Decay of vertebrate characters in hagfish and lamprey (Cyclostomata) and the implications for the vertebrate fossil record

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The timing and sequence of events underlying the origin and early evolution of vertebrates remains poorly understood. The palaeontological evidence should shed light on these issues, but difficulties in interpretation of the non-biomineralized fossil record make this problematic. Here we present an experimental analysis of decay of vertebrate characters based on the extant jawless vertebrates (Lampetra and Myxine). This provides a framework for the interpretation of the anatomy of soft-bodied fossil vertebrates and putative cyclostomes, and a context for reading the fossil record of non-biomineralized vertebrate characters. Decay results in transformation and non-random loss of characters. In both lamprey and hagfish, different types of cartilage decay at different rates, resulting in taphonomic bias towards loss of ‘soft’ cartilages containing vertebrate-specific Col2A1 extracellular matrix proteins; phylogenetically informative soft-tissue characters decay before more plesiomorphic characters. As such, synapomorphic decay bias, previously recognized in early chordates, is more pervasive, and needs to be taken into account when interpreting the anatomy of any non-biomineralized fossil vertebrate, such as Haikouichthys, Mayomyzon and Hardistiella.

Keywords: experimental taphonomy; vertebrate; cyclostomes; decay bias; cartilage evolution

1. INTRODUCTION
Nearly all vertebrate fossils preserve only biomineralized skeletal remains, but the comparatively rare specimens that preserve vertebrate soft-tissue characters are among the most iconic fossils known. This is especially true of remains from the early phase of vertebrate evolution, predating the widespread occurrence of phosphatic skeletal tissues. Most vertebrate crown-group apomorphies are soft-tissue, ultrastructural and embryological characters [1,2]. Without fossil remains of early vertebrates and their soft tissues, we would remain ignorant of the timing and sequence of character acquisition through the vertebrate stem, the base of the gnathostome stem and the cyclostome stem; we would have no temporal or ecological context for early vertebrate evolution and the assembly of the vertebrate body plan. These are landmark events in the history of life that took place against a backdrop of rapid molecular evolution and genome duplication [3,4]: testing the hypothesis of a causal relationship between increasing genomic and morphological complexity requires data from the fossil record [5]. Recent work, for example, has identified the presence of two Col2A1 genes (expressed as type 2 collagen—Col2α1) in lampreys and hagfish as an important innovation in vertebrate skeletal development, possibly linked to genome duplication [6–8]. This work also highlighted the need to combine fossil evidence of cartilages in early vertebrates with developmental data from extant homologues if skeletal development in the vertebrate common ancestor is to be accurately reconstructed [9]. Furthermore, Early Cambrian fossils identified as vertebrates on the basis of non-biomineralized characters [10–12] provide the best constraints on the minimum date of divergence of vertebrates from invertebrates, and deuterostomes from protostomes [13].

Clearly, the remains of non-biomineralized vertebrates have considerable evolutionary significance. This significance is entirely dependent upon correct phylogenetic placement of the fossils, which is in turn contingent upon robust interpretations of anatomical characters, yet this is problematic. Although exceptional preservation of non-biomineralized animals, including vertebrates, provides evidence of fossil anatomy that would otherwise remain unknown, post-mortem decay means that those anatomies are never preserved complete. Determining whether characters are absent from a fossil because they decayed away or because they had yet to evolve is therefore critical, yet difficult [14]. Decay, and in particular decay-induced collapse, also means that the shape and topological relations between characters can change, creating further difficulties for the recognition of homologous anatomical characters, especially if diagenetic stabilization of recalcitrant organic remains [15,16] occurs only after considerable decay. The complex interplay of decay and fossilization represents a double-edged sword, acting both to enhance preservation through bacterial mediation of rapid authigenic mineralization, e.g. [17], and to remove and distort anatomical data through collapse and decomposition of morphological structures.

Understanding decay is therefore of paramount importance to analysis of non-biomineralized fossils and their characters. Decay data can establish a time line,
revealing: (i) characters that are lost so rapidly through decay that they are unlikely ever to become preserved, (ii) characters composed of relatively labile tissues that have the potential to be preserved through rapid authigenic mineralization, and (iii) recalcitrant characters that have the potential to persist in the geological record as diagenetically stabilized organic biomolecules. Previous analyses of organismal decay have focused upon transformation of organisms as a whole in terms of general stages of decay [18–22], but we have developed a different approach: by focusing upon how and when each of the phylogenetically informative morphological characters (synapomorphies) of an organism decay, we are able to distinguish phylogenetic absence from taphonomic loss, and recognize partially decayed characters [23]. This technique, as applied to extant proxies for early chordates (cephalochordate and larval lamprey), has revealed synapomorphic decay biases: plesiomorphic morphological characters were found to be significantly more decay resistant than the more phylogenetically informative characters diagnostic of particular chordate clades [23]. The early decay of diagnostic, synapomorphic characters can result in their non-preservation, and this bias, if unrecognized in fossils, will result in their incorrect phylogenetic placement on the stems of the crown groups to which they truly belong, and thus distort our reading of the record.

Here, we present an experimental investigation of the decomposition of extant representatives of the jawless vertebrates, or cyclostomes (lamprey and hagfish). This allows us to characterize the sequences of morphological decay and loss of synapomorphies, and provide a framework for interpretation of character preservation and loss in non-biomineralized fossil vertebrates. These data also provide a test of whether the synapomorphic decay bias identified in early chordates—'stem-ward slippage'—is a more widespread phenomenon.

Conflicting evidence regarding whether lampreys are more closely related to gnathostomes (generally supported by morphological and physiological data [24–27], cf. [28]) or form a clade with hagfish (supported by molecular data, including nuclear DNA, mtDNA, rDNA and miRNA [29–31], figure 1) is currently obscuring patterns of character acquisition in early vertebrate evolution and results in considerable ambiguity in the placement of important fossil taxa [32]. Better understanding of soft-tissue character combinations and constraints on taphonomic character loss in fossil non-biomineralized vertebrates, including fossil lampreys and hagfish, has the potential to shed new light on this issue.

Figure 1. Phylogeny of vertebrates and hierarchical character classifications. Dashed lines represent alternative resolutions (cyclostome monophyly/paraphyly).

2. MATERIAL AND METHODS

Larval lampreys (Lampetra fluviatilis, 83 individuals, 0.19–2.39 g) at a variety of pre-metamorphosis developmental stages were collected from the River Ure, Yorkshire, in November 2008, by extracting and sifting through river sediments [23]. They were kept overnight in aerated water and transported live to Leicester. Atlantic hagfish (Myxine glutinosa, 48 individuals, 15–41 g) were collected in March 2009, using baited traps in the coastal waters (depth 100–120 m) off West Sweden, near Tjärnö Marine Biological Laboratory. They were kept overnight in circulating sea water, euthanized in the morning and then transported, chilled, to Leicester in under 24 h.

All experimental animals were euthanized using an overdose of tricaine methanesulphonate (MS222; 2 mg ml⁻¹ with buffer). Some previous studies of decay have used asphyxia to kill animals, but MS222 treatment does not adversely affect bacterial flora [33] and our previous results with Branchiostoma demonstrate that using MS222 has no effect on the patterns or rate of decay [19,23].

The decay experiment methodology generally follows that of Sansom et al. [23]. Specimens were placed in individual 1028 or 182 cm³ clear polystyrene containers with lids, filled completely with either artificial sea water solution (hagfish) or filtered de-ionized fresh water (lamprey), reflecting the natural conditions in which organisms lived. In order to minimize the number of experimental variables, no bacterial inocula were added. No attempt was made to disrupt the endogenous bacteria of the specimen. Individual containers were sealed with silicon grease (Ambersil M494), thus limiting oxygen diffusion. No attempt was made to remove oxygen from the water, but irrespective of starting conditions, oxygen concentrations in decay experiments are known to rapidly converge upon anoxia [18,34]. Containers were kept in cooled incubators at 25°C (±1°) and destructively sampled over the course of 200 days, three per interval (or two per interval for hagfish owing to limited specimen numbers). In order to capture early rapid decay, sampling frequency was initially high, reducing as the rate of decay declined. At each sampling interval, each specimen was photographed and pH and weight were measured. Specimens were dissected and the condition of anatomical characters was logged and described. A plastic mesh floor (pores less than 2 mm²) in each container facilitated extraction of specimens; remaining liquid was sieved for small disarticulated anatomical remains.

Hagfish and adult lamprey morphologies are outlined in figure 2a. Anatomical characters were categorized a priori according to the nested, hierarchical clade for which they are synapomorphic (electronic supplementary material, figure S1). For morphological decay, each character for each sample for each interval was scored according to
Figure 2. Anatomy and decay of cyclostomes. (a) Adult lamprey (left) and hagfish (right) anatomy with reconstructions of the morphology at the end of decay stages 1–5. Red represents hard cartilage, blue is soft cartilage, purple is undetermined cartilage type and green is keratinous tissues. (b) Decay sequences for adult lamprey (left) and hagfish (right) through time (days). Observations of the decay of each character (yellow, pristine; orange, decaying; red, onset of loss; terminal point, complete loss) are used to rank them according to last occurrences. For each character, the decay ranks (left) and synapomorphic ranks (right) used to test synapomorphic biases are shown. Asterisk marks skeletal characters with H for hard or S for soft cartilage type where relevant. Profiles of putative fossil vertebrates and cyclostomes are illustrated on the right. Green bars represent described preserved characters in fossil taxon. Thin blue bars represent absent characters which we would expect to find given the decay rank and synapomorphic rank of other characters preserved (light blue for potentially phylogenetically absent synapomorphies, dark blue for absent plesiomorphies).
three defined categories: pristine (same condition as at death), decaying (morphology altered from that condition at death) or lost (no longer observable or recognizable). Characters are ranked according to the timing of their loss.

Correlation between the hierarchical level at which an anatomical character is synapomorphic and the rank of that character in the sequence of loss through decay was tested using Spearman’s rank correlation ($R_s$). The null hypothesis is that there is no correlation between the rank order of decay of characters and the synapomorphic rank order of characters. A $p$-value of less than 0.05 is interpreted as significant.

3. RESULTS

(a) Character loss and stages of decay

General stages of morphological decay are identified on the basis of gross morphological changes and average patterns of character loss observed across the numerous trials. They serve as a narrative tool only. Decay stages are summarized below and illustrated in figure 3. The sequence of morphological character loss through time is shown in figure 2.

(i) Larval lamprey (ammocoetes)

The general stages of decay in the larval lamprey were identified by Sansom et al. [23] and are illustrated in detail in figure 3c. Stage 1 decay, days 0–5: a biofilm forms around the body. Stage 2 decay, days 5–11: the branchial region collapses. Stage 3 decay, days 11–28: the head cartilages (trabecular and otic) are lost. Stage 4 decay, days 28–90: all the remaining features of the head are lost. Stage 5 decay, days 90–130: the ventral surface of the trunk decomposes and fins are lost. Stage 6 decay, day 130 onwards: only the trunk myomeres, notochord and pigmented skin remain.

(ii) Adult lamprey

Stage 1 decay, days 0–15: a biofilm forms and the eyes become clouded. Stage 2 decay, days 15–28: onset of loss of cranial and axial characters. Stage 3 decay, days 28–90: the branchial region softens and collapses. Stage 4 decay, days 90–200: the branchial region is lost and the head begins to collapse. Stage 5, days 200–300: loss of all cartilaginous tissues. Stage 6, day 300 onwards: only the notochord, muscles and keratinous teeth remain. The keratinous teeth from different parts of the lamprey feeding apparatus are affected differently during decay: the lingual teeth become disarticulated at stage 2, while the inferior and superior teeth articulated with the annular cartilage (figure 3b) persist into the later stages of decay, even after the annular cartilage is lost.

(iii) Hagfish

Stage 1 decay, days 0–2: a biofilm forms and the gut and ventral surface of the body leading to loss of the slime glands and oesophagocutaneous duct. Stage 2 decay, days 2–6: the anteriormost part of the head is lost; along the sometimes coiled trunk, skin loss exposes the dorsal musculature; ventral musculature is lost. Stage 3 decay, days 6–15: all trunk soft tissues except the notochord and dorsal nerve cord are lost (e.g. gill pouches, myomere shape); some myomers towards the head remain. Stage 4 decay, days 15–90: the soft tissues associated with the head are lost, exposing articulated skeletal elements. Stage 5 decay, days 90–200: the notochord and more resistant head tissues remain. Stage 6 decay, day 200 onwards: only the keratinous teeth, some of the notochord and parts of the disarticulated lingual cartilage remain. The outer sheath of the dental elements/cusps is more resistant than the pulp, which is softened and lost during decay.

(b) Decay of cartilage characters

Since the earliest detailed anatomical work on cyclostomes, the elements of their endoskeleton have been classified as ‘soft’ and ‘hard’ cartilages based on colour, histology and staining characteristics [35–39]. Our results demonstrate that decay of these cartilages is non-random. In ammocoetes, the soft cartilages (e.g. branchial cartilage) are lost early in decay while the hard cartilages (e.g. otic capsules and skull cartilages) are lost later [23]. In the adult lamprey, the soft cartilages (e.g. pericardial and branchial) are also the first to be lost, while the hard cartilages (e.g. annular, cranial, lingual) are more resistant. Arcualia are the only hard cartilages that are lost before soft cartilages. Hagfish exhibit a similar pattern, with all soft cartilages (e.g. tentacles, prenasal sinus and velar) being lost before hard (e.g. cranial and lingual). Decay in the hagfish thus causes disarticulation of the otic capsules and lingual cartilages, and alteration of the shape of the skull (loss of cornual and branchial arch cartilages, often leaving a disarticulated palatine) and subnasal cartilage (loss of the anterior fork; figure 3c).

Statistical analysis confirms this pattern for adult lamprey (cartilage character $n = 10$) and hagfish (cartilage character $n = 13$). The hypothesis that hard and soft cartilages decay at the same time (based on rank order of decay) can be rejected for both taxa ($p < 0.05$; results significant whether ANOVA or non-parametric tests are employed). Mosaic cartilages (e.g. dentigerous cartilage) are coded as hard because it is the hard part that persists and is the basis for the decay rank assigned. For the lamprey, fin ray and trematic ring cartilages have not been characterized and are thus not included. The ammocoete has too few cartilaginous characters for statistical investigation.

(c) Synapomorphic decay bias

Ammocoetes and Amphioxus exhibit non-random character decay, with a strong synapomorphic decay bias evident from the Spearman rank correlation coefficient.
of decay rank and the phylogenetic rank of a character (amphioxus $R_s = 0.70, p = 0.0018, n = 17$; ammocoete $R_s = 0.70, p = 0.000019, n = 30$) cf. [23]).

The null hypothesis that decay is random with respect to phylogenetic informativeness is also rejected for the adult lamprey: decay rank and synapomorphic rank are significantly correlated ($R_s = 0.38, p = 0.013, n = 42$). Exclusion of characters with skeletal components (i.e. cartilaginous and keratinous tissues) increases the strength of the correlation ($R_s = 0.45, p = 0.018, n = 27$). Testing
character decay data for hagfish (figure 2b) reveals a more complex pattern. Taking all characters into account, decay rank and phylogenetic rank are not significantly correlated ($R_c = 0.37, p = 0.06, n = 32$). However, exclusion of characters with skeletal components (cartilaginous and keratinous) reveals that decay of soft-tissue characters is non-random, with a significant correlation between decay and synapomorphic rank ($R_c = 0.73, p = 0.0011, n = 17$).

4. DISCUSSION
(a) Cartilage decay, skeletal development and genome duplication
The cartilages of lamprey and hagfish were long thought to be non-collagenous [38–41], but recent work has demonstrated the presence of collagen type 2a1 protein as a major component of the cartilaginous extracellular matrix (ECM) in both clades [6–8]. Ammocoetes and tunicates lack these ECM proteins, and the evolution and development of the specialized skeletal system enriched with different ECM proteins is thought to represent a significant innovation in vertebrates. Lampreys and hagfish have two Col2A1 orthologues while Ammocoetes and tunicates have just one ancestral clade A fibrillar collagen gene [8], leading to the hypothesis that innovations in skeletal development, a defining feature of vertebrates, reflect the genome duplication event inferred to have occurred on the vertebrate stem [6–9]. The fossil record of exceptionally preserved non-biomineralized vertebrates has an important role to play in constraining the time of origin of vertebrate cartilages (and thus the genome duplication), and in reconstructing cartilage development in the vertebrate common ancestor [9,42].

Our results have a direct bearing on this issue because gene expression patterns of Col2A1 differ between the hard and soft cartilages of cyclostomes: Col2a1 is a component of soft cartilages, but not hard [7,8]. Recognition in fossils of homologues of skeletal elements which in cyclostomes are composed of soft cartilage thus has the potential to constrain the timing of Col2a1 evolution and genome duplications. The results of our decay experiments, however, sound a note of caution: soft cartilages are more decay prone. Absence of soft cartilage characters from a fossil that contains hard cartilage characters cannot be assumed to have phylogenetic significance or to have predated genome duplication events. It might equally reflect taphonomic loss of soft cartilage. This is especially pertinent when preservation of non-biomineralized taxa does not involve early mineralization and only the more recalcitrant tissues remain, as may be the case in some Burgess Shale-type fossils [43]. The absence of soft cartilage structures in fossil organisms must, therefore, be considered carefully in the light of comparative taphonomic analysis informed by decay data and the mode of preservation in fossils.

Lamprey arcualia, homologues of vertebrae in more derived vertebrates, provide an exception to the pattern of loss of soft cartilage. This is significant because arcualia have been identified among the vertebrate characters present in the Early Cambrian Haikouichthys [11]. Lamprey arcualia are classified as hard, but are more decay prone than some soft cartilages. Interestingly, they are also demonstrated to contain Col2a1 [8], and it would appear that the taphonomic characteristics of cartilages in cyclostomes are more closely linked to their ECM characteristics than to the properties used to designate them as hard and soft.

(b) Synapomorphic decay bias and the vertebrate fossil record
Synapomorphic decay bias is recognized in ammocoetes and amphioxus [23], and it is confirmed here to occur in adult lampreys and the non-skeletal tissues of the hagfish. As such, the process of stem-ward slippage is demonstrated to be phylogenetically pervasive among non-biomineralized chordates. How does this bias affect our understanding of the vertebrate fossil record? Applying our novel taphonomic models of character decay (figure 2) to published morphological fossil descriptions allows us to assess the anatomy, and thus affinity, of putative vertebrates and cyclostomes. The simple null models of vertebrate decay established here can prime visual search strategies when investigating fossil taxa. Using decay resistance to corroborate character interpretation is often based on unspecified assumptions of which characters would decay rapidly. Empirical study can reveal unexpected results however. Robust lamprey cartilages such as the annular cartilage, for example, are lost to decay before more flimsy structures such as the dorsal nerve cord. Using this example, axial structures in non-biomineralized vertebrates need not necessarily represent the gut: potentially, they are the remains of the counterintuitively decay-resistant dorsal nerve cord. Appreciation of the mode of fossil preservation is critical in this context. Rapid post-mortem mineralization of soft tissues has the potential to capture decay-prone characters, such as the gut [15,17], while decay-resistant features are more likely to be organically preserved. So the absence of structures like the nerve cord in a fossil might be a result of misinterpretation of fossil anatomy, lack of taphonomic pathways capable of stabilizing such decay-resistant tissues or more complex taphonomic processes than accounted for, such as alteration of anatomical decay sequences through chemical interaction with sediments.

Here we consider the taphonomic coherency of three examples of putative non-biomineralized vertebrates and cyclostomes. Haikouichthys is a myllokunmingiid from the Lower Cambrian of China [10–12] where the dominant mode of preservation is through the retention of decay-resistant organic remains commonly coated in pyrite [44,45]. Haikouichthys preserves many of the more decay-resistant chordate and vertebrate characters, and as such fits the taphonomic model as a relatively well-preserved total-group vertebrate (figure 2). Most labile characters are missing, but a notable exception is the preservation of the relatively decay prone and potentially Col2a1-bearing arcualia. Furthermore, given that the vertebrate skull is relatively decay resistant (figure 2), its absence in Haikouichthys, which preserves other cartilaginous tissues, is likely to be a result of phylogenetic absence, rather than taphonomic loss; a stem-vertebrate affinity for Haikouichthys is therefore both anatomically and taphonomically consistent. Mayomyzon is from the Carboniferous of Illinois [46–48] where the mode of preservation is less well characterized. Mayomyzon preserves many of the more decay-resistant chordate, vertebrate and petromyzontid synapomorphies (figure 2). The only relatively decay-resistant
petromyzontid characters that are not preserved are the tiny (less than 1 mm scale) tectic rings and oral papillae. *Mayomyzon* is likely, therefore, to represent the remains of a crown-group petromyzontid. *Hardistiella* from the Carboniferous of Montana [49–51] preserves only the most resistant chordate and vertebrate synapomorphies and just one petromyzontid synapomorphy (slanting gills). Comparing the preserved anatomy of *Hardistiella* with the hagfish and lamprey decay models, however, indicates that it is far more consistent with lamprey (figure 2), potentially supporting a petromyzontid affinity.

Application of taphonomic models to described fossil taxa provides support for a stem-vertebrate affinity for *Mayomyzon* and *Hardistiella*, each representing different fidelities of preservation. Reviewing fossil anatomy in light of our new search images and taphonomic models, in combination with considerations of complex modes of preservation, will enable us to further distinguish phylogenetic absence from taphonomic loss. This approach will allow us to test hypotheses of the affinity of these, and other, crucial fossils, aid identification of potential stem cyclostomes and ‘flesh out’ the missing half of the vertebrate fossil record.

All procedures were carried out in accordance with the University of Leicester’s policy on the use of animals in scientific research (http://www2.le.ac.uk/staff/policy/codes-of-practice-and-policy/research/statement) and UK regulations. Brook lampreys were collected from the New Forest with the kind permission of the English Forestry Commission. Practical assistance was provided by Tom Harvey (Leicester/Cambridge). Kim Freedman is thanked for her help with proof-of-concept stages of this work.

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