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*Review*

# Telomeres, species differences, and unusual telomeres in vertebrates: presenting challenges and opportunities to understanding telomere dynamics

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**Abstract:** There has been increasing interest in the use of telomeres as biomarkers of stress, cellular ageing and life-histories. However, the telomere landscape is a diverse feature, with noticeable differences between species, a fact which is highlighted by the unusual telomeres of various vertebrate organisms. We broadly review differences in telomere dynamics among vertebrates, and emphasize the need to understand more about telomere processes and trends across species. As part of these species differences, we review unusual telomeres in vertebrates. This includes mega-telomeres, which are present across a diverse set of organisms, but also focusing on the unusual telomeres traits of marsupials and monotremes, which have seen little to no prior discussion, yet uniquely stand out from other unusual telomere features discovered thus far. Due to the presence of at least two unique telomere features in the marsupial family Dasyuridae, as well as to the presence of physiological strategies semelparity and torpor, which have implications for telomere life-histories in these species, we suggest that this family has a very large potential to uncover novel information on telomere evolution and dynamics.

**Keywords:** telomere; mega-telomere; telomere length dimorphism; non-canonical telomere sequences; marsupial; dasyurid

**Abbreviations:**

TL: telomere length
qPCR: quantitative PCR
FISH: fluorescence in situ hybridisation
ALT: alternative lengthening of telomeres
TRF: telomere restriction fragment
qFISH: quantitative fluorescence in situ hybridisation

STELA: single telomere length analysis PNA: peptide nucleic acid
mya: million years ago

## 1. Introduction

Telomeres, the specialised structures at the ends of chromosomes, often compared to the plastic tips on shoe laces, stop chromosomes from unravelling. They are essential for preventing chromosomes from fusing together, which would result in genetic chaos, and are central to both ageing and cancerous processes [1]. However, telomeres progressively erode with each cell division, exacerbated by oxidative stresses [2–4], until a critically short length is reached and cell division ceases or cell death is initiated [5]. This phenomenon of telomeres shortening with age has generated great interest in using telomere length (TL) as a biomarker for wildlife species in studying stress, life-histories and their association with survival [6]. Unfortunately, our understanding of the mechanisms governing TL maintenance and inheritance for species other than human and mouse is limited. Without a knowledge of telomere biology across divergent taxa, the usefulness of telomeres as biomarkers may be limited. This is highlighted by animals such as the Iberian shrew, chicken and members of the marsupial family Dasyuridae, which have all shown unique telomere biology. The telomere features of these species make commonly used TL assays either inappropriate or unable to give a true indication of TL. These unique features demonstrate the importance of gaining a greater understanding of telomere biology across divergent taxa. Here, we provide a broad review on general species differences of telomere processes in vertebrates, and in particular on unusual telomeres. We highlight the appearance of mega-telomeres across a diverse set of organisms, but also focus on particularly unique telomeric features of non-canonical telomere sequences in Australian marsupials and monotremes, as well as telomere length dimorphism in the marsupial dasyurid family.

### *1.1. Telomeres*

In vertebrates, telomeres are composed of highly conserved $(TTAGGG)_n$ sequences [7,8]. They are instrumental in preserving genomic stability, by preventing chromosomal ends being recognised by the DNA damage machinery as DNA damage breaks and causing erroneous repairs [1]. Telomeres play a central role in cellular ageing mechanisms, and their maintenance is crucial to cell immortalisation and cancer development. Telomeric DNA is gradually lost with each cell division due to incomplete DNA replication [9], further exacerbated by telomeric loss due to oxidative damage [2–4]. This loss is associated with a gradual shortening of TL over time [10]. The shortening of telomeres will eventually reach a critical threshold, at which point cell division either becomes arrested through a p53 dependent pathway, or undergoes apoptosis [5]. In rare cases, cells may bypass this checkpoint, and telomeres will shorten until they trigger cell crisis, causing massive genome instability due to lack of telomeric protection [11]. Importantly, these telomere thresholds are triggered by the shortest telomeres within a cell, rather than average TL [12–14]. To prevent telomere shortening throughout successive generations, telomeres are maintained in germ line cells. This maintenance is mostly attributed to the enzyme telomerase, which synthesises new telomeric repeats onto the ends of telomeres using a complementary RNA template [15]. TL maintenance may also be achieved through alternative lengthening of telomeres (ALT), a pathway which elongates telomeres using homologous recombination and is particularly noted to occur in cancer [16]. There is

some evidence that ALT is also used to help maintain telomeres during embryonic development [17], but it is not clear to what extent this pathway contributes to telomere maintenance.

## 2. Telomeres as biomarkers

Recently, there has been increasing research focused around the use of telomeres as a biomarker of cellular ageing, stress, and life-histories, particularly in an ecological context [18]. Central to this concept is that, (i) within a given species population, individuals with shorter telomeres or increased telomere attrition rates have reduced survival chances [19–23], and (ii) telomere shortening is exacerbated by oxidative damage to telomeres, coinciding with cellular damage and ageing, such that telomere shortening rate is a reflection of cellular ageing [4,6,24]. This cellular ageing in turn reflects organism life-histories such as stress and behavioural decisions that favour short-term over long-term fitness and survival, with increasing telomere attrition rates thought to indicate favouring of short-term strategies at the cost of long-term survival [6,24].

The sampling method of choice when performing telomere measurements has been to use blood samples, with a minority of studies using tissue samples or cultured fibroblasts. For mammalian species, this means measuring telomeres of white blood cell leukocytes due to their non-nucleated red blood cells [10]. Immune cells and hematopoietic stem cells in the blood tend to have relatively high levels of telomerase activity, though not enough to offset telomere loss [25,26]. Non-mammalian vertebrate species possess nucleated red blood cells, and these can be used for telomere measurement from blood samples instead of leukocytes [10]. The vast majority of studies investigating telomeres as biomarkers have used average TL measurement techniques such as quantitative PCR (qPCR) or the southern blot based telomere restriction fragment (TRF) analysis [18]. The qPCR technique operates based on measuring the relative frequency of telomeric repeats compared to a reference sample of DNA with invariable copy number. In this manner, the relative amount of telomere sequences in a sample can be estimated [18]. TRF analysis uses enzymes which cleave non-telomeric DNA, allowing for separation of intact telomeres. These are run through gel electrophoresis, and in conjunction with a DNA size ladder, mean TL can be estimated [27]. The technique used for telomere measurement should be given appropriate consideration, the cheap and fast qPCR may not necessarily provide the most information, and is unable to properly account for interstitial sequences or TL variability. Unlike qPCR, TRF is able to produce smears that give an indication of TL variability, as well as generate different telomere bands, such as when interstitial telomeres of vastly different sizes from the telomeres are present [28]. Fluorescent *in* situ hybridization (FISH) based techniques such as quantitative FISH (qFISH) can offer great information on TL variability. Single telomere length analysis (STELA), a single molecule PCR-based technique, could offer powerful information on individual telomeres and overcome typical disadvantages of TL analysis, but requires subtelomeric sequence for the design of specific primers [29], and may not be appropriate for non-model species at this stage. It is generally assumed that techniques used will be reliable and sufficient to capture information on telomeres. Not all telomeres are equal, however, and in order to properly assess and use telomeres as biomarkers, more needs to be understood about the divergence of telomere dynamics that exists across species and between individuals.

Average TL remains highly species specific, and cannot be used as a direct comparison to lifespan [30]. This has brought into question whether or not TL mediated senescence naturally occurs in species such as the mouse *Mus musculus*, which has increased TL and increased telomerase

activity in somatic cells, but significantly shorter lifespan than humans [31]. Different species often have different attrition rates [32–36], with longer-lived species generally believed to have reduced telomere attrition rates [37]. The role of telomere attrition in species with unequal lifespans and TL cannot either be ignored. For example, canines have average TLs that are generally slightly longer than that of humans (ranging from 12 to 28 kb, depending on breed), but have significantly reduced lifespan [32,38]. However, these also have a roughly ten-fold increase in telomere attrition per year *in vivo*. Given the relationship of larger dog breeds generally having shorter telomeres and lifespans, this has led to the conclusion that canines are likely to employ replicative senescence [32]. Whether or not telomeres act as a barrier to growth within a species natural lifespans is dependent on different factors such as lifespan, TL, and telomere attrition rate. While telomeres retain their ability to limit cell proliferation throughout species [39], the extent to which this naturally occurs in many non-model species is still left to be elucidated. Though this does not diminish the ability to monitor life-histories using telomeres, it highlights the difficulty of assessing the direct effects of telomere shortening on a species. The role of telomeres in predicting survival is still part of an ongoing debate as to the causative nature of the relationship [39]. Are short telomeres directly impacting on physiological processes (e.g. by causing cell senescence), thus leading to reduced fitness, or can telomeres only be considered as biomarkers? Certainly in humans there is evidence to suggest a direct role for TL in ageing [40,41]. Yet if anything can be learned from studies in mice and humans, it's the answer to this question is likely to vary between species.

There are significant differences between species in various telomere dynamics. This includes species differences in telomere maintenance such as telomerase activity [30] and telomere recombination [42,43], differences in heritability of TL [44,45], including within species offspring-sex-linked differences [23], sex differences in attrition [46], as well as species possessing unusual telomere distributions not considered characteristic of most organisms [30,47–49], which may be indicative of differences in several telomere-related pathways or processes. Even within individuals, TLs are not entirely homogeneous [50], presenting a mild challenge to accurately observing TL and attrition. Commonly used techniques such as TRF and especially qPCR have a limited ability to quantify TL heterogeneity within an individual, as these are designed to measure average TL within a population of cells. However, the distribution of telomere lengths is an important property of an individual's telomere landscape, as it is short telomeres that trigger cell senescence [12–14]. Different attrition rates are also observed based on TL [19,51], as well as within different tissues [36], and even between sex chromosomes (possibly related to epigenetic differences) [52].

While it may be possible to describe trends and generalisations on telomere processes to help overcome their complexity across different species and variables, these processes first need to be well understood, as do any exceptions to overall trends. There are still conflicting results which hinder proscribing clear trends, and not much is known on what causes these differences to arise.

One such unclear trend is the relationship between TL and sex. Throughout diverse species, males generally have higher rates of telomere attrition, a phenomenon which is not observed for females [46]. This has been linked to lower mean lifespan in species such as humans, and has been attributed to size dimorphism, with larger males undergoing more cell divisions and thus higher telomere attrition [53]. However, higher male telomere attrition is also observed in species where females have shorter lifespan, or where females have increased body mass [46]. There are many other factors that have been proposed to explain sex driven attrition differences, such as behaviour, effects of hormones on telomeres, differential telomerase expression, effects of heterogametic

expression [46], but none are individually capable of explaining sex differences. If anything, these demonstrate the importance of taking sex into consideration when comparing population TL and attrition rates.

Other trends have emerged which have been more clear-cut, such as the negative relationship between telomerase activity and body mass [30,54]. The prevailing explanation for this is that larger and more longed lived species are more likely to employ telomeres as a barrier to unchecked cell proliferation against cancer, in contrast to smaller and shorter lived species. Exceptions exist, and a minority of large species also display relatively high telomerase activity for their size, such as the Indochinese tiger (*Panthera tigris corbetti*) [30]. In the case of the tiger, it is tempting to link the high karyotypic stability of the cat to some other form of tumour protection that might manifest in the tiger [55]. Nevertheless, the reasons underlying such exceptions remain uncertain.

Related to this is the ongoing debate on which species use replicative senescence, as alluded to earlier. While for humans there has been a strong suit of evidence to indicate this is the case, the opposite appears true for the mouse [39]. Despite this discrepancy, there is evidence to suggest a role for telomeres in mouse survival, as overexpression of the catalytic subunit telomerase reverse transcriptase was shown to increase mean lifespan in mice, and mice longevity could be predicted by the rate of increase of percentage of short telomeres [33]. Nevertheless, the general trend seems to be that smaller and short lived species are less likely to naturally employ telomere mediated senescence, as these often tend to have relatively long TLs and increased telomerase activity [30,39,54]. However, this relationship has not been properly characterised in many non-model species, and exceptions can reasonably be expected to exist.

Understanding processes, trends, and the underlying reasons for observed differences will benefit the assessment of telomeres as biomarkers. For example, there have been several observations of increased attrition on longer telomeres [19,51], coinciding with predictions that oxidative damage would have a greater shortening effect on larger telomeres [56], and observations that telomerase tends to preferentially target short telomeres [12,57,58], which would have a larger protective effect against attrition on short telomeres than long telomeres. Long telomeres were found to provide a better prediction of long-term fitness in two avian species, free-living jackdaws (*Corvus monedula*) [19] and common tern (*Sterna hirundo*) [59]. Could increased attrition of long telomeres provide a more sensitive measurement of stress than average TL attrition? If oxidative stress is more pronounced on longer telomeres, higher attrition in longer telomeres may provide better resolution for detecting significantly different rates of attrition due to life-histories, while simultaneously reducing the effect of telomerase action on attrition rates. The caveat is to what extent long telomeres alone are representative as biomarkers, as well as the added difficulties of separating long and short telomeres during TL measurement. In addition, another study in laboratory mice found that the rate of percentage increase of short telomeres to be a better predictor of survival than average telomere attrition [33]. Are both methods generally more precise than average TL for predicting survival? Are these equally valid across all species, can one of these be determined to be generally more accurate, or is one measurement perhaps a better indicator of survival in some species than others, dependent on underlying telomere biology differences? Could other types of telomere measurement provide more information? Clearly, there remains much work to be done in order to properly understand and optimise the use of TL as a biomarker.

There is a need to increase our understanding of the significance of TL specific processes when assessing TL and attrition, and to learn more about telomeres among vertebrates in order to properly

employ telomeres as biomarkers across species. Some species do not display the "normal" and relatively uniform distributions of TL considered characteristic of most species, containing constructs such as mega-telomeres. Rather than hamper investigation across species, these have driven extra work in investigating telomere dynamics and evolution across species in vertebrates, as well as providing novel perspectives which may help shed light on general telomere processes.

## 3. Unusual telomeres

### *3.1. Mega-telomeres*

The telomere length spreads of mice and human presented a basis for relatively uniform telomere distributions as a benchmark for telomere distributions [50,60]. However, an increasingly large and diverse number of vertebrate organisms contain what can be labelled as mega-telomeres, extremely large tracts of telomeric DNA sequences present at a varying number of chromosome telomeres (Figure 1). These mega-telomeres have generally been observed using telomere FISH based techniques. Though these were identified relatively early on in studies focusing on interstitial telomeric sequences, karyotype and chromosome evolution [61], it is only in the last decade that they have been labelled as mega-telomeres. It should be noted that FISH does not have the sensitivity to exclude blocks of sequences only partially made up of TTAGGG repeats. TTAGGG signals in FISH may not in fact represent continuous telomere tracts. While this complicates the interpretation of what makes up a functional telomere, we can define mega-telomeres as structures on the end of the chromosomes that contain particularly large amounts of telomeric DNA, but need not necessarily be made purely of TTAGGG repeats. For our purposes, we have interpreted as mega-telomeres any particularly large signals of telomeric DNA which are indistinguishable from telomeric signals at the ends of chromosomes.

Mega-telomeres can be observed across a wide range of taxa in vertebrates [61], including birds [62,63], reptiles [64], cetacean [30], bats [65], shrews [49], rodents [66–68], marsupials [69], primates [70], and even in sturgeon in fishes [71]. Most research done on mega-telomeres comes from the chicken (*Gallus gallus domesticus*) and Iberian shrew (*Sorex granarius*). Within the chicken these mega-telomere arrays may contain up to 800 kb of telomeric repeats [28], and in Iberian shrew up to 300 kb of telomeric repeats [49]. The "normal" telomeres for each animal are around 10–40 kb for the chicken and ~4 kb for the shrew respectively [28,49], lengths much more comparable to the human standard of 5–15 kb [72]. In Iberian shrew, the mega-telomeres are specifically associated with ALT activity [73], whereas in chicken there is general evidence of ALT activity in somatic cells [42]. ALT provides a potential pathway for the appearance and maintenance of mega-telomeres, and it is tempting to generally associate mega-telomeres with ALT activity. In both the Iberian shrew and chicken there is significant association between mega-telomeres and ribosomal DNA [48,74], a trait also found in the wood lemming (*Myopus schistocolor*) [75]. However, such a relation is not uncommon even with normal telomeres [76–79], and whether the relationship with mega-telomeres is particularly significant remains to be seen. Mega-telomeres are often associated with microchromosomes in an array of organisms that possess microchromosomes, particularly avians and reptiles [48,62–64,71,80–82]. These microchromosomes have higher rates of recombination than "normal" chromosomes [83], and it has been proposed that the abundance of mega-telomeres on microchromosomes could be associated with this high recombination rate [62]. Different relationships with mega-telomeres are found in other animals. In Iberian shrews, mega-telomeres are

specifically found on the p-arms of acrocentric chromosomes rather than associated with microchromosomes (there are no microchromosomes in mammals) [49], a phenomenon also observed in other animals such as in several lemur species [61,70]. In the chicken, apart from the metacentric W chromosome, mega-telomeres were also situated on the short arm of three acrocentric microchromosomes [48]. In several species mega-telomeres are exclusively found on sex telomeres, such as on the short arm of the X sex chromosomes of the marsupials *Monodelphis kunsi* and *M. brevicaudata* [69], on the W chromosome of the thick-tailed gecko (*Underwoodisaurus milii*) [84] and sand lizard (*Lacerta agilis*) [85], and on the Y chromosome of the red-necked wallaby (*Macropus rufogriseus*) [61]. Mega-telomeres in sex chromosomes coincided with large heterochromatic blocks [69,84–86], a feature also seen on the mega-telomere of the chicken W chromosome [48]. However, some caution needs to be taken when interpreting large fluorescent signals from telomeric probes as mega-telomeres. In the red-necked wallaby, a non-telomeric satellite repeat Mrb0B29 (GGAATTT) is both similar to the telomere repeat and is similarly distributed across the Y chromosome [86], raising questions about any possible cross-hybridisation that may have occurred.

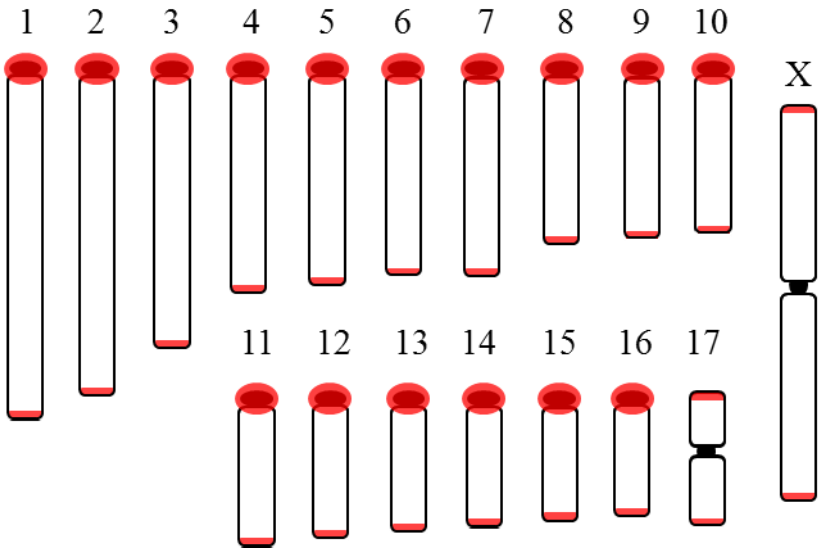


**Figure 1. Illustration of half karyotype and of mega-telomere arrangement in the Iberian shrew.** Telomeres are illustrated in red. Excepting chromosomes 17 and the X sex chromosome, all chromosomes are acrocentric and possess mega-telomeres on their proximal ends. Each autosome is denoted by a number. Note that in other species, mega-telomeres need not be restricted to the short arms of acrocentric chromosomes.

Mega-telomeres do not appear to be highly conserved; the abundance or presence of these is rarely highly conserved between related species, or even within single species. A closely related species to the Iberian shrew, the common shrew (*Sorex araneus*) has no observable mega-telomeres [87]. Brazilian shrew mice (*Blarinomys breciceps*) display noticeable differences in abundance of potential mega-telomeres between individuals, although there is also high karyotypic diversity between individuals of the same species [67]. *Cerradomus subflavus* individuals with different cytotypes also show significant differences in mega-telomere abundance [66]. Within the *Leposoma* genus of squamate reptiles, variance in mega-telomere abundance is observed between three species (*L.*

*osvaldoi, L. guianense, L. scincoides*) [88]. Differences in mega-telomeres abundances are observed between two monitor species (*Varanus salvator macromaculatus*, *V. exanthematicus*) [80]. Even in chickens, mega-telomere presence can be variable between individuals on certain chromosomes [48].

Mega-telomere constructs are not simply "interesting features" that can be ignored during telomere measurements. For example, TRF analysis in the chicken yields several large gel band smears, corresponding to vastly different telomere array sizes. Several of these large bands correspond to mega-telomeres, but other bands also correspond to interstitial telomeric sequences. In chicken, this required demonstrating which TRF bands were digested by Bal 31 exonuclease, an enzyme which cleaves nucleotides from the end of the chain and thus will not target interstitial sequences, to identify if they were located at the terminal ends of chromosomes [28]. Interstitial telomeric sequences are known to interfere with qPCR and TRF measurements, due to the extra telomeric material which is not affected by telomere shortening [89]. Mega-telomeres pose a similar challenge, as these ultra-large telomere arrays will confound any average TL measurements, and their high variability will further compound this problem [48,49]. Telomere qPCR has been the dominant telomere measurement method used in birds [20,21,90–92], followed by TRF [93–95], despite the presence of both mega-telomeres and interstitial telomeric sequences in many avian species [62]. Further work must be done to understand the effect of unusual telomere features, such as mega-telomeres, on common telomere measurement methods.

The dominant majority of mega-telomeres we have identified have been from studies focusing on interstitial telomeric sequences and chromosome evolution. Any role or function to these telomere structures remains to be discovered, and the mechanisms by which mega-telomeres develop are still left to be fully elucidated.

Many other non-vertebrate taxa also demonstrate other very unusual telomeric features, such as *Drosophila* whose telomeric repeats have been superseded by retrotransposons [96], but these are beyond the scope of this review. Within vertebrates, particularly unusual and novel telomeric features are observed in Australasian marsupials and monotremes, and in particular the marsupial Dasyuridae family. These have received almost no real attention thus far, yet their telomeric features are quite distinguishable from mega-telomeres or unusual telomeres seen in other taxa, and thus warrant assessment. Furthermore, a number of dasyurid species possess unique physiological features outside of telomere processes, semelparity and torpor, which are of interest in investigating telomeres and life-histories. For these reasons, we have decided to review their unique telomere features in more detail, and in doing so lay down a basis for future telomere-related studies in these species.

### *3.2. Non-canonical sequences in marsupials and monotremes*

Traditionally our knowledge of telomeres in vertebrates would lead us to believe that they are composed of almost uninterrupted $(TTAGGG)_n$ sequences [97], with very small variation of these sequences due to imperfect telomere synthesis by telomerase [98]. Yet several species of Australian marsupials and even a monotreme have bucked this trend, and feature novel non-canonical telomere sequences.

This inadvertent discovery arose from researchers attempting to measure TL in marsupial and monotreme species using TRF analysis. While generally considered reliable and used as a "gold standard" for telomere measurements [18], TRF analysis has been confounded in Australian marsupials and a monotreme species due to cleaving in telomeric regions (Figure 2). This remained true even when cleaving enzymes were used one at a time, and is observed in organisms where FISH

techniques gave no indication of any significant interstitial telomeric repeats. The obvious conclusion has been that the telomeres of these animals contain non-canonical telomere sequences, which can be targeted by the DNA cleaving enzymes and thus cause telomere cleaving [30,47,99]. This cleaving has been documented in four Australian marsupials: the dasyurid Tasmanian devil (*Sarcophilus harrisii*) [47,99], the red kangaroo (*Macropus rufus*), koala (*Phascolarctos cinereus*), and southern hairy-nosed wombat (*Lasiorhinus* latifrons), as well as in the Australian monotreme the short-beaked echidna (*Tachyglossus aculetatus*) [30]. Interestingly telomere cleaving by TRF analysis is not observed in the American Virginian opossum (*Didelphis virginiana*) [30], suggesting that marsupial non-canonical telomere sequences could be unique to Australasian marsupials. Nor is TRF telomere cleaving observed in another monotreme species, the platypus (*Ornithorynchus anatinus*) [100]. How comparable the echidna's non-canonical telomere sequences are to that of Australasian marsupials is unknown, and it is difficult to speculate a common or divergent origin due to the unknown make-up of these sequences. The lack of such sequences in the Virginian opossum does put in doubt a relationship between marsupial and monotreme non-canonical telomere sequences. It has been suggested that these non-canonical telomere sequences could have arisen and be maintained due to ALT [30], which seems to be the best explanation to explain maintenance of such sequences throughout generations, as exclusive telomerase action might be expected to "clean up' a species" telomeres after sufficient generations. At present, it is unclear exactly what range of marsupials demonstrates this feature. The potential role of these non-canonical telomere sequences is uncertain, and cannot be elucidated until more is known on the make-up of these sequences. Genome assemblies could offer great potential in uncovering these properties.

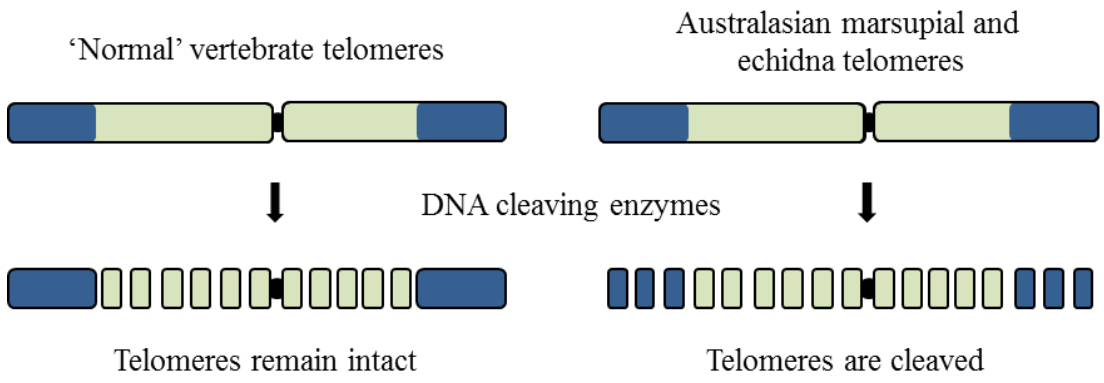


**Figure 2. Telomere cleaving by TRF analysis in marsupial and echidna telomeres.** DNA cleaving enzymes used in TRF analysis normally leave the telomeres intact. TRF analysis on Australian marsupials produces fragmented gel bands, which is attributed to DNA cleaving within the telomeres.

### *3.3. Telomere length dimorphism in the marsupial family Dasyuridae*

Telomere length dimorphism was discovered when investigating telomeres in Tasmanian devils (*Sarcophilus harrisii*, a member of the Dasyuridae family) and devil facial tumour disease using a qFISH technique. The most interesting finding was not on the telomeres of the disease, which were generally short and uniform as in most cancers, but on telomeres of normal Tasmanian devils. Homologous chromosomes had significant discrepancies in TLs which were non-randomly distributed,

essentially forming a bimodal distribution of TL frequency, with each chromosome pair possessing a chromosome homologue with short, and a homologue with long, telomeres (Figure 3) [47]. This is not only in contrast to the unimodal distribution of many other vertebrates [12,47,50], but also in contrast to mega-telomeres of other species which do not tend to follow such a rigid karyotype-wide organisation. Due to the consistency of results across chromosome pairs and individuals, these differences cannot be attributed to random inheritance of long and short telomeres, indicating a process of differential TL regulation is at work [47].

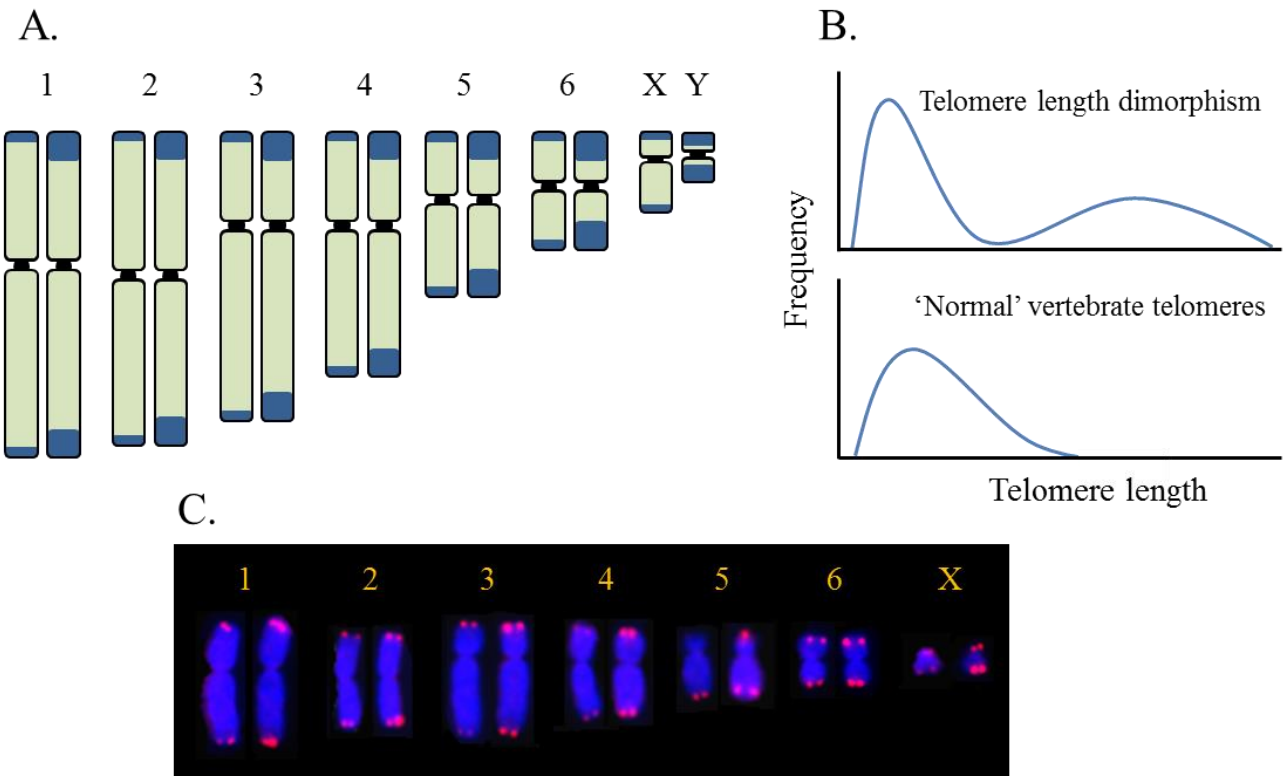


**Figure 3. Illustration of telomere length dimorphism.** (A) Each chromosome pair has two homologues, telomeres are illustrated in dark blue. For each chromosome pair, one homologue has short telomeres, and the other has long telomeres. Numbers are used to indicate chromosome number for a typical Tasmanian devil chromosome spread. All other dasyurid species karyotyped to date share the same diploid number and similar chromosome morphology. (B) Comparison of frequency distribution of TLs between telomere length dimorphism and "normal" vertebrate telomeres. The top graph represents the bimodal frequency distribution of telomere length dimorphism, and the bottom graph represents the unimodal frequency distribution typical of most known vertebrates. (C) Image of female northern quoll (*Dasyurus hallucatus*) chromosomes from a single metaphase, stained with a peptide nucleic acid (PNA) telomeric probe.

Further investigations in related marsupials revealed this feature was specific to the dasyurid family. Telomere length dimorphism is observed in members of both dasyurid subfamilies, Dasyurinae (*S. harrisii* and *Dasyurus maculatus*) and Sminthopsinae (*Sminthopsis crassicaudata, S. macroura, S. douglasi*), but not in other marsupial species of the Diprotodontia or Peramelemorphia

orders (see Figure 4 for phylogeny) [47]. Telomere length dimorphism is not either observed in non-dasyurid marsupials from other studies using telomere FISH; this includes at least 15 members of the American Didelphidae order [30,69,101,102], and two members of the Australian Diprotodontia order [30]. It is unknown if telomere length dimorphism extends to the two other families within the Dasyuromorphia order of marsupials. The only extant species in these families is the endangered numbat (*Myrmecobius fasciatus*) of the Myrmecobiidae family, due to the recent extinction of the thylacine (*Thylacinus cynocephalus*) in the Thylacinidae family.

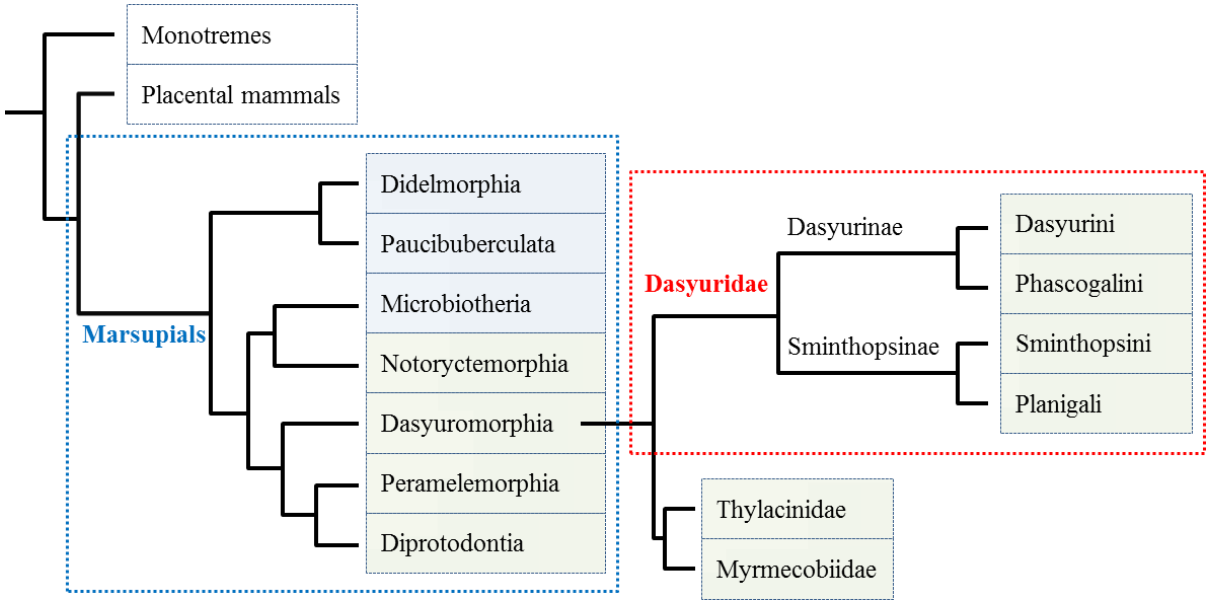


**Figure 4. Broad phylogenetic relationships of marsupial orders and the Dasyuridae family.** All seven marsupial orders are listed, and the dasyurid phylogeny is described up to the tribe level. American marsupial clades are shaded in gray, and Australasian marsupial clades are shaded in green.

### 3.3.1. Parental control of telomere length

If the non-random distribution of short and long TLs indicated that telomere length dimorphism is a regulated process, then it is the sex chromosomes that provided clues to how this regulation is determined. In dasyurid males, Y chromosomes were consistently characterised by long telomeres and the X chromosomes by short telomeres, suggesting that paternally inherited chromosomes correspond to chromosomes with long telomeres. It is this observation that led to the hypothesis of a parental control of TLs in dasyurids: chromosomes of paternal origin carry telomeres which have been elongated, while those of maternal origin carry shortened telomeres [47]. This has been dubbed the parent-of-origin hypothesis, and accounts for the non-random distribution of TLs, as well as the consistently long telomeres on the Y chromosomes.

Bender et al. [47] conceived of two possible timeframes where telomere regulation could occur to drive telomere length dimorphism. The first is that TL regulation occurs differentially in the germ line of both sexes. Under this scenario, telomeres are elongated in the male germ line, and shortened in the female germ line. Thus parents would contribute either only elongated or shortened telomeres to their offspring, dependent on the parent's sex (Figure 5).

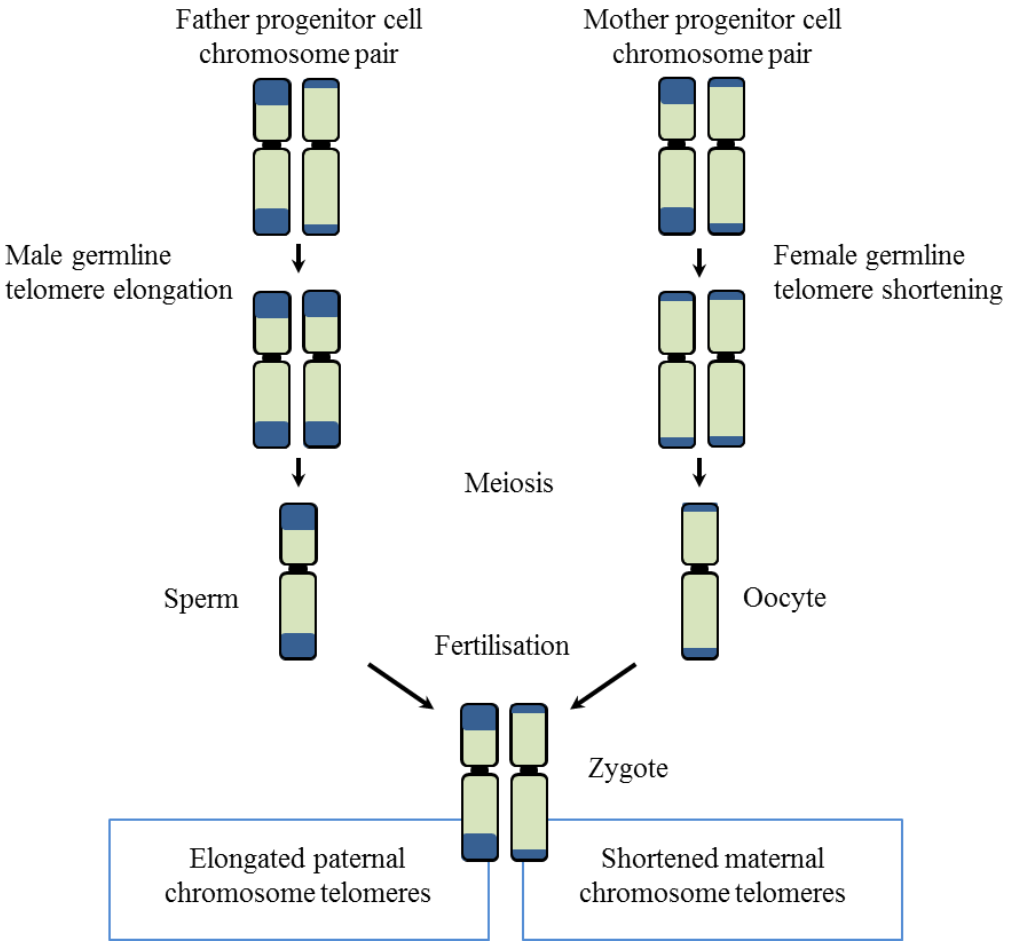


**Figure 5. Potential mechanism of dasyurid telomere length regulation.** During germline regulation, telomeres are either elongated in males or shortened in females. When cells are converted to sperm or oocyte, these only contain elongated or shortened telomeres respectively. The offspring zygote will thus contain one chromosome homologue with elongated telomeres from their father, and one chromosome homologue with shortened telomeres from their mother, for each chromosome pair [47].

The second is that telomere regulation occurs during embryonic development and growth. Specific markers are assigned to chromosomes based on parental origin, and telomere elongation or shortening is targeted based on these markers (Figure 6). Marsupials are characterised by specific epigenetic imprinting on X chromosomes in females which preferentially silences paternally-derived X chromosomes [103,104]. However, to date, apart from selected imprinted genes [105], there is no evidence of widespread and parental origin based epigenetic profiling in marsupials beyond the X chromosome.

Due to the relative simplicity of the first scenario, we currently favour this over the second. However, further investigations are required to test if chromosome parent origin truly is linked to TL beyond the male sex chromosomes, and how such processes are regulated.

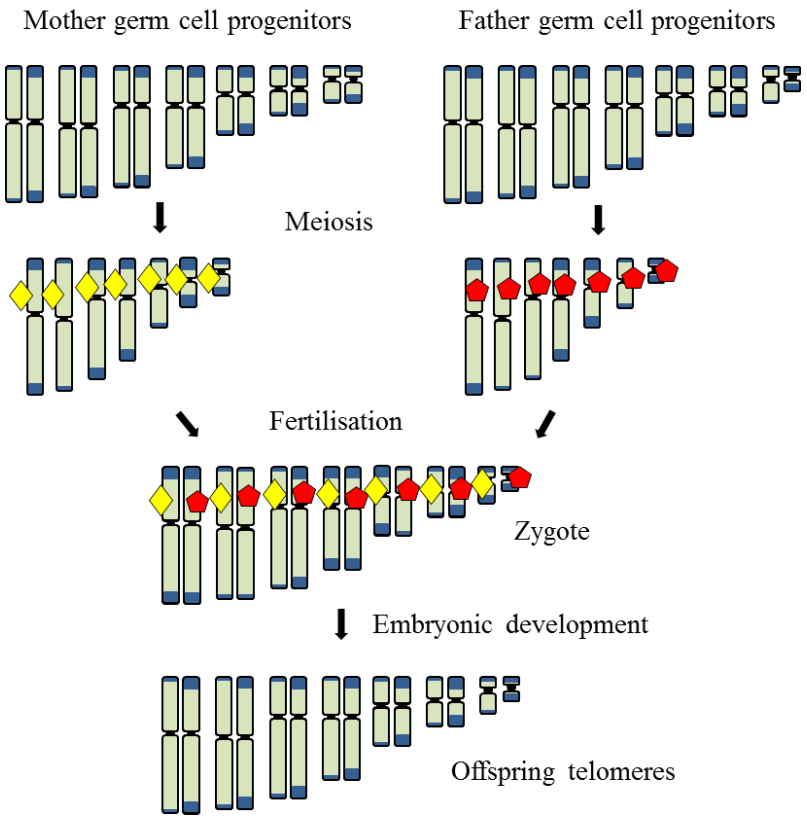


**Figure 6. Potential telomere regulation during embryonic development.** Chromosomes are randomly selected during meiosis, and telomere length remains random at fertilisation. Prior to fertilisation genetic markers are put in place to distinguish maternal from paternal chromosomes. Maternal markers are shown as yellow triangles and paternal markers are shown as red pentagons, though it is possible that only one such parental marker is required. During development, telomeres are either elongated or shortened based on these markers.

### 3.3.2. Telomere length regulation

The parent-of-origin hypothesis assumes that short telomeres can reliably be transformed into long telomeres within one generation, and vice versa for long telomeres being reduced to short telomeres. It is questionable whether the two most familiar methods of telomere elongation and shortening, by the enzyme telomerase and by telomere attrition respectively, are sufficient to create the large differences between short and long telomere subsets within single generations. *S. harrisii* TL estimates indicate differences of tens of kilo base pairs between telomere subsets, with long telomeres often greatly exceeding 50 kb in length [47]. Thus, TL regulatory mechanisms need to be capable of significant changes of many kilo base pairs, and these changes must presumably occur within a small number of cell divisions if they occur during either the germline or embryonic development, putting in doubt the ability of telomere attrition or telomerase action in driving such changes. Since dichotomous lengths are maintained in adulthood, this indicates that normal processes of extension and erosion are unable to homogenize the lengths. Telomere length dimorphism could be regulated by alternative lengthening of telomeres (ALT) and telomere trimming, which are capable of producing large increases or decreases in TL, respectively [106,107]. ALT is consistent

with the extremely heterogeneous size of the long set of telomeres [108], and with the maintenance of non-canonical telomere sequences in Australasian marsupials. The ability of telomere trimming to target elongated telomeres [107] is also consistent with a mode of telomere regulation able to shorten telomeres to below a certain threshold without jeopardising already short telomeres. Telomerase and ALT are not mutually exclusive [109–111], so ALT activity need not be precluded by the presence of telomerase activity in devil testes or other cell types [47].

## 4. Telomeres and dasyurid physiological strategies

The unique telomeres of dasyurids will be of great interest in studying evolutionary dynamics of telomeres, however, this family also enjoys a number of species possessing the physiological strategies of semelparity and torpor, both of which are linked to life-histories and will likely affect TL dynamics in the species that employ them.

### *4.1. Semelparity*

Marsupial semelparous species are defined by an unusual breeding behaviour, which leads to complete male mortality following an intense breeding season, with females surviving long enough to rear their young. This is in stark contrast to the iteroparous strategy which defines most mammals, and allows an individual to breed multiple times within a lifetime. In dasyurids, male die-off is generally surmised to be due to extremely elevated levels of the stress hormone cortisol as well as elevated testosterone levels, which drive the males to mate above all else, and leads to the collapse of their immune system as well as many other symptoms of physical deterioration [112–114]. A decrease in immune system capability has been linked with shortened telomeres [13,115], and the role of stress in an individual's life-history and how that is reflected in telomeres makes this phenomenon particularly interesting in a telomeric context [24]. It would also be interesting to investigate the differences in telomere and survival dynamics affecting both males and females of the same species. Due to the immune system breakdown seen in males during the breeding season, there may be complications using leukocytes from blood to measure telomere length, which may make blood samples inappropriate for semelparous species during this time period.

It is curious to speculate if semelparity and telomere length dimorphism could be linked [47]. The breeding season may represent a period of extreme stress for males which, presumably, will be reflected in their telomere shortening. Could the elongation of male chromosome telomeres represent a form of protection for sperm against telomere shortening due to semelparity? This assumes that the stress incurred in males is sufficient to cause significant telomere shortening in the short time frame that is the breeding season. An iteroparous strategy seems to be the ancestral mode of reproduction within dasyurids, with semelparity having instead independently evolved in several genera [116], dissolving any notions that telomere length dimorphism evolved purely in response to semelparity. It remains possible that telomere length dimorphism is an enabler of semelparity, as very few mammals show this trait, but this raises questions concerning semelparity in marsupial didelphids. Semelparous didelphids are generally considered to have partial or facultative semelparity, where some males are capable of surviving the breeding season [113,117], though it is possible that some didelphid species exhibit complete male die-off [117,118]. Either semelparous stress is not sufficient enough to warrant a form of telomere protection, or didelphids have other mechanisms for coping with semelparous stress. In the semelparous didelphid *Marmosops incanus*, no unusual telomere features similar to

telomere length dimorphism have been observed using telomere FISH (but not qFISH) [101,102,119]. Without knowing more on the direct effects of semelparous stress on telomeres, and the mechanisms of telomere regulation in both dasyurids and didelphids, a relationship between telomere length dimorphism and semelparity in dasyurids cannot be extrapolated.

### *4.2. Torpor*

Comparable to hibernation, torpor is a reduced metabolic state where energy consumption and body temperature are significantly lowered, but occurring for relatively short periods of time [120]. The relation between telomere dynamics, life-histories, and this ectothermic state could be quite interesting, but has not yet been extensively explored, with a very small number of studies on the subject. Results from these studies have not been entirely conclusive [121–123], but seem to indicate that the relationship between TL and torpor relies on its use. This includes frequency and duration of torpor, and is likely affected by other factors such as telomerase expression, antioxidant defense, duration of arousal, minimum metabolic activity in torpor, as well as environmental variables which may in turn affect torpor use. Though not unique to dasyurids among mammals, the inclusion of this strategy among their species bolsters their potential as future telomere and life-history research subjects.

## 5. Future research directions

Distinguishing mega-telomeres from large interstitial telomeric sites situated near chromosome ends based only on fluorescent metaphase images from FISH is challenging. The classification of mega-telomeres may remain contentious for some species and/or chromosomes until further work is done to test their terminal location, though we do not believe this discredits any of the points made. Since interstitial telomeric sequences cannot be shortened in the same manner as telomeres, proving that potential mega-telomeres can be thus shortened is perhaps the first step to resolve this issue. In chicken for example, digestion with an exonuclease was done to demonstrate the true telomeric properties of their mega-telomeres [28].

Telomere length dimorphism and non-canonical telomere sequences provide the opportunity to examine two unusual telomere features within a single diverse group of animals, though whether or not these features are both independent of each other remains unclear. It can be presumed that both features evolved at separate timescales, which may be estimated through species divergence. Corresponding with telomere length dimorphism, the divergence of the order Dasyurimorphia is estimated to have occurred ~65 million years ago (mya), with divergence of the Dasyuridae family occurring ~35 mya [124,125]. Due to the uncertainty surrounding the origin of non-canonical sequences, these may either have arisen in marsupials around the divergence of Australasian and American marsupials ~75–85 mya [124,125], and in monotremes after the divergence of echidna and platypus species ~32 mya [126], or non-canonical sequences may have arisen in both groups prior to the divergence of marsupials and monotremes ~186 mya [126]. While telomere length dimorphism and non-canonical telomere sequences are expected to be typical of all dasyurids, their physiological strategies are found with varying uses throughout the dasyurid species, and represent an “ideal” scenario where their telomeres can be studied with or without a particular strategy.

Telomere length dimorphism and non-canonical sequences may present useful tools with which to investigate telomere and species dynamics. Telomere length dimorphism provides an effective means to investigate how short and long telomeres can be differently affected by various factors, and

provides the means to do so at the level of a single organism or cell. If the parent-of-origin hypothesis proves correct, telomere length dimorphism could potentially be a powerful cytogenetic tool for studying parent-DNA-specific phenomenon, allowing identification of maternal or paternal chromosomes based on TL. Non-canonical telomere sequences may aid in illuminating marsupial evolution and radiation. The monito del monte (*Dromociops gliroides*) is the sole extant species of the marsupial Microbiotheria order. Though geographically situated in South America, genetic studies have proposed the monito del monte is more closely related to Australasian marsupials [124,127,128], and it has been a centrepiece among the discussion of marsupial radiation. It would be of interest to test if this species is also liable to telomere cleaving by TRF analysis, as this could provide evidence of a shared genetic feature beyond DNA content.

It is curious to speculate if telomere length dimorphism could be related to cancer susceptibility. Dasyurids (or certain dasyurid species) are thought to be prone to cancer [129–131]. While telomeres act as a barrier to cell proliferation for potentially cancerous cells, having short telomeres may predispose individuals to cancers, as critically short telomeres that bypass cell arrest may activate cell crisis and trigger genomic instability leading to oncogenesis [132]. The short subset of dasyurid telomeres may be particularly liable to triggering cell crisis. This is especially pertinent with the recent rise of devil facial tumour disease, a contagious cancer that has wiped out most of the Tasmanian devil population [133]. Recent investigations in marsupial genome-wide methylation have indicated that it is the maternal X chromosome that shattered and became extensively rearranged in the devil facial tumour, corresponding to the X chromosome which is expected to have short telomeres [134]. Could shortened telomeres have led to oncogenesis and ultimately the facial tumour through telomere-length dependent cell crisis?

The bimodal distribution of telomere length dimorphism raises some interesting evolutionary questions. What are the benefits of both increasing and decreasing TL, and why would this be done in a parent specific fashion? The semelparity theory gives a potential reason for male telomere lengthening, yet why would this also not apply to females, who still exhibit (less severe) stress, and presumably, associated telomere shortening? The purposeful shortening of telomeres is very much in contrast to the germ-line maintenance of other species, which generally attempt to maintain TL [135,136]. Many dasyurid species are small and short lived, and it is expected these would follow the trend of having relatively long telomeres [30], in contrast to their regulated telomere shortening of a subset of telomeres. While short telomeres may confer tumour protection through replicative senescence, over shortening of telomeres may reverse this effect [132]. If the dasyurid set of short telomeres exists as tumour protection, then why have there been reports of higher rates of cancer incidences in several dasyurid species [129–131]? Do these have some other underlying predisposition to cancer, which their shortened telomeres are used to somewhat offset?

A hurdle in future studies investigating dasyurid telomeres will be telomere measurement. Due to their non-canonical telomere sequences, TRF analysis cannot be used. Other common methods of telomere measurement, such qPCR only measure average TL of a population of cells [18], and cannot properly assess telomere distribution information, yet dasyurid telomeres have two distinct telomere groups with different lengths. Techniques such as qFISH will be needed for proper study and distinction of telomere groups. This may prove to be a blessing in disguise, as the information provided by such techniques may significantly advance our understanding of telomere dynamics. It is after all, only because qFISH was used to examine Tasmanian devil telomeres that telomere length dimorphism was discovered in the first place [47].

## 6. Conclusions

Species with unusual telomeres offer new perspectives with which to investigate telomere dynamics, and have the potential to uncover novel information on telomere dynamics. There has been limited research on mega-telomeres, given the wide diversity of species that possess them. Likewise, little is known of the unique telomere features of Australasian marsupials and monotremes, yet these represent a significant number of species. For these reasons, there is a significant need to learn more about these telomere features and their impacts.

The presence of non-canonical sequences in both Australasian monotreme and marsupial is very interesting from an evolutionary viewpoint. These have not been observed in any other vertebrates so far, and it is tempting to speculate on a common origin, yet the lack of such sequences in the Virginian opossum questions this idea. Uncovering the makeup of such sequences should be a first priority in determining their potential role, and the extent to which these are conserved across species.

Dasyurids are at the crossroads between two unique genetic features, and an unusual set of reproductive and metabolic strategies. There have been more efforts recently to evaluate telomere dynamics from an evolutionary and ecological perspective; in terms of diversity of telomere dynamics, telomere loss due to life-history traits and environmental factors, evolutionary trade-offs between various aspects of telomeres mechanisms and their maintenance, and the significance of telomere lengths within an individual, respective of species [25]. Dasyurids may prove an invaluable research tool in these respects.

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## Conflict of interests

The authors declare that they have no conflicts of interest.

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