J.R., Louchuoarn, P., Neilson, J.D. and Secor, D.H. (2009) 'Interdecadal variation in seawater $\delta^{13}C$ and $\delta^{18}O$ recorded in fish otoliths', *Limnology and Oceanography*, 54(5), pp. 1665–1668. Available at: https://doi.org/10.4319/lo.2009.54.5.1665.

Schmidt, G.A., Bigg, G.R. and Rohling, E.J. (1999) 'Global Seawater Oxygen-18 Database-v1.22'. Available at: https://data.giss.nasa.gov/o18data/.

Schöne, B.R., Pfeiffer, M., Pohlmann, T. and Siegismund, F. (2005) 'A seasonally resolved bottom-water temperature record for the period AD 1866-2002 based on shells of *Arctica islandica* (Mollusca, North Sea)', *International Journal of Climatology*, 25(7), pp. 947–962. Available at: https://doi.org/10.1002/joc.1174.

Schöne, B.R., Zhang, Z., Radermacher, P., Thébault, J., Jacob, D.E., Nunn, E. V. and Maurer, A.F. (2011) 'Sr/Ca and Mg/Ca ratios of ontogenetically old, long-lived bivalve shells (Arctica islandica) and their function as paleotemperature proxies', *Palaeogeography, Palaeoclimatology, Palaeoecology*, 302(1), pp. 52–64. Available at: https://doi.org/10.1016/j.palaeo.2010.03.016.

Schwarcz, H.P., Gao, Y., Campana, S., Browne, D., Knyf, M. and Brand, U. (1998) 'Stable carbon isotope variations in otoliths of Atlantic cod (*Gadus morhua*)', *Canadian Journal of Fisheries and Aquatic Sciences*, 55(8), pp. 1798–1806. Available at: https://doi.org/10.1139/f98-053.

Schwarz, R., Hoving, H.J., Noever, C. and Piatkowski, U. (2019) 'Life histories of Antarctic incirrate octopods (Cephalopoda: Octopoda)', *PLoS ONE*, 14(7), pp. 1–26. Available at:

https://doi.org/10.1371/journal.pone.0219694.

Schwarz, R., Piatkowski, U., Robison, B.H., Laptikhovsky, V. V. and Hoving, H.J. (2020) 'Life history traits of the deep-sea pelagic cephalopods *Japetella diaphana* and *Vampyroteuthis infernalis*', *Deep-Sea Research Part I: Oceanographic Research Papers*, 164(December 2019), p. 103365. Available at: https://doi.org/10.1016/j.dsr.2020.103365.

Seibel, B.A. (2016) 'Cephalopod susceptibility to asphyxiation via ocean incalescence, deoxygenation, and acidification', *Physiology*, 31(6), pp. 418–429. Available at: https://doi.org/10.1152/physiol.00061.2015.

Seibel, B.A. and Childress, J.J. (2000) 'Metabolism of benthic octopods (Cephalopoda) as a function of habitat depth and oxygen concentration', *Deep-Sea Research Part I: Oceanographic Research Papers*, 47(7), pp. 1247–1260. Available at: https://doi.org/10.1016/S0967-0637(99)00103-X.

Seibel, B.A. and Drazen, J.C. (2007) 'The rate of metabolism in marine animals: environmental constraints, ecological demands and energetic opportunities.', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 362(1487), pp. 2061–78. Available at: https://doi.org/10.1098/rstb.2007.2101.

Semmens, J., Doubleday, Z., Hoyle, K. and Pecl, G. (2011) 'A multilevel approach to examining cephalopod growth using *Octopus pallidus* as a model', *Journal of Experimental Biology*, 214(16), pp. 2799–2807. Available at: https://doi.org/10.1242/jeb.051631.

Semmens, J.M., Pecl, G.T., Gillanders, B.M., Waluda, C.M., Shea, E.K., Jouffre, D., Ichii, T., Zumholz, K., Katugin, O.N., Leporati, S.C. and Shaw, P.W. (2007) 'Approaches to resolving cephalopod movement and migration patterns', *Reviews in Fish Biology and Fisheries*, 17(2–3), pp. 401–423. Available at: https://doi.org/10.1007/s11160-007-9048-8.

Semmens, J.M., Pecl, G.T., Villanueva, R., Jouffre, D., Sobrino, I., Wood, J.B. and Rigby, P.R. (2004) 'Understanding octopus growth: Patterns, variability and physiology', *Marine and Freshwater Research*, 55(4), pp. 367–377. Available at: https://doi.org/10.1071/MF03155.

Shirai, K., Otake, T., Amano, Y., Kuroki, M., Ushikubo, T., Kita, N.T., Murayama, M., Tsukamoto, K. and Valley, J.W. (2018) 'Temperature and depth distribution of Japanese eel eggs estimated using otolith oxygen stable isotopes', *Geochimica et Cosmochimica Acta*, 236, pp. 373–383. Available at: https://doi.org/10.1016/j.gca.2018.03.006.

Simpson, G. (2023) 'gratia: Graceful ggplot-Based Graphics and Other Functions for GAMs

Fitted using mgcv', *R package version 0.8.1* [Preprint].

Skrzypek, G. (2013) 'Normalization procedures and reference material selection in stable HCNOS isotope analyses: An overview', *Analytical and Bioanalytical Chemistry*, 405(9), pp. 2815–2823. Available at: https://doi.org/10.1007/s00216-012-6517-2.

Skrzypek, G. and Ford, D. (2014) 'Stable isotope analysis of saline water samples on a cavity ring-down spectroscopy instrument', *Environmental Science and Technology*, 48(5), pp. 2827–2834. Available at: https://doi.org/10.1021/es4049412.

Smale, M.J. (1996) 'Cephalopods as prey. IV. Fishes', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 351(1343), pp. 1067–1081. Available at: https://doi.org/10.1098/rstb.1996.0094.

Smale, M.J. and Buchan, P.R. (1981) 'Biology of *Octopus vulgaris* off the east coast of South Africa', *Marine Biology*, 65(1), pp. 1–12. Available at: https://doi.org/10.1007/BF00397061.

Smith, D.C., Fenton, G.E., Robertson, S.G. and Short, S. a (1995) 'Age determination and growth of orange roughy (*Hoplostethus atlanticus*): A comparison of annulus counts with radiometric ageing', *Canadian Journal of Fisheries and Aquatic Sciences*, 52(2), pp. 391–401. Available at: https://doi.org/otolithe age ageage microstructure radiometrie isotopes.

Solomon, C.T., Weber, P.K., Cech, J.J., Ingram, B.L., Conrad, M.E., Machavaram, M. V., Pogodina, A.R. and Franklin, R.L. (2006) 'Experimental determination of the sources of otolith carbon and associated isotopic fractionation', *Canadian Journal of Fisheries and Aquatic Sciences*, 63(1), pp. 79–89. Available at: https://doi.org/10.1139/f05-200.

Sousa Reis, C. and Fernandes, R. (2002) 'Growth observations on *Octopus vulgaris* cuvier, 1797 from the Portuguese waters: Growth lines in the vestigial shell as possible tools for age determination', *Bulletin of Marine Science*, 71(2), pp. 1099–1103.

Spratt, J.D. (1979) 'Age and growth of the market Squid, *Loligo Opalescens* Berry, from statoliths', *CalCOFI Rep.*, 20, pp. 58–64.

Stephens, P.R. and Young, J.Z. (1978) 'Semicircular canals in squids', *Nature*, 271(5644), pp. 444–445. Available at: https://doi.org/10.1038/271444a0.

Stephens, R.B., Ouimette, A.P., Hobbie, E.A. and Rowe, R.J. (2022) 'Reevaluating trophic discrimination factors ($\Delta\delta^{13}C$ and $\Delta\delta^{15}N$) for diet reconstruction', *Ecological Monographs*, 92(3), pp. 1–20. Available at: https://doi.org/10.1002/ecm.1525.

Stevens, K., Iba, Y., Suzuki, A. and Mutterlose, J. (2015) 'Biological and environmental signals recorded in shells of *Argonauta argo* (Cephalopoda, Octobrachia) from the Sea of Japan', *Marine Biology*, 162(11), pp. 2203–2215. Available at: https://doi.org/10.1007/s00227-015-2750-5.

Stranks, T.N. and Norman, M.D. (1992) 'Review of the *Octopus australis* complex from Australia and New Zealand, with description of a new species (Mollusca: Cephalopoda)', *Memoirs of the National Museum of Victoria*, 53, pp. 345–373.

Sun, D., Gagan, M.K., Cheng, H., Scott-Gagan, H., Dykoski, C.A., Edwards, R.L. and Su, R. (2005) 'Seasonal and interannual variability of the Mid-Holocene East Asian monsoon in coral $\delta^{18}O$ records from the South China Sea', *Earth and Planetary Science Letters*, 237(1–2), pp. 69–84. Available at: https://doi.org/10.1016/j.epsl.2005.06.022.

Surge, D. and Walker, K.J. (2005) 'Oxygen isotope composition of modern and archaeological otoliths from the estuarine hardhead catfish (*Ariopsis felis*) and their potential to record low-latitude climate change', *Palaeogeography, Palaeoclimatology, Palaeoecology*, 228(1–2), pp. 179–191. Available at: https://doi.org/10.1016/j.palaeo.2005.03.051.

Sweeney, M.J., Roper, C.F.E., Mangold, K.M., Clarke, M.R. and von Boletzky, S. (1992) '"Larval" and juvenile cephalopods: a manual for their identification', *Smithsonian Contributions to Zoology*, 513, pp. 1–279. Available at: https://doi.org/10.5479/si.00810282.513.

Taylor, B.E. and Ward, P.D. (1983) 'Stable isotopic studies of *Nautilus macromphalus* sowerby (new Caledonia) and *Nautilus pompilius* L. (Fiji)', *Palaeogeography, Palaeoclimatology, Palaeoecology*, 41(1–2), pp. 1–16. Available at: https://doi.org/10.1016/0031-0182(83)90072-X.

Thorrold, S.R., Campana, S.E., Jones, C.M. and Swart, P.K. (1997) 'Factors determining $\delta^{13}C$ and $\delta^{18}O$ fractionation in aragonitic otoliths of marine fish', *Geochimica et Cosmochimica Acta*, 61(14), pp. 2909–2919. Available at: https://doi.org/10.1016/S0016-7037(97)00141-5.

Tosti, E., di Cosmo, A., Cuomo, A., di Cristo, C. and Gragnaniello, G. (2001) 'Progesterone induces activation in *Octopus vulgaris* spermatozoa', *Molecular Reproduction and Development*, 59(1), pp. 97–105. Available at: https://doi.org/10.1002/mrd.1011.

Trasviña-Carrillo, L.D., Hernández-Herrera, A., Torres-Rojas, Y.E., Galván-Magaña, F., Sánchez-González, A. and Aguíñiga-García, S. (2018) 'Spatial and trophic preferences of jumbo squid *Dosidicus gigas* (D'Orbigny, 1835) in the central Gulf of California: ecological inferences using

stable isotopes', *Rapid Communications in Mass Spectrometry*, 32(15), pp. 1225–1236. Available at: https://doi.org/10.1002/rcm.8147.

Treberg, J.R., Killen, S.S., MacCormack, T.J., Lamarre, S.G. and Enders, E.C. (2016) 'Estimates of metabolic rate and major constituents of metabolic demand in fishes under field conditions: Methods, proxies, and new perspectives', *Comparative Biochemistry and Physiology -Part A : Molecular and Integrative Physiology*, 202, pp. 10–22. Available at: https://doi.org/10.1016/j.cbpa.2016.04.022.

Trueman, C.N. and St John Glew, K. (2019) *Chapter 6 - Isotopic Tracking of Marine Animal Movement*. Second Edi, *Tracking Animal Migration with Stable Isotopes*. Second Edi. Elsevier Inc. Available at: https://doi.org/10.1016/B978-0-12-814723-8.00006-4.

Trumble, S.J., Norman, S.A., Crain, D.D., Mansouri, F., Win, Z.C., Sabin, R., Potter, C.W., Gabriele, C.M. and Usenko, S. (2018) 'Baleen whale corisol levels reveal a physiological response to 20th century whaling', *Nature Communications*, 9(4587). Available at: https://doi.org/10.1038/s41467-018-07044-w.

Trumble, S.J., Robinson, E.M., Berman-Kowalewski, M., Potter, C.W. and Usenko, S. (2013) 'Blue whale earplug reveals lifetime contaminant exposure and hormone profiles', *Proceedings of the National Academy of Sciences of the United States of America*, 110(42), pp. 16922–16926. Available at: https://doi.org/10.1073/pnas.1311418110.

Vighi, M., Borrell, A., Víkingsson, G., Gunnlaugsson, T. and Aguilar, A. (2019) 'Strontium in fin whale baleen: A potential tracer of mysticete movements across the oceans?', *Science of the Total Environment*, 650, pp. 1224–1230. Available at: https://doi.org/10.1016/j.scitotenv.2018.09.103.

Villegas-Bárcenas, G., Perales-Raya, C., Bartolomé, A., Almansa, E. and Rosas, C. (2014) 'Age validation in *Octopus maya* (Voss and Solís, 1966) by counting increments in the beak rostrum sagittal sections of known age individuals', *Fisheries Research*, 152, pp. 93–97. Available at: https://doi.org/10.1016/j.fishres.2013.08.007.

Voss, N.A. and Sweeney, M.J. (1998) 'Systematics and Biogeography of cephalopods. Volume II', *Smithsonian Contributions to Zoology*, I(586), pp. 277–599. Available at: https://doi.org/10.5479/si.00810282.586.277.

Wanamaker, A.D. and Gillikin, D.P. (2019) 'Strontium, magnesium, and barium incorporation in aragonitic shells of juvenile Arctica islandica: insights from temperature controlled

experiments', *Chemical Geology*, 526, pp. 117–129.

Wells, M.J. and Wells, J. (1959) 'Hormonal control of sexual maturity in octopus', *Journal of Experimental Biology*, 36(1), pp. 1–33. Available at: https://doi.org/10.1242/jeb.36.1.1.

Wickham, H. (2011) 'ggplot 2'. Wiley Interdisciplinary Reviews: Computational Statistics, pp. 180–185.

Wood, S.N. (2011) 'Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models', *Journal of the Royal Statistical Society. Series B: Statistical Methodology*, 73(1), pp. 3–36. Available at: https://doi.org/10.1111/j.1467-9868.2010.00749.x.

Wood, S.N. (2017) *Generalized Additive Models: an introduction with R*, *Chapman and Hall/ CRC*.

Wood, S.N., Bravington, M. V. and Hedley, S.L. (2008) 'Soap film smoothing', *Journal of the Royal Statistical Society. Series B: Statistical Methodology*, 70(5), pp. 931–955. Available at: https://doi.org/10.1111/j.1467-9868.2008.00665.x.

Wu, D., Zhang, F., Zhang, X., Li, X., Huang, H., Feng, H. and Zhang, J. (2021) 'Stable isotopes ($\delta^{13}C$ and δ<up>15</sup>N) in black coral as new proxies for environmental record', *Marine Pollution Bulletin*, 164(July 2020), p. 112007. Available at: https://doi.org/10.1016/j.marpolbul.2021.112007.

Xavier, J.C., Golikov, A. V., Queirós, J.P., Perales-Raya, C., Rosas-Luis, R., Abreu, J., Bello, G., Bustamante, P., Capaz, J.C., Dimkovikj, V.H., González, A.F., Guímaro, H., Guerra-Marrero, A., Gomes-Pereira, J.N., Hernández-Urcera, J., Kubodera, T., Laptikhovsky, V., Lefkaditou, E., Lishchenko, F., Luna, A., Liu, B., Pierce, G.J., Pissarra, V., Reveillac, E., Romanov, E. V., Rosa, R., Roscian, M., Rose-Mann, L., Rouget, I., Sánchez, P., Sánchez-Márquez, A., Seixas, S., Souquet, L., Varela, J., Vidal, E.A.G. and Cherel, Y. (2022) 'The significance of cephalopod beaks as a research tool: An update', *Frontiers in Physiology*, 13(November), pp. 1–30. Available at: https://doi.org/10.3389/fphys.2022.1038064.

Yagi, T. (1960) 'On the growth of the shell in *Sepia esculenta* Hoyle caught in Tokyo Bay', *Bulletin of the Japanese Society of Scientific Fisheries*, 26(7), pp. 646–652.

Yasuda, J. (1951) 'Some ecological notes on the cuttlefish, *Sepia esculenta*', *Bulletin of the Japanese Society of Scientific Fisheries*, 16, pp. 350–356.

Young, J.Z. (1960) 'The statocysts of *Octopus vulgaris*', *Proceedings of the Royal Society of London. Series B. Biological Sciences*, 152(946), pp. 3–29. Available at: https://doi.org/10.1093/acprof:oso/9780198547907.003.0342.

Zakharov, Y.D., Shigeta, Y., Smyshlyaeva, O.P., Popov, A.M. and Ignatiev, A. V. (2006) 'Relationship between $\delta^{13}C$ and $\delta^{18}O$ values of the Recent Nautilus and brachiopod shells in the wild and the problem of reconstruction of fossil cephalopod habitat', *Geosciences Journal*, 10(3), pp. 331–345. Available at: https://doi.org/10.1007/BF02910374.

Zielinski, S. and Portner, H.O. (2000) 'Oxidative stress and antioxidative defense in cephalopods: A function of metabolic rate or age?', *Comparative Biochemistry and Physiology Part B*, 125(2), pp. 147–160. Available at: https://doi.org/10.1016/S0305-0491(99)00162-5.

Zumholz, K., Hansteen, T.H., Piatkowski, U. and Croot, P.L. (2007) 'Influence of temperature and salinity on the trace element incorporation into statoliths of the common cuttlefish (Sepia officinalis)', *Marine Biology*, 151(4), pp. 1321–1330. Available at: https://doi.org/10.1007/s00227-006-0564-1.

Zuur, A.F. (2012) *A Beginner's Guide to Generalized Additive Models with R*. Newburgh: Highland Statistics Ltd.

Zuur, A.F., Ieno, E.N. and Elphick, C.S. (2010) 'A protocol for data exploration to avoid common statistical problems', *Methods in Ecology and Evolution*, 1(1), pp. 3–14. Available at: https://doi.org/10.1111/j.2041-210x.2009.00001.x.

Appendix A