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# Age determination in the Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*): novel molecular approaches



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A thesis submitted for the degree of Doctor of Philosophy

February, 2025

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## Statement of the Contribution of Others

This thesis was conducted under the supervision of Dr. Sven Uthicke[1], Prof. Morgan Pratchett[2], Dr. Cecilia Villacorta-Rath[3], and Dr. Alyssa Budd[2, 4]. The detailed contributions from supervisors and other collaborators are provided below.

### Chapter 3

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- Morgan Pratchett[2]: Manuscript preparation
- Sven Uthicke[1]: Conceptualisation / data interpretation / manuscript preparation

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- Alyssa Budd[2, 4]: Conceptualisation / data interpretation / manuscript preparation
- Julia Yun-Hsuan Hung[2]: Laboratory assistance
- Cecilia Villacorta-Rath[3]: Conceptualisation / data interpretation / manuscript preparation
- Sven Uthicke[1]: Conceptualisation / data interpretation / manuscript preparation

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- Morgan Pratchett[2]: Manuscript preparation
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## Abstract

Age is a fundamental parameter in ecological studies, shaping our understanding of population dynamics, life history traits, and the management of species with significant ecological and economic impacts. However, determining the age of invertebrates remains inherently challenging due to their general lack of easily measurable external growth markers and the high plasticity in growth rates influenced by environmental factors. While molecular ageing approaches have shown promise in vertebrates, their applicability to invertebrates remains largely unexplored.

Crown-of-thorns seastar (CoTS, *Acanthaster* spp.) are formidable coral predators that exhibit periodic population irruptions, which cause localised coral loss and significantly exacerbate the degradation of coral reef ecosystems. Several contrasting hypotheses have been proposed to explain the initiation of population irruptions, many of which could be validated through analyses of population age structure. However, the lack of reliable methods to age CoTS has constrained the ability to evaluate these hypotheses effectively, thereby hindering the development of targeted management strategies. This thesis aims to address this gap by developing DNA-based methods to determine the age of CoTS, utilizing specimens of known age that were spawned and reared at the Australian Institute of Marine Science (AIMS). These innovative approaches aim to advance CoTS management and contribute to broader applications of molecular ageing methods in invertebrates.

The first approach tested as a potential age marker in CoTS was telomere length, a biomarker extensively studied in human and wildlife research, characterised by its progressive shortening with age. By establishing a quantitative PCR (qPCR) protocol, I measured relative telomere length across four age groups of CoTS in Chapter 3. I found that adult CoTS generally exhibit shorter telomeres than juveniles, providing the first evidence of age-related telomere attrition in *A.* cf. *solaris*. However, significant variation within age classes,

influenced by factors such as diet, limited its reliability for resolving specific cohorts. Although telomere length may not provide accurate age estimates, it shows potential as a biomarker for assessing stress or overall health status, warranting further investigation.

The second method investigated was DNA methylation, an emerging molecular ageing technique successfully applied to various vertebrate species. To address the limited knowledge of DNA methylation in non-model invertebrates, I used Oxford Nanopore Technology (ONT) to construct the first comprehensive methylome profile of CoTS in Chapter 4, establishing a foundational understanding of their epigenetic landscape. My analysis revealed a mosaic methylation pattern with moderate genome-wide methylation levels (37.7%), positioning CoTS between the hypomethylated genomes of protostomes and the hypermethylated genomes of vertebrates. Additionally, methylation was enriched in gene bodies, suggesting roles in gene regulation and adaptability, while elevated levels in repetitive elements indicated a role in genome defence. These findings advance our understanding of epigenetic mechanisms in deuterostome invertebrates, an area that has been largely underexplored.

Using methylome profiles of CoTS across five age groups, I identified 1,910 CpG sites with age-associated methylation in Chapter 5. These age-related CpG sites served as the foundation for constructing an epigenetic clock, a model that uses DNA methylation patterns to estimate age. The model predicts the age of CoTS with a mean absolute error of 3.7 months, corresponding to approximately 4 - 6% of their lifespan (5 - 8 years). Notably, this is the third epigenetic clock ever developed for invertebrate species, pioneering the use of DNA methylation as an ageing tool beyond vertebrates. Its accuracy enables the differentiation of yearly cohorts of CoTS, offering a reliable and practical approach for monitoring population age structure and testing outbreak initiation hypotheses. Future refinement through the

development of targeted assays and validation with wild specimens will enhance its scalability and efficiency as an ageing tool for CoTS management.

The findings of this thesis have significant implications for both CoTS management and molecular ageing studies. This research reinforces the growing consensus that DNA methylation surpasses telomere length in age prediction, highlighting its potential for broader application in ecological studies and species management. By establishing the first epigenetic clock for CoTS, this thesis opens new avenues for understanding recruitment patterns and refining outbreak predictions, ultimately enhancing management strategies. Importantly, this research is among the first to apply epigenetic ageing techniques to invertebrates, providing a foundation for future studies to refine and extend this approach across a wider range of species. By enabling age estimation when traditional methods fall short, molecular ageing tools could offer unprecedented insights into population dynamics, transforming the management of commercially and ecologically important invertebrate species. Overall, this thesis represents a significant step forward in advancing age determination in invertebrates, with far-reaching implications for research, conservation, and management.

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# Chapter 1: General introduction

## 1.1 Crown-of-thorns seastar

Crown-of-thorns seastars (CoTS; *Acanthaster* spp.) are highly efficient predators of reef-building scleractinian corals (reviewed by Pratchett et al., 2014). During periodic population irruptions, local densities of CoTS can exceed 1,000 individuals per hectare (Chesher, 1969). These population irruptions have caused extensive declines in coral cover, contributing to reef degradation throughout the Indo-Pacific region (Baird et al., 2013; De'ath et al., 2012; Nakamura et al., 2014).

Previously considered a single species, *Acanthaster* spp. are now recognized as a species complex comprising at least four distinct reef-associated clades, each corresponding to a unique mitochondrial lineage (Vogler et al., 2008). These clades are geographically distributed across the Indo-Pacific, including *A. planci* in the northern Indian Ocean, *A. mauritiensis* in the southern Indian Ocean, *A. benziei* in the Red Sea, and *A.* cf. *solaris* in the Pacific (Haszprunar & Spies, 2014). This thesis focuses specifically on the Pacific species, *A.* cf. *solaris* (Figure 1.1), as all specimens were sourced from the Great Barrier Reef (GBR), which lies within the natural range of this clade. Nevertheless, despite geographic variation, all clades share key biological and ecological traits, including their role in coral predation and potential for rapid population outbreaks. Therefore, the findings of this study are likely relevant to the broader species complex, informing the understanding and management of *Acanthaster* outbreaks across their entire range.

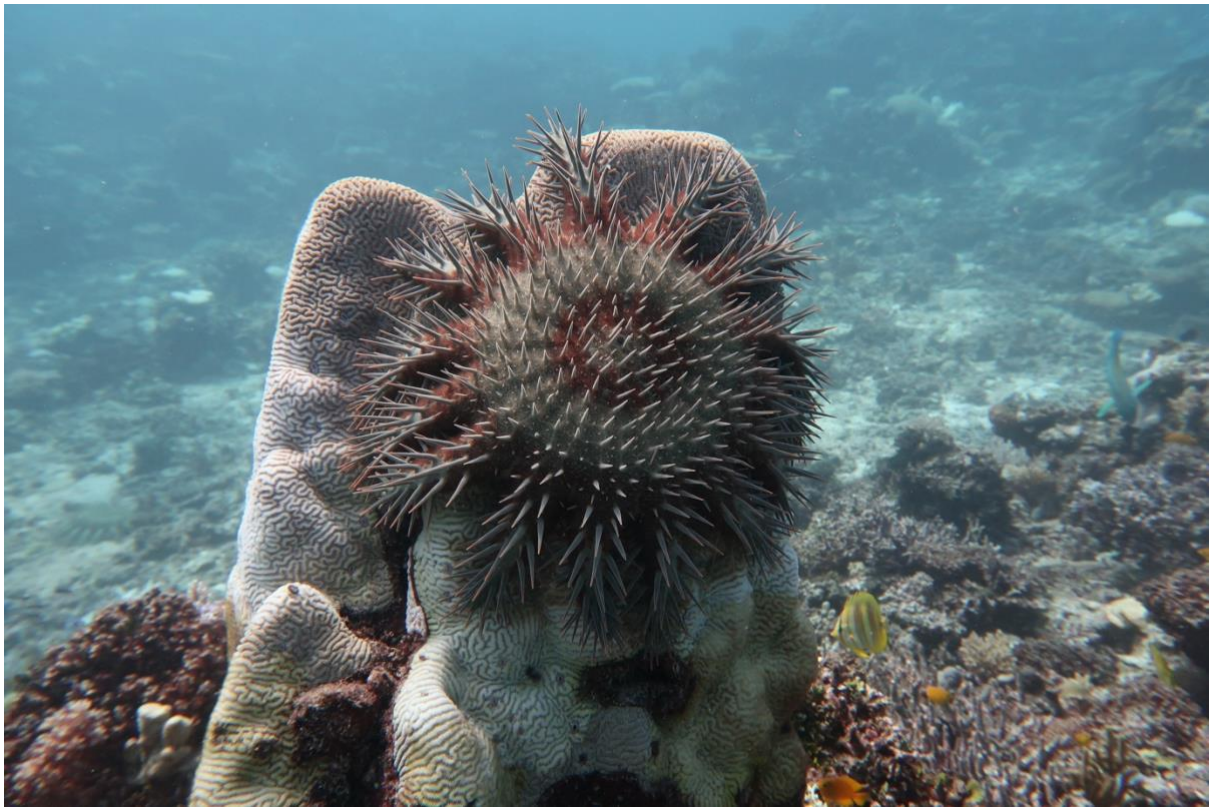


**Figure 1.1** The Pacific crown-of-thorns seastar (*Acanthaster.* cf. *solaris*), one of four distinct clades in the *Acanthaster* species complex. Photo by Deborah Burn.

Since the 1960s, various hypotheses have been put forward to explain the underlying mechanisms of CoTS irruptions. One prominent theory, the "nutrient enrichment hypothesis", posits that terrestrial runoff has introduced excess nutrients to reef ecosystems, enhancing conditions for CoTS larvae survival and proliferation (Birkeland, 1982). Conversely, the "predator removal hypothesis" suggests that the decline of natural predators due to overfishing or habitat degradation has allowed for increases in the size and abundance of adult CoTS (Sweatman, 2008).

Despite extensive research, the cause(s) of CoTS irruptions remain unresolved, in part due to methodological limitations that have hindered the testing of proposed hypotheses (Pratchett et al., 2017). For example, identification of CoTS larvae from plankton samples has been nearly impossible until the recent development of the environmental DNA (eDNA)

method (Doyle et al., 2017), impeding field studies of larval ecology and preventing definitive tests of the nutrient enrichment hypothesis. Furthermore, the inherent plasticity in growth rates of CoTS, in response to external factors such as diet and environmental conditions, complicates efforts to resolve population dynamics based on size structure (Lucas, 1984; MacNeil et al., 2017). Consequently, management strategies have largely relied on manual culling control rather than addressing the root causes of outbreaks (Pratchett et al., 2019). To gain clearer insights into the underlying mechanisms and develop corresponding effective management strategies, innovative approaches are needed to advance our understanding of CoTS biology and ecology.

Molecular ecology is a rapidly expanding field, which involves the application of molecular genetic tools to address ecological questions (Rowe et al., 2017). With increasing accessibility and affordability, these tools have supplemented traditional ecological methodologies by providing information of organisms that is beyond direct observations, such as divergence time between species, genetic diversity, kinship, individual recognition, and dietary analysis. In the case of CoTS, a range of molecular approaches has been applied to improve understanding of population dynamics and ecology. For instance, population connectivity has been explored using mitochondrial DNA and microsatellite markers (Benzie & Stoddart, 1992; Harrison et al., 2017), while predator-prey interactions have been identified through fish faecal and gut content analysis (Kroon et al., 2020). Molecular tools have also enabled the quantification of planktonic larvae to estimate spawning periods (Doyle et al., 2017; Uthicke et al., 2019) and the detection of adult (Uthicke et al., 2018) and juvenile CoTS (Kwong et al., 2021) through eDNA analysis. Beyond these, the recent assembly of the CoTS genome has provided a valuable resource for genetic research (Hall et al., 2017), and transcriptomic studies have provided insights into gene expression during key biological

processes (Morin et al., 2024). The rapid advancement in genetic techniques will continue to create opportunities to address ecological questions that have been intractable in the past.

### *1.1.1 Age data for CoTS management*

Age information is fundamental to ecological studies and species management. Knowing the age of wild animals can improve our understanding of the morphological, physiological, and behavioural features of a species throughout its life cycle (Alexander, 1958). In addition, key demographic rates such as growth, fecundity, and mortality are typically age specific (Caughley, 1977), making age data essential for studying population dynamics.

In the case of CoTS, the ability to determine ages of individual CoTS can enhance understanding in spatial and temporal patterns of recruitment, thereby uncovering whether an outbreak is initiated by gradual accumulation of successive cohorts ("primary outbreak") or a single mass recruitment event ("secondary outbreak") (Endean, 1974). By tracing back the settlement years from age data, it becomes possible to identify key factors that may have triggered mass settlement and subsequent outbreaks, such as excessive nutrient runoff or predator removal. Targeting management efforts toward eradicating primary outbreak reefs could enhance the effectiveness of interventions, preventing the spread of secondary outbreaks.

Knowing the age of CoTS will also allow us to test for differences in demographic rates between non-outbreak and outbreak populations, advancing our knowledge of density-dependent effects on population dynamics (MacNeil et al., 2017). This information can refine our understanding of how recruitment pulses, growth rates, and mortality rates interact under varying ecological conditions. This, in turn, supports more accurate population modelling, leading to improved outbreak predictions and more effective management strategies.

Furthermore, the ability to age CoTS will enable us to test aquarium-based hypotheses *in situ*. For example, Deaker et al. (2020) proposed that CoTS can delay their transition from herbivorous juveniles to coral-eating adults for up to 6.5 years (based on individuals reared in aquaria with known ages) while waiting for favourable condition such as increased coral availability. While this hypothesis has significant implications for understanding CoTS population dynamics and informing management strategies, it has not been tested in the field due the absence of suitable ageing tools.

Age data, therefore, represent a crucial yet underexplored resource for CoTS management. Developing reliable ageing methods for CoTS has the potential to bridge knowledge gaps, support targeted management actions, and ultimately contribute to a more informed and adaptive approach to mitigate CoTS outbreaks. Notably, because CoTS settle on reefs in pulses, with spawning occurring between December to March each year (Uthicke et al., 2019), an ageing tool capable of distinguishing annual cohorts would be sufficient for the establishment of population age structures to address key questions.

### *1.1.2 Challenges in ageing CoTS*

Determining the age of individual CoTS has proven challenging, in part due to their plasticity in growth. Aquarium studies have reported up to a 20-fold difference in size-at-age, depending on whether individuals transitioned from feeding on crustose coralline algae to coral (Deaker et al., 2020; Lucas, 1984). Similar disparities have been observed in the wild, where the timing of dietary shifts in juvenile CoTS has led to significant differences in growth rates and body size among individuals within the same cohort (Wilmes et al., 2016). Furthermore, the asymptotic size of CoTS has been observed to decrease on reefs with higher population densities, indicating that growth may be constrained by intraspecific competition for food during outbreaks (MacNeil et al., 2017). Additional factors, including food quality, water temperature, and wave exposure, have also been shown to influence the growth and

performance of CoTS (Kenchington, 1977; Lucas, 1984). These findings demonstrate that the growth of CoTS is heavily influenced by external factors, obscuring the relationship between size and age, and complicating the differentiation of age structure within populations based on size data.

Traditional tagging and tracking methods, which are commonly used to study natural population demographics (Tourón et al., 2023), also present significant challenges for CoTS. Their cryptic behaviour, high mobility, and the structural complexity of coral reefs hinder effective tagging and long-term monitoring (Pratchett et al., 2020). Additionally, like other soft-bodied echinoderms, CoTS shed physical tags readily (Pratchett et al., 2014), limiting long-term tracking. Fluorescent tagging, such as using tetracycline (Stump & Lucas, 1990), offers an alternative. However, because it does not allow for individual identification, its usefulness for large-scale demographic studies is limited. Without the ability to reliably track individuals over time, it is challenging to directly measure growth rates or validate size-based age estimates. This limitation hinders efforts to establish robust correlations between size and age, further complicating the study of age structure within CoTS populations.

To address these challenges, size-independent proxies of age have been explored. Pigment bands on spines have shown promise but require further validation through medium- to long-term mark-recapture studies to account for temporal and spatial variation in band formation (Stump & Lucas, 1990). Additionally, the assumption that the first band forms during the second year of life remains untested, and the reliability of the method may be compromised by factors such as predation or regeneration history (Souter, 1997). This approach is also unsuitable for small individuals, as their spines are too small to reliably observe and count the growth bands. Together, these limitations highlight the need for further research to establish a reliable and broadly applicable method to determine the age of CoTS.

## 1.2 Nucleic acid-based ageing techniques

In the early 2000s, nucleic acid-based techniques emerged as potential methods for ageing a diverse array of taxa, including mammals, fish, birds, and reptiles (Jarman et al., 2015). These innovative approaches measure changes in DNA or RNA signals that correlate with the chronological age of an organism. In general, specific age-related nucleic acid markers in the study species are first identified, followed by assay development to detect and quantify the nucleic acid signals in individuals of known age (Haussmann et al., 2003a; Polanowski et al., 2014). When a strong and consistent correlation is established between the markers and the known ages of individuals across a representative population, these markers are validated as suitable proxies for ageing.

Nucleic acid-based ageing techniques are increasingly recognized as powerful tools for ecological studies for several reasons (Haussmann & Vleck, 2002; Jarman et al., 2015). Their potential for non-lethal sampling methods make them especially valuable for studying vulnerable or protected species where traditional methods are impractical (Mayne et al., 2021). Additionally, these molecular approaches are highly adaptable for high-throughput age determination, offering an efficient alternative to traditional, often labour-intensive methods such as otolith or growth ring analysis (Anastasiadi & Piferrer, 2020). Furthermore, nucleic acid-based techniques have expanded the scope of age estimation to taxa for which traditional methods are infeasible, such as insects (e.g., mosquitoes) (Cook et al., 2006).

Among the various nucleic acid-based ageing techniques, telomere length and DNA methylation are the two most extensively studied age markers in ecological research (Jarman et al., 2015). The mechanisms and applications of these markers are discussed in detail in **Chapter 2**. Briefly, telomeres are repetitive nucleotide sequences located at the ends of chromosomes that protect genomic integrity (Blackburn, 1991). With each cell division, they progressively shorten, and this gradual attrition over an organism's lifespan makes telomere

length a potential biomarker for age (Haussmann & Vleck, 2002; Vleck et al., 2003). DNA methylation, on the other hand, involves the addition of methyl groups to cytosine bases and exhibits age-related changes at specific genomic sites (Razin & Cedar, 1991). These changes have enabled the development of "epigenetic clocks", which can provide accurate estimates of age across diverse taxa (De Paoli-Iseppi et al., 2017). Given their demonstrated utility, this thesis investigates the potential of telomere length and DNA methylation as molecular markers to determine the age of CoTS.

### 1.3 Thesis outline

The overarching aim of this thesis is to explore the use of molecular approaches to determine the age of individual CoTS. To achieve this, I utilized specimens of known age that were spawned and raised at the Australian Institute of Marine Science (AIMS) National Sea Simulator (SeaSim). In **Chapter 2**, I conducted a comprehensive literature review to evaluate the current state of nucleic acid-based techniques for age estimation in wild animals. While molecular ageing tools have been increasingly utilized across a wide range of vertebrate taxa, their application to invertebrates remains largely unexplored. To address this gap, I examined whether the techniques proven effective in vertebrates could be adapted for use in invertebrates. This review focuses on two well-established molecular age markers, telomere length and DNA methylation. By synthesizing existing knowledge, this chapter provides a foundation for subsequent research into molecular ageing approaches tailored to CoTS.

**Chapter 3** investigated the potential of telomere length as an age proxy in CoTS. By measuring relative telomere length across age groups, this chapter provides preliminary evidence of age-related telomere attrition in CoTS. It also identifies factors, such as diet and tissue type, that influence telomere dynamics. Although telomere length demonstrated potential for classifying CoTS into broad age groups or life stages, individual variation and

environmental factors limited its precision for resolving specific cohorts. Furthermore, no correlation was found between telomere length and traditional ageing features, including diameter, spine length, and pigment bands. This chapter highlights the need for further research to develop more accurate and reliable ageing methods for CoTS.

In **Chapter 4 and 5**, I explored the utility of DNA methylation as a molecular marker for age estimation in CoTS. **Chapter 4** presents the first comprehensive analysis of the CoTS methylome. Leveraging Oxford Nanopore Technology (ONT), I uncovered a moderately methylated genome and mosaic methylation landscape, providing a foundational understanding of the CoTS methylome. This chapter also expands our knowledge of deuterostome invertebrate methylomes through comparative analysis with other taxa. Building on these findings, **Chapter 5** focuses on developing an epigenetic clock for CoTS. By sequencing individuals across five age groups, I identified age-related CpG sites throughout the CoTS genome and constructed predictive models capable of estimating the age of CoTS with high accuracy. Together, these two chapters demonstrate the promise of DNA methylation as a reliable tool for age determination in CoTS and establish a framework for future applications in CoTS management and ecological research.

In **Chapter 6**, I consolidated the findings from all preceding chapters to provide a comprehensive synthesis of the project's contribution to molecular age estimation in CoTS. This chapter summarizes the respective strengths, limitations, and future potential of both telomere length and DNA methylation, with a particular emphasis on the promise of DNA methylation as a practical and scalable ageing tool. I outlined a detailed plan for advancing the development of the CoTS epigenetic clock toward practical implementation, addressing key considerations such as the time, cost, and resources required. This chapter also reflects on the broader implications of this work for invertebrate biology, conservation, and pest

management, emphasizing the value of integrating molecular techniques into ecological research and management strategies.

# Chapter 2: Nucleic acid-based techniques for age estimation in wild animals: from vertebrates to invertebrates


## Abstract

Determining the individual age of wild animals is crucial for understanding population dynamics. Traditional ageing methods, such as counting annual growth bands, have been effective for specific taxa. However, few reliable methods exist for ageing invertebrates. Recent studies have adapted nucleic acid-based ageing tools developed for human biomedical applications for use in ecological research, offering new avenues for age estimation. While these methods show promise, their application has largely been restricted to vertebrates, and their utility for invertebrate species remains uncertain. This review evaluates two dominant nucleic acid-based approaches for age estimation in ecological research: i) telomere length measurement and ii) epigenetic marker detection, specifically DNA methylation. The underlying biological mechanisms are first outlined, providing a foundation for understanding their relevance to age estimation. This is followed by an overview of the laboratory techniques used to measure these markers. Case studies from vertebrate species are then presented to illustrate the accuracy and practical applications of these ageing tools in ecological contexts. Finally, the potential application of these methods to invertebrate species is assessed, highlighting both opportunities and challenges. Overall, nucleic acid-based age estimation techniques hold great potential in providing valuable ecological information for invertebrate research, and is a fruitful area for further work.

## 2.1 Introduction

Accurately determining the age of wild animals is a cornerstone of ecological research and species management. Age data provide critical insights into key life history traits, forming the basis for understanding growth, reproduction, and survival patterns across species (Caughley, 1977). These data are essential for creating accurate population models, needed for the management of endangered species, fisheries, and pest species. As such, there is a strong need for reliable and robust ageing methods for wildlife research and conservation practices (Alexander, 1958). Various direct and indirect methods have been developed to age wild animals. In rare cases, age can be determined by repeated sightings of tagged or recognizable individuals for which birth dates are known. Such individuals are identified as neonates using external markings such as leg bands for birds (Lincoln, 1921) and natural fluke patterns for whales (Katona & Whitehead, 1981). Age can also be indirectly inferred from growth models using methods such as mark-recapture (Pradel, 1996). However, these longitudinal studies are often time-consuming and labour-intensive, and therefore expensive. Alternatively, when lethal or invasive sampling is feasible, or when deceased animals are found, age can be estimated by counting annual growth bands within calcified structures like fish otoliths (Pannella, 1971), mammalian teeth (Laws, 1952), and coral skeletons (Knutson et al., 1972). Additionally, post-mortem biochemical techniques, such as amino acid racemisation, have been used to estimate age in deceased vertebrates or fossilised invertebrate specimens (Goodfriend, 1991).

Established and widely used ageing methods have provided useful information for vertebrates such as mammals (Read et al., 2018), birds (Lincoln, 1921) and fishes (Begg et al., 2005), as well as for a limited number of invertebrate species. For example, growth band counts in skeletal structures have been used to age sea urchins (Gage, 1992) and corals (Knutson et al., 1972). However, age determination in most invertebrates has been hampered

by the general difficulties in tagging or identifying individuals (Martinez et al., 2013) and the lack of calcified structures containing growth bands. In some cases where growth bands appear to be deposited, for example in crustaceans, the relationship between these proxies and verified age are often invalid (Kilada & Driscoll, 2017; Narvaez et al., 2016). For marine invertebrates, age determination is further hindered by the plasticity in growth and variability in life-history strategies, rendering traditional growth models unsuitable in most cases (Hirst & Forster, 2013). As a result, despite the fact that invertebrates constitute over 95% of the world's species fulfilling essential ecosystem services (Wilson, 1987), a lack of broadly applicable and reliable ageing methods remains for this group.

Nucleic acid-based ageing techniques, first introduced in the early 2000s, leverage molecular signals in DNA or RNA that exhibit predictable and consistent changes with an animal's chronological age (Jarman et al., 2015). Developing these tools involves identifying specific age-related molecular markers and designing assays to measure these markers in specimens of known ages. The markers are considered reliable for age estimation when they demonstrate a clear and consistent relationship with age. These methods have been successfully applied across a range of taxa, including mammals, fish, birds, and reptiles (De Paoli-Iseppi et al., 2017).

Early studies of nucleic acid markers focused on age-related shortening of telomere length (Haussmann & Vleck, 2002). However, changes in telomere length provide only coarse estimates of age for some species, as they are often influenced by external factors (Coupé et al., 2019; Hatase et al., 2008). Therefore, more recent studies have capitalized on advances in genomic technology and explore the relationship between age and epigenetic markers such as DNA methylation, messenger RNA (mRNA), and microRNA (miRNA) (Jarman et al., 2015). To date, most of the nucleic acid-based ageing research has focused on humans for forensic or biomedical purposes (Zubakov et al., 2016). While nucleic acid-based

ageing techniques are increasingly popular in ecological studies, their application in invertebrates remains limited.

In this review, I summarize the advancements in nucleic acid-based ageing tools in vertebrates and evaluate their applicability to invertebrates. In particular, I focus on i) telomere length and ii) DNA methylation, which are two of the most tested age markers in wild animals. I also briefly discuss the potential of mRNA and miRNA as age estimators for invertebrates.

## 2.2 Telomeres

Telomeres are tandem repeats of short DNA sequence and protein complexes found at the ends of eukaryotic chromosomes (Blackburn, 1991) (Figure 2.1). The non-coding telomeres act as end caps to prevent inter-chromosomal fusion (van Steensel et al., 1998) and to protect chromosomal DNA from degradation during cell division (Blackburn, 2005). Every time a cell divides, telomeres are shortened while chromosomal DNA stays intact, preserving vital information in the genome (Blackburn, 1991). Eventually, a cell undergoes senescence or apoptosis when its telomeres shorten to a critical limit. While telomere length can be extended and maintained by the ribonucleoprotein enzyme telomerase, activity of this enzyme varies according to cell type, life stage, and species. In vertebrate somatic cells, where telomerase activities are typically low, telomere length continues to shorten throughout life, and may therefore serve as an effective proxy for age determination in wild animals (Haussmann & Vleck, 2002; Vleck et al., 2003).




**Figure 2.1** Telomeres are protective structures found at the end of eukaryotic chromosomes, which shorten with each cell division, eventually leading to cell senescence or apoptosis. In certain cell types such as gamete, stem and tumour cells, telomere lengths are maintained by the ribonucleoprotein enzyme telomerase. [This figure is reproduced from Vaiserman and Krasnienkov (2020).]

### *2.2.1 Measuring telomere length*

The telomere restriction fragment (TRF) assay was the first method developed to measure telomere length (Harley et al., 1990). To conduct a TRF assay, extracted DNA is first digested by restriction enzymes to yield terminal fragments comprised of telomeric repeats, followed by agarose gel electrophoresis, Southern blot hybridization, and image analysis to quantify telomere length distribution (Kimura et al., 2010). TRF is time-consuming and technically demanding (Montpetit et al., 2014). It also requires a large amount of DNA (0.5-10 μg). However, the TRF assay continues to be considered as the standard

method because it provides an estimation of mean telomere length in kilobases (kb), allowing inter-study comparison and new method validation (Kimura et al., 2010).

The quantitative polymerase chain reaction (qPCR) assay is another widely applied method to measure telomere length. Instead of an absolute measurement of telomere length, the qPCR method provides a ratio of the telomeric DNA (T) to the reference gene (N) present in a sample (Cawthon, 2002) (Figure 2.2). The reference gene of non-variable copy number per cell is used to account for the potential difference in the amount of starting genetic material among samples. Although the resulting T/N ratios mean that inter-study comparison is often not possible, qPCR provides a time- and cost-efficient method to determine inter-individual differences in telomere length within a species. Best practice guides on the use of qPCR to measure telomere length in ecology have been put forward to allow researchers to plan and execute telomere studies effectively (Nussey et al., 2014).




**Figure 2.2** Schematic diagram of telomere length measurement using quantitative polymerase chain reaction (qPCR). [This figure is reproduced from Lin et al. (2019).]

### *2.2.2 Telomere-based age proxies in vertebrates*

The concept of using telomere length for age estimation was introduced into ecology by Haussmann and Vleck (2002), a study which found negative correlations between

telomere length and age in a range of avian species. Subsequently, age-related shortening of telomeres was also demonstrated in mammals (McKevitt et al., 2003), reptiles (Scott et al., 2006), and fishes (Hartmann et al., 2009) (Table 2.1). Telomere-based ageing techniques were then applied in natural populations and proven successful in classifying common terns (*Sterna hirundo*) (Haussmann et al., 2003a) and martens (*Martes americana* and *M. caurina*) (Pauli et al., 2011) into broad age-classes (<6, 7-13, 14-25 year for common terns; 0, 1, 2, 3, 4+ year for martens).

Despite a few successful applications of telomere length as age estimators, further research has shown that the negative correlation between age and telomere length cannot be generalized across all species. For example, no age-related changes in telomere length were found in mouse-eared bats (*Myotis myotis*) (Foley et al., 2020) or wire-tailed manakins (*Pipra filicauda*) (Vernasco et al., 2021). In exceptional cases, lengthening of telomeres with age was observed in long-lived species such as Leach's petrels (*Hydrobates leucorhous*) (Haussmann et al., 2003b) and water pythons (*Liasis fuscus*) (Ujvari & Madsen, 2009). These findings indicate that telomere dynamics are highly species-specific, and thus further investigations are needed before telomere length can be developed into a reliable ageing marker for non-model organisms.

In certain cases the telomere-derived age estimation can be unreliable due to the considerable variation in telomere length among individuals of the same age (reviewed by Dunshea et al., 2011). For example, estimated telomere length in liver tissue of juvenile cod (*Gadus morhua*) (114 days post-hatch) ranges from 10 to 14 kb while that of adult cod (900 days post-hatch) ranges from 9 to 14 kb (de Abechuco et al., 2016). As such, it is not possible to differentiate between juvenile and adult cod based on telomere length. Large within-age class variations in telomere length have been observed in numerous species such as the great frigate birds (*Fregata minor*) (Juola et al., 2006), common carps (*Cyprinus carpio*) (Izzo et

al., 2014), sea lions (*Neophoca cinerea*) (Izzo et al., 2011), and big brown bats (*Eptesicus fuscus*) (Ineson et al., 2020), limiting the use of telomeres as a reliable ageing tool.

Variation in telomere length among individuals of the same age can be attributed to a range of factors. For instance, individuals may have inherited different initial telomere lengths (Horn et al., 2011; Njajou et al., 2007; Reichert et al., 2015). Notably, higher maternal age has been associated with the inheritance of shorter telomeres — a phenomenon known as the Lansing effect (Marasco et al., 2019). Additionally, extrinsic factors such as stress and disease can cause oxidative damage to telomeric DNA and thus accelerate telomere attrition rate (Chatelain et al., 2020). For example, a longitudinal study in bats demonstrated that environmental stressors such as high wind speed and rainfall are associated with faster reductions in telomere length (Foley et al., 2020). Other factors that can influence telomere dynamics include social behaviour (Vernasco et al., 2021), reproductive history (Bichet et al., 2020), and antioxidant activity (Angelier et al., 2018). As such, the focus of more recent studies has shifted towards using telomere length as an indicator of the level of stress that an individual has experienced throughout life, i.e., biological age, instead of chronological age.

### *2.2.3 Telomere-based age proxies in invertebrates*

Besides the limitations discussed above, the development of telomere-based ageing tools in invertebrates is faced with additional challenges. For example, continuous telomerase expression in multiple tissues has been reported in invertebrates with regeneration and/or cloning capabilities (e.g., sea stars and sea urchins), resulting in constant telomere restoration and thus a lack of age-associated shortening (Francis et al., 2006; Varney et al., 2017). In addition, telomere dynamics in some invertebrate species appear to exhibit non-linear patterns. It was found in rock oyster (*Saccostrea glomerata*) that telomere length shortens with age only during the early stages of life (< 18 months), and no decrease was observed between the age of 2 – 4 years (Godwin et al., 2011a). Despite these challenges, a clear

relationship has been found between telomere length and shell size (as a proxy of age) in temperate abalone using foot muscle tissue (Ovenden & Godwin, 2011). As telomere dynamics are highly tissue-specific (de Abechuco et al., 2016), identification of the suitable somatic tissue may be key to the successful development of telomere-based age markers in invertebrates.

While telomere-based techniques may not allow for precise age estimation due to the variable nature of telomeres, they can still provide useful information when age data are scarce (e.g., Nehmens et al., 2021), which is often the case in invertebrate species. In particular, the ability to assign individuals to broad age-classes or temporal cohorts is valuable for population monitoring studies. Indeed, Coupé et al. (2019) demonstrated that telomere length could potentially be a relevant marker for the management of sea urchin populations in the Mediterranean. Furthermore, if extrinsic factors affecting telomeres can be delineated, it is possible to establish telomere length as a molecular marker for individual condition and the prediction of remaining lifespan (Monaghan, 2010). Overall, development of telomere markers in invertebrates is worth further investigation, given the potential of such markers to provide valuable information in a relatively low-cost manner.

**Table 2.1** Summary table of research on telomere-based ageing tool for wild animals. Upward/downward arrow represents significant increase/decrease respectively, dash represents no significant change. Studies that support the use of telomere length as chronological age estimator are indicated with an asterisk (*).

| Taxa | Method | Tissue sampled | Sample preservation method | Age-related change in telomere length | Reference |
|---|---|---|---|---|---|
| **Vertebrates** | | | | | |
| Birds | | | | | |
| Zebra finch (*Taeniopygia guttata*) | TRF | Blood | 2% EDTA | ↓ | Haussmann et al. (2003b) |
| Tree swallow (*Tachycineta bicolor*) | TRF | Blood | 2% EDTA | ↓ | |
| Adelie penguin (*Pygoscelis adeliae*) | TRF | Blood | 2% EDTA | ↓ | |
| Leach's petrel (*Oceanodroma leucorhous*) | TRF | Blood | 2% EDTA | ↑ | |
| Common tern (*Sterna hirundo*) | TRF | Blood | 2% EDTA | ↓ | *Haussmann et al. (2003a) |
| Seychelles warbler (*Acrocephalus sechellensis*) | qPCR | Blood | Ethanol | ↓ | Barrett et al. (2013) |

| | | | | | |
|---|---|---|---|---|---|
| Great reed warbler (*Acrocephalus arundinaceus*) | qPCR | Blood | SET-buffer | ↓ | Asghar et al. (2015) |
| <u>Fish</u> | | | | | |
| Sea bass (*Dicentrarchus labrax*) | TRF | Blood | Not reported | - | Horn (2008) |
| Common carp (*Cyprinus carpio*) | qPCR | Muscle | Liquid nitrogen | - | Izzo et al. (2014) |
| | qPCR | Fin | Ethanol | - | |
| Atlantic silverside (*Menidia menidia*) | qPCR | Muscle | -80 °C | - | Gao and Munch (2015) |
| | qPCR | Brain | -80 °C | - | |
| Cod (*Gadus morhua*) | TRF | Brain | RNAlater at -80 °C | ↓ | de Abechuco et al. (2016) |
| | TRF | Liver | RNAlater at -80 °C | ↓ | |
| | TRF | Muscle | RNAlater at -80 °C | - | |
| | TRF | Skin | RNAlater at -80 °C | - | |
| Sturgeon (*Acipenser baerii*) | qPCR | Blood | Ethanol | ↓ | Simide et al. (2016) |
| | qPCR | Fin | Ethanol | ↓ | |
| <u>Mammals</u> | | | | | |
| Australian sea lion (*Neophoca cinerea*) | qPCR | Flipper biopsy | Ethanol | ↓ | Izzo et al. (2011) |
| Marten (*Martes americana, M. caurina*) | qPCR | Muscle | - 40 °C | ↓ | *Pauli et al. (2011) |

| | | | | | |
|---|---|---|---|---|---|
| Dugong (*Dugong dugon*) | qPCR | Skin | Ethanol | ↓ | *Cherdsukjai et al. (2020) |
| Little brown bat (*Myotis lucifugus*) | qPCR<br>qPCR | Wing<br>Buccal cells | RNAlater at -80 °C<br>RNAlater at -80 °C | -<br>- | Ineson et al. (2020) |
| <u>Reptiles</u> | | | | | |
| Loggerhead turtle (*Caretta caretta*) | qPCR<br>qPCR | Blood<br>Epidermis | TNES buffer<br>Ethanol | -<br>- | Hatase et al. (2008) |
| Water python (*Liasis fuscus*) | TRF | Blood | Ethanol | ↑ | Ujvari and Madsen (2009) |
| **Invertebrates** | | | | | |
| <u>Echinoderms</u> | | | | | |
| Sea urchin (*Strongylocentrotus franciscanus*) | TRF | Gonads, intestine, esophagus, Aristotle's lantern muscle, ampullae, tube feet | -70 °C | - | Francis et al. (2006) |
| Sea star (*Luidia clathrata*) | qPCR | Tube feet | Ethanol | ↓ | Varney et al. (2017) |
| Sea star (*Acanthaster* cf. *solaris*) | qPCR | Pyloric caeca | Ethanol | ↓ | Kwong et al. (2023) |
| Sea urchin (*Paracentrotus lividus*) | qPCR | Spine/whole organism | Ethanol | ↓ | *Coupé et al. (2019) |

| | | | | | |
|---|---|---|---|---|---|
| Molluscs | | | | | |
| Rock oyster (*Saccostrea glomerata*) | TRF | Adductor tissue | Liquid nitrogen | ↓ | Ovenden and Godwin (2011) |
| Abalone (*Haliotis* spp.) | TRF | Foot muscle | Liquid nitrogen | ↓ | |
| Planarians | | | | | |
| Freshwater flatworm (Dendrocoelidae) | qPCR | Anterior end of the body | Not reported | - | Koroleva et al. (2020) |

## 2.3 DNA methylation

Epigenetics is the study of the biological processes that regulate gene expression without altering the DNA sequence (Portela & Esteller, 2010). DNA methylation is a major epigenetic mechanism which involves the addition of methyl groups to DNA strands, typically occurring at the carbon-5 position of the cytosine (C) base (Razin & Cedar, 1991) (Figure 2.3). In vertebrates, this conversion of cytosine to 5-methylcytosine (5mC) is almost exclusively found at cytosine-phosphate-guanine (CpG) sites (Bird, 1987). In vertebrates, research on DNA methylation has mainly focused on CpG-rich promoter regions, where higher methylation levels are typically linked to gene repression (Al Adhami et al., 2022). Changes in global or site-specific methylation levels have been shown to correlate with chronological age in a variety of vertebrate taxa such as mammals, birds, and reptiles, and thus have been used to develop ageing tools in recent ecological studies (De Paoli-Iseppi et al., 2017).




**Figure 2.3** (A) DNA methylation typically occurs at the carbon-5 position of the cytosine base. (B) Methylation of cytosine-phosphate-guanine (CpG) sites is commonly associated with gene repression in vertebrates. [This figure is reproduced from Gillespie et al. (2019).]

### *2.3.1 Measuring DNA methylation levels*

Bisulfite sequencing is one of the most widely used methods to detect methylated cytosines (5-methylcytosine) in the genome (Frommer et al., 1992). Genomic DNA is first treated with sodium bisulfite, which converts unmethylated cytosines to uracil via deamination, leaving methylated cytosines intact. The bisulfite treated DNA, which retains only methylated cytosines is then PCR-amplified, during which the uracils are amplified as thymines. The resulting PCR products are sequenced, and aligned to the reference DNA to

determine methylation status. Methylation levels can be assessed at the whole-genome scale (whole genome bisulfite sequencing; WGBS), at CG-rich parts of the genome (reduced representation bisulfite sequencing; RRBS), or specific genes of interest (bisulfite amplicon sequencing; BSAS). The major drawback of bisulfite sequencing approaches is that they generally require a large amount of high quality DNA (~0.1 μg) to account for extensive DNA fragmentation during bisulfite treatment (Ehrich et al., 2007). They also require sophisticated bioinformatic analysis techniques (Yong et al., 2016), and may generate false positive methylation detection if the denaturation of DNA is incomplete, as bisulfite conversion only works on single-stranded DNA (Fraga & Esteller, 2002).

Owing to the drawbacks of bisulfite sequencing, recent studies have advocated Oxford Nanopore Technologies (ONT) as an alternative method to detect DNA cytosine methylation (Sansom & Sassoubre, 2017; Simpson et al., 2017). As a third-generation sequencing approach, ONT works by monitoring changes in electrical current as long DNA or RNA fragments pass through the protein Nanopore (Wiggin et al., 2008). It enables real-time detection of methylated cytosines based on electrical readout, eliminating the need for a large amount of bisulfite treated DNA as input. While the use of ONT for epigenetics research is still at an early stage of development, there is great potential for advances in the coming years as protocols continue to be developed (Schatz, 2017).

### *2.3.2 Epigenetic clocks in vertebrates*

Motivated by forensic applications and ageing studies, highly accurate epigenetic clocks (i.e., age estimation models based on DNA methylation levels at a set of CpG sites) have been developed for chronological age estimation in humans over the last decade (Bekaert et al., 2015; Eipel et al., 2016; Horvath, 2013). For example, based on the methylation status at 353 CpG sites, Horvath's epigenetic clock can predict the age of an individual human with a median absolute error of ± 3.6 years (Horvath, 2013). Given that the

extent and patterns of DNA methylation are well conserved in closely related species (Jiang et al., 2014), Horvath's epigenetic clock has also been successfully applied in chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*) (Horvath, 2013). Subsequently, such techniques have been adopted in ecological studies to estimate the age of other vertebrates such as mice (Han et al., 2018), bats (Wright et al., 2018), whales (Bors et al., 2021; Polanowski et al., 2014), birds (De Paoli-Iseppi et al., 2019), and fishes (Anastasiadi & Piferrer, 2020) (Table 2.2).

Epigenetic clocks can provide accurate age estimations in wild animals. For example, using the methylation status of 23 CpG sites across the genome, chronological age of beluga whales (*Delphinapterus leucas*) can be predicted with a mean absolute error of ± 2.9 years, which is 5% of the species' lifespan (Bors et al., 2021). Similarly, Anastasiadi and Piferrer (2020) constructed an epigenetic clock that can accurately predict the age of European sea bass (*Dicentrarchus labrax*) (mean absolute error ± 2.1 years; approximately 10% of the species' lifespan) without being influenced by environmental perturbations and indeterminate growth. These accurate epigenetic clocks are of great value for species conservation and fisheries management, where age data are crucial but often lacking. While DNA methylation-based biomarkers have only been developed for a small number of vertebrate species to date, it is predicted that the application will continue to be broadened in the next decade enabled by the advancement in genomic technologies, such as reduced sequencing costs and improved accessibility of bioinformatics tools (Parrott & Bertucci, 2019). Given that DNA methylation-based biomarkers hold potential as promising ageing tools, it is of great interest to explore if accurate epigenetic clocks can be constructed across a wide range of taxa.

Despite the purported accuracy of epigenetic clocks (Table 2.2), they are not without limitations and uncertainty. In particular, the influence of genetic and environmental factors on DNA methylation levels is not fully resolved (Bell et al., 2019). There is strong evidence

suggesting that stress and disease can accelerate epigenetic ageing, and thus introducing bias in epigenetic clocks if not corrected for (Horvath & Raj, 2018). It has also been suggested that early life exposures to stress factors may have great influence in epigenetic ageing (Vaiserman, 2018), affecting the timing of key life history events (Parrott & Bertucci, 2019). Further studies examining samples from a variety of environmental contexts are needed to reveal the underlying mechanism and drivers of epigenetic ageing. Delineating the chronological and biological components of epigenetic clocks will lead to further improvement in the accuracy of age estimation, as well as deepen our understanding of biological ageing.

### *2.3.3 Epigenetic clocks in invertebrates*

Due to the limited availability of fully annotated reference genomes, epigenetic studies in invertebrates to date have mainly focused on model species such as the nematode *Caenorhabditis elegans* and the planktonic crustacean *Daphnia magna*, typically in the context of genome evolution or ageing mechanisms rather than ecology (Harris et al., 2012; Yu et al., 2019). To date, only two invertebrate species have had epigenetic clocks developed: the European lobster (*Homarus gammarus*) (Fairfield et al., 2021) and the jewel wasp (*Nasonia vitripennis*) (Brink et al., 2024). Despite this limited progress, there is promising evidence for age-related changes in DNA methylation in invertebrates. For example, global methylation levels were found to be significantly different between adult and juvenile freshwater crustaceans *Gammarus fossarum* (Cribiu et al., 2018), while development-associated changes in methylation patterns have been demonstrated in the purple sea urchin (*Strongylocentrotus purpuratus*) (Fronk et al., 1992). More recently, Hearn et al. (2021) successfully identified 61 age-related CpG sites in *D. magna*, demonstrating the potential of epigenetic clock development for invertebrates.

The approach to construct epigenetic clocks for invertebrates, however, is likely to be different from that for vertebrates. This is because the DNA methylation profiles of invertebrates are broadly different to that of vertebrates. Vertebrates are known to exhibit a "global" methylation pattern, characterised by widespread CpG hypermethylation throughout the genome, except in certain CpG-rich regions (Suzuki & Bird, 2008). This results in relatively high genome-wide CpG methylation levels, typically ranging from 60% to 80% (Klughammer et al., 2023). While vertebrate-like hypermethylation has been demonstrated in the demosponge *Amphimedon queenslandica* (de Mendoza et al., 2019), this landscape has been lost in most prebilaterians and protostome invertebrates. These groups typically exhibit a “mosaic” methylation pattern, characterized by alternating regions of hypermethylation and unmethylation (Suzuki & Bird, 2008), resulting in generally low methylation levels. Some invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* have little (<1%) to no methylation (Rae & Steele, 1979; Simpson et al., 1986), while more evolutionarily derived groups exhibit higher genome-wide methylation levels, ranging from 10% to 20% (Klughammer et al., 2023).

The DNA methylation patterns across the invertebrate-vertebrate boundary is still under investigation, but preliminary evidence suggests a reacquisition of methylation complexity in some deuterostome invertebrates. For example, the basal chordate vase tunicate (*Ciona intestinalis*) and the purple sea urchin (*Strongylocentrotus purpuratus*) exhibit genome-wide methylation levels of 30% and 27%, respectively, which are of a similar magnitude to those of vertebrates (Klughammer et al., 2023; Skvortsova et al., 2022). Further investigation into the methylation patterns of other deuterostomes, such as echinoderms, will provide important insights into the evolutionary origin of the differentiation in DNA methylation patterns between invertebrates and vertebrates.

Due to potential differences in methylation profiles, common approaches used for epigenetic research in vertebrates may not be suitable for invertebrates. For example, RRBS primarily targets CpG-rich regions, which are not the major methylation sites in invertebrates. Instead, a whole-genome approach may be required to capture their sparse methylation patterns. Among whole-genome approaches, ONT may be a promising option, as it allows direct methylation detection without the DNA damage associated with bisulfite treatment. Additionally, it simultaneously generates genomic sequences, making it particularly suitable for non-model invertebrates, where background genomic information is often limited. Considerably more foundational work is required before epigenetic clocks can be applied as a reliable ageing tool for invertebrates. Further research on DNA methylation patterns in a variety of invertebrate taxa will not only enable the construction of epigenetic clocks, but also generate critical knowledge on epigenetic mechanisms and genome evolution.

**Table 2.2** Summary table of research on epigenetics clocks for wild animals, including only those reporting mean absolute error to provide a standardised basis for comparison. (RRBS = reduced representation bisulfite sequencing; WGBS = whole genome bisulfite sequencing; DREAM = digital restriction enzyme analysis of methylation).

| Taxa | Method | Tissue sampled | Sample preservation method | ± Mean absolute error (years) | Maximum possible lifespan (years) | Reference |
|---|---|---|---|---|---|---|
| **Vertebrates** | | | | | | |
| Amphibians | | | | | | |
| African clawed frog (*Xenopus laevis*) | Methylation array | Blood, Brain, Skin, Liver, Muscle, Toe | Liquid nitrogen | 3.24 | 30.3 | Zoller et al. (2024) |
| Western clawed frog (*Xenopus tropicalis*) | Methylation array | Blood, Brain, Skin, Liver, Muscle, Toe | Liquid nitrogen | 0.95 | 16 | |
| Birds | | | | | | |
| Short-tailed shearwaters (*Ardenna tenuirostris*) | DREAM | Blood | Whatman FTA Micro cards | 2.8 | 39 | De Paoli-Iseppi et al. (2019) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Gould's petrel (*Pterodroma leucoptera*) | Targeted bisulphite sequencing | Blood | Whatman FTA Micro cards | 2.24 | 40 | Roman et al. (2024) |
| <u>Fish</u> | | | | | | |
| Sea bass (*Dicentrarchus labrax*) | RRBS | Muscle | Liquid nitrogen | 2.149 | 15 | Anastasiadi and Piferrer (2020) |
| Lungfish (*Neoceratodus forsteri*) | RRBS | Fin | Not reported | 0.86 | ~100 | Mayne et al. (2021) |
| Cod (*Maccullochella* spp.) | RRBS | Fin | Not reported | 0.34 | 48 | |
| Golden perch (*Macquaria ambigua*) | Targeted bisulphite sequencing | Fin | Ethanol | 0.2 | 27 | Mayne et al. (2023) |
| Cownose ray (*Rhinoptera bonasus*) | ECrad-seq (Enzymatically-converted restriction site-associated DNA sequencing) | Fin clips, | DMSO | 0.68 years | 21 | Nick Weber et al. (2024) |
| | | Whole blood | RNAlater | 0.74 years | | |
| <u>Mammals</u> | | | | | | |
| Humpback whale (*Megaptera novaeangliae*) | Bisulfite pyrosequencing | Skin | Ethanol / RNA later ICE | 2.991 | 95 | Polanowski et al. (2014) |
| Chimpanzee (*Pan troglodytes*) | Bisulfite pyrosequencing | Blood | -20 °C | 5.41 | 60 | Ito et al. (2018) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Bechstein's bat (*Myotis bechsteinii*) | Bisulfite pyrosequencing | Wing | Ethanol | 1.52 | 20 | Wright et al. (2018) |
| Wood mouse (*Apodemus sylvaticus*) | Nanopore | Ear punch | Not reported | 0.16 | 1.4 | Little et al. (2020) |
| Naked mole-rat (*Heterocephalus glaber*) | RRBS | Liver | Liquid nitrogen | 3.1 | 31 | Lowe et al., 2020 |
| Beluga whale (*Delphinapterus leucas*) | RRBS | Skin | DMSO / liquid nitrogen | 2.9 | 60 - 70 | Bors et al. (2021) |
| Cattle (*Bos taurus*) | Nanopore | Hair | Not reported | 1.4 | 16 | Hayes et al. (2021) |
| Opossum (*Monodelphis domestica*) | Methylation array | Ear, liver, tail | -20 °C | 0.44 | 4.2 | Horvath et al. (2022) |
| Bottlenose dolphin (*Tursiops aduncus*) | Methylation array | Skin | RNAlater / DMSO | 2.1 | 40 | Peters et al. (2023) |
| Sea lion, walrus, seal (*Zalophus californianus, Odobenus rosmarus divergens, Phoca vitulina*) | Methylation array | Blood and skin | EDTA | 1.36 | 48 | Robeck et al. (2023) |
| Brown bear (*Ursus arctos*) | Bisulfite pyrosequencing | Blood | EDTA | 1.3 | 30 | Nakamura et al. (2023) |

| | | | | | | |
|---|---|---|---|---|---|---|
| White-tailed deer (*Odocoileus virginianus*) | Methylation array | Skin, muscle, blood | -20 °C | 0.29 | 10.5 | Czajka et al. (2024) |
| Black bear (*Ursus americanus*) | Methylation array | Skin, fibroblast | -20 °C | 1.33 | 19 | |
| Mountain goat (*Oreamnos americanus*) | Methylation array | Skin, muscle | -20 °C | 0.61 | 12 | |
| Reptiles | | | | | | |
| Green turtle (*Chelonia mydas*) | RRBS | Skin | Liquid nitrogen | 2.1 | 75 | Mayne et al. (2022) |
| **Invertebrates** | | | | | | |
| Arthropods | | | | | | |
| European lobster (*Homarus gammarus*) | Targeted bisulphite sequencing | Claws or antennae | Not reported | 0.14 | 42 - 72 | Fairfield et al. (2021) |
| Jewel wasp (*Nasonia vitripennis*) | WGBS | Whole body | Liquid nitrogen | 0.39 days | 17 - 20 days | Brink et al. (2024) |

## 2.4 Other nucleic acid-based ageing techniques

### *2.4.1 Messenger RNA*

Messenger RNA can be used as an age estimator because gene expression levels change with age in both vertebrates and invertebrates (Arbeitman et al., 2002; de Magalhães et al., 2009). A common method to establish mRNA age markers is to examine the transcriptional profiles of known-age individuals to identify a set of genes that are expressed differentially across life stages (Petzold et al., 2013). Quantitative reverse transcriptase PCR (qRT-PCR) assays are then developed to isolate these gene transcripts from study organisms, followed by age estimation based on a calibrated model. In the case of non-model species, this can be achieved by referring to the transcriptional profiles of closely related species, as age-informative genes are likely to be conserved across phylogenetically proximate taxa (Cook et al., 2006).

Messenger RNA age markers have been applied in humans for forensic purposes, providing age estimation with mean absolute error of ± 9.2 years (Zubakov et al., 2016). For wildlife, the only application to date is for the management of mosquito-transmitted diseases such as malaria and dengue. Using transcriptional profiling data from the model invertebrate species *Drosophila melanogaster*, Cook et al. (2006) identified a set of genes that display age-dependent expression in the disease-transmitting mosquito *Aedes aegypti*. The mRNA markers were then tested in the field, providing estimation of mosquito age with mean absolute error of ± 6 days (lifespan ~30 days) (Hugo et al., 2010).

Surprisingly, despite the successful application of mRNA age markers in mosquitoes, similar findings have not been reported in other wild animals. This is perhaps due to the prioritization of the development of DNA methylation age markers, which has been shown to typically provide higher age predictive power in humans (Maulani & Auerkari, 2020). Additionally, mRNA is inherently less stable than DNA, being susceptible to rapid

degradation by ribonucleases and environmental factors such as temperature and pH (Houseley & Tollervey, 2009). This instability may pose challenges for field-based studies where sample preservation is difficult, potentially limiting the broader application of mRNA-based age estimation in wildlife research. Nonetheless, mRNA could be an attractive alternative ageing method in some cases; for example when identification of age-related DNA methylation markers fails, or when transcriptional profiles of closely related species are available.

### *2.4.2 MicroRNA*

MicroRNAs are short (20 - 25 nucleotides) single-stranded non-coding RNA molecules that regulate gene expression by repressing mRNA translation (Lee et al., 1993). Similar to other epigenetic markers, miRNA levels have been found to be correlated with age in model species such as human (Noren Hooten et al., 2010), fruit fly (*Drosophila*) (Liu et al., 2012), and nematode (*Caenorhabditis elegans*) (Pincus & Slack, 2010). The majority of miRNAs decrease in abundance with age, and are associated with ageing processes and disease state (Dimmeler & Nicotera, 2013). Because miRNAs can be detected in extracellular fluids, they have been proposed as biomarkers for a variety of human diseases such as cancer and cardiovascular disorders (Wang et al., 2016).

While no miRNA-based age markers have been developed for wild animals yet, miRNA has been advocated as a promising molecular ecological tool for several reasons (Bonin et al., 2019). In particular, most miRNAs are highly conserved in sequence and function among metazoans, making it possible to transfer knowledge from human biomedical research to non-model species (Bartel, 2018). Furthermore, the bioinformatics workflow of miRNA is relatively amenable as compared with other epigenetic markers, due to the small number of genomic miRNAs (Lussier et al., 2012). MicroRNAs are also more stable than other RNAs (e.g., mRNA) and can be detected in extracellular fluids in most animals, making

it a practical tool for ecological studies (Turchinovich et al., 2011). Due to the above advantages, miRNA has emerged as a molecular tool for understanding environmental stressor responses in recent years (Bonin et al., 2019). Further research is needed to test whether miRNAs can be used for age estimation in wild animals.

## 2.5 Conclusion

Nucleic acid-based ageing research is a growing field that offers promising methods for estimating age in wild animals, providing valuable insights for ecological studies. While telomere-based tools are low-cost biomarkers, they often lack accuracy and are highly species-specific. In contrast, more recent studies using epigenetic clocks have demonstrated high accuracy. Despite their success in vertebrates, the applicability of epigenetic clocks to invertebrates remains underexplored, highlighting the need for more extensive research to assess the effectiveness of these tools. Furthermore, adapting these tools for invertebrates presents challenges due to differences in their epigenetic profiles.

For instance, invertebrates often exhibit sparse methylation patterns, which are poorly captured by methods like RRBS that target CpG-rich promoter regions. Instead, approaches that provide more comprehensive genome-wide methylation data, such as ONT, may be better suited for invertebrates. Overall, nucleic acid-based age estimation techniques hold great potential in providing valuable ecological information for wildlife research. Continued development and adaptation of these tools across taxa will remain a fruitful area for future work.

# Chapter 3: Telomere dynamics in the Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*): effect of age, diet, and tissue type

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## Abstract

Population irruptions of crown-of-thorns seastar (CoTS, *Acanthaster* spp.) represent a perennial threat to Indo-Pacific coral reefs. Age determination of CoTS is challenging, thereby hindering understanding and management of this nuisance species. Telomeres, which are protective DNA structure found at the ends of eukaryotic chromosomes that shorten at each cell division, have been used to estimate age in wild animals. To investigate the use of telomeres in CoTS, we optimized a quantitative PCR protocol to measure relative telomere length (rTL) in CoTS for the first time. Comparing rTL among four age groups (4, 7, 16, >24 months post-settlement), we found that adult CoTS generally exhibit shorter rTL than juveniles, which is the first evidence of age-related telomere attrition in CoTS. However, there was large within-age class variation, and no significant relationships were found between adult CoTS rTL and potential age-indicating external features. Furthermore, we found accelerated telomere attrition under sub-optimal diet, where individuals that were fed crustose coralline algae for 16 months exhibited shorter rTL than their counterparts fed on coral. A positive correlation was found between rTL of tube feet and pyloric caeca, suggesting synchronization of telomere dynamics across somatic tissues in CoTS. Overall, our results suggest that rTL could be used to classify CoTS into broad age groups, though individual variation constrains the ability to resolve specific cohorts. The present study contributes to the understanding of telomere dynamics in marine invertebrates, while laying

the groundwork for future research into rTL as biomarker for age and potentially stress for CoTS.

## 3.1 Introduction

Crown-of-thorns seastars (CoTS; *Acanthaster* spp.) are highly efficient predators of reef-building scleractinian corals (Pratchett et al., 2014). During periodic population irruptions, local densities of CoTS can exceed 1,000 individuals per hectare (Chesher, 1969). These population irruptions have caused extensive decline in coral cover, contributing to reef degradation throughout the Indo-Pacific region (De'ath et al., 2012; Nakamura et al., 2014). Despite considerable research effort since the 1960s, the causes of irruptions remain uncertain due to the inability to resolve some critical research questions, hampering the development of corresponding management strategies (Pratchett et al., 2021).

One of the knowledge gaps that remains in CoTS research is the lack of a validated method to determine their age. For example, their size-at-age was found to be highly plastic, dependent on food available and population density (Lucas, 1984; MacNeil et al., 2017), and thus the differentiation of cohorts based on size-structure has been contentious. On the other hand, the count of visible pigment bands on the spines of CoTS have been considered as reasonably promising size-independent proxies of age (Stump & Lucas, 1990). However, this method still needs to be further validated through medium- to long-term mark-recapture studies to elucidate temporal or spatial variation in band formation (Souter, 1997; Stump & Lucas, 1999). Additionally, it is possible that these bands reflect the time since transition to corallivory, rather than the time since larval settlement, as substantial spine and body growth typically occur only after the dietary shift to coral feeding. However, this remains untested and requires further investigation. Overall, the inability to age individual CoTS has hindered the understanding of population dynamics and the processes that initiate irruptions. It is, therefore, of interest to search for new approaches for age determination in CoTS.

Telomeres are tandem repeats of short DNA sequence (5'-$T_xA_yG_z$-3') and protein complexes found at the ends of eukaryotic chromosomes (Blackburn, 1991). The non-coding

telomeres act as end caps to prevent inter-chromosomal fusion (van Steensel et al., 1998) and to protect chromosomal DNA from degradation during cell division (Blackburn, 2005). At each cell division, telomeres are shortened while chromosomal DNA stays intact, preserving vital information in the genome (Blackburn, 1991). While telomere length can be extended and maintained by the ribonucleoprotein enzyme telomerase, activity of this enzyme varies according to cell type, life stage, and species (Blackburn, 2005). In somatic tissues, where telomerase activities are typically low, a cell undergoes senescence or apoptosis when its telomeres shorten to a critical limit. Since the first description of telomeres in the 1930s, extensive research effort has been devoted to the understanding of their structure and function. To date, it is widely recognized that telomeres play a central role in cellular ageing (Armanios & Blackburn, 2012), while telomere attrition is considered a major hallmark of ageing in humans (López-Otín et al., 2013).

As telomeric DNA sequence is widely conserved across eukaryotes (Gomes et al., 2010), studies in the past two decades have drawn on experience gained in human biomedical research and introduced telomere length as a biomarker into the ecological field (reviewed by Louzon et al., 2019). Notably, telomere length has been proposed as a proxy for chronological age in wild animals, based on the fact that age-associated telomere shortening has been found in various taxa such as birds (Haussmann & Vleck, 2002), reptiles (Scott et al., 2006), fishes (Hartmann et al., 2009), as well as invertebrates from both protostome (e.g., molluscs; Godwin et al., 2011a) and deuterostome (e.g., echinoderms; Varney et al., 2017) groups. Molecular ageing techniques have been advocated as powerful tools for ecological studies, as they offer versatile alternatives to traditional methods (e.g., repeated sighting of tagged individuals, annual growth bands count on calcified structures) in obtaining age information from wild populations. Thus far, several studies have demonstrated the potential

of using telomere-based technique to classify wild animals into broad age groups (Haussmann et al., 2003a; Pauli et al., 2011).

The emergence of telomere length as a biomarker may present a unique opportunity to improve the understanding of CoTS biology and ecology. However, there is considerable work to be done before telomere length can be developed into a reliable ageing tool for marine invertebrates. Despite studies that have demonstrated coarse age indication in some marine invertebrates using telomere-based technique (e.g., sea urchin, Coupé et al., 2019), other research has shown that the negative correlation between age and telomere length cannot be generalized across all species. For example, no age-related changes in telomere length were found in red sea urchin (*Mesocentrotus franciscanus*) (Francis et al., 2006) or spiny lobsters (*Sagmariasus verreauxi* and *Jasus edwardsii*) (Godwin et al., 2011b). These incongruent findings suggest that telomere dynamics may be species-specific, and thus careful investigations are needed to establish the relationship between telomere length and age in non-model or new study species.

To establish a robust ageing tool, the influence of age-independent factors on telomere dynamics must be considered. For example, recent research has shown that extrinsic factors such as stress and diet can cause oxidative damage to telomeric DNA and thus accelerate telomere attrition rate (Chatelain et al., 2020). In the case of CoTS, it has been established that their growth is highly dependent on diet, where exponential growth has been recorded following the ontogenetic shift of diet from crustose coralline algae (CCA) to coral at 4 to 12 months post-settlement (Neil et al., 2022; Wilmes et al., 2020; Yamaguchi, 1974). However, it has recently been hypothesized that CoTS can prolong their herbivorous phase and delay growth for at least six years while waiting for favourable conditions (e.g., availability of coral) to arise ("hidden army hypothesis"; Deaker et al., 2020). While this hypothesis has important implications in the understanding of CoTS population dynamics, little is known

about the physiological consequences of the delay in diet transition, and the proposed trophic flexibility is yet to be tested in natural environments due to the lack of a reliable ageing tool. Here we set up treatment groups fed on CCA only, thus mimicking the "hidden army", to investigate the influence of diet on telomere dynamics in CoTS. In addition, as telomere dynamics can be tissue specific (de Abechuco et al., 2016), it is important to identify the suitable tissue type representing the overall telomere dynamics in CoTS.

The present study seeks to investigate the potential of telomere length as a biomarker to enhance the understanding of CoTS population irruptions. We set out to adopt and optimize the quantitative PCR (qPCR) protocol developed for humans (Cawthon, 2002) to measure telomere length in CoTS for the first time. To explore age-associated telomere dynamics in CoTS, telomere lengths of individuals from four age groups were measured and correlated with potential age-indicators including diameter, spine length, and pigment band count. Effect of diet on telomere dynamics was determined by raising juvenile CoTS under two diet conditions, where one batch shifted diet ontogenetically from CCA to coral, and the other was fed only with CCA since settlement. As a test of tissue effect, telomere lengths measured from pyloric caeca and tube feet were compared.

## 3.2 Materials and methods

### *3.2.1 Sample collection*

Adult Pacific crown-of-thorns seastars (CoTS; *Acanthaster* cf. *solaris*) were spawned in December 2020 and 2021 at the Australian Institute of Marine Science (AIMS) National Sea Simulator (SeaSim) following Uthicke et al. (2015). The larvae were settled and raised as described by Balu et al. (2021). The two cohorts were sampled at two distinct time points, constituting three age groups in total (4-, 7-, and 16-month post-settlement; Table 3.1). To assess the effect of diet on telomere length, seastars were divided into two diet groups: coral and CCA. The coral diet group was fed only with corals once individuals had shifted diet

from CCA to coral at 4 months post-settlement. This represents a "typical" situation where CoTS exhibit ontogenetic diet shift while transitioning from juvenile to adult (Wilmes et al., 2020). On the other hand, the CCA diet group was raised entirely on CCA, and was not exposed to corals for the 16 months experimental period, mimicking the "hidden army" hypothesis situation where juveniles exhibit a prolonged herbivorous phase (Deaker et al., 2020). As a result, there were five treatment groups of juvenile CoTS in total (Table 3.1). During sampling, diameters of the seastars were recorded (ranged from 3.5 to 51.9 mm; measured as the maximum arm spread), followed by whole specimen preservation in individual tubes with 95% ethanol. Samples were stored at -20 °C until DNA extraction.

**Table 3.1** Juvenile crown-of-thorns seastar (CoTS; *Acanthaster* cf. *solaris*) specimens used in this study. CCA = crustose coralline algae

| Age group | Settlement date | Sampling date | Diet | Sample size |
|---|---|---|---|---|
| 4-month | 10 Dec 2021 | 12 Apr 2022 | CCA | 15 |
| 7-month | 14 Dec 2020 | 30 Jul 2021 | CCA | 13 |
| | | | Coral | 15 |
| 16-month | 14 Dec 2020 | 12 Apr 2022 | CCA | 15 |
| | | | Coral | 14 |

Adult CoTS specimens were collected from the central section of the Great Barrier Reef by Pacific Marine Group, a CoTS culling operator. The first batch of samples (n = 14) was collected from Wheeler Reef (-18.79756, 147.52681) on 20 Apr 2022, and the second batch of samples (n = 15) was collected from Hopkinson Reef (-18.54816, 147.19429) on 16 June 2022. The CoTS were transported alive to AIMS and kept at mesocosm until sampling within a week. Although the age of these field samples was unknown and the two batches of

samples were presumably composed of different temporal cohorts, it has been established that CoTS larger than 200 mm are likely older than 2 years and are classified as adults (Pratchett et al., 2014). While the growth of CoTS is highly plastic and their juvenile phase could potentially be prolonged under certain conditions (Deaker et al., 2020), it is unlikely for individuals below 2 years old to attain a diameter above 200 mm, due to inherent constraints in growth rates (Wilmes et al., 2020). As such, field collected specimens (diameter ranged from 210 to 380 mm) were grouped together and hereafter referred to as the > 24 months adult group. Tube feet and pyloric caeca tissue were collected from each individual and preserved in in 95% ethanol at -20 °C until DNA extraction. In addition, the longest aboral spine from four arms were collected for spine length measurement and pigment band count, following Stump and Lucas (1990) and MacNeil et al. (2017).

### *3.2.2 DNA extraction*

Genomic DNA was extracted from CoTS samples using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. DNA integrity of all samples was examined by electrophoresis on a 1% agarose gel, while the DNA purity (260/280 absorbance ratio) was assessed using NanoPhotometer N60 (Implen). DNA concentration was measured by Qubit 3.0 Fluorometer (Thermo Fisher Scientific) using a Qubit dsDNA BR (Broad-Range) Assay Kit (Thermo Fisher Scientific), and subsequently standardized to 1 ng/μL before qPCR assays.

### *3.2.3 Quantitative PCR assay*

Telomere length measurement was conducted using the qPCR method developed by Cawthon (2002). In brief, qPCR reactions were performed respectively to quantify telomeric (T) and reference (S; gene that is non-variable in copy number) DNA. Subsequently, the T/S ratio of each sample was calculated, representing the relative telomere length (rTL). Because telomere sequence is conserved among deuterostomes (Gomes et al., 2010), the primers used

for the amplification of CoTS telomeric DNA were the same as those used for humans (forward 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'; reverse 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3') (Epel et al., 2004). Among the candidate reference genes that were examined, 18S ribosomal RNA (rRNA) demonstrated the most consistent amplification profile as well as clean single-peak melt curves, and thus was selected as the reference gene in this study. The suitability of 18S rRNA as a reference gene is also supported by previous research in echinoderms (Dettleff et al., 2020; Ragusa et al., 2013). Primers for the 18S rRNA gene are: forward 5'-ACTCAACACGGGAAACCTCA-3'; reverse 5'-AACCAGACAAATCGCTCCAC-3'.

Quantitative PCR reactions for telomeres and 18S rRNA were set up on separate 72-well Rotor-Disc (Qiagen) using a QIAgility (Qiagen) liquid handling robot. Each reaction consisted of 10 μL of PowerUp SYBR Green Master Mix (Thermo Fisher Scientific), 1000 nM of telomere primers or 500 nM of 18S rRNA primers (Integrated DNA Technologies), 5 μL of template DNA, and nuclease-free water in a total volume of 20 μL. For experimental samples, the template DNA concentration was 1 ng/μL. The amplification efficiency of each run was evaluated by a standard curve derived from 5-fold serial dilution of an arbitrarily assigned reference sample, yielding five concentrations ranging from 0.016 to 10 ng/μL. In addition, each run contained a no template control (substituting nuclease-free water for template DNA) for contamination detection, and a calibrator sample at 1 ng/μL (an identical sample repeated on all runs) to account for inter-run variation. All reactions were performed in technical triplicate using Rotor-Gene Q (Qiagen) with the following conditions: 50 °C for 2 min (uracil-DNA glycosylase activation); 95 °C for 2 min (polymerase activation); 45 cycles of 95 °C for 15 s and 60 °C (for telomeres) or 55 °C (for 18S rRNA) for 1 min; followed by a melt curve analysis at the end of each run to check for primer dimerization and non-specific amplification.

The quantification cycle (Cq) data were exported for analysis from the Rotor-Gene Q software (Qiagen) after baseline correction. Mean amplification efficiencies (± standard deviation) were 1.03 ± 0.04 for telomere and 1.02 ± 0.05 for 18S rRNA. Samples were identified as technical outliers and excluded from further analysis if the standard deviation of Cq values between technical triplicates were > 0.2.

The relative telomere length (rTL) of each sample was calculated using the framework by Hellemans et al. (2007). In brief, the mean Cq values across technical triplicate were converted to normalized relative quantities (NRQ) according to Pfaffl (2001):

$$NRQ = [(E_{telomere})^{\Delta Cq\ telomere}] / [(E_{18s\ rRNA})^{\Delta Cq\ 18s\ rRNA}]$$

Where E is gene-specific amplification efficiency, and ΔCq represents the difference between the mean Cq value across all samples within a single run and the mean sample Cq value. Subsequently, inter-run calibration was conducted based on the NRQ of the calibrator sample of each run using formula 13' and 15' in Hellemans et al. (2007).

### *3.2.4 Statistical analyses*

Differences in rTL between treatment groups were assessed with Kruskal-Wallis test (Kruskal & Wallis, 1952). This non-parametric test was chosen because the assumptions of normality and homogeneity of variance were not met. Post-hoc analysis was conducted using Dunn's multiple comparisons test with p-values adjusted by the Benjamini-Hochberg correction method (Benjamini & Hochberg, 1995). Pearson's correlation test was used to determine whether rTL was correlated between tube feet and pyloric caeca tissue in adult CoTS. To investigate factors affecting rTL in field collected CoTS, a generalized linear mixed model (GLMM) was constructed with the package glmmTMB (Brooks et al., 2017). The model was built with rTL as response variable; diameter, spine length and pigment band

count as fixed factors; and reef as a random effect to account for different collection locations. The initial model was constructed with all possible two-way interactions, followed by stepwise elimination of non-significant terms and selection of the best fitted model using Akaike information criteria (AIC). The variance inflation factors (VIF) for all predictors in the final model were below 5, indicating no violations of collinearity. For model validations, residual diagnostics were performed using the DHARMa package (Hartig, 2017). The level of significance of all tests was set at $p < 0.05$. All statistical analyses were performed using R version 4.1.1 (R Core Team, 2022).

### 3.3 Results

To investigate telomere dynamics in CoTS, rTL were compared among four age groups. The median rTL value was the highest in the 4-month juvenile group at 4.23, and the lowest in the 16-month CCA diet group at 2.04. Significant differences in rTL among groups were detected by Kruskal-Wallis test ($X^2 = 30.495$, df = 5, $p < 0.001$). Pairwise comparisons revealed that apart from the 16-month CCA diet group, the juveniles exhibited significantly longer rTL than the adults (presumed > 24 months), while the rTL among other juvenile groups were not significantly different from each other (Dunn's multiple comparisons test; Benjamini-Hochberg adjusted *p*, significance level = 0.05) (Figure 3.1). Overall, there was large within-age class variation, with some adults showing similar rTL as the juveniles. For example, the rTL of the adult group ranged from 0.11 to 5.35, while the rTL of the 4-month group ranged from 2.10 to 8.51. With regards to diet treatment, the 16-month CCA diet group showed significantly lower median rTL (2.04) compared to the 16-month coral diet group (3.34) ($p = 0.02$); while no significant differences were found between the 7-month CCA and coral diet group. A significant positive correlation was found between the rTL measured from adult tube feet and pyloric caeca tissue (Pearson's $r = 0.73$, $p < 0.05$) (Figure 3.2). Finally, there were no significant relationships between field collected adult CoTS rTL and the

diameter of the seastar, the length of the spines, or the count of pigment bands on spines (GLMM; Table 3.2, Figure 3.3).

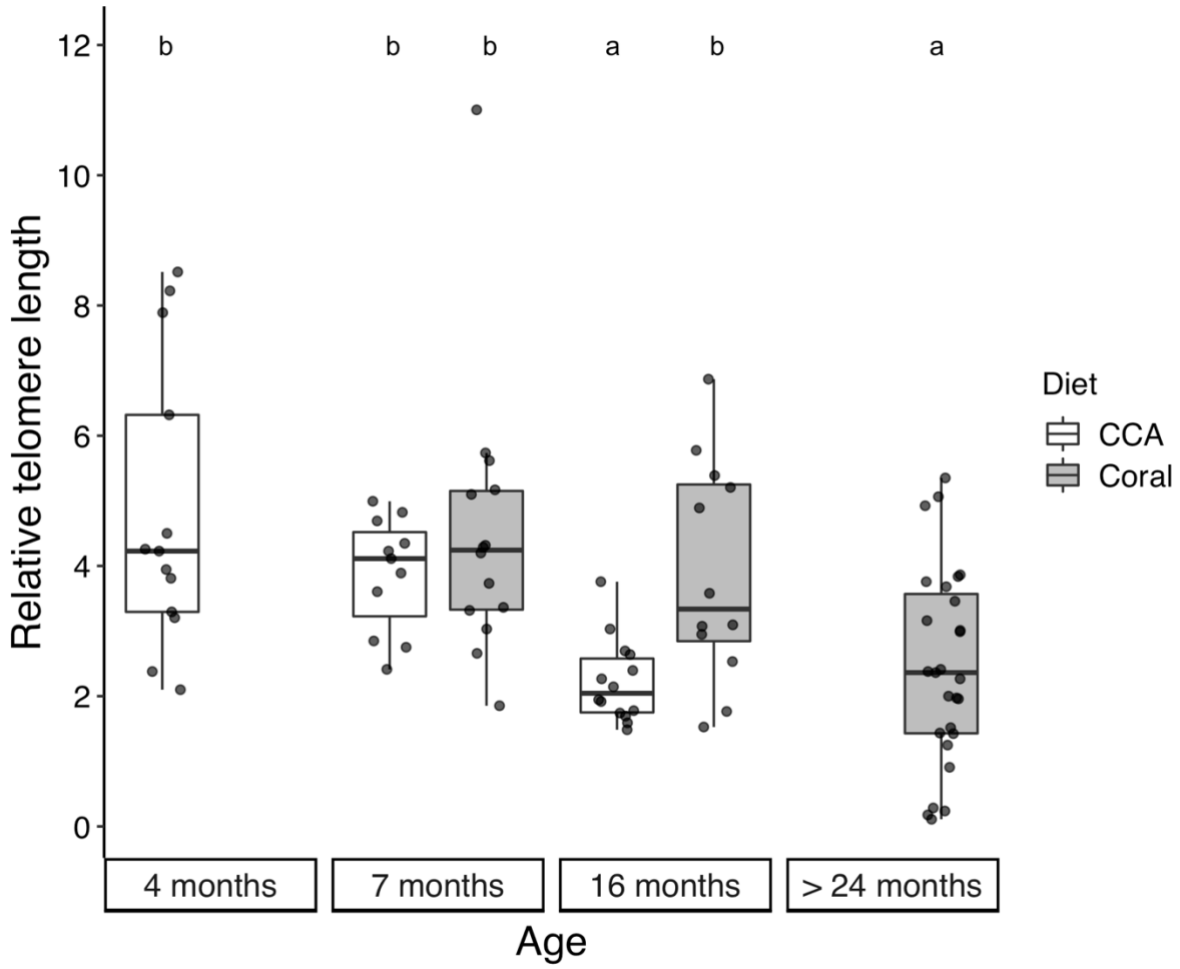



**Figure 3.1** Relative telomere length (rTL) in crown-of-thorns seastar (CoTS; *Acanthaster* cf. *solaris*) by age (months after settlement) and diet. Boxes represent interquartile range of the distribution; horizonal lines within the boxes represent median; vertical extending lines represent maximum and minimum values within 1.5 interquartile range; each dot represents a sample. Different letters indicate statistically significant differences between groups (Kruskal-Wallis test followed by Dunn's multiple comparisons, Benjamini-Hochberg adjusted $p < 0.05$). CCA = crustose coralline algae.

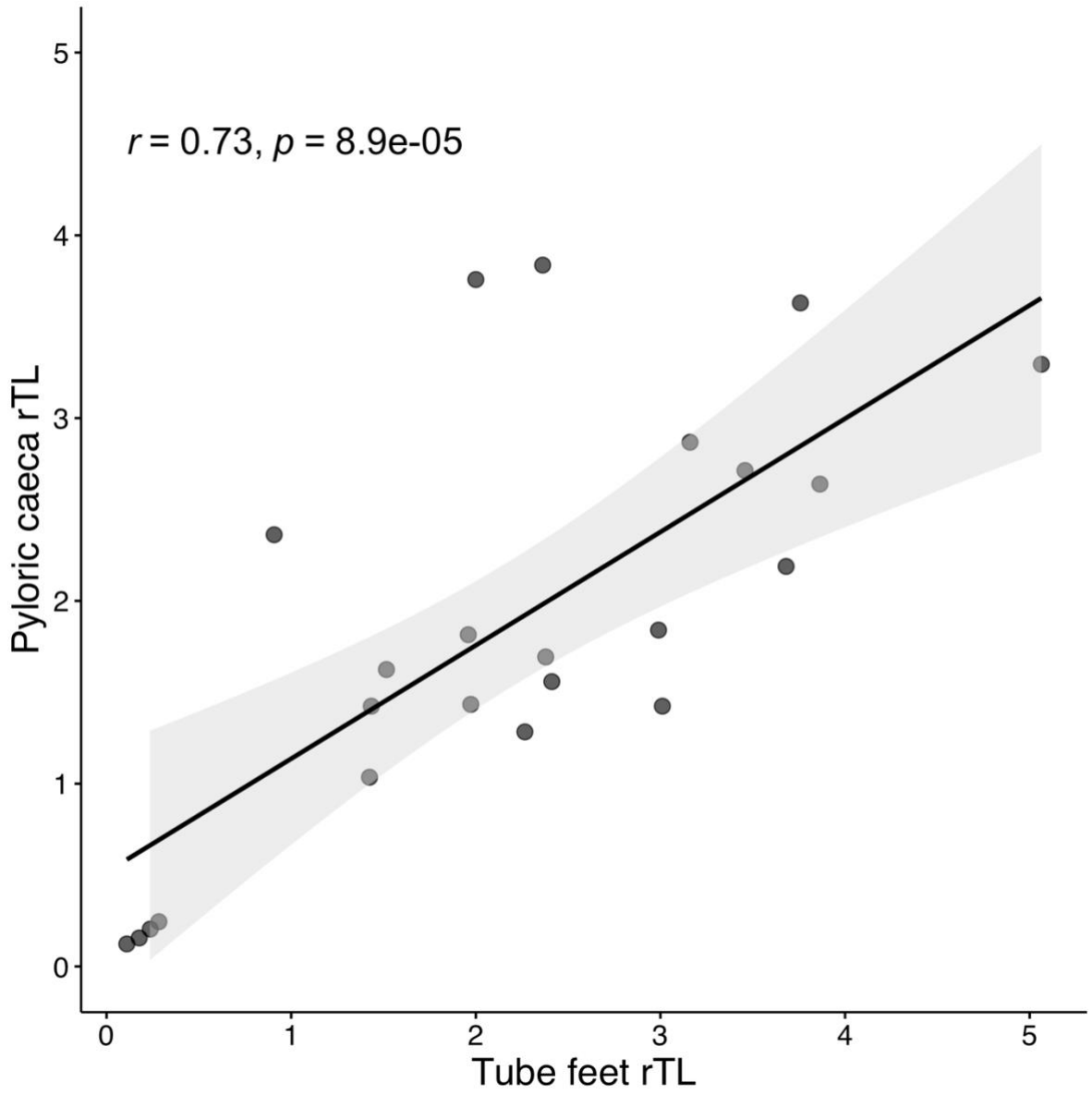



**Figure 3.2** Correlation between tube feet and pyloric caeca tissue relative telomere length (rTL) in adult crown-of-thorns seastar (CoTS; *Acanthaster* cf. *solaris*).

**Table 3.2** Summary table of generalized linear mixed model (GLMM) examining factors affecting relative telomere length in crown-of-thorns seastar (CoTS; *Acanthaster* cf. *solaris*).

| **Fixed effects** | **Estimate** | **Std Error** | **z value** | ***p* value** |
|---|---|---|---|---|
| Diameter | 0.007 | 0.005 | 1.351 | 0.177 |
| Spine length | - 0.012 | 0.160 | - 0.072 | 0.943 |
| Pigment band count | - 0.075 | 0.075 | - 1.007 | 0.314 |

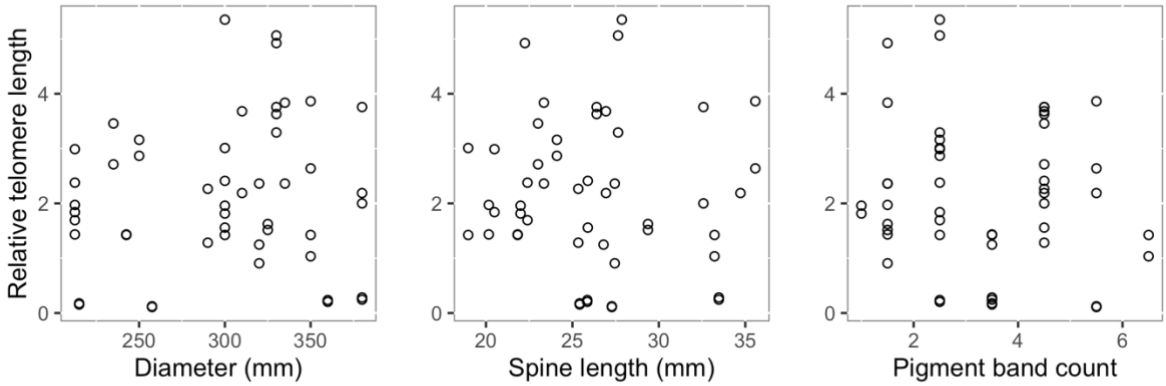



**Figure 3.3** Relationships between relative telomere length and diameter, spine length, and pigment band count of adult crown-of-thorns seastar (CoTS; *Acanthaster* cf. *solaris*).

## 3.4 Discussion

The present study is the first report of telomere measurement in the Pacific crown-of-thorns seastar (CoTS; *Acanthaster* cf. *solaris*). We demonstrated that the qPCR method allowed CoTS relative telomere length (rTL) to be measured in a high-throughput manner, providing a cost- and time-efficient approach for future research in CoTS telomere biology. As an exploratory study, our findings offer important insight into the potential factors influencing telomere dynamics in CoTS, while raising the possibility that telomere length could be a relevant biomarker to enhance the understanding of the population ecology of CoTS. More specifically, knowledge of the age structure of CoTS will help resolve persistent controversy regarding the putative cause(s) of population irruptions (Pratchett et al. 2017). The ability to age CoTS is for example, critical for testing whether juvenile seastar can defer ontogenetic shifts to withstand periods of resource limitation (Deaker et al. 2020).

### *3.4.1 Age-related telomere dynamics*

Our results indicate that adult CoTS in general exhibit shorter rTL than juveniles, which is the first evidence of age-related telomere attrition in CoTS. These findings are in line with the general pattern observed in vertebrates (reviewed by Remot et al., 2021), while adding to the few examples of age-related telomere attrition in marine invertebrates (e.g., seastar and sea urchin; Coupé et al., 2019; Varney et al., 2017). Our observation contributes to the understanding of the relationship between age and telomere length in marine invertebrates, where inconsistent results have been reported from a limited number of existing studies. However, with juvenile and adult CoTS sourced from different growing environments (aquaria vs field), these findings must be interpreted with caution, as the age-rTL relationship could be obscured by extrinsic factors (see below).

Our data illustrate that juvenile CoTS (< 16 months) fed on their natural diet (i.e., CCA followed by coral) can retain their telomere length, likely through energy demanding

repair mechanisms. Previous research has established that telomere dynamics of a species is closely associated with the activity of telomerase, the enzyme that is involved in the repair and maintenance of telomeres (Blackburn, 2005). For example, continuous telomerase expression has been reported in invertebrates with regeneration capacity (e.g., sea urchin; Francis et al., 2006) and/or indeterminate growth (e.g., lobster; Klapper et al., 1998), resulting in constant telomere restoration and thus a lack of age-associated shortening pattern. CoTS also exhibit strong regenerative abilities, including arm regrowth and tissue repair, which may indicate the presence of sustained telomerase activity during certain life stages or under specific physiological conditions (Messmer et al., 2013). Whilst telomerase activities were not measured directly in this study, the telomere dynamics observed here appear to suggest that the telomerase expression pattern in CoTS is more similar to those observed in vertebrates: being stable during early development, followed by downregulation in later life stages (Gomes et al., 2010) or under stressful situations such as sub-optimal diet (see below). This is a compelling area for further research as telomerase activity is closely linked to the process of senescence, which is poorly understood in CoTS. In this context, future research should be undertaken to measure telomerase activity in CoTS using assays such as the telomeric repeat amplification protocol (TRAP; Kim et al., 1994).

### *3.4.2 Effects of diet on telomere dynamics*

We found that among the 16-month-old juvenile CoTS, individuals that were fed on CCA diet (the situation suggested by the "hidden army hypothesis") exhibited significantly shorter average rTL than their counterparts that were allowed to diet transition to coral at 4-month post-settlement (the "typical" situation). Previous research has proposed that telomere dynamics are likely influenced by a combination of factors (Haussmann & Marchetto, 2010). Our results suggest that diet could be one of such factors that influences rTL in CoTS. It is well established in human research that nutritional status is positively associated with

telomere length (Paul, 2011), while a similar pattern has also been observed in animals such as birds and fishes (Badás et al., 2015; McLennan et al., 2021). It is proposed that sub-optimal diet is linked to a deficiency of dietary antioxidant intake, leading to a weakening in defence against oxidative damage to telomere DNA (Paul, 2011). In addition, malnourished animals might not have excess energy to produce telomerase, compromising the ability to repair and maintain telomere length. Energy shortage of CCA-fed juveniles is supported by the fact that their growth was stunted, indicating that they have no "scope for growth" in their energy budget. Hence, it could conceivably be hypothesized that the shorter rTL observed here might be associated with inadequate levels of nutrients or energy in the CCA diet for CoTS that naturally would have transitioned to coral. Further research might explore whether this damage can be reversed if CoTS switch to a coral diet later in life, and whether these energy-deficit individuals would undergo sexual maturation and be able to reproduce.

Our results, while preliminary, suggest that even though juvenile CoTS are seemingly able to survive on CCA diet for a prolonged period (Deaker et al., 2020), the delay in diet transition might have imposed oxidative stress and undermined their body conditions, which is manifested as accelerated telomere attrition. However, it is important to note that generalizability of our results could be limited by the scale of the study, and further investigation is required to prove our hypothesis. For example, measuring the rTL of juvenile-sized CoTS collected from the field would be an essential next step to validate our findings *in situ*.

### *3.4.3 Effects of tissue type on telomere dynamics*

Significant positive correlation was found between rTL measured from tube feet and pyloric caeca tissue. While it is likely that the cell proliferative activity of pyloric caeca is higher than that of tube feet due to the continuous growth and replacement of digestive epithelium (Van der Plas et al., 1983), previous research found that telomere dynamics across

somatic tissues in an individual is in general synchronized, regardless of proliferative status (Daniali et al., 2013). The same observation has been made in various taxa including invertebrates such as oysters (Debes et al., 2016; Godwin et al., 2011a; Power et al., 2021). On the other hand, while regeneration history of our samples is unknown, it has been demonstrated that telomere lengths do not differ between regenerated and non-regenerated arm tissues in the seastar *Asterias rubens* (Hernroth et al., 2010). Overall, our results broadly corroborate earlier findings which suggest no tissue effect in general, supporting the use of tube feet rTL as a proxy for overall telomere dynamics in CoTS. These findings are of interest to future sample collection, as tube feet tissue is easily accessible and convenient to collect from adult CoTS without requiring dissection.

### *3.4.4 Other age-independent factors*

Contrary to expectations, this study did not find a significant relationship between the rTL of adult CoTS and the diameter of the seastar, the length of the spines, or the count of pigment bands on spines. While these physical measurements and features have not been fully validated as age proxies in CoTS, there is general consensus that they could, to some degree, be used to distinguish broad age classes, especially within the same population (Pratchett et al., 2014). As such, our results might point to the possibility that rTL in CoTS are considerably influenced by additional factors than age. An example of such factors is environmental stress, which has been demonstrated to cause oxidative damage to telomeric DNA and thus accelerate telomere attrition rate (Monaghan, 2014; Von Zglinicki, 2002). It is well established that CoTS development and growth are strongly influenced by environmental conditions such as food availability and temperature (Kamya et al., 2014; Lucas, 1984; MacNeil et al., 2017). It is therefore reasonable to hypothesize that exposure histories to unfavourable conditions may be reflected in the rTL of CoTS, obscuring the age-related telomere attrition pattern. In addition, variation among parents, including inherited

telomere length and physiological condition, may also contribute to inter-individual differences. There are, nonetheless, other possible explanations that are worth further investigation, such as reproductive history, pathogen infection, and competition (reviewed by Chatelain et al., 2020).

As our adult seastar samples were collected from the wild where individuals' growth histories were unclear and presumably complicated, it was intractable to disentangle the effects of various factors on rTL. Furthermore, the largest individual included in this study was 380 mm in diameter, while CoTS can grow to > 600 mm in the wild (Stump, 1996). As such, future studies with samples across a wider size range, ideally from more controlled environment such as mesocosm experiments will be needed if a full picture of age-rTL association is to be developed.

### *3.4.5 Telomere length as biomarker for age determination in CoTS*

Our results suggest that rTL could potentially be used as a tool to distinguish CoTS into broad age groups or life stages, though individual variation constrains the ability to resolve specific cohorts. This conclusion is in line with that of Dunshea et al. (2011), who found overall loose relationships between age and rTL across a range of invertebrates. To improve the utility of telomere length as an ageing tool, further research accounting for the influence of extrinsic factors will need to be undertaken. While a broad-scale ageing tool is nonetheless valuable and relevant for CoTS research, considerably more work is needed to tighten the age-rTL relationship in CoTS before such tool can be developed.

When comparing rTL to conventional ageing methods such as size-at-age, it appears that both approaches might be susceptible to the influence of extrinsic factors, particularly food availability. Consequently, like size-at-age, the effectiveness of using rTL as an ageing tool may be constrained if these extrinsic factors are not accounted for. However, it is worth noting that rTL presents a unique advantage in directly assessing the physiological conditions

of CoTS. Therefore, there is abundant ground for future research to explore rTL as biomarker for stress or health status in CoTS (Haussmann & Marchetto, 2010). For example, our results indicated that sub-optimal diet could be a potential stress factor accelerating rTL attrition in juvenile CoTS. If this pattern holds true *in situ*, rTL can be used as a tool to test the aquarium-based "hidden army hypothesis", confirming whether CoTS exhibit prolonged delay in diet transition to coral in natural settings (Deaker et al., 2020). Furthermore, through measurement of rTL from CoTS populations experiencing varying stages of outbreak, we might be able to better understand the process of population collapse at the end of the outbreak cycle. For example, rTL might reflect stress related to starvation and over-crowding at the end of outbreaks, which is a yet-to-be-tested mechanism leading to population collapse. Nonetheless, due to the variable nature of telomeres, extensive field sampling as well as carefully controlled experiments will be required before rTL can be used as a relevant biomarker for CoTS management.

### 3.5 Conclusions

Here we established the first evidence of age-related telomere attrition in CoTS. However, telomere dynamics in CoTS are likely influenced by various age-unrelated factors such as diet and environmental stressors, limiting the use of telomere length as a precise chronological age indicator in this species. Overall, this study contributes to the understanding of telomere dynamics in marine invertebrates, while laying the groundwork for future research into rTL as a molecular biomarker for CoTS management. This is a compelling area for further investigation, given the potential of rTL to provide valuable biological information in a relatively low-cost and high-throughput manner.

# Chapter 4: Methylome profiling of a deuterostome invertebrate using Oxford Nanopore Technology (ONT)

Submitted as: Kwong, S. L. T., Budd, A., Hung, J., Villacorta-Rath, C., Uthicke, S. (2025). Methylome profiling of a deuterostome invertebrate using Oxford Nanopore Technology (ONT). Under review in *Molecular Ecology Resources*

## Abstract

DNA methylation is crucial for genome regulation and provides key insights into the interaction between genetics and environmental factors, offering valuable perspectives for ecological research. However, knowledge of DNA methylation patterns in non-model invertebrates remains limited. The present study addresses this knowledge gap by conducting the first comprehensive methylome profiling of the Pacific crown-of-thorns seastar (CoTS; *Acanthaster* cf. *solaris*), a coral-eating species that aggravates the decline of Indo-Pacific coral reefs. Using Oxford Nanopore Technology (ONT) V14 chemistry we generated high-quality, long-read sequences covering over 90% of CpG dinucleotides in the CoTS genome. Our analysis revealed a mosaic methylation landscape with moderate genome-wide methylation levels of 37.7%. Comparative analysis highlights the intermediate methylation state in deuterostome invertebrates, positioning them between the hypomethylated genomes of protostomes and the hypermethylated genomes of vertebrates. Methylation in CoTS was predominantly localized within gene bodies, especially in intronic regions, enabling modulation of gene expression and potentially supporting fitness of CoTS in dynamic marine environments. Additionally, elevated methylation in repetitive elements suggests a role in genome defence. The present study demonstrates the effectiveness of ONT for comprehensive methylome analysis in ecologically important non-model species, and deepens our understanding of the epigenetic landscape in deuterostome invertebrates. The

workflow and comprehensive methylome profiles generated here serve as foundational resources for future research, enabling in-depth investigations into DNA methylation functions, offering potential applications for CoTS outbreak management, and facilitating comparative studies across diverse lineages.

## 4.1 Introduction

DNA methylation, a key epigenetic mechanism, plays a pivotal role in regulating gene expression and chromatin structure (Buitrago et al., 2021). It involves the addition of methyl groups to DNA, typically at cytosine bases within cytosine-phosphate-guanine (CpG) sites, thereby modulating genomic activity without altering the underlying DNA sequence (Moore et al., 2013). In ecology, methylation studies are emerging as a valuable approach to reveal an additional layer of information about the complex interplay between genetic code and environmental factors, and how this interaction shapes phenotypic traits (Budd et al., 2022; Kilvitis et al., 2014; Verhoeven et al., 2016). For instance, DNA methylation plays a crucial role in stress responses and adaptation to environmental changes across various taxa from plants to marine invertebrates (Hawes et al., 2018a; Kilvitis et al., 2017; Zhang et al., 2013). The exploration of DNA methylation has proven valuable, offering novel molecular insights and opening avenues for species management and conservation (Balard et al., 2024).

Despite advances in DNA methylation research, studies have predominantly targeted vertebrate species, creating a significant gap in our understanding of these processes in invertebrates (de Mendoza et al., 2020). Vertebrates are widely recognized to follow a "global" methylation pattern, with most CpG sites hypermethylated across the genome except in specific CpG-rich regions (Suzuki & Bird, 2008), resulting in high average genome-wide CpG methylation levels of  60% to 80% (Klughammer et al., 2023). In contrast, invertebrates exhibit a "mosaic" methylation pattern, alternating between hypermethylated and unmethylated regions (Suzuki & Bird, 2008). Their genomes tend to be hypomethylated, with *Drosophila melanogaster* and *Caenorhabditis elegans* displaying little (< 1%) to no methylation (Rae & Steele, 1979; Simpson et al., 1986). Meanwhile, more evolutionarily derived invertebrates, such as bivalves and cephalopods, often show higher genome-wide methylation levels, ranging from 10% to 40% (Klughammer et al., 2023). This has led to the

hypothesis that hypomethylation is the ancestral state of the epigenome (Tweedie et al., 1997), with the exception of the demosponge *Amphimedon queenslandica*, which exhibits vertebrate-like hypermethylation, suggesting that this state may have evolved independently in some early metazoans (de Mendoza et al., 2019).

Many invertebrate species studied for their methylomes, such as the Western honeybee (*Apis mellifera)* and the Pacific oyster (*Crassostrea gigas*), belong to the Protostomia (Šrut, 2021). In contrast, deuterostome invertebrates - including echinoderms, hemichordates, cephalochordates, and tunicates - remain understudied despite being phylogenetically closer to vertebrates. Profiling the methylomes of deuterostome invertebrates therefore presents an opportunity to advance our understanding of DNA methylation across different lineages. Positioned phylogenetically between protostomes and vertebrates, deuterostome methylomes can shed light on evolutionary trajectories of the DNA methylation landscape across metazoans. Furthermore, while previous studies on deuterostome invertebrates have often focused on selected genomic regions (Han et al., 2021; Strader et al., 2019), comprehensive methylome profiling provides significantly more information.

Identifying targets of DNA methylation can provide insights into its functional role, which remains poorly characterized in invertebrates. In vertebrates, DNA methylation is predominantly studied in CpG-rich promoter regions, where hypermethylation typically leads to gene silencing (Al Adhami et al., 2022). The functional significance of DNA methylation in other genomic regions is less clear. Invertebrate methylation is primarily observed within gene bodies, where it is thought to play a crucial role in regulating splicing and preventing undesirable transcription from intragenic promoters (Sarda et al., 2012). Additionally, invertebrate DNA methylation is thought to contribute to genome defence by silencing transposable elements and other repetitive sequences, protecting the genome from potential instability and mutagenesis (Ying et al., 2022). Expanding research to include various

invertebrates and thoroughly characterizing their methylation profiles, including the identification of methylated genes and genomic regions, will enable a better understanding of the common principles and unique adaptations in the functional roles of DNA methylation across different lineages.

In recent years, Oxford Nanopore Technology (ONT) has emerged as a powerful tool for methylation analysis (Simpson et al., 2017). ONT works by measuring changes in electrical current as single DNA molecules pass through a nanopore, allowing direct detection of modified bases. This eliminates the need for bisulfite treatment, a method traditionally used to assess DNA methylation, thereby preserving DNA integrity and reducing errors from incomplete conversion (Sigurpalsdottir et al., 2024). In addition, the long-read sequencing capability provides a transformative, contiguous view of both the genome and methylome (Jain et al., 2018), while the portability of ONT allow for real-time, on-site sequencing in diverse environments, benefiting field-based ecological studies (Runtuwene et al., 2019). Although ONT initially faced high error rates, raw read accuracy now reaches 99.75% for super accuracy (SUP) basecalling and 98.81% for cytosine methylation detection when using the recently released V14 chemistry (Lerminiaux et al., 2024; Oxford Nanopore Technologies, 2023). Building on the limited research that has utilized ONT to investigate methylation in non-model invertebrates (Dimond et al., 2021; Perez et al., 2023), the present study is among the first to employ the updated chemistry for such purposes. By extending the application of these sequencing advancements beyond model organisms and biomedical research (González-Recio et al., 2023; Sigurpalsdottir et al., 2024), we seek to evaluate the potential of ONT as a practical tool for ecological methylation studies.

Our study focuses on the Pacific crown-of-thorns seastar (CoTS; *Acanthaster* cf. *solaris*), a species notorious for its extensive coral consumption during population outbreaks, posing significant threat to Indo-Pacific coral reefs (De'ath et al., 2012; Pisapia et al., 2016).

Due to its impact, extensive research has been conducted on CoTS to develop local management strategies to control outbreaks and mitigate coral loss amid increasing climate change pressures (Pratchett et al., 2017). Despite these substantial efforts, numerous aspects of CoTS biology remain poorly understood, hindering effective management of population outbreaks (Pratchett et al., 2021). This indicates a pressing need for a more comprehensive understanding of the species, alongside new perspectives and innovative approaches. While the genome of CoTS has been annotated and is currently under investigation (Hall et al., 2017; Popovic et al., 2024), its methylome remains unexplored. Establishing a comprehensive profile of the CoTS methylome provides a foundation for understanding of its epigenetic landscape. This can guide future research into critical epigenetic processes such as gene regulation, development, and responses to environmental stress, potentially uncovering intervention points for controlling CoTS outbreaks.

The objective of this study is to leverage the ONT V14 chemistry advancements to profile the methylome of CoTS for the first time. We aim to evaluate the utility of ONT for methylation detection in non-model species, supplementing the limited existing research in this area. The detailed workflow presented here, from high molecular weight DNA extraction to bioinformatics analysis, will support future methylation studies. In addition, we seek to establish a comprehensive methylome profile of CoTS, detailing methylation patterns and identifying methylation targets. By comparing the CoTS methylome with those of other taxa, we aim to better understand the evolutionary and functional implications of DNA methylation in deuterostome invertebrates.

## 4.2 Materials and methods

### *4.2.1 Specimens*

Twelve field collected adult CoTS (6 females and 6 males) were spawned at the Australian Institute of Marine Science (AIMS) National Sea Simulator (SeaSim) in

December 2020, using the method described in Uthicke et al. (2015). The seastars were settled and raised in aquaria, with an ontogenetic shift from crustose coralline algae to coral diet starting from four months post-settlement (Neil et al., 2022). In December 2022, when the samples were 2 years old, three biological replicates were randomly selected for sampling. We used laboratory-reared individuals to ensure biological replicates were from the same cohort and of the same age, minimizing between-sample variability. Three replicates were used to reduce noise and distinguish biological signals from random variation, enhancing the reliability of the results. Pyloric caeca tissue was collected from each specimen, snap-frozen with liquid nitrogen, and stored at -80°C until DNA extraction.

### *4.2.2 High molecular weight DNA extraction*

Genomic DNA was extracted using a modified phenol-chloroform protocol (Sambrook & Russell, 2006) to enhance the yield of high molecular weight DNA (Appendix A). SPRIselect beads (Beckman Coulter) were used at 0.4x the sample volume to remove short DNA fragments (< 500 bp) prior to library preparation, to ensure optimal performance of Nanopore sequencing. The manufacturer's protocol was modified to ensure the complete removal of short DNA fragments (Appendix A). Due to the limited yield of high molecular weight DNA (~150 ng per sample), extraction and size selection were performed on six samples per individual. DNA from the same individual was subsequently pooled for sequencing. The purity of the extracted DNA was assessed using the NanoDrop One Spectrophotometer (Thermo Fisher Scientific), and the DNA concentration was measured with the QuantiFluor dsDNA System (Promega). The integrity of the extracted DNA was visualized through 1% agarose gel electrophoresis.

### *4.2.3 Nanopore sequencing*

Nanopore sequencing libraries were prepared using the Native Barcoding Kit 24 V14 (SQK-NBD114-24) following the manufacturer's protocol. Sequencing was conducted on the

MinION Mk1C platform implemented with MinKNOW v22.12.5, using the R10.4.1 flow cells (FLO-MIN114). Each sequencing run spanned a duration of 72 hours.

In this study, "methylation" refers specifically to the modification of cytosine bases to 5-methylcytosine (5mC), unless otherwise stated. Post-hoc canonical and modified (5mC) basecalling was simultaneously conducted using the GPU version of Guppy v6.5.7 on a Linux computer with an NVIDIA A100 graphics card. The SUP model dna_r10.4.1_e8.2_260bps_modbases_5mc_cg_sup was applied with demultiplexing, barcode trimming, and alignment features enabled. This basecalling process resulted in modbam files, which are enhanced version of BAM files that includes the location and confidence score for base modifications. These modbam files contain reads with mean Phred quality score above Q10, aligned to the CoTS OKI-Apl_1.0 reference genome (Hall et al., 2017). Herein, any mention of the CoTS reference genome refers specifically to the OKI-Apl_1.0 assembly (GCF_001949145.1). To summarize methylation status at each called CpG dinucleotide, the modbam files were converted to bedMethyl files using Modkit v0.2.1 with the "traditional" preset. The pass confidence threshold value for the conversion was established at 99.4%, ensuring only reliable modification calls are considered. A minimum read depth of 5x was applied as threshold for downstream analysis.

### *4.2.4 Data analyses*

To assess the genetic relatedness among individuals, we conducted identity-by-state (IBS) analysis using ANGSD (Korneliussen et al., 2014). This approach calculates pairwise genetic similarity based on shared alleles across loci.

Methylome analyses were performed using R version 4.3.2 (R Core Team, 2022). The methylation level of individual CpG dinucleotides was defined as the ratio of methylated cytosines to the total number of reads covering that site. For broader genomic regions, the methylation level was determined by calculating the mean methylation across all CpG

dinucleotides within the specified region. To visualize the overall methylation landscape, a Circos plot (Krzywinski et al., 2009) was generated to show the distribution of methylation levels, transposable elements, and gene density across the first ten scaffolds of the CoTS reference genome (NW_019091355.1 to NW_019091364.1). These scaffolds were selected to highlight patterns without overcrowding the plot by including all scaffolds. To assess the correlation between specimens, we utilized the getCorrelation function from the methylKit package v1.28.0 (Akalin et al., 2012) to generate methylation distribution profiles, pairwise Pearson's correlation coefficients, and corresponding correlation plots. The Integrative Genomics Viewer (IGV) v2.16.2 (Thorvaldsdóttir et al., 2013) was used to visualize methylation patterns, with a representative snapshot captured from the beginning of the first scaffold of the CoTS reference genome (NW_019091355.1:1 – 200,000). To compare the CoTS methylome with other taxa, genome-wide methylation levels were compiled and averaged from a diverse array of published studies including data from prebilaterian (cnidarian, poriferan), protostome (mollusc, annelid, arthropod), deuterostome invertebrate (tunicate, echinoderm, hemichordate), and vertebrate (mammal, bird, fish) species. The specific references for these data are listed in the legend of Figure 4.4 in the Results section. An illustrative phylogenetic tree was constructed using the fifth edition of the TimeTree of Life (TToL5), which synthesizes divergence time estimates from multiple independent molecular clock analyses based on genetic data and fossil calibrations (Kumar et al., 2022).

To identify primary targets of methylation in the CoTS genome, various genomic regions were defined based on the National Center for Biotechnology Information (NCBI) RefSeq annotation of the CoTS reference genome (NCBI *Acanthaster planci* Annotation Release 100 - August 7, 2017). Gene bodies were categorized into intronic and exonic regions, while promoters were defined as 2 kilobase pairs (kbp) upstream of the transcription start site (TSS). The remaining non-promoter regions were classified as intergenic. Repetitive

elements were identified using the soft-masked regions of the CoTS reference genome. The GenomicRanges package v1.54.1 (Lawrence et al., 2013) was employed to isolate these genomic regions to assess the distribution of CpG dinucleotides and their corresponding methylation levels. Methylation levels across gene bodies and their 2 kbp flanking regions were profiled using the plotProfile function in deepTools2 (Ramírez et al., 2016).

## 4.3 Results

### *4.3.1 Sequencing performance*

Three Nanopore sequencing runs were conducted, one for each of the three CoTS specimens. These runs generated a total of 22.32 gigabase pairs (Gbp) of data, encompassed by 9,634,060 reads (Table 4.1). This corresponds to an average coverage of 16x to 26x among specimens, given the size of the CoTS reference genome at 383.8 megabase pairs (Mbp). The maximum read length was 365,628 base pairs (bp). The read length N50 ranged from 3,048 to 3,572 bp, and the mean read length ranged from 2,006 to 2,694 bp. The mean Phred quality score across all reads was Q17.7, indicating an average accuracy of 98.3%.

Reads from each specimen were aligned to the CoTS reference genome, resulting in mapping efficiencies from 83.8% to 85.0%. Of these aligned reads, 77.3% to 80.4% were uniquely mapped. Coverage analysis showed that 77.6% to 94.5% of the genome was covered at least 10x. The average mapping quality (MAPQ) score was 55.5 to 55.7, reflecting reliable alignment quality. The count of called CpG dinucleotides at read depth greater than 5x ranged from 8,916,925 to 9,325,272, representing coverage of 91.9% to 96.1% of the 9,705,479 CpG dinucleotides present in the CoTS reference genome.

**Table 4.1** Summary of three Nanopore sequencing runs, each corresponding to an individual Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*) specimen

| Specimen | Total reads | Total base pairs (bp) | Max read length (bp) | Mean read length (bp) | Read length N50 (bp) |
|---|---|---|---|---|---|
| A | 4,246,594 | 9,854,114,797 | 159,049 | 2320 | 3098 |
| B | 2,969,361 | 5,956,556,065 | 230,970 | 2006 | 3048 |
| C | 2,418,105 | 6,513,051,432 | 365,628 | 2694 | 3572 |

### *4.3.2 Methylation landscape*

The pairwise 1-IBS distances indicate that specimens A and B (0.338), as well as B and C (0.339), are potentially full or half-siblings. In contrast, specimens A and C exhibit a greater genetic distance (0.381), suggesting they could be half-siblings or from different parents. Examination of CoTS methylation profiles revealed a consistent pattern across specimens, with a moderately methylated genome and a patchy distribution of methylated regions. These features were visualized using a Circos plot (Figure 4.1). In line with these observations, we found intermediate levels of genome-wide methylation, ranging from 37.2% to 38.1% among the three individuals (Figure 4.2a). Over half (~56%) of the CpG dinucleotides in each specimen were completely unmethylated, with 34%, 8%, and 2% having high (> 80%), medium (20-80%), and low (> 0 and < 20%) methylation levels, respectively (Figure 4.2a). Pairwise analysis showed that the methylation profiles were consistent among specimens, with Pearson's correlation coefficients ranging from 0.97 to 0.98 (Figure 4.2b). The methylation distribution was bimodal, with most CpG sites exhibiting either no methylation or near 100% methylation (Figure 4.2b). In addition, mosaic methylation patterns were observed throughout the genome, characterized by alternating segments of heavily methylated and unmethylated regions (Figure 4.3).

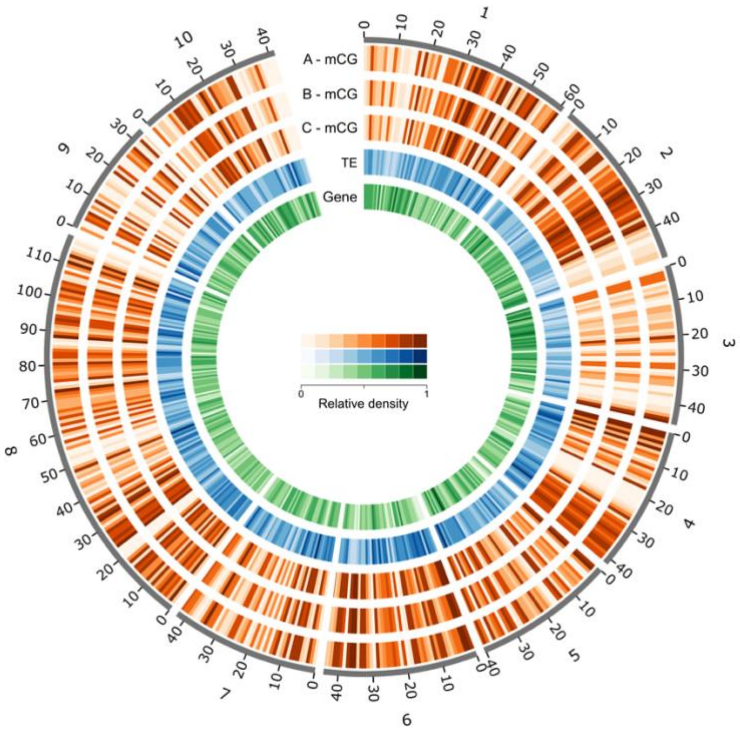



**Figure 4.1** Circos plot showing DNA methylation levels (mCG) in Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*) specimens A, B, and C, along with transposable element (TE) density and gene density, across the first ten scaffolds of the CoTS OKI-Apl_1.0 reference genome (NW_019091355.1 to NW_019091364.1).

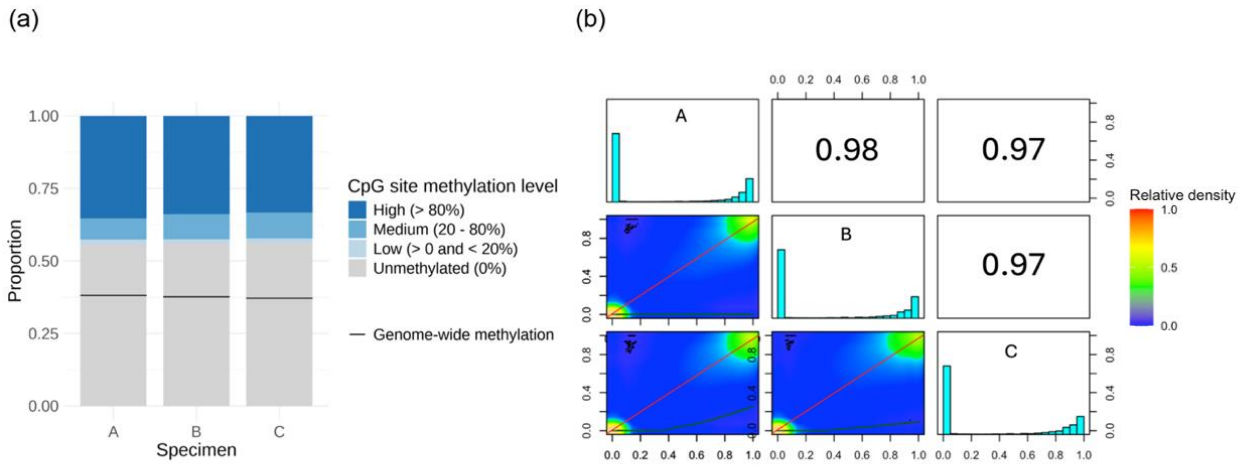



**Figure 4.2** DNA methylation landscape of the Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*) (a) Stacked bar plot showing the proportional distribution of CpG dinucleotide methylation levels and genome-wide methylation across three specimens. (b) Pairwise comparison of methylation levels among specimens. Diagonal panels are histograms indicating distribution of methylation levels for each specimen. Upper right panels display pairwise Pearson's correlation coefficients. Lower left panels are density plots of methylation levels between pairs of specimens, with warmer colours representing higher levels of similarity.

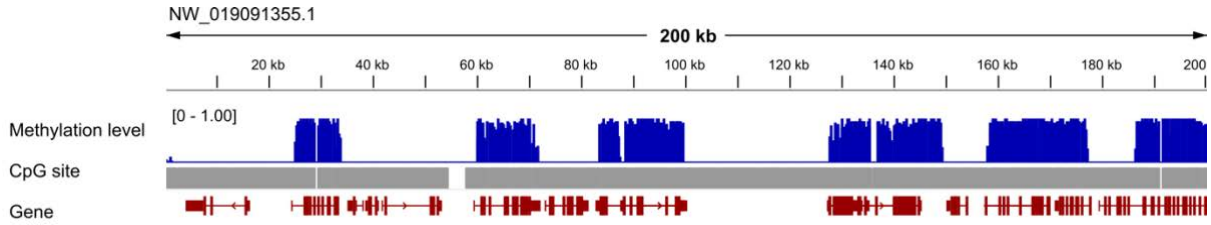



**Figure 4.3** Genome browser snapshot illustrating mosaic DNA methylation patterns in the Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*) genome, using specimen A, scaffold NW_019091355.1 region 0 - 200 kb as an example.

The genome-wide methylation level in CoTS, compared to a diverse array of taxa, was visualized using a phylogenetic tree (Figure 4.4). The methylation level in CoTS was within the range of approximately 30% to 40%, aligning with other deuterostome invertebrates (vase tunicate, purple sea urchin, acorn worm; Figure 4.4). This level was higher than that observed in protostome invertebrates (Pacific oyster, tubeworm, western honeybee), which exhibited a broader range of methylation levels, from 1% in arthropods to 21% in annelids. In comparison to prebilaterians, CoTS showed higher methylation levels than cnidarians (14% in the starlet sea anemone and 20% in *Acropora* coral), but demosponges stood out as a notable exception to all other groups with a distinctively high methylation level (81%). Vertebrates demonstrated the highest methylation levels across the taxa, reaching nearly 80%. This phylogenetic analysis positioned CoTS and deuterostome invertebrates as intermediates in the evolutionary spectrum of DNA methylation.

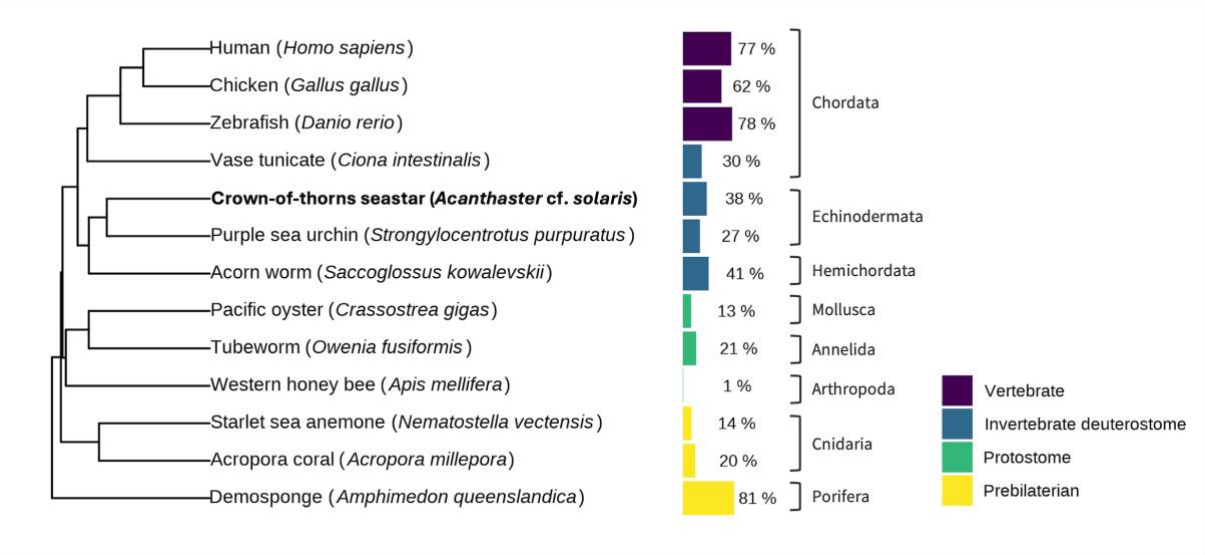



**Figure 4.4** Phylogenetic tree comparing average genome-wide DNA methylation across a diverse array of taxa, with methylation levels for each species indicated by bar plots. References: Chordata - *Homo sapiens* (Schultz et al., 2015), *Gallus gallus* (Zhang et al., 2017), *Danio rerio* (de Mendoza et al., 2021), *Ciona intestinalis* (Klughammer et al., 2023); Echinodermata - *Strongylocentrotus purpuratus* (Skvortsova et al., 2022); Hemichordata - *Saccoglossus kowalevskii* (Klughammer et al., 2023*)*; Mollusca - *Crassostrea gigas* (Klughammer et al., 2023); Annelida - *Owenia fusiformis* (Guynes et al., 2024); Arthropoda - *Apis mellifera* (Xu et al., 2019); Cnidaria - *Nematostella vectensis* and *Acropora millepora* (Ying et al., 2022); Porifera - *Amphimedon queenslandica* (de Mendoza et al., 2021)

### *4.3.3 Primary targets of methylation*

We assessed the methylation status across various genomic regions to identify key methylation targets within the CoTS genome. Our analysis revealed that most methylated sites were located within gene bodies: 65% in intronic regions and 26% in exonic regions (Figure 4.5a). Only a small fraction of methylated dinucleotides was found within intergenic (8%) and promoter regions (1%). Genic regions exhibited relatively high methylation levels, with introns showing the highest level (48.4% to 49.8%), followed by exons (44.1% to 45.2%) (Figure 4.5b). These levels were four to ten fold higher than those observed in non-

genic regions, where intergenic regions exhibited methylation levels of 11.5% to 12%, and promoter regions had the lowest level at 4.9% to 5.1% (Figure 4.5b).

Profiling of genes and their flanking regions further revealed that methylation levels were higher within gene bodies compared to region 2kbp up and downstream (Figure 4.5c). Methylation levels sharply increased following the TSS, remained elevated throughout the gene body, and then declined towards the TES. Additionally, we found that repetitive elements consistently exhibited higher methylation compared to the non-repetitive background methylation in both intronic and intergenic regions (Figure 4.5d). In intronic regions, background methylation level averaged 46.8%, whereas repetitive elements showed higher levels at 59.5%. In intergenic regions, the overall methylation level was lower, with background methylation averaging 10% and repetitive elements showing a slight elevated level of 11.8% (Figure 4.5d).

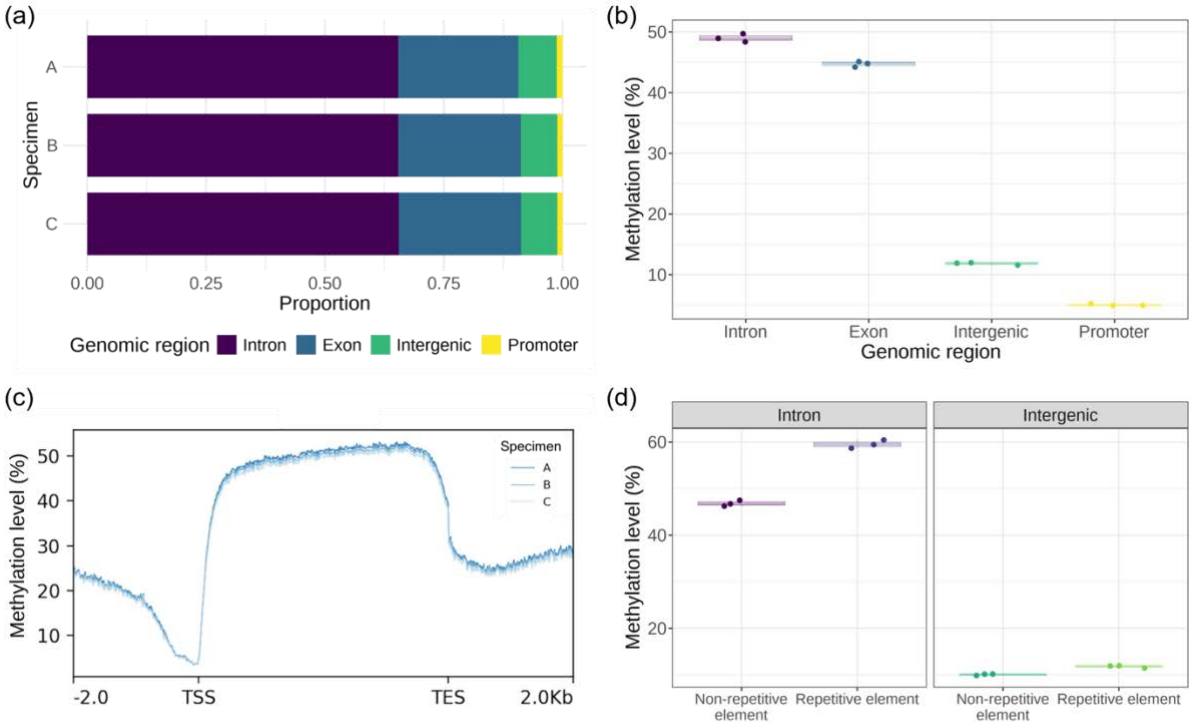



**Figure 4.5** DNA methylation targets in the CoTS genome (a) Stacked bar plot illustrating the distribution of methylated CpG dinucleotides in each specimen. (b) Boxplot comparing methylation levels across genomic regions. (c) Average methylation profiles across gene bodies and 2 kb upstream and downstream flanking regions. Gene body lengths were normalised and aligned at the transcription start site (TSS) and transcription end site (TES). Methylation levels were averaged across all genes. (d) Boxplot comparing methylation levels in non-repetitive and repetitive elements, within intron and intergenic regions.

## 4.4 Discussion

The present study provides the first comprehensive methylome profile of CoTS, a coral-eating deuterostome invertebrate that plays a significant role in the decline of Indo-Pacific coral reefs. Using ONT, we generated high-quality long-read sequences that capture over 90% of CpG dinucleotides within the CoTS genome. Our analysis revealed a mosaic

methylation landscape characterized by a moderate genome-wide methylation level of 37.7%, with gene bodies and repetitive elements being the primary targets of methylation.

Here, we demonstrate the utility of ONT as a robust method for comprehensive genomic and methylation analysis in non-model species. While our aim was to sequence the CoTS methylome, we also generated long-read genomic data averaging over 2 kbp. This was made possible by optimizing the extraction and size selection protocol to overcome the challenges of obtaining high molecular weight DNA from marine invertebrates (Panova et al., 2016). These long reads will facilitate future efforts to improve genome assembly and analysis of complex regions such as repeats, structural variants, and GC rich regions that are challenging to resolve using short read sequences. By covering over 90% of all CpG dinucleotides in the CoTS reference genome (Hall et al., 2017), we provide a genome-wide perspective on the methylation profile, surpassing traditional methods such as Reduced Representation Bisulfite Sequencing (RRBS) and microarray, which capture less than 10% of all CpG sites and are biased towards GC-rich regions (Sun et al., 2015). While WGBS also offers genome-wide coverage, ONT presents distinct advantages, such as avoiding destructive bisulfite treatment, simultaneous genetic sequencing, and distinguishing between DNA modification types. There has been limited exploration of ONT for methylation studies in non-model invertebrates, with very few examples such as deep-sea polychaetes (Perez et al., 2023) and sea anemones (Dimond et al., 2021). Our research not only contributes to the growing body of work validating ONT for ecological methylation studies, but also marks significant progress in efficiently generating reliable methylation data, facilitated by the use of the latest tools with improved accuracy. By applying the protocols established here, we anticipate that future studies will continue to profile high-quality methylomes across various taxa, advancing our understanding of methylation patterns and their functions in natural populations.

The CoTS methylome landscape observed in this study provides empirical evidence supporting the intermediate position of deuterostome invertebrates, situated between the hypomethylated genomes of most protostome invertebrates and the hypermethylated genomes of vertebrates. This evidence aligns with previous observations using CpG density as a proxy for methylation (Okamura et al., 2010) and enhances our understanding of the evolutionary dynamics of DNA methylation. While vertebrate-like hypermethylation has been demonstrated in a species of demosponge (de Mendoza et al., 2019), this landscape has been lost in most prebilaterians and protostome invertebrates, which display mosaic methylation primarily in gene bodies and repetitive elements interspersed among hypomethylated regions (Feng et al., 2010). Our data, combined with the limited deuterostome methylation data available (Feng et al., 2010; Klughammer et al., 2023; Zemach et al., 2010), suggest an evolutionary reacquisition of methylation complexity in deuterostome invertebrates. This intermediate state may represent a unique balance between the flexibility of mosaic methylation and the regulatory robustness of hypermethylation. Overall, our observations are consistent with the hypothesis that methylation patterns have evolved modularly, with diverse strategies employed by different lineages to adapt their methylation landscapes to specific evolutionary pressures (Suzuki & Bird, 2008).

Analysis of CoTS methylation profiles performed here revealed a moderate and mosaic pattern, potentially indicating a role in facilitating biological plasticity. CoTS is known for its ability to survive under certain unfavourable conditions such as food scarcity. For instance, juvenile CoTS can delay diet transition and survive on suboptimal nutrient sources while awaiting improved conditions (Deaker et al., 2020). Similarly, adult CoTS can survive for extended periods without food (Chesher, 1969). The conservation of relatively low methylation levels across invertebrate genomes suggests a strategy prioritizing gene expression flexibility over rigid regulatory mechanisms. This configuration is linked to

phenotypic plasticity and environmental adaptability, as it allows more room for inducible methylation during stress (Ardura et al., 2017; Trigg et al., 2022). For example, rapid global DNA methylation changes in response to environmental stressors have been observed in the ascidian *Didemnum vexillum*, aiding its success as an invasive species (Hawes et al., 2018b). In CoTS, previous research demonstrated immediate changes in gene expression in response to environmental stressors (Morin et al., 2023), suggesting a potentially flexible and dynamic epigenome that may contribute to its success as a coral reef pest. The comprehensive methylation profiles generated in this study provide a foundational dataset for investigating how environmental factors influence epigenetic processes, which will deepen our understanding of the ability of CoTS to adapt to different environments and establish outbreak populations.

In our exploration of DNA methylation targets within the CoTS genome, we identified a prominent pattern of gene body methylation. This observation is consistent with findings from previous studies on both vertebrate and invertebrate species, supporting the hypothesis that gene body methylation is a conserved regulatory mechanism likely predating the divergence of plants and animals (Feng et al., 2010; Zemach et al., 2010). However, while the plants and animals studied in Feng et al. (2010) predominantly exhibit exonic methylation, our results reveal enhanced intronic methylation in CoTS. Intronic methylation is less understood compared to exonic methylation, but it is being increasingly recognized for its role in fine-tuning gene expression (Blattler et al., 2014). Although intronic regions are non-coding segments that are typically spliced out during mRNA processing, their methylation status can affect the binding of regulatory proteins and the accessibility of splicing machinery, thereby modulating mRNA isoform production (Back & Walther, 2021; Espinas et al., 2020; Nam et al., 2020). Notably, enhanced intronic methylation has also been observed in other marine invertebrates, such as corals and anemones (Liew et al., 2018; Ying

et al., 2022). This observation warrants further investigation to determine if intronic methylation represents an evolutionary adaptation essential for the development and survival of organisms in dynamic marine environments.

The CoTS genome exhibits elevated methylation within repetitive elements, aligning with a broader evolutionary strategy observed across diverse lineages. While earlier studies suggested that transposons in invertebrate genomes were generally not specifically targeted by DNA methylation (Regev et al., 1998), more recent findings have challenged this view. For example, Ying et al. (2022) demonstrated that in cnidarians, DNA methylation predominantly targets transposons, particularly the younger and potentially more active elements. Similarly, Wang et al. (2014) documented that in the Pacific oyster *Crassostrea gigas*, DNA methylation is directed towards young repetitive elements. Our finding of elevated methylation in repetitive elements in CoTS adds to the growing evidence that DNA methylation acts as a defence mechanism in invertebrates and is potentially more prevalent than previously thought. Methylation is crucial for suppressing the expression and transposition of transposable elements, thereby preserving genome stability and integrity (Bestor, 2007). Without this protective function, unchecked transposition could lead to harmful consequences, including gene disruption, mutations, and chromosomal rearrangement (Fedoroff, 2012). The consistent targeting of transposons and repetitive elements across various lineages underscores a fundamental role of DNA methylation in genomic defence, reflecting a common evolutionary solution to the shared challenges posed by transposable elements.

While significant progress has been made in characterising the CoTS epigenome in the current study, much remains to be explored. Future research should focus on how these methylation patterns influence gene expression dynamics under varying environmental stressors and contribute to the species' resilience. For instance, research on the highly

invasive tunicate *Ciona robusta* has demonstrated that DNA methylation aids in retaining stress adaptations and enhancing performance under fluctuating conditions (Fu et al., 2021). Conducting similar research on CoTS may lead to the development of more effective intervention strategies by better understanding the species' epigenetic stress response mechanism. Additionally, future work should incorporate genetically diverse individuals to better capture population-level variation in methylation and avoid potential biases from closely related samples, as used in the present study. This would strengthen the generalisability of observed patterns and help clarify how epigenetic variation is shaped by both genetic background and environmental factors. Furthermore, developing epigenetic biomarkers for monitoring CoTS populations could be highly beneficial. For example, epigenetic clocks, which estimate the age of organisms based on DNA methylation levels at age-related CpG sites, have shown promise in fisheries management by providing a high-resolution and efficient way to assess age structure (Piferrer & Anastasiadi, 2023). This is particularly relevant for CoTS management, as the size-age relationship in CoTS is highly plastic (MacNeil et al., 2017), and no validated method currently exists to accurately age wild specimens. This limitation hinders our understanding of population age structure and outbreak origins. Overall, further investigation into the epigenetics of CoTS could provide a deeper understanding of the biological adaptability driving population outbreaks and lead to the development of effective tools for population monitoring and management.

## 4.5 Conclusion

Our study demonstrates the robustness of ONT for comprehensive genomic and methylation analysis in non-model species, using CoTS as a case study. By optimizing DNA extraction and size selection protocols, we achieved long reads averaging over 2 kbp, establishing a valuable framework for future long-read sequencing of species that face challenges with high molecular weight DNA extractions. Our data cover over 90% of CpG

dinucleotides in the CoTS reference genome, providing the first genome-wide perspective of the CoTS methylome. The methylation landscape of CoTS provides empirical evidence for an intermediate position of deuterostome invertebrates in the evolutionary spectrum of DNA methylation, balancing gene expression plasticity with regulatory robustness. The CoTS genome is moderately methylated, primarily targeting gene bodies and repetitive elements, suggesting conserved mechanisms in genome regulation and defence. Additionally, we observed unique features such as intronic methylation, which may have important implications for the biological adaptability of CoTS. Overall, our comprehensive methylation profiles not only establish a foundational understanding of the CoTS epigenetic landscape but also offer a critical dataset and related protocols for future functional epigenome and comparative analyses, contributing to our understanding of DNA methylation in natural populations.

**Data availability statement**

The raw sequencing data, along with associated sample and sequencing metadata, have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1251702. Methylome data for all samples are available on Figshare (http://doi.org/10.6084/m9.figshare.27068959). The workflow guide and bioinformatics scripts are accessible on GitHub (https://github.com/sarahkwong/cots_methylome/).

# Chapter 5: A DNA methylation-based epigenetic clock for the Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*)

Intended submission: Kwong, S. L. T., Budd, A., Gomez-Cabrera, M., Hung, J., Pratchett, M., Villacorta-Rath, C., Uthicke, S. (2025). A DNA methylation-based epigenetic clock for the Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*). *PLoS genetics*

## Abstract

Accurate and reliable ageing tools are essential for effective wildlife research, conservation, and management. In recent years, DNA methylation emerged as a promising tool for age estimation in vertebrates, though its utility for invertebrates is uncertain. Here, we present a DNA methylation-based epigenetic clock for the Pacific crown-of-thorns seastar (CoTS; *Acanthaster* cf. *solaris*), a coral predator exacerbating the decline of Indo-Pacific reefs. Using Oxford Nanopore Technologies (ONT), we generated whole-genome methylation profiles for CoTS of known ages, ranging from 1 to 3.5 years. Our analysis revealed that methylation levels (the proportion of 5-methylcytosine reads) at 1,910 CpG sites were significantly associated with age, representing 0.02% of all CpG sites in the CoTS genome. Notably, fewer than 5% of age-related sites were found within CpG islands, and none were found within rDNA regions. This highlights the value of genome-wide methylation profiling in uncovering dispersed age-related sites in invertebrates, which are often overlooked by traditional RRBS and rDNA approaches. Using elastic net regression and leave-one-out cross-validation (LOOCV), we constructed age prediction models with a mean absolute error of 0.31 years (3.7 months), corresponding to approximately 4 - 6% of the lifespan of CoTS (5 - 8 years). This level of accuracy allows for clear differentiation of yearly cohorts, offering significant potential for management applications and enabling testing of outbreak initiation hypotheses. A final epigenetic clock was developed using 14 CpG sites, selected based on their consistent

association with age across model iterations, providing an important framework for practical implementation. Our study demonstrates the potential of DNA methylation as an ageing tool for organisms beyond vertebrates, offering a promising avenue to enhance the management of invertebrate species and address ecological challenges.

## 5.1 Introduction

Age information is fundamental to ecological studies and species management. Understanding the age structure of a population can reveal critical information about population health, stability, and recovery capacity, enabling assessments of its ecological status and predictions of future trends (Alexander, 1958). Furthermore, modelling key population parameters, including growth, fecundity, and mortality, requires knowledge of individual age (Caughley, 1977). Age data are therefore essential for accurate population modelling, which is integral to the effective management of endangered, fisheries, or pest species (Begg et al., 2005; Beissinger & Westphal, 1998). Given the importance of age information, accurate and reliable ageing tools are highly desirable in wildlife research and management.

Age determination in invertebrates remains a significant challenge. While some vertebrates can be aged through repeated sightings of tagged or recognizable individuals, such as birds with leg bands (Lincoln, 1921) or whales with distinct fluke patterns (Katona & Whitehead, 1981), applying this approach to invertebrates is generally more challenging. For example, soft-bodied echinoderms tend to shed tags readily (Nadalini et al., 2024; Reichenbach, 1999), and crustaceans can lose exoskeleton markings after moulting (Linnane & Mercer, 1998). Additionally, calcified structures with growth bands, such as otoliths (Pannella, 1971), teeth (Laws, 1952), and vertebrae (Simpfendorfer, 1993) are commonly used to age vertebrates but are often absent or unreliable in invertebrates due to damage, obscuration, or inconsistency (Moltschaniwskyj & Cappo, 2009; Narvaez et al., 2016). Although growth bands have been used to model growth of some invertebrate species, such as cephalopods and crustaceans, their correlation with age remains uncertain (Arkhipkin et al., 2018; Kilada & Driscoll, 2017). In echinoderms, asexual reproduction, such as transverse fission in sea cucumbers (Crozier, 1917; Uthicke & Conand, 2024) or "comet" formation in

seastars (regrowth of an individual from a single arm; Haeckel, 1878), makes size-based age estimation unreliable. Consequently, unconventional ageing methods like bomb radiocarbon dating (Ebert & Southon, 2003) and genetic fingerprinting combined with recapture of individuals (Uthicke et al., 2004) have been used. Additionally, the plasticity in growth and variability in life-history strategies across invertebrates renders traditional growth models inapplicable (Hirst & Forster, 2013). As a result, there is a lack of broadly applicable and reliable ageing methods for invertebrates, hindering effective management and conservation efforts.

In recent years, epigenetic clocks have emerged as a powerful method for age determination. This approach is typically based on DNA methylation, a biochemical process where methyl groups are attached to DNA, most frequently at cytosine-phosphate-guanine (CpG) sites (Razin & Cedar, 1991). As organisms age, methylation levels at specific CpG sites change predictably, enabling the construction of models, referred to as epigenetic clocks, to estimate chronological age accurately (Horvath, 2013). Initially developed for humans (Horvath, 2013), epigenetic clocks have since been applied in ecological studies to age various vertebrate species, including whales (Polanowski et al., 2014), fish (Anastasiadi & Piferrer, 2020), and sea turtles (Mayne et al., 2022). These clocks have demonstrated high accuracy; for instance, the methylation levels of 23 CpG sites can predict the age of beluga whales (*Delphinapterus leucas*) with a mean absolute error (MAE) of 2.9 years, equivalent to 5% of the species' lifespan (Bors et al., 2021). Furthermore, epigenetic clocks are non-invasive and enable high-throughput age estimation, making them likely to become an invaluable tool for wildlife management. However, their application in invertebrates remains limited, leaving it uncertain whether this approach can be effectively extended to a broad range of invertebrate species. To date, the European lobster (*Homarus gammarus*) and the

jewel wasp (*Nasonia vitripennis*) are the only invertebrates with developed epigenetic clocks (Brink et al., 2024; Fairfield et al., 2021).

Previous approaches to building epigenetic clocks often rely on identifying orthologous age-related genes or conserved CpG sites from closely related species (Mayne et al., 2021; Polanowski et al., 2014), using Reduced Representation Bisulfite Sequencing (RRBS) to target CpG-rich regions (Mayne et al., 2022), or focusing on rDNA regions, which show conservation across certain taxa (Fairfield et al., 2021). However, these methods may be less effective for invertebrates due to limited genomic resources in non-model species, or the lack of well-studied related species. Additionally, invertebrates often exhibit sparse methylation patterns (Suzuki & Bird, 2008), and targeted approaches like RRBS risk missing biologically relevant sites dispersed throughout the genome (Paun et al., 2019). As such, a whole-genome approach offers a more reliable solution, as it captures all CpG sites and increases the likelihood of detecting dispersed age-related patterns. Among whole-genome approaches, Oxford Nanopore Technology (ONT) is particularly promising because it directly detects methylated cytosines and efficiently generates high-quality long-read methylome data, beneficial for non-model species lacking reference genomes (Chapter 4). ONT has so far only been used to develop an epigenetic clock for cattle (Hayes et al., 2021), demonstrating its capability for on-site and real-time sequencing. Expanding its application to invertebrates could pave the way for a more accessible and standardized approach to epigenetic clock development across diverse taxa.

This study focuses on the crown-of-thorns seastar (CoTS; *Acanthaster* spp.), a formidable and effective coral predator, for which periodic population irruptions have caused sustained coral loss on reefs throughout the Indo-Pacific (De'ath et al., 2012). Specifically, we examined *A.* cf. *solaris*, the Pacific clade within the *Acanthaster* species complex, as all specimens were sourced from the Great Barrier Reef (GBR). Despite extensive research, the

initiation of CoTS population irruptions remains unclear, partly owing to challenges in effectively resolving population age structures. Age data are critical for identifying the timing and frequency of recruitment pulses, allowing researchers to trace back to the year of recruitment and gain crucial insights into the environmental conditions that caused or facilitated irruptions. For example, recruitment events may coincide with periods of excessive nutrient runoff (Birkeland, 1982) or favourable oceanographic conditions (Miller et al., 2015). Without reliable age data, it is nearly impossible to pinpoint and mitigate outbreak drivers effectively. However, determining the age of individual CoTS is challenging due to their growth plasticity (Lucas, 1984), which obscures the size-age relationship, making cohort differentiation based on size structure contentious. Furthermore, juvenile CoTS can delay their transition to a coral-based diet, remaining small until more coral becomes available (Deaker et al., 2020), further complicating size-based age estimations. Although spine pigment bands have been proposed as age proxies, their reliability is limited by factors such as predation or regeneration history (Souter, 1997), and the inability to count growth bands in juveniles or small individuals. Developing a more robust ageing tool is essential for refining our understanding of CoTS population dynamics, improving population modelling, and enhancing outbreak predictions.

The aim of this study is to assess whether CpG methylation can be used to estimate the age of CoTS, effectively proving an epigenetic clock. To achieve this, we reared CoTS in captivity, providing access to specimens of known age, which is a rare and invaluable resource. The process required significant time, effort, and resources, making each specimen uniquely valuable. Furthermore, known-age CoTS are unattainable from wild populations, and no equivalent dataset exists in the literature to date, further emphasizing its rarity and importance. Whole-genome methylation profiles were generated across five age groups to identify age-related CpG sites, which were used to construct age predictive models. We then

evaluated the performance of these models in estimating the chronological age of CoTS and showed that this approach holds significant promise for advancing age determination in invertebrates.

## 5.2 Materials and methods

### *5.2.1 Specimen collection, DNA extraction, and Nanopore sequencing workflow*

The procedures for specimen collection, high molecular weight DNA extraction, Nanopore sequencing, and modified basecalling are detailed in Chapter 4. In brief, twelve adult CoTS (6 females and 6 males) were collected from mid-shelf reefs in the central GBR and spawned at the Australian Institute of Marine Science (AIMS) National Sea Simulator (SeaSim) in December 2020 and 2021. The offspring were settled and raised in aquaria, with an ontogenetic dietary shift from crustose coralline algae to coral beginning at four months post-settlement. Samples were collected at 1, 2, 2.5, 3, and 3.5 years of age, each in triplicate. High molecular weight DNA was extracted from snap-frozen pyloric caeca (digestive gland) tissue using a modified phenol-chloroform extraction protocol, followed by size selection with SPRIselect beads (Beckman Coulter). Sequencing libraries were prepared using the Native Barcoding Kit 24 V14 (SQK-NBD114-24) and sequenced on the MinION Mk1C platform implemented with MinKNOW v22.12.5, using the R10.4.1 flow cells (FLO-MIN114). Basecalling was conducted using the GPU version of Guppy v6.5.7, using the super accuracy (SUP) model dna_r10.4.1_e8.2_260bps_modbases_5mc_cg_sup.

### *5.2.2 Age-related CpG sites*

All analyses were performed using R version 4.3.2 (R Core Team, 2022). The methylation dataset generated from basecalling was filtered to retain CpG sites that were common across all specimens and had a minimum 5-fold coverage. The methylation level of each CpG dinucleotide was defined as the proportion of methylated cytosines to the total number of reads at that site. To identify age-related CpG sites, the DSS package (Feng et al.,

2014) was used to fit linear models to each site, with age as the explanatory variable. The significance of the relationship between methylation level and age was assessed using the Wald test. CpG sites with a false discovery rate (FDR) < 0.05 were classified as age-related and used in downstream model development for the epigenetic clock.

### *5.2.3 Genomic distribution of age-related CpG sites*

Understanding the genomic distribution of age-related CpG sites is essential for uncovering their functional relevance and identifying potential epigenetic regulatory mechanisms. To achieve this, age-related CpG sites were mapped to genomic regions based on the National Center for Biotechnology Information (NCBI) RefSeq annotation of the CoTS reference genome (NCBI *Acanthaster planci* Annotation Release 100 - August 7, 2017), following the same approach described in Chapter 4. Gene bodies were categorized into intronic and exonic regions, while promoters were defined as 2 kilobase pairs (kbp) upstream of the transcription start site (TSS). Non-promoter regions outside gene bodies were classified as intergenic. The GenomicRanges package (Lawrence et al., 2013) was used to assign CpG sites to these regions. The average number of methylated CpG sites per region was calculated across all specimens, serving as a background reference to evaluate the distribution of age-related CpG sites. This comparison identified regions where age-related CpG sites were over- or under-represented, revealing genomic regions potentially involved in age-associated processes.

### *5.2.4 Gene set enrichment analysis*

To assess the functional relevance of age-related CpG sites, those located within gene bodies (as defined in Section 5.2.3) were analysed to identify the genes they were associated with, and the corresponding gene IDs were extracted. These gene IDs were then used for gene set enrichment analysis using the g:GOSt tool on the g:Profiler online analysis platform (Kolberg et al., 2023). The statistical significance of enriched terms was evaluated using

g:Profiler's default g:SCS algorithm for multiple testing correction, which accounts for dependency between terms by considering their overlap.

### *5.2.5 Epigenetic clock construction*

The epigenetic clock was developed using an elastic net penalized regression approach, implemented with the glmnet package v 4.1-8 (Friedman et al., 2010). To ensure reliable estimates of predictive accuracy on unseen data, an outer leave-one-out cross-validation (LOOCV) approach was used to model age using DNA methylation. With this approach, each data point was sequentially held out as the test set, while the remaining data were used to train the model. This process generated 15 distinct models, each evaluated on its corresponding hold-out test point. Additionally, 5-fold cross-validation was performed, following a similar process but partitioning the dataset into five parts and isolating a single part for each of five models instead of isolating individual data points. This approach evaluated the models on larger test sets, enabling comparison of model performance on train and test sets and complementing the single-point testing of LOOCV.

For both approaches, internal 10-fold cross-validation was performed to optimize the lambda parameter, which determines the strength of the penalty applied during regularization. The lambda value within one standard error of the minimum (lambda.1se) was used to balance between model complexity and performance. The alpha parameter, which controls the mixing ratio between ridge regression and LASSO regression, was set to 0.5 to balance both methods. The trained models were then used to predict the ages of the corresponding test sets. To assess the predictive performance of the models, we evaluated the relationship between the predicted and known age using Pearson's correlation. Additionally, we computed the absolute error for each prediction to assess accuracy across ages and the mean absolute error (MAE) to quantify overall prediction accuracy.

Elastic net regression performs feature selection such that during the model-building process, some CpG site coefficients were penalized to 0 and dropped from the model, retaining only the most informative sites. Common CpG sites across LOOCV iterations were identified to assess model stability and highlight those sites that contain key age-related signals. The final model (i.e., the epigenetic clock) was constructed using the common sites shared by all LOOCV models. As per the cross-validation steps, the lambda parameter was tuned using 10-fold inner cross-validation, and the alpha parameter was set at 0.5.

### *5.2.6 Evaluating targeted genomic approaches for age-related CpG sites detection*

Previous studies using targeted approaches have demonstrated that epigenetic clocks can be constructed by focusing on specific genomic regions. Two commonly used methods are rDNA, which targets rDNA regions; and RRBS, which targets CpG islands (short genomic regions rich in CG dinucleotides, often associated with gene promoters). To assess the feasibility of these targeted approaches for CoTS and related species, we examined whether the age-related sites identified in our study were located within rDNA regions or CpG islands, using the GenomicRanges package (Lawrence et al., 2013). Potential rDNA regions within the CoTS genome were identified through BLAST searches against rDNA sequences, including the 5S, 18S, and 28S regions of the purple sea urchin (*Strongylocentrotus purpuratus*) and the European lobster (*Homarus gammarus*), while CpG islands were identified using the EMBOSS cpgplot tool (Rice et al., 2000). Additionally, age estimation models were constructed using all CpG sites within the identified rDNA regions, following the same procedure described in Section 5.2.5.

## 5.3 Results

### *5.3.1 Nanopore sequencing performance*

A total of 14 Nanopore sequencing runs, each on a dedicated flow cell, were conducted to sequence 15 specimens spanning five age groups. In three of the runs, two

specimens were multiplexed on a single flow cell, while the remining runs were allocated to individual specimens to ensure sufficient sequencing output (Table B1, Appendix B). Flow cells were discarded after a single use. Collectively, the sequencing efforts generated 37,983,539 reads and 96.2 gigabase pairs (Gbp) of data, yielding an average coverage of 8x to 26x across specimens, given the size of the CoTS reference genome at 383.8 megabase pairs (Mbp). The read length N50 ranged from 2,419 to 5,241 base pairs (bp), and the mean read length ranged from 1,555 to 3,861 bp. The maximum read length was 525,161 bp. Between 6,364,935 to 9,368,699 CpG sites were covered at depth greater than 5x across specimens, representing 66 to 97% of the 9,705,479 CpG dinucleotides in the CoTS reference genome.

### *5.3.2 Age-related CpG sites*

To assess age-related patterns in CoTS, we conducted linear regression to examine the relationship between methylation levels and known ages at each CpG site. Although some sites may exhibit non-linear patterns, most age-related sites are expected to follow linear trends, and the elastic net model used in this study focuses on identifying linear relationships. After quality filtering, 3,479,059 CpG sites were included in the analysis. Of these, 1,910 sites exhibited significant associations with age (Wald test, FDR < 0.05), representing 0.02% of all CpG sites in the CoTS genome. Among the significant sites, 127 exhibited positive associations with age, with a mean effect size of 19.4% per year (Figure 5.1). In contrast, 1,783 showed negative associations, with a mean effect size of -14.9% per year. These 1,910 CpG sites are hereafter referred to as “age-related CpG sites”.

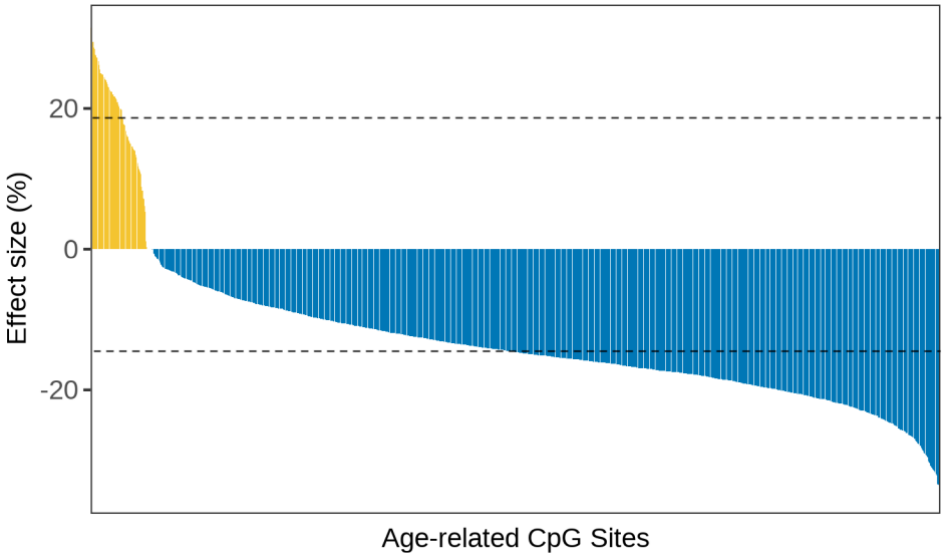



**Figure 5.1** Bar plot illustrating the effect size (% change in methylation per year of age) for 1,910 age-related CpG sites in the Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*). Yellow bars represent positive changes, and blue bars represent negative changes. Horizonal bars represent mean effect size.

### *5.3.3 Genomic distribution of age-related CpG sites*

Genomic distribution analysis revealed that the majority of age-related CpG sites were located within gene bodies, with 67.3% in introns and 20.3% in exons. Fewer sites were found in intergenic (10.4%) and promoter regions (2%). While the distribution of age-related CpG sites generally aligned with the distribution of background methylated sites, it was 5.6% lower in exons and 0.9 – 2.8% higher in other regions (Figure 5.2).

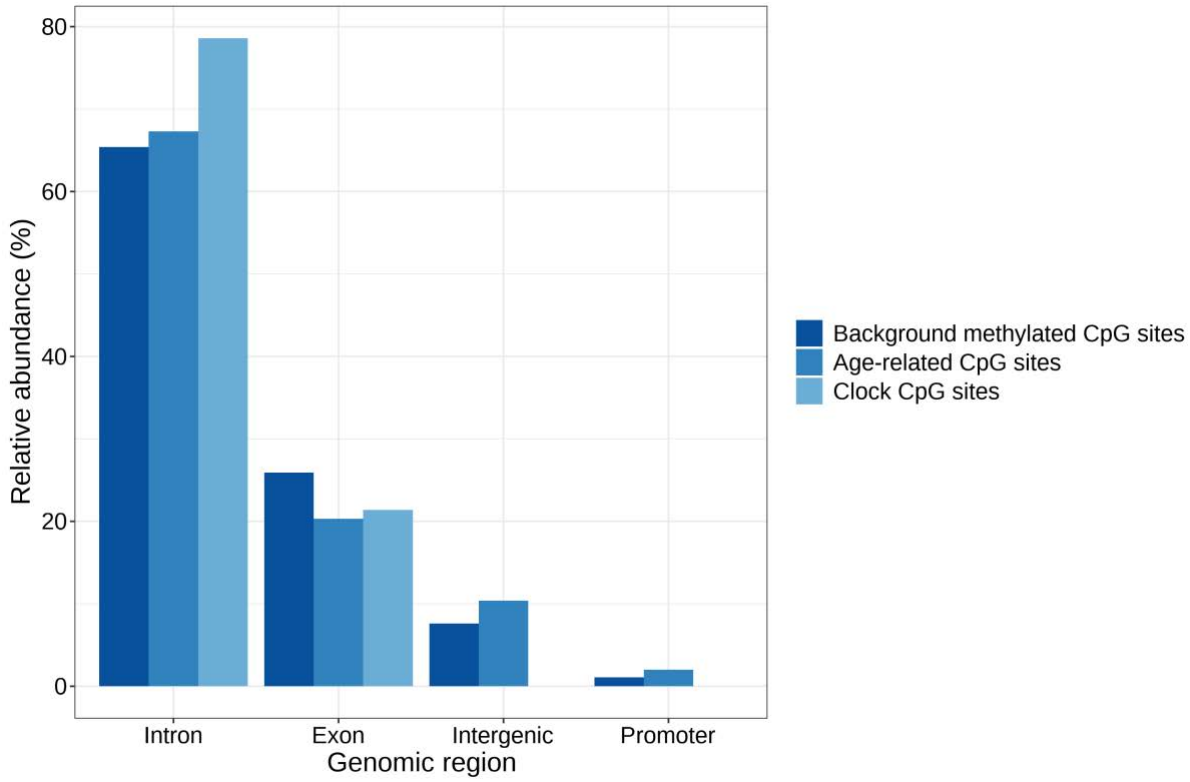



**Figure 5.2** Distribution of age-related and clock CpG sites across genomic regions in the Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*).

### *5.3.4 Gene set enrichment analysis*

To investigate epigenetic mechanisms associated with ageing, age-related CpG sites were mapped to genes. The 127 CpGs with positive age associations were mapped to 28 genes. However, functional enrichment analysis did not yield any statistically significant results, likely due to the small number of genes. The 1783 negatively age-associated CpGs were mapped to 474 genes. The three genes with the highest number of mapped sites were LOC110990087 (uncharacterized) with 50 sites, LOC110988520 (uncharacterized) with 49 sites, and LOC110973333 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1-like) with 29 sites. Basic Local Alignment Search Tool (BLAST) analysis against Deuterostomia (taxid: 33511) suggested that LOC110990087

shares potential homology with NLRC5, an immune-related protein involves in regulating proinflammatory pathways and supporting immune responses. LOC110988520 is classified as a long non-coding RNA (lncRNA) and therefore yielded no informative protein-level hits. LOC110973333 is likely associated with a SWI/SNF chromatin-remodelling protein, which regulates gene expression by modifying DNA accessibility.

Function enrichment analysis of age-related CpG sites revealed significant enrichment across 22 molecular function (MF), 14 biological process (BP), and 3 cellular component (CC) terms (Figure B1, Appendix B). Among these, 5 MF, 6 MP, and 1 CC terms were identified as driver terms, which are key representative categories selected to reduce redundancy and highlight the most biologically meaningful enriched terms (Figure 5.3). The top MF driver terms included binding (GO:0005488), GTPase regulator activity (GO:0030695), and kinase activity (GO:0016301). For BP, the top driver terms were phosphorylation (GO:0016310), intracellular signal transduction (GO:0035556), and protein kinase C-activating G protein-coupled receptor signalling pathway (GO:0007205). The single CC driver term was dendrite (GO:0030425). These findings suggested that age-related CpG sites are linked to enzymatic regulation, molecular signalling, and structural components involved in cellular connectivity and morphology.

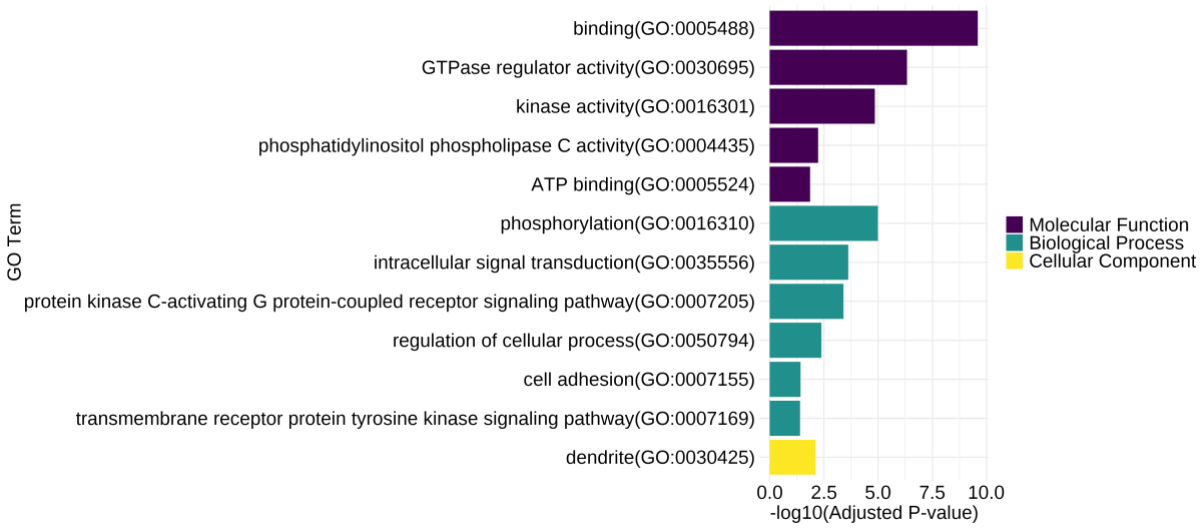



**Figure 5.3** Driver gene ontology (GO) terms ranked by significance (-log10 adjusted p-value) from functional enrichment analysis of 501 genes associated with 1,910 age-related CpG sites in the Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*).

### *5.3.5 Epigenetic clock*

Using a LOOCV approach, 15 elastic net models were constructed and evaluated. The number of CpG sites included in the models ranged from 40 to 54, with 16 sites shared across all iterations. The LOOCV models demonstrated high predictive performance, with an $R^2$ of 0.86 between predicted and observed ages (Figure 5.4a) and an MAE of 0.31 ± 0.22 years (Figure 5.4b). Assessment of MAE across the age groups indicated that age prediction was less accurate at the extremes of the age range, with a tendency to overestimate younger ages and underestimate older ages. The 5-fold cross-validation approach resulted in comparable predictive performance to LOOCV (Figure B2, Appendix B).

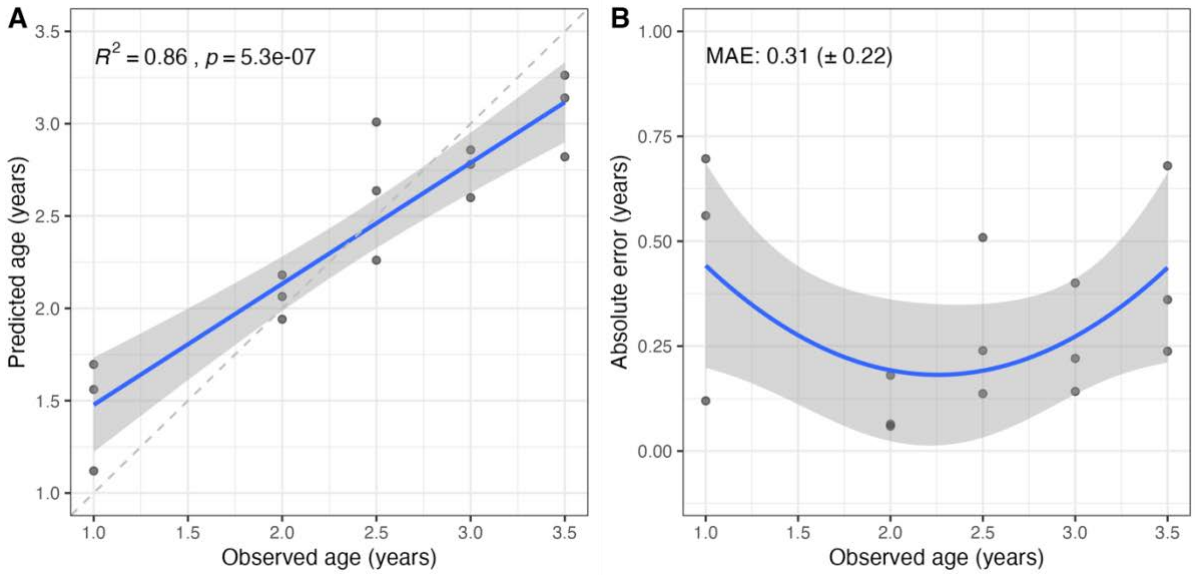



**Figure 5.4** Performance of elastic net models using nested leave-one-out cross-validation (LOOCV) to predict the chronological age of n = 15 Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*). (a) Regression plot of predicted age against chronological age. (b) Absolute error of predictions across chronological ages

The final epigenetic clock was constructed using the 16 CpG sites common to all LOOCV models, which likely capture key biological signals related to ageing. During the elastic net model fitting process, less informative sites were penalised and subsequently omitted, resulting in 14 CpG sites being retained in the final model, all of which exhibited age-related hypomethylation (Table 5.1). The relationship between predicted age ($\hat{Y}$) and methylation levels ($x$) at these sites is defined by the linear equation $\hat{Y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_p x_p$ where $\beta_0$ is the intercept, and $\beta$ values are the model coefficients for each CpG sites. Notably, all model coefficients are negative, reflecting the consistent decline in methylation with age. These 14 CpG sites are hereafter referred to as "clock CpG sites".

The 14 CpG sites identified in the final mode were found exclusively within gene bodies, with 78.6% in introns (13.2% above the expected background), indicating an overrepresentation in intronic regions. In contrast, 21.4% were in in exons, 4.5% below the

expected background (Figure 5.2). Additionally, the clock sites were mapped to 15 genes, with one site mapped to two genes due to overlapping gene regions (Table 5.1). Gene set enrichment analysis did not reveal any significant results for the clock CpG sites.

### *5.3.6 Evaluating targeted genomic approaches for age-related CpG sites detection*

To assess the feasibility of building epigenetic clocks for CoTS and related species using rDNA and RRBS approaches, we examined whether age-related and clock CpG sites were located within the targeted rDNA regions or CpG islands. None of the age-related CpG sites were found within rDNA regions, while 4.3% (83 sites) were located within CpG islands. None of the 14 clock sites were located within either rDNA regions or CpG islands. The age prediction models constructed using CpG sites within rDNA regions resulted in a $R^2$ of 0.088 (Figure B3a, Appendix B) and an MAE of 0.59 ± 41 years (Figure B3b, Appendix B), reflecting limited predictive power and substantial variability in error, indicating that the rDNA models are unsuitable for age estimation. This illustrates the importance of screening the entire methylome for age-related CpG sites.

**Table 5.1** Details of the Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*) epigenetic clock model, listing CpG sites with their genomic positions, model coefficients, Pearson's correlation values, and associated gene information. The model intercept ($\beta_0$) is 4.5356384. Gene descriptions marked with an asterisk (*) are based on BLAST analysis against Deuterostomia (taxid: 33511).

| Scaffold | Position | Model coefficient | Pearson's correlation | Associated gene ID | Gene description |
|---|---|---|---|---|---|
| NW_019091396.1 | 146450 | -0.0019125 | -0.91 | LOC110978098 | sodium-dependent phosphate transporter 1-like, transcript variant X2 |
| NW_019091403.1 | 82170 | -0.0021052 | -0.92 | LOC110978830 | protein quaking-A-like, transcript variant X6 |
| NW_019091406.1 | 902728 | -0.0052218 | -0.90 | LOC110979391 | putative sodium-coupled neutral amino acid transporter 11, transcript variant X4 |
| NW_019091416.1 | 1359840 | -0.0016686 | -0.94 | LOC110980267 | trinucleotide repeat-containing gene 6C protein-like, transcript variant X3 |
| NW_019091418.1 | 1514787 | -0.0050775 | -0.95 | LOC110980516 | neuronal PAS domain-containing protein 1-like, transcript variant X1 |
| NW_019091432.1 | 1221032 | -0.0035928 | -0.90 | LOC110981791 | *glycoprotein endo-alpha-1,2-mannosidase-like |
| | | | | LOC110981794 | uncharacterized LOC110981794, transcript variant X4 |
| NW_019091439.1 | 1007463 | -0.0059026 | -0.93 | LOC110982326 | clustered mitochondria protein homolog |
| NW_019091443.1 | 569800 | -0.0033427 | -0.92 | LOC110982539 | ubiquitin carboxyl-terminal hydrolase 34-like |
| NW_019091457.1 | 403167 | -0.0029115 | -0.93 | LOC110983384 | disintegrin and metalloproteinase domain-containing protein 12-like, transcript variant X3 |

| | | | | | |
|---|---|---|---|---|---|
| NW_019091477.1 | 211551 | -0.0023903 | -0.92 | LOC110984471 | PDZ and LIM domain protein 1-like, transcript variant X1 |
| NW_019091535.1 | 56884 | -0.0004317 | -0.92 | LOC110987042 | *GAS2-like protein |
| NW_019091655.1 | 169350 | -0.0002948 | -0.92 | LOC110989598 | N(G),N(G)-dimethylarginine dimethylaminohydrolase 1-like, transcript variant X3 |
| NW_019091851.1 | 16502 | -0.0102344 | -0.91 | LOC110990785 | uncharacterized LOC110990785, transcript variant X7 |
| NW_019091938.1 | 24528 | -0.0040874 | -0.94 | LOC110990872 | uncharacterized LOC110990872 |

## 5.4 Discussion

This study demonstrated the feasibility of using CpG methylation to predict chronological age in Pacific CoTS, *Acanthaster* cf. *solaris*. Our models achieved an MAE of 0.31 ± 0.22 years, corresponding to approximately 4 – 6% of the CoTS lifespan (estimated at 5 to 8+ years, Pratchett et al., 2014) and within 9% of the tested age range. This level of accuracy enables the differentiation of yearly cohorts, providing effective distinction of population age structures necessary to inform population dynamics and corresponding management strategies. By identifying 14 key CpG sites for constructing the CoTS epigenetic clock, we established a workflow for sequencing, age-related sites selection, and epigenetic clock construction, providing a scalable and transferable framework for invertebrate research and management.

Our study contributes to the expanding field of molecular ageing as the second epigenetic clock developed for any marine invertebrate, utilizing a more comprehensive whole-genome approach compared to the targeted rDNA method used for the European lobster (Fairfield et al., 2021). Despite challenges inherent to marine systems, such as complex life histories and phenotypic plasticity, our findings suggest that DNA methylation holds potential as a molecular ageing marker. However, this remains a proof-of-concept constrained by a limited age range and small sample size, and further research is needed to evaluate its utility across broader biological and environmental contexts. Previous studies have shown that many age-related CpG sites are conserved across taxa, enabling the development of epigenetic clocks for closely related species without the need to repeat expensive whole methylome sequencing steps (Mayne et al., 2021; Polanowski et al., 2014). As such, our whole-methylome data provide a basis for exploring conserved age-associated CpG sites in related taxa, including commercially and ecologically important species such as sea cucumbers, sea urchins, and other asteroid species, potentially extending the use of

epigenetic clocks beyond model organisms and across marine invertebrates. This extension could advance invertebrate management, which currently relies on length- or weight-based models, an approach prone to inaccuracies. In contrast, age-based models offer more precise insights into key biological parameters (Punt et al., 2020). Reliable epigenetic ageing models can refine stock assessments and inform harvest strategies in fisheries, while also enhancing the management of invasive and other pest species through improved population age structure data (Piferrer & Anastasiadi, 2023). With such potential, continued exploration of epigenetic clocks is both necessary and promising.

A whole-genome approach, though less common than targeted methods, was used in this study and proved essential to address the dispersed methylation landscape in CoTS. In vertebrates, methylation patterns often concentrate around CpG islands or regulatory regions, making targeted methods such as RRBS effective. However, methylation landscapes in invertebrates are often sparser and more dispersed across the genome (Suzuki & Bird, 2008). Brink et al. (2024), who developed one of the two existing invertebrate epigenetic clocks, serve as an example of employing a whole-genome approach for such patterns. On the other hand, while rDNA regions are conserved across certain taxa such as humans and mice, and have been targeted to build epigenetic clocks (Fairfield et al., 2021; Wang & Lemos, 2019), it remains uncertain whether this conserved pattern can be applied more broadly. Our findings reveal that most age-related CpG sites in CoTS fall outside rDNA regions and CpG islands, requiring a genome-wide approach to capture them effectively. Furthermore, our attempt to construct age prediction models using rDNA regions failed to identify sufficient age-predictive sites, suggesting that rDNA may have limited utility across taxa. Together, our findings highlight the value of whole-genome approaches, which may be essential for the development of epigenetic clocks in other invertebrates or taxa with similarly dispersed methylation landscapes.

Our work holds considerable promise for enhancing CoTS management. The age estimation models achieve an MAE of less than six months, providing a level of accuracy well-suited to management needs. Given that CoTS on the GBR settle in annual cohorts due to their spawning season from December to March (Uthicke et al., 2019), the ability to differentiate annual cohorts is sufficient for effectively discerning population age structures, addressing knowledge gaps related to initiation processes and population dynamics. With further refinement (discussed below), this approach could enable high-throughput, quick-turnaround, and even on-site age determination, which could be incorporated into ongoing culling efforts to facilitate efficient sample collection for age analysis. On the GBR, management strategies rely heavily on manual culling, with approximately 50,000 CoTS culled per year (Great Barrier Reef Marine Park Authority, 2024), presenting ample opportunity to collect tissue samples in batches for age analysis. This approach would provide substantial data for population monitoring, offering managers a scalable and practical tool to track age dynamics and tailor outbreak interventions effectively. For instance, targeting primary outbreak reefs, where multiple age cohorts accumulate, could help disrupt outbreak cycles and prevent further spread across the reef system.

We found that the majority of age-related CpG sites in CoTS exhibited reduced methylation with age, with their overall distribution aligning closely with the background methylation trends across the genome, primarily concentrated within gene bodies. This pattern is consistent with observations in humans and other taxa, such as the beluga whale (*Delphinapterus leucas*), where age-related sites predominantly exhibit hypomethylation over time (Bors et al., 2021; Johansson et al., 2013). A possible explanation is that hypomethylation may upregulate genes critical for cellular maintenance and repair in ageing organisms, potentially mitigating age-related declines in cellular function (Marttila et al., 2015). Our gene set enrichment analysis lends support to this idea, identifying enriched

pathways related to signal transduction and cellular communication, which are processes fundamental to sustaining cellular function. Furthermore, genes with high densities of age-related CpG sites suggest potential roles in immune regulation and epigenetic remodelling during ageing. Notably, the concentration of age-related CpG sites at LOC110973333, a gene involved in chromatin remodelling, aligns with the growing focus in epigenetic clock research on PRC2-associated genes (chromatin regulators) as key sites for age-related methylation changes, emphasizing the potential significance of chromatin remodelling in epigenetic ageing (Moqri et al., 2024). Alternatively, the dispersed age-associated hypomethylation pattern may result from a passive decline in methylation fidelity during cell divisions, leading to a less targeted, genome-wide shift in methylation over time (Keith et al., 2022). Together, these findings suggest that age-associated hypomethylation may represent a common epigenetic signature of ageing across vertebrates and invertebrates, contributing to the understanding of the ageing process.

While our study establishes a foundational epigenetic clock for CoTS, further refinement is required for practical applications. Our model captured consistent age-associated methylation trends, though some CpG sites may be influenced by intraspecific variation. Importantly, the clock was trained on individuals aged up to 3.5 years, so its accuracy beyond this range remains untested. Broader population testing will be important to assess the clock's robustness and generalisability. For example, testing on wild-caught CoTS is necessary to evaluate the model's performance in natural settings. Although obtaining known-age wild specimens remains unfeasible due to the inability to track individuals from settlement and the lack of external age markers, size-based testing offers an alternative. For instance, in optimal environments, individuals under 200 mm in diameter are highly likely less than 2 years old, while those over 350 mm are older than 5 years (Pratchett et al., 2014). Model predictions aligning with these expectations would support its applicability to natural

populations. However, it should be noted that in CoTS with limited access to coral prey, the relationship between size and age can be substantially obscured (Lucas, 1984). To overcome this, sequence data from CoTS reared under different aquarium conditions (e.g., diet, temperature, or pH) could be used to retrain the model to ensure that age-associated sites are not influenced by environmental conditions. A further step toward practical utility is eliminating the need of genome-wide sequencing to age new samples. To achieve this, the most strongly associated age-related sites could be targeted for the development of a more cost-effective assay, ensuring efficient and scalable age determination. This may involve targeted methods such as primers for multiplex bisulfite amplicon sequencing PCR or Nanopore adaptive sequencing (Loose et al., 2016). Once validated, such targeted assays will enable high-throughput age determination, facilitating large-scale analyses across broader sample pools and enhancing the practical utility of the epigenetic clock.

## 5.5 Conclusions

This study presents an epigenetic clock for CoTS, highlighting the potential of DNA methylation as a reliable marker for age determination in marine invertebrates. Using whole-genome sequencing, we identified age-related sites and demonstrated their utility in distinguishing yearly cohorts, offering a promising tool for CoTS management. Our study sets a precedent for developing epigenetic clocks in marine invertebrates, with the identified age-related CpG sites serving as a valuable reference for related taxa. By demonstrating the feasibility of epigenetic ageing methods, we anticipate that our findings will inspire further investigation across diverse invertebrate species, paving the way for the broader application of this approach in ecological research and management.

# Chapter 6: General discussion

Accurately determining the age of animals is essential for understanding their population dynamics and life history traits (Alexander, 1958; Caughley, 1977). However, traditional ageing techniques are often unsuitable for invertebrates due to the absence of consistent and reliable external growth markers (Arkhipkin et al., 2018; Kilada & Driscoll, 2017; Moltschaniwskyj & Cappo, 2009). While molecular ageing tools have emerged as a promising approach, their application has predominantly focused on vertebrates, leaving uncertainty about their broader applicability to invertebrates (**Chapter 2**). For crown-of-thorns seastars (CoTS; *Acanthaster* cf. *solaris*), age data are crucial for managing population outbreaks, yet there has been a lack of reliable methods for age determination. This thesis addresses these knowledge gaps by investigating molecular approaches to determine the chronological age of CoTS. Specifically, it examines telomere length (**Chapter 3**) and DNA methylation (**Chapter 4 & 5**), the two most widely applied molecular age markers in ecological research.

A key contribution of this thesis is the foundation it establishes for the development of a practical ageing tool for CoTS. Specifically, it demonstrates the feasibility of DNA methylation as a robust method for age estimation in CoTS, offering a reliable approach to infer population age structures and inform management strategies. In exploring age markers, this thesis also reveals new insights into various aspects of CoTS biology, including telomere dynamics and their epigenetic landscape, further enriching our understanding of this ecologically significant pest species. Beyond CoTS, these findings contribute to advancing molecular age estimation, a field previously dominated by research on vertebrates.

## 6.1 Molecular ageing tool for CoTS

### *6.1.1 Telomere length*

Telomere length has been extensively studied as a molecular age marker in taxa such as mammals and birds, where it shows promise as an indicator of age in certain cases (Haussmann et al., 2003a; Pauli et al., 2011). Its relatively low cost and high-throughput sample processing via qPCR make it an attractive biomarker. However, in **Chapter 3**, I found that telomere length analysis does not provide the level of accuracy needed to differentiate annual cohorts in CoTS. This chapter provides the first evidence of telomere attrition in CoTS, demonstrating that adult CoTS generally exhibit shorter telomere lengths compared to juveniles. While this suggests some potential for using telomere length to distinguish broad age classes or life stages, its utility for resolving yearly cohorts is limited by significant variation within age classes and the lack of fine-scale age patterns. This high variability is likely attributed to telomere length being influenced by various age-independent factors. Through controlled experiments, I identified diet as one such factor, where individuals on suboptimal diets exhibited shorter telomere lengths, potentially due to oxidative stress. This illustrates the limitations of telomere length as a precise ageing tool for CoTS. However, the results suggest it could serve as a biomarker for factors such as health or stress status, which are worth investigating due to their potential relevance for management and the method's affordability (further discussed in section 6.4).

### *6.1.2 DNA methylation*

In **Chapter 4**, I established the first methylome profile for CoTS, providing the foundation for age prediction modelling. My analysis revealed a mosaic methylation pattern with moderate genome-wide methylation levels, positioning CoTS between the hypomethylated genomes of protostomes and the hypermethylated genomes of vertebrates. These findings address important knowledge gaps in deuterostome invertebrates, a group for which epigenetic data remain scarce. Building on this, in **Chapter 5**, by analysing age-associated methylation changes across cytosine-phosphate-guanine (CpG) sites in laboratory-

reared individuals of known age, I demonstrated that DNA methylation is a reliable indicator of age in CoTS. The age prediction models achieve a mean absolute error of 3.7 months, providing sufficient accuracy to differentiation annual cohorts, addressing a critical gap in CoTS population studies. This represents a significant advancement, offering a novel and powerful ageing approach that enables access to previously unattainable age data. While this is a foundational epigenetic clock based on a whole-methylome approach, which is currently time- and resource-intensive, further development of targeted assays will enable high-throughput and cost-effective age estimation for larger sample pools. Combined with future validation across broader population and age ranges, the groundwork established here can be used to develop a practical and widely applicable ageing tool for CoTS.

### *6.1.3 Comparison*

While both telomere length and DNA methylation were evaluated as molecular ageing markers for CoTS, and both showed an association with age, my findings indicate that DNA methylation is a more accurate and reliable predictor. Telomere length was assessed in juveniles from 4 to 16 months to capture early-life dynamics, as telomere attrition is often most pronounced during rapid juvenile growth (Gomes et al., 2010). However, despite being raised under controlled conditions, juveniles within the same age group exhibited substantial variation in telomere length, and there was significant overlap between juveniles and adults. In contrast, DNA methylation was studied from 1 to 3.5 years to capture cumulative ageing signals. DNA methylation-based age predictions demonstrated no overlap between age classes separated by one year, achieving a level of resolution relevant to management that telomere length could not provide.

While telomere length was explicitly tested for age-independent factors, namely diet and tissue type, these tests were not extended to DNA methylation due to resource constraints, primarily the high cost of whole-methylome sequencing at this stage of development.

However, studies in vertebrates suggest that epigenetic clocks remain stable for age prediction across environmental conditions (Bertucci et al., 2021), supporting the expectation of similar robustness in CoTS, pending further validation. Nevertheless, my findings demonstrate when tested on captive-raised specimens under controlled conditions, DNA methylation provided superior accuracy and resolution compared to telomere length. This advantage likely stems from the DNA methylation model integrating multiple age-related loci across the genome, reducing the influence of individual outliers. This multi-locus approach enhances statistical stability by ensuring that age predictions rely on collective methylation patterns rather than a single variable susceptible to extrinsic factors. Biologically, DNA methylation changes are tightly regulated by DNA methyltransferases (DNMTs) to maintain organismal function (Moore et al., 2013), reflecting molecular processes related to ageing, such as gene regulation and cellular maintenance. In contrast, telomere attrition is driven by cell division and oxidative stress, with the latter being influenced by external factors, making telomere length more variable and environmentally sensitive. Overall, the findings of this thesis indicate the superiority of DNA methylation over telomere length as an ageing marker for CoTS, warranting further refinement and development to enhance its scalability as a practical ageing tool.

## 6.2 Implications for molecular ageing research

This thesis represents a major advancement in molecular ageing research, establishing the third known epigenetic clock for an invertebrate species. It pioneers the extension of these tools beyond vertebrates, addressing the longstanding challenge of ageing invertebrates. By demonstrating the feasibility of DNA methylation-based age estimation in CoTS, this research paves the way for broader applications of this tool in other invertebrate species. The whole-genome methylation data generated here identifies age-associated CpG sites, offering a basis for exploring conserved markers in related species and facilitating the development of their

epigenetic clocks. Additionally, the workflow established, which leverages ONT for methylome profiling and epigenetic clock construction, offers an adaptable framework for other non-model invertebrates.

Together, this thesis facilitates the shift away from traditional size- and weight-based ageing methods, which are often unreliable in invertebrates due to plastic growth rates, environmental influences, and variable life histories. Molecular approaches offer a scalable and reliable alternative, enabling age estimation when conventional methods are insufficient. The ability to age invertebrates has far-reaching implications, with the potential to transform the management of ecologically and commercially important species. Access to previously unattainable age data can enhance population assessments and optimize stock management, providing valuable insights to inform conservation and fisheries strategies.

The findings from this thesis align with the general trend in the field of molecular ageing research. While telomere length was one of the first molecular age markers to be studied, the field is now shifting towards DNA methylation and other biomarkers as the preferred approach for age estimation (Jarman et al., 2015). My findings reinforce that while age-related telomere attrition can be observed in some species, it remains an unreliable ageing tool due to environmental sensitivity and species-specific dynamics (Dunshea et al., 2011). In contrast, DNA methylation offers more accurate age estimation, often achieving a mean absolute error (MAE) within 5 – 10% of an organism's lifespan (Anastasiadi & Piferrer, 2020; Bors et al., 2021). For CoTS, the MAE falls within this range at 4 – 6%. As such, although the DNA methylation-based ageing approach requires more advanced sequencing technologies, bioinformatics expertise, and higher costs compared to telomere analysis, its superior accuracy and reliability make it a worthwhile investment. The initial effort required to develop an epigenetic clock will ultimately enable scalable, high-throughput applications, significantly benefiting ecological research and management. For researchers aiming to build

an age estimation tool for their study species, prioritising DNA methylation over telomere length is strongly recommended, as the latter has repeatedly shown high variability and limited predictive power. Given the rapid advancements in sequencing technology and analytical methods, DNA methylation will likely emerge as the dominant molecular ageing tool, offering transformative potential for age estimation across a wide range of taxa.

The transition from telomere length to DNA methylation has been made possible by developments in genetic methodologies that enable the identification and analysis of intricate patterns like CpG methylation. As research tools continue to evolve, the construction of epigenetic clocks is anticipated to become increasingly accessible and cost-effective. **Chapter 4** of this thesis contributes to this progress by demonstrating the efficacy of ONT for methylome profiling. The ability of ONT to directly detect methylation patterns eliminates the need for bisulfite conversion, thereby reducing DNA degradation, avoiding biases from incomplete conversion, and enhancing data reliability. Moreover, the use of ONT is particularly suited for non-model organisms, as it can generate high-quality genomic and epigenomic data simultaneously, overcoming the limitations of insufficient pre-existing genomic resources. As ONT continues to improve in read accuracy and throughput, it is likely to become a key tool in epigenetics research, facilitating the application of molecular ageing approaches to a broader array of species. This thesis serves as a foundational example demonstrating the potential of ONT in this area.

### 6.3 Implications for CoTS outbreak management

Using molecular ageing tools to establish the age structure of CoTS populations will provide unprecedented insights into population dynamics, especially the initiation of population irruptions. Critically, it is currently challenging to determine whether CoTS outbreaks have developed from the continuous accumulation of cohorts over time or from mass settlement of larvae originating at upstream reefs, except by assessing size structure

(Pratchett et al., 2014). This distinction is crucial for management because primary outbreaks, where recruitment events regularly establish new cohorts, are often the sources of larvae that leads to secondary outbreaks (Endean, 1974). With further development, molecular ageing tools could help clarify these dynamics by providing information on the timing and frequency of recruitment pulses. Combined with other lines of evidence, such as coral cover, these tools could help detect delayed growth or diet transition and support earlier identification of potential primary outbreak reefs earlier in the outbreak cycle. Although the complex and flexible nature of the CoTS larval stage limits the ability to precisely time settlement events, molecular ageing tools remain valuable for detecting broader recruitment trends and shifts in population structure. These insights can indirectly guide more effective and timely interventions.

Molecular ageing tools also have the potential to improve the accuracy of outbreak prediction models, which were previously based on assumptions about population dynamics and lacked precise demographic data for validation. For example, fecundity has been estimated based on size, with the fecundity-at-age relationship remains poorly understood. This knowledge gap is critical, as reproductive output directly influences population growth and outbreak potential. By determining the age of individuals, molecular ageing tools enable direct measurement of key demographic rates, rather than relying on inferred estimates. Additionally, knowing the age structure of a population refines data on recruitment timing, population turnover, and population trajectories. Incorporating improved demographic data into population models enables managers to more accurately predict the timing and location of future outbreaks, allowing for more proactive and targeted interventions. Without reliable age data, fundamental population parameters remain rough approximations. Molecular ageing tools therefore represent a major step forward in refining outbreak prediction models and enhancing targeted control strategies.

Once validated across broader age ranges, molecular ageing tools could enable monitoring of the age structure of CoTS populations over time, providing feedback on whether culling efforts are breaking recruitment cycles or merely suppressing adult populations temporarily. A current knowledge gap in CoTS management is determining whether newly detected individuals after culling are new recruits or previously hidden individuals emerging from reef structures or deeper waters. With a reliable ageing tool, managers can assess whether younger CoTS consistently dominate a population despite ongoing culling. If this is the case, it may indicate that recruitment pressure remains high and that recruitment events are outpacing culling efforts. This would highlight the need to reassess strategies and address potential upstream larval sources sustaining the outbreak. In contrast, a shift toward an older population age structure, with fewer young individuals appearing over time, would suggest that recruitment events are becoming less frequent or less intense, indicating that culling efforts are having a lasting impact on population dynamics. By providing timely insights into population trends, molecular ageing tools can help managers optimize resource use, reducing reliance on short-term solutions and promoting a more sustainable, long-term approach to CoTS control.

### 6.4 Future directions

Although telomere length did not appear suitable as a reliable age marker, findings from **Chapter 3** suggest that it may serve as an indicator of physiological state. For instance, individuals subjected to suboptimal diets exhibited shorter telomere lengths, supporting the idea that telomeres could reflect stress and body condition (Haussmann & Marchetto, 2010). Further research on telomere dynamics as a physiological marker may help address key knowledge gaps related to CoTS outbreak cycle. For instance, comparing the conditions of outbreak and non-outbreak populations could help clarify the dynamics during periods of population stability, contributing to a more comprehensive understanding of the entire

outbreak cycle. Furthermore, the physiological state of CoTS at the end of an outbreak remains poorly understood, despite being a critical phase where stress factors like starvation and over-crowding are believed to drive population collapse. Investigating telomere length as a molecular marker of stress could provide valuable insights into the processes underlying CoTS population transitions from outbreak to collapse. To achieve these goals, future studies should focus on carefully controlled experiments simulating stressors such as food deprivation or increased population density, to disentangle the relationships between telomere dynamics, physiological state, and environmental conditions.

While the epigenetic clock constructed in this thesis represents a significant step forward, further validation and refinement are required to enhance its practical utility. A key limitation of the current model is its reliance on captive-reared CoTS, which may experience elevated and chronic stress levels compared to their wild counterparts. These factors could potentially influence physiological ageing processes and in turn affect DNA methylation patterns. This raises the possibility that ageing signatures observed in captivity may not fully reflect those in wild populations. Validating the model in natural populations is therefore a critical next step. Although determining the exact age of wild CoTS is not currently feasible, hence the need for molecular ageing tools, approximate age classes can be inferred from size under known environmental conditions. If model predictions for wild-caught individuals align broadly with these expectations, it would support the model's applicability beyond captive settings. In that case, the next step is to develop a more practical assay. This would involve ensuring efficiency, cost-effectiveness, and scalability, potentially through a PCR-based approach, as outlined in **Chapter 5**.

However, if significant deviations in age prediction accuracy are found, this may indicate the need for a more diverse training dataset and thus the necessity of overcoming challenges associated with obtaining wild samples of known age. One potential solution

is fluorescent tagging, a technique where a fluorescent marker is incorporated into calcified structures and is visible under UV light (Stump & Lucas, 1990). While this method does not allow for individual identification, it could still be useful for generating a limited set of known-age specimens to refine the epigenetic clock. For example, CoTS approximately 5 – 10 cm in diameter, which can be reasonably assumed to be around one year old, could be captured from the wild, or known-age individuals reared at aquaria could be used. These would be tagged and released back into the wild. Periodic recapture and sampling over time would generate a set of known-age samples. However, this approach would require a long-term study, considerable effort, and approval from regulatory bodies. Additionally, practical considerations, such as conducting tagging in isolated reefs or areas with natural barriers to minimize migration, would be necessary to enhance the likelihood of recapture.

Another constraint of the current model is that the applicability is limited to 3.5 years, as epigenetic clocks cannot be extrapolated. Extending the age range of CoTS specimens used for model training would require overcoming logistical challenges, such as raising cohorts of CoTS in captivity for longer durations, which is resource intensive, including the requirement for large amounts of live coral feed. While addressing these challenges requires significant effort, they are essential for improving the generalizability of the epigenetic clock for application in natural populations.

The effect of diet on DNA methylation is an intriguing subject for future exploration. While this thesis demonstrates that diet influences telomere length, its impact on DNA methylation is yet to be tested. To enhance model robustness, future studies could train epigenetic clocks using CoTS reared under different dietary conditions, with one treatment group transitioning to a coral diet while the other remains a crustose coralline algae (CCA) diet. This approach would ensure that the model accounts for dietary variability, producing a highly robust ageing tool that could be applied to test the aquarium-based “hidden army”

hypothesis. This hypothesis proposes that CoTS can delay their diet transition from herbivorous juveniles to coral-eating adults, remaining on the reef as a concealed population while waiting for coral cover to increase (Deaker et al., 2020). This hypothesis has significant implications for CoTS management but is currently untestable due to the lack of a suitable ageing tool. Further investigation into the effects of diet on DNA methylation would thus be critical, potentially enabling the determination of whether the small size of wild individuals reflects young age or represents an older, concealed population.

Similarly, while the effect of tissue type has been tested for telomere length, it has not yet been examined for DNA methylation. This limitation arises from the small size of some individuals (diameter ~5 cm), with pyloric caeca being the only viable tissue available to provide sufficient material for DNA extraction. Additionally, the significant cost of whole-methylome sequencing further restricts the feasibility of testing multiple tissues. Consequently, the epigenetic clock was constructed using a single tissue. Future studies could compare DNA methylation patterns across different tissues within the same individual of known age (provided the individual is large enough to yield sufficient material for DNA extraction from multiple tissues) to assess whether the age-related sites exhibit consistent methylation levels across tissue types. This could enhance our understanding of tissue-specific effects and improve the utility of the epigenetic clock. For instance, if age-related DNA methylation signals are found to be synchronized across tissue types, as observed in **Chapter 3** for telomere length, tube feet would be particularly advantageous for ageing adult CoTS, as they are the easiest tissue to obtain.

In addition to telomere length and DNA methylation, several emerging biomarkers present exciting opportunities for future research. For instance, by analysing transcriptional profiles of known-age individuals and identifying genes with age-dependent expression, messenger RNA (mRNA) can be established as an age marker (de Magalhães et al., 2009). In

non-model species, transcriptional profiles of closely related taxa can provide a reference, as many age-informative genes are conserved (Cook et al., 2006). Although mRNA may not provide the same level of accuracy as DNA methylation, it remains a valuable biomarker to explore as an affordable method, particularly when transcriptional data are readily available. Additionally, this exploration can enhance our understanding of age-related gene expression and the underlying ageing processes. Similarly, microRNA (miRNA) has been shown to correlate with age in model species (Liu et al., 2012; Noren Hooten et al., 2010), although it has yet to be developed as an age marker for ecological studies. The exploration of miRNA is attractive due to its conservation across species, stability in extracellular fluids, and the manageable number of genomic miRNAs, making it a potentially feasible and transferable age marker (Bonin et al., 2019). These features, combined with miRNA's resilience to degradation and potential to reveal broader gene regulatory mechanisms, make it a compelling candidate for further investigation.

## 6.5 Conclusions

This thesis presents the first in-depth investigation of telomere length and DNA methylation as age markers for CoTS, leading to the development of an epigenetic clock for this ecologically significant invertebrate species. Beyond advancing our understanding of CoTS biology, including telomere dynamics and epigenetic landscape, this work lays the foundation for integrating molecular age estimation into outbreak management, enabling more effective and targeted control measures. Importantly, this thesis contributes to a shift in the methods for age estimation for invertebrates, demonstrating that molecular ageing tools are a feasible alternative to traditional methods in a field long constrained by a lack of suitable techniques. My findings strengthen the growing consensus that DNA methylation outperforms telomere length in predictive accuracy, highlighting its greater potential as a molecular ageing biomarker for widespread application in future work. Moving forward, as

molecular technologies continue to advance, the integration of epigenetic ageing tools into ecological research and species management will become increasingly feasible, expanding opportunities for understanding population dynamics. By demonstrating the feasibility of molecular ageing in a species and taxonomic group that is notoriously challenging to age, this thesis sets a precedent for future studies. Collectively, the work in this thesis advances molecular ageing research and will enable enhanced management strategies for invertebrates in the face of intensifying global climate pressures.

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## Appendix A: Supplementary information for Chapter 4

Extraction and size selection of high molecular weight genomic DNA from the Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*)

### Overview

This protocol was developed after unsuccessful attempts to obtain high molecular weight DNA using the Pacbio Nanobind Tissue Kit and Qiagen Genomic-tip 20/G. Gentle handling of DNA is crucial throughout this protocol - vortexing, vigorous agitation, tube shaking, rapid or excessive pipetting should be avoided. After extraction, high molecular weight DNA between 10 – 20 kbp should be obtained, along with some small DNA fragments (< 500 bp), which are to be removed during size selection steps.

### Equipment and consumables

Reagents

- MilliQ water
- 1M Tris-HCl (pH 8.0)
- 0.5M EDTA (pH 8.0)
- 5M NaCl
- 10% SDS
- Proteinase K (20 mg/mL)
- RNaseA (10 mg/mL)
- Phenol:Choloroform:Isoamyl alcohol (25:24:1)
- Chloroform:Isoamyl alcohol (24:1)
- Freshly prepared 70% and 85% ethanol
- 100% isopropanol
- Elution buffer
- SPRIselect beads (Beckman Coulter)

Consumables

- 1.5 mL and 2 mL microcentrifuge tubes

Equipment

- P1000 pipette and tips
- P200 pipette and tips

- P20 pipette and tips
- Incubator with rocking platform for heating at 65ºC
- Centrifuge
- Vortex mixer
- Magnetic rack
- Fume hood

**Procedure**

**Tissue lysis**

1. Prepare the lysis buffer by combining the following in a 2 mL tube:
    - 243 µL MilliQ water
    - 60 µL 1M Tris-HCl (pH 8.0)
    - 180 µL 0.5 M EDTA (pH 8.0)
    - 12 µL 5M NaCl
    - 60 µL 10% SDS
    - 45 µL Proteinase K (20 mg/mL)
    - 6 µL RNaseA (10 mg/mL)
2. Preheat lysis buffer at 65 ºC.
3. Add 20 mg of snap frozen pyloric caeca tissue to the preheated lysis buffer.
4. Incubate at 65ºC overnight on a rocking platform for gentle agitation. At the start of the incubation, invert the tube every 10 minutes until all visible pieces are dissolved, which should take approximately 60 minutes.

**DNA extraction**

Safety consideration - All steps involving phenol/chloroform extraction must be performed in a fume hood while wearing appropriate personal protective equipment.

5. Phenol:Choloroform:Isoamyl alcohol extraction
    - Add an equal volume (600 µL) of Phenol:Choloroform:Isoamyl alcohol (25:24:1) to the sample.
    - Mix by inverting the tube gently.
    - Centrifuge at 16,000 x g for 10 minutes.

- Recover the aqueous phase (top layer, 550 µL) into a new 2 mL tube.

6. Repeat Phenol:Choloroform:Isoamyl alcohol extraction
    - Add an equal volume (550 µL) of Phenol:Choloroform:Isoamyl alcohol (25:24:1) to the sample.
    - Mix by inverting the tube gently.
    - Centrifuge at 16,000 x g for 10 minutes.
    - Recover aqueous phase (top layer, 500 µL) into a new 2 mL tube.
7. Chloroform:Isoamyl alcohol extraction
    - Add an equal volume (500 µL) of Chloroform:Isoamyl alcohol (24:1) to the sample.
    - Mix by inverting the tube gently.
    - Centrifuge at 16,000 x g for 10 minutes.
    - Recover the aqueous phase (top layer, 450 µL) into a new 2 mL tube.
8. Add 1/10 volume (45 µL) of 3M sodium acetate (NaAc) to the tube and mix by inversion.
9. Add an equal volume (495 µL) of isopropanol to precipitate the DNA. Let the tube sit at room temperature for 3 hours to allow DNA precipitation.

**DNA purification**

10. Centrifuge at 20,000 x g for 30 minutes at 4ºC to pellet the DNA.
11. Carefully decant the supernatant. The DNA pellet, though barely visible, should appear as a small transparent spot attached to the wall of the tube.
12. Add 1 mL of 70% ethanol to the pellet and gently flick the tube to mix.
13. Centrifuge at 18,000 x g for 10 minutes at 4ºC and carefully decant the supernatant.
14. Repeat steps 12 and 13 for a second ethanol wash.
15. Let the pellet air dry until all ethanol has evaporated, which should take approximately 10 minutes.
16. Resuspend the pellet in 30 µL of elution buffer.

**Size selection**

17. Vortex the SPRIselect beads bottle thoroughly to ensure resuspension.
18. Add 10 µL of SPRIselect beads to 25 µL of the DNA sample to achieve a 0.4x ratio.
19. Gently flick the tube to mix the beads and sample, incubate at room temperature for 30 seconds.

20. Place the tube on a magnetic rack and allow the beads to settle at the magnet. Once the supernatant becomes clear, promptly remove and discard the supernatant.
    - Note: leaving the beads in contact with the sample for too long may result in the carryover of small fragments. Remove the supernatant as soon as it becomes clear.
21. With the tube still on the magnetic rack, add 180 µL of 85% ethanol to wash the beads. Incubate at room temperature for 30 seconds, then remove and discard the ethanol supernatant.
22. Remove the tube from the magnetic rack and add 20 µL of elution buffer to the beads. Flick the tube gently to resuspend the beads.
23. Incubate the tube at 40ºC for 10 minutes to elute the high molecular weight DNA.
24. Place the tube on a magnetic rack and allow the beads to settle at the magnet.
25. Transfer the clear eluate to a new tube for storage until ready for library preparation and sequencing.

**Disclaimer**

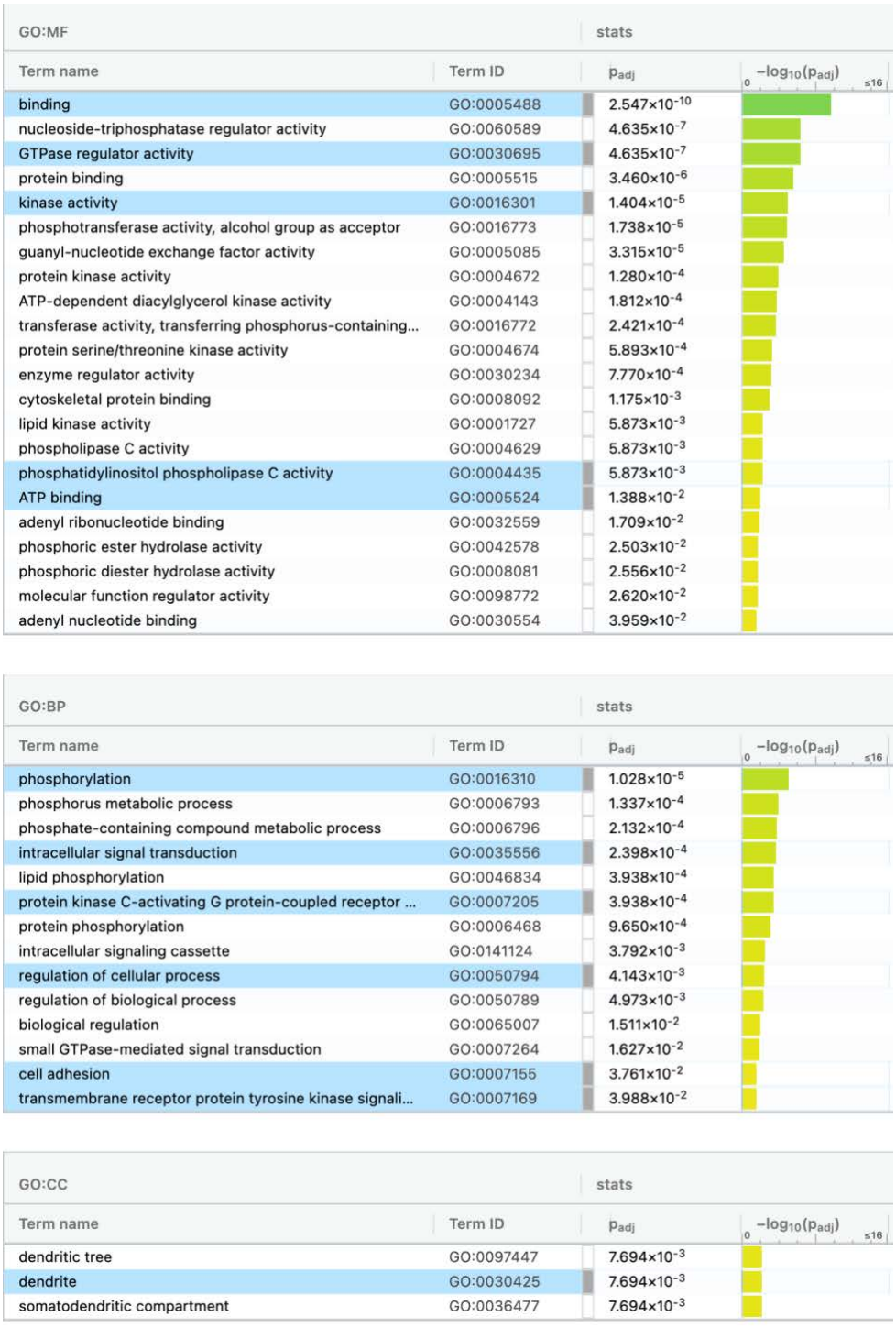
The content provided in this protocol is for informational purposes only and does not constitute legal, medical, clinical, or safety advice. This protocol is not a substitute for independent professional judgment or advice. Any actions you take or retrain from taking based on the information in this protocol are solely at your own risk. Neither the authors, contributors, administrators, nor anyone else associated with this protocol or related services can be held liable for your use or reliance on the information contained herein.

## Appendix B: Supplementary information for Chapter 5

**Table B1** Summary of 14 Nanopore sequencing runs for Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*) specimens, with each run conducted on a dedicated flow cell.

| Specimen | Age (year) | Run | Total reads | Total base pairs (bp) | Max read length (bp) | Mean read length (bp) | Read length N50 (bp) |
|---|---|---|---|---|---|---|---|
| A1 | 1 | 1 | 1,248,285 | 4,793,391,666 | 194,161 | 3,840 | 5,241 |
| A2 | 1 | 2, 5 | 2,741,933 | 5,132,018,865 | 183,499 | 1,872 | 3,508 |
| A3 | 1 | 3 | 3,919,646 | 8,833,836,513 | 267,319 | 2,254 | 3,576 |
| B1 | 2 | 9 | 4,246,594 | 9,854,114,797 | 159,049 | 2,320 | 3,091 |
| B2 | 2 | 10 | 2,969,361 | 5,956,556,065 | 230,970 | 2,006 | 3,040 |
| B3 | 2 | 11 | 2,418,105 | 6,513,051,432 | 365,628 | 2,694 | 3,553 |
| C1 | 2.5 | 1 | 1,225,492 | 3,190,908,086 | 166,888 | 2,604 | 4,143 |
| C2 | 2.5 | 2, 5 | 1,913,201 | 4,962,453,584 | 300,271 | 2,594 | 4,237 |
| C3 | 2.5 | 4 | 1,967,293 | 3,059,297,690 | 188,883 | 1,555 | 2,419 |
| D1 | 3 | 6 | 2,140,936 | 5,016,044,532 | 257,050 | 2,343 | 3,723 |
| D2 | 3 | 7 | 1,690,330 | 6,526,512,560 | 306,028 | 3,861 | 4,935 |
| D3 | 3 | 8 | 2,321,647 | 7,280,233,812 | 392,186 | 3,136 | 4,467 |
| E1 | 3.5 | 12 | 2,893,907 | 10,021,345,437 | 525,161 | 3,463 | 4,622 |
| E2 | 3.5 | 13 | 3,452,458 | 8,919,130,075 | 321,978 | 2,583 | 3,665 |
| E3 | 3.5 | 14 | 2,834,351 | 6,158,627,936 | 300,651 | 2,173 | 3,468 |

**Figure B1** Summary of enriched gene ontology (GO) terms from function enrichment analysis of 501 genes associated with 1,910 age-related CpG sites in the Pacific crown-of-thorns seastar (Acanthaster cf. solaris). Terms are grouped into molecular function (MF), biological process (BP), and cellular component (CC), and ranked by significance (-log10 adjusted p-values). Driver terms are highlighted in blue.



GO:MF

| Term name | Term ID | stats: $p_{adj}$ | stats: $-\log_{10}(p_{adj})$ (0 … ≤16) |
|---|---|---|---|
| binding | GO:0005488 | $2.547\times10^{-10}$ | |
| nucleoside-triphosphatase regulator activity | GO:0060589 | $4.635\times10^{-7}$ | |
| GTPase regulator activity | GO:0030695 | $4.635\times10^{-7}$ | |
| protein binding | GO:0005515 | $3.460\times10^{-6}$ | |
| kinase activity | GO:0016301 | $1.404\times10^{-5}$ | |
| phosphotransferase activity, alcohol group as acceptor | GO:0016773 | $1.738\times10^{-5}$ | |
| guanyl-nucleotide exchange factor activity | GO:0005085 | $3.315\times10^{-5}$ | |
| protein kinase activity | GO:0004672 | $1.280\times10^{-4}$ | |
| ATP-dependent diacylglycerol kinase activity | GO:0004143 | $1.812\times10^{-4}$ | |
| transferase activity, transferring phosphorus-containing... | GO:0016772 | $2.421\times10^{-4}$ | |
| protein serine/threonine kinase activity | GO:0004674 | $5.893\times10^{-4}$ | |
| enzyme regulator activity | GO:0030234 | $7.770\times10^{-4}$ | |
| cytoskeletal protein binding | GO:0008092 | $1.175\times10^{-3}$ | |
| lipid kinase activity | GO:0001727 | $5.873\times10^{-3}$ | |
| phospholipase C activity | GO:0004629 | $5.873\times10^{-3}$ | |
| phosphatidylinositol phospholipase C activity | GO:0004435 | $5.873\times10^{-3}$ | |
| ATP binding | GO:0005524 | $1.388\times10^{-2}$ | |
| adenyl ribonucleotide binding | GO:0032559 | $1.709\times10^{-2}$ | |
| phosphoric ester hydrolase activity | GO:0042578 | $2.503\times10^{-2}$ | |
| phosphoric diester hydrolase activity | GO:0008081 | $2.556\times10^{-2}$ | |
| molecular function regulator activity | GO:0098772 | $2.620\times10^{-2}$ | |
| adenyl nucleotide binding | GO:0030554 | $3.959\times10^{-2}$ | |

GO:BP

| Term name | Term ID | stats: $p_{adj}$ | stats: $-\log_{10}(p_{adj})$ (0 … ≤16) |
|---|---|---|---|
| phosphorylation | GO:0016310 | $1.028\times10^{-5}$ | |
| phosphorus metabolic process | GO:0006793 | $1.337\times10^{-4}$ | |
| phosphate-containing compound metabolic process | GO:0006796 | $2.132\times10^{-4}$ | |
| intracellular signal transduction | GO:0035556 | $2.398\times10^{-4}$ | |
| lipid phosphorylation | GO:0046834 | $3.938\times10^{-4}$ | |
| protein kinase C-activating G protein-coupled receptor ... | GO:0007205 | $3.938\times10^{-4}$ | |
| protein phosphorylation | GO:0006468 | $9.650\times10^{-4}$ | |
| intracellular signaling cassette | GO:0141124 | $3.792\times10^{-3}$ | |
| regulation of cellular process | GO:0050794 | $4.143\times10^{-3}$ | |
| regulation of biological process | GO:0050789 | $4.973\times10^{-3}$ | |
| biological regulation | GO:0065007 | $1.511\times10^{-2}$ | |
| small GTPase-mediated signal transduction | GO:0007264 | $1.627\times10^{-2}$ | |
| cell adhesion | GO:0007155 | $3.761\times10^{-2}$ | |
| transmembrane receptor protein tyrosine kinase signali... | GO:0007169 | $3.988\times10^{-2}$ | |

GO:CC

| Term name | Term ID | stats: $p_{adj}$ | stats: $-\log_{10}(p_{adj})$ (0 … ≤16) |
|---|---|---|---|
| dendritic tree | GO:0097447 | $7.694\times10^{-3}$ | |
| dendrite | GO:0030425 | $7.694\times10^{-3}$ | |
| somatodendritic compartment | GO:0036477 | $7.694\times10^{-3}$ | |

**Figure B2** Performance of elastic net models using nested 5-fold cross-validation to predict the chronological age of n = 15 Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*). (a) Regression plot of predicted against chronological age across five cross-validation folds. (b) Box plots comparing the absolute error between training and test datasets.

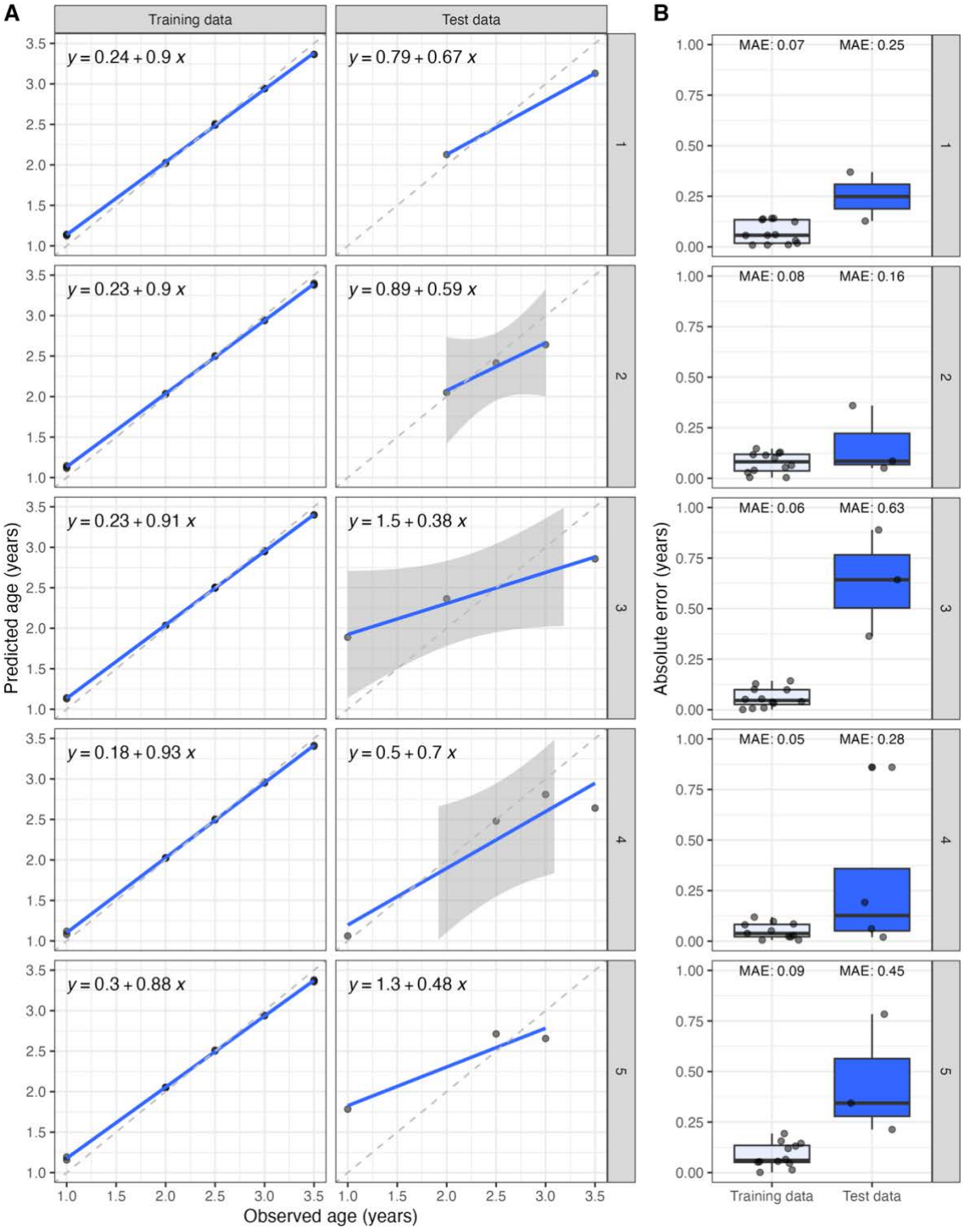
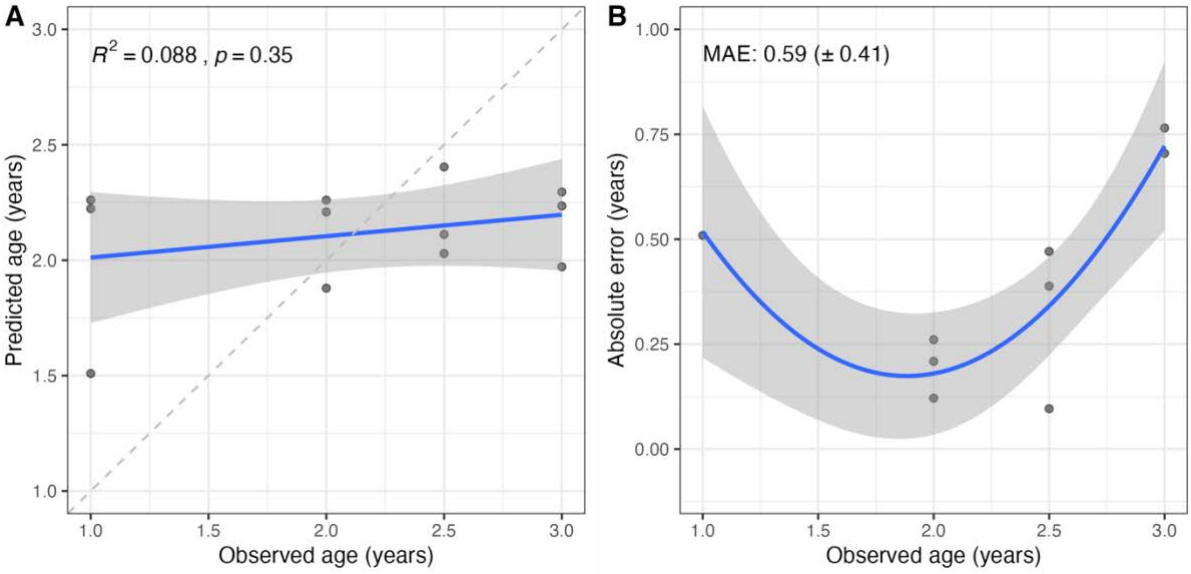


**Figure B3** Performance of elastic net models using nested leave-one-out cross-validation (LOOCV) to predict the chronological age of n = 15 Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*) based on CpG sites within rDNA regions. (a) Regression plot of predicted age against chronological age. (b) Absolute error of predictions across chronological ages.



## Appendix C: Additional publications during candidature

Chandler, J. F., Burn, D., Caballes, C. F., Doll, P. C., **Kwong, S. L.**, Lang, B. J., Pacey, K.I., & Pratchett, M. S. (2023). Increasing densities of Pacific crown-of-thorns starfish (Acanthaster cf. solaris) at Lizard Island, northern Great Barrier Reef, resolved using a novel survey method. *Scientific Reports*, *13*(1), 19306

Pratchett, M. S., Chandler, J. F., Choukroun, S. M., Doll, P. C., Lang, B. J., **Kwong, S.**, Chen, C.C., Emslie, M.J., Caballes, C.F., Uthicke, S. & Matthews, S. A. (2024). Biophysical Processes Involved in the Initiation and Spread of Population Irruptions of Crown-of-Thorns Starfish on the Great Barrier Reef. In *Oceanographic Processes of Coral Reefs* (pp. 290-305). CRC Press.

Wolfe, K., Desbiens, A. A., Patel, F., **Kwong, S.**, Fisher, E., Mumby, P. J., & Uthicke, S. (2025). eDNA confirms lower trophic interactions help to modulate population outbreaks of the notorious crown-of-thorns sea star. *Proceedings of the National Academy of Sciences*, *122*(11), e2424560122.