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## Mammaliaform extinctions as a driver of the morphological radiation of Cenozoic mammals

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Abstract:	<p>Adaptive radiations are hypothesised as a generating mechanism for much of the morphological diversity of extant species <sup>1,2,3,4,5,6,7</sup>. The Cenozoic radiation of placental mammals, the foundational example of this concept <sup>8,9</sup>, gave rise to much of the morphological disparity of extant mammals, and is generally attributed to relaxed evolutionary constraints following the extinction of non-avian dinosaurs <sup>10,11,12,13</sup>. However, study of this and other radiations has focussed on variation in evolutionary rates <sup>4,5,7,14</sup>, leaving the extent to which relaxation of constraints enabled the origin of novel phenotypes less well-characterised <sup>15,16,17</sup>. We evaluate constraints on morphological evolution among mammaliaforms (mammals and their closest relatives) using a new method that quantifies the capacity of evolutionary change to generate phenotypic novelty. We find that Mesozoic crown-group therians, which include the ancestors of placental mammals, were significantly more constrained than other mammaliaforms. Relaxation of these constraints occurred in the mid-Paleocene, post-dating the extinction of non-avian dinosaurs at the K/Pg boundary, instead coinciding with important environmental shifts and with declining ecomorphological diversity in non-theriimorph mammaliaforms. This relaxation occurred even in small-bodied Cenozoic mammals weighing &lt;100g, which are unlikely to have competed with dinosaurs. Instead, our findings support a more complex model whereby Mesozoic crown therian evolution was in-part constrained by co-occurrence with disparate mammaliaforms, as well as by presence of dinosaurs, within-lineage incumbency effects and environmental factors. Our results demonstrate that variation in evolutionary constraints can occur independently of variation in evolutionary rate; and that both make important contributions to the understanding of adaptive radiations.</p>

Mammaliaform extinctions as a driver of the morphological radiation of Cenozoic mammals

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## Summary

Adaptive radiations are hypothesised as a generating mechanism for much of the morphological diversity of extant species<sup>1,2,3,4,5,6,7</sup>. The Cenozoic radiation of placental mammals, the foundational example of this concept<sup>8,9</sup>, gave rise to much of the morphological disparity of extant mammals, and is generally attributed to relaxed evolutionary constraints following the extinction of non-avian dinosaurs<sup>10,11,12,13</sup>. However, study of this and other radiations has focussed on variation in evolutionary rates<sup>4,5,7,14</sup>, leaving the extent to which relaxation of constraints enabled the origin of novel phenotypes less well-characterised<sup>15,16,17</sup>. We evaluate constraints on morphological evolution among mammaliaforms (mammals and their closest relatives) using a new method that quantifies the capacity of evolutionary change to generate phenotypic novelty. We find that Mesozoic crown-group therians, which include the ancestors of placental mammals, were significantly more constrained than other mammaliaforms. Relaxation of these constraints occurred in the mid-Paleocene, post-dating the extinction of non-avian dinosaurs at the K/Pg boundary, instead coinciding with important environmental shifts and with declining ecomorphological diversity in non-theriimorph mammaliaforms. This relaxation occurred even in small-bodied Cenozoic mammals weighing <100g, which are unlikely to have competed with dinosaurs. Instead, our findings support a more complex model whereby Mesozoic crown therian evolution was in-part constrained by co-occurrence with disparate mammaliaforms, as well as by presence of dinosaurs, within-lineage incumbency effects and environmental factors. Our results demonstrate that variation in evolutionary constraints can occur independently of variation in evolutionary rate; and that both make important contributions to the understanding of adaptive radiations.

**Keywords:** Adaptive Radiation; Constraint; Mammal; Mesozoic; K/Pg Mass Extinction

## 51   **Results**

52           Discrete character state matrices provide a characterisation of morphological variation  
53   that summarises observations from across the skeleton. Analytical methods for this data type  
54   can make use of incompletely-sampled datasets, which are common in fossil record studies.  
55   These have previously been deployed to study patterns of disparity and evolutionary rates in a  
56   wide range of groups<sup>18,19,20</sup> but are relatively underexploited for the study of evolutionary  
57   constraints (but see<sup>21</sup>). Character state matrices provide information about constraints on  
58   phenotypic evolution through the concept of character saturation<sup>21,22,23</sup>, which describes the  
59   effective size of character state space or the limit of novel phenotypes available to a lineage  
60   for a given set of characters. Once this limit is attained, further evolutionary change (i.e.  
61   additional character state transitions), no matter the rate, does not result in expansion of  
62   morphospace<sup>21,22,23</sup>.

63           Character saturation occurs at different levels in different groups, indicating variation  
64   in constraints on morphological evolution among groups and through time. We quantify  
65   levels of character saturation by comparing the morphological dissimilarity of taxon pairs  
66   (pairwise character state dissimilarity) to the inferred character state changes between those  
67   taxa measured along the branches of the phylogeny ('patristic distances'), a measure of the  
68   amount of evolutionary change since divergence from their most recent common ancestor  
69   (see<sup>22,23</sup> for comparable approaches; and see<sup>24</sup> for similar concepts in DNA). Taxon pairs  
70   separated by less evolutionary change (lower patristic distance) are more similar to each other  
71   (lower morphological dissimilarity) and dissimilarity generally increases with increasing  
72   evolutionary change (see Figure S1A for method schematic). However, this relationship  
73   begins to asymptote as further character state transitions are added (patristic distance  
74   increases), an effect of character state saturation. Greater morphological dissimilarity at the  
75   asymptote indicates more relaxed constraints (Figure S1B).

We analysed several large datasets of Mesozoic mammaliaforms<sup>25,26,27,28</sup>. Our analyses reveal considerable variation in constraint among lineages when comparing non-theriimorphs (i.e. stem mammals and Australosphenida) to stem- and crown-therians. Theriimorpha is the total-group of therian mammals<sup>29</sup> comprising stem therians (species more closely related to therian mammals than to Australosphenida) and crown-group therians (marsupials, placentals, their more recent common ancestor and all of its descendants<sup>29</sup>). Loess regression curves used to characterise morphological constraints within non-theriimorph mammaliaforms and within stem therians suggest that neither grouping reached character saturation (Figure 1B). This is confirmed by evaluation of their predicted asymptotic levels of character saturation, quantified as the asymptote ( $V_{\max}$ ) parameter of a Michaelis-Menten curve fitted to the points. Neither stem therians nor non-theriimorphs have reached their respective levels (Table 1), suggesting that both continued to explore novel morphologies throughout their evolutionary histories.

In contrast, Mesozoic crown-group therians show strong evidence of having reached character state saturation (Figure 1B). Furthermore, Mesozoic therian disparity asymptotes at considerably lower morphological dissimilarity than the predicted asymptotes of other mammaliaforms, indicating a more constrained pattern of morphological evolution. The crown therian asymptote is significantly lower than the null expectation derived from simulating character evolution under a uniform, pan-mammaliaform equal-rates model (Table 1), whereas stem therians and non-theriimorphs exceed it.

We conducted additional analysis to address the possibility that high constraint in Mesozoic crown therian evolution resulted from uneven taxon sampling, which focussed on pre-Cretaceous taxa in source matrices based on Zhou et al.<sup>25</sup>. When comparing two taxon partitions of equal size from that matrix, the non-theriimorph Haramyida and the crown therian Eutheria (n=15), eutherians are still found to be under significantly greater

constraint than haramiyids (Table S3). Moreover, simulation studies show that incomplete sampling does reduce the observed  $V_{\max}$  of a dataset or partition (Supplementary Results). However, this is accounted for by our null simulations, which show a broadening the range of expected  $V_{\max}$  values, therefore becoming more conservative with decreasing sample size (Figure S4). This suggests that the risk of false positives due to low sample size is low (Supplementary Results).

Evidence of high constraint in Mesozoic crown therians emerges consistently from analysis of multiple datasets with different taxonomic foci (see Supplementary Methods), including a broad-based matrix of theriomorphs and non-theriomorphs<sup>25</sup>, matrices with a larger sampling of stem-group therians<sup>26</sup> or crown therians<sup>27</sup>, as well as a recent alternative topology for non-theriomorph mammals<sup>28</sup> (Figure 1C; Supplementary Results; Table S3). Assembly of these datasets focussed on characterising morphological variation in Mesozoic mammals but excludes or incompletely characterises the morphology of younger lineages. We therefore focus our interpretations from these matrices on Mesozoic events. These analyses vary in their implications for relative degrees of constraint between non-theriomorphs and stem therians, but generally indicate increased constraint in Mesozoic crown therians where sampling of that group is sufficient to provide reasonable estimates (Figure 1C). The only exception to this occurs in an analysis of Krause et al.<sup>26</sup>, which recovers similar asymptotic disparities for crown therians and non-theriomorphs, with extrapolated asymptotic disparities that fall within the bounds of null simulations (Table S3). Nevertheless, stem therians asymptote at a greater disparity than these, exceeding the null expectation, consistent with the conclusion from other datasets that therians were under enhanced evolutionary constraint when compared to their stem lineage.

Highly constrained morphological evolution in crown therians compared to stem therians is found independently across cranial, dental, and postcranial partitions (Figure S2,

Table S3). Constrained evolution in Mesozoic therians compared to both non-theriimorphs and stem therians is found in cranial and dental partitions. These findings suggest that our results, particularly for comparisons of therians to their stem-group, are not artefacts of the distribution of characters or missing data, or of the varying extent of ecological signal among anatomical partitions.

Variation in the level of constraint (character saturation) depends more strongly on group identity (i.e. crown-therian | stem-therian | non-theriimorph) than on the inferred divergence dates between taxon pairs. Divergence time interval can only explain approximately 5% of the variance in morphological dissimilarity, according to a non-linear generalised least squares analysis (Figure 2; Table S4). This suggests that earlier phylogenetic splits resulted in greater phenotypic divergence than later splits, but that this effect is weak compared to the differences between groups.

Various mammalian radiations have been hypothesised before the diversification of placentals following the K/Pg mass extinction. These include a crown therian radiation associated with the Cretaceous Terrestrial Revolution<sup>30,31,32</sup> and an earlier, mid-Jurassic radiation of crown mammals<sup>8,33</sup>. We find little evidence that either was associated with a substantial relaxation of evolutionary constraints compared to other studied intervals. The divergence time between pairs of taxa explains less than 5% of the variance in morphological dissimilarity when encoded as a binary split, before and after each event (Table S4; Figure. 2), compared to more than 50% explained by patristic distance (Figure 2). Our analyses therefore find no evidence for a relaxation of constraints on overall skeletal evolution among therian mammals following the mid-Cretaceous Terrestrial Revolution and initial diversification of flowering plants (Figure 2C). This contrasts with previous evidence for an expansion of ecological diversity among Late Cretaceous crown-group therians<sup>30,31,32</sup>. However, we note that even the existing datasets of ecologically-relevant mandibular and

dental traits suggest this Late Cretaceous expansion was small compared to later expansions of therian disparity, and was quantitatively a replacement of the ecological disparity of Early Cretaceous non-theriimorphs and stem therians<sup>12,13,30</sup>. Our findings suggest a decoupling of this early episode in therian ecological diversification from their overall patterns of skeletal evolution, which do not show evidence for substantial anatomical novelty.

Patterns of constraint in eutherian (including placental) mammals across the K/Pg boundary were evaluated using comprehensive eutherian data matrices<sup>34,35</sup>. These matrices focussed on characterising morphological variation in late Mesozoic and early Cenozoic eutherians, encompassing the early radiation of placental mammals, and provide strong evidence for relaxed constraints on Cenozoic eutherian (i.e. placental mammal) evolution compared to Mesozoic eutherians (Figure 3A). However, the time-slicing of divergence times before pairwise comparison shows that this shift post-dated the K/Pg boundary extinction of non-avian dinosaurs. Eutherian divergences during the Danian (early Paleocene, 66-61.6 million years ago) asymptote at a similar morphological dissimilarity to those of Mesozoic eutherians, and both are lower than expected from uniform, equal-rate null simulations (Figure 3A, Figure S3, Table 1). Our results show that the release in constraint instead occurred during the latter half of the Paleocene, when projected asymptotic dissimilarity falls within expectation from null simulations (Table 1). Crucially, late Paleocene taxa have not reached character saturation (their curve has not reached its asymptote), despite already attaining greater morphological disparity than in the early Paleocene (Figure 3A). These patterns of variation in constraint through time differ from patterns obtained for rates of morphological evolution by analysis of the same dataset, which were elevated during the earliest Paleocene<sup>11</sup>.

We also classified eutherian mammals into distinct size classes (small, body mass < 100 g; medium, 100 g – 5 kg; large, > 5 kg; see Methods) and examined the morphological

constraints within these different classes. Cenozoic eutherians within all three size classes reach character state saturation at a higher morphological dissimilarity than Mesozoic eutherians (Figure 3B). Furthermore, small-bodied eutherians show the largest relaxation of morphological constraint compared to null simulations; large and medium-sized Cenozoic eutherians both reach character saturation at lower morphological dissimilarity than expected from null simulations, whereas small-bodied Cenozoic eutherians are within the expected range (Table 1). This relaxation of constraints on morphological evolution in small-bodied mammal cannot readily be explained by extinctions of non-avian dinosaurs, which had minimum body sizes of approximately 400g<sup>36</sup>. Furthermore, ecosystem models predict that the competitive effects of adult and juvenile dinosaurs were very weak for non-dinosaurian species weighing less than 100g<sup>37</sup>. If non-avian dinosaurs were solely responsible for constraining the morphological evolution of Mesozoic eutherians, with no role for non-theriimorph mammaliaforms, then the effects of the K/Pg should instead have manifested predominantly as a release of constraints on large and possibly medium-sized eutherians.

Multituberculates (a lineage of stem therians that survived and radiated during the Paleocene) exhibit the inverse pattern of constraint to those observed in eutherians, when analysing a matrix that comprehensively samples Mesozoic and Paleocene taxa<sup>38</sup>. Cretaceous and Danian multituberculates show low levels of constraint as indicated by  $V_{\max}$  values of 0.94 and 0.82 respectively (Figure S3, Table S3). Constraints on multituberculate evolution strengthened substantially during the latter half of the Paleocene, when the observed  $V_{\max}$  falls to 0.69 (Figure S3; Table S3). These shifts are not found to be significant in the null simulations (Table S3), potentially due to the smaller number of characters sampled (130 characters), leading to a wide envelope of simulated null values.

Metatherians, analysed using the matrix of Williamson et al.<sup>39</sup>, show no major shifts in constraint across the K/Pg boundary; character saturation in the Mesozoic and Cenozoic

occurs at similar morphological dissimilarities within the range expected from null simulations (Table S3). Nevertheless, it is not clear whether this is due to a difference in patterns of metatherian evolution compared to that of eutherians (including placentals), rather than more limited sampling of Cenozoic Metatheria and the extremely small number of characters sampled in the studied matrix. Analysis of Mesozoic eutherians and metatherians separately using the matrix of Zhou et al.<sup>25</sup> shows eutherians still under significantly increased constraints but produces a  $V_{\max}$  value for metatherians only just within the limits of the null simulations (Table S3). This may be a result of metatherians being under reduced constraints relative to eutherians during the Mesozoic<sup>30</sup> or due to the smaller taxon sample of Mesozoic metatherians in this matrix leading to broader null estimates (see above)

## Discussion

Our findings suggest that Mesozoic crown-group therians were substantially more constrained than other mammaliaforms, occupying only a small portion of the total mammaliaform character state space. We show that relaxation of this constraint played a central role in the Cenozoic radiation of placental mammals, but that this did not occur until after the Danian, during the second half of the Paleocene, several million years after the extinction of non-avian dinosaurs. This is evident even in small-bodied therians, and from analysis of character states from across the skeleton (Figure 1B) as well as within individual anatomical compartments with more specific ecological relationships such as the teeth, crania and postcrania (Table S3, Figure S2). At least two hypotheses could explain variation in patterns of character state saturation among groups and through time, either (1) intrinsic factors such as inherited developmental or functional limitations on the range of possible forms and evolutionary changes; or (2) extrinsic factors such as ecological or selective forces<sup>20,40,41</sup>. The occurrence of a rapid, time-correlated shift as found here (rather than a

clade-specific shift) cannot readily be explained by relaxation of intrinsic developmental or functional limitations. We therefore focus on examining extrinsic, ecological explanations for this prominent phase-shift in mammalian phenotypic evolution.

A widespread explanation for constrained mammalian evolution in the Mesozoic invokes competition with, or predation by, non-avian dinosaurs. Under this hypothesis, the extinction of non-avian dinosaurs during the K/Pg mass extinction allowed ecological release of mammalian phenotypes<sup>8,16</sup>. This provides only a partial explanation of patterns of mammal evolution. For example, it can explain the attainment of large, dinosaur-like body sizes<sup>42,43</sup>, and the occurrence of phenotypic novelty in large–medium sized mammals during the early Cenozoic. However, it does not explain the different patterns of constraint observed here among Mesozoic crown-group therians compared to other mammaliaforms (Figure 1). Nor does it explain the delay in the release of the constraints on eutherian evolution until the late Paleocene (Figure 3A), or the fact that constraints were released even among the smallest therians, weighing less than 100 g (Figure 3B). Instead, these patterns suggest that Mesozoic crown-group therians were also constrained by the ecological incumbency of other mammaliaform lineages that radiated prior to the origin of the therian crown, excluding crown therians from a broader range of phenotypes. The early Cenozoic radiation of placental mammals can therefore be cast at least in part as an example of an incumbent-replacement dynamic in the aftermath of a mass extinction (e.g.<sup>14,44,45</sup>).

Mesozoic mammaliaforms document the first two-thirds of mammalian evolution and exhibited great ecomorphological diversity, including fossorial, arboreal, aquatic, gliding and predatory forms with a wide range of body masses (mostly < 1 kg, but extending up to ~14 kg), diversifying particularly along locomotor axes<sup>32,46</sup>. The two crown therian clades Eutheria and Metatheria diverged during the Middle-Late Jurassic in the northern hemisphere<sup>33</sup>. A major turnover event during the mid-Cretaceous, coinciding with the

diversification and rise to dominance of angiosperms<sup>30,31,32</sup>, and resulted in extinctions of many non-theriiform groups in the northern hemisphere. This gave rise to a Late Cretaceous mammaliaform assemblage in Laurasia comprising species-rich crown therians co-occurring with ecologically disparate stem therian multituberculates<sup>47,48,49</sup>. Although our dataset is nominally global in scope, most of the record of late Mesozoic and early Cenozoic mammals represents this northern hemisphere assemblage, and especially North America.

Therians and multituberculates both underwent a Late Cretaceous expansion of dental ecomorphology and body size related to the origin of grinding and plant-related diets<sup>30,47,48</sup>. Both groups also continued to diversify in the immediate aftermath of the K/Pg mass extinction<sup>12,30,47</sup>. Multituberculates retained high abundance in North American fossil assemblages<sup>50</sup>, and achieved peak morphological diversity in the early Danian (Pueran)<sup>47,51</sup> with a substantial disparity of ecologies, including arboreal forms<sup>52</sup>, folivores, frugivores<sup>47,53</sup>, omnivores and faunivores<sup>47</sup>, and an expanded range of body sizes<sup>47</sup>. In contrast, therians experienced a delay in their expansion of morphological diversity until after the early Paleocene, based on dental morphology (late Danian<sup>48</sup>) and our analyses of discrete characters. Morphological and inferred ecological disparity of multituberculates declined after the Danian, followed by large decreases in richness during the late Paleocene and extinction in the late Eocene<sup>47,51,54</sup>. The timing of early decreases in the ecological diversity of multituberculates coincides with the timing of release of constraints on therian evolution shown here (Figure S3, Table S3), suggesting that the two events may have been linked. We discuss several hypothesised mechanisms for this (see below), but the most direct is via an incumbent-replacement dynamic between multituberculates and small-bodied therians. This may have been particularly relevant for smaller-body-sized arboreal and plant-based placentals such as early primates and rodents. Indeed, ecological interactions between

multituberculates and placentals have also been proposed during the geographic expansion of rodents into North America in the latest Paleocene and early Eocene (e.g.<sup>50,55</sup> but see<sup>56</sup>).

Mesozoic–Paleocene therians were rare on the southern continents, which generally present a more poorly-sampled fossil record. Instead, Late Cretaceous assemblages of the southern continents were dominated by gondwanatherians and dryolestids<sup>12,26,57,58</sup>. Evidence from South America and Madagascar indicates that gondwanatherians persisted into the middle Eocene<sup>59</sup> and dryolestids into the Miocene<sup>58</sup>. Nevertheless, both groups experienced substantial reductions in their diversity during the K/Pg boundary interval<sup>13,58,60</sup>. Eutherian and metatherian mammals appeared rapidly in the southern continents during the early Cenozoic<sup>61,62</sup> before diversifying in situ into a range of groups with varying ecologies and morphologies, undergoing stepwise radiations with geographic variation in timing and extent, as observed by Osborn<sup>8</sup>. This pattern suggests that relaxation of the geographical incumbency of gondwanatherians and dryolestids allowed geographic expansion of therians in to the southern continents after the K/Pg. This is consistent with our hypothesis that the presence of mammaliaform groups outside the therian crown imposed constraints on the evolutionary diversification of Mesozoic eutherians and metatherians via incumbency, but is much less consistent with a hypothesis of dinosaur incumbency.

This delayed radiation of eutherians, with heterogeneous patterns occurring in different areas, is inconsistent with the extinction of the non-avian dinosaurs being the sole or dominant driver in the release of placental mammals. It should be noted that the hypothesis that extinctions among non-theriimorph mammaliaforms facilitated the Cenozoic placental radiation is not mutually exclusive of others. These include evidence for a more limited ecomorphological expansion of crown therians during the Late Cretaceous<sup>12,13,29</sup>, that extinctions of non-avian dinosaurs at the K/Pg boundary allowed mammals to diversify into a wider range of body sizes<sup>16</sup>, or that the diversification of angiosperms or other environmental

shifts placed important controls on the timing of ecological diversification in mammals for a long interval spanning the Late Cretaceous and Paleogene<sup>31,46,62</sup>. It is also possible that the late Paleocene release of constraints in Eutheria may have been influenced by the climatic warming and the expansion of more productive, angiosperm-dominated forest ecosystems at low and mid latitudes<sup>63,64</sup>. This environmental shift coincided with a major turnover in early Cenozoic mammalian assemblages, including not only the decline of multituberculates, but also of early-diversifying therian groups with more generalised dietary ecomorphology such as phenacodontids and arctocyonids, and the emergence of specialised herbivores such as artiodactyls, perissodactyls and rodents<sup>56,65,66</sup>. Divergent evolutionary responses to environmental change combined with a trend of increasing specialisation might therefore explain some of the patterns shown here even without invoking incumbency effects.

Analyses of rates of evolutionary change have dominated discussion of adaptive radiations since their inception<sup>14</sup> and many recent analyses have focused on identifying early bursts of high rates which then decrease through time as niches are filled<sup>4,5,6,7</sup>. However, although high rates provide the potential for rapid phenotypic divergence, the relaxation of evolutionary constraints must also play an important role in permitting the evolution of novel phenotypes<sup>15,16</sup>. In this framework, ‘rate’ (or tempo) is a driver of evolutionary change along individual lineages, and ‘constraint’ (an aspect of the ‘mode’ of evolution) describes the extent to which those changes result in an overall expansion of morphospace<sup>15,16,17</sup>. Without relaxation of constraints, large amounts of evolutionary change (i.e. high rates) can result in the repeated exploration of a limited morphospace (i.e. convergent evolution), with only rare development of novel phenotypes<sup>15</sup>. In contrast, when constraints are relaxed, then evolutionary change is expected to more frequently generate novel phenotypes.

Despite its clear importance in controlling the development of novel phenotypes, relatively little attention has been given to patterns of constraint and how they vary between

groups and through time. The method presented here provides a new approach for quantifying the evolution of morphological constraints, using data drawn from the entire skeleton. Our results illustrate the importance of variation in constraint among groups and through time in permitting major evolutionary radiations. Furthermore, we find that patterns of variation in constraint are at least partially independent of variation in evolutionary rate; increased rates at the origin of therians co-occurred with strong constraints and did not lead to increased disparity<sup>12,30</sup>. Moreover, the Cenozoic radiation of eutherian mammals is characterised by increased rates during the earliest Cenozoic<sup>11</sup>, but relaxation of constraints did not occur until slightly later, during the late Paleocene (Figure 3A). The contrast between these two events, and our findings regarding constraints on the Cenozoic mammal radiation, both highlight the importance of a more complete characterisation of phenotypic macroevolution for understanding the origins of the great diversity of organismal form.

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351 **Author contributions**

352 Conceptualisation: NB, RBJB

353 Methodology: NB, RBJB

354 Data: NB, EP, GLB

355 Analysis: NB

356 Writing: NB, EP, GLB, RBJB

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358 **Declaration of interests**

359 The authors declare no competing interests.

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361

362 **Figures**363 Figure 1: **Patterns of Constraint in Mesozoic Mammaliaformes**

364 A) Phylogeny of Mesozoic mammals used in analyses with branch lengths scaled to the

365 number of character changes, based on the character matrix of Zhou *et al.*<sup>25</sup>. Node labelled I

366 = Mammaliaformes; II = Australosphenida; III = Theriimorpha; IV = Crown Theria; V =

367 Metatheria; VI = Eutheria. Colours represent the subsets used in analyses: black = non-

368 theriimorph mammals; red = stem Theria; blue= crown Theria. Lilac used in Figures 2 and 3

369 for Eutheria only. B) Comparison of patristic distances and morphological dissimilarities

370 between pairs of Mesozoic mammaliaforms, calculated from the character matrix of Zhou *et*371 *al.*<sup>25</sup>. Each point represents a pairwise comparison of two taxa, and the lines represent loess

372 fitted regression curves. Black squares represent pairs of Australosphenidans. C)

373 Comparisons of the Loess regression curves produced by analyses of different matrices.

374 Numbers indicate reference of dataset used in each study. Analysis marked \* had the

phylogeny constrained so that haramiyidans were stem therians. Silhouettes from  
 phylopic.org shared under Creative Commons Liscence  
<https://creativecommons.org/licenses/by-nc/3.0/> (*Juramaia*, *Castorocauda*, *Cimolodonta* –  
 Nobu Tamura), Public Domain Dedication 1.0 license (*Sinodelphys*), or No Copyright  
 (*Fruitafossor*)

**Figure 2: Variation in Patterns of Constraint in Mesozoic Mammaliaformes Through Time**

Comparison of patristic distances and morphological dissimilarities between pairs of  
 mammaliaforms, calculated from the matrix of Zhou et al.<sup>25</sup>, separating pairs by divergence  
 dates. Darker colours represent more recent divergence dates. A) Non-theriimorph  
 mammaliaforms; B) Stem therian mammals; C) Crown therian mammals. See also Table S2

**Figure 3: Patterns of Constraint in Cretaceous and Cenozoic Eutherians**

Comparison of patristic distances and morphological dissimilarities, calculated from the  
 matrix of Halliday *et al.*<sup>34</sup>, between pairs of eutherian mammals, separating pairs by A)  
 divergence date, and B) size category. Mesozoic Eutheria are all small apart from two  
 medium sized taxa (not included). Silhouette from phylopic.org (no copyright). See also  
 Figure S3, Table S3

## Tables

**Table 1: Point of character saturation in different mammaliaform lineages**

Morphological dissimilarity between pairs of taxa at the point of character saturation, estimated as the  $V_{\max}$  parameter of a Michaelis-Menten curve fitted to comparisons of patristic distances and morphological dissimilarities between the pairs. Null expectation calculated by simulating character evolution over a phylogeny under an equal rates models. See also Table S1

Data-set	Partition	Observed $V_{\max}$	$V_{\max}$ values expected from null simulation: Median (range)
25	Non-theriimorph mammaliaforms	1.11	0.70 (0.68-0.73)
25	Stem therian mammals	0.93	0.85 (0.80-0.90)
25	Crown therian mammals	0.64	0.75 (0.69-0.80)
34	Mesozoic Eutheria	0.72	0.80 (0.77-0.83)
34	Danian Eutheria	0.74	0.79 (0.76-0.81)
34	Late Paleocene Eutheria	0.85	0.86 (0.82-0.90)
34	Eocene-Recent Eutheria	0.84	0.83 (0.81-0.85)
34	Mesozoic small Eutheria	0.62	0.86 (0.81-0.89)
34	Paleogene small Eutheria	0.87	0.91 (0.83-0.98)
34	Paleogene medium Eutheria	0.80	0.90 (0.87-0.94)
34	Paleogene large Eutheria	0.76	0.91 (0.86-0.95)

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411 **STAR METHODS**

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413

414 **RESOURCE AVAILABILITY**

415 *Lead Contact* – Further information and requests for resources and reagents should be  
416 directed to and will be fulfilled by the Lead Contact: Neil Brocklehurst

417 ([neil.brocklehurst@earth.ox.ac.uk](mailto:neil.brocklehurst@earth.ox.ac.uk))

418

419 *Materials Availability*

420 This study did not use or generate any new material.

421

422 *Data and Code Availability*

423 The Nexus files employed are presented in Supplementary Data 1-8. All analyses were  
424 carried out in MrBayes and R<sup>67,68</sup> using functions from packages cited in the Key Resources  
425 Table. The script is available in Supplementary Data 12. These have been deposited in Dryad  
426 (<https://doi.org/10.5061/dryad.bk3j9kdbt>)

427

428 *Experimental Models and Subject Details*

429 Datasets employed were taken from references<sup>12,26,27,28,34,35,38,39</sup>. The nexus files employed  
430 are found in Supplementary Data 1-10

431

432 **METHOD DETAILS**

433

## 434 *Data*

435       The morphological character matrices under study represent extensive samplings of  
 436 morphological data from Mesozoic and Cenozoic mammaliaforms. Four matrices, which  
 437 between them contain the largest available sample of taxa across a broad range of Mesozoic  
 438 mammaliaform clades, were chosen to compare patterns between Mesozoic crown therians,  
 439 stem therians and non-theriimorph mammaliaforms, indicated in Figure 1C as “Zhou et al.  
 440 2019”<sup>25</sup>, “Krause et al. 2020”<sup>26</sup>, “Bi et al. 2018”<sup>27</sup>, and “King and Beck 2020”<sup>28</sup>. The Zhou et  
 441 al.<sup>25</sup> matrix (Taxa – 126, Characters 556) contains a broad coverage of the three  
 442 mammaliform subgroups (non-theriimorphs, stem therians and crown therians), but is limited  
 443 in its sampling of certain stem mammal clades such as dryolestids and multituberculates, and  
 444 late Cretaceous crown therians. The Bi et al.<sup>27</sup> matrix (Taxa – 64, Characters – 401) contains  
 445 no taxa outside theriimorphs, but a broader sampling of crown and stem therian taxa. Krause  
 446 et al.<sup>26</sup> (Taxa – 82, Characters – 530) contains a more restricted sampling of crown Theria,  
 447 but a more extensive sampling of stem therians, in particular multituberculates and  
 448 Gondwanatheria. The King and Beck<sup>28</sup> matrix (Taxa – 96; Characters – 507) contains a very  
 449 similar sampling to the Zhou et al.<sup>25</sup> matrix, but their analysis using the Fossilised Birth  
 450 Death model produced a novel topology with polyphyletic haramiyidans, and so analysis was  
 451 performed with phylogenetic relationships constrained to this topology. Although no  
 452 individual matrix present an even sample of morphological variation among Mesozoic  
 453 mammals, our finding of similar patterns regardless of dataset choice suggests that these  
 454 biases do not overwhelm the underlying signal of constraint in Mesozoic therians compared  
 455 to other groups.

456       To compare patterns of constraint in crown therians across the Cretaceous/Paleogene  
 457 boundary, two comprehensive datasets covering Eutheria were used: Manz et al.<sup>35</sup> and  
 458 Halliday et al.<sup>34</sup>. The Manz et al. study (Taxa – 81; Characters 415) is a recent iteration of the

matrix of Wible et al.<sup>75</sup>, designed to resolve the relationships of Cretaceous eutherians. Halliday et al.<sup>34</sup> (Taxa – 177; Characters – 681) sampled a substantially greater number of Paleocene taxa. The results from these were compared to those from Williamson et al.<sup>39</sup>, which broadly samples Mesozoic and early Cenozoic Metatheria (Taxa – 95; Characters – 85), and Wang et al.<sup>38</sup>, which samples multituberculates over a similar interval (Taxa – 51; Characters – 130).

To generate the phylogenetic framework for the analyses, the matrices were subjected to a Bayesian phylogenetic analysis in MrBayes v3.2.6<sup>67</sup>. The analyses were run for 10,000,000 generations, sampling every 1000. Four runs, each with four chains, were employed to ensure convergence. A variable rates model of character evolution, with rates drawn from a gamma distribution, was used. The analysis was undated so that branch lengths would represent number of character transitions rather than time. The maximum clade credibility (MCC) tree, with 25% of the posterior sample of trees discarded as burn in, was used in subsequent analyses. Non-mammaliaform taxa were dropped from the tree (we adopt Mammaliaformes as comprising the most recent common ancestor of *Sinoconodon* and modern mammals, and its descendants<sup>76</sup>). Cenozoic mammals were dropped from the trees produced using the Mesozoic mammaliaform matrices because these contained only a limited and non-representative sampling of mostly extant Cenozoic mammals and their relevant characters (see Table S1 for taxa included), and document events in morphological evolution extending after the end of our study interval. Note that the King & Beck<sup>28</sup> analysis produced a novel set of relationships not seen in other studies by analysing their matrix using the Fossilised Birth Death Model; the relationships in the analysis of their matrix were constrained to this novel topology.

#### *Character Saturation*

Pairwise patristic distances between each taxon in the MCC tree were calculated in R 3.6.1<sup>68</sup> using the *distTips()* function in the package *adephylo*<sup>70</sup>. The morphological dissimilarity between the pairs of taxa were calculated using the *MorphDistMatrix()* function in the package *Claddis*<sup>20</sup>, using the maximum observable rescaled distance (MORD) metric. A loess regression line was calculated in R comparing the pairwise patristic distances and morphological dissimilarities (See Figure S1 for method schematic).

#### *Temporal partitions*

We used a time-slicing approach based on node ages to test whether more ancient divergences of Mesozoic mammals accumulated more morphological variation, and the effects of the Cretaceous Terrestrial Revolution on crown therian evolution. Our approach subdivides the pairwise comparisons of patristic and morphological distances calculated from the character matrix of Zhou et al.<sup>25</sup>, subdividing according to the interval in which the lineages diverged (i.e. the ‘node age’, or divergence date). Divergence dates were calculated in the R package *paleotree*<sup>69</sup> using the minimum branch length (MBL) method, with a minimum branch length of 1 million years. The intention here was to obtain approximate dates that can be used to assign divergence times to relatively coarse, epoch-level time bins. The same approach was used to assess the eutherian, metatherian and multituberculate matrices<sup>34,35,38,39</sup>, subdividing time into Cretaceous, Danian, Late Paleocene, and Eocene-Recent.

#### *Anatomical Partitions*

To examine whether the patterns of constraint are consistent across anatomical partitions, and thus show whether the distribution of characters and missing data may have affected the inferences, the characters were all assigned to one of three partitions: Skull, Dental and

Postcranial. For each partition, a matrix restricted to the characters within the partition was analysed in MrBayes in using the same protocols and parameters as described in the main text, but constraining the tree topology to that found in the analysis of all characters. This produces a tree whose topology is identical to that of Figure 1, but whose branch lengths represent only character changes within the relevant partition. Character saturation was then assessed as described (Figure S2).

### *Body size partitions*

Patterns of constraint within different size classes were assessed in Eutheria using the matrix of Halliday *et al.*<sup>34</sup>. Body masses of Mesozoic and Paleogene taxa were estimated using published regressions of body mass on m1 measurements derived from extant species<sup>77,78</sup>. Two separate regressions were used to account for the differences in these relationships in artiodactyls and perissodactyls<sup>37,78</sup> and in all other mammals<sup>77</sup>. This follows the approach used by other studies of mammalian body size evolution in the fossil record<sup>42</sup>.

Only m1 length was used to estimate body mass in artiodactyls and perissodactyls based on previous studies that showed this measurement correlates best with body mass in extant species<sup>42,78</sup>, according to equation 1:

$$\text{Equation 1. } \ln(\text{massm1}) = 1.24 + (3.11 * (\ln(\text{lengthm1})))$$

A generic body mass regression for all mammals<sup>77</sup> was applied to all species in the dataset except artiodactyls and perissodactyls:

$$\text{Equation 2. } \ln(\text{massm1}) = 1.81 + (1.827 * (\ln(\text{lengthm1} * \text{widthm1})))$$

Body mass estimates were used to categorize species in our dataset as ‘small’, ‘medium’, or ‘large’. The cut off size for each size class was based on the distribution of eutherian mammal body sizes throughout the Mesozoic. ‘Small’ mammals are equivalent in mass to the most common Mesozoic mammals (below 100 g). Mammals of this mass are small enough that they are unlikely to have directly competed with non-avian dinosaurs in the Cretaceous; the smallest adult specimen of a non-avian dinosaur known has been estimated at 400 g<sup>36</sup>. ‘Medium’ mammals have body masses from 100 g to 5 kg. This represents body sizes which are rare but present, both in non-avian dinosaurs and Mesozoic mammaliaforms, and includes the largest Mesozoic eutherians that may have competed both with dinosaurs and other Mesozoic mammaliaforms. ‘Large’ mammals are larger than 5 kg, and by definition include only Cenozoic eutherian species, representing an almost exclusively novel body size class for Cenozoic mammals. Strict conceptions of the dinosaur-incumbency hypothesis predicts that most morphological novelty should occur among large, and potentially medium, bodied mammals.

## QUANTIFICATION AND STATISTICAL ANALYSIS

To provide an estimate of the point of character saturation of different groups, a Michaelis-Menten curve (which tends to an asymptote) was fit to the pairwise comparisons of taxa using the *drm()* function in the R package *drc*<sup>74</sup>. The  $V_{\max}$  parameter was used as an estimate of the morphological dissimilarity at which character saturation occurred.

The observed point of character saturation identified by the Michaelis-Menten curve fittings were compared to null simulations of character evolution. For each character an estimate of the instantaneous probability of change was fit to the phylogeny using the *fitMk()*

function in the R package *phytools*<sup>71</sup>, assuming an equal rates model. Using these probabilities, an evolutionary history of each character was simulated over the phylogeny, producing a set of character scores for each tip. These were used to calculate morphological dissimilarities between the taxa as above. The null morphological dissimilarities were compared to the patristic distances again by fitting a Michaelis-Menten curve. This process was repeated 1000 times, producing a null distribution of character saturation estimates which can then be compared to the observed.

Finally, the relative impact of divergence time and character change frequency on the morphological dissimilarities between pairs of taxa was tested using generalised least squares, comparing morphological dissimilarity to the square root of the patristic distance (root transformed since the curve should tend to an asymptote) and divergence time. This was carried out using the *gls()* function in the R package *nlme*<sup>72</sup>. The relative importance of the variables was calculated by averaging  $R^2$  contributions over ordering of regressors<sup>79</sup>, implemented in the R package *relaimpo*<sup>73</sup>. Three models were tested: one where divergence time was a continuous character; one where it was treated as binary: diverging before or after the Early Jurassic, at which point a significant rate increase occurred in mammals<sup>33</sup>; and one where it was treated as binary diverging before or after the end of the Cretaceous Terrestrial Revolution (80 mya)<sup>30</sup>.

## SIMULATION ANALYSIS

As discussed above, incomplete taxon sampling has the potential to reduce the observed character state space. It might be expected, therefore, that incomplete taxon sampling within a dataset or partition might be expected to lead to inference of increased morphological constraint. To examine this possibility, we carried out further analyses on the Krause et al.<sup>26</sup> matrix. The entire taxon sample produced an observed  $V_{\max}$  (0.86) within the

range of values observed in null simulations (0.83-0.88) i.e. significantly strengthened/relaxed constraints are not supported. To test whether incomplete taxon sampling could result in a false inference of significantly strengthened constraints (i.e. false positives), taxa were deleted at random. The observed  $V_{\max}$  and range of null simulations were calculated using the new taxon sample. This process was carried out with 25%, 50 % and 75% of taxa deleted, with 1000 replicates each time.

Simulations of incomplete taxon sampling show little evidence that this leads to false positive inferences of strengthened constraint. Only one out of the 1000 simulations with 25% of taxa deleted falsely indicated significantly strengthened constraint. With 50% deleted the number was four, and with 75% the number was 12. It is true that the observed  $V_{\max}$  values showed considerable variation and were usually lower when large numbers of taxa were deleted. However, under such circumstances the null values were also lower and the range of simulated values was wider (Figure S4), making the inference of significant strengthening of constraints more conservative. This set of simulations validates the approach of using null simulations to test for significant variation in constraints, even when there are concerns about taxon sampling within a dataset or partition. However, these results do also indicate that the observed  $V_{\max}$  values should not be used alone as an indicator of constraint; they are likely not directly comparable between different datasets or partitions. Rather they must be used in conjunction with null simulations.

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Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited Data		
Zhou et al character/taxon matrix	12	Supplementary data file 1
Krause et al character/taxon matrix	26	Supplementary data file 2
Bi et al. character/taxon matrix	27	Supplementary data file 3
King and Beck character/taxon matrix	28	Supplementary data file 4
Halliday et al. character/taxon matrix	34	Supplementary data file 5
Manz et al. character/taxon matrix	35	Supplementary data file 6
Williamson et al. character/taxon matrix	39	Supplementary data file 7
Wang et al. character/taxon matrix	38	Supplementary data file 7
Body size data	This Paper	Table S5
Software and Algorithms		
MrBayes	67	3.2.6
R	68	3.6.1
paleotree (R package)	69	3.3.0
adephylo (R package)	70	1.1.7
Claddis	20	0.3.0
phytools (R package)	71	0.6
nlme (R package)	72	3.1
relaimpo (R package)	73	2.2.3
drc (R package)	74	3.0.1
Analysis script	This paper	Supplementary data file 12





