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Direct age determination with validation for commercially important Australian lobster and crab species: Western, Eastern, Southern and Ornate Rock Lobsters and Crystal, Giant and Mud Crabs

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Executive Summary

This research project was undertaken by a national collaboration of government and academic scientists representing key Australian crustacean fisheries. The collaborating institutions were the: Marine Ecology Research Centre – Southern Cross University, Department of Fisheries Western Australia, Institute for Marine and Antarctic Studies – University of Tasmania, New South Wales Department of Primary Industries – Fisheries, Northern Territory Department of Primary Industry and Fisheries, South Australian Research and Development Institute and James Cook University. The project was initiated in response to the need for validated age information for crustacean fisheries management. We applied a novel direct age-determination method to seven commercially important Australian crustaceans sourced from tropical to temperate habitats, shallow to deep water and including both short- and long-lived species. Similar to fish ageing, the direct ageing method applied here involves cross-sectioning gastric ossicles (i.e. semi-calcified structures within the stomach) to enable the extraction of a chronological record (i.e. by counting growth marks) for subsequent growth modelling. For the first time, we have demonstrated the widespread applicability of direct ageing to Australian crustaceans and validated that ossicular growth marks in Western, Eastern and Ornate Rock Lobster and Crystal Crab ossicles are deposited annually. Validation of the direct ageing method, allowed for the construction of the world's first directly determined growth models for any Rock Lobster, with most comparisons to existing indirect estimates corroborating annual periodicity.

Background

The ability to procure accurate age information is important for any sustainable fisheries management plan. Age information underpins growth and productivity estimates and also informs the selection of input control regulations (e.g. minimum legal size). For many fin fish and invertebrate species, age determination is relatively straightforward and involves counting growth increments in calcified structures. Because crustaceans grow via consecutive moult events, it was always presumed that their hard parts could not retain a chronological growth record and fisheries scientist have relied solely on less-accurate indirect methods (e.g. tag-and-recapture) that infer age. However, recent studies have demonstrated that crustacean ossicles contain growth marks that can be used for direct age determination, but species-specific periodicity validation (i.e. proof of accuracy) is needed before widespread use of the method occurs. The need for a validated direct ageing method for crustaceans was recognised throughout Australia and resulted in this project being strongly supported by relevant industry bodies, state government fisheries departments and academic institutions. Although indirect techniques provide useful information, a validated direct ageing method is highly desirable and could substantially increase the resolution of age-related data for crustacean fisheries management in Australia.

Objectives

The specific objectives of this research project were to: 1) assess the relationship between estimated age and size, compared with existing growth models for Western and Eastern Rock Lobster, 2) evaluate growth mark periodicity for Western and Eastern Rock Lobster and Crystal Crab by vital staining and long-term grow-out, 3) investigate the applicability of direct ageing methods to other commercially important crustaceans (Western, Eastern, Southern and Ornate Rock Lobsters and Giant, Crystal and Mud Crabs) – validated with laser ablation induction-coupled plasma mass spectroscopy and known-age individuals and 4) establish a network of Australian government and academic fisheries researchers that can consistently apply direct ageing methods to decapod crustaceans.

Methodology

Objective 1 – Size-at-putative-age assessment – Western and Eastern Rock Lobster: Wild-caught Western and Eastern Rock Lobster for putative age estimation were sourced from commercial fishers, sampled under permit, or purchased from retail outlets. Western Rock Lobster were sourced from 'shallow' and 'deep' sites at a single location (Lancelin, Western Australia – WA). Eastern Rock Lobster were sampled from three New South Wales (NSW) locations (i.e. Coffs Harbour, South West Rocks and Jervis Bay). For both species, the number of primary growth marks in sectioned ossicles was recorded for each individual, before being used to construct von Bertalanffy growth models for comparison with existing indirectly obtained estimates from tag-and-recapture studies.

Objective 2 – Evaluation of growth mark periodicity – Western and Eastern Rock Lobster and Crystal Crab: Wild-caught Western and Eastern Rock Lobster and Crystal Crab (from Carnarvon, WA) were sourced (as above) and treated with a chemical stain (i.e. calcein), before being reared in research or commercial aquaculture facilities and sacrificed after 6, 12 or 18 months. The calcein stain produces a known-date artificial mark, for subsequent determination of growth mark deposition during the intervening period between staining and sacrifice. For rock lobster, two separate calcein-staining experiments were done to assess growth mark periodicity in both temperate (i.e. Perth, WA) and subtropical (i.e. Coffs Harbour, NSW) locations. For Crystal Crab, a deep-sea species, the validation experiment was done (i.e. in Perth, WA) under controlled conditions simulating their cold-water environment (i.e. at 6°C). After sacrifice, the calcein-stained ossicles were imaged under confocal fluorescence microscopy for subsequent determination of growth mark formation beyond the artificial mark.

Objective 3 – Applicability to other crustacean species – with laser ablation and known-age individual validation: In addition to the primary project species (i.e. Western and Eastern Rock Lobster and Crystal Crab – Objective 1 and 2), a small number (n = 3–7 of each) of other commercially important wild-caught rock lobster (i.e. Southern Rock Lobster – from Tasmania and South Australia, and Ornate Rock Lobster –

from Queensland) and crab (i.e. Giant Crab and Mud Crab – from South Australia and the Northern Territory, respectively) were provided by project co-investigators to investigate the applicability of direct ageing methods and apply other less-expensive validation techniques that require relatively few individuals. For all species (except Giant Crab), a putative age estimate (as above – Objective 1) was assigned to each individual and laser ablation-inductively coupled plasma mass spectrometry (LA-ICPMS) was used to quantify changes in primary growth mark composition that could correspond to annual cycles (e.g. minimum and maximum temperatures). Recently settled young-of-the-year rock lobster (i.e. of known age) were sourced from the wild and reared under natural (i.e. Ornate Rock Lobster – up to 1.4 years) or indoor conditions (i.e. Western Rock Lobster – up to 3.2 years), before they were sacrificed and the number of primary growth marks deposited during the grow-out was determined. For Tasmanian Southern Rock Lobster and Ornate Rock Lobster (from Torres Strait, Queensland), a preliminary growth model was generated for comparison with existing estimates from the same (broad) source locations.

Objective 4 – Direct ageing network – workshop: To address this objective, a Crustacean Ageing Workshop was convened by the Principal Investigator (J. Leland) at Southern Cross University's Marine Ecology Research Centre during 2016. Participants included several project co-investigators and other interested academic and government fisheries researchers.

Key findings

Objective 1 – Size-at-putative-age assessment – Western and Eastern Rock Lobster: For Western and Eastern Rock Lobster, sectioned ossicles contain regular primary growth marks that are positively correlated with body size. Ossicular growth mark counts were converted to putative age estimates and used to generate von Bertalanffy growth models that were not significantly different to those from comparable tag-and-recapture studies. For Western Rock Lobster, the directly determined putative ages closely agreed with indirect longevity estimates and the age at fishery-specific milestones (i.e. minimum legal size and size-at-sexual maturity), with the relationship between direct and indirect age (i.e. derived from both wild-caught and known-age individuals) being approximately 1:1 and providing strong corroborative support for annual periodicity. For Eastern Rock Lobster, the directly determined putative ages broadly agreed with indirect maximum longevity estimates, but yielded consistently older ages at fishery-specific milestones (i.e. minimum legal size, size-at-sexual maturity and maximum legal size), with the relationship between direct and indirect age estimates for some locations being approximately 1:1 (i.e. providing support for annual periodicity), but for others it was markedly different (i.e. for Jervis Bay and some Coffs Harbour individuals). For both rock lobster species, the directly determined putative age corresponded to known biological and ecological patterns (e.g. juvenile growth or ontogenetic movement), with differences between the direct and indirect estimates being attributable to other factors (e.g. temperature or density) that are known to influence crustacean growth.

Objective 2 – Evaluation of growth mark periodicity – Western and Eastern Rock Lobster and Crystal Crab: For Crystal Crab, there was ossicular extension during the 18 month grow-out, with primary growth mark formation occurring during the inter-moult. Irrespective of the sampling period, most Crystal Crab deposited one new-formed primary growth mark (n = 12) during the grow-out. For Western Rock Lobster (n = 1), the periodicity evaluation indicated that a single primary growth mark was deposited during the 18 month grow-out. For Eastern Rock Lobster (n = 1), the periodicity evaluation indicated that a single primary growth mark was deposited during the 12 month grow-out. For both rock lobster species, there were other ossicles that had material deposited beyond the calcein stain, but were without an identifiable growth mark. For all species, the common outcome of the periodicity evaluation was that a single new growth mark was deposited during the grow-out, indicating that the primary marks are deposited annually.

Objective 3 – Applicability to other crustacean species – with LA-ICPMS and known-age individual validation: The direct ageing method was readily applied to Ornate Rock Lobster, Southern Rock Lobster, Mud Crab and Crystal Crab ossicles. Giant Crab (n = 3 individuals) ossicles contained some primary growth marks, but complete counts were not possible. For Ornate Rock Lobster (n = 5) and Southern Rock Lobster (n = 5), the direct ageing method allowed for the rapid estimation of preliminary von Bertalanffy growth parameters that were not significantly different to those derived from tag-and-recapture studies at the same location. For all project species (i.e. except Giant Crab), we confirmed that the visually identified primary growth marks generally corresponded with quantifiable physico-chemical variations in ossicular mineral composition, with the number of localised minima or maxima for elemental ratios (i.e. in the endocuticle) increasing with putative age. Some LA-ICPMS results (e.g. for Mud Crab and Western and Eastern Rock Lobster) could be interpreted as supporting annual periodicity, but emerging uncertainties around ossicular decalcification and potential re-deposition of mineral features precluded a positive validation outcome. The direct ageing method was also validated by the use of known-age Ornate Rock Lobster (n = 13) and Western Rock Lobster (n = 3).

Objective 4 – Direct ageing network – workshop: A round-table discussion held at the Crustacean Ageing Workshop (i.e. held at the approximate midpoint of the project) identified several cross-jurisdictional research priorities. These included further research aimed at: i) developing crustacean age validation techniques, ii) procuring species-specific validations, iii) first growth mark formation, iv) construction of standard ossicle collections and v) understanding the processes governing growth mark deposition. These priorities were addressed to varying degrees in this project, but further research is needed to expand on the knowledge advances presented here.

Implications

The immediate impact from this project will be jurisdiction- and species-specific, because each state fisheries department has different needs, priorities and validation expectations. However, the ability to

directly determine (i.e. and validate) crustacean age provides another tool for fisheries scientists to enhance the resolution of current growth models, while decreasing research costs. Validation of the ageing method for Western, Eastern and Ornate Rock Lobster and Crystal Crab also opens the way for preliminary trials using the technique in stock assessments. Further, the validated technique will allow for rapid location-specific growth assessments and more accurate longevity estimates. This will be particularly important for long-lived species that present difficulties for tagging studies (e.g. Crystal Crab and Tasmanian Southern Rock Lobster) and would be useful for securing fishery sustainability certifications (e.g. Marine Stewardship Council). For shorter-lived species (e.g. Ornate Rock Lobster and Mud Crab), direct ageing could improve the assessment of population dynamics. The financial gains are difficult to quantify, but even a 1% improvement in decision making, and/or decrease in research costs (i.e. across multiple valuable fisheries), would equate to a substantial return of investment from this project. Such gains will translate into improved sustainability among Australia's crustacean fisheries, with flow-on benefits to the relevant fishing industry and across other sectors.

Recommendations

The broad-ranging nature (i.e. in terms of species and fisheries jurisdictions) of this project made definitive recommendations difficult. However, further species-specific research should: i) validate periodicity across the entire age range, ii) determine the age at first growth mark formation and iii) assess ageing accuracy. Concurrent studies trialling the direct ageing method during ongoing stock monitoring programs would be beneficial. This would allow for direct methodological comparison and growth model construction for the exact same location(s) and temporal period. For some species (e.g. Eastern Rock Lobster), the direct ageing method should be used to assess the potential for location-specific differences in growth. Application of the direct method to Crystal Crab is needed to provide the first solid (i.e. non-preliminary) assessment of growth and longevity for this species. Such research should encompass the relevant priorities for further development, particularly the requirement for concurrent species-specific precision assessments. The provision of this report to the relevant state fisheries departments is expected to initiate further jurisdiction- and stock-specific recommendations that will form the basis for further research and development applications.

Keywords: calcein, Crystal Crab (*Chaceon albus*), direct age determination, Eastern Rock Lobster (*Sagmariasus verreauxi*), Giant Crab (*Psuedocarcinus gigas*), growth, Mud Crab (*Scylla serrata*), Ornate Rock Lobster (*Panulirus ornatus*), rock lobster, Southern Rock Lobster (*Jasus edwardsii*), spiny lobster, validation, von Bertalanffy, Western Rock Lobster (*Panulirus cygnus*)

1.0 Introduction

1.1 Background

This research project stemmed from the principal investigator's (J. Leland) pioneering crustacean ageing research at Southern Cross University (see Leland et al., 2011), for which he was awarded the Department of Agriculture, Fisheries and Forestry 2013 Science and Innovation Award (Fisheries category). The associated Fisheries Research and Development Corporation (FRDC) funded grant initiated a one year research project into direct age-determination studies for two short-lived Australian crustaceans (i.e. the Mud Crab, *Scylla serrata* and Redclaw Crayfish, *Cherax quadricarinatus*). That project allowed J. Leland to refine his direct ageing protocols and resulted in one scientific publication (Leland et al., 2015) and an Honours thesis (Sarapuk, 2014) that demonstrated the methods applicability to short-lived subtropical decapods. During that time, co-investigator S. de Lestang was investigating the utility of direct methods for ageing Western Rock Lobster (*Panulirus cygnus* – see Rudd, 2013), while applying for FRDC funding (along with other co-investigators) to support further research. This project represents a combination of the two initiatives. Recognition of the value of developing direct methods for ageing crustaceans, while extending Australian leadership in this field has led to this collaborative effort. This project aligns with five FRDC Strategic Priority Areas: Program 1 (Environment), Program 2 (Industry), Program 3 (Communities), Program 4 (People) and Program 5 (Adoption).

From a fisheries management perspective, age information is an important biological variable, because of its importance for calculating recruitment, growth and mortality (Campana, 2001). Global recognition of this, combined with the requirements for effective sustainable management, has led to the development of methodologies that can be used to directly assign individual ages. Since the early fish ageing studies, direct ageing methodologies have expanded to include a diverse range of calcified structures that are present in invertebrate and vertebrate taxa, including gastropod statoliths (e.g. Chatzinikolaou and Richardson, 2007), polychaete mandibles (e.g. Leland, 2009), octopus stylets (e.g. Leporati and Hart, 2015), and the more commonly known fish otoliths (e.g. Piddocke et al., 2015a). Such structures are retained for life and record easily extractable age information. During the past few decades, finfish ageing studies have become somewhat routine, but analogous methods for crustaceans have been lacking (Leland et al., 2011; 2015).

Crustacean growth occurs through consecutive moults (Hartnoll, 2001). The shedding of the old exoskeleton presumably caused scientists to overlook the potential for crustacean hard parts to contain any chronological growth record (Leland et al., 2011; 2015). Because of this, fisheries scientists have relied solely on indirect methods for ageing harvested decapods and in contrast with finfish, crustacean

longevity remains poorly understood. Indirect methods for crustacean age determination include captive rearing of known-age individuals, length-frequency analysis and tag-and-recapture in wild-caught populations (Hartnoll, 2001). In the absence of direct ageing methods for crustaceans, these methodologies provide useful age information, but are known to incorporate at least some intrinsic biases (Hartnoll, 2001; Vogt, 2012). For example, the captive rearing of known-age individuals can provide accurate longevity information, but occurs under unnatural (and usually optimal) conditions thereby reducing their representative value for wild populations (Vogt, 2012). Size frequency analysis and tag-and-recapture studies can provide relatively accurate wild growth information, but the efficacy of these methods can decrease with increased longevity (Hartnoll, 2001; Vogt, 2012). One alternative ageing technique involves the accumulation of an age-related pigment lipofuscin in the crustacean brain (Hartnoll, 2001). Some disadvantages of this method are that it requires extensive expertise and technological support and also requires location-specific calibration (Vogt, 2012).

The gastric mill comprises a set of semi-calcified toothed ossicles that are housed within the decapod foregut and used to masticate food (Holdich and Lowery, 1988; Factor, 1995). Leland et al. (2011) reported that, similar to fish otoliths, cross-sectioned gastric ossicles from five Australian crayfish, crab and lobster species contain concentric growth marks that can be used for direct age estimation. However, like otolith studies, the use of crustacean ossicles for direct ageing requires periodicity validation as a prerequisite (Campana, 2001; Leland et al., 2011). A subsequent study by Kilada et al. (2012), applied the direct method to both internal (i.e. gastric ossicles) and external (i.e. eyestalks) cuticular structures from four North American species, providing evidence for annual growth mark formation in known-age American Lobster (*Homarus americanus*) and demonstrating that periodicity validation is possible by calcein staining. Leland et al. (2015) used a short-lived (i.e. 3 years) model species (the Redclaw Crayfish) in a one year calcein-staining validation study (following Kilada et al., 2012) to demonstrate that gastric ossicles sequentially record past events, providing perhaps the strongest evidence to date supporting annual periodicity. Other direct crustacean ageing studies have paired corroboration methods such as length-frequency analysis and known-age individuals, with age estimates to provide further evidence for annual periodicity (Kilada and Acuna, 2015; Kilada et al., 2015). Such studies have reported that the number of primary growth marks generally agrees with the individual known age, or length-frequency based estimation (Kilada et al., 2012; Kilada and Acuna, 2015; Kilada et al., 2015; Kilada et al., 2017a). Most recent studies note that directly determined ages corroborate previously known life-history milestones and/or longevity estimates for short-lived (< 12 years) crustaceans (Kilada et al., 2012; Sarapuk, 2014; Kilada and Acuna, 2015; Kilada et al., 2015; Leland et al., 2015; Krafft et al., 2016; Kilada et al., 2017a).

Past history in fisheries biology has highlighted the crucial importance of validating age estimates (Beamish and McFarlane, 1983). Because of this, stringent requirements were developed regarding

best-practice for validation approaches in finfish, with rigorous studies applying at least two methods (Campana, 2001). One age validation method that is commonly applied to finfish involves the vital staining of calcified structures (e.g. vertebrae, scales or otoliths) for subsequent re-examination of material deposited beyond the artificial mark (Campana, 2001; Piddocke et al., 2015b), allowing for quantification of the number of growth marks formed per unit of time (i.e. termed 'periodicity'). The fluorescent dye calcein is effective for staining a range of ageing structures (e.g. Cameron et al., 2011; van der Geest et al., 2011) and is the only chemical tag that is known to be retained (i.e. after consecutive moults) in crustacean ossicles for up to one year (Kilada et al., 2012; Sarapuk, 2014; Leland et al., 2015). Another useful age validation method is the direct age determination of captive-reared individuals. This approach requires only a few individuals, but carries the risk that captive growth increments can be markedly different to that of wild specimens (Campana, 2001). Laser ablation-inductively coupled plasma mass spectrometry (LA-ICPMS) can be applied to increment-bearing structures to analyse isotopic ratio changes that coincide with visual features and provide further support for periodicity assessments. Strontium and boron deposition into aragonitic structures fluctuate with temperature at the time of formation (Bath et al., 2000; Naik and Naidu, 2014) and can be used to link optically identified features (i.e. growth increments) with seasonal cycles (Hale et al., 2006; Scharer et al., 2012).

Like the external crustacean exoskeleton, gastric ossicles comprise four distinct cuticular layers: the epicuticle, exocuticle, endocuticle and the membranous layer (Raz et al., 2002; Kilada et al., 2012). Leland et al. (2011) identified the presence of a primary and secondary growth mark series within the ossicular endocuticle (also see Kilada et al., 2012). Other subsequent studies have reported that eyestalk endocuticle also contains primary and secondary growth marks (e.g. Kilada et al., 2012; Krafft et al., 2016; Kilada et al., 2017b). To date, all relevant ageing studies have hypothesised that the primary growth marks in both ossicle and eyestalk endocuticle are deposited annually, with this series being more prominent and having substantially wider spacing than either the secondary marks or structural lamellae (e.g. Kilada et al., 2012; Sarapuk, 2014; Kilada and Acuna, 2015; Kilada et al., 2015; Leland et al., 2015; Krafft et al., 2016).

During the project application phase (~early 2013–2014), there was a paucity of detailed information on whether gastric ossicles are moulted along with the external exoskeleton. Despite numerous studies detailing gastric mill functional morphology and other important aspects, no study had ever (to our knowledge) comprehensively ascertained the structure's fate during ecdysis. At that time, there was a general international consensus that the gastric mill could be retained during moulting, with multiple authors providing some supporting evidence (Kilada et al., 2012; Sarapuk et al., 2014; Leland et al., 2015). However, Leland et al. (2015) also noted methodological limitations and identified the need for

more detailed (i.e. specifically tailored) studies to definitively determine if gastric ossicles are retained for life (also see Weldon et al., 2015).

Since this project began, three authors have reported that gastric ossicles are either entirely (Vatcher et al., 2015; Sheridan et al., 2016), or partially moulted (Brosing, 2014). Brosing (2014) described species-specific differences in ossicle retention and noted that some moulted structures appeared decalcified, but others did not. In contrast, Vatcher et al. (2015) and Sheridan et al. (2016) reported that Blue Crab (*Callinectes sapidus*) and Norway Lobster (*Nephrops norvegicus*) completely moult their gastric ossicles, with the latter study reporting that complete and wholly calcified ossicles are moulted. As a result, there is substantial uncertainty whether gastric ossicles might be retained for life, or if they are moulted (i.e. either partially or entirely) and if the process is consistent across all species (also see Krafft et al., 2016) and individual moult events. Leland et al. (2015) demonstrated that a known-to-be moulted ossicular component, the mesocardiac tooth plate, sequentially added new external information (i.e. additional urocardiac ridges) to the structure and hypothesised that a similar process could result in the deposition of additional internal features (i.e. ossicular growth marks) over time. Regardless of the moulting debate, the number of primary growth marks within the endocuticle corroborates other known longevity estimates for over 14 decapod species (Kilada et al., 2012; Sarapuk, 2014; Kilada and Acuna, 2015; Kilada et al., 2015; Leland et al., 2015; Krafft et al., 2016).

This project applied direct ageing methodologies to seven commercially important Australian lobster and crab species that sustain high-value fisheries within (and/or across) their respective jurisdictions. These were the: Eastern Rock Lobster (*Sagmariasus verreauxi*), Ornate Rock Lobster (*Panulirus ornatus*), Southern Rock Lobster (*Jasus edwardsii*), Western Rock Lobster (*P. cygnus*), Crystal Crab (*Chaceon albus*), Giant Crab (*Pseudocarcinus gigas*) and Mud Crab (*S. serrata*). For both achelate lobsters and brachyuran crabs, the applicability of the direct ageing method was investigated across tropical to temperate regions, and deep-sea to relatively shallow waters, with sampling being focussed on areas with existing indirectly obtained growth and/or longevity information for comparison. The animals selected ranged from short- to long-lived taxa (e.g. Ornate Rock Lobster and Eastern Rock Lobster) and included both well-studied (i.e. Western Rock Lobster, Southern Rock Lobster and Mud Crab) and relatively data-poor species (i.e. Giant Crab and Crystal Crab), allowing for construction of a holistic applicability assessment across a wide habitat and size (and age) range.

Campana (2001) reported that the four key steps towards developing and sustaining a successful ageing program are: i) development of an ageing method, ii) age validation (i.e. periodicity determination), iii) preparation of a reference collection and iv) quality control monitoring. Further, Campana (2001) noted that the first two steps (i.e. i and ii above) are prerequisites for the second two (i.e. iii and iv above), with the latter being most important for high-volume applications with ongoing

age determinations being made. Because of this, combined with the relative immaturity of direct crustacean ageing, this project was focussed on: i) development of the crustacean ageing method and ii) age validation. This allowed for the initiation of: iii) a reference collection for Western and Eastern Rock Lobster ossicles, with the project conclusions informing recommendations for further research before iv) quality control monitoring can be adequately addressed. This project utilised a national collaborative network of government and academic fisheries scientists to facilitate benefit maximisation (and sharing) across all Australian jurisdictions, while ensuring that the knowledge generated can be consistently applied in future research and development projects.

1.2 Need

In broad terms, a validated ageing method would benefit most crustacean fisheries through improved stock assessment information, with the actual benefit being directly proportional to the economic and geographic scale. However, the need within each fishery corresponds to species-specific biological (e.g. relative longevity) and ecological patterns (e.g. ontogenetic migration), combined with managerial factors (e.g. history, approach and research funding) and the present state of age and growth knowledge in general. For example, for fisheries with a long management history (e.g. > 60 years for Western Rock Lobster) and a relatively rich biological and ecological understanding of the target species, direct ageing methods can provide a corroboration of existing indirect estimates, particularly at important fishery milestones (e.g. minimum legal size and size-at-sexual-maturity). In fisheries that restrict harvesting of long-lived species with a maximum legal size (e.g. approximately 30 years for Eastern Rock Lobster), at which bias from indirect age estimation is probably compounded by slow growth rates, a validated ageing method could help resolve growth estimates among the older individuals.

Indirect age estimates derived from tagging programs are expensive and require substantial time for data collection. In some Tasmanian regions, Southern Rock Lobster tagging programs have been running for > 30 years, but sufficient data for a reliable parameterisation of a growth matrix does not exist. Further, lobster tagging leads to some tag-induced mortality (and reduction in product quality), which carries some annual productivity loss. Despite substantial financial and time investments, and some negative impacts, in some jurisdictions (e.g. Southern Rock Lobster in Tasmania) tagging growth data can be inadequate for stock assessments, because of numerous technical reasons (e.g. uncertainty of moult timing). In relatively underdeveloped fisheries, some decapods harvested from temperate waters are particularly slow growing (e.g. Giant and Crystal Crab), with long inter-moult durations (e.g. 4–7 years for Giant Crab) compounding the challenges associated with measuring growth and resulting in an absence of reliable (or any) longevity estimates. In contrast, relatively reliable indirect age estimates exist for fast-growing tropical species (e.g. Ornate Rock Lobster and

Mud Crab), but fisheries scientists would benefit from the ability to use age (i.e. rather than size) to more-accurately assess fishery demographics. Irrespective of the specific fishery, crustacean researchers lack methodologies that would enable accurate measurement of growth alterations through time (e.g. in response to climate change). A validated method for ageing Australian crustaceans would overcome the challenges identified here, while fulfilling complex fishery-specific needs.

2.0 Objectives

1) Assess the relationship between estimated age and size, compared with existing growth models for Western and Eastern Rock Lobster.

2) Evaluate growth mark periodicity for Western and Eastern Rock Lobsters and Crystal Crab by vital staining and long-term grow-out.

3) Investigate the applicability of direct ageing methods to other commercially important crustaceans (Western, Eastern, Southern and Ornate Rock Lobsters and Giant, Crystal and Mud Crabs) – validated with laser ablation induction-coupled plasma mass spectroscopy and known-age individuals.

4) Establish a network of Australian government and academic fisheries researchers that can consistently apply direct ageing methods to decapod crustaceans.

3.0 Methodology

3.1 OBJECTIVE 1. Size-at-putative-age assessment – Western and Eastern Rock Lobster

3.1.1 Specimen procurement and measurement

Wild-caught Western and Eastern Rock Lobster were sourced from commercial fishers or caught by project staff during 2014. For both species, this included trapped and hand-collected individuals (of both sexes) spanning the full accessible size range. Eastern Rock Lobster were sourced from three locations spanning sub-tropical and temperate waters in New South Wales. This included two relatively lower-latitude locations (i.e. termed 'Northern' locations – Coffs Harbour and South West Rocks) and a single higher-latitude location (i.e. termed 'Southern' location – Jervis Bay). Western Rock Lobster were caught from a single location (Lancelin, Western Australia) that included both 'shallow' and 'deep' water (~2–4 and 36–66 m depth), allowing for an alternative view of lobster carapace length-at-putative-age data. Sex and carapace length (CL) was recorded for Western (nearest 0.1 mm) and Eastern Rock Lobster (nearest 1 mm).

3.1.2 Sample preparation

Gastric mills were extracted whole (Figure 1) and placed into 70% ethanol for transport, before being transferred into a storage medium of ethanol, glycerol and distilled water with a 60:30:10 ratio (Leland et al., 2015). Any remaining organic material was manually removed from the ossicles, before they were rinsed with distilled water and disarticulated. Previous studies have demonstrated that both ptero- and mesocardiac ossicles are suitable for direct ageing of lobster (Kilada et al., 2012; Leland et al., 2015), with Rudd (2013) demonstrating that for Western Rock Lobster both structures yielded sections of equally good clarity. In this study, after a preliminary comparison of sectioned ptero- and mesocardiac ossicles, the former was chosen for Western and Eastern Rock Lobster age estimation. All pterocardiac ossicles were rinsed with 100% ethanol and air dried, before being embedded in clear-casting polyester resin (Leland et al., 2015).

3.1.3 Sectioning, age estimation and protocols

For both species, transverse sections were cut (200 µm thickness) from pterocardiac ossicles (i.e. at the approximate midpoint) using a Buehler Isomet slow-speed saw (Figure 2). Ossicular sections were hand thinned (to ~70–100 µm thickness) using 1200 grit wet sandpaper (~2 m) to increase light transmission through the structure. All sections were wetted with distilled water for viewing on a

glass slide (i.e. without a cover slip or mounting medium), before being dried and stored (Leland et al., 2015). A DP12 digital camera fitted to an Olympus CX40 compound microscope (running analySIS Five software, Life Science) was used to generate a photographic record (at 100–400×) of all sectioned ossicles.

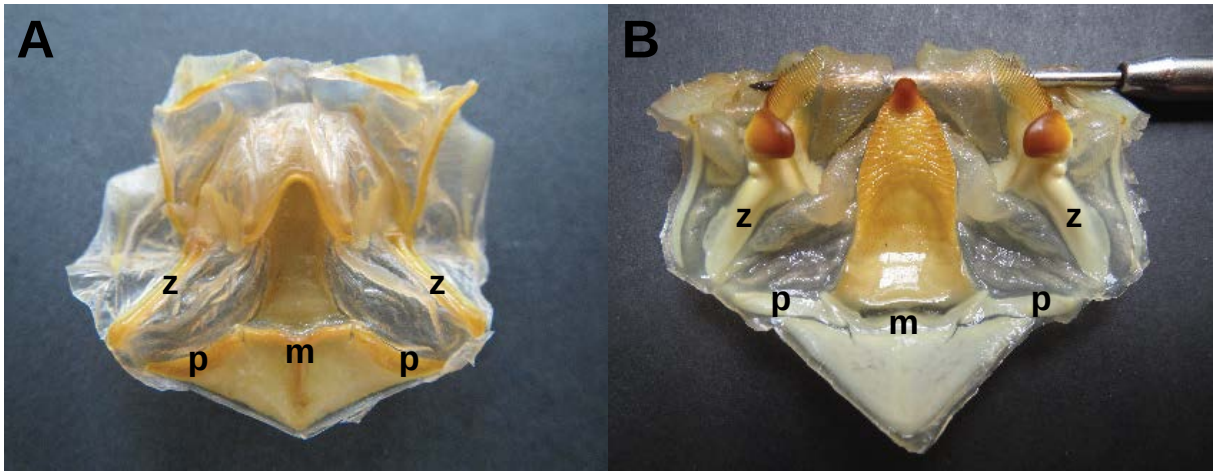

Figure 1. Extracted gastric mills with the foregut mostly removed to expose the mesocardiac (m), pterocardiac (p) and zygocardiac (z) ossicles. A: Western Rock Lobster mill (in dorsal view) showing that the pterocardiac ossicles are essentially an extension of the mesocardiac ossicle ridge. B: Eastern Rock Lobster mill (in ventral view) with fully articulated pterocardiac ossicles.

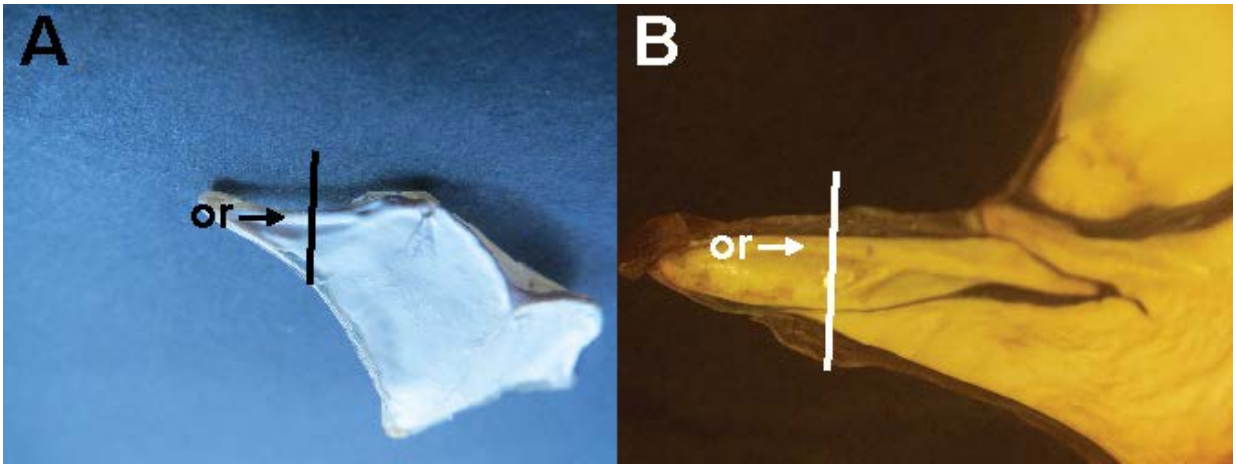

Figure 2. Pterocardiac ossicles showing the approximate sectioning position and plane (indicated by the black line) relative to the main ossicular ridge (or). A: Western Rock Lobster. B: Eastern Rock Lobster. The sections were cut perpendicular to the main ossicular ridge.

Crustacean ossicles contain a primary growth mark series that appear as alternating light and dark bands within the endocuticle (Leland et al., 2011; Kilada et al., 2012). In this study, the number of primary growth marks (i.e. the 'dark' bands) occurring within the endocuticle was counted for each

individual. The cuticular boundary was identified visually and was the zero point for all counts. Pending the outcome of validation studies, the first primary growth mark occurring within the endocuticle was assumed as representing the first winter (see Leland et al., 2015). Presumed winter deposition and annual formation thereafter (i.e. during minimum water temperature in September), was extended to all subsequent primary growth marks. Based on these assumptions, all counts were converted to days, before being adjusted to account for the individual collection date using a nominated mid-season settlement month as a 'birthdate' (e.g. Leland et al., 2015). An October birthdate was assigned to all Eastern Rock Lobster and to Western Rock Lobster with counts that were > 7. For Western Rock Lobster with counts that were < 7, a January birthdate was used to account for the recent (i.e. 2007) shift in the peak settlement timing in Lancelin, Western Australia. The adjusted counts were then divided by 365, yielding a fractional 'putative age' (i.e. total estimated days alive).

All counts were done using a high resolution computer screen displaying the digital images (at 100–400×) without knowledge of individual CL. The outermost growth mark was only counted if there was subsequent material deposited beyond it (i.e. it was fully formed). Where possible, counts were made along a single reading plane, sections that required multiple reading planes were noted (see below). Apparently double growth marks that merged together (i.e. combined to form one) were only counted once. In some ossicles, particularly those from larger and older individuals, optically dense zones that obstructed the count were present in otherwise easily read samples. Because the primary growth marks series generally comprises very consistent spacing, in these instances additional growth marks (i.e. to a maximum of two) were assumed following the same increment width as adjacent marks (termed 'optical density adjustment'), allowing for the conservation of valuable samples. Sections requiring optical density adjustments of > 2 were rejected (see below). For both Western and Eastern Rock Lobster, the CL for the lobster aged in this project were used to calculate the corresponding putative age for comparison to other published models, with a 1:1 ratio indicating a perfect relationship. For these comparisons, the data were constrained (using the published L_∞ value) to the CL range (and putative ages) common to both models.

Individual ossicle readability was categorised using the following criteria: i) sections read along a single plane, without any potential series ambiguity (i.e. between the primary and secondary) or need for optical density adjustment were 'good', ii) sections with very light structure (i.e. less well defined growth marks) and/or potential series ambiguity, that required multiple reading planes or optical density adjustment were 'intermediate' and iii) sections with obvious series ambiguity and/or broad optically dense zones that are likely to bias counts were 'poor'. The criteria violated for all intermediate and poor sections were recorded. All sections categorised as poor and those with obvious decalcification (total $n = 5$), morphological aberration (total $n = 3$) or processing artefacts such as large cracks (total $n = 6$) were excluded from the analyses.

3.1.4 Ossicular measurements

For both species, pterocardiac ossicles were measured for length (i.e. defined as the distance between the proximal and distal tips – Figure 3) using electronic Mitutoyo digital calipers (nearest 0.01 mm). Total ossicle width and exocuticle width were measured (nearest 0.1 µm) across the widest point of the calcified layers (i.e. excluding the membranous layer) using the arbitrary distance function in analysis FIVE software (Life Science). Endocuticle width was calculated as total ossicle width minus exocuticle width.

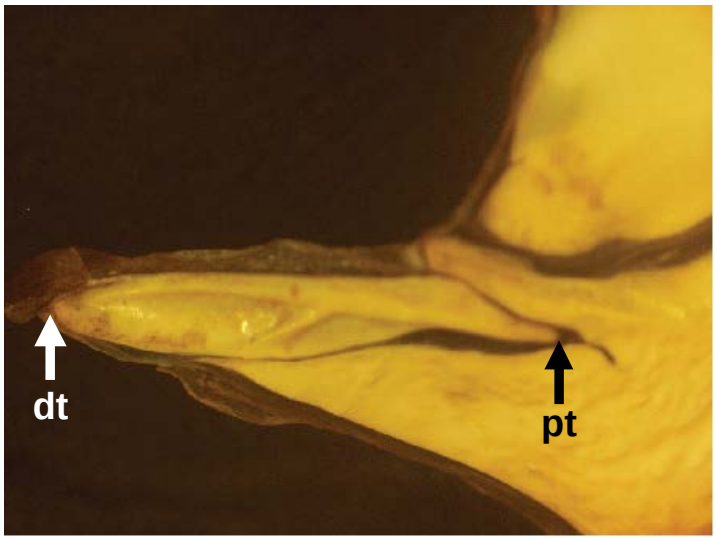

Figure 3. Pterocardiac ossicle length: the distance between the proximal (pt) and distal tip (dt).

3.1.5 Data analyses and growth modelling

The non-linear regression function in the Statistical Package for Social Sciences (SPSS – version 22, IBM Corporation) was used to fit the von Bertalanffy (1938) growth model to the Western and Eastern Rock Lobster carapace-length-at-putative-age data where: $CLt = L_\infty [1 - e^{-K(t - t_0)}]$, where L_∞ is the asymptotic CL at infinite age, K is the Brody growth constant affecting curvature and t_0 is the hypothetical age at zero CL. Starting values for the iterative process were estimated from an examination of a carapace-length-at-putative-age scatterplot. For each model, adjusting the nominated iteration starting values for CL_∞ (i.e. using the maximum observed CL) and K (i.e. 0) did not affect the final estimates. The value for t_0 was fixed at 0, to reflect the relatively small CL at settlement (e.g. 9 mm for Western Rock Lobster) compared with the maximum observed size. The regression coefficient (R^2) for each von Bertalanffy growth model was calculated in SPSS. For Western Rock Lobster, two separate growth models were estimated using the full data (i.e. comprising all ages – termed the 'complete model') and partial data sets (i.e. comprising individuals with a putative age estimate ≤ 6 years – termed the 'partial model'). Three separate parameter estimates were constructed for Eastern Rock Lobster, including one for the two northern locations (i.e. termed 'Northern' model),

one for the southern location (i.e. termed 'Southern' model) and a model with all locations combined (i.e. termed 'combined' model), for comparison with an existing model that was derived from tag-and-recapture data from across a similar latitudinal range (i.e. Evans Head–Narooma – see Montgomery et al., 2009).

Carapace length and putative age data were log-transformed in Microsoft Excel (v. 15.0). General Linear Models (GLM) were applied using SPSS to test for statistically significant differences, with the null hypotheses being rejected at $p < 0.05$. We tested the hypotheses of no differences in species-specific: CL, putative age, pterocardiac ossicle length and endocuticle width. Fixed factors included: 'depth' (i.e. shallow or deep – for Western Rock Lobster only), 'sex' and 'readability category' (i.e. good or intermediate) and the appropriate interactions. For the GLM testing the influence of depth on the relationship between Western Rock Lobster CL and putative age, the data were constrained to ages common to both shallow- and deep-water samples (1.9–6.9 years). The terminology used to describe correlation strength follows Dancey and Reidy (2004).

3.2 OBJECTIVE 2. Evaluation of growth mark periodicity – Western and Eastern Rock Lobster and Crystal Crab

3.2.1 Calcein staining and grow-out

Wild-caught Western and Eastern Rock Lobster and Crystal Crab were sourced from commercial fishers or caught by project staff for the calcein staining experiment. Individuals captured for this experiment were sourced from either single or multiple locations, using one or more collection methods, before being immediately transported to the grow-out facility, where they were placed into their respective aquaria (Table 1).

All three species were stained by immersion (for three days) in a static bath of pH adjusted (to 7.0) calcein and seawater (at 500 mg calcein l^{-1}) following Leland et al. (2015) (also see Kilada et al., 2012). Each species was stained in three consecutive batches (i.e. over nine days) comprising both sexes and a wide size range (Table 2). For Western and Eastern Rock Lobster, the staining medium was influenced by the ambient temperature (~20°C), but for Crystal Crab the water temperature was controlled (at ~6°C). All stained individuals were rinsed thoroughly, before being sexed, measured (CL) and tagged with a Hallprint cable tie or t-bar tag (Table 1). After this process, all individuals were placed into their respective grow-out aquaria and reared for up to 18 months (termed the 'grow-out period). Subsets of the calcein-stained Eastern Rock Lobster and Crystal Crab were sampled after 6, 12 or 18 months had elapsed (Table 3). Substantial numbers of the calcein-stained Western Rock Lobster were lost due to equipment failure (from a single tank) and cannibalism. Because of this, all

remaining Western Rock Lobster – i.e., those that did not experience any adverse conditions – were sampled after 18-months (Table 3). For all species, the initial and final CL was recorded (i.e. at the start and end of the grow-out), with the CL measurement presented being the final value. Western Rock Lobster and Crystal Crab were re-measured for CL after each moult. For Eastern Rock Lobster, the number of times an individual moulted was collected by periodic checking for external tags. A Gulland-Holt plot was constructed to estimate L_∞ and K for the calcein-stained Eastern Rock Lobster (i.e. during captive rearing) and compared with those parameters estimated from other methods applied to wild-caught individuals.

3.2.2 Growth mark identification

All calcein-stained ossicles were extracted, stored in light-proof containers and prepared as described above (Objective 1 methodology – see section 3.1.2). For all species, images of the stained-ossicle sections were captured using a compound microscope (with transmitted light at 100–400×) fitted with a digital camera. These images were displayed on a large computer screen and the outermost growth marks were identified and labelled 'blind' (i.e. without knowledge of individual size or the calcein position), for subsequent comparison with the position of the calcein stain. All calcein-stained ossicles were assigned to a readability category using the same method as that for age estimation (see section 3.1.3), except that the criteria were only applied to the outermost marks needed for confident determination of the primary series. The minimum number of consecutive primary growth marks needed to confidently determine the primary series spacing was nominated as four for both lobster species (i.e. except for one Western Rock Lobster with only three marks) and five for Crystal Crab. This approach precluded any discarding of valuable samples that had interpretable growth marks within the region of interest. One calcein-stained Eastern Rock Lobster ossicle was decalcified, but was retained for the analysis, for completeness and because the primary growth marks were easily identifiable.

3.2.3 Calcein detection and interpretation

Calcein-stained ossicle sections were also viewed on a Nikon A1R confocal microscope (100–400×) to detect and produce digital images of the calcein in three separate channels (termed 'blue', 'green' and 'red channel'). For each sample, the Auto Gain function (NIS Elements AR410.00) was used to determine the optimal levels for viewing the calcein using the green channel, before the blue and red channel settings were adjusted to equal that of the green. This procedure subtracts any natural ossicular autofluorescence occurring in the blue and red channels, producing an image comprising the green channel only (i.e. termed the 'final' calcein image) for interpretation and presentation (see Kilada et al., 2012 for a similar procedure). The final calcein image was overlain on a greyscale transmitted light image to facilitate edge identification. For some sections, other NIS functions (i.e.

Detect Peak and Edge Effects) were used to further enhance identification of the artificial mark or the ossicle edge.

The final calcein image was displayed on a large computer screen and material deposited beyond the stain (termed the 'captive growth increment' – CGI) was measured using Mitutoyo IP66 digital calipers (nearest 0.01 mm), before being adjusted to account for magnification. Because ossicular morphology naturally exhibits a tapering effect (particularly in larger individuals), with increment width narrowing on either side of a maximum, the CGI width was measured at the widest point that was representative of the entire sample. The position of the calcein stain was transferred (i.e. using the CGI width) onto the pre-made light microscope image (i.e. with the outermost growth marks pre-labelled) and the number of growth marks identified beyond the artificial mark was recorded. The width of the previous complete cycle (i.e. adjacent to the calcein stain and comprising one light and one dark zone) was measured (nearest 0.01 mm) and used to calculate the relative CGI increase (as a percentage of the previous increment width). Each ossicle was categorised (i.e. visually) based on the approximate amount of calcein penetration throughout the structure where: 0–33% is 'minor', 34–66% is 'moderate' and 67–100% is 'complete'. The mean CGI for ossicles sampled after the 6, 12 or 18 month grow-out were tested for significant differences using ANOVA.

3.2.4 Captive-growth modelling

For the calcein-stained Eastern Rock Lobster, the mean CL (i.e. calculated from the initial and final CL) was plotted against the change in CL to estimate growth parameters for the captive-reared individuals. The von Bertalanffy growth parameters L_∞ and K were estimated from the regression line-of-best fit equation: $y = mx + c$, where m is $-K$ and the x intercept is L_∞.

Table 1. Summary of the source locations, collection methods, grow-out facility, aquaria type and housing status of the Western and Eastern Rock Lobster and Crystal Crab sourced for the calcein-staining experiment.

Taxa	Location(s)	Collection method(s)	Grow-out facility	Aquaria type	Housing status	Tag type
Western Rock Lobster	Lancelin	Trapped and hand collected	Western Australian Marine Research Laboratories, Hillarys, Perth Western Australia 6025	Flow-through	Caged[a]	Cable tie
Eastern Rock Lobster	Coffs Harbour, Southwest Rocks, Forster-Tuncurry	Trapped and hand collected	National Marine Science Centre, Coffs Harbour New South Wales 2450	Flow-through	Free range[b]	Cable tie and t-bar
Crystal Crab	Carnarvon[c]	Trapped	Chaceon Pty Ltd, Osbourne Park, Perth Western Australia 6017	Recirculating	Caged[a]	Cable tie

[a]Western Rock Lobster and Crystal Crab were initially free ranging, but were transferred into individual cages to exclude cannibalism during the remaining grow-out.

[b]Eastern Rock Lobster were distributed among three replicate 3 000 litre aquaria, with each containing approximately equal sex ratios and size ranges.

[c]Crystal Crab were sourced from waters northwest of Carnarvon (approximately 22° 35.1 S, 113° 28.0 E).

Table 2. Summary of the total number (*n*) stained for the 18-month grow-out, with the start date, carapace length (CL) range, sex ratio and mortality given for each species. 'Procedural mortality' is that which occurred immediately after staining and tagging (i.e. within 24 h) and is exclusive of any other mortality. M = male, F = female.

Taxa	*n*	Start date	CL range (mm)	Sex ratio (M:F)	Procedural mortality (%)
Western Rock Lobster	45	Sept. 13, 2014	53–101	1:5	0
Eastern Rock Lobster	60	Oct. 10, 2014	105–237	1:1	< 1[a]
Crystal Crab	48	Sept. 13, 2014	79–154	1:7	0

[a]This individual was immediately replaced with another calcein-stained lobster.

Table 3. The total number (*n*) of calcein-stained individuals sampled after each time interval.

Taxa	6 month (*n*)	12 month (*n*)	18 month (*n*)	Total (*n*)
Western Rock Lobster	-	-	16	16
Eastern Rock Lobster	9	12	9	30
Crystal Crab	10	9	10	29

3.3 OBJECTIVE 3. Applicability to other crustacean species – with LA-ICPMS and known-age individual validation

3.3.1 Applicability assessment

Wild-caught lobster and crab were sourced from around Australia to assess the applicability of direct ageing methods across tropical, subtropical and temperate regions (Table 4). This included: Ornate Rock Lobster, Southern Rock Lobster, Mud Crab, Crystal Crab and Giant Crab. Eastern Rock Lobster and Western Rock Lobster were excluded from this sub-section, because the applicability of direct ageing methods to these species was comprehensively described under Objective 1. For most species assessed here (all except Mud Crab), there were insufficient numbers to determine whether the relationships between CL and putative age (see below) were statistically significant. Nevertheless, in each instance, the R^2 value was used to indicate the correlation strength.

All ossicles were prepared and sectioned as described under Objective 1 (Methodology – see section 3.1.2). For Ornate and Southern Rock Lobster, pterocardiac ossicles were selected for age estimation (Leland et al., 2015). For Mud and Crystal Crab, the zygocardiac ossicle was selected for age estimation (Kilada et al., 2015), with zygocardiac ossicles being sectioned transversely at ~3–6 mm from the proximal tip (see Sarapuk, 2014) (Figure 4). For Giant Crab, both pterocardiac and zygocardiac ossicles were examined for endocuticular growth marks.

For Ornate and Southern Rock Lobster, the adjusted growth mark count was converted to a fractional putative age using a nominated mid-season settlement birthdate (July 15 and July 1 respectively) and the sample collection date – under the assumptions described for Objective 1 (see section 3.1.3). For these species, the CL-at-putative-age data was used to generate preliminary von Bertalanffy growth parameters (see section 3.1.5) for comparison with indirectly determined growth estimates. Only one Southern Rock Lobster (from Tasmania) ossicle was categorised as poor (see section 3.1.3) and excluded from the preliminary model. The identification of pre-moult Ornate Rock Lobster was determined by the presence of new-forming ossicular tooth plates.

Like for the other species, Mud Crab counts were adjusted (i.e. using a December 15 birthdate), before being fitted with linear and von Bertalanffy models to describe the relationship between CL and putative age. Because Crystal Crab spawning occurs throughout the year (Smith et al., 2004), and detailed settlement timing information is lacking, growth mark counts for this species were not adjusted and putative ages are presented as whole numbers only.

3.3.2 Elemental composition analysis

For all project species, the elemental composition of sectioned ossicles (Table 4) was analysed using a LA-ICPMS unit comprising an NW 213 nm laser coupled with an Agilent (7700) ICPMS. To maximise resolution and ensure adequate data acquisition, a slow moving (5 µm s^{-1}) 10 µm laser spot with a constant energy density of 40 J $(cm^2)^{-1}$ and a 10 Hz pulse rate was used. The resultant plasma was transported (under helium flow) to the ICPMS inlet, where it was mixed with argon carrier gas, before entering the argon plasma. Three evenly-spaced linear transects (i.e. 5–50 µm apart) were ablated across each ossicular section (i.e. from the membranous layer to the opposing edge), with the central one traversing the cuticular boundary midline and a replicate being on either side (i.e. 'Region A' – Figure 6). Only Southern Rock Lobster sourced from South Australia (Table 4) were analysed across another ossicular region (i.e. 'Region B' – Figure 5), because this was where counts were made.

Figure 4. Zygocardiac ossicle from a Crystal Crab showing the sectioning positioning and plane (indicated by the black line) for this ossicle type. Pt = proximal tip.

A typical scan series was run as follows. Signals for each of the measured elemental isotopes (Calcium (^{43}Ca and ^{44}Ca), barium (^{137}Ba), boron (^{11}B), copper (^{63}Cu), magnesium (^{24}Mg), manganese (^{55}Mn), phosphorous (^{31}P) and strontium (^{88}Sr)) were acquired at approximately 1 point s^{-1}. Initial and final baseline levels were recorded for each mass. The baseline region was followed by measurements on NIST 611 and 613 glass standards, after which the triplicate transects were measured. These were followed by final scans of the NIST standards and acquisition of the final baseline levels. The baseline levels (including any baseline drift) were subtracted from each mass channel. Instrumental drift was accounted for by calculating the difference between the first and last standard (i.e. using NIST 611) and appropriately correcting signal levels. Concentration data were calculated by comparison with the NIST 611 standard and accounting for isotope mass and natural abundance.

For each sample, the isotopic data from all three transects was visually assessed for homogeneity (i.e. using line graphs). Data acquired from the central transect was always used in the analysis (and presentation), unless there was an apparent aberration within the set. In some instances, an adjacent track was selected because of a high-amplitude peak that was not present in either replicate and probably explained by some procedural artefact. To normalise ion intensities between analytes, all isotopic data are expressed as molar ratios to ^{44}Ca. This provides a better index of elemental composition, by compensating for potential topographical effects and ablation efficiency variations (Reish and Mason, 2003). One ossicle from a pre-moult individual (Figure 6), displayed visual differences in endocuticular calcification that might correspond with reduced calcium concentration.

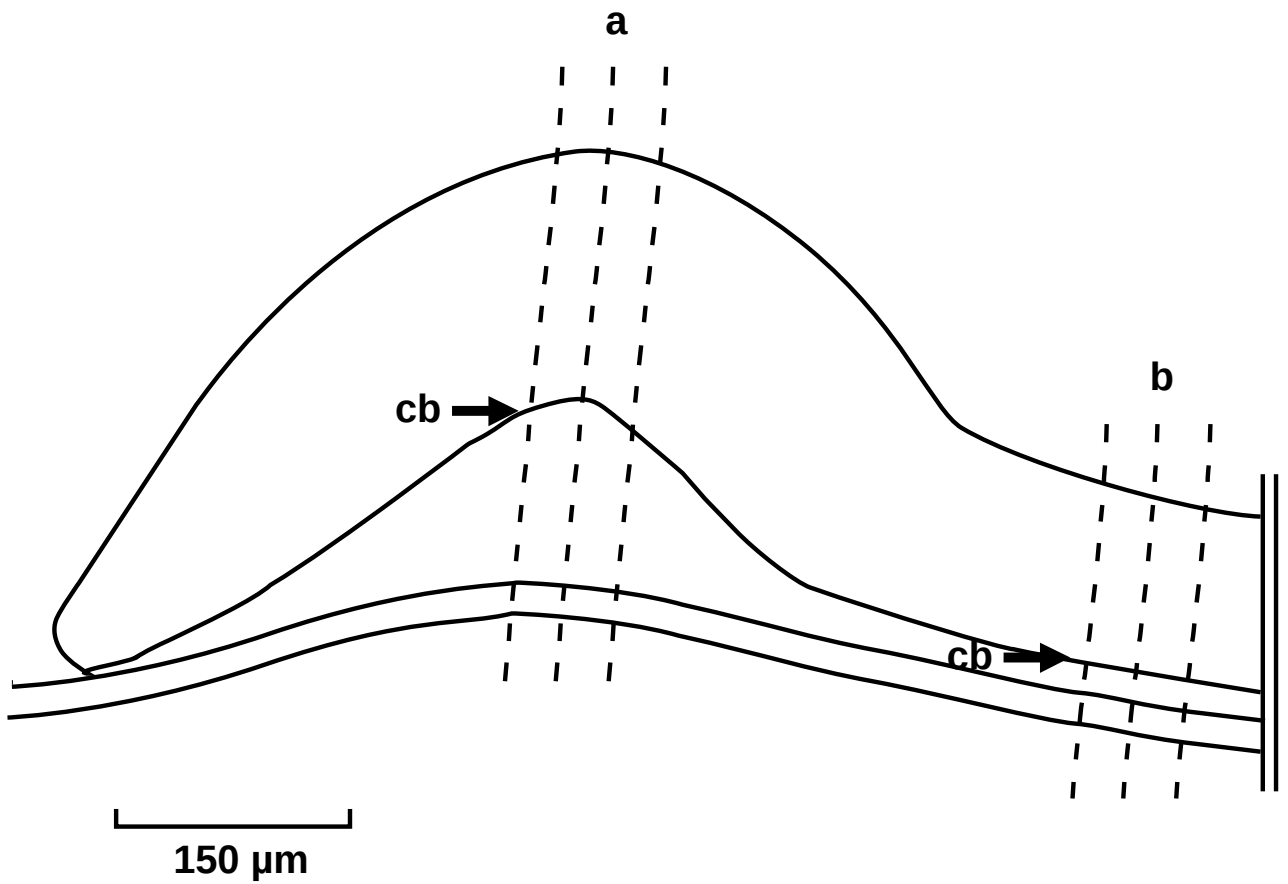

Figure 5. Illustration of the standardised transect position and spacing for the replicate LA-ICPMS transects (indicated by the dotted line) traversing the entire ossicle, including the cuticular boundary (cb). For most ossicles, Region A (a) was the primary area of interest, with a small subset being measured across Region B (b).

Table 4. Summary data for all samples analysed with LA-ICMPS, with the sample identifier (ID), collection date, source location, sex (M = male, F = female), carapace length (CL), ossicle type and transect spacing given. ORL = Ornate Rock Lobster, MC = Mud Crab, SRL = Southern Rock Lobster, CC = Crystal Crab, WRL = Western Rock Lobster, ERL = Eastern Rock Lobster, TAS = Tasmania, SA = South Australia.

Sample ID	Collection date (DD-MM-YY)	Source location	Sex (M, F)	CL (mm)	Ossicle type	Transect spacing (µm)
ORL-1	06-11-14	Torres Strait (Southeast Zone), Queensland	M	85	Pterocardiac	5
ORL-2	06-11-14	Torres Strait (Southeast Zone), Queensland	M	103	Pterocardiac	50
ORL-3	06-11-14	Torres Strait (Southeast Zone), Queensland	M	114	Pterocardiac	50
MC-4	01-10-14	Darwin (Darwin Harbour), Northern Territory	M	97	Zygocardiac	5
MC-5	01-10-14	Darwin (Darwin Harbour), Northern Territory	M	109	Zygocardiac	50
MC-6	20-03-15	Darwin (Shoal Bay), Northern Territory	M	125	Zygocardiac	50
SRL-TAS-7	01-02-14	Taroona Waters Research Area, Tasmania	F	59	Pterocardiac	20
SRL-TAS-8	01-02-14	Taroona Waters Research Area, Tasmania	M	88	Pterocardiac	50
SRL-SA-11	26-02-15	Robe, South Australia	M	122	Pterocardiac	50
SRL-SA-12	26-02-15	Robe, South Australia	M	171	Pterocardiac	50
CC-16	06-12-14	Carnarvon, Western Australia	F	73	Zygocardiac	50
CC-17	06-12-14	Carnarvon, Western Australia	M	115	Zygocardiac	50
CC-18	06-12-14	Carnarvon, Western Australia	M	146	Zygocardiac	50
WRL-19	30-12-14	Lancelin, Western Australia	F	27	Pterocardiac	20
WRL-20	24-10-14	Lancelin, Western Australia	M	77	Pterocardiac	50
WRL-21	01-10-14	Lancelin, Western Australia	M	124	Pterocardiac	50
ERL-22	03-11-2014	Coffs Harbour, New South Wales	M	65	Pterocardiac	20
ERL-23	28-10-14	Coffs Harbour, New South Wales	M	114	Pterocardiac	50
ERL-24	04-12-14	Coffs Harbour, New South Wales	M	205	Pterocardiac	50

For this sample, ^{44}Ca detector count data (i.e. for ^{44}Ca alone) were acquired to assess calcification differences between apparently dissimilar ‘inner’ and ‘outer’ endocuticular zones (i.e. change in total count vs. position), with three replicate samples being taken in each (Figure 6). Mean ^{44}Ca detector count data for inner and outer zones were tested for statistically significant differences using a paired *t*-test.

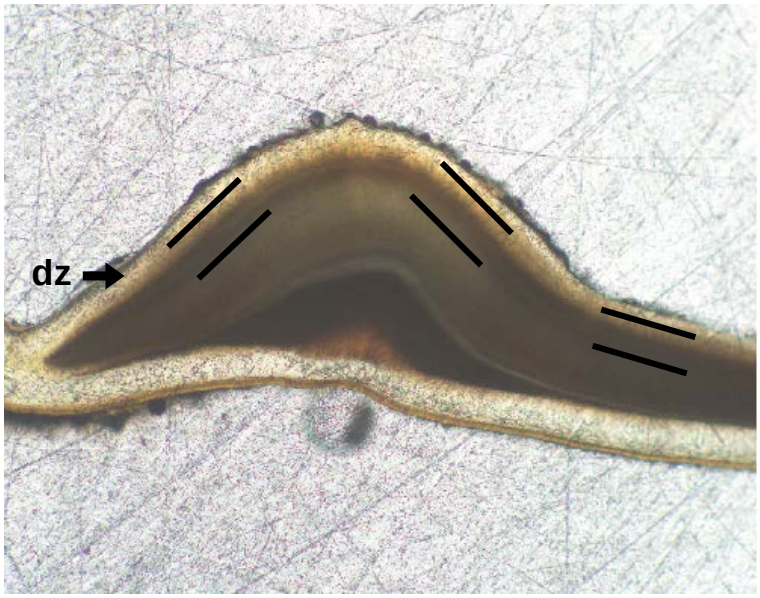

Figure 6. Pterocardiac ossicle showing a broad decalcified zone (dz) extending around the entire ossicle. The approximate positioning of the replicate LA-ICPMS transects (indicated by the black lines) used to assess differences in inner and outer endocuticular calcification.

For all samples, the position of the cuticular boundary and each primary growth mark was measured (i.e. relative to the edge) on printed digital images using Mitutoyo digital calipers (nearest 0.01 mm). This information was then transposed onto the corresponding ion intensity graphs for presentation (prepared in Microsoft Excel). Spatial patterns in the intensity ratios (vs. distance) were evaluated against the primary growth mark position to determine if the visual signal corresponded to cyclical compositional changes. For each sample, all elemental ratios were assigned a 'coincidence percentage', defined as the proportion of primary growth marks that corresponded with a local (or overall) maxima. Local maxima correspondence was considered positive if a 90° vertical transect (i.e. from the growth mark) intercepted the peak at approximately 50% of the total height. For samples CC-17 and CC-18, accurate transposition of the entire primary series was not possible, because some marks that were read in an alternate plane could not be confidently identified along the LA-ICPMS transect. In these two instances, only the growth marks that were identifiable along the LA-ICPMS transect were presented for evaluation against changes in elemental composition.

3.3.3 Known-age individuals

Young-of-the-year Ornate Rock Lobster and Western Rock Lobster (i.e. immediate post puerulus) were captured for comparison of the primary growth mark count (i.e. putative age) and 'known age' (i.e. actual age since collection). For both species, the known age was calculated using the collection and sacrifice date, before being converted to a fractional age (see Objective 1 – section 3.1.3). All known-age pterocardiac sections were assigned to a readability category, with partially decalcified

ossicles being retained to conserve valuable samples. Before sacrifice, the final CL and sex were recorded for all known-age individuals.

Ornate Rock Lobster

Forty known-age Ornate Rock Lobster puerulus (assigned birthdate of September 23, 2014) were caught and transported to the Marine Fisheries and Aquaculture Development Centre in Lombok (Indonesia), before being reared in outdoor sea cages with natural photoperiod and temperature. A randomly selected subset of the known-age Ornate Rock Lobster (i.e. spanning the full CL range) ossicles were read after 1.1 (n = 3) and 1.4 years (n = 10). All known-age Ornate Rock Lobster ossicles were stored, prepared, sectioned and photographed using the methodology described under Objective 1 (see section 3.1.2). Two Ornate Rock Lobster ossicles were categorised as poor and excluded from the analysis.

Western Rock Lobster

Known-age Western Rock Lobster puerulus were also collected (i.e. during 2013–2015 years), before being reared in small indoor aquaria (i.e. with modified ambient photoperiod and temperature) at the Western Australian Marine Research Laboratories in Perth. In total, five Western Rock Lobster of differing size (and age) were sacrificed for interpretation. All ossicles from known-age individuals were read by a primary (J. Leland) and secondary reader (L. Rudd) – both without knowledge of CL or age. All Western Rock Lobster known-age ossicles were stored, prepared, sectioned and photographed using the methodology described by Rudd (2013). For this species, all sections were categorised as poor, but were assigned putative ages to conserve valuable samples.

3.4 OBJECTIVE 4. Direct ageing network – workshop

A workshop on direct age determination methodologies for crustaceans was convened at Southern Cross University on March 16 and 17, 2016. The workshop included presentations by J. Leland summarising the project: i) background, ii) preparation methods, iii) validation methods and iv) preliminary results. Co-investigator D. Bucher presented on the physical structure of crustacean cuticle with a focus on the nature of ossicular growth marks. Laboratory sessions included demonstrations of ossicular preparation, sectioning and interpretation. Because there were no specific data associated with this objective, there is no corresponding results section. However, a summary of the workshop and the research priorities identified are presented in the Objective 4 Discussion.

4.0 Results

4.1 OBJECTIVE 1. Size-at-putative-age assessment – Western and Eastern Rock Lobster

4.1.1 Age estimation

Ossicles from 95 Western Rock Lobster (26.7–129.1 mm CL) and 97 Eastern Rock Lobster (58–254 mm CL) were sampled for putative age estimation. Pterocardiac ossicle length was significantly correlated with CL for both Western Rock Lobster ($y = 0.0887x + 0.96$; $p < 0.001$; $n = 72$; $R^2 = 0.85$) and Eastern Rock Lobster ($y = 0.0596x + 0.36$; $p < 0.001$; $n = 108$; $R^2 = 0.97$) (Figure 7A and D). The pterocardiac ossicle width was strongly correlated with that of the endocuticle for both Western Rock Lobster ($y = 0.6758x + 13.754$; $n = 5$; $R^2 = 1.00$) and Eastern Rock Lobster ($y = 0.6897x – 29.499$; $n = 5$; $R^2 = 0.99$) (Figure 7B and E). Endocuticle width was also strongly correlated with CL for Western Rock Lobster ($y = 2.4068x + 58.102$; $n = 5$; $R^2 = 0.93$) and Eastern Rock Lobster ($y = 1.3423x + 34.331$; $n = 5$; $R^2 = 0.83$) (Figure 7C and F). The relationship between pterocardiac ossicle length and Western Rock Lobster CL was not significantly influenced by depth ($p = 0.95$; $n = 62$; $R^2 = 0.88$) or sex ($p = 0.16$; $n = 69$; $R^2 = 0.88$). Similarly, for Eastern Rock Lobster the relationship between pterocardiac ossicle length and CL was not significantly influenced by sex ($p = 0.53$; $n = 109$; $R^2 = 0.96$).

For both species, the cuticular boundary was easily identified in all ossicles examined using transmitted light microscopy. The primary growth marks appeared as paired light and dark zones within the endocuticle of Western Rock Lobster (Figure 8) and Eastern Rock Lobster ossicles (Figure 9). For both species, the distance to the first primary growth mark was consistently the widest increment irrespective of CL or putative age. Successive primary marks were progressively narrower, becoming more evenly spaced towards the growing edge in most ossicles (Figure 8 and 9).

Some Western Rock Lobster ossicles (18.6% of the entire sample), were visually distinct to the other samples. These ossicles had a wide (and grey coloured) zone before the first and/or second primary growth mark (Figure 10A) that sometimes contained unusually pronounced secondary growth marks (Figure 10B and C). These ossicles comprised both good ($n = 5$) and intermediate ($n = 11$) categorisations and were restricted to a relatively narrow putative age range (3.8–5.8 years). For both species, there was substantial variation in the individual ossicle appearance, but readability was mostly categorised as good or intermediate, with some being categorised as poor (Table 5). The total percentages categorised as good and intermediate were similar for both lobster species.

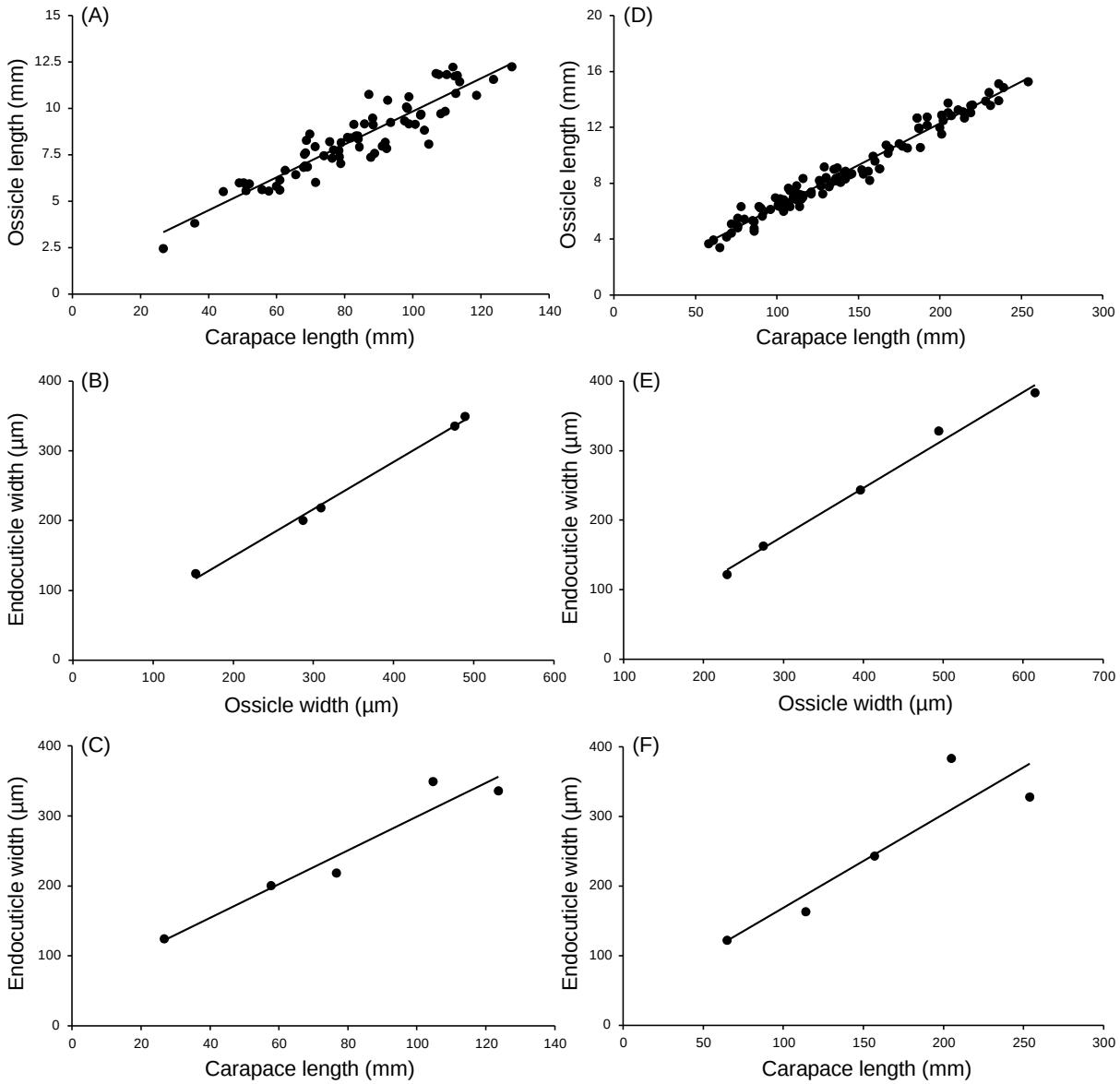

Figure 7. Relationships between pterocardiac ossicle morphometrics and carapace length for Western Rock Lobster (A, B and C) and Eastern Rock Lobster (D, E and F).

Compared with Western Rock Lobster, a greater proportion of Eastern Rock Lobster ossicles were rejected (9.5 and 19.6%, respectively – Table 5). The categorisation of Western Rock Lobster ossicles as good or intermediate was not significantly influenced by CL or putative age ($p = 0.23$; $n = 86$; $R^2 = 0.52$). Similarly, Eastern Rock Lobster CL and putative age did not significantly influence the readability categorisation for good and intermediate sections ($p = 0.94$; $n = 78$; $R^2 = 0.80$).

Most of the Western Rock Lobster and Eastern Rock Lobster ossicles categorised as intermediate (66 and 70%, respectively) only violated a single readability criterion, with the most common being that for potential series ambiguity (Table 6). For Western Rock Lobster, the most commonly violated criteria combination was very light structure with potential series ambiguity, while that for Eastern Rock Lobster was multiple reading planes requiring optical density adjustment (Table 7).

4.1.2 Growth modelling

For Western Rock Lobster sampled from shallow water, CL was not significantly correlated with putative age ($y = 0.4225x + 53.847$; $p = 0.76$; $n = 27$; $R^2 = 0.00$), but for those sampled from deep water CL was significantly correlated with putative age ($y = 3.1029x + 62.38$; $p < 0.001$; $n = 34$; $R^2 = 0.41$) (Figure 11). The Western Rock Lobster CL-at-putative-age relationship was significantly influenced by depth ($p = 0.03$; $n = 61$; $R^2 = 0.45$), with individuals in shallow water being generally smaller for a given age (Figure 11). Sex did not significantly influence the relationship between CL and putative age for Western Rock Lobster ($p = 0.80$; $n = 32$; $R^2 = 0.45$) or Eastern Rock Lobster ($p = 0.39$; $n = 78$; $R^2 = 0.78$). For both species, the von Bertalanffy equation provided an adequate description of the directly determined CL-at-putative-age data, allowing for the estimation of growth parameters for Western Rock Lobster (Table 8). Three separate parameter estimates were constructed for Eastern Rock Lobster, including one for the two Northern locations, one for the Southern location and a combined model (Table 9). For both Western (Figure 12 and 13) and Eastern Rock Lobster (Figure 14), the directly determined growth parameters yielded estimates that were broadly similar to that from other published models derived from indirect ageing methods.

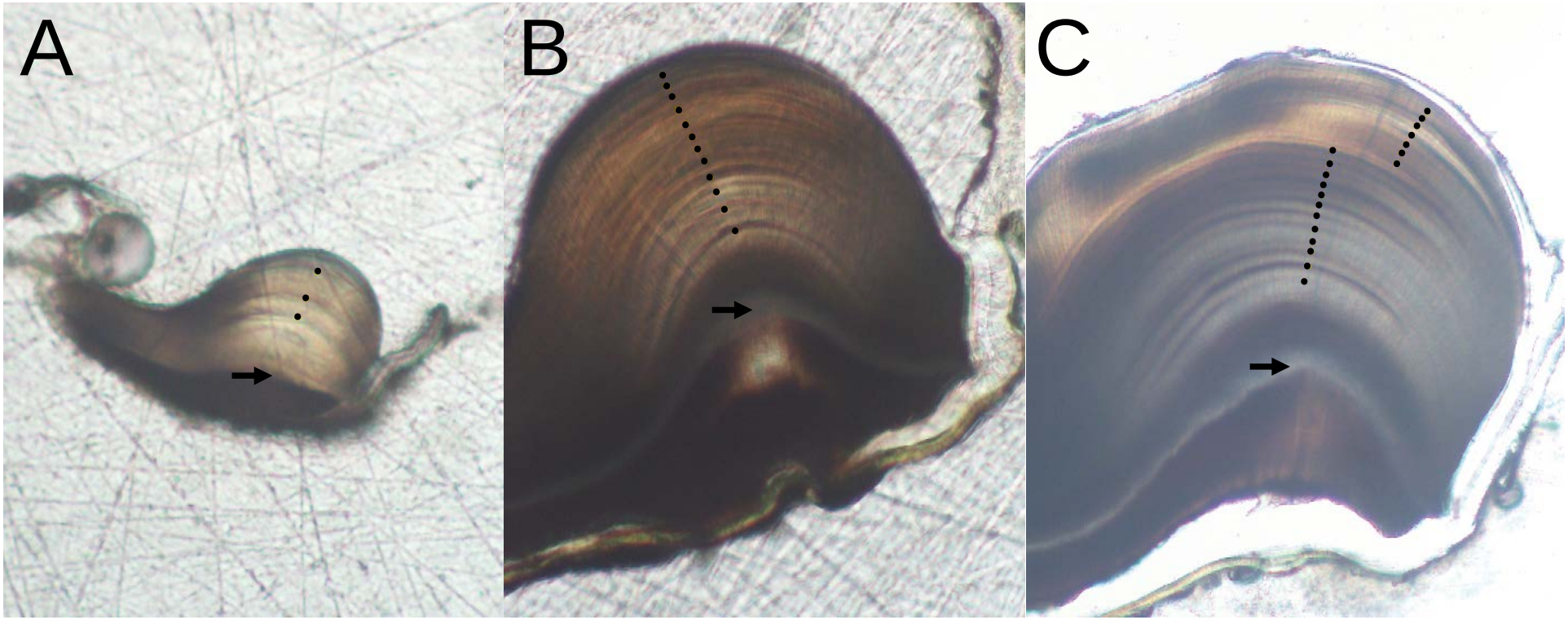

Figure 8. Transverse cross sections of pterocardiac ossicles showing the primary growth marks (black dots) used to assign putative ages to Western Rock Lobster. The three sections presented here were categorised as good. The black arrow indicates the cuticular boundary. Note that the distance to the first primary growth mark is widest, with successive marks being generally narrower and regularly spaced. A: 27 mm CL female with 3 marks (putative age = 2.9 years). B: 84 mm CL male with 12 marks (putative age = 12.0 years). C: 124 mm CL male with 19 marks (putative age = 19.0 years).

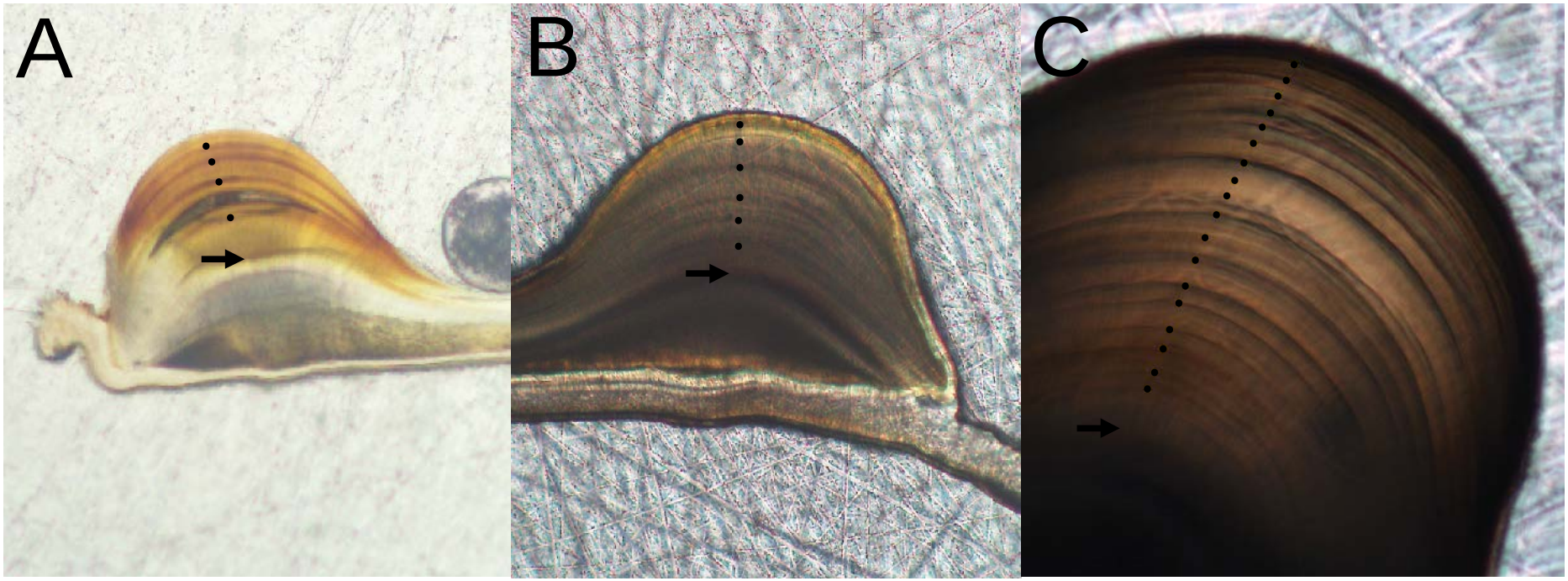

Figure 9. Transverse cross sections of pterocardiac ossicles showing the primary growth marks (black dots) used to assign putative ages to Eastern Rock Lobster. The three sections presented here were categorised as good. The black arrow indicates the cuticular boundary. Note that the distance to the first primary growth mark is widest, with successive marks being generally narrower and regularly spaced. A: 65 mm CL male with 4 marks (putative age = 4.1 years). B: 104 mm CL male with 6 marks (putative age = 6.1 years). C: 177 mm CL male with 18 marks (putative age = 18.1 years).

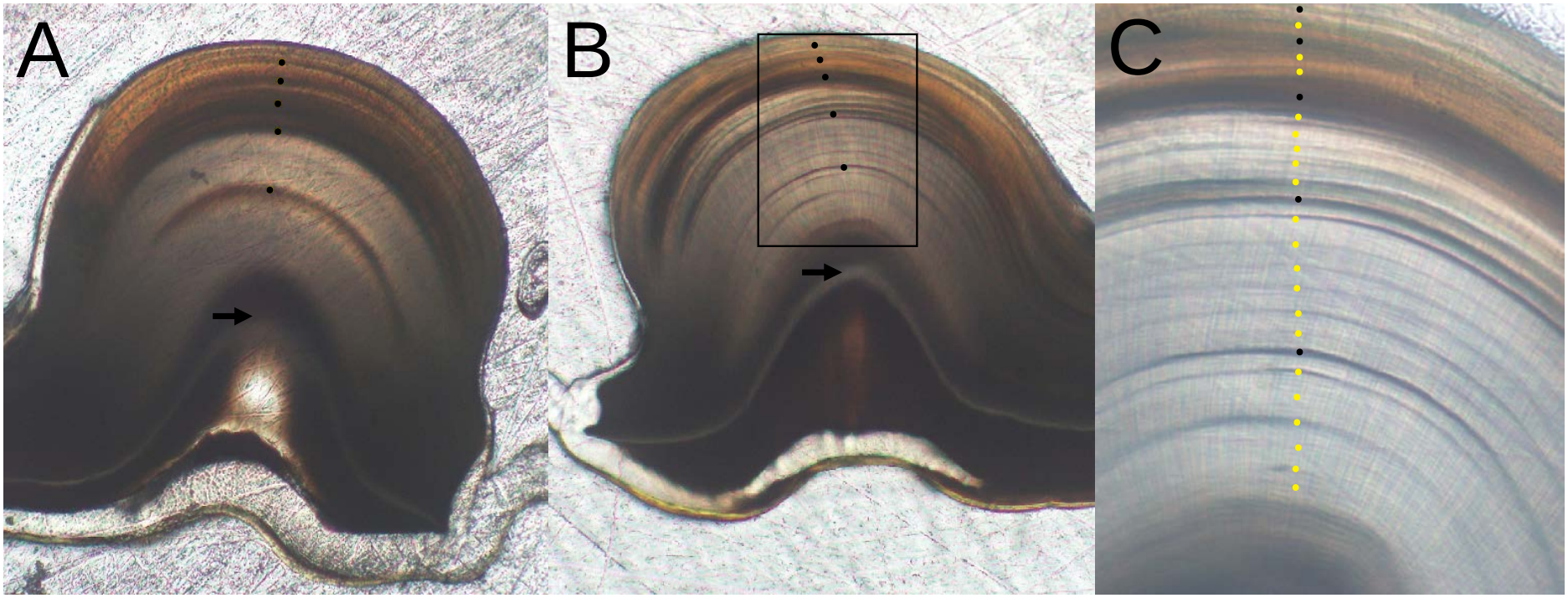

Figure 10. Visually distinct Western Rock Lobster ossicles showing the broad grey zones before the first two primary growth marks (black dots), with the cuticular boundary demarcated by the black arrow. A: 109.5 mm CL female without a clear secondary series (putative age = 4.8). B: 84.3 mm CL female (putative age = 4.8) with an unusually prominent secondary series. C: Inset of B with both the primary (black dots) and secondary (yellow dots) series labelled.

Table 5. Number (*n*), carapace length (CL), sex ratio (M:F), putative age and total (%) for all Western and Eastern Rock Lobster readability categorisations. The total percentage expresses the relative contributions of each category to the total sample. The numbers in parentheses indicate the number of individuals with unknown (u) sex.

Taxa	**Category**	***n***	**CL (mm)**	**M:F (u)**	**Putative age (years)**	**Total (%)**
Western Rock Lobster	Good	39	26.7–129.1	19:18 (2)	1.8–19.0	41.1
	Intermediate	47	40.4–113.1	20:26 (1)	1.8–18.0	49.4
	Poor	9	49.5–110.0	4:5	-	9.5
Eastern Rock Lobster	Good	38	58–219	20:18	2.1–18.1	39.2
	Intermediate	40	69–236	20:20	3.1–26.1	41.2
	Poor	19	89–254	10:9	-	19.6

Table 6. Number of Western and Eastern Rock Lobster ossicles categorised as intermediate and the criterion violated. VLS = very light structure, PSA = potential series ambiguity, MRP = multiple reading planes, ODA = optical density adjustment.

	Criterion violated			
Taxa	**VLS**	**PSA**	**MRP**	**ODA**
Western Rock Lobster	5	22	2	2
Eastern Rock Lobster	11	11	5	1

Table 7. Number of Western and Eastern Rock Lobster ossicles categorised as intermediate and the two criteria violated. VLS = very light structure, PSA = potential series ambiguity, MRP = multiple reading planes, ODA = optical density adjustment.

	Violated criteria combination					
Taxa	**VLS×PSA**	**MRP×ODA**	**MRP×VLS**	**ODA×PSA**	**VLS×ODA**	**PSA×MRP**
Western Rock Lobster	9	2	1	2	2	0
Eastern Rock Lobster	3	4	2	1	1	1

Table 8. The von Bertalanffy growth parameters calculated for Western Rock Lobster sourced from Lancelin, Western Australia. For this project, the complete model included all individuals (with combined sexes and depths), but the partial model was estimated using only lobster with a putative age ≤ 6 years. The parameters of Cheng and Kuk (2002), Phillips et al. (1992) and Chittleborough (1976) are included for comparison. Where available the 95% confidence intervals are given in parentheses. '-' indicates data not provided.

Source	**Method**	L_∞	K	t_0	R^2
This project (complete model)	Direct ageing	104.17 (94.74–113.61)	0.26 (0.20–0.32)	0[a]	0.49
Cheng and Kuk, 2002[b]	Tag-and-recapture	111.92 (106.94–116.90)[c]	0.37 (0.28–0.46)[c]	0.21	-
Phillips et al., 1992[d]	Tag-and-recapture	102.92	0.22	0	-
This project (partial model)	Direct ageing	90.20 (64.42–115.97)	0.36 (0.13–0.60)	0	0.20
Chittleborough, 1976[e]	Laboratory rearing	113.47	0.46	1.05	-

[a]The t_0 value for this model was fixed at zero (see Methodology – section 3.1.5).

[b]Cheng and Kuk (2002) estimated growth parameters (i.e. using the maximum likelihood method) for female Western Rock Lobster from the Kalbarri region in Western Australia.

[c]The confidence intervals presented here (i.e. for L_∞ and K) were converted from the standard error reported by Cheng and Kuk (2002).

[d]Phillips et al. (1992) estimated growth parameters (i.e. using the Fabens method) for male Western Rock Lobster from Cliff Head and Seven Mile Beach in Western Australia.

[e]Chittleborough (1976) estimated growth parameters from six juvenile Western Rock Lobster (i.e. of known age) that were reared under optimal laboratory conditions (i.e. constant 25°C water temperature) for six years.

Table 9. The von Bertalanffy growth parameters estimated for Eastern Rock Lobster (combined sexes) sourced from Coffs Harbour, South West Rocks and Jervis Bay, New South Wales. The parameters of Montgomery et al. (2009) are included for comparison. The number ranges in parentheses are the 95% confidence intervals. '-' indicates data not provided.

Source	**Method**	L_∞	K	t_0	R^2
This project (Northern model)	Direct ageing	226.35 (200.16–252.54)	0.11 (0.08–0.14)	0[a]	0.78
This project (Southern model)	Direct ageing	147.20 (130.18–164.21)	0.23 (0.15–0.31)	0[a]	0.55
This project (Combined model)	Direct ageing	218.69 (194.48–242.91)	0.11 (0.09–0.14)	0[a]	0.73
Montgomery et al., 2009 (male)[b]	Tag-and-recapture	246.10 (244.69–280.70)[c]	0.14 (0.12–0.15)[c]	0.40[c]	-
Montgomery et al., 2009 (female)[b]	Tag-and-recapture	239.77 (224.37–243.51)[c]	0.13 (0.12–0.16)[c]	0.37[c]	-

[a]The t_0 value for this model was fixed at zero (see Methodology – section 3.1.5).

[b]Montgomery et al. (2009) was a multi-year study conducted across a similar latitudinal range (i.e. Evans Head–Narooma) in New South Wales that presented sex-specific Eastern Rock Lobster growth curves (that were not significantly different).

[c]The parameter estimates of Montgomery et al. (2009), are those calculated from their full data set, with first-order corrected bootstrap confidence intervals (where relevant).

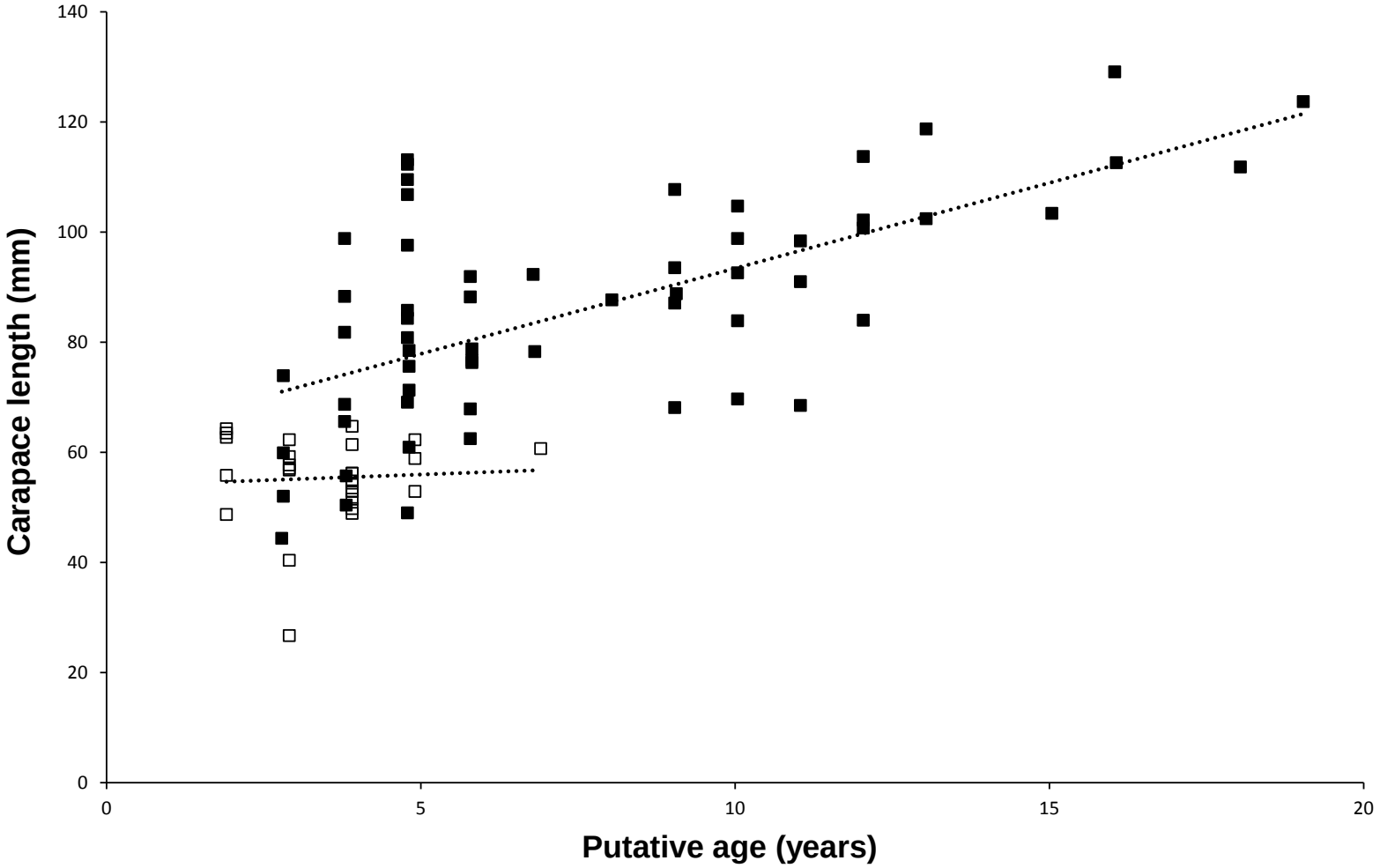

Figure 11. Relationships between CL and putative age for Western Rock Lobster (sexes combined) sampled from shallow (white squares) and deep (black squares) water at Lancelin, Western Australia. Individuals in shallow water were generally smaller for a given putative age, with depth significantly (p = 0.03) influencing the CL-putative-age relationship.

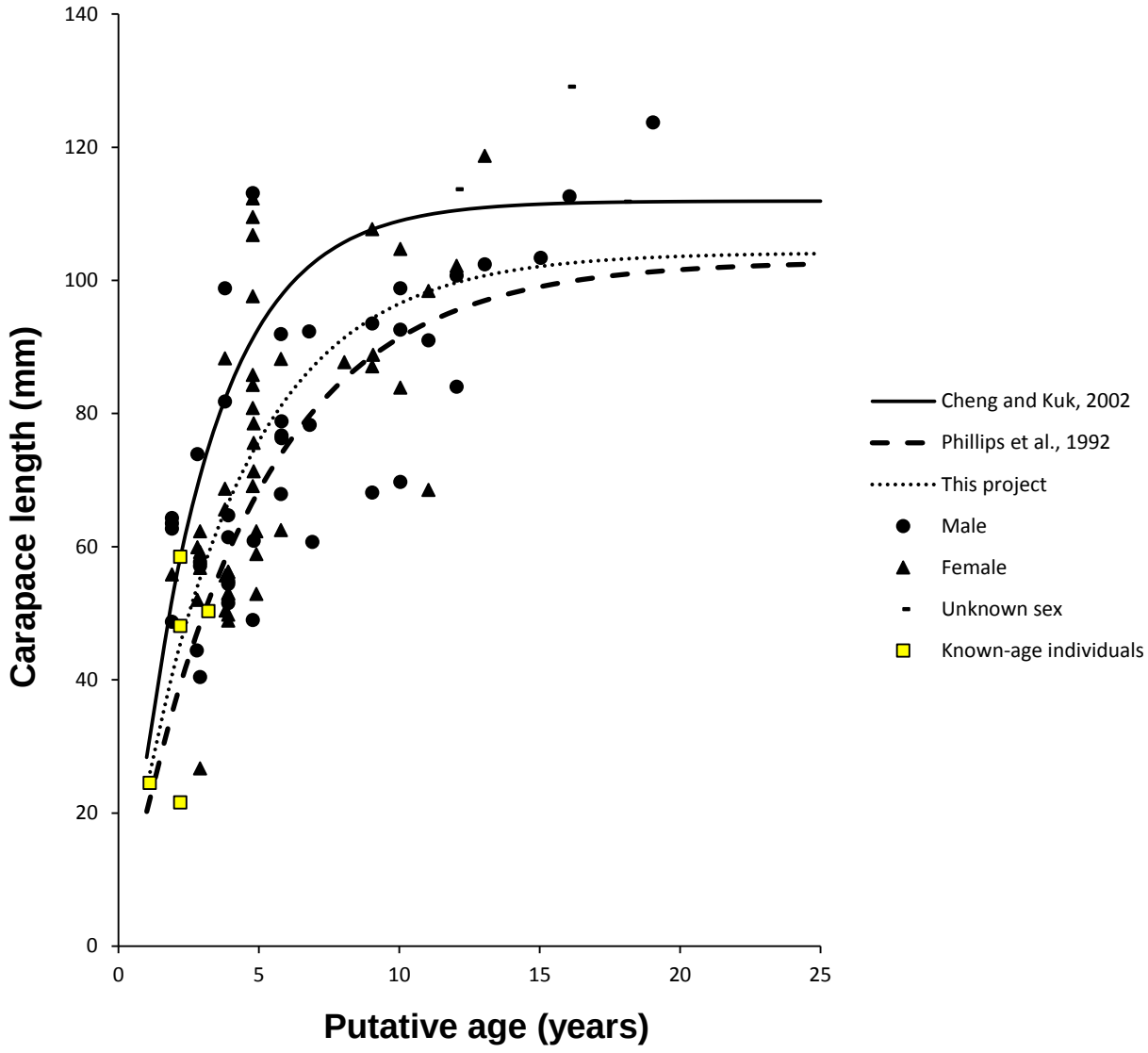

Figure 12. The von Bertalanffy growth equation fitted to all CL-at-putative-age data (sexes combined) from Western Rock Lobster (total *n* = 86) sourced from Lancelin, Western Australia, compared with the curve published by Cheng and Kuk (2002 – for females from the Kalbarri region, *n* = 42) and Phillips et al. (1992 – for males from Cliff Head and Seven Mile Beach, *n* = 175). The individual data points for the five known-age individuals reared during this project are presented for comparison.

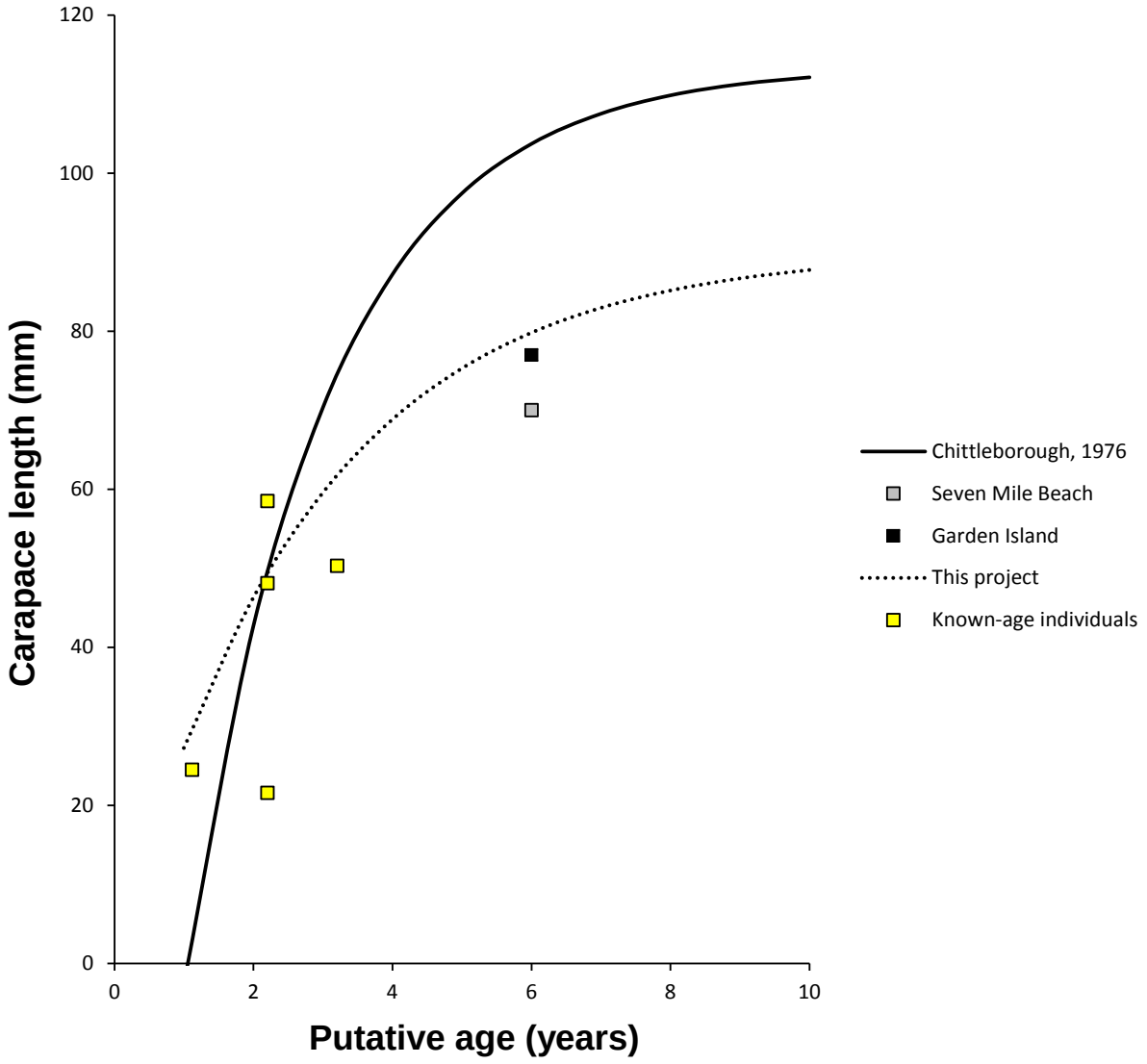

Figure 13. The von Bertalanffy growth model fitted to only young Western Rock Lobster (i.e. with a putative age estimate ≤ 6 years) sourced in this project from Lancelin, Western Australia (n = 58; L_∞ = 90.20; K = 0.36, t_0 = 0), compared with the curve published by Chittleborough (1976) for lobsters reared under optimal laboratory conditions for 6 years. The individual data points for Seven Mile Beach and Garden Island (in Western Australia) were adapted from Chittleborough (1976 – see Figure 6) and are modal values determined by cohort length analysis of wild-caught Western Rock Lobster at those locations. The individual data points for the five known-age individuals reared during this project are presented for comparison.

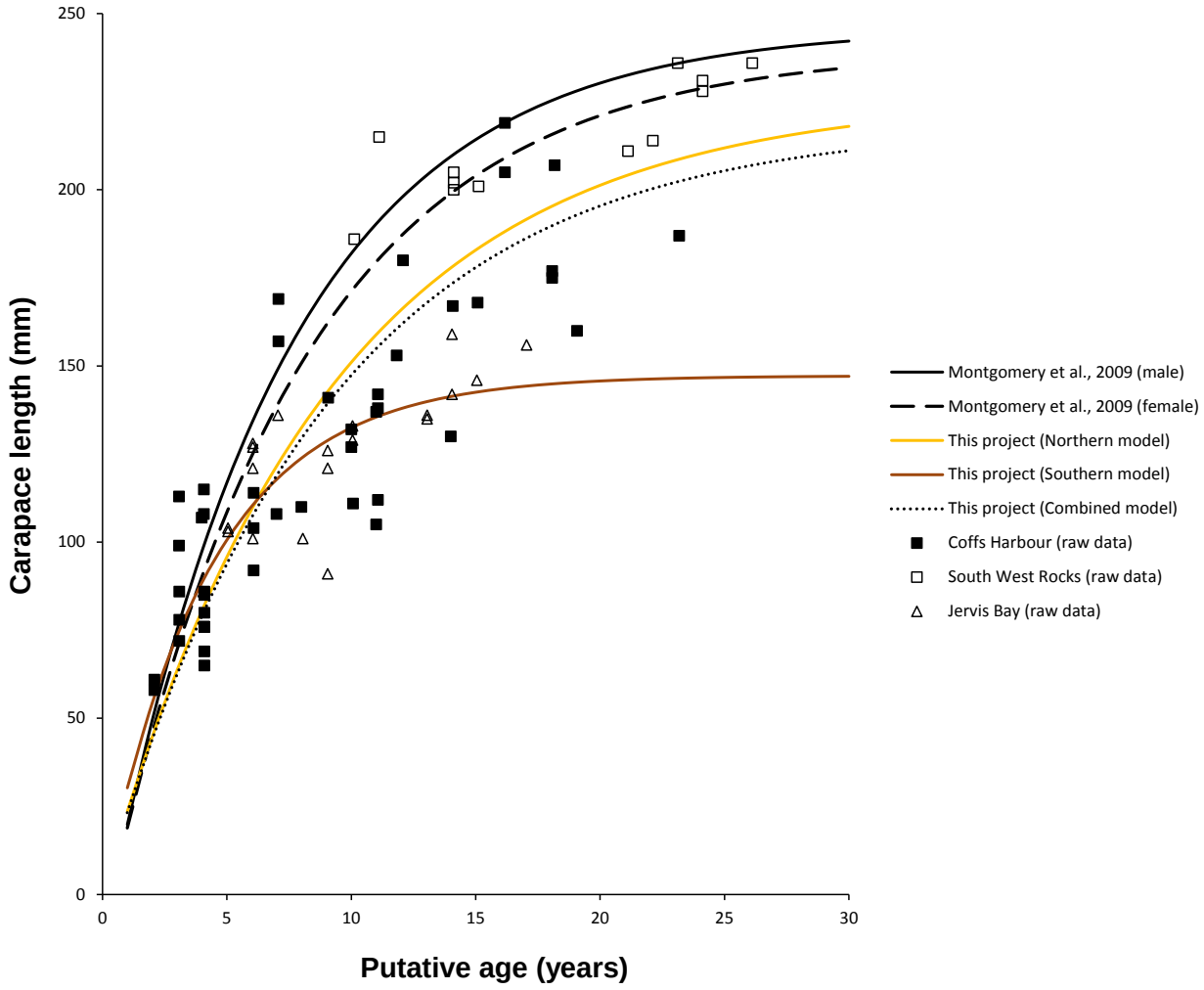

Figure 14. The von Bertalanffy growth model fitted to the CL-at-putative-age data (combined sexes, total *n* = 78) for Eastern Rock Lobster sampled from Coffs Harbour and South West Rocks (northern locations) and Jervis Bay (southern location) in NSW. The Montgomery et al. (2009) models were derived from tag-and-recapture data that was collected over a similar latitudinal extent as that in this project and are provided for comparison.

4.2 OBJECTIVE 2. Evaluation of growth mark periodicity – Western and Eastern Rock Lobster and Crystal Crab

4.2.1 Calcein detection and interpretation

Crystal Crab

Grow-out mortality was low among the calcein-stained Crystal Crab (< 5%). In total, twenty-nine Crystal Crab (92.1–153.9 mm CL) were sacrificed 6, 12 or 18 months after staining. The calcein-stained sections were mostly categorised as having good (56%) or intermediate (33%) readability, but one (11%) was categorised as poor and excluded, because the primary growth marks could not be confidently identified (i.e. before or beyond the calcein stain). One calcein-stained section was substantially damaged (i.e. in the region of interest) during processing and was discarded.

The only individual (female, 92.7 mm initial CL) for which the calcein was indistinguishable from the natural ossicular autofluorescence had moulted once during 18 months and had a moult increment of 13.2 mm CL. None of the other Crystal Crab moulted during the grow-out period and the calcein stain was identifiable within the endocuticle of all other ossicles examined after 6, 12 and 18 months. In these ossicles, the calcein stain was present as either a sharp (i.e. narrow) or relatively diffuse (i.e. broad) mark. The degree of calcein penetration into each ossicle varied with most being categorised as minor (78%), followed by those that were moderate (11%) and complete (11%), but this did not impede differentiation between the new-formed (i.e. unstained) and calcein-stained material. Most Crystal Crab (96%) had new-formed ossicular material deposited beyond the calcein stain, but one individual (4%) did not. There was no significant difference between the mean captive growth increment (CGI – see Methodology section 3.2.3) for Crystal Crab reared for 6, 12 or 18 months after staining (ANOVA, $p = 0.49$, $df = 2, 18$; $F = 3.55$). For all grow-out durations combined, the CGI was not correlated with individual CL ($y = 0.3555x + 94.19$; $n = 26$; $R^2 = 0.01$).

For most Crystal Crab (68%), the calcein stain was positioned along a primary growth mark, but for others (32%) it was incorporated approximately halfway between two adjacent marks. The number of primary growth marks identified beyond the calcein stain was 1 or 0 (Figure 15). Some Crystal Crab (32%) had ossicular material deposited beyond the calcein stain, with CGIs ranging from 33–129% (Figure 15), but there was no identifiable primary growth mark within this zone (Figure 16). For most Crystal Crab (52%), one clear primary growth mark was identified beyond the calcein stain (Figure 17), with CGIs ranging from 46–212%. Four individuals (16%) that were reared for 6 or 12 months had broad extension zones beyond the calcein stain (Figure 18), with CGIs ranging from 234–265%. In these ossicles, the endocuticular region beyond the calcein stain was apparently different to that in

other ossicles (i.e. those with 1 or 0 marks) and did not contain primary growth marks (compare Figure 17 vs. 18).

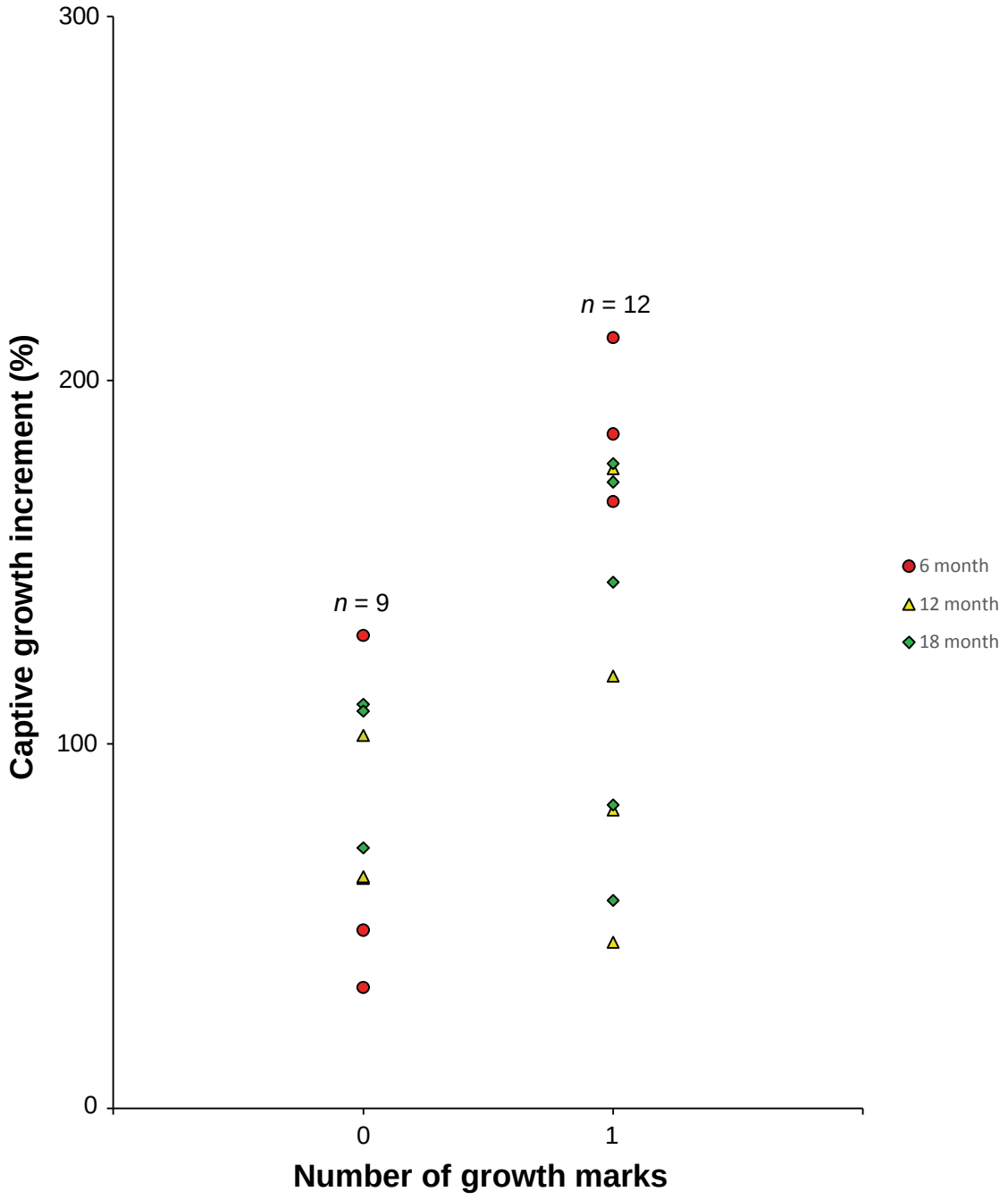

Figure 15. The Crystal Crab captive growth increment (CGI) plotted against the number of growth marks identified beyond the calcein stain after the 6, 12 or 18 month grow-out. Each data point represents one individual (*n* = 21), with only ossicles categorised as good and intermediate being presented.

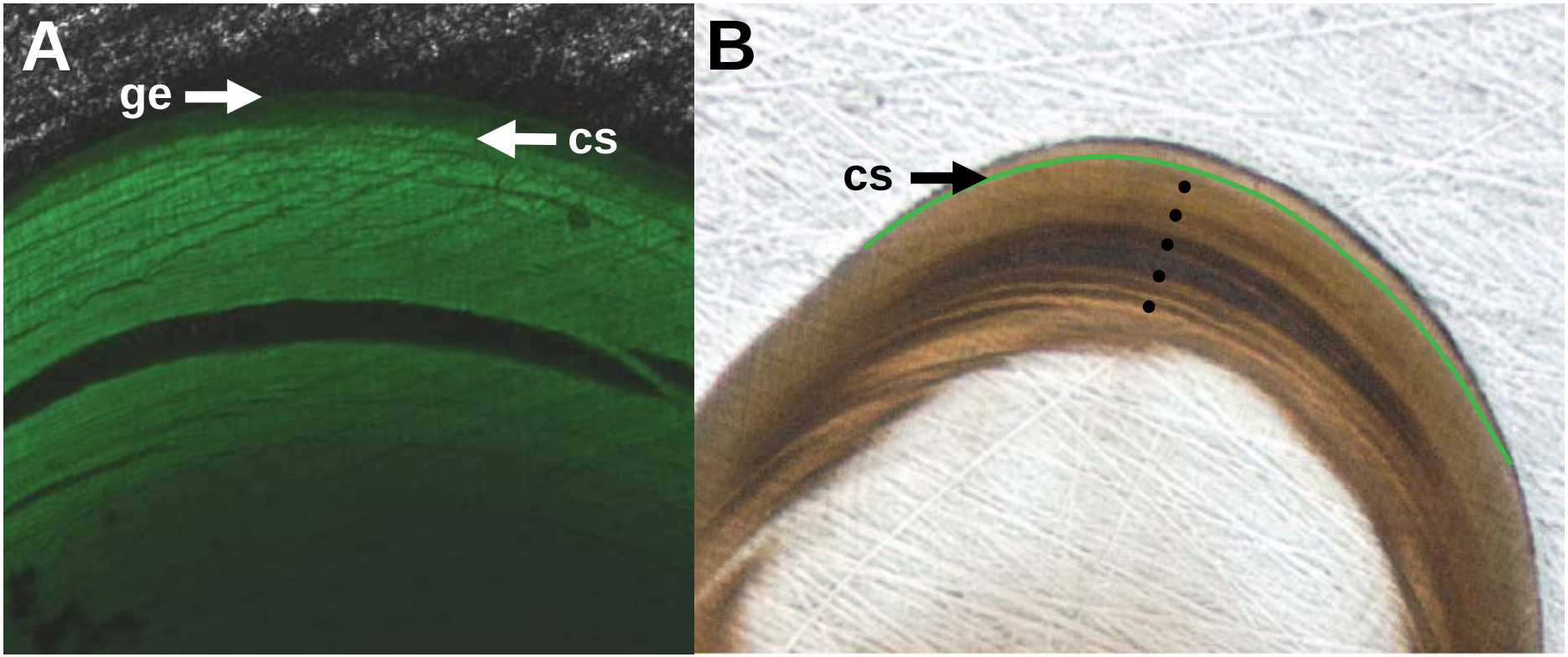

Figure 16. Ossicular section from a female Crystal Crab (112 mm CL) that did not have an identifiable primary growth mark beyond the calcein stain after a 12 month grow-out. A: Confocal microscopy image showing the position of the calcein stain (cs) relative to the growing edge (ge). B: Illustration of the calcein stain (cs – green line) position relative to the primary growth mark series (black dots).

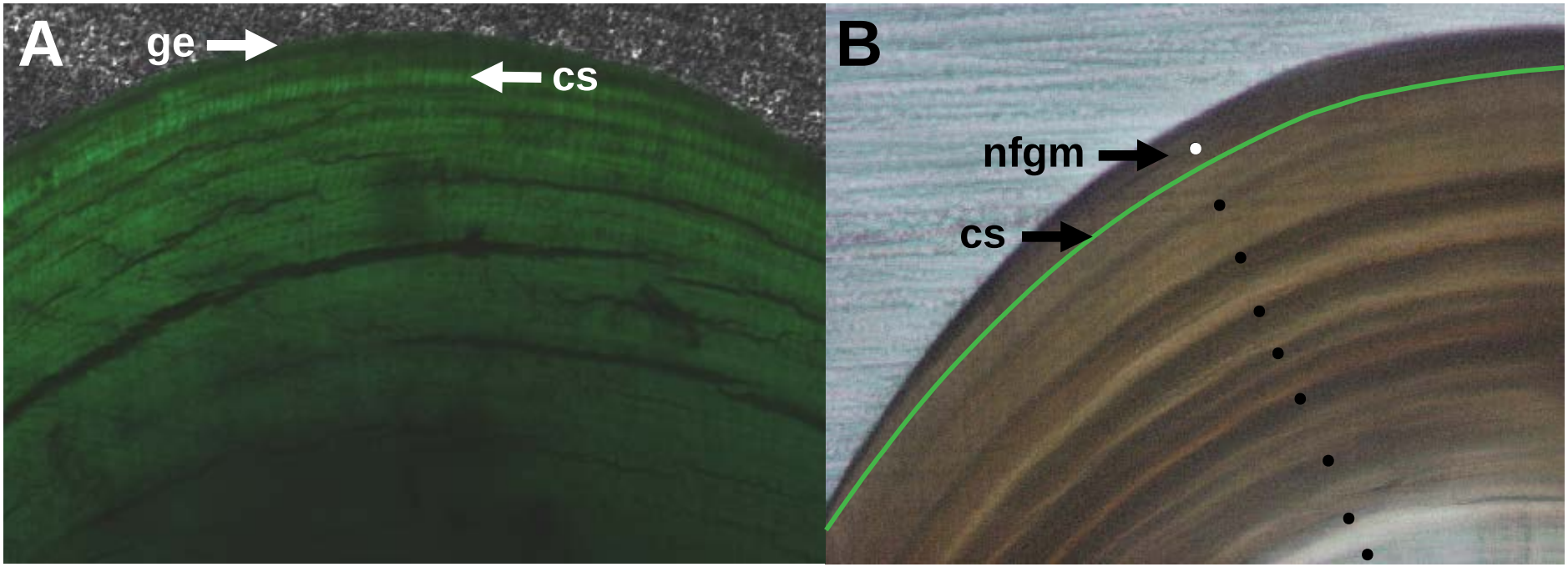

Figure 17. Ossicular section from a female Crystal Crab (129 mm CL) that had one new-formed primary growth mark beyond the calcein stain after a 12 month grow-out. A: Confocal microscopy image showing the position of the calcein stain (cs) relative to the growing edge (ge). B: Illustration of the calcein stain (cs – green line) position relative to the primary series (black dots) and the single new-formed growth mark (nfgm – white dot). Note that the calcein stain is positioned between the two outermost primary growth marks.

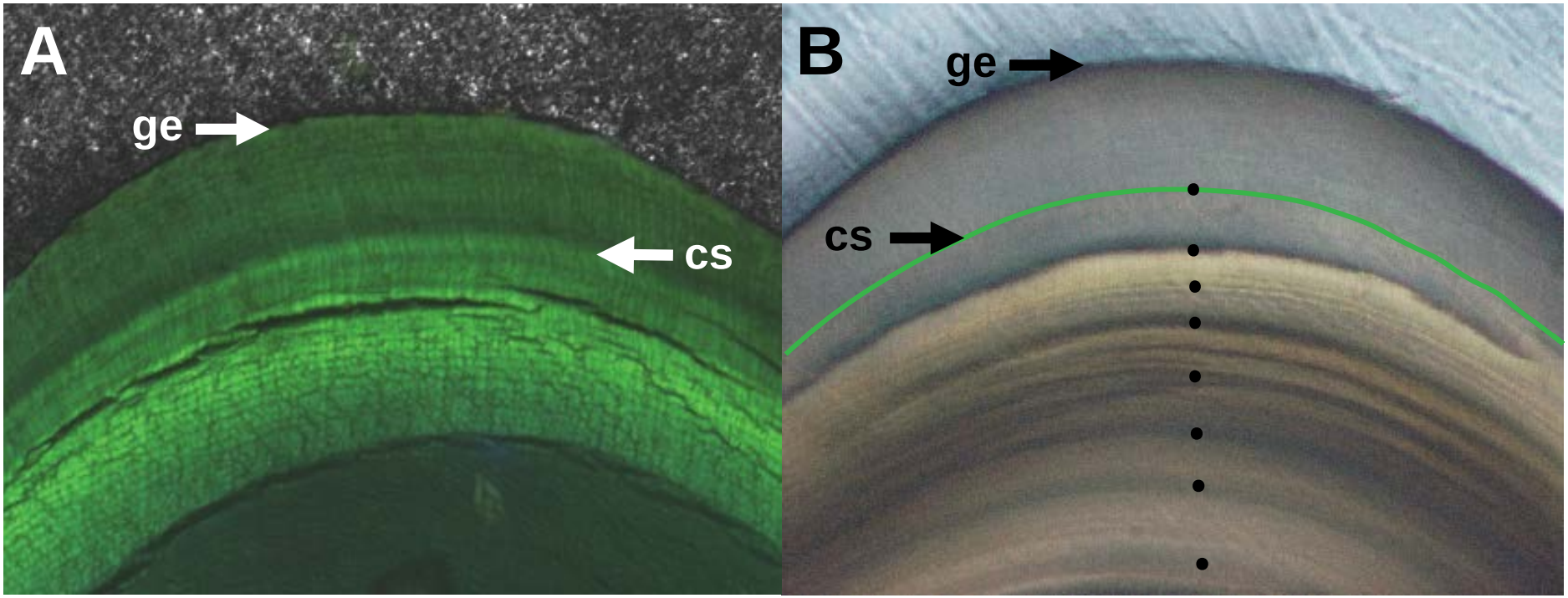

Figure 18. Ossicular section from a female Crystal Crab (111 mm CL) with a broad extension zone beyond the calcein stain after a 6 month grow-out. A: Confocal microscopy image showing the position of the calcein stain (cs – green line) relative to the growing edge (ge). B: Illustration of the calcein stain (cs) position relative to the primary series (black dots) and the growing edge. The calcein stain is positioned directly adjacent to a primary growth mark. Note that the new-formed material is distinctly-coloured and apparantly different to the other ossicles (compare with Figure 16B and 17B).

Western Rock Lobster

Sixteen calcein-stained Western Rock Lobster (62.6–100.7 mm CL) that had moulted (from 2–4 times) during the 18 month grow-out were sacrificed for examination. Of these, three individuals were in immediate pre- or post-moult condition and had decalcified gastric ossicles that were not suitable for sectioning (i.e. because they were too soft), reducing the total *n* for the confocal analysis to 13. The calcein stain was retained in all ossicles, with each having some decalcification of the exocuticle and/or outermost endocuticular layer. In these individuals, the calcein stain was present as either a sharp (i.e. narrow – 30%) or diffuse (i.e. broad – 70%) artificial mark within the endocuticle.

Eight Western Rock Lobster did not have any identifiable ossicular material beyond the calcein stain, with the stain being distributed from the outermost growth mark to the edge (Figure 19). One Western Rock Lobster (70.6 mm CL male), with a CGI of 61%, had a single new-formed primary growth mark identified beyond the calcein stain during the blind count (Figure 20). Another individual (75.4 mm CL female) had material deposited beyond the artificial mark (CGI of 95%), but no new-formed growth mark. The remaining four Western Rock Lobster (62.6–76.7 mm CL) had CGIs of 95, 157, 183 and 198%. For these individuals, a growth mark was not identified beyond the calcein stain during the blind count. However, subsequent re-examination of these ossicles indicated that decalcification along the growing edge impeded confident edge interpretation and made determination of complete mark formation difficult (Figure 21).

Most calcein-stained Western Rock Lobster lost some appendages during regular grow-out maintenance and handling, because of their relatively high propensity for limb autotomy. After the grow-out completion, their Δ CL values ranged from –0.7 to 3.8, with no significant linear relationship being found between the Δ CL and the mean CL ($y = –0.0891x + 7.439$; $p < 0.16$; $n = 12$; $R^2 = 0.19$).

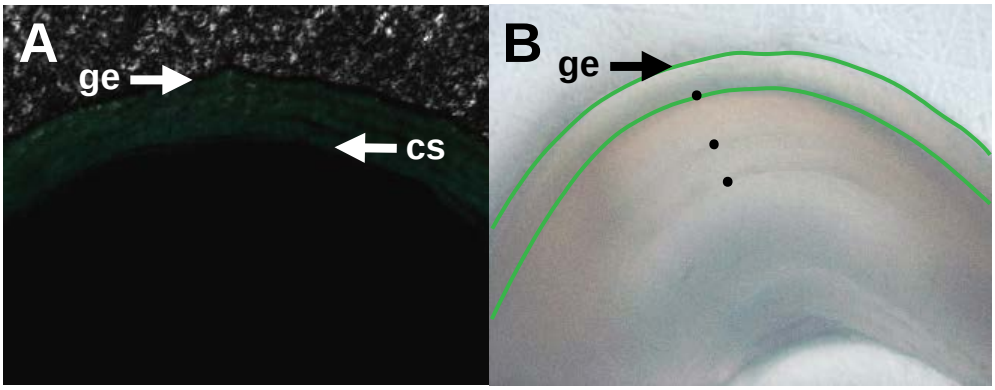

Figure 19. Western Rock Lobster (71.3 mm CL male) ossicle without identifiable deposition beyond the broad calcein-stained zone. A: Confocal microscopy image showing the position of the calcein stain (cs) relative to the growing edge (ge). B: Illustration of the inner- and outermost extent of the calcein stain (cs – green line) relative to the primary growth mark series (black dots).

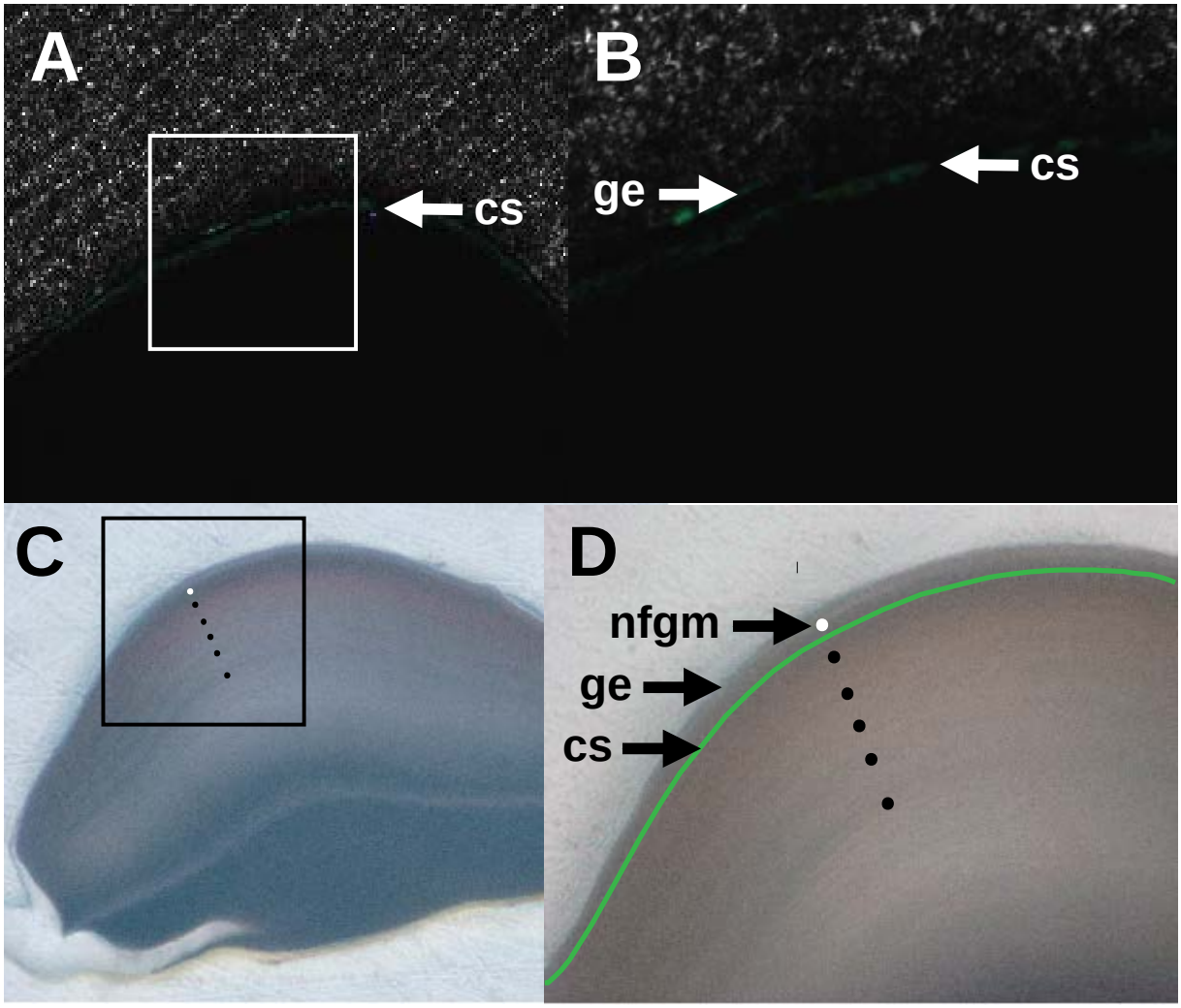

Figure 20. Calcein-stained Western Rock Lobster (70.6 mm CL male) with one primary growth mark identified beyond the artificial mark. A: Confocal microscopy image showing the calcein stain (cs). B: Inset of A showing the position of the calcein stain relative to the growing edge (ge). C: Light-microscopy image showing the primary growth mark series (black dots) and single new-formed growth mark (nfgm – white dot). D: Inset of C with an illustration of the calcein stain (green line) position relative to the primary series and the new-formed growth mark.

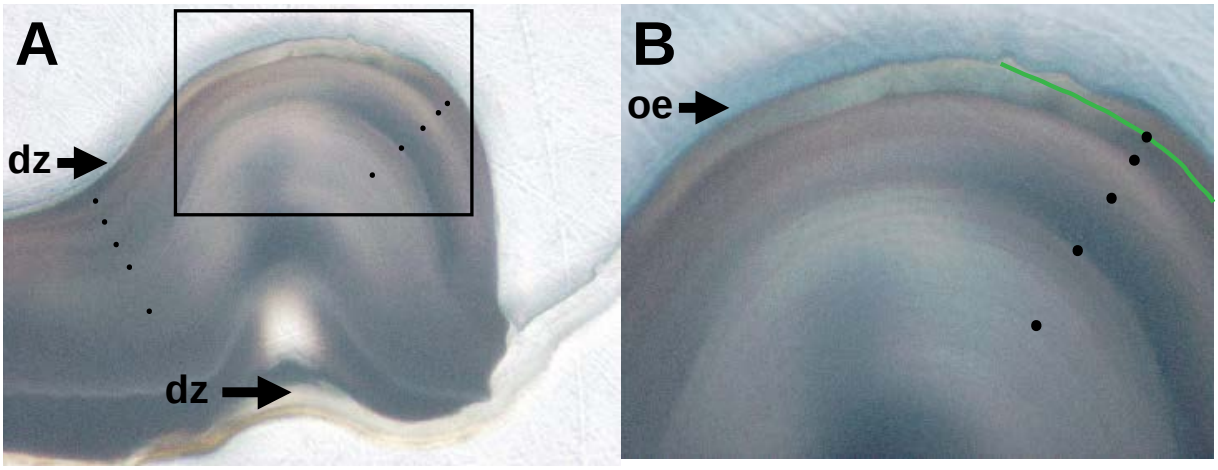

Figure 21. Calcein-stained Western Rock Lobster (76.0 mm CL female) ossicle. A: Image showing the decalcified zones identified within the exo- (lower) and endocuticular layer (upper) relative to the primary growth mark series (black dots). Note that the outer edge of the decalcified zone in the endocuticle (upper) is difficult to identify compared with that of the adjacent layer. B: Inset of A showing the outer edge (oe) of the decalcified endocuticle and an illustration of an irregular calcein stain (green line) mark, with asymmetric ossicular extension beyond it.

Eastern Rock Lobster

A total of 30 Eastern Rock Lobster (118–240 mm CL) were sacrificed for periodicity evaluation. This comprised an equal sex ratio (15:15) of juvenile to mature individuals. Only one mortality (<2%) occurred immediately after the calcein staining procedure. Some other fatalities (20%) occurred during the subsequent grow-out, with some being attributed to pre- or post-moult cannibalism, while others were unexplained. All Eastern Rock Lobster moulted (at least 1–3 times) during the 12 and 18 month grow-out. Irrespective of the sampling interval, the majority of the calcein-stained ossicles (81%) showed some decalcification, but this did not impede interpretation.

Most calcein-stained Eastern Rock Lobster ossicles were categorised as having good readability (63%), with others being intermediate (11%) and some poor (26%). The individuals whose ossicles were rejected for having poor readability ranged from 118–171 mm CL and comprised both male and female (3:2) lobster. The calcein stain was present in all rejected samples, but none had any growth beyond the artificial mark.

For most (61%) of the ossicles sampled after 6 months, the calcein stain was distributed throughout the entire endocuticle, but there was no measurable growth beyond the artificially marked material (Figure 22). However, two Eastern Rock Lobster (39%) that were reared for 6 months showed ossicular extension beyond the calcein stain, but there was no primary growth mark identified within the new-

formed material in the blind count. For the smaller individual (156 mm CL male), the calcein stain penetrated the entire ossicular endocuticle (and exocuticle – Figure 23A), but the new-formed material beyond the artificial mark was relatively unstained (Figure 23B). For the larger individual (170 mm CL male), the calcein also penetrated the entire endocuticle, but was still identifiable as two distinct bands along the two outermost full-formed growth marks (Figure 24). The CGI for both individuals was 88%.

The calcein stain was also present in Eastern Rock Lobster ossicles examined after 12 months. For most ossicles (72%) the calcein was concentrated around the growing edge and there was no measurable growth beyond the artificial mark. However, two individuals (28%) showed ossicular extension beyond the calcein stain. The larger individual (237 mm CL male) did not have a growth mark identified beyond the calcein in the blind count and had a CGI of 106%. For the smaller Eastern Rock Lobster (130 mm CL female) sampled after 12 months, one newly formed growth mark was identified during the blind count (Figure 25), with this individual having a CGI of 94%.

For all individuals sampled after 18 months (132–183 mm CL), the calcein stain was very faint relative to the natural ossicular autofluorescence. After the subtraction process, calcein could not be identified within the ossicular endocuticle, with the artificial stain only being present (i.e. but very faint) along the membranous layer of the growing edge (Figure 26).

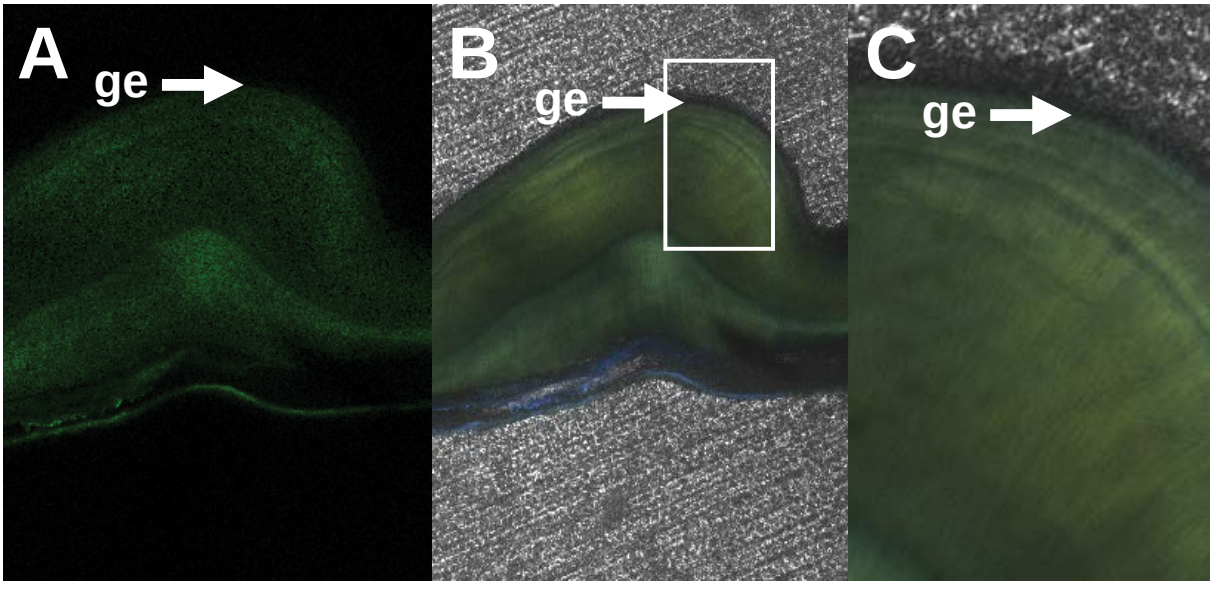

Figure 22. Microscopy images of an Eastern Rock Lobster (142 mm CL male) ossicle sampled 6 months after staining with calcein. A: Confocal image of calcein stain throughout the ossicular endocuticle. B: Confocal image superimposed onto a transmitted light image. C: Inset of B showing the growing edge (ge) without any measurable growth beyond the calcein stain.

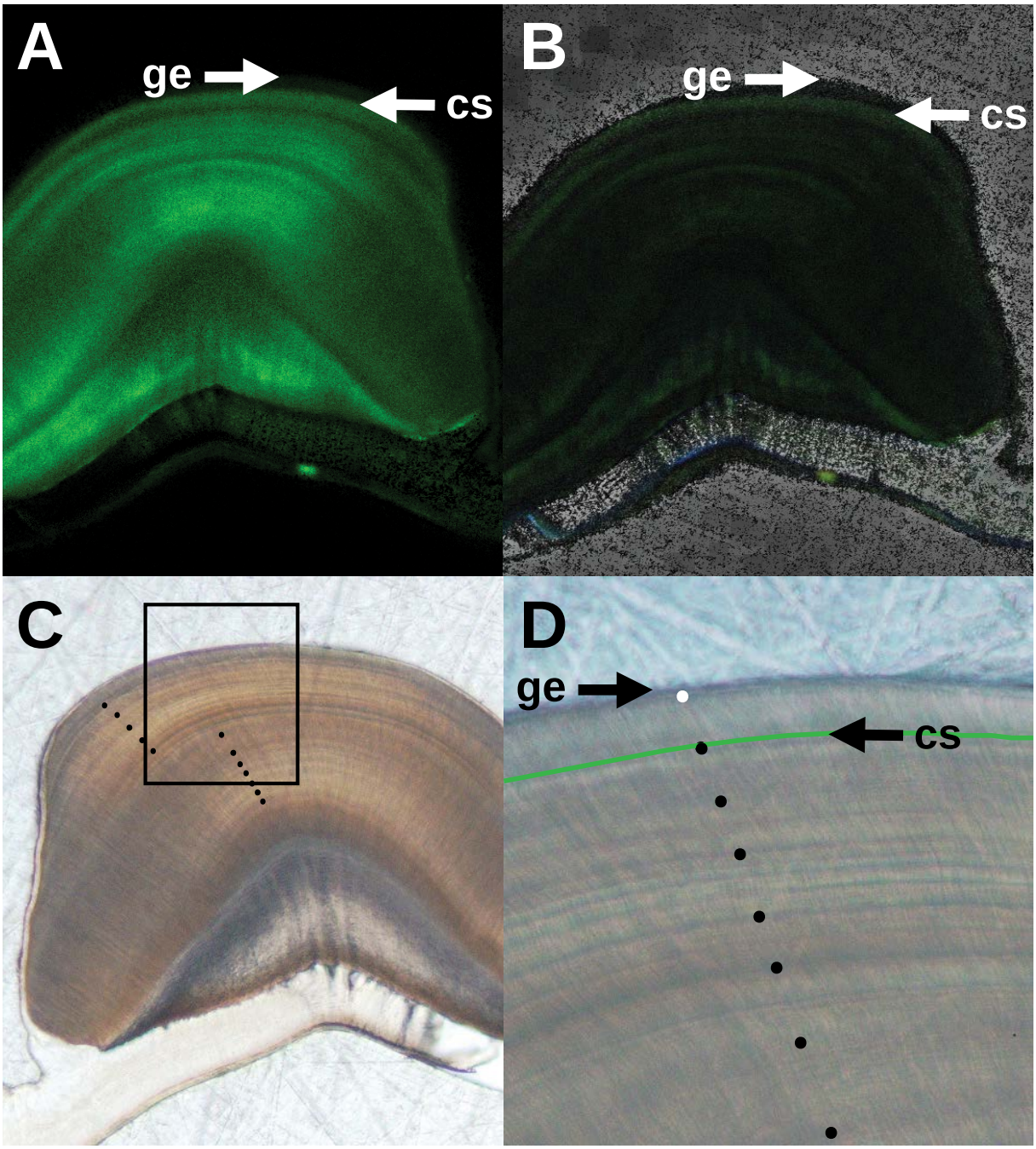

Figure 23. Microscopy images of an Eastern Rock Lobster (156 mm CL male) ossicle sampled 6 months after staining with calcein. A: Confocal image of calcein stain (cs) throughout the entire endocuticle, with peak intensity occurring near the growing edge (ge). B: Peak intensity-adjusted confocal image superimposed onto a transmitted light image. C: Light microscopy image. D: Inset of C showing the position of the calcein stain (green line) relative to the primary growth mark series (black dots) and the interface between the membranous layer and endocuticle (white dot).

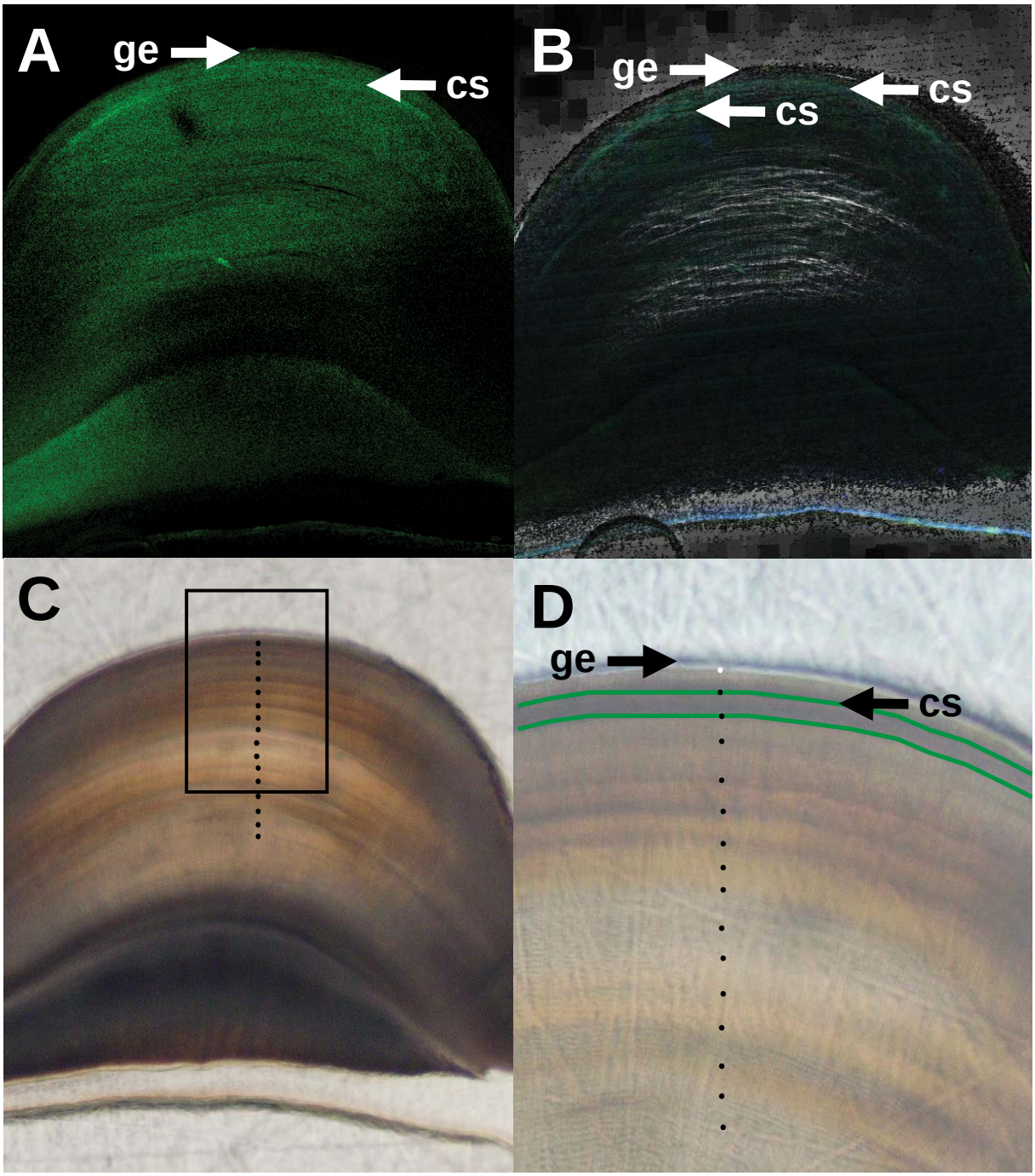

Figure 24. Microscopy images of an Eastern Rock Lobster (170 mm CL male) ossicle sampled 6 months after staining with calcein. A: Confocal image of calcein stain (cs) throughout the ossicular endocuticle, with peak intensity occurring near the growing edge (ge). B: Peak intensity-adjusted confocal image superimposed onto a transmitted light microscopy image. Note that the calcein was incorporated along the two adjacent outermost growth marks. C: Light microscopy image. D: Inset of C showing the position of the calcein stain (green line) relative to the primary growth mark series (black dots) and the interface between the membranous layer and endocuticle (white dot).

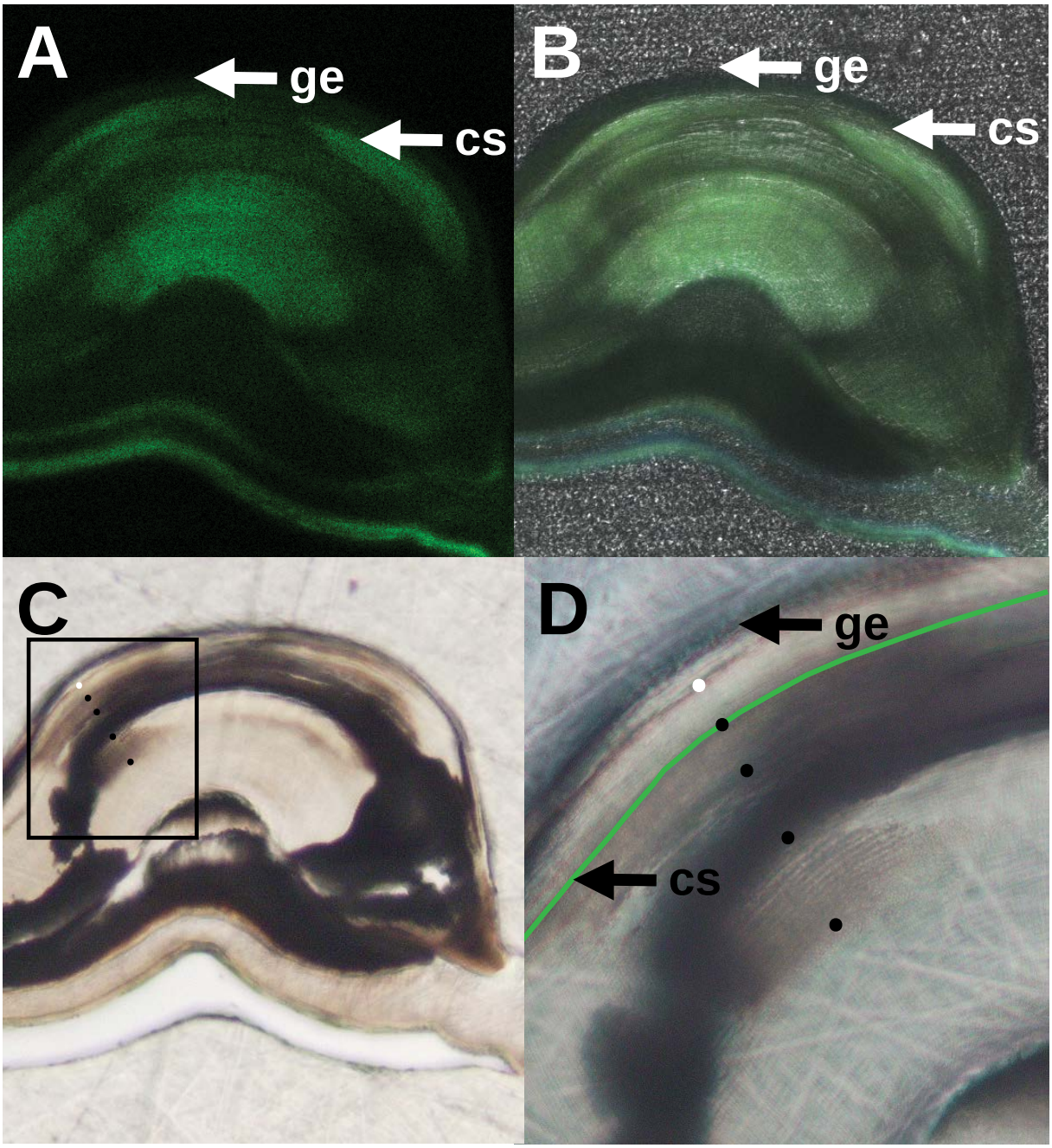

Figure 25. Microscopy images of an Eastern Rock Lobster (130 mm CL female) ossicle sampled 12 months after staining with calcein. A: Confocal image of calcein stain (cs) throughout the ossicular endocuticle, with peak intensity occurring near the growing edge (ge). B: Peak intensity-adjusted confocal image superimposed onto a transmitted light microscopy image. Note that this ossicle was partially decalcified. C: Light microscopy image. D: Inset of C showing the position of the calcein stain (green line) relative to the primary growth mark series (black dots) with one new-formed growth mark that was identified during the blind count.

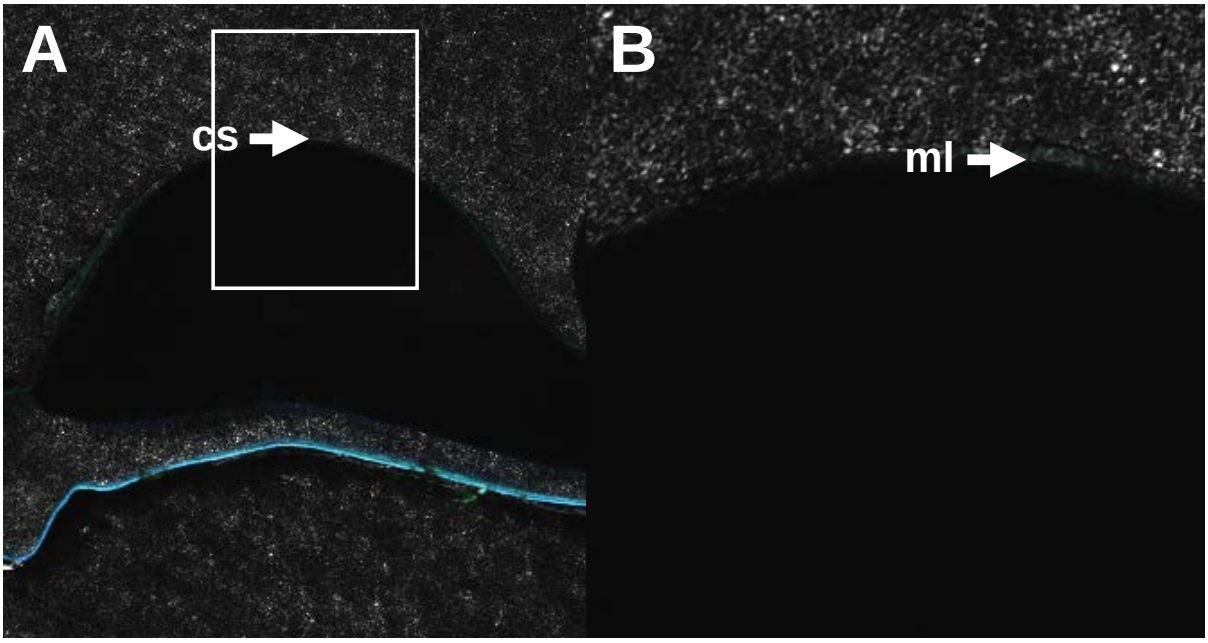

Figure 26. Confocal microscopy images of an Eastern Rock Lobster (183 mm CL female) ossicle sectioned 18 months after staining with calcein. A: Any calcein stain (cs) retained in the 18 month samples was restricted to a very faint mark incorporated into the membranous layer at the growing edge. B: Inset of A identifying the calcein position along the membranous layer (ml) and demonstrating the absence of an artificial mark within the ossicular endocuticle. This individual moulted twice.

4.2.2 Captive-growth modelling

For Eastern Rock Lobster, there was a significant negative linear relationship between mean CL during the grow-out period and the Δ CL during the 18 months ($p < 0.001$; $n = 22$; $R^2 = 0.61$ – Figure 27). The von Bertalanffy growth parameter estimates for the calcein-stained ERL that were reared in captivity ($L_\infty = 219.49$ mm CL, $K = 0.15$) were broadly similar to those that were directly determined in this project (Figure 28) and published by Montgomery et al. (2009).

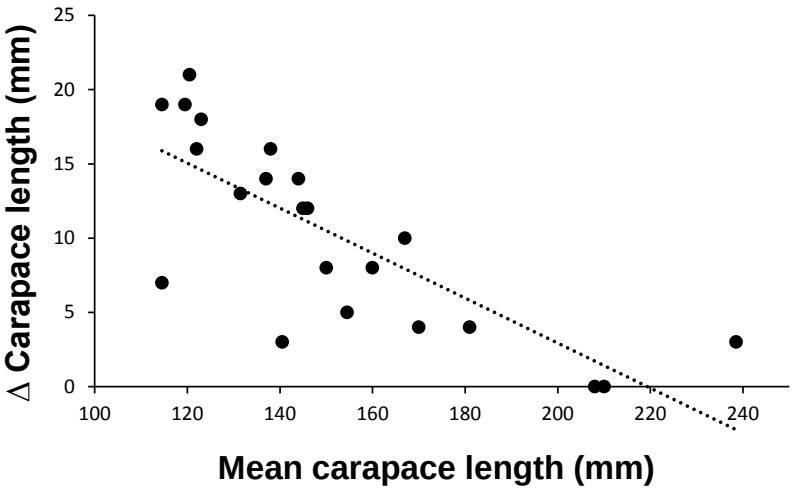

Figure 27. The significant linear relationship (i.e. Gulland-Holt plot) between Eastern Rock Lobster mean carapace length and the change in this measure during the 18 month grow-out period. The equation for the line-of-best-fit is: $y = -0.1514x + 33.23$ ($p < 0.001$; $n = 22$; $R^2 = 0.61$).

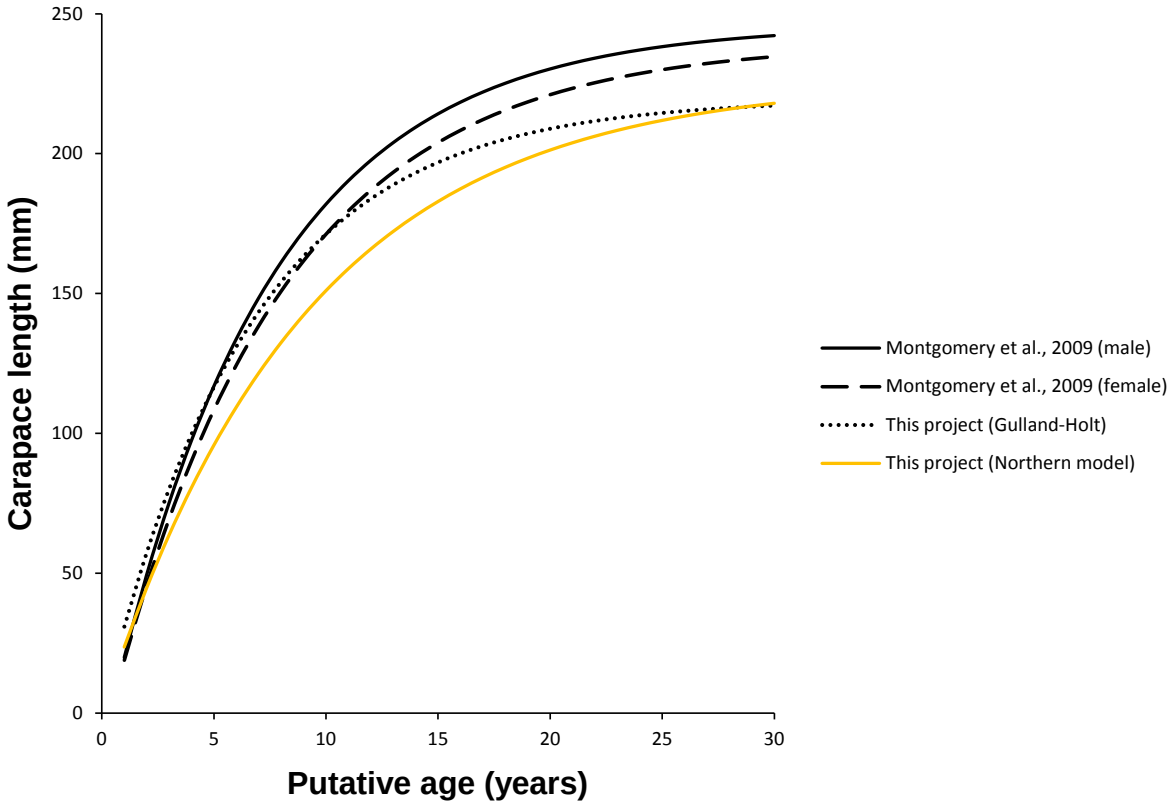

Figure 28. Von Bertalanffy growth curve derived from the Gulland-Holt plot in Figure 27 for the calcein-stained Eastern Rock Lobster (combined sexes, $n = 22$) reared for 18 months. The von Bertalanffy growth model for wild-caught individuals from the northern locations (Coffs Harbour and South West Rocks) in this project (combined sexes, $n = 78$) and the tag-and-recapture models of Montgomery et al. (2009) are provided for comparison.

4.3 OBJECTIVE 3. Applicability to other crustacean species – with LA-ICPMS and known-age individual validation

4.3.1 Applicability assessment

Sectioned ossicles from Ornate and Southern Rock Lobster and Crystal, Mud, Crystal and Giant Crab all revealed the presence of ossicular growth marks like those observed for Western and Eastern Rock Lobster. For all five species, the cuticular boundary was easily identifiable and the sectioned pterocardiac or zygocardiac ossicles contained primary growth marks that were distinguishable from endocuticular lamellae. For all species except Giant Crab, the primary growth mark series was countable (i.e. in Region A or Region B – Figure 5) across the entire endocuticle and allowed for putative age estimation.

Ornate Rock Lobster

Macroscopic differences in calcification were apparent in some whole and sectioned Ornate Rock Lobster ossicles (Figure 29), but did not impede growth mark counts, with the primary series still being identifiable within the less-calcified zone. Laser ablation-ICPMS analysis of a pre-moult Ornate Rock Lobster ossicle identified a significant difference between the mean ^{44}Ca detector count for inner (i.e. calcified) and outer (i.e. decalcified) endocuticular zones (paired *t*-test, $p = 0.003$, $df = 2$), with that for the latter being less than the former (Figure 30). For male Ornate Rock Lobster (85–122 mm CL) from Torres Strait (Southeast Zone – Table 4), putative age ranged from 1.7–4.7 years (Figure 31) and was positively correlated with CL ($y = 11.19x + 69.148$; $n = 5$; $R^2 = 0.72$). The Ornate Rock Lobster CL-at-putative-age data allowed for estimation of the von Bertalanffy growth parameters L_∞ and K for comparison with those from other published curves (Table 10) and the size-at-known-age data for captive-reared individuals in this project (Figure 32).

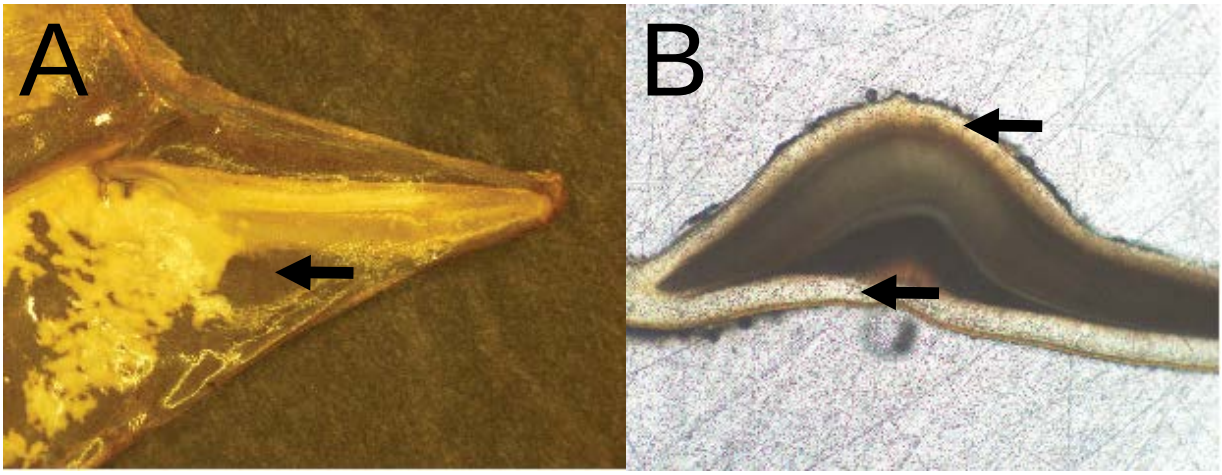

Figure 29. A: Pterocardiac ossicle from a pre-moult Ornate Rock Lobster showing the macroscopically visible decalcification (black arrow). B: Sectioned pterocardiac ossicle (depicted in A) showing apparent endo- and exo-cuticular decalcification (black arrows) extending around the structures edge.

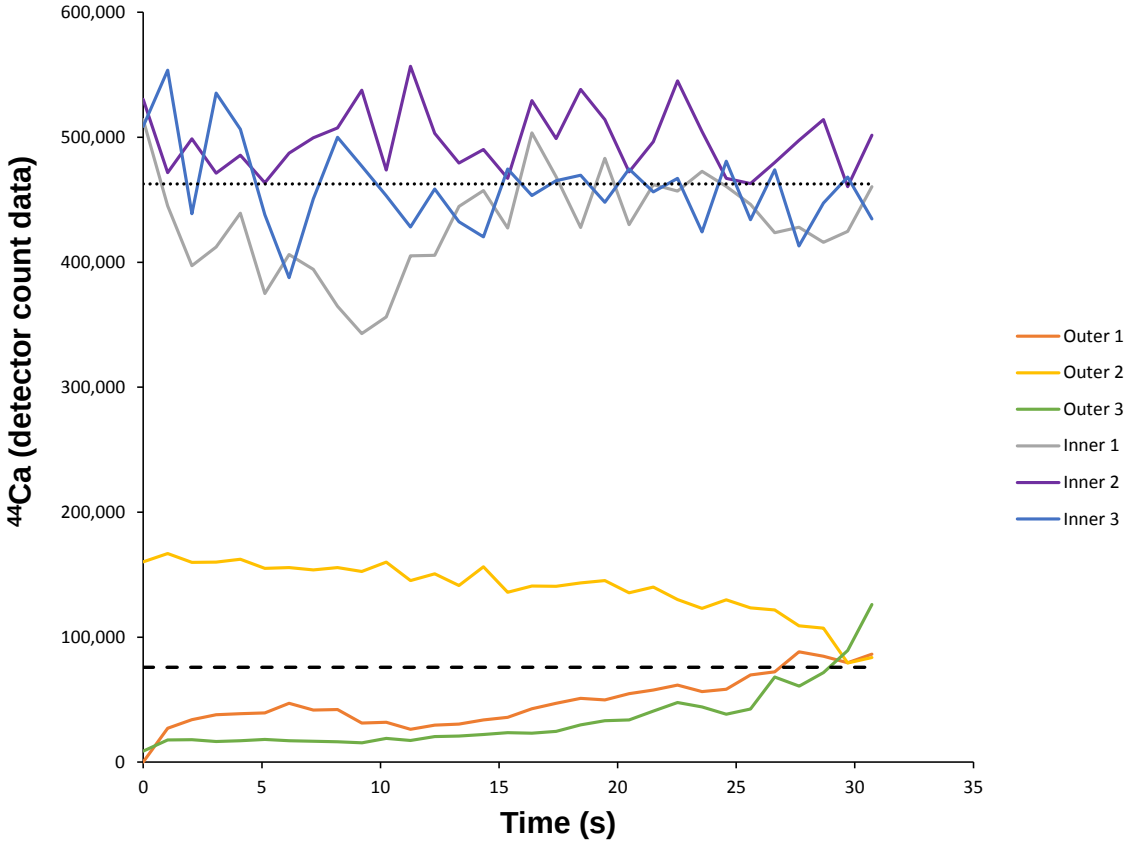

Figure 30. ^{44}Ca detector data from replicate LA-ICPMS transects across an Ornate Rock Lobster ossicle (n = 1) showing the significantly different (p = 0.003) counts between visibly decalcified (outer) and calcified (inner) endocuticular zones. The dotted and dashed line indicate the respective means.

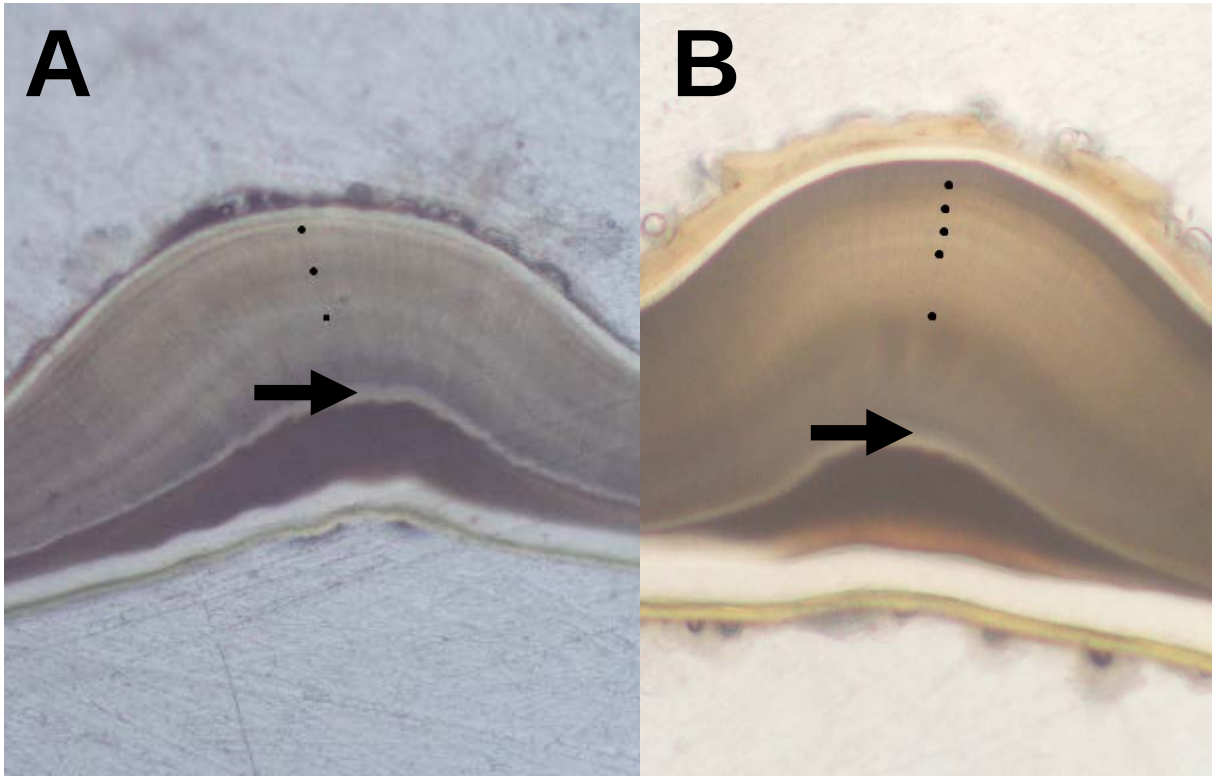

Figure 31. Pterocardiac ossicle sections from Ornate Rock Lobster showing the primary growth marks (black dots) used to assign putative ages. The black arrow indicates the cuticular boundary. A: 103 mm CL male with 3 marks (putative age = 2.7 years). B: 122 mm CL male with 5 marks (putative age = 4.7 years).

Table 10. The von Bertalanffy growth parameters estimated for male Ornate Rock Lobster sourced from the Southeast Zone in Torres Strait. The parameters of Phillips et al. (1992) and Skewes et al. (1997) are included for comparison. Where available the 95% confidence intervals are given in parentheses. '-' indicates data not provided.

Source	**Method**	L_∞	K	t_0	R^2
This project	Direct ageing	122.48 (71.40–173.57)	0.66 (–0.19–1.52)	0[a]	0.53
Phillips et al., 1992[b]	Tag-and-recapture	150.67 (115.00–175.00)[c]	0.57	0	-
Skewes et al., 1997[d]	Tag-and-recapture	150.67 (115.00–175.00)[c]	0.44	0.42[e]	-

[a]The t_0 value for this model was fixed at zero (see Methodology – section 3.1.5).

[b]Phillips et al. (1992) estimated growth parameters (i.e. using the method of Palmer et al., 1991) for male Ornate Rock Lobster from Torres Strait.

[c]The confidence intervals presented here were approximated from Phillips et al. (1992 – Figure 7b).

[d]Skewes et al. (1997) estimated growth parameters (using the L_∞ of Phillips et al., 1992) for 1 and 2+ male Ornate Rock Lobster (identified with size-modal analysis) from Torres Strait and found that a lower K value better described growth data for the Southeast Zone.

[e]The Skewes et al. (1997) t_0 value equates to the time elapsed from spawning to settlement.

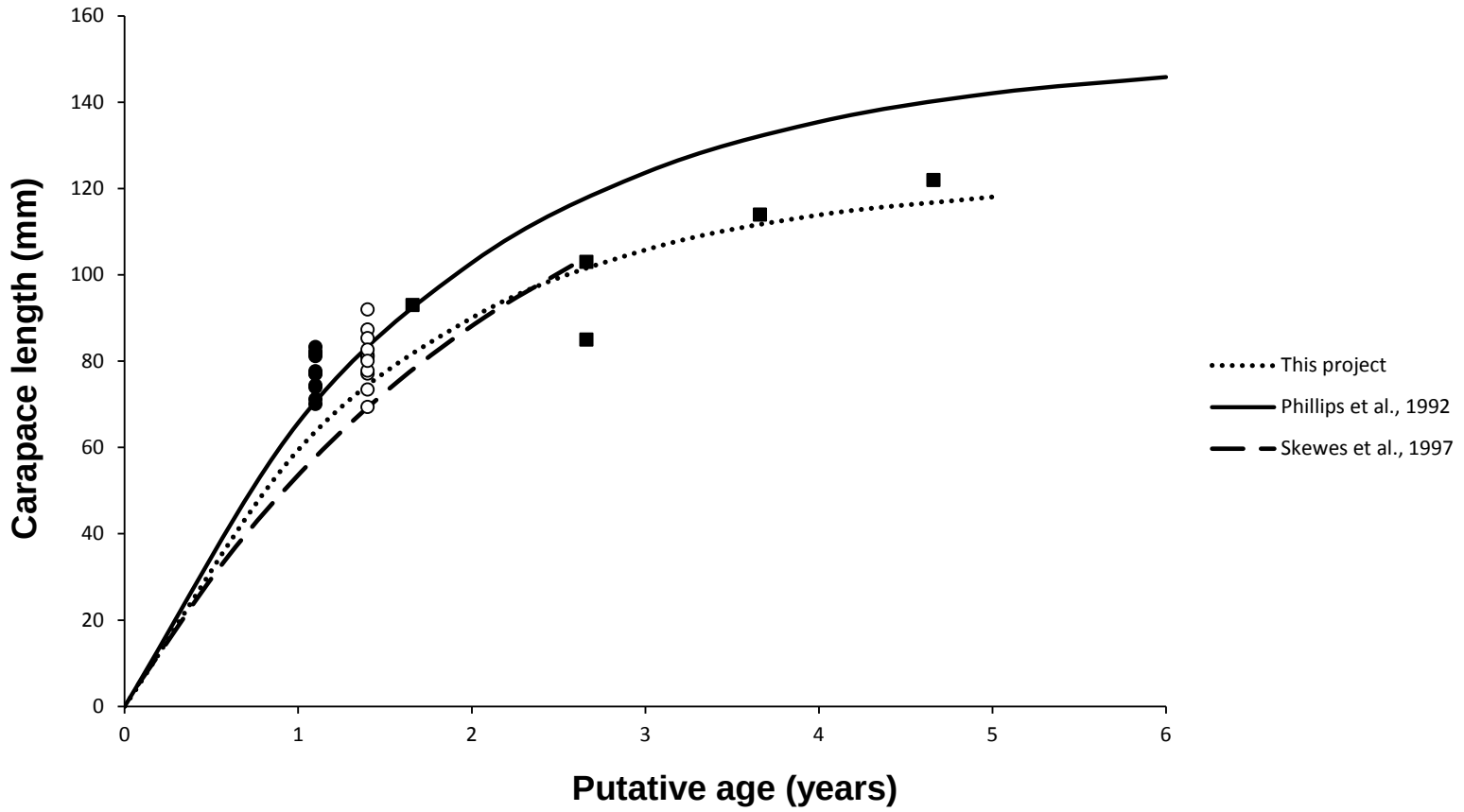

Figure 32. Comparison of von Bertalanffy growth curves for male Ornate Rock Lobster from Torres Strait. The curve for this project (black squares) was generated from directly determined putative age estimates (n = 5) for lobster from the Southeast Zone. The Phillips et al. (1992) model estimated growth parameters using the method of Palmer et al. (1991) (n = 69). The Skewes et al. (1997) curve used the L_∞ of Phillips et al. (1992), but reduced K to better describe slower growth for 1 and 2+ lobster from the Southeast Zone. Circles represent size-at-known-age for young-of-the-year Ornate Rock Lobster (reared in Lombok, Indonesia for this project) sampled after 1.1 years (black circles, n = 12) and 1.4 years (white circles, n = 12). Note: The Skewes et al. (1997) curve reproduced here was repositioned (by –0.44 years) to align settlement times.

Southern Rock Lobster

For Southern Rock Lobster (59–170 mm CL) sourced from Taroona Waters, Tasmania (Table 5), growth marks were counted within Region A, with putative age ranging from 1.6–16.6 years (Figure 34) and being positively correlated with CL ($y = 7.4149x + 54.223$; $n = 4$; $R^2 = 0.94$). For Southern Rock Lobster from Robe, South Australia (121–171 mm CL) (Table 5), complete growth mark counts (i.e. uninterrupted across the entire endocuticle) were only possible in Region B, with putative age ranging from 11.6–16.6 years (Figure 35) and being positively correlated with CL ($y = 4.8x + 66.373$; $n = 3$; $R^2 = 0.24$). For all Southern Rock Lobster combined, putative age was positively correlated with CL ($y = 5.7572x + 62.712$; $n = 7$; $R^2 = 0.74$). The CL-at-putative-age data for Tasmanian Southern Rock Lobster allowed for estimation of the von Bertalanffy growth parameters L_∞ (172.19 mm CL) and K (0.22) for comparison with those published by Gardner and van Putten (2008) (Table 12; Figure 36). The narrow size and age range in the South Australian Southern Rock Lobster precluded the estimation of von Bertalanffy parameters for that location.

Mud Crab

For male Mud Crab (87–125 mm CL) sourced from the Northern Territory (Table 4), putative age ranged from 2.8–4.2 (Figure 36) and was significantly and positively correlated with CL ($p = 0.003$; $y = 22.163x + 24.48$; $n = 7$; $R^2 = 0.81$). Biologically meaningful von Bertalanffy growth parameters could not be estimated for Mud Crab, because of the narrow putative age range sampled.

Crystal Crab

For male Crystal Crab (91.5–145.9 mm CL) sourced from Carnarvon in Western Australia (Table 4), putative age ranged from 18–33 years (Figure 37). The only female Crystal Crab (72.6 mm CL) was assigned a putative age of 37 (Figure 37). The male Crystal Crab CL-at-putative-age data was variable and, combined with the lack of younger putative age classes (>18 years – Figure 38) and the small sample size, precluded estimation of biologically meaningful von Bertalanffy growth parameters. Similarly, a linear model provided a very poor description of the male CL-at-putative-age data ($y = 1.4495x + 84.075$; $n = 4$; $R^2 = 0.17$).

Giant Crab

For Giant Crab (150–219 mm CL) sourced from Kangaroo Island in South Australia (Table 4), only some of the more prominent primary growth marks could be confidently identified (in both zygocardiac and pterocardiac ossicles). Because of this, complete counts (i.e. uninterrupted across the entire endocuticle) were not possible for this species (Figure 39), but putative ages were noted as ranging from >9–21 and Giant Crab was excluded from the elemental composition analysis.

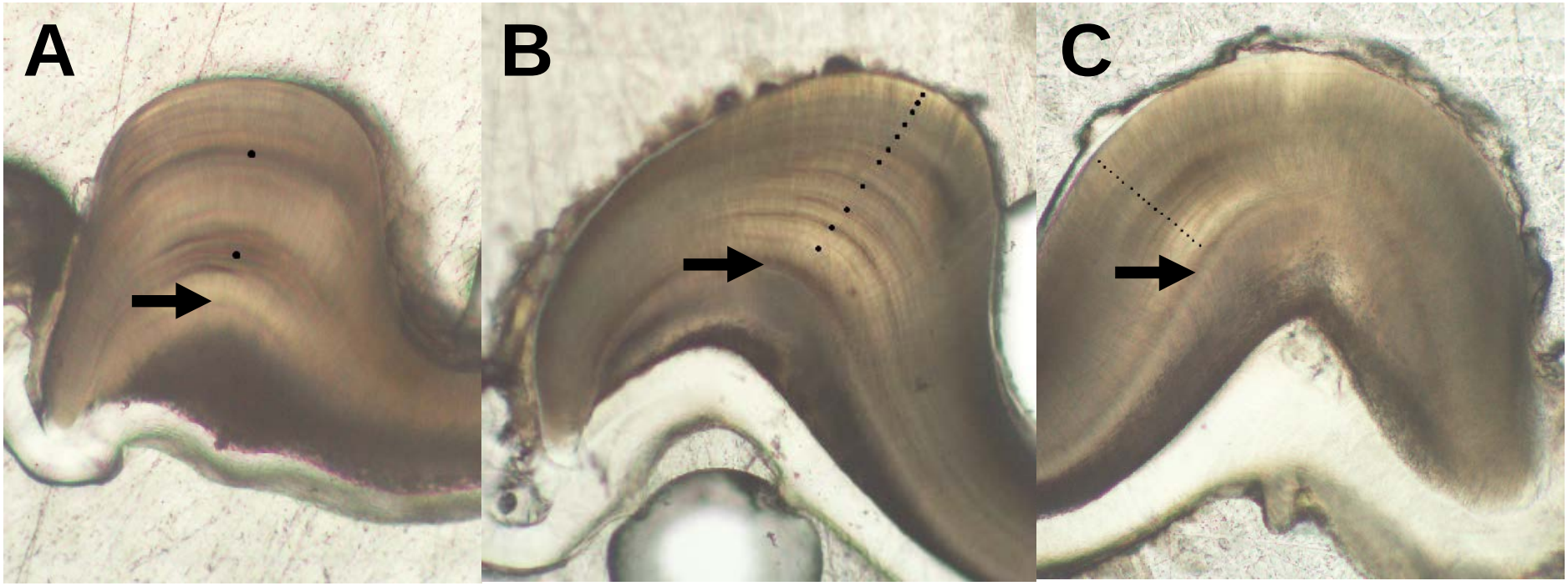

Figure 33. Pterocardiac ossicles showing the primary growth marks (black dots) in Region A used to assign putative ages to Tasmanian Southern Rock Lobster. The black arrow indicates the cuticular boundary. A: 59 mm CL female with 2 marks (putative age = 1.6 years). B: 152 mm CL male with 11 marks (putative age = 10.6 years). C: 170 mm CL male with 17 marks (putative age = 16.6 years).

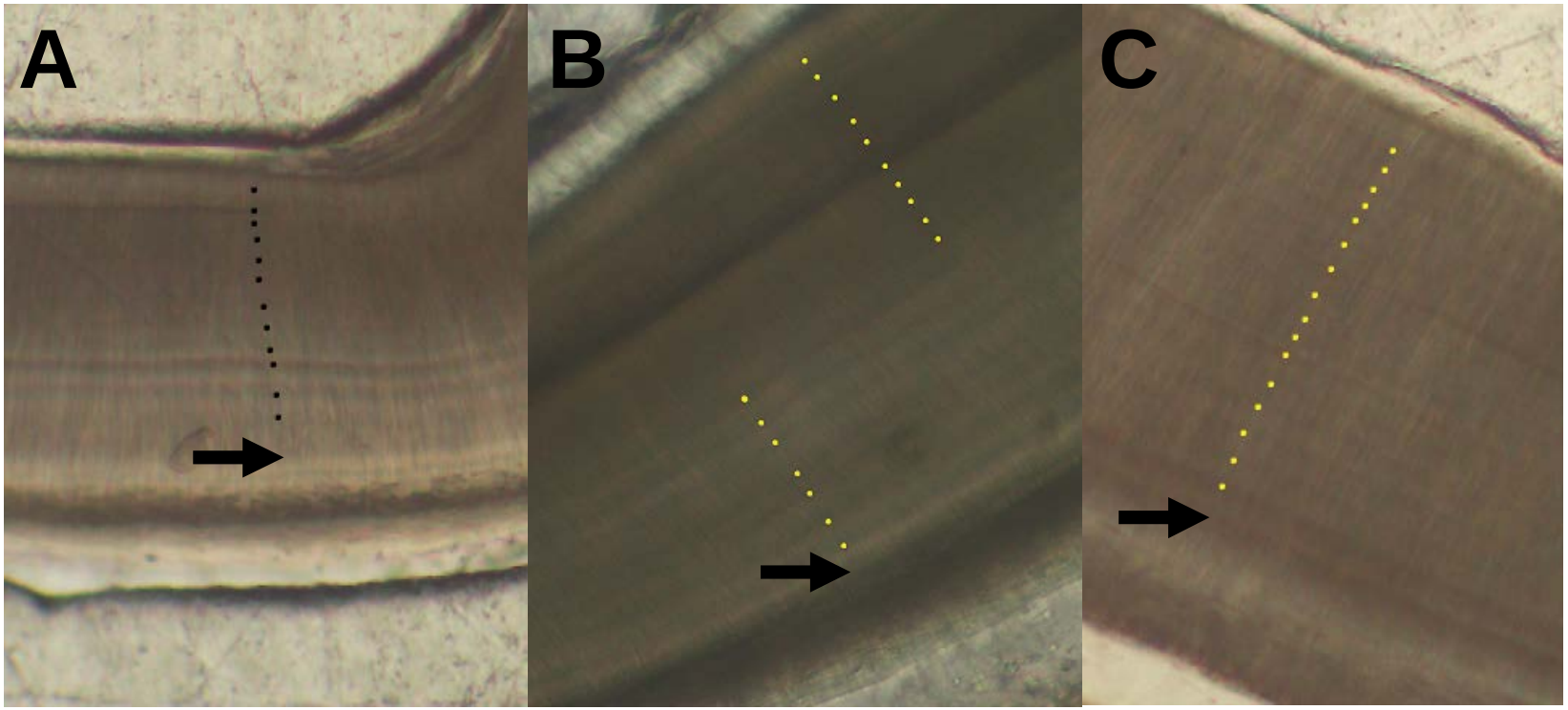

Figure 34. Pterocardiac ossicles showing the primary growth marks (black or yellow dots) in Region B used to assign putative ages to South Australian Southern Rock Lobster. The black arrow indicates the cuticular boundary. A: 122 mm CL male with 12 marks (putative age = 11.6 years). B: 121 mm CL male with 17 marks (putative age = 16.6 years). C: 171 mm CL male with 17 marks (putative age = 16.6 years).

Table 11. The von Bertalanffy growth parameters estimated for Southern Rock Lobster sourced from Taroona Waters, Tasmania. The parameters of Gardner and van Putten (2008) for Taroona Waters (male only) and other shallow-water (<40 meters depth) Tasmanian sites are included for comparison. Where available the 95% confidence intervals are given in parentheses. '-' indicates data not provided.

Source	**Method**	**Location**	L_∞	K	t_0	R^2
This project	Direct ageing	Taroona Waters	172.19 (136.45–207.92)	0.22 (0.09–0.35)	0[a]	0.99
Gardner and van Putten, 2008	Tag-and-recapture	Taroona Waters	182.44	0.23	0	-
		Maria Island	122.67	0.30	0	-
		King Island	184.26	0.26	0	-

[a]The t_0 value for this model was fixed at zero (see Methodology – section 3.1.5).

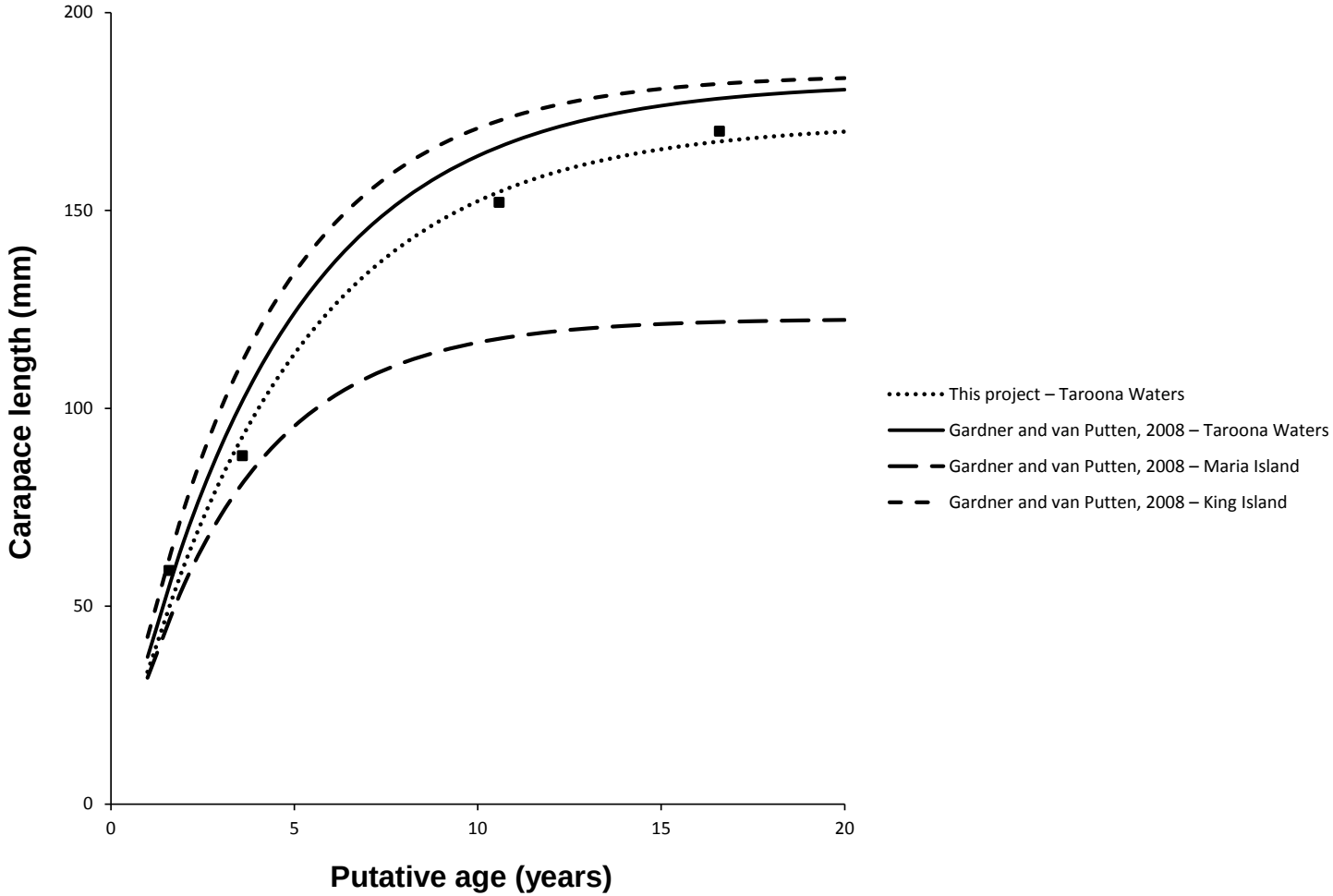

Figure 35. Comparison of von Bertalanffy growth models for Southern Rock Lobster from Tasmania. The curve for this project was generated from directly determined putative age estimates (black squares, n = 4; R^2 = 0.99) for mostly male lobster (i.e. only the smallest individual was female). The Gardner and van Putten (2008) models were derived from tag-and-recapture data for male lobster from Taroona Waters (n = 7,413), Maria Island (n = 366) and King Island (n = 472).

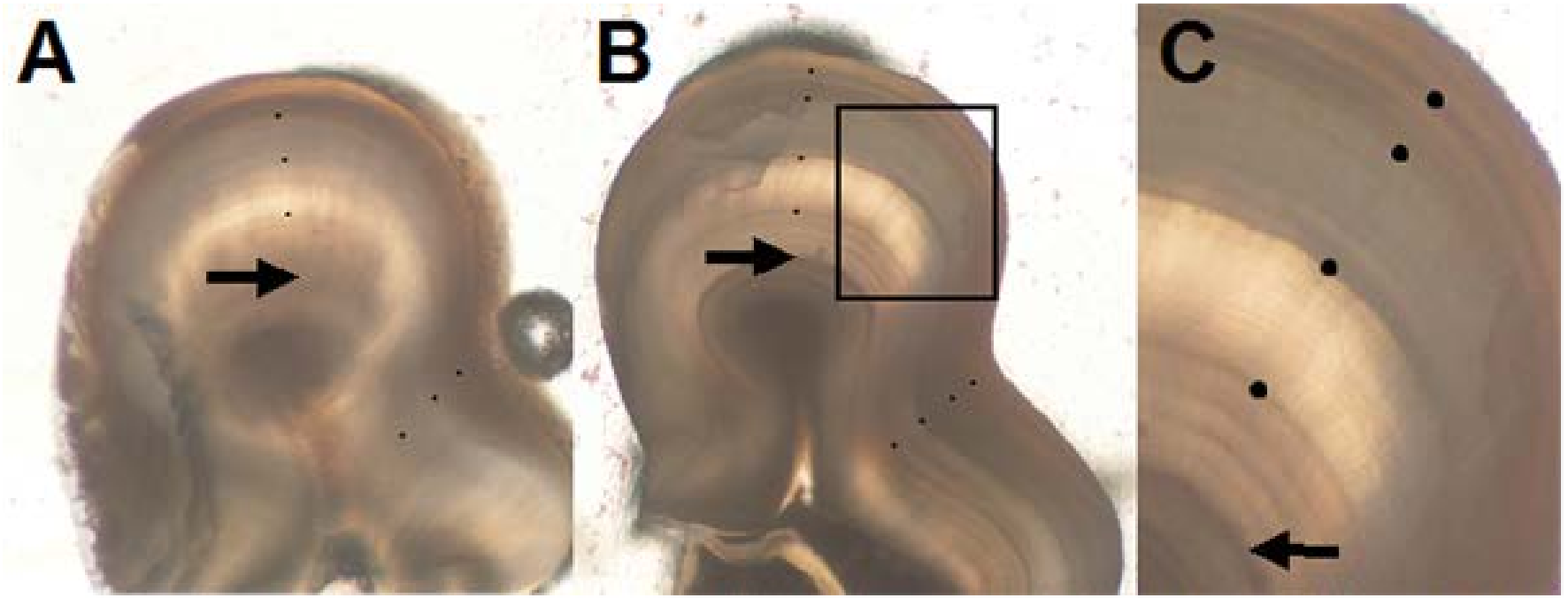

Figure 36. Zygocardiac ossicles showing the primary growth marks (black dots) used to assign putative ages to male Mud Crab. The black arrow indicates the cuticular boundary. A: 87 mm CL male with 3 marks (putative age = 2.8 years). B: 97 mm CL male with 4 marks (putative age = 3.8 years). C: Inset of B, showing the presence of strong secondary marks between the cuticular boundary and the first growth mark and structural lamellae visible between the first and second.

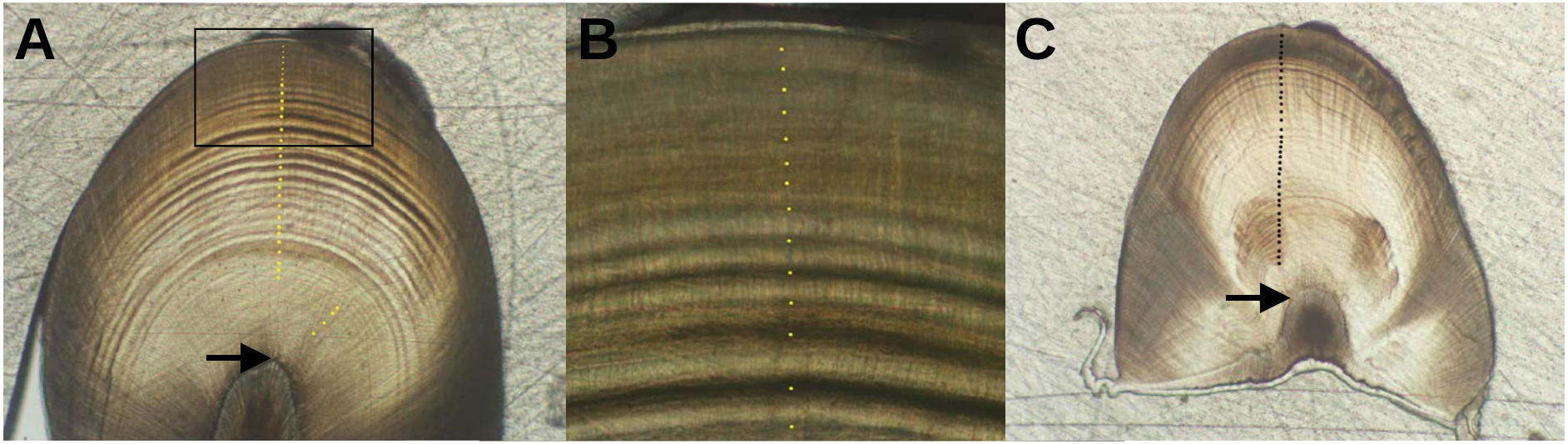

Figure 37. Zygocardiac ossicles showing the primary growth marks (yellow or black dots) used to assign putative ages to Crystal Crab. The black arrow indicates the cuticular boundary. A: 130.5 mm CL male with 33 marks (putative age = 33 years). B: Inset of C, showing the outermost 14 marks. C: 72.6 mm CL female with 37 marks (putative age = 37 years).

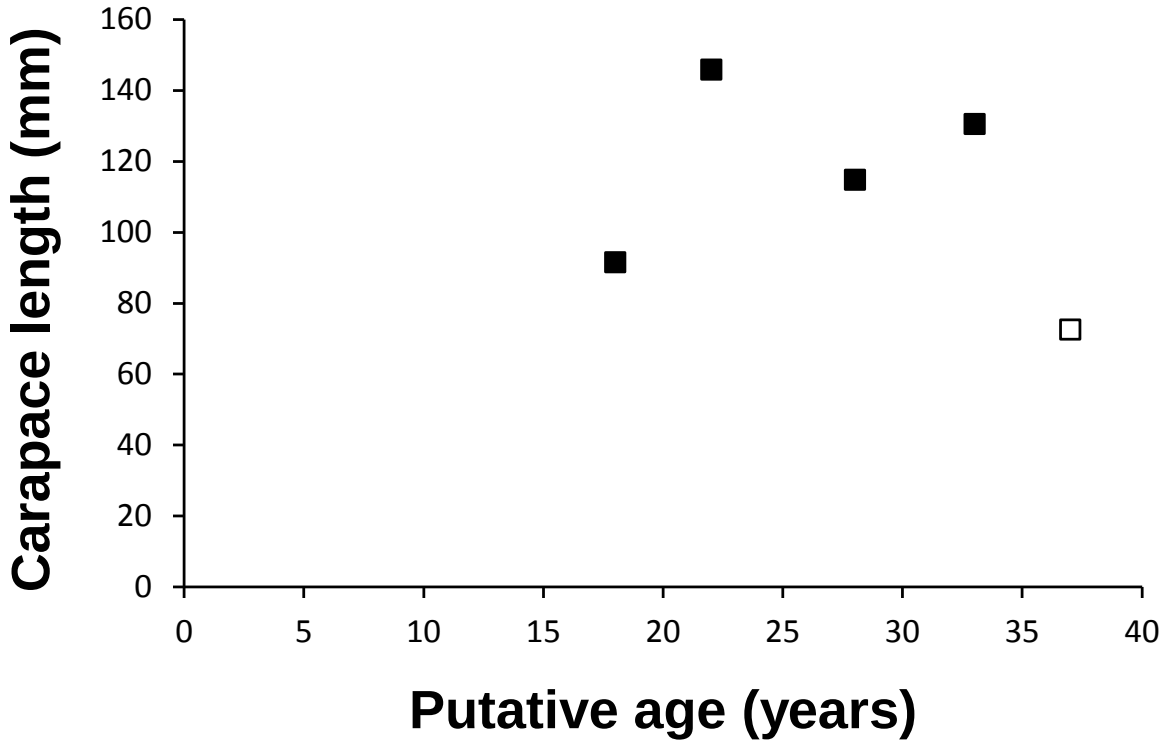

Figure 38. The CL-at-putative-age data for male (black squares) and female (white square) Crystal Crab sourced from Carnarvon, Western Australia (*n* = 5).

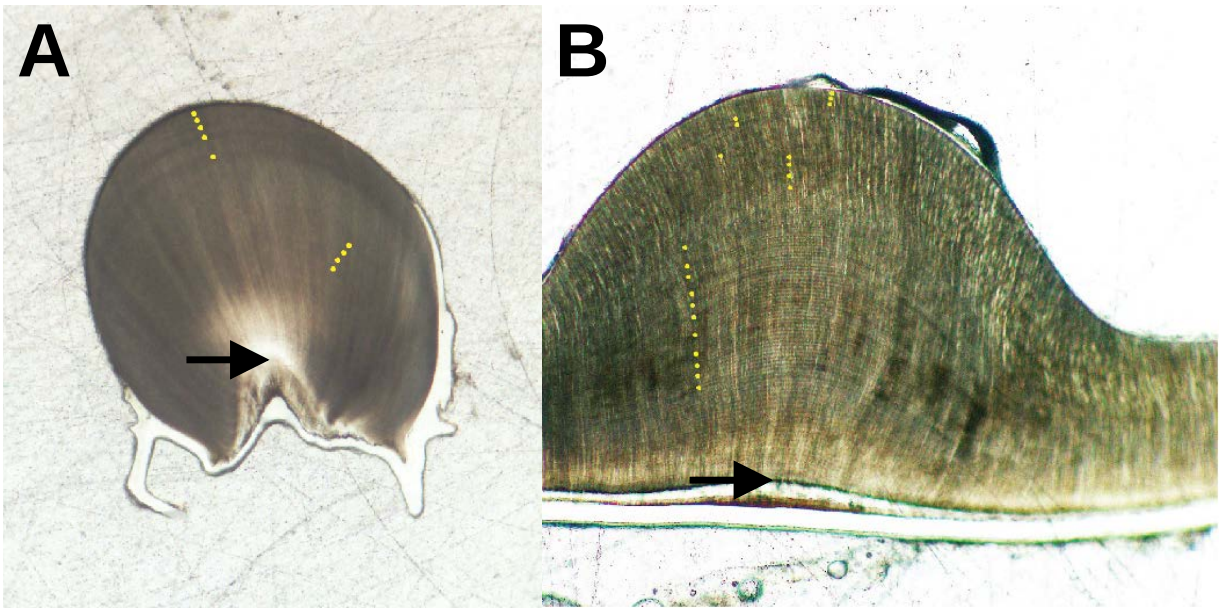

Figure 39. Sectioned Giant Crab ossicles showing the lack of continuity in primary growth marks (yellow dots) within the endocuticle. A: Zygocardiac ossicle from 150 mm CL male with >9 marks. B: Pterocardiac ossicle from a 205 mm CL male with >21 growth marks. The black arrow indicates the cuticular boundary.

4.3.2 Elemental composition analysis

Western Rock Lobster

Laser ablation-ICPMS analysis of three Western Rock Lobster ossicles revealed that the local maxima frequency (i.e. the number of peaks) for Sr:Ca, B:Ca and Mn:Ca increased with the number of primary growth marks (compare Figure 40–42), with the Sr:Ca ratio having the most consistent baseline position. For the youngest individual (WRL-19, with 3 primary growth marks), the mark position mostly coincided with local maxima in Sr:Ca and B:Ca and minima in Mn:Ca (100, 66.6 and 100% coincidence, respectively – Figure 40B–D). For the intermediately aged Western Rock Lobster (WRL-20, with 6 primary growth marks), the mark position sometimes coincided with Sr:Ca and B:Ca local maxima (50 and 83.3% coincidence, respectively – Figure 41B and C). For the oldest individual (WRL-21, with 19 primary growth marks), the mark position mostly coincided with Sr:Ca and B:Ca local maxima (89.5 and 73.7% coincidence, respectively – Figure 42B and C). For this individual, the presence of cross-cuticular compositional changes in Mn:Ca ratio with substantially greater amplitude than the local cycle made coincidence assessment difficult (e.g. Figure 42D). In all three ossicles, the B:Ca and Mn:Ca ratios increased towards the growing edge and there local maxima present before the first primary growth mark for all elemental ratios (Figure 40–41). In some samples, Sr:Ca and B:Ca local maxima were present that were not attributed to a growth mark (e.g. Figure 40C and D).

Eastern Rock Lobster

Eastern Rock Lobster endocuticle showed an elemental composition pattern similar to that of the Western Rock Lobster, with the local maxima frequency for Sr:Ca, B:Ca and Mn:Ca increasing with the number of primary growth marks (compare Figure 43–45) and the Sr:Ca ratio generally having the most consistent baseline position. For the youngest individual (ERL-22, with 4 primary growth marks), the mark position mostly coincided with local maxima for Sr:Ca and B:Ca and local minima for Mn:Ca (100, 75 and 75% coincidence, respectively – Figure 43B–D). For the intermediately aged Eastern Rock Lobster (ERL-23, with 6 primary growth marks), the mark position mostly coincided with Sr:Ca and B:Ca local maxima (83.3 and 66.6% coincidence, respectively – Figure 44B and C). For the oldest individual (ERL-24, with 16 primary growth marks), the mark position sometimes coincided with a Sr:Ca and B:Ca local maxima (48.8 and 93.8% coincidence, respectively – Figure 45B and C). In some samples, Sr:Ca and B:Ca local maxima were present that were not attributed to a growth mark (e.g. Figure 43B and C). Compared with the Sr:Ca ratio, that for B:Ca and Mn:Ca showed greater amplitude fluctuations across the endocuticle, with the overall peak intensity occurring near the growing edge (e.g. Figure 44C and D) or cuticular boundary (e.g. Figure 45C and D) in younger and older individuals, respectively.

Ornate Rock Lobster

The Ornate Rock Lobster putative age range (from 3–4 primary growth marks) for the elemental composition analysis was much narrower than that for Western and Eastern Rock Lobster. For the first individual (ORL-1, with three primary growth marks), the mark position always (100%) coincided with a Sr:Ca, B:Ca or Mn:Ca local maxima (Figure 46B–D). For the second Ornate Rock Lobster (ORL-2, with three primary growth marks), the mark position mostly coincided with a Sr:Ca, B:Ca or Mn:Ca local maxima (100, 100 and 66.6% coincidence, respectively – Figure 47B–D). Sample ORL-1 and ORL-2 also had Sr:Ca and B:Ca local maxima present that were not directly associated with a primary growth mark (e.g. Figure 46B and C). For the third individual (ORL-3, with four primary growth marks), the mark position mostly coincided with a Sr:Ca local maxima (75% coincidence – Figure 48B). In this ossicle, the B:Ca and Mn:Ca ratios were affected by an unnaturally steep increase in ^{43}Ca:^{44}Ca at the fourth mark (Figure 48C and D) that was indicative of a measurement artefact.

Southern Rock Lobster

Compared with the Western and Eastern Rock Lobster data, the elemental composition analysis for Southern Rock Lobster sourced from Tasmania and South Australia, generally showed substantially poorer coincidence with primary growth marks (Figure 49–52). For the first Southern Rock Lobster (SRL-TAS-7, with two primary growth marks), the mark position sometimes coincided with a Sr:Ca and B:Ca local maxima (100 and 50% coincidence, respectively – Figure 49B and C), but there were 8 other similar peaks that were not associated with a growth mark. Similarly, for the second Southern Rock Lobster (SRL-TAS-8, with four primary growth marks), the mark position sometimes coincided with a Sr:Ca, B:Ca or Mn:Ca local maxima (100, 25 and 75% coincidence, respectively – Figure 50B–D), but there were several (between 6–8) other similar peaks that were not associated with a growth mark. For the third Southern Rock Lobster (SRL-SA-11, with 12 primary growth marks), the mark position sometimes coincided with a Mn:Ca local maxima (41.7% coincidence – Figure 51D), but a greater proportion did not. For this individual, Sr:Ca and Mn:Ca were an order of magnitude less than that for Tasmanian Southern Rock Lobster, with low counts (and high variability) probably affecting the reliability of the data (Figure 51B and D). Further, the B:Ca ratio showed only negative values (Figure 51C), indicating low reliability and these results were discounted. For the fourth Southern Rock Lobster (SRL-SA-12, with 17 primary growth marks), all elemental ratios were affected by an unnaturally steep increase in ^{43}Ca:^{44}Ca after the fifteenth mark (Figure 52A–D) that was indicative of a measurement artefact.

Mud Crab

The three Mud Crab ossicles used for the elemental composition analysis all had four primary growth marks (Figure 53–55) that generally coincided with the position of larger-amplitude Sr:Ca and B:Ca overall maxima. For the first Mud Crab (MC-4), the primary growth mark position mostly coincided with Sr:Ca (i.e. but localized only), B:Ca and Mn:Ca overall maxima (75, 100 and 75% coincidence, respectively – Figure 53B–D). For the second Mud Crab (MC-5), the primary growth mark position always (100%) coincided with a Sr:Ca overall maxima (Figure 54B). In that sample, the mark position only sometimes (50%) coincided with a Mn:Ca overall maxima (Figure 54D) and the B:Ca ratio was discounted due to a measurement artefact occurring before the fourth mark (Figure 54C). For the third Mud Crab (MC-6), the primary growth mark position always (100%) coincided with an overall Sr:Ca, B:Ca maxima or local Mn:Ca maxima (Figure 55B–D).

Crystal Crab

For all three Crystal Crab ossicles used for the elemental composition analysis, the Mn:Ca ratio showed greater amplitude fluctuations (compared with Sr:Ca and B:Ca) across the endocuticle, with overall peak intensity occurring at the cuticular boundary and growing edge making interpretation difficult (Figure 56D, 57D and 58D). For the first Crystal Crab (CC-16, with 37 primary growth marks), the mark position mostly coincided with a Sr:Ca and B:Ca local maxima (59.5 and 62.2% coincidence, respectively – Figure 56B and C). For the second Crystal Crab (CC-17), the mark position mostly coincided with a Sr:Ca and B:Ca local maxima (87.0 and 69.6% coincidence, respectively – Figure 57B and C). For the third Crystal Crab (CC-18), the mark position mostly coincided with a Sr:Ca and B:Ca local maxima (88.2 and 76.5% coincidence, respectively – Figure 58B and C). In all three ossicles, there were Sr:Ca, B:Ca and Mn:Ca local maxima present before the first primary growth mark (Figure 56–58).

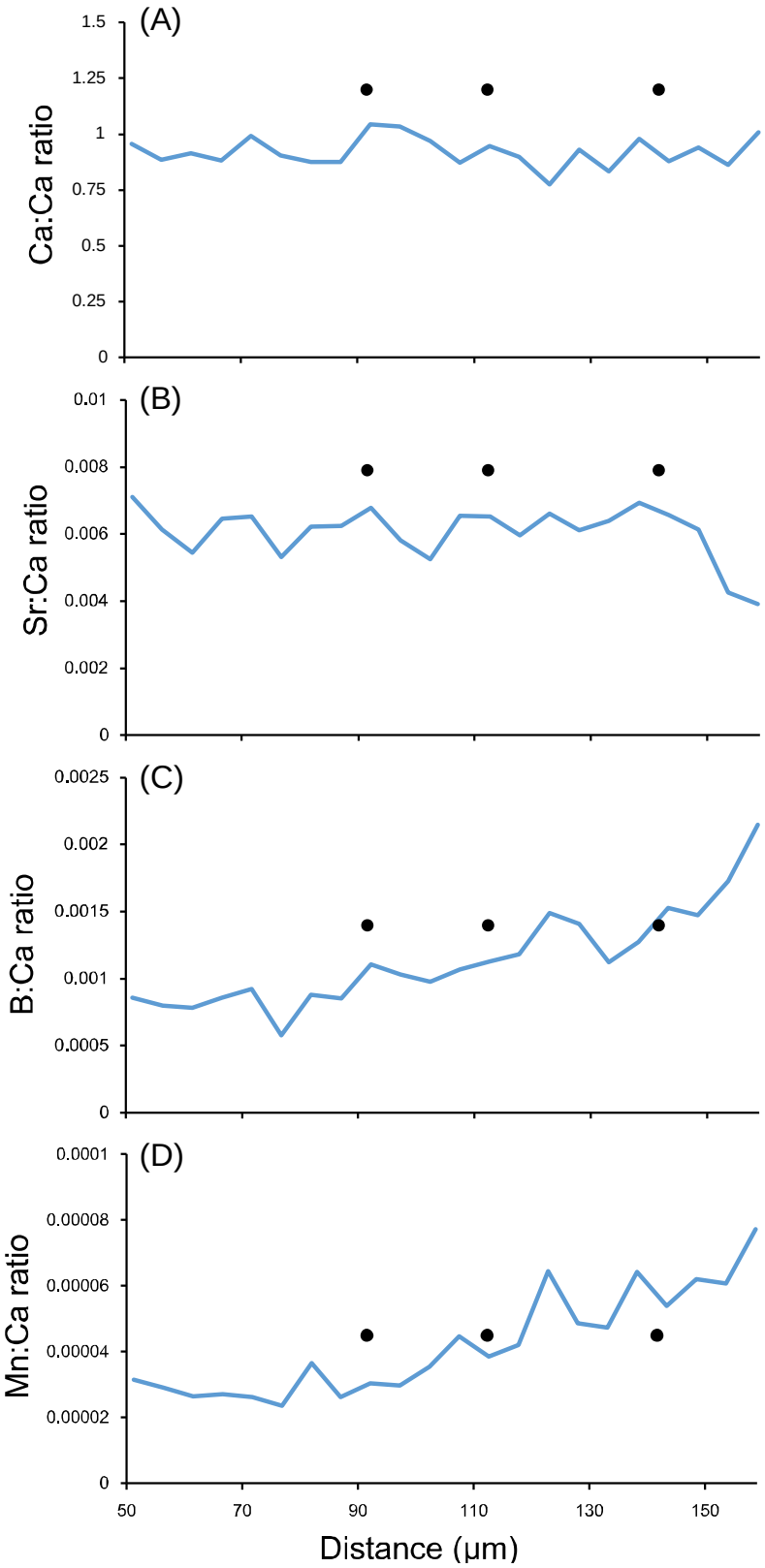

Figure 40. Ion ratios showing compositional changes (blue line) measured across a Western Rock Lobster ossicle (WRL-19). The relative position of the three primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

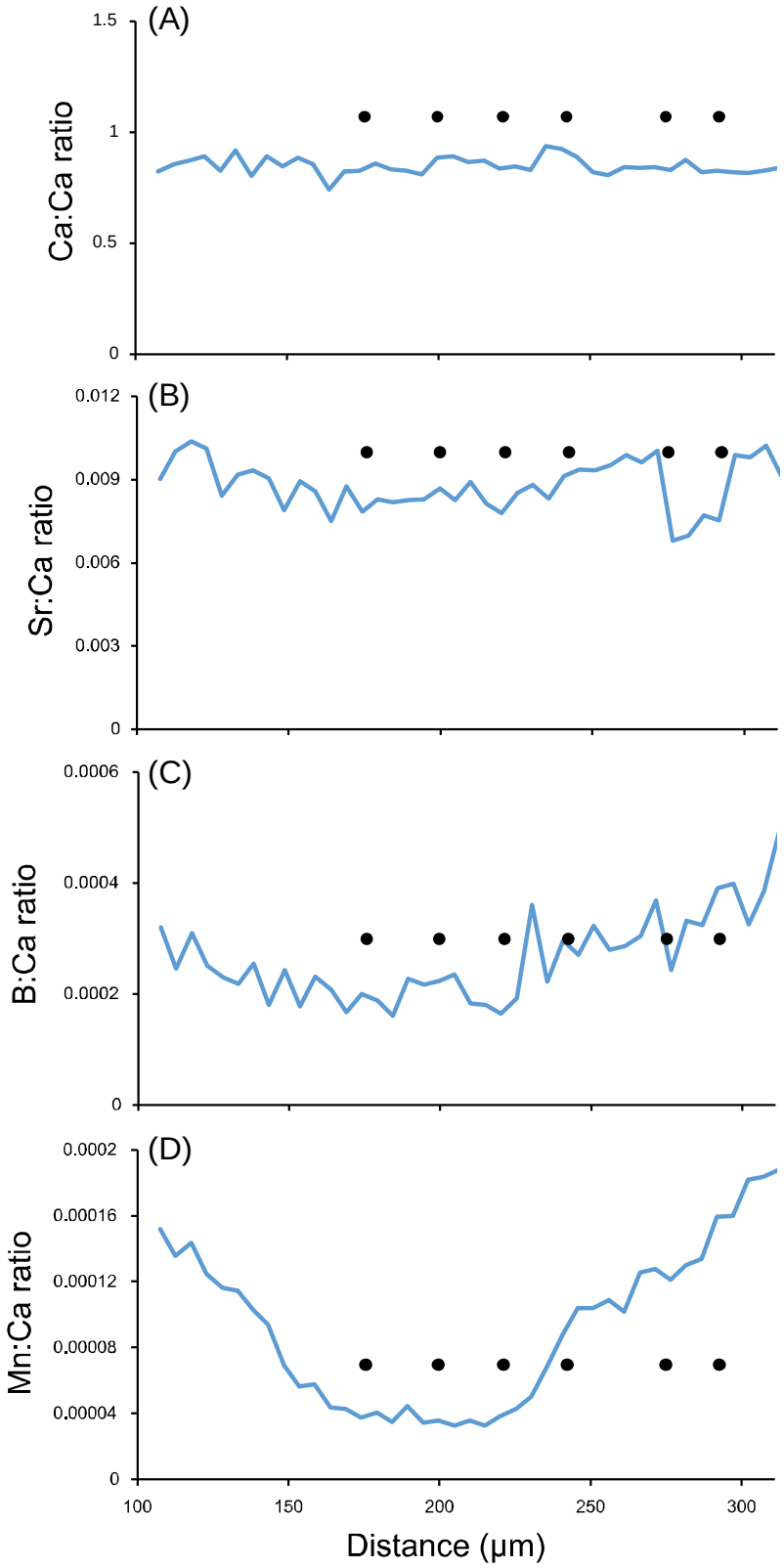

Figure 41. Ion ratios showing compositional changes (blue line) measured across a Western Rock Lobster ossicle (WRL-20). The relative position of the six primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

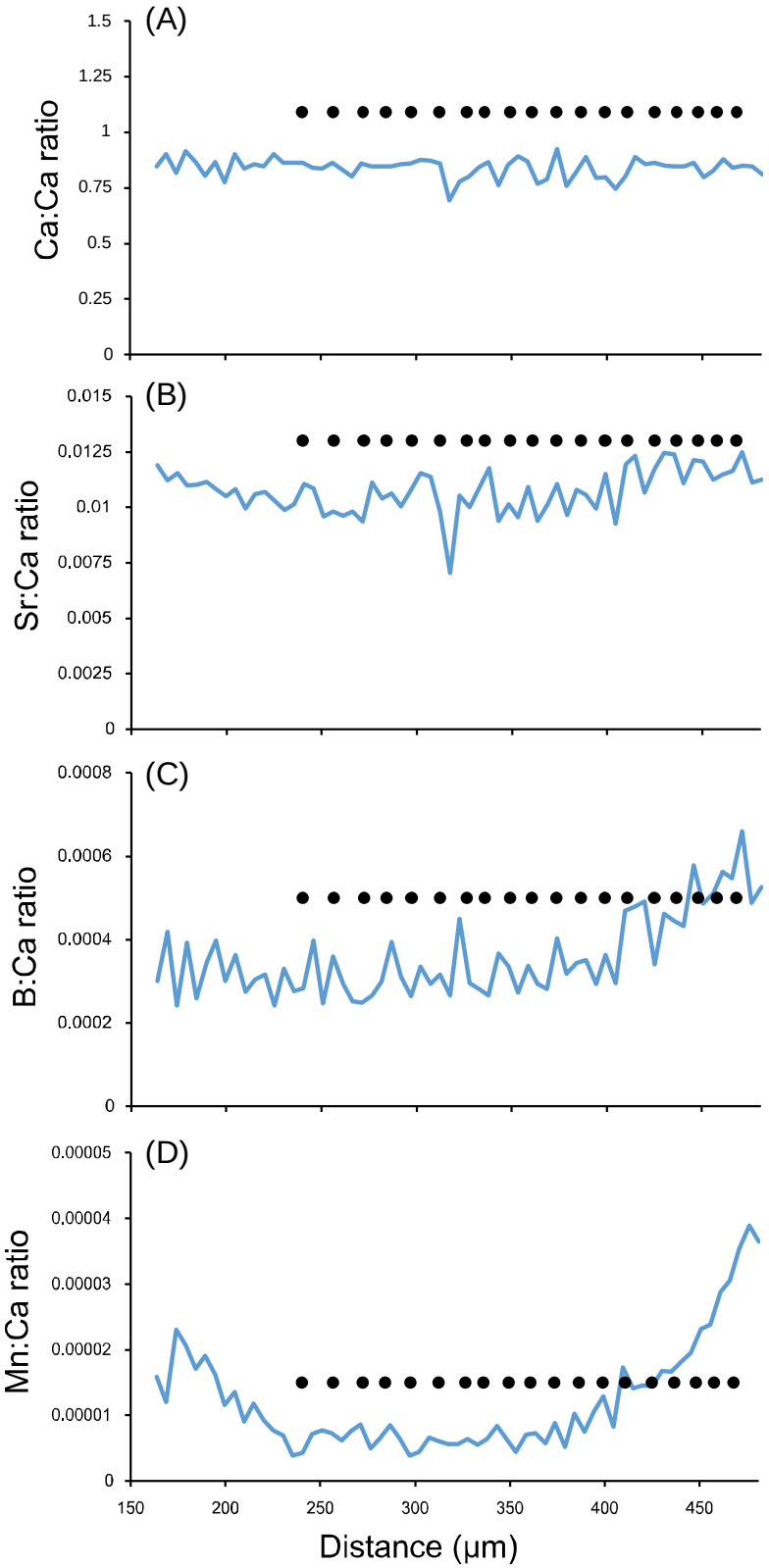

Figure 42. Ion ratios showing compositional changes (blue line) measured across a Western Rock Lobster ossicle (WRL-21). The relative position of the nineteen primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

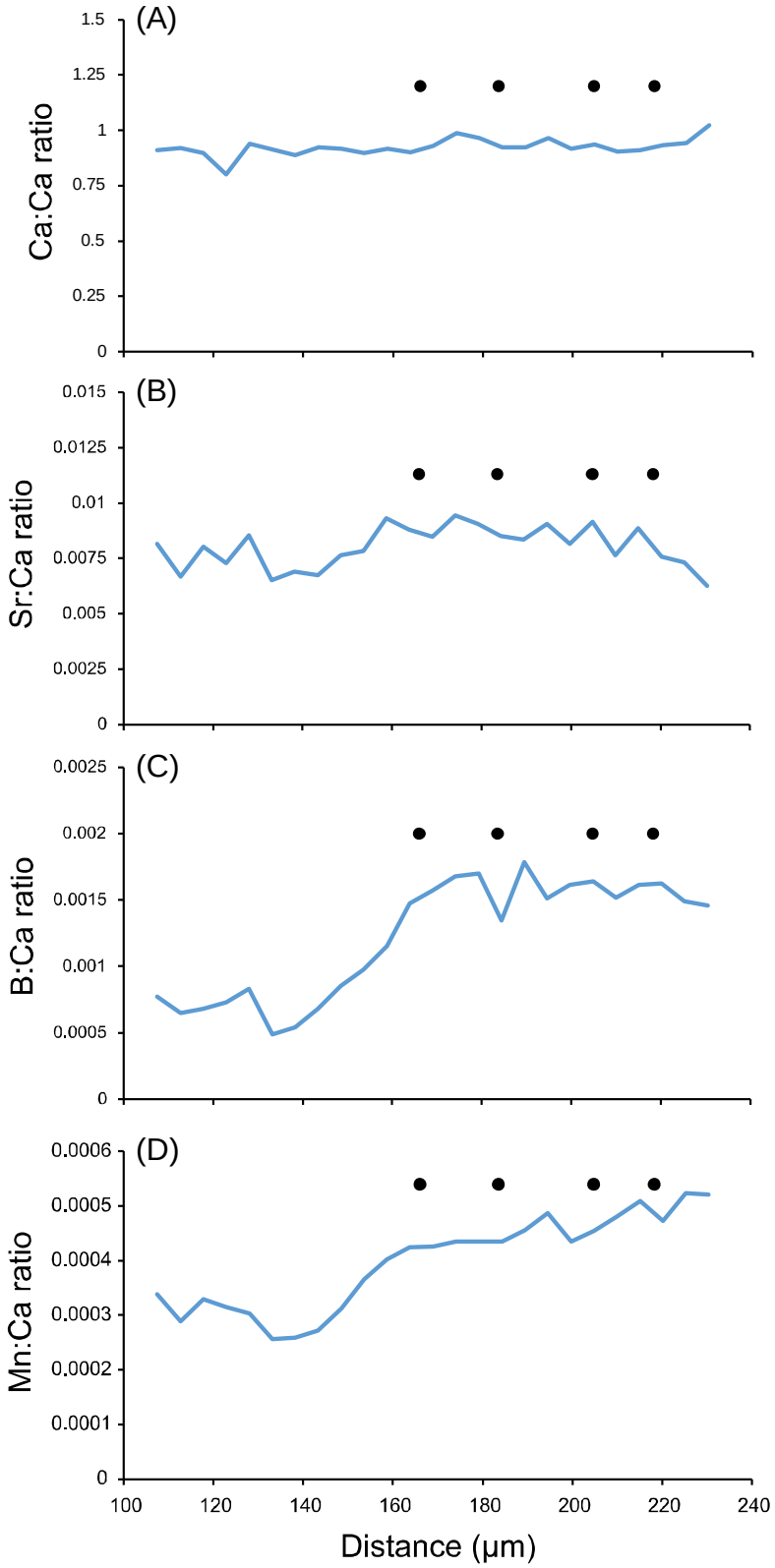

Figure 43. Ion ratios showing compositional changes (blue line) measured across an Eastern Rock Lobster ossicle (ERL-22). The relative position of the four primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

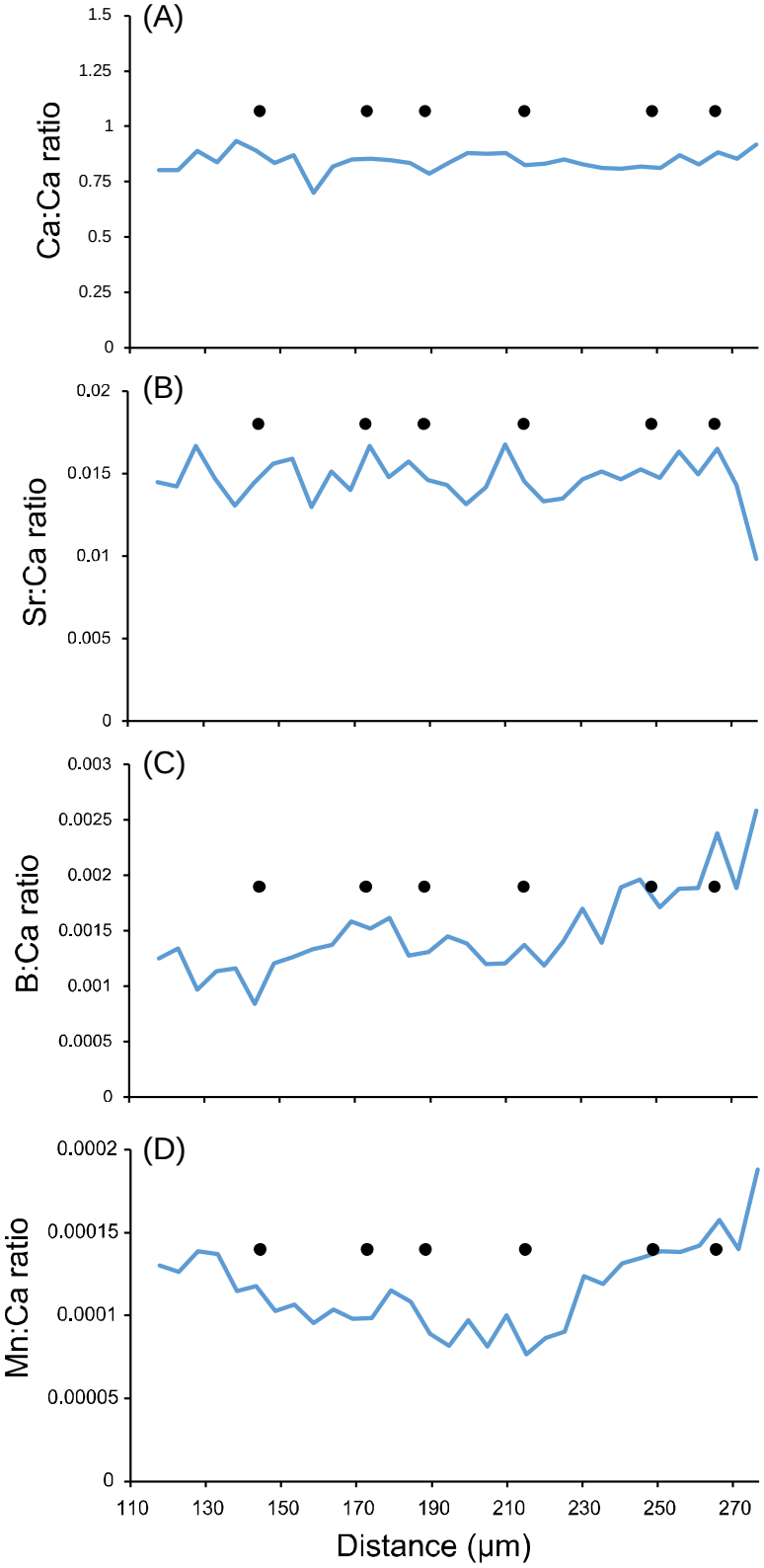

Figure 44. Ion ratios showing compositional changes (blue line) measured across an Eastern Rock Lobster ossicle (ERL-23). The relative position of the six primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

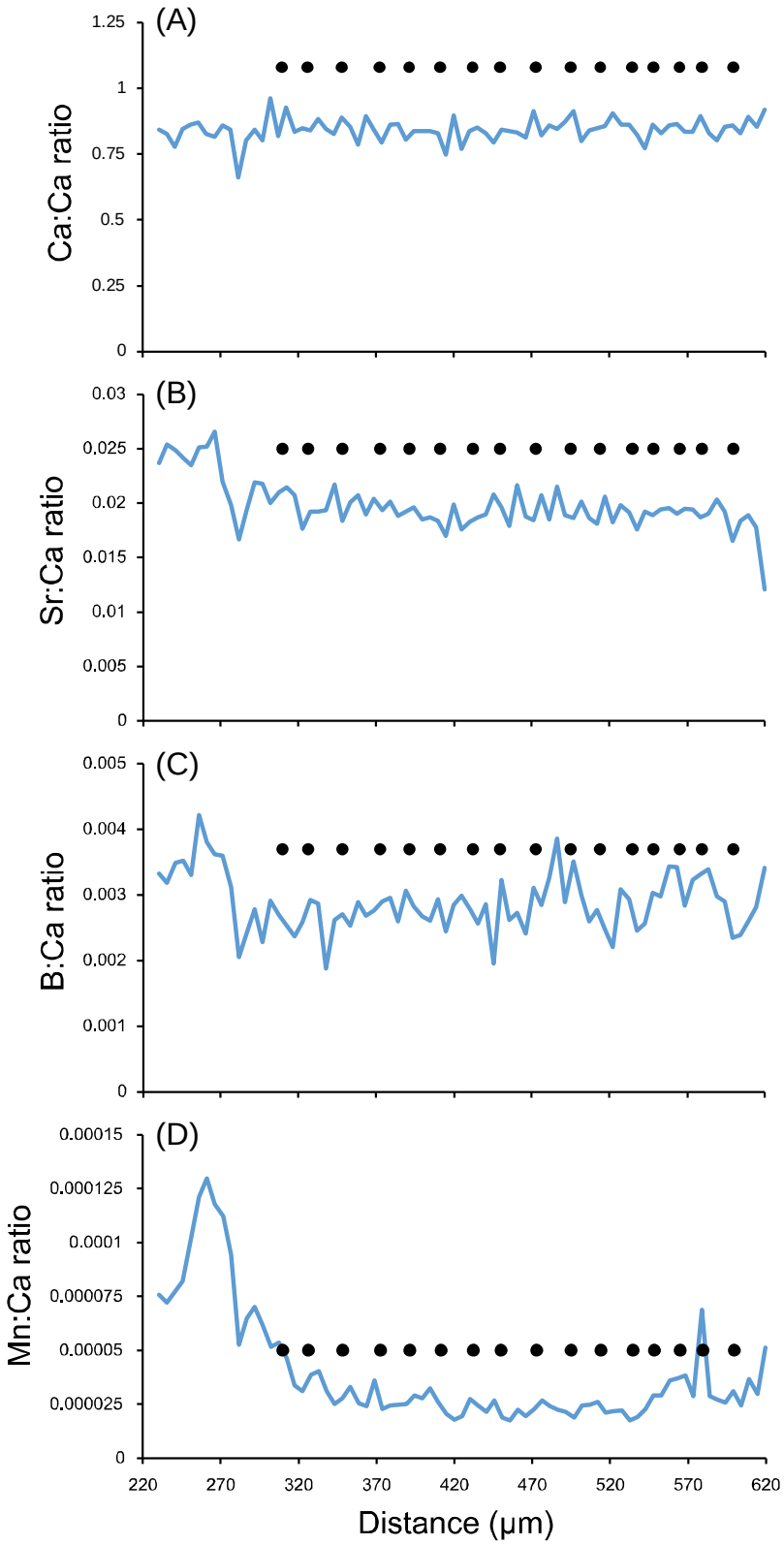

Figure 45. Ion ratios showing compositional changes (blue line) measured across an Eastern Rock Lobster ossicle (ERL-24). The relative position of the sixteen primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

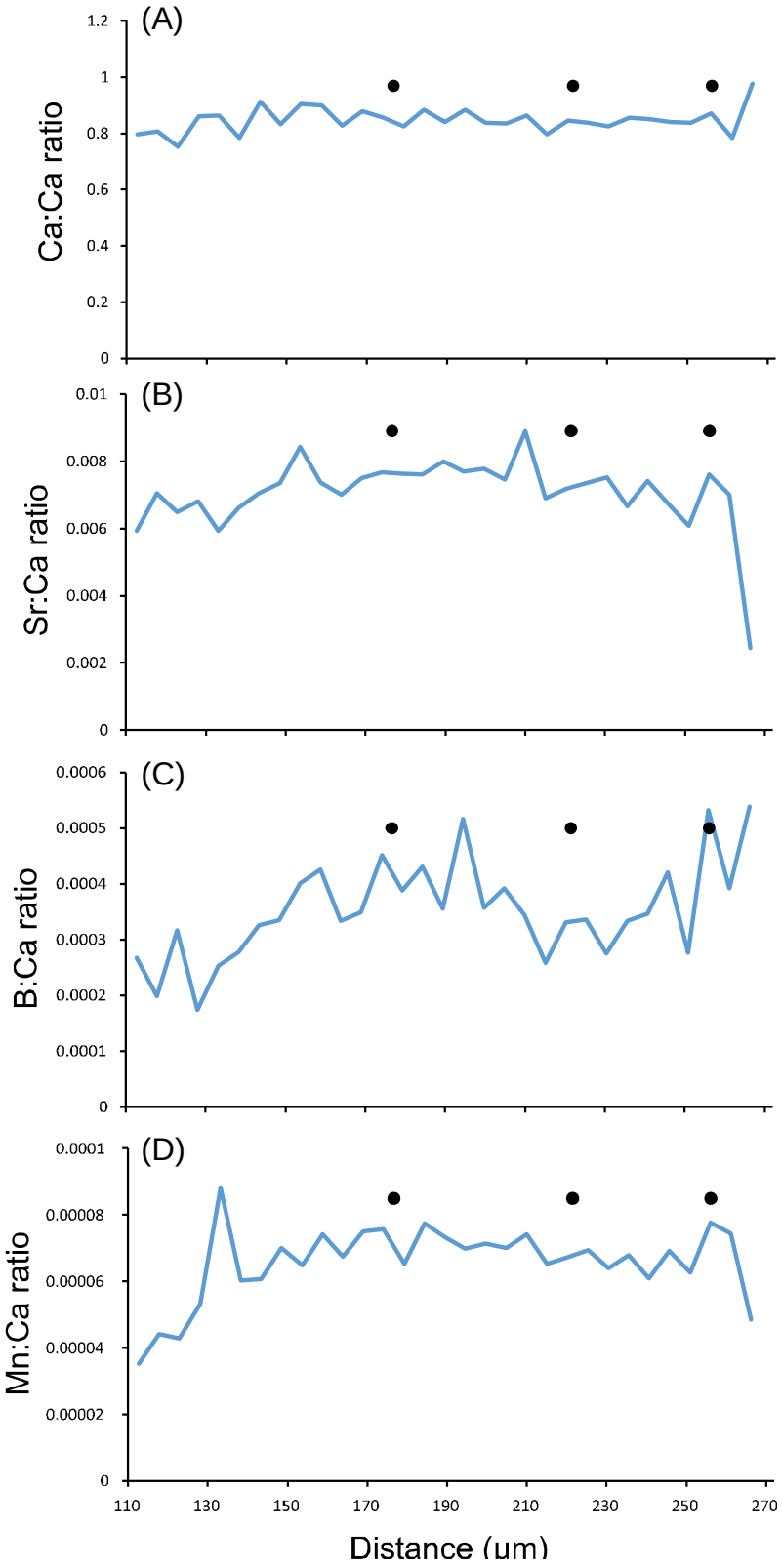

Figure 46. Ion ratios showing compositional changes (blue line) measured across an Ornate Rock Lobster ossicle (ORL-1). The relative position of the three primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

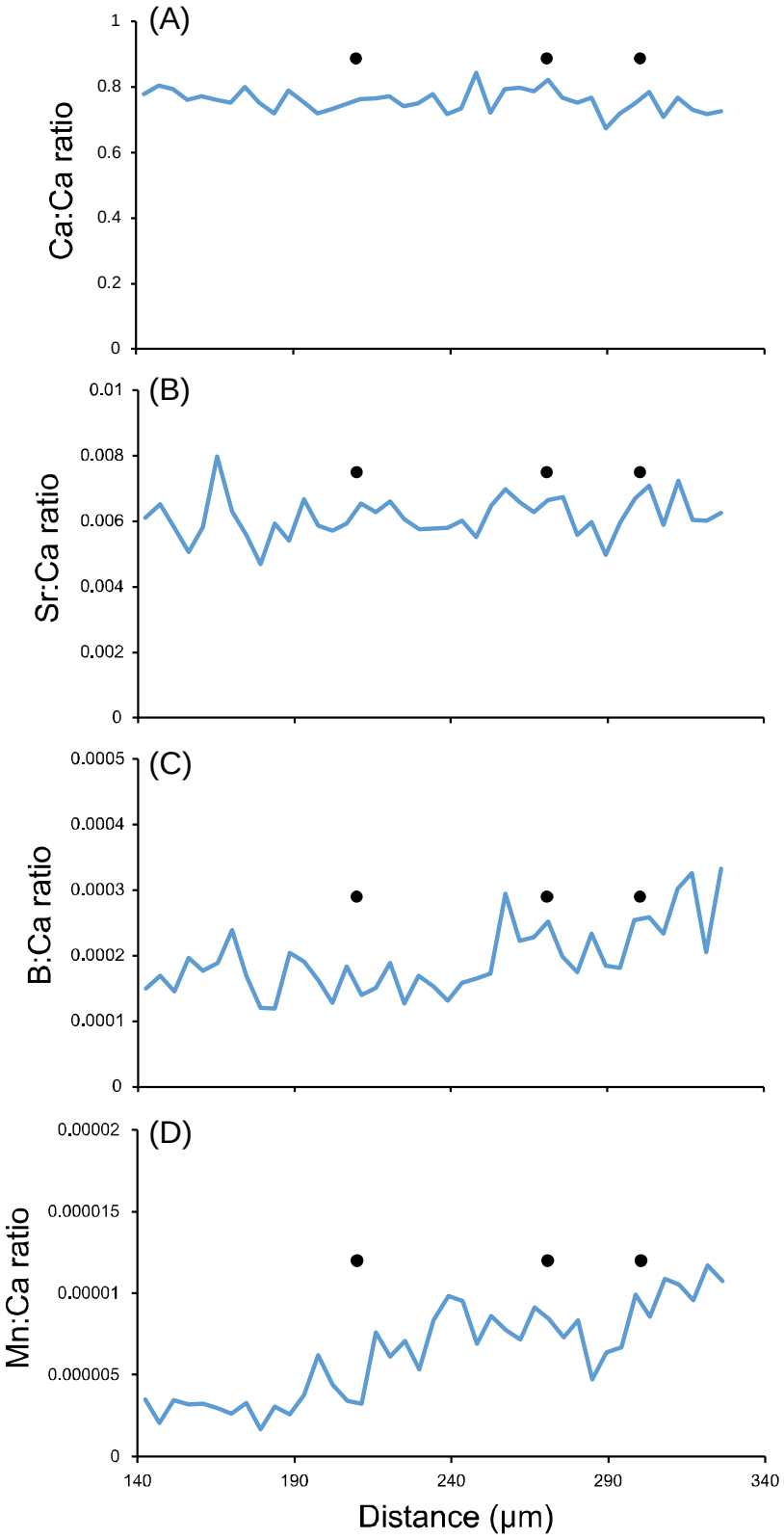

Figure 47. Ion ratios showing compositional changes (blue line) measured across an Ornate Rock Lobster ossicle (ORL-2). The relative position of the three primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

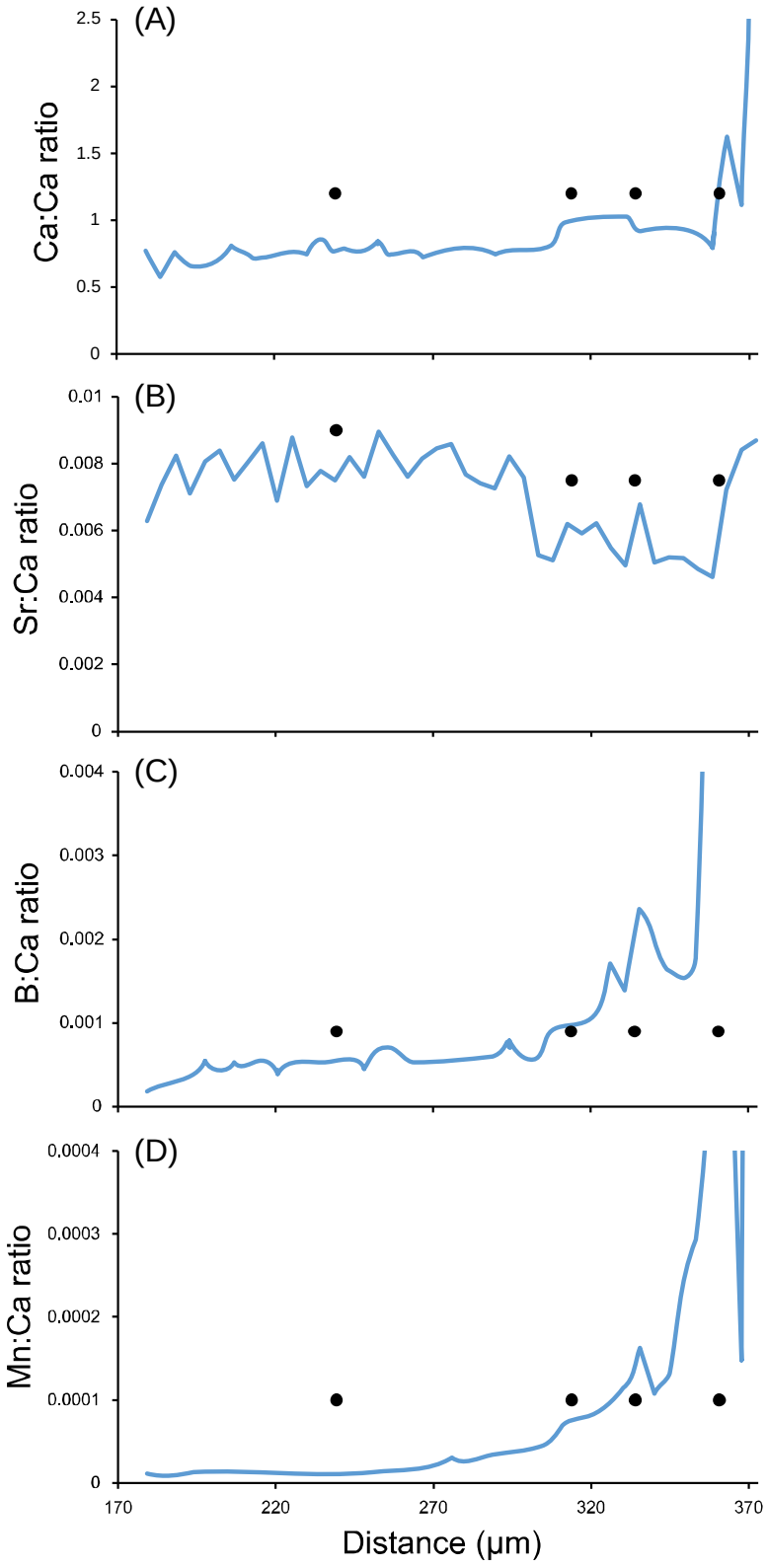

Figure 48. Ion ratios showing compositional changes (blue line) measured across an Ornate Rock Lobster ossicle (ORL-3). The relative position of the four primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

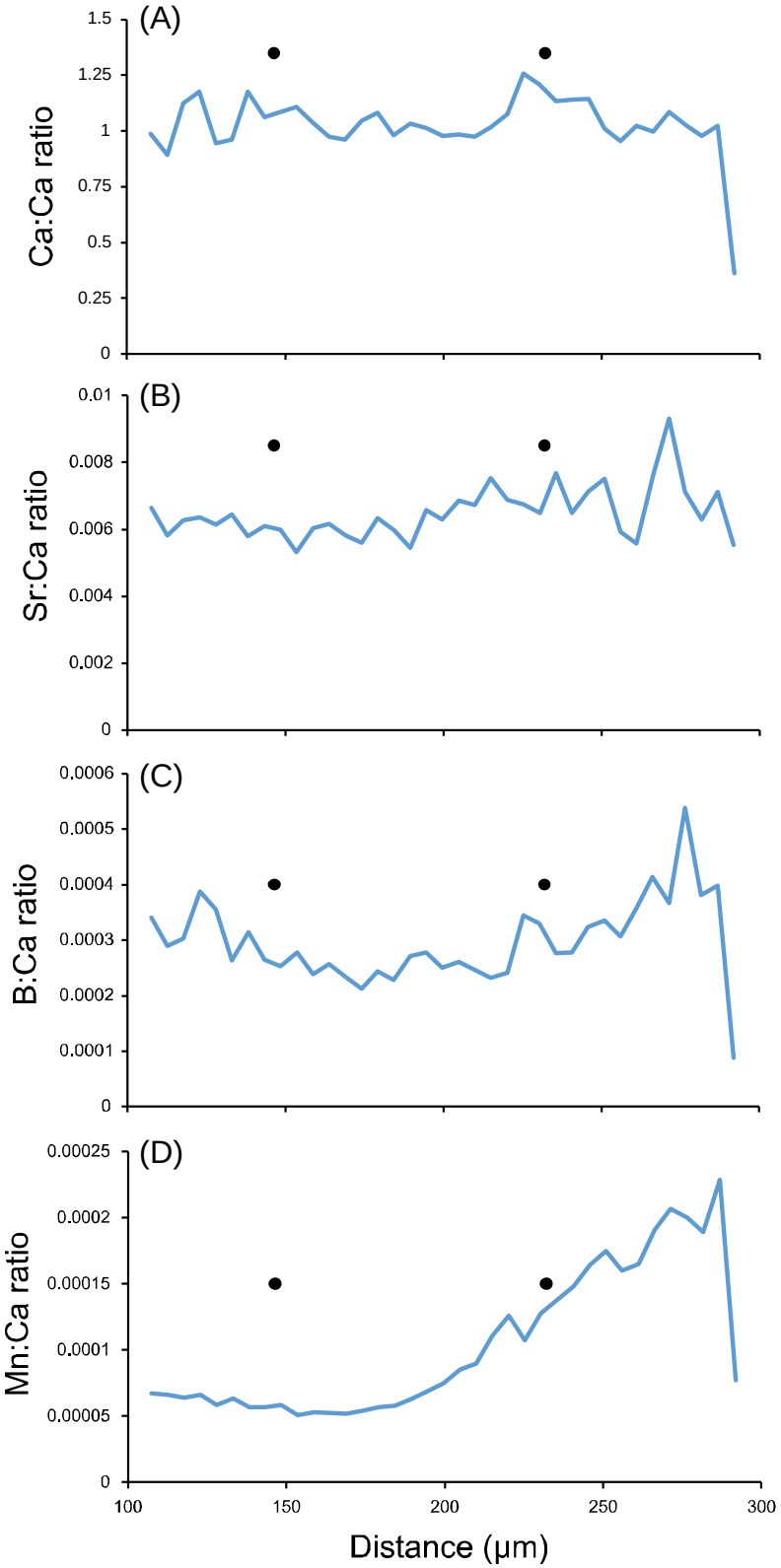

Figure 49. Ion ratios showing compositional changes (blue line) measured across a Southern Rock Lobster ossicle (SRL-TAS-7). The relative position of the two primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

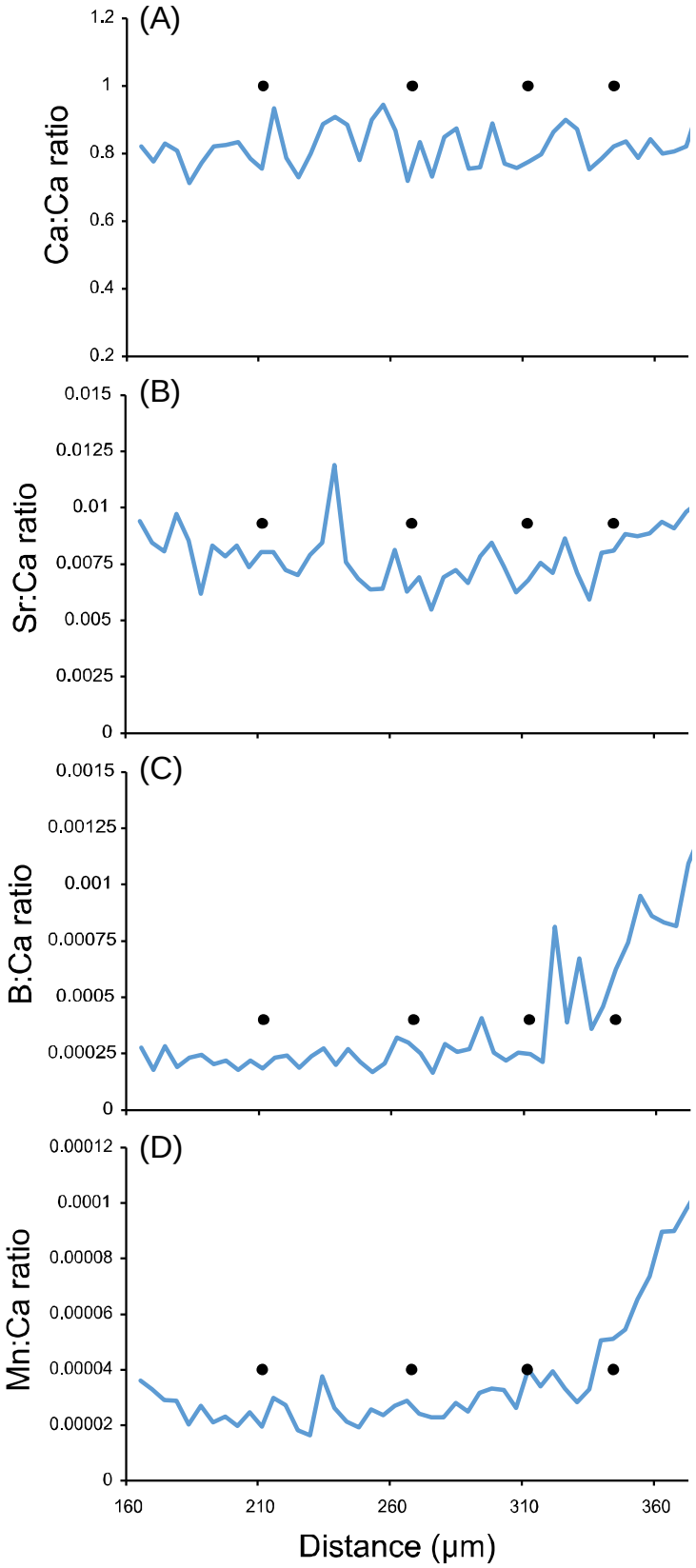

Figure 50. Ion ratios showing compositional changes (blue line) measured across a Southern Rock Lobster ossicle (SRL-TAS-8). The relative position of the two primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

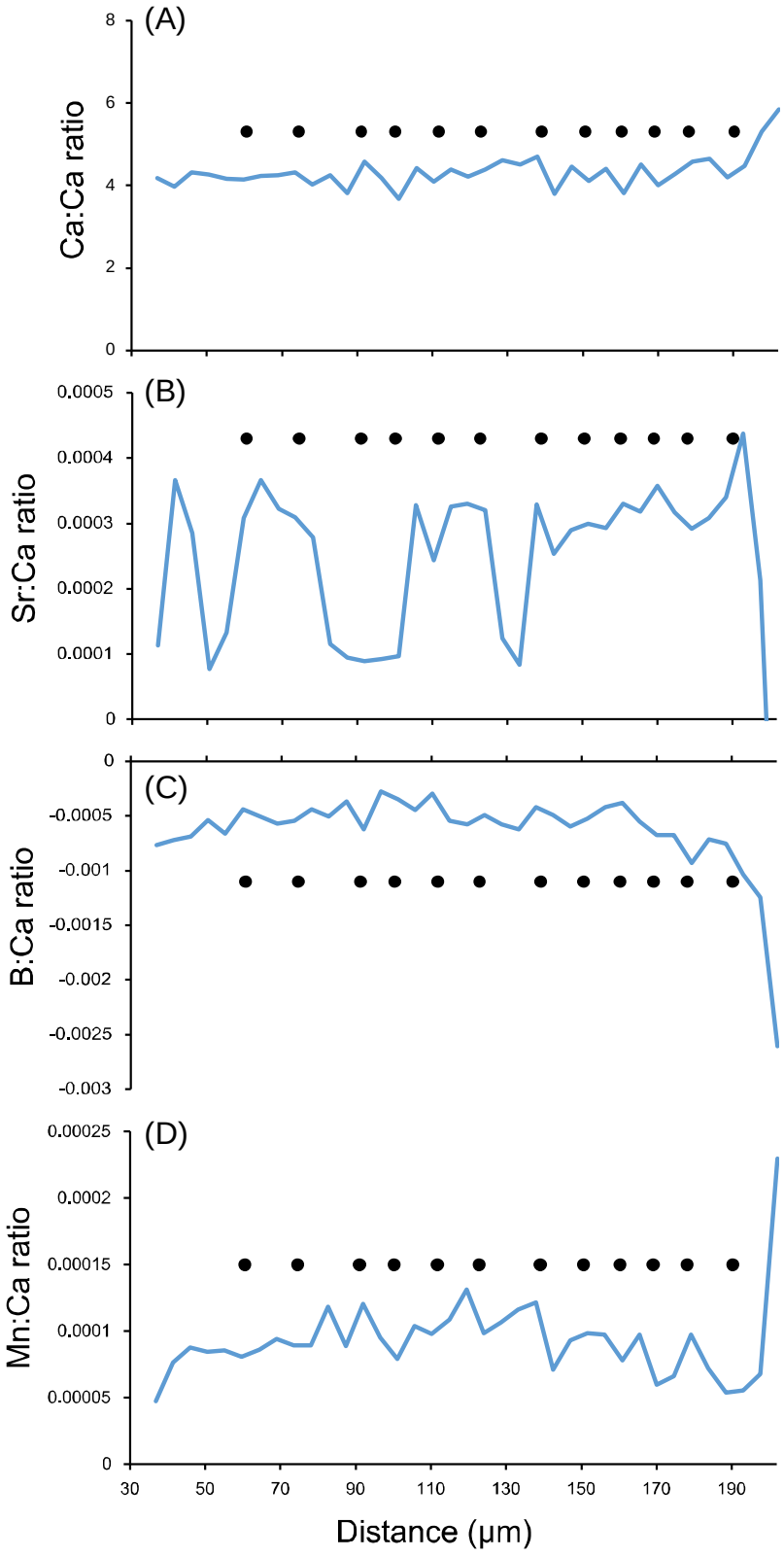

Figure 51. Ion ratios showing compositional changes (blue line) measured across a Southern Rock Lobster ossicle (SRL-SA-11). The relative position of the twelve primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

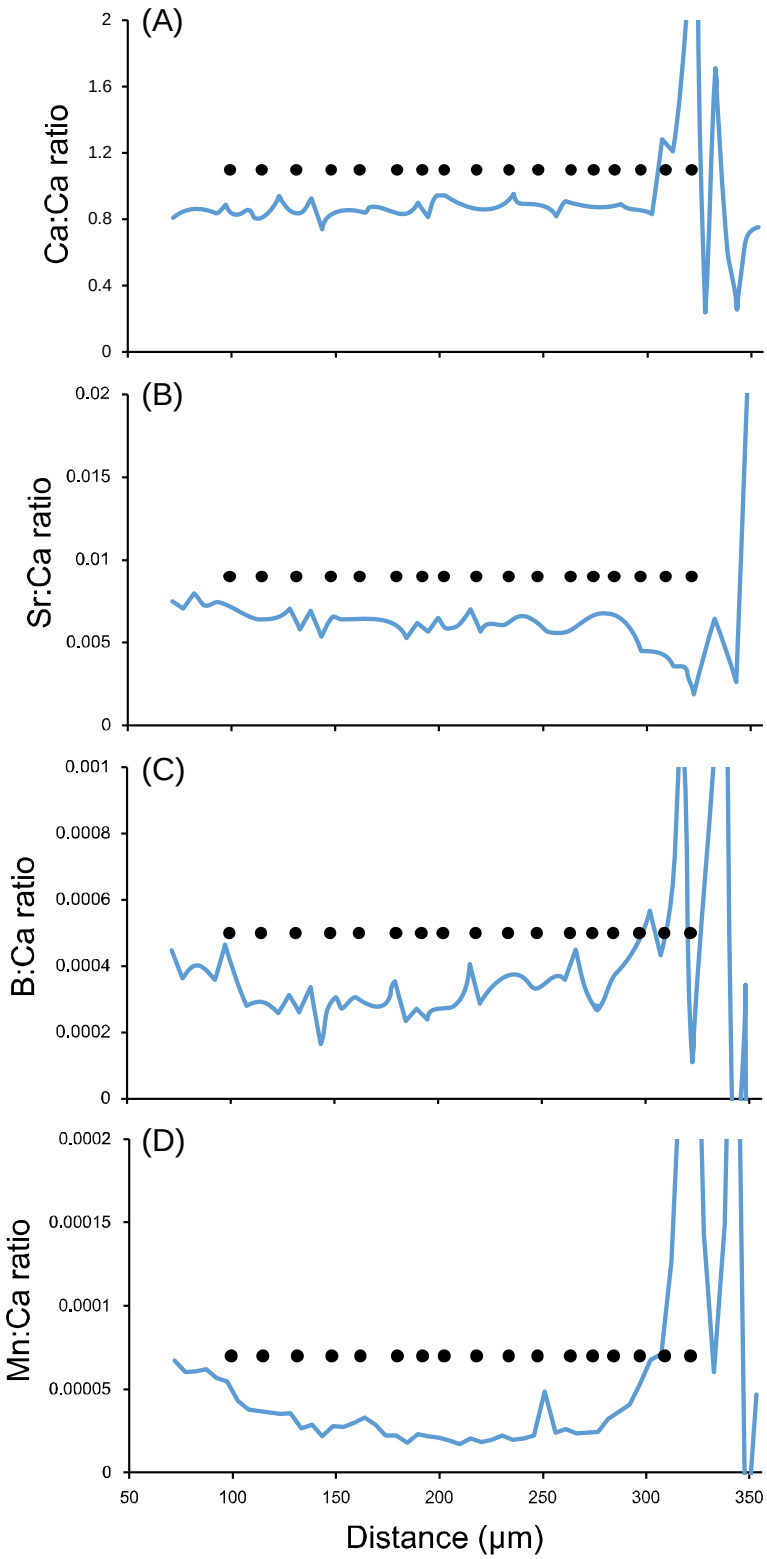

Figure 52. Ion ratios showing compositional changes (blue line) measured across a Southern Rock Lobster ossicle (SRL-SA-12). The relative position of the seventeen primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

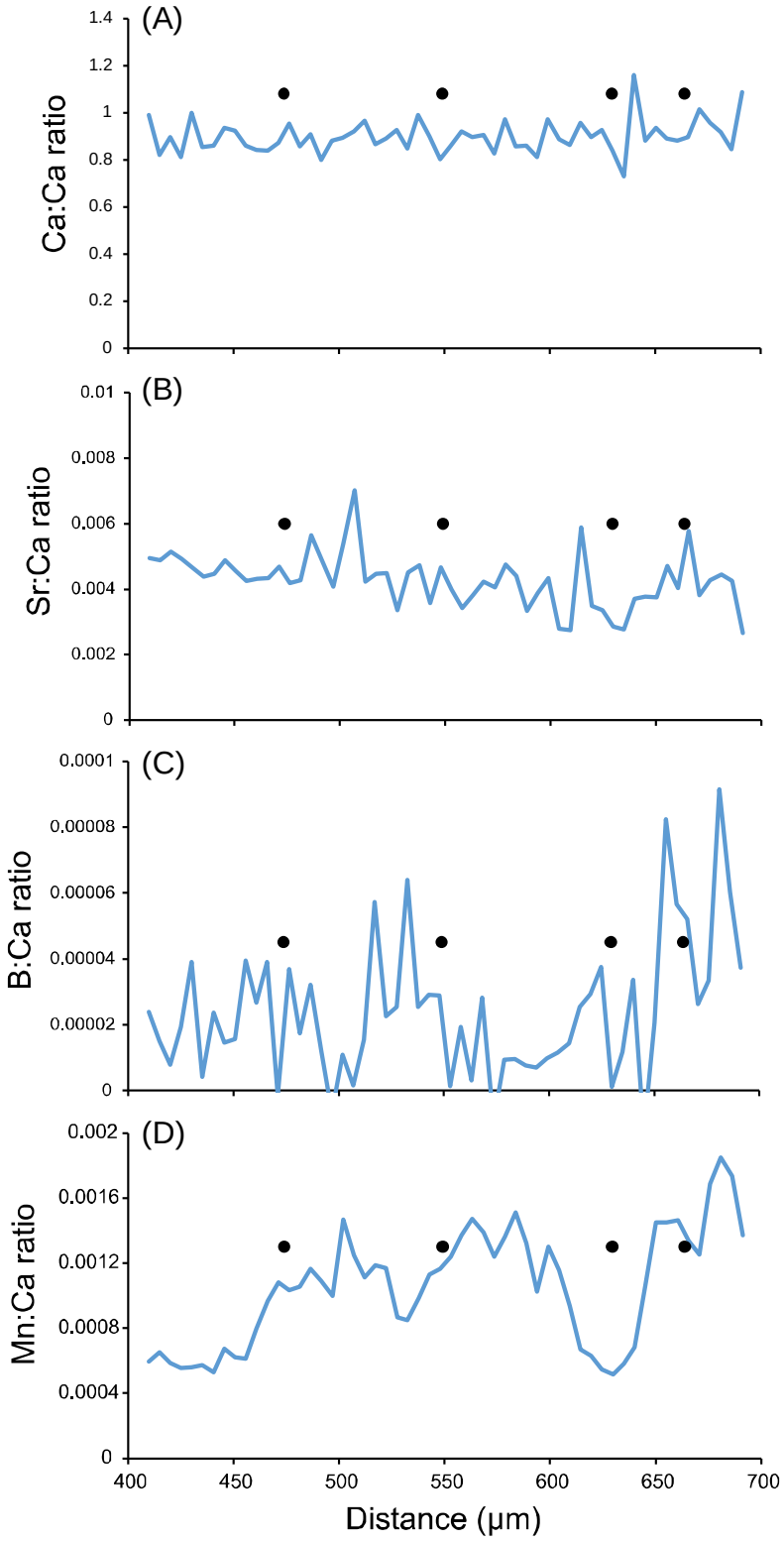

Figure 53. Ion ratios showing compositional changes (blue line) measured across a Mud Crab ossicle (MC-4). The relative position of the four primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

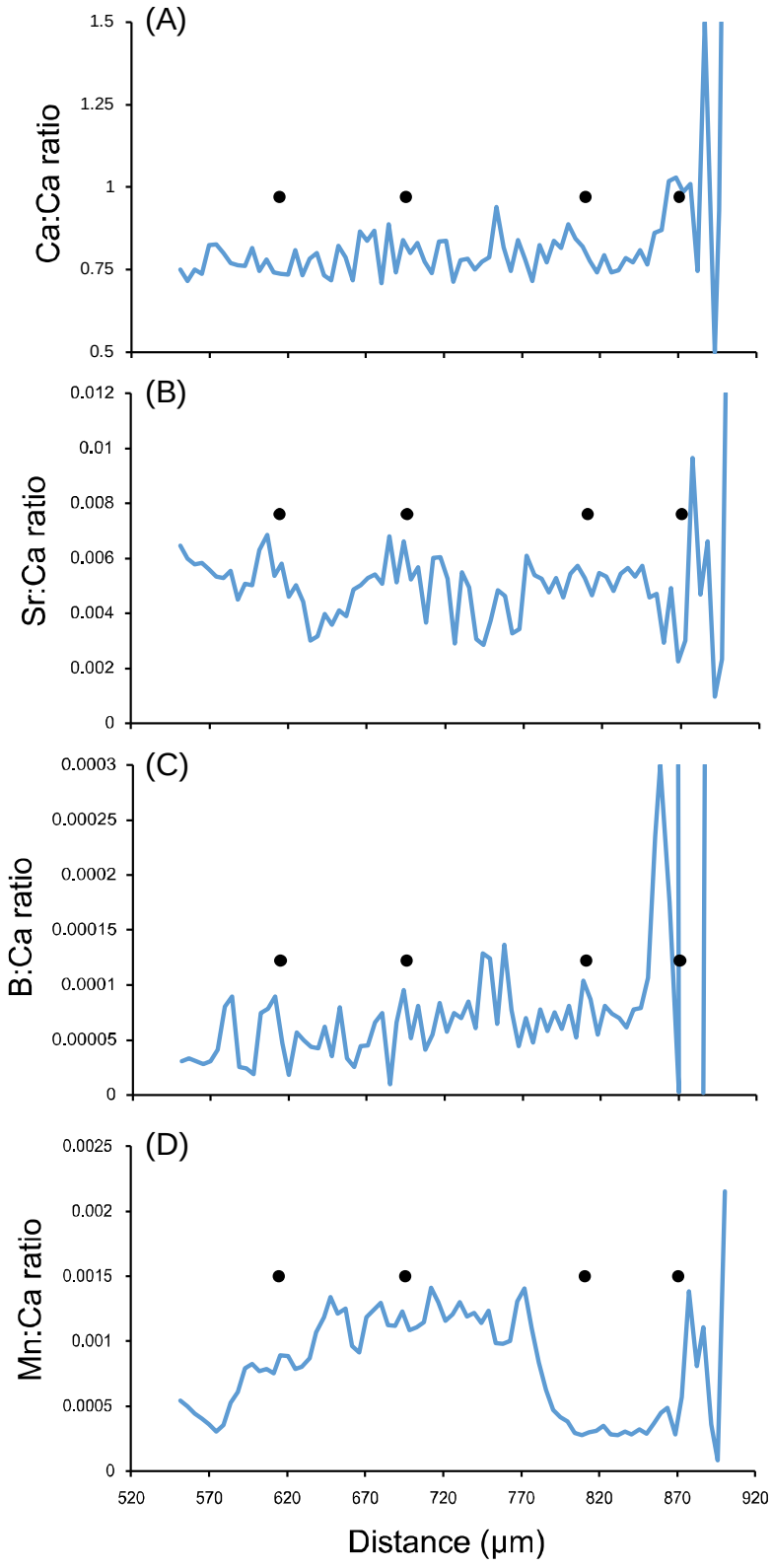

Figure 54. Ion ratios showing compositional changes (blue line) measured across a Mud Crab ossicle (MC-5). The relative position of the four primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

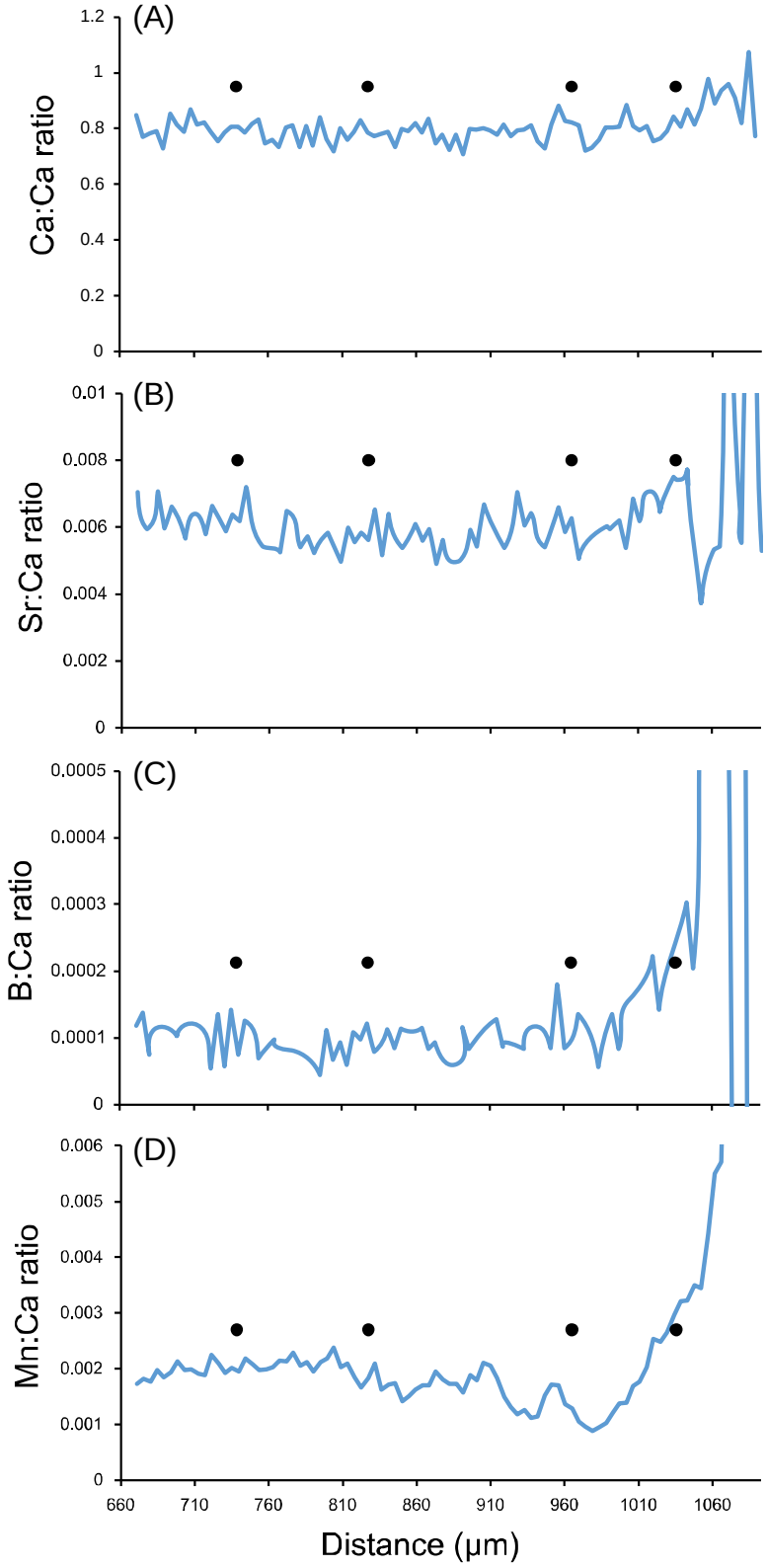

Figure 55. Ion ratios showing compositional changes (blue line) measured across a Mud Crab ossicle (MC-6). The relative position of the four primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

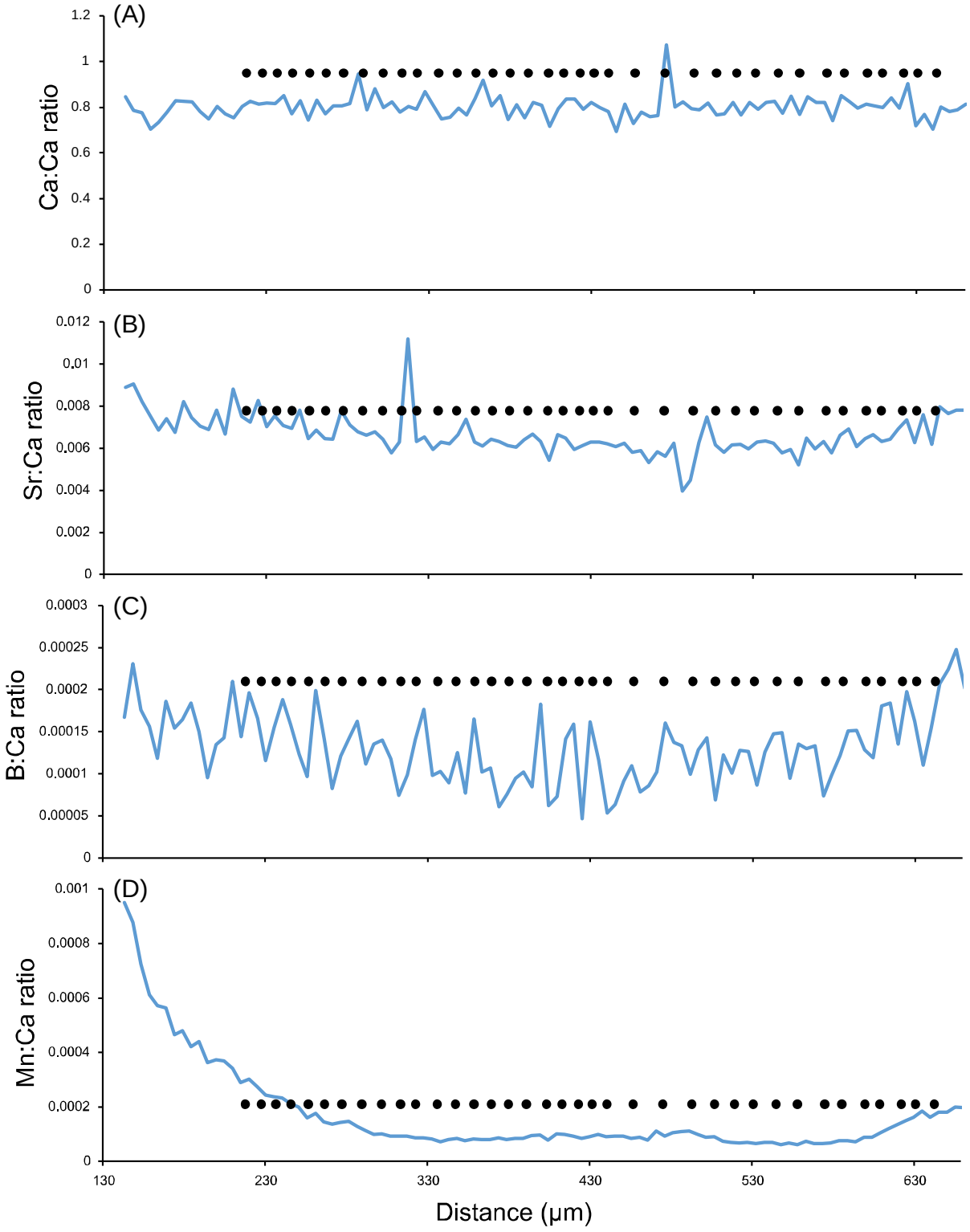

Figure 56. Ion ratios showing compositional changes (blue line) measured across a Crystal Crab ossicle (CC-16). The relative position of the thirty-seven primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively.

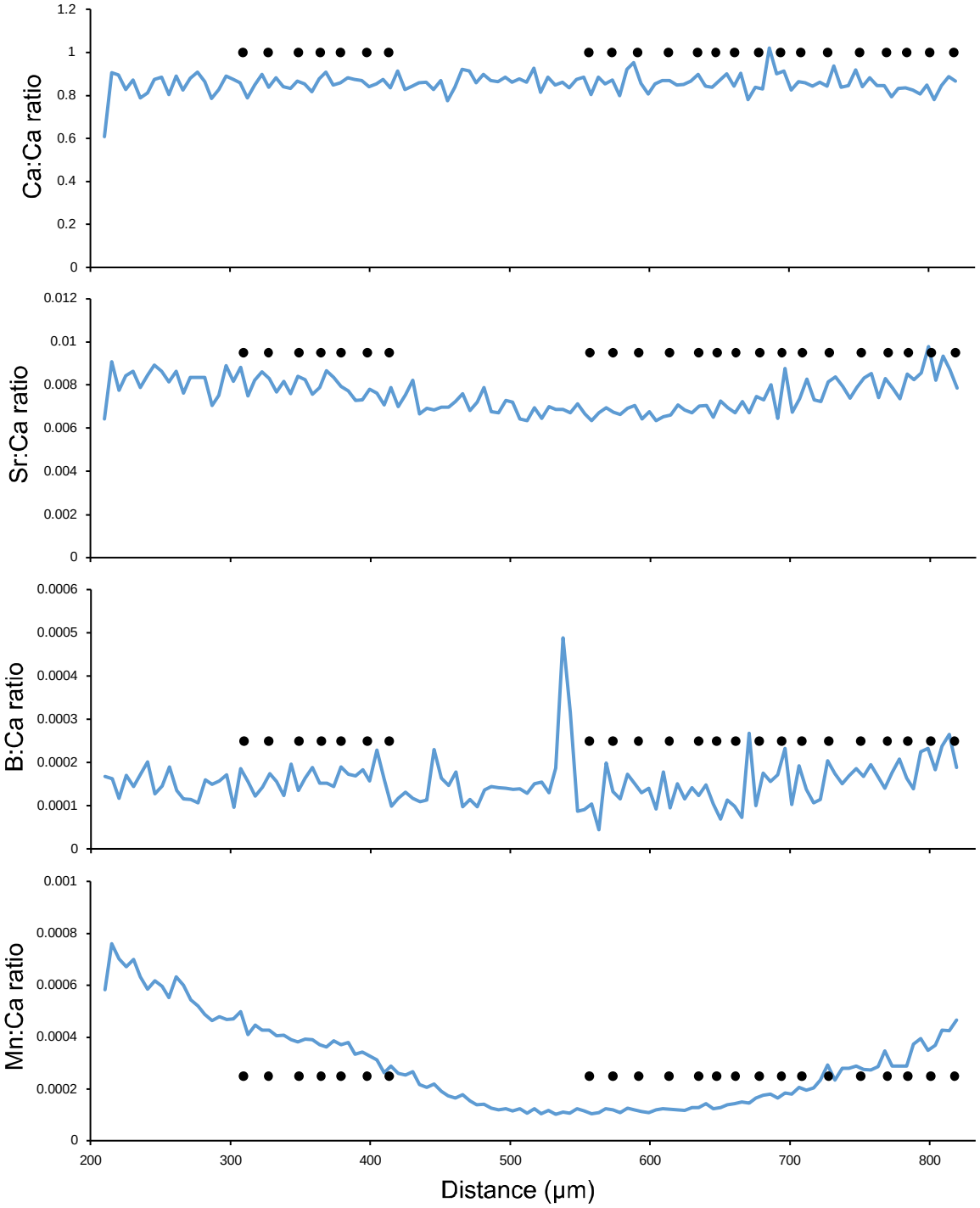

Figure 57. Ion ratios showing compositional changes (blue line) measured across a Crystal Crab ossicle (CC-17). The relative position of twenty-three primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively. Note: The gap between the black dots was a region where the primary growth marks could not be visually identified.

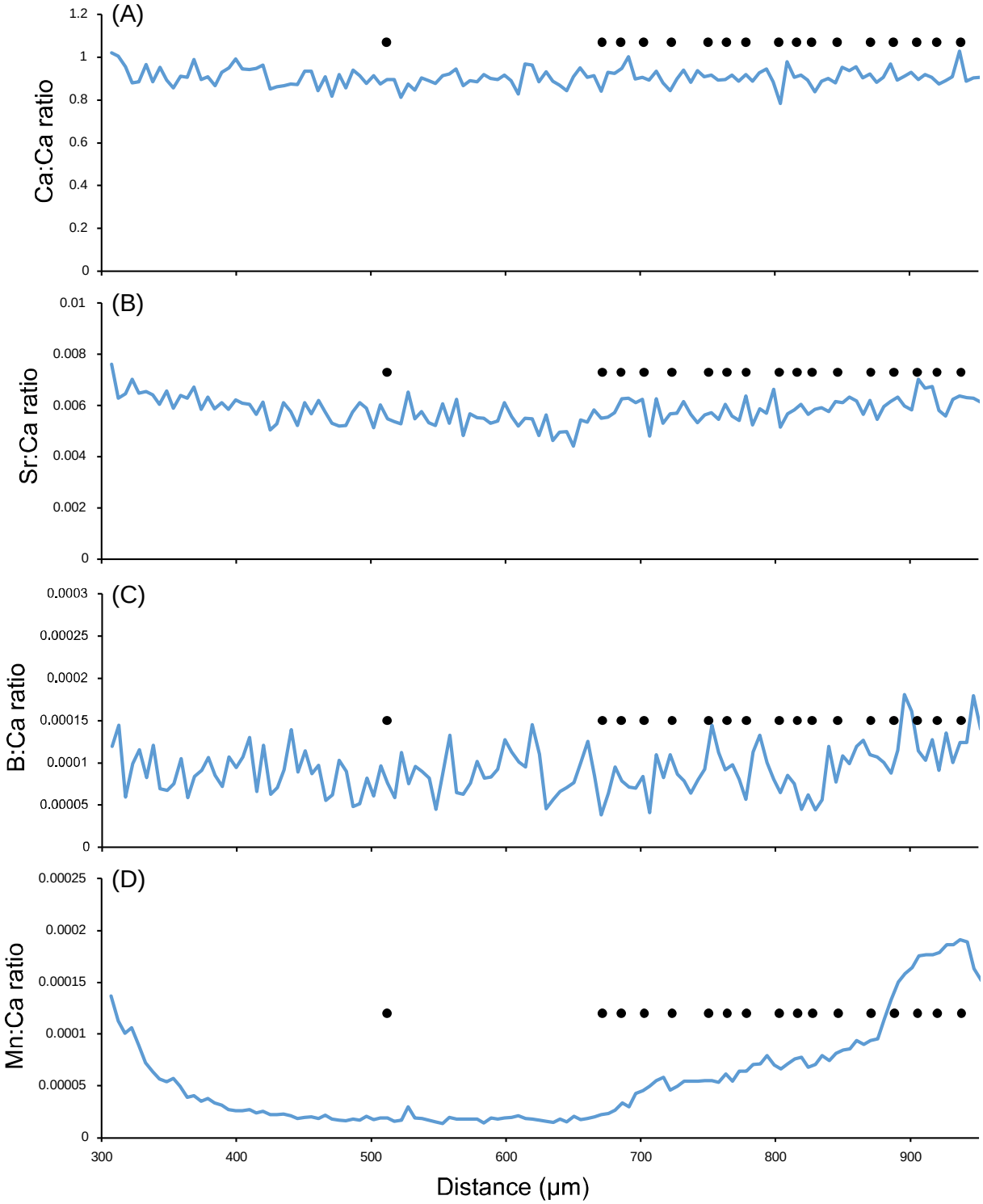

Figure 58. Ion ratios showing compositional changes (blue line) measured across a Crystal Crab ossicle (CC-18). The relative position of seventeen primary growth marks are indicated by the black dots, with the first-formed mark being positioned to the left of the plot. The ion ratio data approximately begin and end at the cuticular boundary (left) and growing edge (right), respectively. Note: The gap between the first and second black dot was a region where the primary growth marks could not be visually identified.

4.3.3 Known-age individuals

Ornate Rock Lobster

All 13 known-age Ornate Rock Lobster (69.4–92.0 mm CL) sacrificed after 1.1 and 1.4 years had one primary growth mark identified within the endocuticle, with subsequent growth beyond it (Figure 59 A–C). All sections were classified as having intermediate readability, with most having very light structure (62%) or requiring some series differentiation (62%). Two 1.4 year old individuals (8%) had 2–3 visually distinctly coloured regions within the endocuticular material deposited beyond the first primary mark (Figure 59C). The Ornate Rock Lobster CL-at-known-age data showed substantial variation (Figure 60).

Western Rock Lobster

All five pterocardiac ossicles from known-age Western Rock Lobster were categorised as poor, because in these sections the secondary series was markedly prominent and made confident identification of the primary series difficult (Figure 61). The primary and secondary reader's putative ages were always within ± 1 year of the actual known age, but there was substantial variation between the two estimates (Table 12). The primary reader mostly underestimated age, while the secondary usually overestimated. Similar to that for Ornate Rock Lobster, the Western Rock Lobster CL-at-known-age data showed substantial variation (Figure 62).

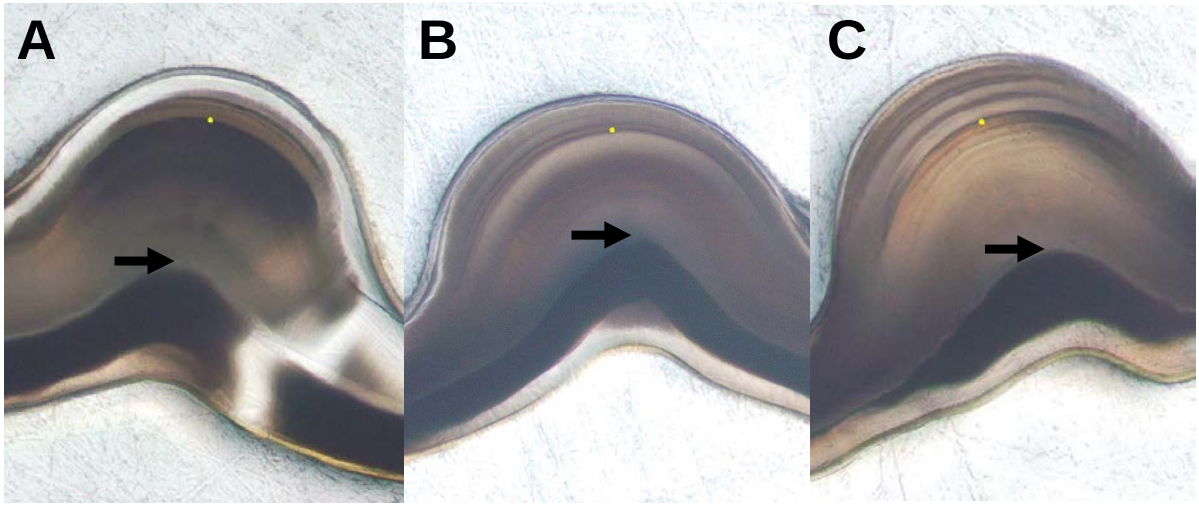

Figure 59. Pterocardiac ossicles from known-age Ornate Rock Lobster showing the cuticular boundary (black arrow) and single primary growth mark (yellow dot). Theoretically the cuticular boundary should be formed at settlement, with both endo- and exo-cuticular layers being present. A: 74.4 mm CL male with one mark (known age 1.1 years). B: 81.3 mm CL male with one mark (known age = 1.4 years). C: 81.0 mm CL male with one primary growth mark (known age = 1.4 years) and other visually distinctly coloured bands deposited within the endocuticle. The distinctly coloured bands were only present within the ossicles from two individuals and were not representative of the entire sample, but have been included for completeness.

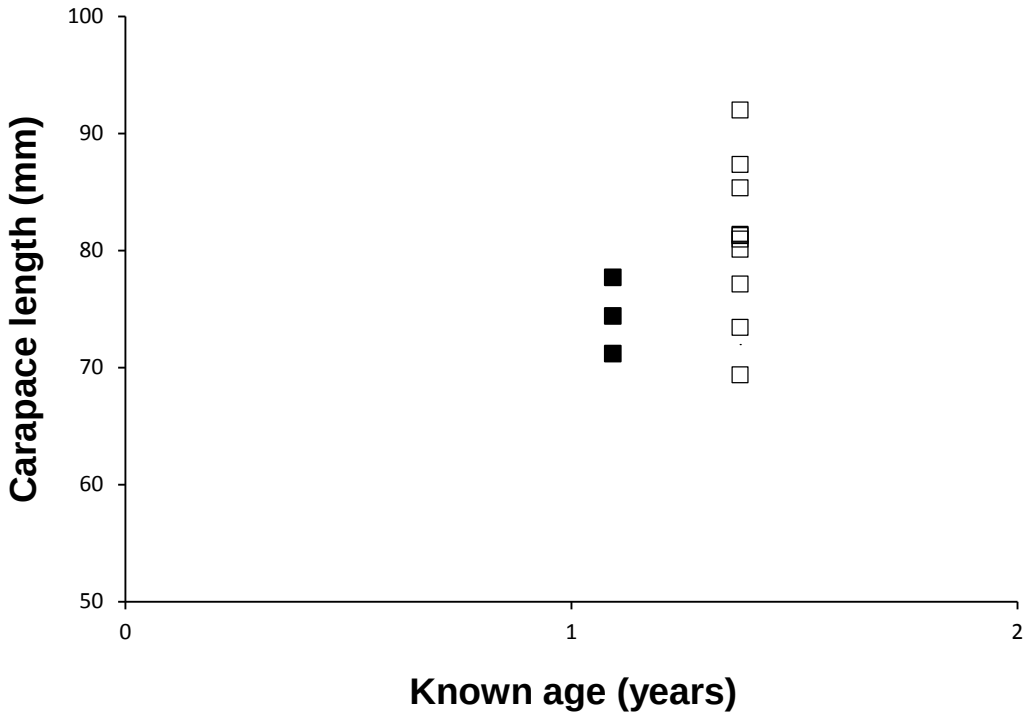

Figure 60. The Ornate Rock Lobster carapace-length-at-known-age data showing the variation among 1.1 and 1.4 year old individuals (*n* = 13).

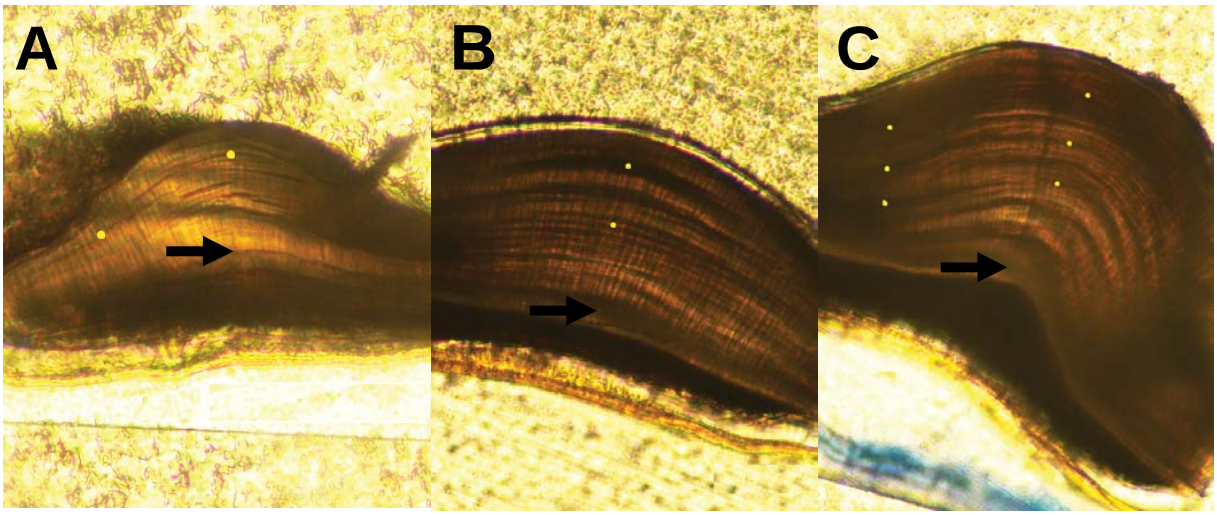

Figure 61. Known-age Western Rock Lobster ossicles (all categorised as poor) showing the primary growth marks (yellow dots), with the unusually prominent secondary series impeding interpretation. The cuticular boundary is indicated by the black arrow. A: 24.5 mm CL female assigned a putative age of 1.2 years by the secondary reader (known age = 1.1 years). B: 58.5 mm CL male assigned a putative age of 2.2 years by the secondary reader (known age = 2.2 years). C: 50.3 mm CL female assigned a putative age of 3.2 years by the primary reader (known age = 3.2 years).

Table 12. Western Rock Lobster (WRL) known age compared with directly determined putative age calculated from blind counts by the primary and secondary reader. The number in parentheses indicates the difference between the putative and known age. CL = carapace length.

				Putative age (years)	
Sample ID	**Category**	**CL (mm)**	**Known age (years)**	**Primary reader**	**Secondary reader**
WRL-1	Poor	58.5	2.2	1.2 (–1.0)	2.2 (0.0)
WRL-2	Poor	24.5	1.1	2.2 (+1.1)	1.2 (+0.1)
WRL-3	Poor	48.1	2.2	1.2 (–1.0)	3.2 (+1.0)
WRL-4	Intermediate[a]	50.3	3.2	3.2 (0.0)	4.2 (+1.0)
WRL-5	Poor	21.6	2.2	1.2 (–1.0)	1.2 (–1.0)

[a]All readability categorisations were made by the primary reader.

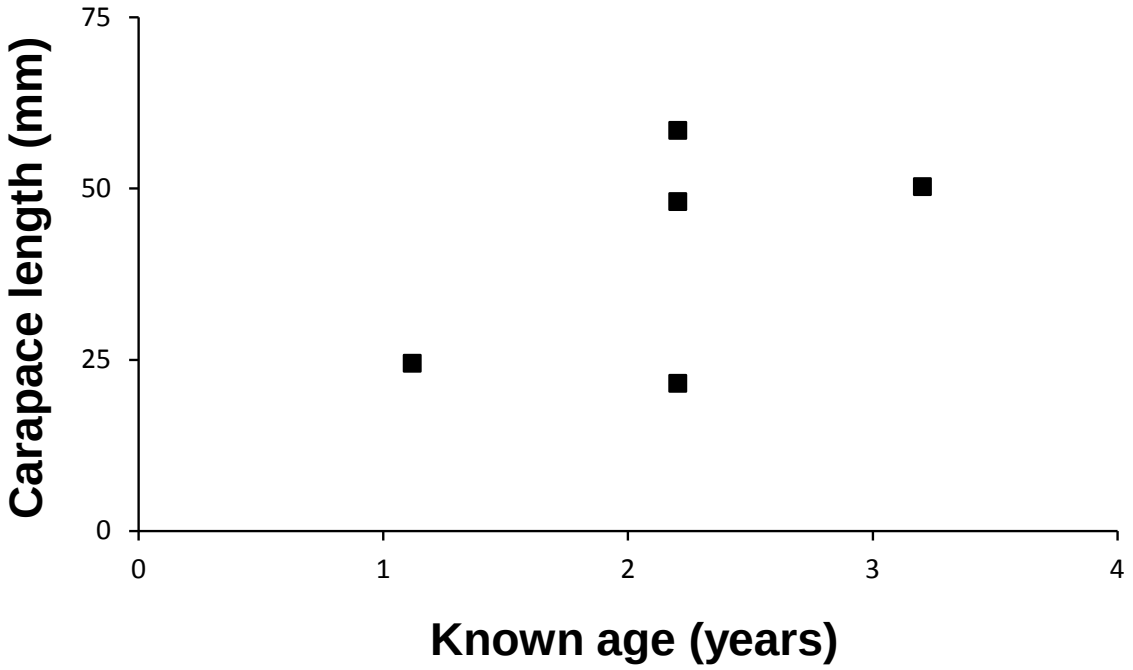

Figure 62. The Western Rock Lobster carapace-length-at-known-age data showing the variation among 1.1–3.2 year old individuals (n = 5).

5.0 Discussion

In this project, we have applied ossicular ageing methods to seven commercially important Australian crustaceans. This included some relatively short- and long-lived species and resulted in a substantial expansion of the methods applicability across a wide range of latitudes and habitat types (i.e. from tropical–temperate and coastal–deep sea). We present the world's first directly obtained growth models for any palinurid lobster. This project represents the most comprehensive assessment of ossicular growth mark periodicity ever undertaken and provided strong evidence for annual deposition for three crustacean species, with supplementary corroborative evidence from indirect methods supporting the validation experiment outcomes. Because this field is relatively immature, and to increase transparency and completeness, some information that might have otherwise been excluded (i.e. in a scientific journal article) was retained because it might prove useful for later research questions. The 'General discussion' provides a cross-objective (and -species) discussion of the project results and their relevance to the broader field of direct crustacean ageing.

5.1 OBJECTIVE 1. Size-at-putative-age assessment – Western and Eastern Rock Lobster

5.1.1 Age estimation

The Western and Eastern Rock Lobster ossicles examined during this project contained a primary growth mark series that was used to estimate individual putative age. For both species, the clear overall trend was that small lobster generally had relatively few primary marks, while large individuals had substantially more (e.g. Figure 8 and 9). The cuticular boundary was easily identified and the distance to the first growth mark was consistently the widest for both lobster species. The initial growth of Western and Eastern Rock Lobster phyllosoma occurs during an extended planktonic larval phase (i.e. ~9–11 and 9 months duration, respectively), before they metamorphose and settle as puerului (i.e. miniature adults – Liggins, 2014; de Lestang et al., 2015) with a fully formed gastric mill (see Factor, 1995 – for American Lobster). The strong relationships identified between pterocardiac ossicle morphometrics and CL (Figure 7) demonstrated that in Western and Eastern Rock Lobster the ossicular endocuticle grows isometrically – i.e., the ratio between endocuticle and exocuticle thickness remains constant with CL (and age) increase. This supports our use of the cuticular boundary as the zero point for primary growth mark counts, because at age zero (i.e. taken as immediately after the metamorphic moult) both exo- and endocuticle layers must be present, with additional material being subsequently deposited beyond the boundary (also see Leland et al., 2015).

For both Western and Eastern Rock Lobster, the individual appearance of different ossicles showed substantial variation. In this study, most Western and Eastern Rock Lobster ossicles were categorised as having good or intermediate readability (Table 5), with neither category being significantly influenced by CL or putative age. Compared with other rejection proportions from fish ageing studies (e.g. 2% – Ewing et al., 2003), that for Western and Eastern Rock Lobster (9.5 and 19.6%, respectively) in this project was quite high. For both species, the primary factor influencing rejection (i.e. potential series ambiguity), was mostly observed in ossicles from inter-moult individuals and was therefore probably not related to pre- or post-moult status. For ossicles rejected because of the potential for series ambiguity, the primary and secondary series were difficult to distinguish from each other and interpretation of the structure would carry a clear risk of substantial age overestimation (e.g. 4.8 vs. 24.8 years – Figure 10B and C). Some retained ossicles also had an unusually prominent secondary series, but an experienced reader could confidently identify that of the primary with the support of reference collection material (e.g. compare Figure 10A and B). In this project, Eastern Rock Lobster were sampled during a discrete two-month period (~early October–late November) that approximately coincided with peak moulting (Montgomery et al., 2009). Similarly, Western Rock Lobster were sampled just prior to peak moulting season (Chubb et al., 1999). For both species, it is possible that fine-scale chemical changes in ossicular composition during the inter-moult or early pre-moult might have influenced readability. Further research into the potential for temporal effects on ossicle readability is needed to assess whether targeted sample acquisition schedules can maximise ossicle readability and retention.

The age estimates in this project were made from high resolution digital images. Subsequent re-examination of individual ossicle sections (~ 1 year later) showed that some structures showed signs of distortion (e.g. cracking and shrinkage) that rendered them useless for interpretive purposes. In this project, all ossicles were stored dry. The structures were only wetted (i.e. with distilled water) for thinning and photographing, before being dried again for storage. Other studies have used the same storage and impermanent mounting methods (Kilada et al., 2012; Sarapuk, et al., 2014; Leland et al., 2011; 2015). Unlike fish otoliths, crustacean ossicles comprise a semi calcified organic-chitin matrix (Stewart, 2016). Because of this, the structures may be more susceptible to long-term drying effects. Kilada et al. (2017b) suggested that extensive drying can contribute to increased structural degradation during processing. Creating a digital reference record can overcome the issue, and has other benefits, but ideally 100% of the original reference material should be suitable for subsequent re-examinations. Further research into possible storage and mounting medium alternatives are needed to facilitate long-term sample preservation.

5.1.2 Growth modelling

Western Rock Lobster

In this project, the growth of Western Rock Lobster sourced from Lancelin, Western Australia was adequately described using the von Bertalanffy growth equation. This allowed for comparison with other published von Bertalanffy growth parameter estimates from indirect ageing methods. Phillips et al. (1992) estimated growth parameters for wild-caught male Western Rock Lobster from Cliff Head and Seven Mile Beach in Western Australia. Cheng and Kuk (2002) modelled growth for female Western Rock Lobster from the Kalbarri region in Western Australia. Chittleborough (1976) estimated growth parameters from six juvenile Western Rock Lobster (i.e. of known age) that were reared under optimal laboratory conditions (i.e. constant 25°C water temperature) for six years. Based on the confidence interval overlap for L_∞ and K, our parameter estimates for the complete model were not significantly different to that published by Cheng and Kuk (2002). Phillips et al. (1992) and Chittleborough (1976) did not present confidence intervals, but because their L_∞ and K estimates fall within those for this project (Table 8), we infer that there was no significant difference in parameter estimates between the studies. Phillips et al. (1992) reported a maximum longevity of 23 years for a captive-reared Western Rock Lobster. In this project, we observed a maximum individual putative age of 19 years, with the directly determined complete model predicting that Western Rock Lobster reach their maximum size after approximately 15 years and the upper L_∞ confidence interval being less than the maximum observed size (113.6 vs 129.1 mm CL).

In the past, directly determined ages for Western Rock Lobster were not available. Because of this, there is some variation around indirect age estimates at particular life history milestones. For example, the relationship between puerulus settlement and subsequent catches has been used for decades as a predictive management tool, with Western Rock Lobster recruiting to the fishery approximately 3–4 years later (Phillips, 1986; de Lestang, 2009). However, other sources estimate that the minimum legal size (i.e. 76 mm CL), which coincides with the mean size-at-sexual-maturity (range: 65–80 mm CL), is reached after ~5–7 years depending on the location (Thompson et al., 1996; de Lestang, 2014). Chittleborough (1974) reported that known-age Western Rock Lobster reared under laboratory conditions reached sexual maturity after 4.9–5.7 years. In this project, both the complete and partial models predicted an average age of approximately 5 years at the minimum legal size (Figure 12 and 13) – closely agreeing with existing indirect estimates. The CL-at-age data for the known-age Western Rock Lobster reared during this project showed substantial variation (e.g. from 21.6–58.5 mm CL after 2.2 years – Figure 62), but were closely aligned with both the complete and partial growth models.

The von Bertalanffy growth parameters for the partial model (i.e. comprising only Western Rock Lobster with a putative age estimate ≤ 6 years), were not significantly different to those published by

Chittleborough (1976), but our curve for wild-caught lobster estimated consistently slower growth (Table 8). Given that the Chittleborough (1976) model was derived from similarly aged known-age juveniles (n = 6) reared under optimal temperature and feed regimes, it is not surprising that our model for wild-caught lobster experiencing natural influences (e.g. seasonal cycles, competition and density pressure) estimated slower growth. The CL-at-age data for the known-age individuals reared in this project (under controlled conditions) were closely aligned with both the Chittleborough (1976) curve and our partial model for wild-caught lobster (Figure 13). However, the age estimates presented by Chittleborough (1976) for wild-caught Western Rock Lobster from Seven Mile Beach and Garden Island, were closer to our wild-caught model, rather than their curve for laboratory-reared individuals. Compared with the mean predicted CL-at-known-age from Chittleborough (1976), the line-of-best-fit slope for our directly determined individual putative age was similar to a 1:1 relationship, but began diverging (slightly) after approximately 4+ years (Figure 63).

Recently settled Western Rock Lobster experience relatively fast growth during their first year on shallow-water nursery reefs, partly because they do not compete directly with older individuals (Chittleborough, 1976). However, from ~2–6 years of age, competition increases with the growth of young juveniles being depressed, before they move to deeper water (at ~ 4–7 years of age), where growth can accelerate because of reduced competition (Chittleborough, 1976; Phillips et al., 1980). In this project, water depth significantly influenced the Western Rock Lobster CL-at-putative-age relationship, with individuals from the same year class being consistently larger (at a given age) in deeper waters (Figure 11 and 63) and demonstrating that shallow nursery reefs generally house < 5 year old lobster (with an extreme of 6.9 years). This is in close agreement with the indirect age estimates associated with previously known biological and ecological patterns identified for juvenile Western Rock Lobster.

Beyond the broad similarities between the complete model parameter estimates for Western Rock Lobster in this project and those presented for comparison (Table 8), there were some differences (albeit not significant ones) that warrant consideration. Western Rock Lobster growth varies substantially between different locations (Chubb et al., 1999), with limiting factors often including temperature, population density and food availability (Chittleborough, 1976; Phillips et al., 1980). Different spatial combinations of these factors can sometimes result in sex-specific growth differences (Phillips et al., 1992; Chubb et al., 1999). However, Chittleborough (1976) found that during years with abundant food (or alternately extreme shortages) sex-specific differences in Western Rock Lobster growth was absent. This might explain why, in this project we found no significant difference between male and female growth. The Cheng and Kuk (2002) female-only model for the Kalbarri region estimated faster growth than that of our combined-sexes complete model (Figure 12) and the male-only model of Phillips et al. (1992). Crustacean growth is temperature dependant, with higher

temperatures increasing moult increments (Hartnoll, 2001). Direct comparison of our individual putative ages with the mean predicted CL-at-putative-age from the indirect model of Cheng and Kuk (2002), showed initial similarity until about age 3–4+. After approximately 5 years, the line-of-best-fit slope for our putative ages diverged strongly from the 1:1 relationship (Figure 64). In the Kalbarri region, the mean sea temperature is substantially warmer than the Western Rock Lobster source location for this project at Lancelin, Western Australia (22–24°C vs. 20–22°C, respectively) (BOM, 2016). This probably explains why the Cheng and Kuk (2002) female-only parameter estimates predict faster growth than our combined sex model (Figure 12) and the abrupt divergence from the expected 1:1 relationship between the direct and indirect putative age estimates.

If primary growth marks are deposited annually in Western Rock Lobster ossicles, a strong similarity between directly and indirectly determined putative ages (i.e. the rate of increase) for animals from comparable locations would be expected. In this project, we estimated putative ages for wild-caught Western Rock Lobster from Lancelin, Western Australia. Phillips et al. (1992) estimated this species growth at a comparable source location (i.e. Cliff Head and Seven Mile Beach, Western Australia). Unlike the Kalbarri region, lobster at our source location would have experienced similar mean annual water temperatures as those in Phillips et al. (1992) (20–22°C – BOM, 2016). In this project, the line-of-best-fit for the directly determined putative ages increased at a 1:1 rate with the mean predicted CL-at-putative-age of Phillips et al. (1992), with our estimates being consistently about 7 months less (Figure 65). This indicates that the primary growth mark series in Western Rock Lobster ossicles are probably deposited annually.

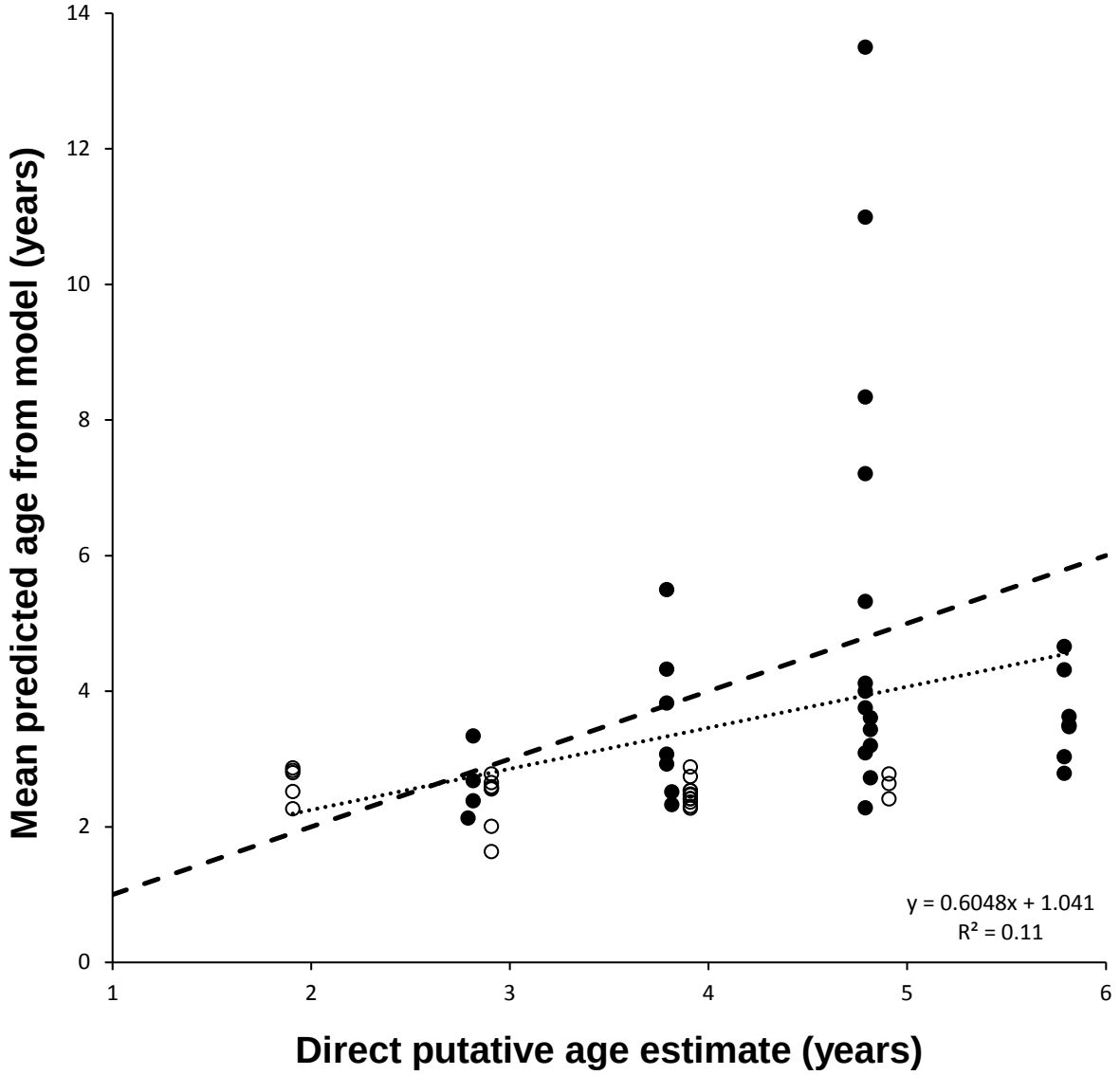

Figure 63. Comparison of the directly determined putative age estimates from this project (i.e. for ≤ 6 year old lobster) and the mean predicted age (i.e. at equal CL) from the Chittleborough (1976) model for known-age Western Rock Lobster reared under optimal conditions. The data were constrained by the Chittleborough (1976) L_∞ parameter (113.47 mm CL). Individuals sampled in this project from shallow (white circles) and deep (black circles) waters are indicated. The line-of-best-fit (dotted line) and 1:1 relationship (dashed line) are given. The linear correlation was statistically significant (p = 0.01). Note: The Chittleborough (1976) estimates are influenced by a positive t_0 (i.e. 1.05 years – possibly including the planktonic duration), but that for this project was fixed at zero.

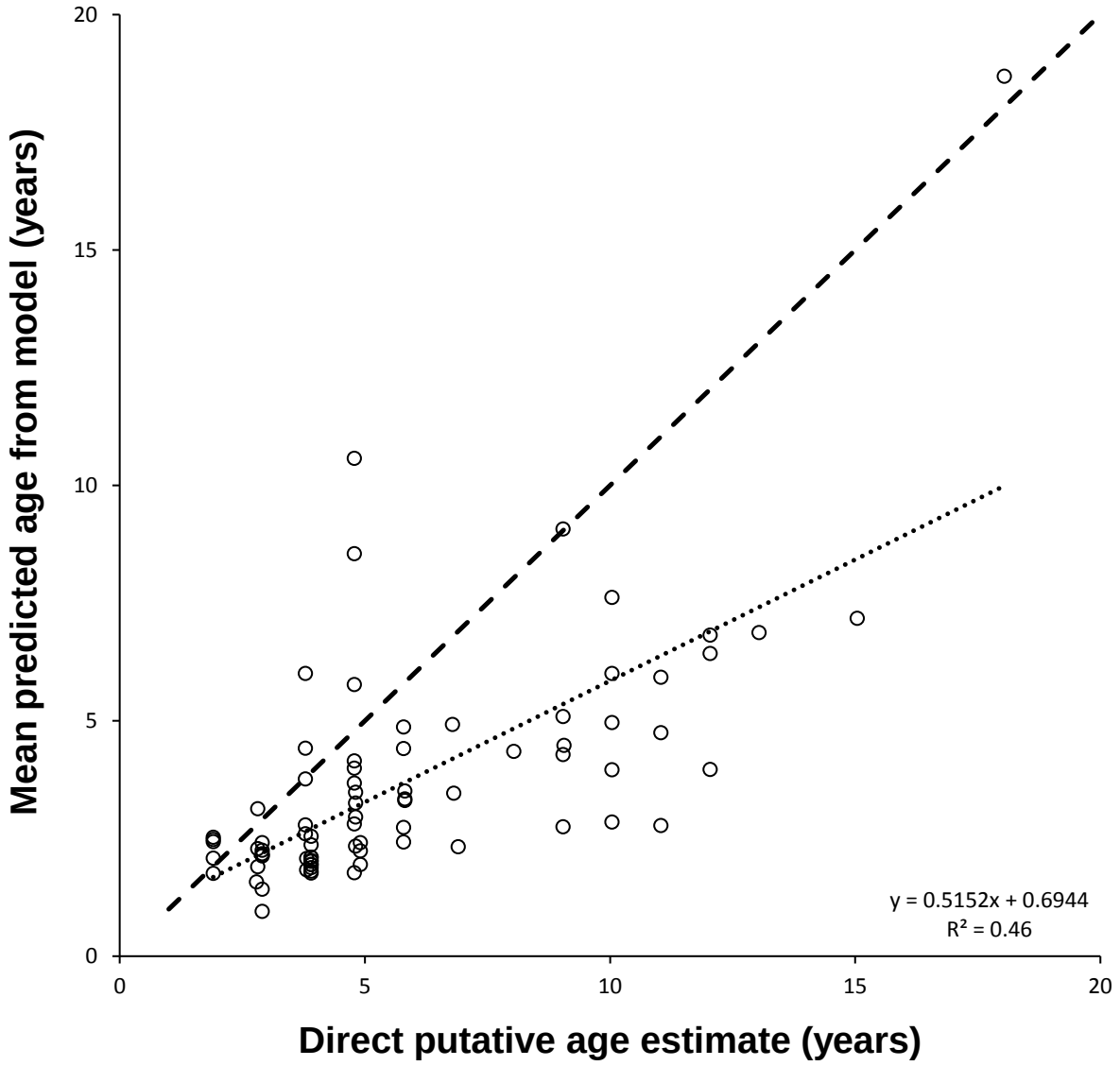

Figure 64. Comparison of the directly determined putative age estimates from this project (i.e. for all ages) and the mean predicted age (i.e. at equal CL) from the Cheng and Kuk (2002) tag-and-recapture model for female Western Rock Lobster from Kalbarri, Western Australia. The data were constrained by the Cheng and Kuk (2002) L_∞ parameter (111.92 mm CL). The line-of-best-fit (dotted line) and 1:1 relationship (dashed line) are given. The linear correlation was statistically significant (p = < 0.001). Note: The Cheng and Kuk (2002) estimates are influenced by a positive t_0 (0.21 years), but that for this project was fixed at zero.

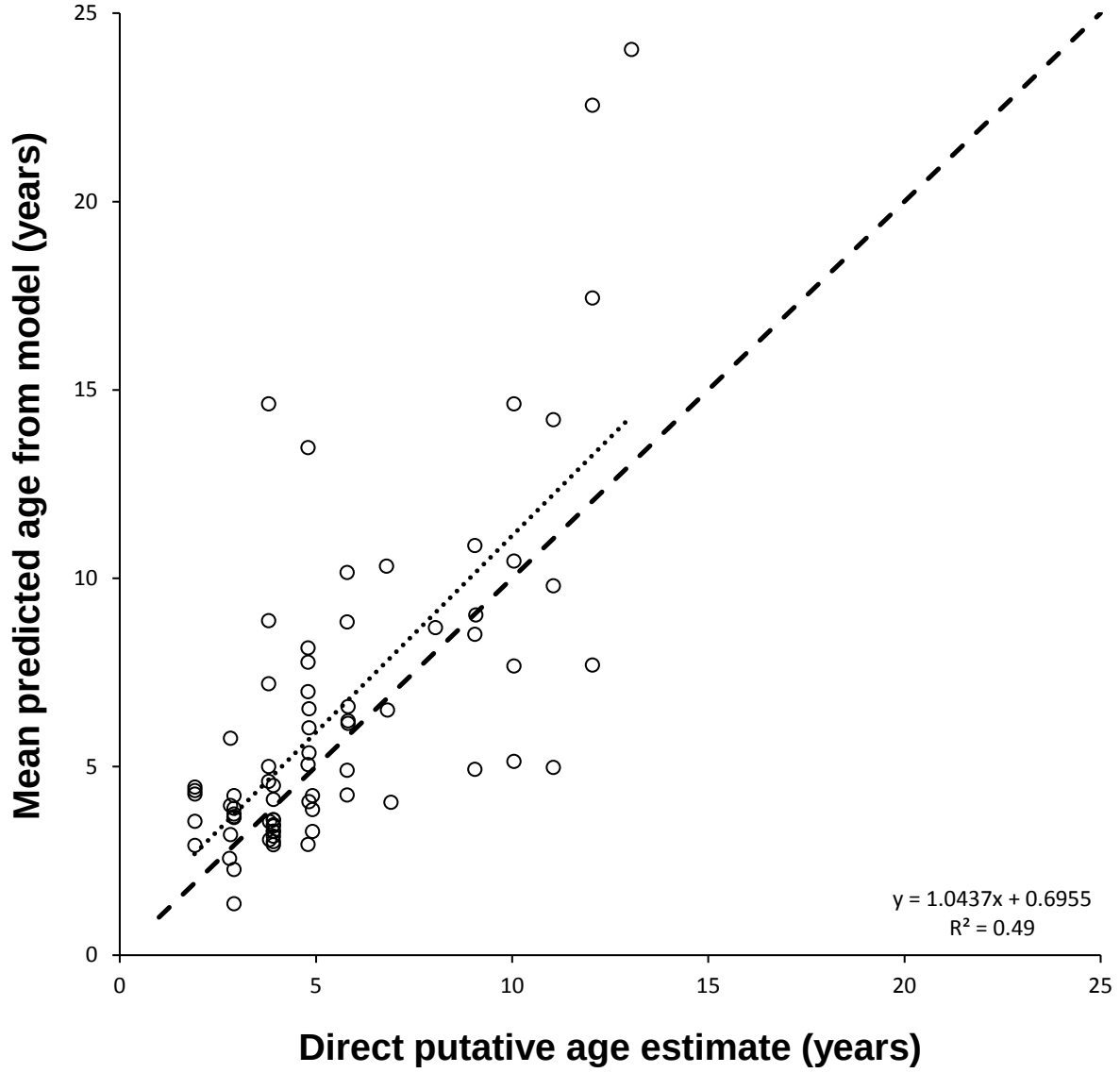

Figure 65. Comparison of the directly determined putative age estimates from this project (i.e. for all ages) and the mean predicted age (i.e. at equal CL) from the Phillips et al. (1992) tag-and-recapture model for male Western Rock Lobster from Cliff Head and Seven Mile Beach in Western Australia. The data were constrained by the Phillips et al. (1992) L_∞ parameter (102.92 mm CL). The line-of-best-fit (dotted line) and 1:1 relationship (dashed line) are given. The linear correlation was statistically significant (p = < 0.001). Note: The Phillips et al. (1992) t_0 was fixed at zero (i.e. like that in this project), but the corresponding size was positive (~10 mm CL), whereas that in this project was neutral (0 mm CL).

Eastern Rock Lobster

The von Bertalanffy growth equation provided an adequate description of the directly determined CL-at-putative-age-data for Eastern Rock Lobster sourced from the three NSW locations (i.e. Coffs Harbour, South West Rocks and Jervis Bay). This allowed for comparisons with the only other primary-literature published study (i.e. Montgomery et al., 2009) describing the species growth in NSW. Montgomery et al. (2009) was a multi-year study conducted across a similar latitudinal range (i.e. Evans Head–Narooma) in New South Wales that presented sex-specific Eastern Rock Lobster growth curves (that were not significantly different). Based on the confidence interval overlap, both our combined (i.e. with all three locations) and Northern (i.e. Coffs Harbour and South West Rocks only) model parameter estimates were (mostly) not significantly different from those of Montgomery et al. (2009). The only exception, was our combined model estimate for L_{∞}, which was significantly different to the male-only Montgomery et al. (2009) parameter, because their confidence intervals were asymmetric, with the lower bound being very similar to their estimate (i.e. 244.69 vs. 246.10 mm CL, respectively) and the upper bound (i.e. 280.70 mm CL) being substantially larger than the maximum observed size in wild specimens (Table 9). Like Montgomery et al. (2009), we found no significant difference in Eastern Rock Lobster growth between the sexes.

The directly determined von Bertalanffy growth parameters generated in this project estimated that Eastern Rock Lobster reach their maximum size after approximately 30 years. This maximum longevity estimate was equal to that of Montgomery et al. (2009), with our oldest individual observed age in this project being 26.1 years. Compared with our combined model, and those of Montgomery et al. (2009), the Northern model parameter estimates yielded an upper L_{∞} confidence interval (253 mm CL) that was closer to the maximum size occurring in nature (i.e. 254 mm CL – observed during this project). Despite the broad similarities between our Northern model and those of Montgomery et al. (2009), our mean predicted putative-age estimates (i.e. for a given CL) were consistently greater at important fishery-related milestones including the minimum legal size (104 mm CL, 4.5 vs. 5.6 years), female size-at-sexual-maturation (167 mm CL, 9.5 vs. 12.2 years) and maximum legal size (180 mm CL, 10.4 vs. 14.4 years).

If the direct and indirect methods are yielding equivalent Eastern Rock Lobster age estimates, and therefore supporting the annual periodicity hypothesis, the line-of-best-fit slope should be similar to a 1:1 relationship (e.g. see Western Rock Lobster – Figure 65). Trajectories that abruptly deviate from the 1:1 ratio would probably indicate the influence of an unquantified environmental factor. There was an apparent (but qualitative) difference between the location-specific line-of-best-fit slopes and their relative similarity to a 1:1 relationship (Figure 66). The line-of-best-fit slope for Eastern Rock Lobster sourced from South West Rocks was very similar to 1:1 and provided strong corroboration of

our direct putative ages for some of the oldest individuals in this project (see Campana, 2001). Alternately, the line-of-best-fit slope for individuals sourced from Jervis Bay and Coffs Harbour had greater deviation from 1:1. For the Coffs Harbour individuals, approximately half of the data appeared to be more closely aligned with a 1:1 relationship (and the South West Rocks lobster), but for the remainder the line-of-best-fit trajectory was more similar to that for Jervis Bay (Figure 66).

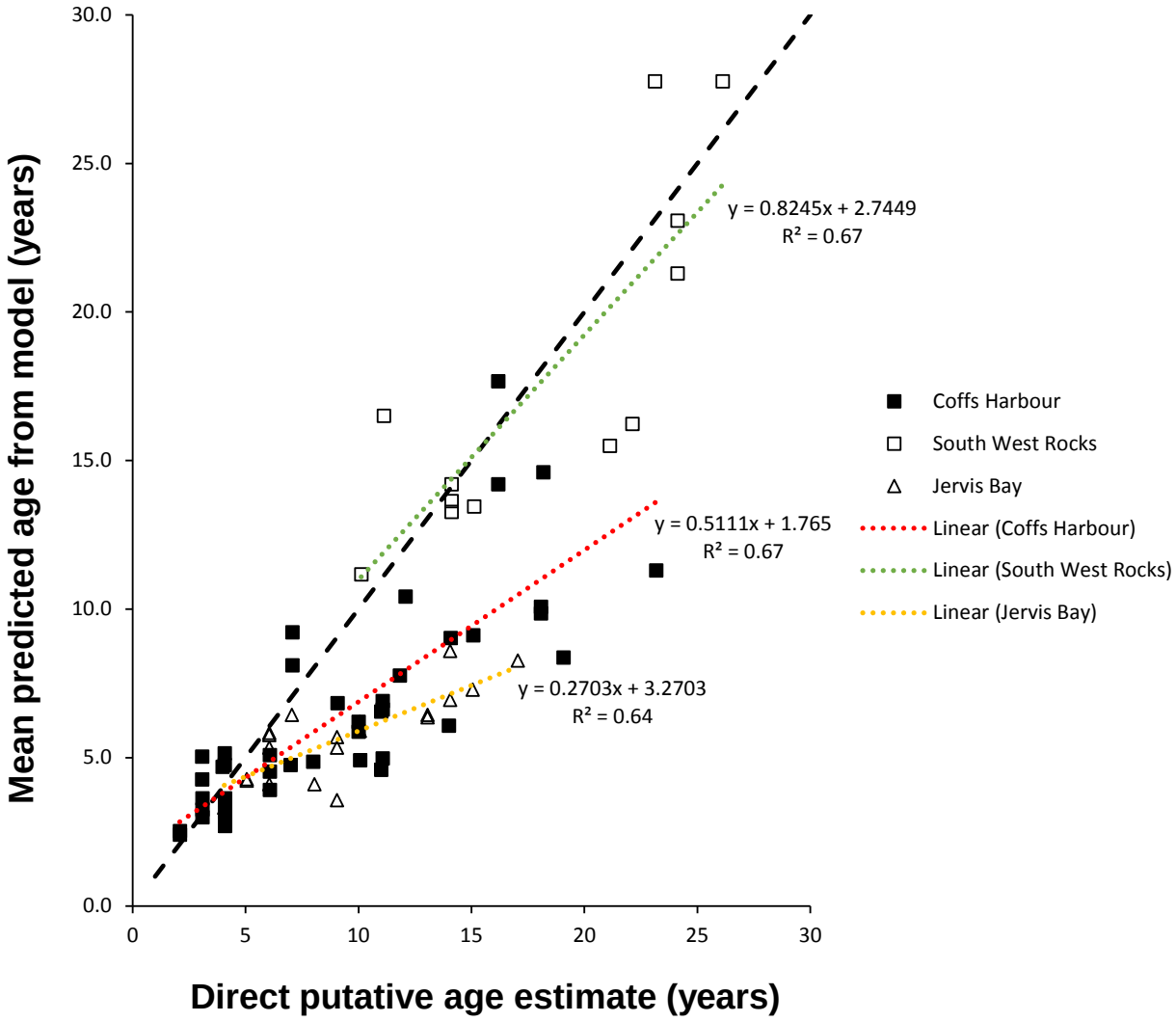

Figure 66. Comparison of the directly determined putative age estimates from this project (i.e. for all locations) and the mean predicted age (i.e. at equal CL) from the Montgomery et al. (2009) tag-and-recapture model for Eastern Rock Lobster sourced from New South Wales. The location-specific line-of-best-fit (dotted lines) and 1:1 relationship (dashed line) are given. The data were constrained by the male and female Montgomery et al. (2009) L_∞ parameters (246.10 and 239.77 mm CL, respectively), with the final predicted age presented here being the between-sexes mean. All linear correlations were statistically significant (p = < 0.001). Note: The Montgomery et al. (2009) data are influenced by a positive t_0 (male = 0.40, female = 0.37 years), but that for this project was fixed at zero.

In the NSW fishery, Eastern Rock Lobster are considered a single population, with individuals sampled south of Wollongong, NSW rarely reaching >167 mm CL, before they generally begin moving northwards along the coast (see Booth, 1984; Montgomery et al., 2009). Given the positive relationship between increased temperature and crustacean growth (Hartnoll, 2001), combined with other local variables (e.g. temperature and food availability) and inshore–offshore movement patterns (Montgomery et al., 2009), it is likely that Eastern Rock Lobster growth varies between Coffs Harbour and Jervis Bay. Compared with Jervis Bay, the Coffs Harbour and South West Rocks mean annual water temperatures are substantially different (i.e. 18–20 and 22–24°C, respectively) (BOM, 2016) and provide the most likely explanation for the deviation (i.e. from 1:1) in the line-of-best-fit slope for the individuals sourced from Jervis Bay (Figure 66). The Eastern Rock Lobster growth parameters estimated for the Southern model (i.e. for the Jervis Bay location) were probably also heavily influenced by size-specific ontogenetic migration, with the upper L_{∞} confidence interval (164.21 mm CL) reflecting the region-specific maximum size (i.e. 167 mm CL). Compared with the Northern model parameters, the Southern estimates were possibly artificially depressed, because fast-growing individuals may migrate first, leaving an increasing proportion of relatively slow-growing lobster in older age classes.

In this project, the Northern and Southern models estimated approximately equal growth rates during the first seven years (Figure 14). However, at approximately age 7+ (i.e. and 125 mm CL) the growth models diverge, with some apparent segregation in the Coffs Harbour samples occurring beyond that point – i.e., most of the CL-at-putative-age data are distributed either ‘above’ or ‘below’ the von Bertalanffy growth curve for the Northern (or combined model – Figure 14). Specifically, the CL-at-putative-age data for the Coffs Harbour samples appear more closely aligned with the respective Montgomery et al. (2009) or Southern model (Figure 14). Eastern Rock Lobster puerulus settlement occurs at all the NSW locations sampled in this project. A possible explanation for the split-distribution pattern in the Coffs Harbour CL-at-putative-age data is that our Northern model includes lobster that settled there and also some relatively recent migrants from the higher-latitude locations (e.g. Jervis Bay). In addition, the known movement of lobster from higher to lower latitudes (see Booth, 1984; Montgomery et al., 2009) would influence individual growth rates. This might also contribute to the split-distribution in the putative ages for Coffs Harbour lobster, with post-migration size differences being offset by greater mean temperatures and becoming less distinguishable over time. Our observations about the potential for location-specific age and growth differences fit with the existing knowledge about Eastern Rock Lobster settlement and migration patterns, but require further quantitative research to support them.

5.2 OBJECTIVE 2. Evaluation of growth mark periodicity – Western and Eastern Rock Lobster and Crystal Crab

5.2.1 Crystal Crab

Compared with other Australian crustaceans (e.g. rock lobster), Crystal Crab are poorly understood, perhaps because they occupy less-accessible habitats (450–1220 m depth – Smith et al., 2004) and have been harvested for a relatively short time. At these depths, the sea temperature generally ranges from 4–6.5°C (Smith et al., 2004) and presumably results in Crystal Crab having relatively slow growth, late maturation and long lifespan like that reported for other deep-sea crustaceans and bony fish (Morales-Nin and Panfili, 2005; Vogt, 2012). Other deep-sea crustaceans are known to have long inter-moult periods (e.g. 4–7 years), with concomitant reductions in growth (Lux et al., 1982; Levings et al., 2001). Because crustacean growth occurs via moulting (Hartnoll, 2001), all previous direct ageing studies on crustacean validation have only considered evaluating periodicity in ossicles from calcein-stained individuals that moulted after staining (Kilada et al., 2012; Sarapuk, 2014; Leland et al., 2015).

In this project, the long-term rearing of calcein-stained Crystal Crab provided the first direct information on their inter-moult duration (i.e. ≥ 18 months), with only one crab moulting (once) during the 18 month grow-out period. In this individual, the calcein could not be distinguished from the natural autofluorescence, indicating a temporal limitation to using this chemical tag for long-term crustacean validation studies. Examination of the ossicles from individuals that did not moult allowed for a unique evaluation of ossicular periodicity during the inter-moult. Similar to some other crustacean validation studies (Kilada et al., 2012; Sarapuk, 2014), in this project the calcein penetrated into the ossicles to varying degrees, because of inter-layer connectivity via structural transport canals (see Roer and Dillaman, 1984). However, almost all Crystal Crab had an identifiable artificial mark, with stain-free material deposited beyond it (see Leland et al., 2015) indicating that some ossicular extension (i.e. growth) occurred during the inter-moult period. To our knowledge, ossicular extension during the inter-moult has never been reported for any crustacean. However, Kilada et al. (2012) reported moult-independent primary growth mark formation in Snow Crab eyestalks (*Chionoecetes opilio*) and it is well known that crustaceans synthesize other materials (i.e. muscle proteins) during the inter-moult period (El Haj et al., 1996; Grubert et al., 2012).

The timing of ossicular growth mark formation has never been comprehensively investigated. In this project, the calcein stain was consistently located in one of two positions relative to the primary growth mark series in Crystal Crab ossicles. Preliminary marginal increment analysis for Redclaw Crayfish has indicated that the deposition of a new-formed primary growth mark was complete during

the late austral spring–summer period (Stewart, 2016). Leland et al. (2015) noted that Redclaw Crayfish stained with calcein during late autumn (i.e. April 26) had an artificial mark incorporated directly adjacent to a primary growth mark and inferred possible winter formation. In this project, Crystal Crab were stained in early spring (i.e. September 13), with approximately half of the individuals showing the same pattern as that observed by Leland et al. (2015) – i.e., calcein incorporation directly adjacent to (or along) a primary growth mark. However, for the other Crystal Crab in this project, the calcein stain was positioned between two successive primary growth marks (Figure 17), with the outermost being formed during the grow-out period. In fish otoliths, annual periodicity is often linked with temperature (Ewing et al., 2003) and/or reproduction (Morales-Nin and Panfili, 2005; Neat et al., 2008). However, deep-sea species like Crystal Crab experience low seasonal temperature variation (Smith et al., 2004) and primary growth mark formation may therefore be expected to correspond to other biological factors (e.g. reproduction) interacting with seasonal changes in food supply (Morales-Nin and Panfili, 2005). In this project, the calcein-stained Crystal Crab were predominantly female (sex ratio of 4:1) and were reared under captive conditions with constant temperature (~6°C) and food availability. Our observation on the relative position of the calcein stain may indicate asynchronous growth mark formation in Crystal Crab ossicles. Given that Crystal Crab are deep-sea asynchronous spawners that reproduce throughout the year (Smith et al., 2004), it is possible that growth variations could be linked to individual reproductive cycles.

For two individuals (103.0 and 121.0 mm CL) sampled after 6 months, the calcein stain was incorporated between the outermost primary growth mark and the growing edge, with both CGIs approaching 50% (i.e. 33 and 49%). If the primary series is deposited annually, and ossicular growth during the inter-moult is linear, this would indicate that these individuals were stained approximately six months after depositing their last growth mark (~March–April), before depositing an approximately equal amount of ossicular material during the grow-out period. Another Crystal Crab (153.9 mm CL) sampled after 6 months had a CGI of 130%, but a new growth mark was not identifiable yet. Three other individuals (97.9, 106.0 and 107.0 mm CL) sampled after 6 months had deposited one primary growth mark beyond the calcein stain. These individuals also had additional material subsequent to the new-formed mark and CGIs that were >150% (i.e. 167–211%). Some Crystal Crab (n = 3) sampled after 6 months had apparently different endocuticle zones beyond the calcein stain (i.e. with high CGIs), but no primary marks were identified within them (Figure 18). We could not explain the dissimilarity in appearance for these samples; perhaps they represent some artefact of the captive-rearing process (see Vogt, 2012). Another possible explanation for the obvious difference in the endocuticular appearance might be that these individuals were entering the pre-moult phase, with concomitant changes in ossicular chemistry.

Four of the Crystal Crab (92.1–145.0 mm CL) sampled after 12 months had formed a new primary growth mark beyond the calcein stain, with some CGIs approaching 100% (i.e. 46 and 82%) and others being >100% (i.e. 119 and 176%) and all individuals having subsequent material deposited beyond the new-formed mark. The other three individuals (93.0–112 mm CL) reared for 12 months also showed ossicular extension beyond the calcein stain, with their CGIs approaching or exceeding 100% (i.e. 63, 63 and 102%), but a new-formed growth mark was not yet identifiable on the growing edge. Like those sampled after 6 months, one individual had an apparently different outer endocuticle, but no primary growth marks were identified beyond the calcein stain.

Some similar patterns were observed for the Crystal Crab sampled after 18 months. Five individuals (100.8–135.0 mm CL) formed one new primary growth mark during the grow-out period (Figure 15 and 17). For some individuals, the CGI was consistent with the expected value if the primary growth marks are deposited annually (i.e. 172 and 177% after 1.5 years), but for others it was still approaching 100% (i.e. 57 and 84%), demonstrating clear differences in relative growth rates between individuals. Three other Crystal Crab (95.5, 96.5 and 109.1 mm CL) had ossicular extension beyond the calcein stain (i.e. CGIs of 72, 109 and 111%), but a new-formed growth mark was not identifiable on the growing edge.

Irrespective of the grow-out duration, there was considerable variation in the amount of ossicular material deposited after staining. One disadvantage of captive-rearing validation studies is that the altered environment can produce growth increments that are markedly different to those in wild individuals (Campana, 2001; Vogt, 2012). For example, an unnatural captive diet, with *ad libitum* feeding (i.e. and no competition) throughout the project, presumably raised consumption above normal for deep-sea crustaceans, while removing any temporal availability restrictions (see Morales-Nin and Panfili, 2005 for bony fish analogue) and might have positively influenced growth. Conversely, other individuals with lower than expected CGIs, might have been experiencing physiological stress from captive rearing (e.g. Taylor et al., 2009), or caging (e.g. Leland et al., 2013a), with subsequent negative impacts on growth.

The CGIs recorded for Crystal Crab might have also been influenced by other relatively poorly-understood factors. The novelty of ossicular extension during the inter-moult period introduces some uncertainty into any explanation for the observed CGI patterns. The substantial variation in Crystal Crab CGIs across the 6, 12 or 18 month grow-out period was presumably partially attributable to individual differences in growth rate. However, if ossicular extension during the ≥ 18 month inter-moult period occurred at a non-linear rate for all, or part of the duration, the CGI would be heavily influenced by the amount of time elapsed since the previous moult. Uncertainties around the influence of endogenous and exogenous factors, coupled with probable asynchronous primary growth mark

deposition made it challenging to explain the entire range of observed outcomes. However, irrespective of the sampling period, most Crystal Crab (including both sexes) deposited a single new-formed primary growth mark during the 18 month grow-out (Table 13). This provided evidence that a single primary growth mark is deposited annually (i.e. during the inter-moult period) in Crystal Crab ossicles.

Table 13. The number (*n*) of Crystal Crab that either: i) formed a single new primary growth mark, or ii) did not form a new mark. The number in parenthesis indicates the relative percentage of the entire sample.

	Crystal Crab (*n*)	
Grow-out duration	**0 marks**	**1 mark**
6 month	3 (50)	3 (50)
12 month	3 (43)	4 (57)
18 month	3 (37.5)	5 (62.5)

5.2.2 Western Rock Lobster

In this project, the calcein stain was present in all Western Rock Lobster ossicles examined after 18 months (i.e. after 2–4 moults), but it was relatively faint compared with the samples taken after 6 and 8 months. Compared with Eastern Rock Lobster, Western Rock Lobster are more likely to autotomise appendages during measurement and aquaria maintenance, with limb-loss generally being linked to reduced palinurid growth (Dubula et al., 2005; Leland et al., 2013b). In this project most individuals lost some appendages (i.e. during consecutive handling events), with multiple lobsters being lost due to cannibalism before they were transferred to individual cages for the remainder of the grow-out period. This paired with a qualitative assessment of the individual moult increments (i.e. with some being negative) indicated that growth among some of the calcein-stained Western Rock Lobster was probably retarded under the conditions in the grow-out aquaria.

For Western Rock Lobster, the artificial mark (i.e. applied on September 13) was positioned either directly adjacent to a primary growth mark, or approximately midway between two successive marks (Figure 20), with the outermost being deposited during the 18 month grow-out. Only one individual had a single new-formed primary growth mark that was confidently identified beyond the calcein stain during the initial blind count, with the CGI (61%) being substantially less than the expected value (~150%) for annual periodicity. Four other Western Rock Lobster had CGIs that were more similar to the expected value (95, 157, 183 and 198%), but a new-formed primary growth mark was not identified during the blind count. Subsequent re-examination of these ossicles showed that

decalcification along the interface between the endocuticle and membranous layer (i.e. the growing edge) made confident edge determination difficult. Because of this, interpreting these ossicles could produce ambiguous results. If the edge was identified as the outermost decalcified material, then all four individuals would be taken to have deposited a single new-formed growth mark.

5.2.3 Eastern Rock Lobster

The calcein-stained Eastern Rock Lobster reared during this project were free-ranging under optimal conditions with *ad libitum* feeding and controlled tank density. The von Bertalanffy parameters derived from the Gulland-Holt plot for the captive-reared individuals (Figure 28) were similar to that of our Northern model (and that of Montgomery et al., 2009). This demonstrated that, compared with our wild-caught estimate, Eastern Rock Lobster growth during the captive rearing was slightly faster. Elevated growth is often reported for crustaceans reared under optimal conditions (e.g. Chittleborough, 1976; Barki and Karplus, 2004).

Examination of the calcein-stained Eastern Rock Lobster ossicles after 18 months (i.e. and 1–3 moults) showed that the artificial mark faded over time. However, the calcein stain was present in all ossicles sampled after 6 and 12 months, with the position of the artificial mark (i.e. applied on October 10) being consistently adjacent to a primary growth mark. Leland et al. (2015) observed similar calcein positioning in Redclaw Crayfish ossicles. Two Eastern Rock Lobster sampled after 6 months had measurable ossicular extension beyond the calcein stain, with their CGIs being greater than the expected value (i.e. 88 vs. ~50%), but neither having a new-formed primary growth mark. Two Eastern Rock Lobster sampled after 12 months also had ossicular material deposited beyond the calcein. Of these, both individuals had CGIs (94 and 106%) that were approximately equal to the previous presumed annual cycle, but only one had a new-formed primary growth mark identified beyond the calcein stain (Figure 25).

5.3 OBJECTIVE 3. Applicability to other crustacean species – with LA-ICPMS and known-age individual validation

5.3.1 Applicability assessment

Ossicular growth marks were observed in the sectioned ossicles of all seven project species and allowed for the direct estimation of putative age. The direct ageing method has previously been used to estimate age for Mud Crab (Sarapuk, 2014) and other brachyuran crabs (Kilada et al., 2012), but this project has provided the first extension to any palinurid (spiny) rock lobster. Similarly, previous research has demonstrated the methods applicability to crustaceans from temperate (Kilada et al.,

2012) and sub-tropical freshwater and estuarine environments (Sarapuk, 2014; Leland et al., 2015), but this project is the first to extend the feasibility of using direct ageing techniques on species from tropical and deep-sea environments. The direct ageing method (Leland et al., 2011) was validated using known-age juvenile Ornate and Western Rock Lobster. Comparison of directly obtained preliminary growth parameters for Ornate and Southern Rock Lobster with other independent estimates (i.e. from existing tag-and-recapture models) corroborated the validity and accuracy of using primary growth marks to determine age.

The ability to assign direct putative age estimates to Ornate Rock Lobster and Southern Rock Lobster allowed for the rapid generation of preliminary von Bertalanffy growth parameters for comparative purposes. Although based on a very small sample size (i.e. $n = 5$ for each model), the growth parameters estimated for Ornate and Southern Rock Lobster were not significantly different (i.e. based on the observed or inferred confidence interval overlap, respectively) to those published in other studies at the same broad location (Table 10 and 11). For example, Phillips et al. (1992) estimated growth parameters for male Ornate Rock Lobster from Torres Strait. Skewes et al. (1997) estimated growth parameters for 1 and 2+ (i.e. identified with size-modal analysis) male Ornate Rock Lobster from Torres Strait, reporting that reducing *K* resulted in a better fit to the Southeast Zone data. The male Ornate Rock Lobster used to estimate our preliminary growth parameters were sourced from the same location (i.e. the Southeast Zone), with our curve being more closely aligned with the Skewes et al. (1997) model (Figure 32) and application of the direct ageing method enabling the identification of four distinct year classes. Further, the CL-at-known-age data for the Ornate Rock Lobster reared under optimal conditions during this project (in Lombok, Indonesia) were closely aligned (i.e. but slightly elevated) with our model estimate for wild-caught lobster. Similarly, the preliminary growth parameters for Southern Rock Lobster from Taroona Waters, Tasmania (Figure 35) were consistent with those published by Gardner and van Putten (2008) for the same location. Despite the small sample sizes, the similarities between the direct and indirect growth estimates for Ornate and Southern Rock Lobster support the annual deposition hypothesis and demonstrate the utility of extracting age information from palinurid ossicles.

Mud Crab were the only project species for which both indirect and direct age estimates already exist. Indirect longevity estimates from the Northern Territory and southeast Queensland indicate that the species lives for approximately 3–4 years (Heasman, 1980; Knuckey, 1999). The only directly estimated longevity information (i.e. for NSW Mud Crab), also indicates that the species lives for up to 3.9 years (Leland unpublished data – also see Sarapuk, 2014). In this project, the maximum putative age estimate for Mud Crab (i.e. 4.2 years) sourced from the Northern Territory was broadly consistent with previous longevity information, but our ages were greater for a given size. For example, Knuckey (1999) reported that in the Northern Territory Mud Crab reach 130 mm CW (i.e.

equivalent to 88 mm CL) after approximately 1 year (i.e. based on tag-and-recapture data). In contrast, we aged two individuals at 92 and 97 mm CL at 2.8 and 3.8 years, respectively. In this project, the small sample number, combined with a lack of very young individuals, precluded the estimation of biologically meaningful von Bertalanffy growth parameters, with the predicted theoretical maximum length being substantially greater than that observed in nature (e.g. L_∞ = 261.16 mm CL and K = 0.13). Knuckey (1999) a described similar challenge with estimating growth parameters from tag-and-recapture data for female Mud Crab (e.g. L_∞ = 530.7 mm CL and K = 0.30). Further research is needed to investigate the reasons for the differences between the direct and indirect estimates for this species.

Compared with the other project species, the growth and longevity of Crystal Crab is less-well understood. The only existing indirect size-at-age estimate for Crystal Crab (i.e. for males only) estimated slow growth, with males becoming sexually mature (~94 mm CL) and reaching the minimum legal size (120 mm CL), after approximately 17 and 19 years, respectively (see 6°C model in Melville-Smith et al., 2007). In this project component we examined very few Crystal Crab ossicles (n = 5), but our directly determined putative ages were always greater than that predicted by Melville-Smith et al. (2007). For example, in this project a male crab (at 91.5 mm CL) approaching the size-at-maturity was aged as being approximately 18 years old (i.e. vs. ~13 – Melville-Smith et al., 2007). Similarly, two other male Crystal Crab (114.8 and 130.5 mm CL) that were less and greater than the minimum legal size (i.e. 120 mm CL) were assigned putative ages that were almost double (i.e. 28 vs. 15 and 33 vs. 18 years, respectively) that predicted by Melville-Smith et al. (2007). No indirect size-at-age estimates exist for female Crystal Crab, but they are known to have smaller moult increments and reach a smaller maximum size (Melville-Smith et al., 2007). The only female Crystal Crab sampled in this project was both the smallest (72.6 mm CL) and oldest (~37 years) individual (Figure 38). Melville-Smith et al. (2007) noted that their size-at-age estimates were preliminary and based on numerous unverified assumptions that integrated uncertainty into their model. In this project, we have provided strong evidence that primary growth marks are deposited annually in Crystal Crab ossicles. Further, our validated age estimates incorporate only a single unverified assumption – i.e., that primary growth marks are deposited annually in the ossicles of small juveniles not considered in this project (i.e. < 92 mm CL). For these reasons, it appears likely that the previous estimates for Crystal Crab predicted substantially faster growth than actually occurs in nature.

Similar to Crystal Crab, the growth and longevity of Giant Crab is poorly understood. Giant Crab inhabit deep cold-water habitats and can reach a substantial maximum size (> 200 mm CL and ~10 kg – Gardner and Quintana, 1998; Levings et al., 2001). Although formal indirect age estimates for the species do not exist, Giant Crab are presumed to be slow growing (i.e. with 4–7 year moult intervals) and long lived (Levings et al., 2001; Hartmann et al., 2014). One study reported that laboratory-reared

juvenile Giant Crab took two years to reach 25 mm CL (Gardner and Welsford, 2003). In this project, Giant Crab was the only species for which applying the direct ageing methodology did not allow for the rapid generation of putative age estimates. Complete counts were not possible from either pterocardiac or zygocardiac ossicles, with both structures having endocuticular zones where the primary growth marks could not be confidently identified. To our knowledge, no other direct ageing study has reported this for any other crustacean. The utility of particular fin fish ageing structures can be species specific (Campana, 2001). Given that only a small number of ossicles (i.e. n = 3 for each type) were examined during this project, further investigation into optimal Giant Crab ageing structures (e.g. other ossicles or eyestalks) is needed to confirm our preliminary finding. Despite the above, the presence of at least some identifiable primary growth marks in Giant Crab ossicles allowed for the first preliminary minimum estimation of age (Figure 39). For example, one large Giant Crab (205 mm CL male – Figure 39B) ossicle had > 21 primary growth marks. Presuming a relatively constant primary series spacing (i.e. as observed in all other species) within the uninterpretable zones, this individual would exceed 30 years of age.

5.3.2 Elemental composition analysis

Elemental incorporation into biogenic calcium minerals can correspond to ambient environmental conditions during deposition. The ratios at which particular elements are incorporated into the calcified matrix varies according to environmental factors including temperature (Bath et al., 2000) and salinity (Scharer et al., 2012). However, other factors such as the organic content (Takesue et al., 2008) can regulate the relative affinity of a particular element to the structure, or alter the selectivity of calcium ions during the calcification process thereby affecting the deposition rate (Carre et al., 2006). Irrespective of the driving factor, elemental ratios often vary on a seasonal basis and cyclical patterns that correspond to growth increments can be used to validate periodicity (e.g. Scharer et al., 2012; Beaver et al., 2016).

During the project planning phase LA-ICPMS analysis was identified as a relatively simple (e.g. compared with calcein staining) validation method that would require few samples and allow for a broader assessment of primary growth mark periodicity in all seven project species. However, novel data acquired during the project revealed previously unidentified challenges in using LA-ICPMS for decapod age validation. During the pre-moult stage, the mineralized cuticle of decapod crustaceans is progressively decalcified until moulting occurs (Greenaway, 1985). In this project, whole extracted ossicles from Ornate Rock Lobster, Western Rock Lobster and Eastern Rock Lobster were noted to have apparently decalcified zones. This has never been reported for any decapod. The LA-ICPMS analysis of macroscopically detected decalcified zones in Ornate Rock Lobster ossicles (Figure 29) confirmed that multiple gastric mill structures (i.e. the meso-, zygo- and pterocardiac ossicles) are

decalcified during the pre-moult stage (Figure 30). Therefore, regardless of whether gastric ossicles are moulted or not, pre-moult decalcification (i.e. for spiny lobster at least) definitely mobilizes some inorganic cuticular components associated with the calcified material, with subsequent post-moult re-deposition being likely (Greenaway, 1985; also see Sheridan et al., 2016).

Given the above, the elemental ratios in ossicular endocuticle might not be expected to show cyclical variations corresponding to the ambient environmental conditions during primary growth mark formation. Instead, any observed elemental pattern should reflect the periodic (and episodic) re-deposition of mineral content. For all species examined however, some elemental ratios (e.g. Sr:Ca and B:Ca) did show regular cyclical variation across the entire endocuticle, with the number of maxima (generally) corresponding to the visually observed primary growth marks. This pattern was clearest for relatively short-lived species (e.g. Mud Crab – Figure 54 and 555) or young animals (e.g. 3–4 years old – Figure 40 and 43), because their primary growth marks are spaced further apart.

Until detailed species-specific information exists for the exact fate of gastric ossicles during ecdysis, making any detailed inferences (i.e. for validation purposes) from the LA-ICPMS data would be too speculative. For example, the strong cyclical patterns quantified in Mud Crab ossicles (e.g. for Sr:Ca in MC-5 and MC-6) could be interpreted as supporting annual periodicity, with the ratio maxima associated with each primary growth mark being linked with seasonal patterns such as salinity fluctuations during wet or dry seasons (see Scharer et al., 2012). However, such an interpretation would assume that for this species the mineral content is: i) never mobilized or ii) re-deposited with the observed cyclical pattern being preserved. Kilada et al. (2012) demonstrated that in American Lobster some cuticular mineral features are retained through moulting. Astrop et al. (2015) reported that the outer surface of the carapace in Clam Shrimp (Branchiopoda: Spinicaudata) is retained after moulting and contains an ontogenetic growth record. It is possible that some mineral content remains integrated within the decalcified organic matrix of the ossicle, rather than being mobilized with the calcium. Another potential explanation could be that the organic matrix provides a template that controls re-formation and results in the reproduction of previous physico-chemical features that are linked with cyclical patterns (see Leland et al., 2015).

Further detailed studies into decalcification and re-deposition of cuticular mineral features are needed, with particular attention being paid to whether gastric ossicles are wholly (see Sheridan et al., 2016), or partially moulted (see Astrop et al., 2015), and if all moult events are equal (i.e. are ossicles moulted every time the external exoskeleton is shed?). Because of the above uncertainties, the quantified cyclical changes in elemental ratios associated with the primary growth marks in crab and lobster (e.g. WRL-19 and ERL-22) ossicles cannot be used for age validation at present, but remain a fertile area for further investigation. In this project however, we have confirmed for the first time that the visually

identified primary growth marks correspond with real physico-chemical variations in ossicular mineral composition.

5.3.3 Known-age individuals

Three previous studies have used known-age individuals to validate that the number of primary growth marks in American Lobster and European Lobster (*Homarus gammarus*) equates to chronological age (i.e. using up to 5 year old animals) (see Kilada et al., 2012; 2015; 2017a). In this project, all ossicles from the known-age (i.e. at 1.1 and 1.4 years old) Ornate Rock Lobster (n = 13) consistently had a single primary growth mark identified within their ossicular endocuticle. We noted that two Ornate Rock Lobster had other visually distinct bands beyond the first annual growth mark (Figure 59), but these were obviously inconsistent with the normal appearance of the primary series and were possibly attributable to a captive rearing artefact (Campana, 2001; Vogt, 2012), or pre-moult-related effect (see Vatcher et al., 2015).

The known-age ossicles from Western Rock Lobster were relatively difficult to interpret, because of the presence of a very prominent secondary series (Figure 61). Because of this, most known-age Western Rock Lobster ossicles were categorised as having poor readability (n = 4), with a high potential for series ambiguity. Ideally, all poor sections would have been discarded. However, to conserve valuable samples and to illustrate some of the challenges associated with unusually prominent secondary series, we retained all known-age ossicles for their comparative value. The only known-age individual categorised as having intermediate readability was correctly aged (i.e. at 3+ years) by the primary reader. Two other known-age Western Rock Lobster were correctly aged by the secondary reader (i.e. at 1+ and 2+). For both readers, the number of primary growth marks was always within ±1 of the known-age value, but the two readers rarely agreed on any particular count (Table 12), because of their differing interpretations of the primary and secondary series.

The Ornate Rock Lobster were reared under natural photoperiod and temperature (i.e. in outdoor sea-pens), but the Western Rock Lobster were reared in indoor aquaria (i.e. under artificial conditions). It is conceivable, that the rearing conditions might have influenced the appearance of the Western Rock Lobster ossicles (Campana, 2001; Vogt, 2012). Further, the known age Western Rock Lobster ossicles were the only samples in this project that were not prepared by the principal investigator and it is possible that slight methodological alterations might have contributed to the substantial differences in relative appearance (compare Figure 59 and 61). Irrespective of the relative differences, both species provided a validation of the direct ageing method (i.e. using juvenile animals), with the number of primary growth marks being equal to the known chronological age in most individuals examined. Further assessment of older known-age individuals (> 4 years) would be beneficial, particularly for

Western Rock Lobster. As an overarching caveat, we acknowledge that the interpretation of the known-age ossicles in this study incorporated some non-excludable potential for reader bias due to: i) ossicle diameter and any associated age expectation and ii) knowledge of maximum grow-out duration.

5.4 OBJECTIVE 4. Direct ageing network – workshop

Eleven participants including project co-investigators and other fisheries scientists, attended the Crustacean Ageing Workshop held at Southern Cross University (Lismore campus) on March 16 and 17, 2016. The participants and their respective institutions were: Jesse Leland and Daniel Bucher (SCU), Mark Grubert (Northern Territory Department of Primary Industry and Fisheries), Graeme Ewing (Institute for Marine and Antarctic Studies – University of Tasmania), Geoff Liggins (New South Wales Department of Primary Industries – Fisheries), Simon de Lestang and Jason How (Department of Fisheries Western Australia), Wayne Sumpton and Jason McGilvray (Queensland Government – Department of Agriculture and Fisheries) and Peter Hawthorne and Lachlan McLeay (South Australian Research and Development Institute). Co-investigator Paul Butcher (NSW DPI – Fisheries) registered to attend, but was a last-minute apology.

The SCU workshop included detailed laboratory instruction on direct ageing methodologies, with all presentations being provided to the participants. Hands-on laboratory sessions that included methodological and theoretical explanations, cross-sectioning, cuticular boundary identification and ossicle interpretation (for Western Rock Lobster, Eastern Rock Lobster and Crystal Crab), stimulated discussion on the similarities and differences between crustacean ossicles and fish otoliths, with a focus on areas where technique cross-over might be beneficial. Wayne Sumpton displayed pre-prepared Spanner Crab ossicle sections, with participants discussing their interpretation and agreeing that the method seems applicable to that species, but further assessment is needed. The workshop concluded with an open discussion on future research priorities, with all participants providing valuable contributions. The five specific research needs that were identified are summarized below and incorporated into the General Discussion.

1. Further research and development into the application of validation techniques to crustacean ossicles is needed.
2. A definitive validation is needed before direct ageing methods are used in large-scale applications (e.g. stock assessments).
3. Further validation studies should investigate the formation of the first growth mark using known-age individuals. Such research should also assess the seasonality of growth mark

deposition and the potential for alterations to the original spacing of early growth marks (i.e. apparent compression with increased age).

4. For each species, the construction of a standard ossicle reference collection is needed for reader training and subsequent precision assessment.
5. A better understanding of the processes governing growth mark deposition is important, with a particular focus on moult cycle influences.

5.5 General Discussion

The rationale behind this project involved providing a validation for the direct ageing method, while developing and extending the technique within a national applicability assessment using some of Australia's most valuable commercial crustaceans. This broad-scale approach was adopted with a view towards completing obligatory first steps (i.e. a methodological validation), while building confidence in the direct ageing method and future research capacity via the national collaborative network established during the project (i.e. Objective 4). For almost 100 years, the collective efforts of fisheries biologists around the world have focussed on developing validated ageing methods for fin fish. Despite this mammoth effort, the underlying exogenous and endogenous causes for growth increment formation are not completely understood, with successive advances revealing further complexity and uncertainty (Gronkjaer, 2016). Less than a decade ago, Leland et al. (2011) pioneered the field of direct crustacean ageing. This developing field has benefitted from the breadth of previous knowledge from fin fish ageing studies, but faces additional challenges (e.g. the moult cycle) for which there is no prior information to draw on (i.e. with regard to ageing).

At present, the mechanism governing the accrual of primary growth marks in crustacean endocuticle is not well understood (but see Kilada et al., 2012 and Leland et al., 2015) and can result in low confidence for direct ageing methods – i.e., expressed as uptake and/or research inertia. However, there is a mounting (global) body of evidence that has consistently demonstrated that the number of primary growth marks in the endocuticle of decapod crustaceans equates to chronological age (Kilada et al., 2012; Sarapuk, 2014; Kilada and Acuna, 2015; Kilada et al., 2015; Leland et al., 2015; Krafft et al., 2016; Kilada et al., 2017a). In this project, we have provided multiple lines of evidence demonstrating annual periodicity for four species and found that applying the direct method consistently yields ages that agree with previous indirect longevity estimates (Table 14). This project has raised the global verified methodological applicability total to > 23 species, while making substantial advances in applying the method to long-lived crustaceans. Further, we have demonstrated that, like otolith increments, the formation of ossicular growth marks is ubiquitous across tropical to temperate regions and deep-sea to relatively shallow waters (Morales-Nin and Panfili, 2005; Gronkjaer, 2016).

5.5.1 Moulting and uncertainty

One question raised at the Crustacean Ageing Workshop, was if ossicular growth marks might simply comprise a record of growth via moult events (i.e. a total instar record). However, Kilada et al. (2012) provided the first evidence for moult-independent growth mark formation in Snow Crab eyestalks. Similarly, in this project, we have demonstrated that growth marks in Crystal Crab ossicles are formed during the inter-moult period. Given the above, and the fact that our maximum putative ages for the seven species examined in this project was consistently aligned with maximum longevity (i.e. rather than moult history) (Table 14), the primary growth mark series does not equate to an instar record (also see Kilada et al., 2012; Leland et al., 2015). For example, the known-age Ornate Rock Lobster moulted ~20–24 times (C. Jones, pers. com.) during the captive grow-out period, but only had a single primary growth mark.

Currently, it is uncertain if gastric ossicles are retained for life, or if these structures are wholly or partially moulted. However, known-to-be moulted crustacean exoskeleton components (e.g. eyestalks – Krafft et al., 2016) contain primary growth marks, with the observed number in external structures being equal to those in ossicles (Kilada et al., 2012). Because of this, explanations for the existence or interpretation of the primary growth mark series should be developed irrespective of the current moulting debate. If future research reveals that decapod ossicles are retained for life, no further explanation for preservation of the growth record will be needed. However, if gastric ossicles are wholly or partially moulted (i.e. like eyestalks), then any investigation into endocuticular growth marks will need to consider the mechanism for growth record retention in moulted structures (e.g. like eyestalks – Krafft, 2016; Kilada et al., 2012; 2017a). Given the above, in this report we have only discussed the potential for moult-related effects that are definitively known (i.e. ossicular decalcification).

Ossicles and otoliths are quite different in terms of their physical structure, composition and stability. Because of such differences, some analyses (i.e. and in-built assumptions) that were originally developed for otolith studies might not be applicable to ossicles. For example, otolith structure is stable and compositional variations can be assumed to reflect differences in environmental conditions during deposition. Ossicles are unstable by comparison, because the elemental composition can be altered (i.e. decalcified) subsequent to the initial deposition. For the rock lobster species in this project, there was an apparent difference between the primary growth mark spacing in very young and old individuals, with successive widths consistently decreasing with increasing age (Figure 8 and 9) – i.e., the primary series seems to be compressed over time. A similar relationship was observed for the width of endocuticular lamellae. Any re-working of ossicles during moulting could potentially affect the position of early growth marks. This indicates that using ossicular growth marks for back-calculated growth estimates might not be possible.

Table 14. Summary table comparing the carapace length (CL) and putative age range examined in this project with the corresponding data from indirect estimates (*n* = number of individuals, max. = maximum). '-' indicates data not available.

Taxa (*n*)	CL (mm) in this project	Direct putative age (years)	CL-at-maturity (mm)	Max. CL (mm)	Indirect age-at-maturity (years)	Indirect max. longevity (years)	Source(s)
Western Rock Lobster (86)	26.7–129.1	1.9–19.0	65–80[a]	>150	5–7	20+	de Lestang (2014)
Eastern Rock Lobster (78)	58–236	2.1–26.1	~167	260	~9.5[b]	30+	Liggins (2014), Montgomery et al. (2009)
Ornate Rock Lobster (5)	85–122	1.7–4.7	~100	>150	2–3	3–5+	Flood and Roelofs (2014)
Southern Rock Lobster (8)	59–171[c]	1.6–16.6	59–122[a]	>200	-	20+	Linnane et al. (2014)
Mud Crab (7)	87–125	2.8–4.2	81–103[a,d]	>140[d]	1.5–2.0	3–4+	Grubert et al. (2014), Kailola et al. (1993)
Crystal Crab (5)	72.6–145.9	18–37[e]	94	>180	~17[f]	30+	Melville-Smith et al. (2007)
Giant Crab (3)	150–219	>9–21[e]	125–140[a]	>200	-	30+	Hartmann et al. (2014)

[a] Region dependant.

[b] This value is the predicted mean age (i.e. at 167 mm CL) calculated using the Montgomery et al. (2009) female growth parameters.

[c] Southern Rock Lobster sourced from Tasmania and South Australia are combined in this table.

[d] Mud crab CL-at-maturity and maximum CL was converted from CW using the equation, CL = CW – 7.8881 ÷ 1.3832 (Leland unpublished data, R^2 = 0.98; *n* = 690).

[e] Primary growth mark count – not adjusted for birthdate or sampling date. Note: Complete counts were not possible for Giant Crab, estimates provided here are for discussion only.

[f] Value derived from the 6°C model (Table 6.1).

5.5.2 Growth mark formation

In this project, we have experimentally demonstrated the formation of ossicular growth marks both during the inter-moult period (i.e. for Crystal Crab) and after moulting (i.e. for Western and Eastern Rock Lobster), with the latter including formation of the first primary mark (i.e. for known-age Ornate Rock Lobster). In locusts the exoskeletal endocuticle is deposited after each moult (Neville, 1965). Neville (1965) documented the daily deposition of paired endocuticular features (i.e. lamellate and non-lamellate layers – termed "growth layers") during the locust inter-moult period (also see Nesbit-Noble, 1963). Further, Neville (1965) demonstrated that endocuticular growth layer formation (i.e. up to 20 daily increments) in the locust exoskeleton directly corresponded to temperature and light cycles, with experimental manipulation of circadian factors consistently altering normal deposition.

Leland et al. (2015) demonstrated the sequential addition of new information (i.e. urocardiac ridges) to a known-to-be moulted structure (i.e. the mesocardiac tooth plate), hypothesising that "previously recorded internal growth-rate variations could be duplicated, before being added to over time" and inferring that a similar process might control the accrual of primary growth marks. The work of Neville (1965) confirms that cyclical growth-rate variations (i.e. with constant periodicity) can be recorded in arthropod endocuticle. A more recent study has verified that endocuticular features (i.e. structural lamellae) are sequentially added over time in Ghost Shrimp, *Neotrypaea californiensis*, ossicles (i.e. at 4.9 lamellae yr^{-1} – K. Bosley, pers. com.). Further, that study involved rearing known-age Ghost Shrimp for up to five years and found that the number of endocuticular lamellae showed a clear modal progression that closely agreed with the actual known age and lipofuscin-based age estimate (K. Bosley pers. com.). This clearly demonstrates that even if Ghost Shrimp ossicles are moulted (i.e. which is currently unknown), internal features deposited in their ossicular endocuticle persist through time and can represent chronological age. Although the exact formation mechanism remains unknown, existing knowledge for other arthropods (Nesbit-Noble, 1963; Neville, 1965) and crustaceans (Leland et al., 2015), combined with the results of this study, support using ossicular growth marks for crustacean age determination and provide a theoretical basis for the investigation of growth mark formation.

5.5.3 Calcein staining

In this project, we used a relatively high calcein concentration (500 mg l^{-1}) and immersion duration (3 days), because previous studies have demonstrated that this treatment effectively marked crustacean ossicles for up to one year (Kilada et al., 2012; Sarapuk et al., 2014; Leland et al., 2015). These studies presumed that ossicular extension only occurred after moulting, lengthening the immersion duration to ensure adequate time for incorporation into the growing edge and to account for the uncertainty around the moulting–retention–time nexus. In hindsight, given that ossicular extension

also occurs during the inter-moult, the staining duration could probably have been reduced. Effective calcein staining requires a balance between concentration and duration, with both parameters influencing the potential for stress responses (van der Geest et al., 2011).

Post-staining responses to calcein can be species specific, but generally studies report high survival for treatments ranging from 80–640 mg l^{-1} (Kaehler and McQuaid, 1999; van der Geest et al., 2011). In this project, we observed low mortality among artificially-stained Western Rock Lobster, Eastern Rock Lobster and Crystal Crab (i.e. 0, < 2 and 5%, respectively), indicating that, like other decapods, (Kilada et al., 2012; Sarapuk, 2014; Leland et al., 2015), those in this project were generally tolerant to the calcein treatment used. Other calcein-marking studies on aquatic organisms (e.g. fin fish and bivalves) have reported similar maximum mortality (e.g. 0–5%), without any significant impacts on calcification (or growth) rate or body condition (Riascos et al., 2007; Cameron et al., 2011; van der Geest et al., 2011). Despite the low mortality in this project, the potential for some sub-lethal effect on ossicular extension could not be ruled out and could conceivably have influenced the CGIs recorded during the grow-out. However, given that there were no visible stress-induced marks associated with the calcein stain, the likelihood for this was considered to be low.

Previous studies using fluorochrome stains on crustaceans (Kilada et al., 2012; Sarapuk, 2014; Leland et al., 2015) and other organisms (Pirker and Schiel, 1993; Day et al., 1995) have noted variability in mark incorporation and the potential for natural autofluorescence to mask artificial marks. Crustacean cuticular layers are interconnected by structural transport canals (see Roer and Dillaman, 1984). Combined with the relatively high concentration and duration parameters, such connectivity probably explains the general tendency for calcein to penetrate most (or all) ossicular layers. However, similar to Leland et al. (2015), Kilada et al. (2012) and Sarapuk (2014), we also observed that for some ossicles the calcein was only incorporated along the growing edge (or outermost layer). Given the range of staining patterns and variable propensity for calcein penetration in individual ossicles (i.e. across all three species), it appears likely that unknown factors (e.g. fine-scale changes during the inter-moult) are influencing the efficacy of the artificial mark.

The maximum time period reported in the literature for calcein retention in crustacean ossicles is one year (Leland et al., 2015). In this project, the calcein was retained in Eastern Rock Lobster ossicles for one year (i.e. after 1–3 moults), but after 18 months the artificial mark was difficult to distinguish from natural autofluorescence. For Western Rock Lobster, the calcein was identifiable after 18 months (i.e. after 2–4 moults), but was notably fainter (i.e. relative to the natural autofluorescence) than that in samples examined shortly after staining. Alternately, the calcein was retained in Crystal Crab ossicles for 18 months without any apparent fading, but was absent from a single individual that moulted (see Sheridan et al., 2016). Leips et al. (2001) reported short-term calcein fading for external fin fish

ageing structures (i.e. scales and fin rays) and inferred that the most likely cause was ultraviolet degradation. However, given that the gastric mill is housed within the cardiac stomach, and the extracted ossicles were appropriately stored in lightproof containers, ultraviolet degradation cannot explain the reduced calcein intensity over time. Ideally, artificial marks for crustacean age validation studies should persist for multiple years. Given the clear temporal limitation on the efficacy of calcein as a long-term (> 18 month) artificial tag, further research is needed to identify alternative stains that can be used to mark the organic component of the ossicular framework. An artificial mark that effectively labels the organic matrix would facilitate a definitive assessment of whether it is retained after moulting and might facilitate a more objective determination of the stain boundary. Similarly, a marker (e.g. strontium chloride) that can be quantitatively assessed (i.e. for presence or absence) might improve objectivity in determining the stain boundary.

5.5.4 Growth modelling

In the past, application of direct ageing methods was limited to relatively short-lived taxa, or younger age classes of long-lived species. Kilada et al. (2012) generated sex-specific CL-at-age relationships for three Atlantic species (i.e. Snow Crab, American Lobster and Northern Shrimp, *Pandalus borealis*), with estimated individual ages ranging from 1–12+ years. Leland et al. (2015) demonstrated that ossicular growth records can be easily extracted for rapid growth modelling and provided the world's first directly determined (i.e. with validatory evidence) von Bertalanffy growth parameter estimates using the short-lived (i.e. 0–3+ years) Redclaw Crayfish. In this project, we have demonstrated the applicability of direct ageing methods to both short- and long-lived palinurid rock lobster (i.e. range of 1–26+ years – Table 14) and found that their growth can be adequately described with the von Bertalanffy (1938) equation. This represents a substantial advance and allowed for the generation of easily comparable growth models. However, we note that the palinurid growth models presented here were primarily constructed for their corroborative value, with financial and time constraints affecting the sampling design, sample number and other important factors (see 'Precision' sub-section – 5.5.6 below).

The immediate importance of estimating growth parameters from directly determined ages will vary across fisheries jurisdictions and according to managerial approaches. In some jurisdictions, further study may be needed to rigorously assess the potential for region-specific growth differences (e.g. for Eastern Rock Lobster) and construct directly determined growth models that can be used to inform management decisions. In other jurisdictions, the growth model (e.g. for Western and Ornate Rock Lobster) constructed in this project provided a validation of the existing indirect ageing methods used for stock assessment and regulatory purposes. In Tasmania, there are particular fishery regions (i.e. for Southern Rock Lobster) where tagging programs have run for >20 years, but a reliable growth matrix

does not exist. The ability to produce directly determined (i.e. and validated) models could overcome such challenges for Southern Rock Lobster, while allowing for rapid location-specific assessments to identify optimal zones for growth enhancement via lobster translocation.

Size is generally a poor predictor of crustacean age. In this project, the directly determined putative ages for Western and Eastern Rock Lobster were strongly correlated with CL, but there was substantial variation among individual year classes. Other direct ageing studies have identified similar CL-at-putative-age variation (e.g. Kilada et al., 2012; Kilada and Acuna, 2015). The CL-at-known-age data for Western Rock Lobster (Figure 62) in this project, illustrated the capacity for substantial variation (i.e. in CL or age) among individuals. Further, for young known-age Western Rock Lobster (i.e. 1– 3+ years) the CL-at-age variation was similar among captive-reared and wild-caught individuals (Figure 12). For other crustacean species, CL-at-putative-age variation generally increases with longevity, with short-lived taxa having less than long-lived species (e.g. Kilada et al., 2012; Kilada and Acuna, 2015; Leland et al., 2015).

Crustacean exoskeletal growth is commonly referred to as being a function of the moult increment and duration (Hartnoll, 2001). However, any individual's final size reflects not only their cumulative lifetime sum of these parameters, but also the influence of many exogenous factors (e.g. food availability) that vary both spatially and temporally (O'Malley, 2009). In addition, physical impacts can also affect crustacean growth. For example, crustaceans that autotomise appendages (i.e. to avoid predation) generally have reduced growth, presumably because of the energetic demand for regenerative processes (Dubula et al., 2005; Leland et al., 2013b). Crustaceans also have endogenous controls for active growth moderation in response to changing environmental (Hartnoll, 2001).

Palinurid tagging studies commonly report substantial variation in individual moult increments (Phillips et al., 1992; Montgomery et al., 2009; O'Malley, 2009). The primary limitation of tag-and-recapture is that this method only quantifies size increase during a discrete time period (i.e. during the time-at-liberty) and measurement of any past (i.e. occurring from birth to before first capture) growth-rate variation is impossible. However, our directly determined CL-at-putative-age data for Western and Eastern Rock Lobster contain the sum of all past growth-rate variations (i.e. from exogenous and endogenous factors) that have occurred over each animal's entire lifetime (i.e. from settlement to sacrifice). Given the wide range of factors that influence crustacean growth, it is not surprising that long-lived species like Western and Eastern Rock Lobster show substantial variation in CL-at-putative-age.

5.5.5 Method validation and corroboration

Rigorous age validation is needed before direct crustacean ageing methods can be incorporated into programs that inform managerial decisions (Leland et al., 2011). Past history has demonstrated the effects of using un-validated fin fish ages (Beamish and McFarlane, 1983; Campana, 2001), which can translate into substantial negative environmental, economic and social costs. Because of this, we have evaluated the validation results from this project against best-known practice and using standard methodological approaches as described by Campana (2001) for fin fish. Ideally, any ageing method should be validated using two (or more) methods to confirm the accuracy of increment interpretations (Campana, 2001). In this project, we have provided substantial evidence for annual periodicity in multiple crustaceans using validation methods that are routinely applied to fin fish (Table 15). Further, for all four Australian rock lobster species the corroborative evidence (i.e. the direct and indirect ageing comparisons) indicates that our interpretations were accurate (Table 15), with the LA-ICPMS results demonstrating that primary growth marks correspond with real physico-chemical variations in ossicular composition. However, like other recent studies (Kilada et al., 2012; Kilada et al., 2015; Leland et al., 2015) that have provided validatory evidence (e.g. using known-age individuals and calcein staining), our results were somewhat limited by small sample size (i.e. despite meeting the minimum validation method criteria – Table 15) and a single evidence line for some species (e.g. Crystal Crab).

Identification of the first growth increment is important for ageing accuracy (Campana, 2001). In this project we consistently identified the first primary growth mark in known-age Ornate Rock Lobster, providing the strongest result to date using this method. Although species-specific identification of the first growth mark would be ideal, the known-age Ornate Rock Lobster results nonetheless support our assumption for all rock lobster that the first primary mark beyond the cuticular boundary is deposited during the first year.

A few previous studies have used length-frequency analysis to corroborate their directly determined putative ages for relatively short-lived crustaceans (Kilada et al., 2012; Kilada and Acuna, 2015; Kilada et al., 2015). Our estimation of directly determined von Bertalanffy growth parameters enabled the world's first direct comparison between mean predicted indirect and direct age estimates over the majority of a species lifespan. For both Western and Eastern Rock Lobster, the apparent similarity between the rates of increase between the two methods strongly indicates that our putative age estimates are accurate and that primary growth marks are deposited annually. Further, the strong similarities between the direct and indirect von Bertalanffy growth parameters for Ornate and Southern Rock Lobster (i.e. at specific locations) provides preliminary corroborations for these species.

Like the known-age individuals, the calcein-staining evaluations in this project also indicated that the primary growth marks are annual (Table 15). For Crystal Crab, a single new-formed primary growth mark was deposited during the inter-moult period. Because of this, the potential for ossicular decalcification (i.e. with concomitant calcium and calcein mobilisation) has no immediate bearing on the validatory evidence presented for this species. However, a reasonable interpretation of the calcein results for animals that moulted after staining (i.e. Western and Eastern Rock Lobster) requires some caution. One caveat for the rock lobster calcein-staining results is that the interpretation presented here contains unverified assumptions. Specifically, because our interpretation was dependent on positively identifying an artificial mark (i.e. of known date) for subsequent re-examination (i.e. after 1–4 moult events) and measurement of ossicular growth beyond the stain, our validation evidence presupposes that either: i) ossicles are not moulted, or ii) ossicles are only partially moulted with the calcein being retained in the original incorporation position (i.e. either in the mineral or organic matrices). In this project, we observed that the calcein stain was present in almost completely decalcified ossicles (e.g. Figure 25). This suggests that the artificial mark can also be retained within the organic template, which could be retained after moulting (see Leland et al., 2015 for hypothesis).

In this project, we have provided a methodological validation (i.e. for four species) for the direct ageing technique (Table 15). The calcein staining experiments, and known-age lobster, provided validation of both periodicity and absolute age (respectively), with all corroborative evidence being consistent with annual deposition (see Campana, 2001). We acknowledge that other important steps in the validation process (i.e. for individual species) exist and are a priority for further research. Further research should: i) validate periodicity across the entire age range, ii) determine the age at first growth mark formation and iii) assess ageing accuracy.

5.5.6 Precision

This project was focussed on developing and validating the direct ageing method for crustaceans. As such, we have only taken the first step towards addressing potential ageing error (i.e. by providing a methodological validation). Beyond the other validation requirements (see section 5.5.5 above), assessing the reproducibility of age estimates (i.e. termed ‘precision’) is equally important for any ageing program. Only few studies (e.g. Leland et al., 2015; Krafft et al., 2016; Kilada et al., 2017a) have assessed the precision of endocuticular growth mark counts, with both being focussed on relatively short-lived species (i.e. Redclaw Crayfish and Antarctic Krill, *Euphausia superba*). Krafft et al. (2016) found no significant difference between endocuticular growth mark counts (i.e. $n = 51$) from multiple readers (i.e. 2–4), with six being the maximum count. Similarly, Kilada et al. (2017a) found that direct age estimates for Antarctic Krill were highly reproducible and yielded acceptable variation levels. Leland et al. (2015) reported 87% agreement (i.e. $n = 15$) between two independent readers,

but noted that the ±1 year maximum difference was a substantial proportion of total age (i.e. 3+ years). In this project, the comparison of counts for the known-age Western Rock Lobster showed similar variation (±1 year). Given the poor quality of these samples however, this was not surprising.

Table 15. The species-specific validation and corroboration method outcomes achieved in this project, with the number of individuals given in parenthesis. A = agreed with annual periodicity, D = determined absolute age, na = not assessed in this project, U = undetermined.

Taxa	Validation method		Corroboration method	
	Calcein staining	**Known-age individuals**	**Indirect growth model**	**Maximum longevity**
Western Rock Lobster	A (1+)[a]	A, D (3)[a]	A (86)	A
Eastern Rock Lobster	A (1+)[a]	na	A (>34)[b]	A
Ornate Rock Lobster	na	A, D (13)[a]	A (5)	A
Southern Rock Lobster	na	na	A (5)	A
Mud Crab	na	na	U[c]	A
Crystal Crab	A (12+)[a]	na	U[c]	A[d]

[a]The sample size required for validation by calcein staining and known-age individuals is > 1 (Campana, 2001). The numeric value indicates the *n* for positive growth mark deposition during the grow-out and the '+' denotes the presence of multiple ossicles for which the CGI was approximately equal to the previous annual cycle.

[b]Sample size excludes lobster from Jervis Bay and some Coffs Harbour individuals. The positive corroboration is based on the inference that the 1:1 divergence for the excluded individuals was attributable to temperature.

[c]Direct models for comparison were not constructed for these species during this project.

[d]The direct ages for Crystal Crab were broadly consistent (but always greater) with the only other existing preliminary estimate from Melville-Smith et al. (2007).

After procuring a methodological validation, precision assessments form an important quality control measure to manage accuracy (Campana, 2001). The importance of standard reference collections for training purposes is well known and was identified as a priority during the Crustacean Ageing Workshop. This project has generated initial digital reference collections for Western and Eastern Rock Lobster that can be added to over time. Leland et al. (2015) found that some ossicles have a disproportionally prominent secondary series that can impede interpretation. Similarly, in this project we found that the most commonly violated readability criterion for Western and Eastern Rock Lobster was the potential for series ambiguity, which triggered the high ossicle rejection rates. Further species-specific assessments are needed to quantify the potential for precision bias in direct crustacean ageing studies, particularly for long-lived species.

6.0 Conclusion

Completion of the project objectives has yielded substantial advances in the field of direct crustacean ageing. Assessment of the relationship between size and age allowed for the world's first estimation of von Bertalanffy growth parameters for any palinurid rock lobster and direct comparison between indirect and direct age estimates. For all four rock lobsters, the von Bertalanffy growth parameters estimated during this project were not significantly different to previous indirect estimates. The evaluation of primary growth mark periodicity via calcein-staining and long-term grow-out, added further support for a methodological validation for Western, Eastern Rock Lobster and Crystal Crab, demonstrating that ossicular growth marks were deposited annually in some individuals. The direct ageing methodology was readily applied to most (i.e. all except Giant Crab) project species, with validatory evidence from known-age Ornate and Western Rock Lobster also agreeing with annual periodicity. Some of the patterns quantified with the LA-ICPMS analysis could be interpreted as verifying annual periodicity, but current theoretical uncertainties (i.e. around decalcification and moulting) precluded making a definitive conclusion at this point in time. Despite the methodological validation achieved here, further research is needed to procure species-specific age validations that encompass the entire age range, before the direct method can be routinely used for ageing project species. To make it clear, the specific conclusions for each experimental objective (i.e. Objectives 1–3) are listed below.

OBJECTIVE 1. Size-at-putative-age assessment – Western and Eastern Rock Lobster

1) For Western and Eastern Rock Lobster, sectioned ossicles contain regular primary growth marks that can be used to assign putative ages. Rejection rates were higher than anticipated, but might be reduced via strategic sampling.
2) For Western and Eastern Rock Lobster, the von Bertalanffy growth parameters estimates derived from directly determined putative ages were not significantly different to those from tag-and-recapture studies.
3) For Western Rock Lobster, the directly determined putative ages closely agree with indirect longevity estimates and the age at fishery-specific milestones (i.e. minimum legal size and size-at-sexual maturity). For lobsters sampled from comparable locations, the relationship between direct and indirect (i.e. derived from wild-caught and known-age individuals) age estimates was approximately 1:1, providing strong support for annual periodicity.
4) For Eastern Rock Lobster, the directly determined putative ages agree with indirect longevity estimates, but yielded consistently older age estimates at fishery-specific milestones (i.e.

minimum legal size, size-at-sexual maturity and maximum legal size). For some locations, the relationship between some direct and indirect age estimates was approximately 1:1 (i.e. providing support for annual periodicity), but for others it was markedly different.

5) In all instances, the directly determined putative age corresponded to known biological and ecological patterns (e.g. juvenile growth or ontogenetic movement), with differences between the direct and indirect estimates being attributable to other factors (e.g. temperature or density) that are known to influence crustacean growth.

OBJECTIVE 2. Evaluation of growth mark periodicity – Western and Eastern Rock Lobster and Crystal Crab

1) For Crystal Crab, there was ossicular extension during their ≥ 18 month inter-moult period, with primary growth mark formation occurring in individuals that did not moult during the grow-out.
2) Irrespective of the sampling period, most Crystal Crab (including both sexes) deposited a single new-formed primary growth mark (n = 12) during the grow-out.
3) For Western Rock Lobster (n = 1), the periodicity evaluation indicated that a single primary growth mark was deposited during the 18 month grow-out. Ossicles from other individuals (n = 4) could have been interpreted as having deposited a single new-formed growth mark, but were excluded because decalcification made confident edge determination difficult.
4) For Eastern Rock Lobster (n = 1), the periodicity evaluation indicated that a single primary growth mark was deposited during the 12 month grow-out period. Another individual had ossicular extension that was approximately equal to the previous cycle, but a new-formed primary growth mark was not identified.
5) The calcein was retained in gastric ossicles for either 12 (Eastern Rock Lobster) or 18 months (Western Rock Lobster and Crystal Crab).

OBJECTIVE 3. Applicability to other crustacean species – with LA-ICPMS and known-age individual validation

1) The direct ageing method was readily applied to Ornate Rock Lobster, Southern Rock Lobster, Mud Crab and Crystal Crab ossicles.
2) Giant Crab ossicles contained some primary growth marks, but complete counts across the entire endocuticle were not possible for the small number of individuals examined (n = 3). Further research is needed to assess the methods applicability using a larger sample size and should also investigate using alternative ageing structures (i.e. other ossicles and eyestalks).

3) For Ornate Rock Lobster and Southern Rock Lobster, the direct ageing method allowed for the rapid estimation of preliminary (n = 5) von Bertalanffy growth parameters that were not significantly different to those derived from tag-and-recapture studies.
4) For most project species (i.e. except Giant Crab), we confirmed that the visually identified primary growth marks generally corresponded with real physico-chemical variations in ossicular mineral composition, with the number of localised minima or maxima for elemental ratios (i.e. in the endocuticle) increasing with putative age. Some LA-ICPMS results (e.g. for Mud Crab and Western and Eastern Rock Lobster) could be interpreted as supporting annual periodicity, but emerging uncertainties around ossicular decalcification and potential re-deposition of mineral features precluded a positive validation outcome.
5) The direct ageing method was validated by the use of known-age Ornate Rock Lobster (n = 13) and Western Rock Lobster (n = 3).

Implications

The immediate impact from this project will be jurisdiction- and species-specific, because each state fisheries department has different needs, priorities and validation expectations. However, the ability to directly determine (i.e. and validate) crustacean age provides another tool for fisheries scientists to enhance the resolution of current growth models, while decreasing research costs. Validation of the ageing method for Western, Eastern and Ornate Rock Lobster and Crystal Crab also opens the way for preliminary trials using the technique in stock assessments. Further, the validated technique will allow for rapid location-specific growth assessments and more accurate longevity estimates. This will be particularly important for long-lived species that present difficulties for tagging studies (e.g. Crystal Crab and Tasmanian Southern Rock Lobster) and would be useful for securing fishery sustainability certifications (e.g. Marine Stewardship Council). For shorter-lived species (e.g. Ornate Rock Lobster and Mud Crab), direct ageing could improve the assessment of population dynamics. The financial gains are difficult to quantify, but even a 1% improvement in decision making, and/or decrease in research costs (i.e. across multiple valuable fisheries), would equate to a substantial return of investment from this project. Such gains will translate into improved sustainability among Australia's crustacean fisheries, with flow-on benefits to the relevant fishing industry and across other sectors.

Recommendations

The broad-ranging nature (i.e. in terms of species and fisheries jurisdictions) of this project made definitive recommendations difficult. However, further studies trialling the direct ageing method during ongoing stock monitoring programs would be beneficial. This would allow for direct methodological comparison and growth model construction for the exact same location(s) and temporal period. For some species (e.g. Eastern Rock Lobster), the direct ageing method should be used to assess the potential for location-specific differences in growth. Application of the direct method to Crystal Crab is needed to provide the first solid (i.e. non-preliminary) assessment of growth and longevity for this species. Such research should encompass the relevant priorities for further development given below, particularly the requirement for concurrent species-specific precision assessments. The provision of this report to the relevant state fisheries departments is expected to initiate further jurisdiction- and stock-specific recommendations that will form the basis for further research and development applications.

Further development

This report will be distributed among the state fisheries departments in all Australian states and the Northern Territory, via the network of government and academic fisheries scientists developed during

this project. This will enable fisheries managers in each jurisdiction to consider the findings and any managerial responses that may be required and promote useful follow-up projects. The report will also be provided to the many industry groups that supported the project and interested scientists from the Crustacean Ageing Workshop. This approach will maximise further development of the direct ageing method within each jurisdiction. Specific priorities for further development are:

1) The development of species-specific reference collections (and ageing protocols) for reader training.
2) Precision assessments for Western, Eastern and Ornate Rock Lobster and Crystal Crab.
3) Assessment of temporal influences on ossicle readability and techniques for long-term ossicle mounting and storage.
4) Investigations into other non-lethal stains that effectively stain the organic matrix and detailed assessments of ossicular retention (or otherwise).
5) Species-specific identification of the first primary growth mark in ossicles from known-age crustaceans. Where possible, such research should also include a wider age range (e.g. 1–10).
6) Periodicity evaluations for Southern Rock Lobster and Mud Crab.
7) Further assessment of elemental composition using methods that can provide greater resolution (i.e. at the nanometre scale). For example, X-ray fluorescence microscopy (at the Australian Synchrotron), or nanoSIMS (at the University of Western Australia).
8) Further assessment of the direct ageing methods applicability to Giant Crab ossicles.

Extension and Adoption

During the project, the Crustacean Ageing Workshop was used to extend the methods and to-date findings to fisheries scientists from most Australian jurisdictions including: Western Australia, Tasmania, New South Wales, Queensland, South Australia and the Northern Territory (see Objective 4 sections for further details). Co-investigators Simon de Lestang and Jason How presented project results at multiple Annual Management Meetings including those for: i) Deep Sea Crab fishers, in Fremantle WA (May 12, 2015) and Perth WA (June 2, 2016), ii) Commercial Western Rock Lobster fishers, in Fremantle WA (June 11, 2015 and June 13, 2016) and Geraldton WA (June 18, 2015 and June 16, 2016).

After the project completion, the results will be internationally disseminated via scientific publications in high-impact journals, with the authors anticipating the construction of 2–3 articles. J. Leland is currently liaising with North American colleagues regarding further project extension via another Crustacean Ageing Workshop to be held in the United States. Beyond that, this final report will be distributed to the relevant departmental heads, fishery managers, beneficiaries, all workshop

participants and numerous overseas academic researchers. J. Leland will also take further opportunities to respond to distribution-initiated queries as they arise.

Project coverage

During the project, several media, industry and government research articles (below) were published to promote knowledge of the project and benefit of direct ageing techniques for crustaceans. To make it clear, the article link, title and citation (where relevant) are provided below.

Upon the project funding announcement, Southern Cross University (SCU) published a Community News article describing the project aims and objectives, with a follow-up interview being given by J. Leland for ABC Mid-North Coast Radio. During the project, J. Leland and D. Bucher (along with manuscript co-author Jason Coughran) published an online open-access journal article in PLOS ONE. The paper is on crustacean age determination (using Redclaw Crayfish as a model) and reports research that J. Leland completed with funding delivered through the FRDC-sponsored 2013 Department of Agriculture Fisheries and Forestry's Science and Innovation Award for Young People. Because the publication is relevant to this project, J. Leland utilized a SCU publication media release to communicate the FRDC project aims to the Australian public. That SCU article generated further media interest, with linked articles being posted by SCIMEX and The Northern Star, and interviews being given by J. Leland for both radio (The Big Fish, ABC Sydney) and print (Brisbane Times). J. Leland also authored an article explaining the project value, aims and progress-to-date that was published in the Tasmanian Seafood Industry News – Fishing Today to promote awareness of the project and within a relevant industry readership. Another similar article (i.e. a project summary) was published in the FRDC periodical FISH. A second Northern Star Article was used to promote the research at a local marine science community promotional event. Co-investigator D. Bucher summarised the project need and importance for the 2015 Australian 5-Minute Research Pitch National Final and was awarded: i) 1st in the Science and Health category, ii) 1st across all categories and iii) the People's Choice Award, with the SCU Community New publishing a subsequent article.

1) SCU Community News: http://discover.scu.edu.au/2014-02-february/fisheries-funding/ – Fisheries research projects attract funding.
2) PLOS ONE article: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0134966 – Leland, J.C., Bucher, D.J., Coughran, J., 2015. Direct age determination of a subtropical freshwater crayfish (Redclaw, *Cherax quadricarinatus*) using ossicular growth marks. PLOS ONE, 10: e0134966. doi:10.1371/journal.pone.0134966.

3) SCU release: http://scu.edu.au/news/media.php?item_id=13221&action=show_item – The way to a crayfish's age is through its stomach: new research confirms groundbreaking method for ageing crustaceans.
4) SCIMEX: https://www.scimex.org/newsfeed/the-way-to-a-crustaceans-age-is-through-its-stomach – Crustacean agein'? How to tell.
5) The Northern Star: http://www.northernstar.com.au/news/way-crayfishs-age-through-its-stomach/2753124/ – The way to a crayfish's age is through its stomach.
6) Fishing Today: http://dpipwe.tas.gov.au/Documents/10222_FT_Aug-Sept_2015_WEB.pdf – Leland, J.C., 2015. Ageing Australian crustaceans: past, present and future. Fishing Today, 28: 11.
7) FISH magazine: http://www.frdc.com.au/knowledge/publications/fish/Documents/Fish_1512%20%28LowRes%29.pdf – Leland, J.C., 2015. A stomach for history. FISH Magazine, 23: 22–23.
8) The Northern Star: http://www.northernstar.com.au/news/lobster-dating-for-the-ages/2505561/ – Lobster dating for the ages.
9) SCU Community News (5-Minute Research Pitch Final): http://discover.scu.edu.au/2015-11-november/legend-of-arthurs-grail-helps-secure-win-for-scu-academic-at-research-pitch-competition/ –Legend of Arthur's grail helps secure win for SCU academic at research pitch competition.

Project materials developed

Four presentations were given at the Crustacean Ageing Workshop. Please contact the principal investigator (J. Leland) to obtain a copy of these presentations or to arrange another crustacean ageing workshop. The initial digital reference collections for the ossicles examined during this project are held by the principal investigator as the nominated data custodian. Any scientific journal articles published after this report will be provided to the FRDC upon completion.

Appendix 1: Project Staff

All project staff with their respective institutions and expertise contribution area are listed below (in alphabetical order).

Name	Institution	Position	Expertise contribution area
Dr Adrian Linnane	South Australian Research and Development Institute	Offshore Crustaceans Program Leader	Southern Rock Lobster
Mr Ben Hebiton	Department of Fisheries Western Australia	Technical Officer	Animal husbandry and sampling
Dr Caleb Gardner	University of Tasmania	Professor	Southern Rock Lobster/Giant Crab
Dr Clive Jones	James Cook University	Principal Research Fellow	Ornate Rock Lobster
Dr Daniel Bucher	Southern Cross University	Associate Professor	Direct crustacean ageing
Dr Geoff Liggins	New South Wales Department of Primary Industries – Fisheries	Supervising Scientist – Rock Lobster	Eastern Rock Lobster
Dr Jason How	Department of Fisheries Western Australia	Research Scientist	Western Rock Lobster/Crystal Crab
Ms Jenine Dempster	Southern Cross University	Laboratory assistant	Sample preparation
Dr Jesse Leland	Southern Cross University	Postdoctoral researcher	Direct crustacean ageing
Mr Kelvin Rushworth	Department of Fisheries Western Australia	Technical Officer	Animal husbandry and sampling
Dr Lachlan McLeay	South Australian Research and Development Institute	Research Scientist	Southern Rock Lobster
Dr Mark Grubert	Northern Territory Department of Primary Industry and Fisheries	Senior Fisheries Scientist	Mud Crab
Dr Matthew Tonge	Southern Cross University	Research Technician	LA-ICPMS
Mr Mitch Burns	Department of Fisheries Western Australia	Technical Officer	Animal husbandry and sampling
Dr Paul Butcher	New South Wales Department of Primary Industries – Fisheries	Senior Research Scientist	Eastern Rock Lobster
Mr Peter Hawthorne	South Australian Research and Development Institute	Research Officer	Southern Rock Lobster
Dr Renaud Joannes-Boyau	Southern Cross University	Senior Researcher	LA-ICPMS
Dr Simon de Lestang	Department of Fisheries Western Australia	Principal Rock Lobster Scientist	Western Rock Lobster
Dr Toby Piddocke	Southern Cross University – National Marine Science Centre	Research Technician	Animal husbandry

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