Correlation between Hox code and vertebral morphology in archosaurs

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The relationship between developmental genes and phenotypic variation is of central interest in evolutionary biology. An excellent example is the role of Hox genes in the anteroposterior regionalization of the vertebral column in vertebrates. Archosaurs (crocodiles, dinosaurs including birds) are highly variable both in vertebral morphology and number. Nevertheless, functionally equivalent Hox genes are active in the axial skeleton during embryonic development, indicating that the morphological variation across taxa is likely owing to modifications in the pattern of Hox gene expression. By using geometric morphometrics, we demonstrate a correlation between vertebral Hox code and quantifiable vertebral morphology in modern archosaurs, in which the boundaries between morphological subgroups of vertebrae can be linked to anterior Hox gene expression boundaries. Our findings reveal homologous units of cervical vertebrae in modern archosaurs, each with their specific Hox gene pattern, enabling us to trace these homologies in the extinct sauropodomorph dinosaurs, a group with highly variable vertebral counts. Based on the quantifiable vertebral morphology, this allows us to infer the underlying genetic mechanisms in vertebral evolution in fossils, which represents not only an important case study, but will lead to a better understanding of the origin of morphological disparity in recent archosaur vertebral columns.

1. Introduction

The regionalization of the axial skeleton into a cervical, dorsal, sacral and caudal compartments is a key attribute of amniotes, reflecting an enhanced specialization of the vertebral column to perform different functions. The vertebral column and its associated structures, such as ribs, play a large variety of roles in animal functional morphology and physiology, including in breathing, in sustaining the body posture, in locomotion and in food acquisition. Vertebral morphology and number have thus far-reaching consequences for organismal function and ecology. The form of the axial column is suited to accommodate a broad range of functional roles, receiving multiple mechanical stimuli simultaneously [1], resulting in a high variability of anatomical structures across species. Whereas mammalian presacral count and axial regionalization are very conservative [2–5], reptiles, including dinosaurs and birds, display a high variability in vertebral count [6,7].

The total number of postembryonic vertebrae is determined by the process of somitogenesis [8–13]. The rhythmic formation of somites continues until the total species-specific number of transient embryonic segments is reached [12,14–16]. Subsequently, the vertebral precursors differentiate through resegmentation into vertebrae, exhibiting distinct morphologies depending on their position along the anteroposterior body axis [12,14,15,17,18].

Since the pioneering discovery of homeotic genes, intensive work spanning three decades has shown that the specific temporal and spatial expression pattern of highly conserved Hox genes mediates the anteroposterior organization and segmentation of all metazoans, including chordates [9,10,19–21].

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**Hox genes** are key determinants of vertebral identity [8–12,22–26] and it has been proposed that a unique or highly distinctive Hox code expressed in each somite specifies different vertebral morphologies [27]. Vertebral Hox codes have been established for actinopterygian fish [28], mammals [22,23], squamates [23,29–31] and birds [23], but not yet fully for reptiles. In crocodiles, only a partial Hox code (eight out of 39 Hox genes) for the American alligator (*Alligator mississippiensis*) has been proposed so far [32]. Previous analyses have shown that the vertebral Hox code in amniotes is highly conserved, and several Hox gene expression boundaries can be used as markers for different regions of the axial skeleton [23]. For example, the expression of Hox-5 and Hox-6 genes governs the cervico-thoracic transition in a variety of vertebrate species that differ in cervical number [23]. Likewise, Hox-10 and Hox-11 paralogues regulate the formation of the lumbosacral boundary in amniotes [33]. The variation in relative vertebral count appears to be owing to modifications in the pattern of the Hox gene activity [6,34]. Although the interactions between Hox genes, their target genes and respective mechanisms of activity for vertebral specification are complex and not yet fully understood, knockdown and misexpression experiments have further elucidated the important role of Hox gene expression in determining proper vertebral morphology [25,33,35–38].

Studies of Hox gene expression patterns thus have the potential to reveal homology between vertebrate body plans and constitute an additional set of characters to homologize segments between organisms [23]. Because adult morphological similarity within an individual vertebral column seems to be related to early Hox gene expression [39], the study of morphological variation of vertebrae as an expression pattern proxy therefore potentially provides an opportunity to re-examine long-problematic aspects of morphology, such as the establishing of the exact homologies of different body sections in related taxa with varying vertebral counts.

Here, we analysed the Hox gene expression in the cervical vertebral column of the Nile crocodile (*Crocodylus niloticus*) in order to complement and extend a previous examination in the alligator. The correlation between anterior Hox gene expression limits and quantifiable changes in vertebral morphology is tested in the cervical vertebral column of extant archosaurs. As a result, the correlation observed in modern crocodiles and birds may allow a reconstruction of the vertebral Hox code in extinct relatives such as the sauropodomorph dinosaur *Plateosaurus*.

### 2. Material and methods

#### (a) Hox gene expression analysis

In order to establish the extant phylogenetic bracket for extinct archosaurs [40], we analysed Hox gene expression patterns in crocodilians and birds. This approach allows inference of the likelihood of unknown traits in fossils based on conditions in the two closest extant relatives (crocodilian and bird) bracketing extinct members of this clade (dinosaur). Besides a literature survey, whole-mount *in situ* hybridizations (WISH) were performed in order to complete the cervical Hox code for crocodilians. Nile crocodile eggs were collected at the crocodile farm ‘La Ferme aux Crocodiles’ in Pierrelatte (France). Embryos were harvested after 9–15 days of development, dissected in 1× PBS and fixed overnight in 4% paraformaldehyde at 4°C followed by serial dehydration to 100% ethanol. Hybridization was done using digoxigenin (DIG)-labelled riboprobes for the cervical Hox genes (*HoxA*-4, *B*-4, *C*-4, *D*-4 and *A*-5, *B*-5, *C*-5). The RNA probes were detected with NBT/BCIP. The applied WISH protocol is based on that described by Har粒川 al. [41] with some modifications. Further details are described in the electronic supplementary material. All novel sequences generated in this study have been deposited in the European Nucleotide Archive (http://ebi.ac.uk/ena; accession nos LN809999–LN810008). All alignments used in this study are freely available at OpenDataLMU (http://dx.doi.org/10.5282/ubm/data68). After WISH, the embryos were sequentially dehydrated into 100% ethanol and photographs were taken immediately with the M165 FC microscope (Leica).

#### (b) Morphological analysis

Morphological variability within the cervical vertebral column of alligator, crocodile, chicken and the dinosaur *Plateosaurus* was evaluated by a combined morphological analysis. First, qualitative characters were collected and coded as binary or multistate characters. These characters include the presence and absence of osteological features, such as a ventral keel, a bifurcated neural spine and muscle insertion points that vary within each cervical series and could not be captured by homologous landmarks. Second, the morphological differences between the vertebrae within a cervical vertebral column were quantitatively analysed via three-dimensional landmark-based geometric morphometrics. Applying the software *Landmark* v. 3.0 [42], a series of 17 homologous landmarks were digitized on the three-dimensional scans of the cervical vertebrae (figure 1). The homologous points abstract the vertebral shape and characterize important osteological features, such as the articulation facets of the cervical ribs (diaphyses and parapophyses), which correlate with the corresponding articulating structures of the ribs (tuberculum and capitulum). The first cervical vertebra (atlas) is not included in the geometric morphometric analysis as it is highly modified and lacks specific serial homologies with postatlantal cervicals, and thus, several landmarks cannot be applied to it. The same three-dimensional landmark sets were applied to all analysed taxa in order to provide a comparable basis for the morphological study. Although there are transverse processes connecting the cervical ribs with the vertebral centrum, landmarks (LM) 13 and 14 are not applied in the analysis of chicken because their placement is not exactly repeatable owing to fusion of the ribs to the centra. Geometric morphometric data were processed using the software *Morphologika* [43] with the following procedures. The coordinates of all landmark sets were superimposed using general procrustes analysis. The Relative Warps (RW) analysis summarized the multi-dimensional information. With the applied settings, this method is equivalent to a principal components analysis. The shape differences were visualized with three-dimensional thin-plate splines. Using the software PAST [44], both datasets were assembled to one data matrix that served as basis for Principal Coordinates (PCO) Analysis. In order to find the similarity relationships among the vertebrae for each taxon, the superimposed three-dimensional landmark coordinates assembled with the qualitative character matrix were analysed with a PCO Analysis applying the Gower index [45,46]. Via the cluster analysis using the single linkage algorithm in combination with the Gower similarity index, the vertebrae were joined based on the smallest distance between them. This resulted in the establishment of morphological subregion patterns of the cervical series for the analysed taxa. Further details are provided in the electronic supplementary material.

#### (c) Comparison of genetic and morphological data

To establish phylogenetic homology [47] between Hox gene expression in recent archosaurs, we compared Hox gene expression patterns in relation to vertebral morphology in crocodiles and birds. Given the sister-taxon relationship of these two groups,
the finding that the same \textit{Hox} gene expression boundaries coincide with vertebral subregions is most parsimoniously explained as implying homology between these subregions. These results from recent archosaurs can then be used as a phylogenetic bracket to hypothesize \textit{Hox} gene expression patterns from vertebral morphology in the most recent common ancestor of birds and crocodiles, and in fossil stem-line representatives of these clades [47]. To test the applicability of the results to fossil representatives, we used the basal sauropodomorph dinosaur \textit{Plateosaurus}, as this taxon is represented by numerous, well-preserved skeletons, including complete vertebral columns [48,49].

3. Results

(a) Cervical \textit{Hox} gene expression in the Nile crocodile

The gene expression analysis (figure 2) showed that \textit{C. niloticus} expresses the same \textit{Hox} genes (\textit{HoxA-4, B-4, C-4, D-4} as well as \textit{A-5, C-5}) found in the neck of other tetrapods [22,23,29–32, 50,51]. In crocodiles, which generally have nine cervicals, the anterior expression limit of \textit{HoxA-4} and \textit{C-4} is at the fifth cervical vertebra (C5), extending to the thoracic region (figure 2c,d). The expression of \textit{HoxB-4} and \textit{D-4} begins at the third cervical vertebra (figure 2a,b). \textit{HoxB-4} is only active until C6, whereas \textit{HoxD-4} is expressed to the end of the neck. Whereas the expression of \textit{HoxB-5} already starts at C2 [23], the anterior expression boundary of \textit{HoxA-5} is at the last cervical vertebra (C9) (figure 2f). \textit{HoxC-5} is expressed at the last two cervical vertebrae (figure 2e).

(b) Morphological variation within the cervical vertebral column of modern and fossil archosaurs

The analysis of qualitative characters of the vertebrae revealed significant morphological variation within the cervical series in crocodilians and chickens (refer to electronic supplementary material, figure S1). The distribution of osteological features indicates morphological differentiation of the cervical vertebral region in each taxon (refer to electronic supplementary material, tables S1–S3). Although there are minor differences between the Nile crocodile and the American alligator, the variation in qualitative characters indicates four morphological subregions in the crocodilian neck. Five cervical subunits are recognized in the chicken.

The landmark-based geometric morphometric study allowed us to quantitatively assess the varying morphology of the cervical vertebrae, to gain additional insights into the regionalization of the neck. The RW analysis summarized the vertebral shape differences and three-dimensional thin-plate splines allowed visualization of the morphological changes from the average (figure 3). The first two RWs explain about 70–90% of the variation in the sample for each examined taxon. In all examined archosaurs, the morphological groups separate along the axes. The morphologically clearly distinct second cervical vertebra always occupies a unique region of the morphospace. A group of following anterior cervicals clusters separately from posterior vertebrae. In between is a cluster of middle cervical vertebrae. In general, the morphological differences within each cervical region involve variation in the shape of the vertebral centrum, the pre- and postzygapophysis and the neural spine. Differences in the relative position of the diapophysis and the parapophysis are only detected in crocodilians, because the vertebrae of the chicken lack unambiguous diapophyseal and parapophyseal landmarks. The morphological differences of the cervical vertebrae, observed along the RW axes, are not a function of size. The size regression analysis (log centroid size versus RWs) revealed no significant correlation between shape variation and size in all analysed taxa.

Our combined quantitative and qualitative morphological analysis allowed discrimination of vertebrae in different cervical regions (figure 4). The morphospace occupation along the first two PCOs (which account for about 70–90% of the explained variation) for each examined archosaur shows substantial differences among cervical vertebrae. The archosaur neck (excluding the atlas) can be subdivided into 3, 4 or 5 morphological subregions. The general units are the axis complex,
Figure 2. WISH results. *Hox* gene expression in the somites (so) of Nile crocodile embryos (ED 10–14). Arrowheads indicate the anterior and posterior expression boundary. (a) *HoxB-4* has an anterior limit at C3 (so 7/8) and extends to C6 (so 10/11). (b) *HoxD-4* expression starts at C3 (so 7/8) and fades out posteriorly at D1 (so 14/15). (c) *HoxA-4* is expressed from C5 (so 9/10) to D3 (so 16/17). (d) *HoxC-4* has an anterior boundary at C5 (so 9/10) and extends to C9 (so 13/14). (e) *HoxC-5* expression starts at C8 (so 12/13) and fades out posteriorly at D1 (so 14/15). (f) *HoxA-5* is expressed from C9 (so 13/14) to D4 (so 17/18).

Figure 3. RW analysis results. Each plot shows the shape differences of the cervical vertebrae along RW 1 and RW 2 for (a) crocodile, (b) alligator, (c) chicken and (d) *Plateosaurus*. Thin-plate splines (three-dimensional in left lateral view) visualize the variation between landmark configurations of the vertebrae from the respective average shape (zero point).
an anterior section and a posterior group. The main difference between crocodilians and chicken is an additional subregion that can be recognized in the mid-cervical series in the latter. The analysis recovered four subregions for the crocodilian neck (nine cervical vertebrae), corresponding to the axis, two anterior, three middle and two posterior cervical vertebrae as morphological subregions (figure 4a,b). The relatively long neck of chicken (14 cervical vertebrae) is subdivided into five morphological subregions (figure 4c). Additional to the axis, three anterior, two middle and two posterior cervical vertebrae, there is also a midposterior cervical compartment, comprising C8 to C12. Another difference between the morphological pattern of crocodiles and chickens is that the number of cervical vertebrae that form the anterior subregion is higher and that of the middle subregion is lower in birds. The geometric distance between C2 and C3 is high in the crocodilians and smallest in the chicken (figure 4).

Examination of qualitative characteristics of the vertebrae in the extinct dinosaur *Plateosaurus* (10 cervical vertebrae) revealed distinct morphological differences within the cervical region (see electronic supplementary material, figure S2 and table S4). The distribution of osteological features indicates morphological differentiation of the cervical vertebral region. The regionalization of the dinosaur cervical vertebrae is also indicated by quantitative shape differences, as revealed by landmark analysis (figure 3d). The first two RWs explain about 80% of the variation in the vertebrae of *Plateosaurus*. As previously observed for the modern archosaurs, the morphological groups separate along the axes. The morphologically unique second cervical vertebra is distant from the other vertebrae in the morphospace. There is a cluster of anterior cervicals that is separate from the posterior vertebrae. In between, there is a group of middle cervical vertebrae. The morphological differences of the cervical vertebrae in *Plateosaurus*, observed along the RW axes, are not a function of size. The size regression analysis (log centroid size versus RWs) revealed no significant correlation between shape variation and size.

Combining the qualitative and quantitative morphological data via PCA showed substantial differences between the cervical vertebrae of *Plateosaurus*. The morphospace occupation along the first two PCOs (accounting for almost 80% of the explained variation) indicates four morphological subregions in the neck: the axis, three anterior, three middle and two posterior cervical vertebrae (figure 4d).

4. Discussion

(a) Cervical *Hox* gene expression in modern archosaurs

*In situ* hybridization results revealed that the gene expression pattern of *HoxB-4*, C-4, D-4 and *HoxA-5* in the Nile crocodile is identical to that found in the American alligator [32]. The exact expression of *HoxB-5* is only known for the alligator and *HoxC-5* was only investigated in the crocodile. In comparison, the expression of *HoxA-4* and C-4 in chicken [23], which possess 14 cervical vertebrae, is posteriorly shifted by one vertebra and, thus, begins at the sixth cervical (figure 5). The anterior expression limit of chicken *HoxB-4*...
and D-4 is at the second cervical vertebra (axis) and both end at C10 [23]. HoxA-5 is expressed at the eighth cervical vertebra [32]. It is shifted anteriorly by one vertebra in comparison to the crocodilian pattern. The anterior expression limit of Hox B-5 is at the axis (C2), as previously observed in crocodilians [32]. The HoxC-5 expression pattern [23] is also similar to that of crocodiles.

The pattern of cervical expression differs between model species of crocodilians and birds (figure 5). Although the general expression pattern of the Hox 4 paralogues is relatively similar in all taxa, there is some variation in their anterior expression limits, in relation to the number of cervical vertebrae. The same is seen in the Hox 5 paralogues, with HoxA-5 showing the highest variability.

(b) Hox gene expression correlates with morphological subregion pattern in the cervical vertebral column of modern archosaurs

The individual vertebral morphology clearly differs between crocodilians and chicken (see electronic supplementary material, figure S1). The combined morphological analysis detected a corresponding pattern of units of vertebrae based on morphological similarity within a cervical series. There is a striking correspondence between anterior expression boundaries of Hox genes and distinct vertebral regions along the body axis of modern archosaurs (figure 6). Comparing the Hox gene expression pattern with the morphological subregions of the archosaur neck reveals that vertebrae that form clusters in the morphological analysis have identical patterns of Hox gene expression. Thus, distinct shape changes in cervical vertebrae (that is between the last vertebra of one subregion and the first vertebra of the following subregion) coincide with differences in the activity of cervical Hox genes.

In all analysed archosaurs, the combined morphological analysis revealed that the second cervical vertebra is separate from a group of following anterior cervicals (C3 and C4 in crocodilians; C3, C4 and C5 in chicken) (figure 4). The morphological pattern is reflected in the Hox gene expression pattern as the anterior expression boundaries of HoxA-4 and D-4 are at the third cervical vertebra in crocodile (this study), alligator [32] and chicken [23,32] (figure 6). Even though it is distinct, the measured shape difference between C2 and C3 in the chicken is not as high as in the crocodilians (figure 4). In contrast to crocodilians, in which the anterior expression boundary of HoxD-4 is clear (this study, [32]), the published expression of HoxD-4 in chicken shows a slightly weaker signal and appears to have a relatively unclear anterior boundary [23]. This may be due to inefficient probes. Alternatively, it may indicate a graded anterior expression boundary of HoxD-4, which may have an influence on the lower degree of shape change between the second and third cervical vertebra in the chicken.

Although there is one discrepancy in crocodilians regarding the association of HoxA-5 expression and the posterior cervical vertebrae (figure 6), this correlation strongly indicates that changes in segmental organization are driven by changes in function of Hox genes. HoxA-5 is the most variable in its expression compared with the other Hox genes expressed in the cervical vertebral column of the crocodile (this study), alligator and chicken [32]. This may strengthen its previously suggested important role in the patterning of the axial skeleton, which might allow the adaptation to varying functions of the vertebral column in different animals [25,32]. In contrast to both, crocodile and alligator, the expression of HoxA-5 is anteriorly shifted in the chicken and correlates with the morphological midposterior subregion of the avian neck (figure 6).

In summary, changes in the number of vertebrae are associated with changes in the morphological grouping
of the cervical region. The Hox gene expression pattern significantly correlates with the pattern of the morphological subregions in the neck of the analysed archosaur taxa. The expression of each Hox gene maintains a definite, gene-specific anterior limit that is associated with distinct shape changes within a cervical vertebral column.

(c) Modification of Hox gene expression associated with vertebral evolution

Based on the above, the morphological pattern may serve as a proxy for the underlying Hox code in taxa where the genetic information is not available, especially in fossils. Based on these results, and applying the concept of the extant phylogenetic bracket [40], it should be possible to hypothesize changes in the Hox code responsible for modifications during vertebral evolution from morphological data, including fossil taxa such as the basal sauropodomorph dinosaur Plateosaurus.

In Plateosaurus, our analyses showed four morphological subregions in the neck, as seen in crocodilians, but with the difference that the anterior cervical subregion is expanded by one vertebra, as observed in the chicken (figure 4). On the basis of the correlation noted above, a hypothetical Hox code for the extinct dinosaur can be established (figure 6). Our results indicate that the posterior shift of the expression boundary of HoxA-4 and HoxC-4 seen in modern birds is already present in this basal saurischian dinosaur, whereas the anterior shift of the expression boundary of HoxA-5 cannot yet be recognized. The morphological results for

Figure 6. Phylogenetic distribution of morphological subregions and Hox gene expression pattern in the neck among extant and extinct archosaurs. The correlation between Hox code and vertebral morphology in modern crocodiles and birds allows a reconstruction of the Hox code in the extinct archosaur Plateosaurus on the basis of the morphological subregion pattern.
Plateosaurus thus display an intermediate state between the representatives of the extant phylogenetic bracket. This study showed that the anterior Hox gene expression limits shift together with the displacement of cervical subregions. This provides a likely mechanism to explain evolutionary changes along the axial column.

Applying the concept of the extant phylogenetic bracket, and considering that the increase of cervical vertebral number is an evolutionary novelty in birds, the ancestral archosaur Hox code was probably similar to that of crocodilians. The general morphological groups of the neck in archosaurs include: the atlas–axis complex, which is specialized for facilitating mobility of the head, the anterior and the middle subregion, and the posterior subregion, which forms the junction of the highly mobile cervical column to the relatively stiff thoracic column. With the evolutionary elongation of the neck, the Hox gene expression patterns were expanded and shifted in relation to each other, respectively. The first step towards the elongation of the cervical vertebral column as seen in chicken in comparison to crocodiles may have been the addition of one vertebra to the anterior section of the neck, as indicated by the presence of this shift, but not other modifications, in the basal dinosaur Plateosaurus. The next step may have involved the further addition of vertebrae to the middle region as a result of an expanded Hox gene expression domain. Changes in the length of cervical vertebral column associated with changes in the morphological subregions of the neck suggest that important modifications in the expression of Hox genes have occurred during archosaur evolution. Among other aspects, this may have facilitated the extraordinary evolution of extremely long necks with up to 19 cervical vertebrae in the sauropodomorph dinosaurs that remain unsurpassed in all other terrestrial animals [52].

5. Conclusion
Determination of the number and morphological identity of vertebrae is of major importance in interpreting the evolution of amniotes. The highly conserved Hox genes play a fundamental role in the development of the axial column, because they mediate specification of vertebral shape and thus are responsible for the regionalization of the primary body axis.

The WISH experiments revealed that the same Hox-4 and Hox-5 paralogue genes are active in the cervical columns of recent archosaurs, exemplified by crocodilians and chicken. By comparing the anterior expression boundaries of the Hox genes in modern archosaurs, a correlation between the Hox gene expression limits and the boundaries of morphologically distinct subregions within each cervical column is found. Neck elongation is a prominent feature in the evolution of ornithodiran archosaurs, both on the lineage towards modern birds and also in sauropodomorph dinosaurs, in which the extremely elongated neck has been directly linked to their ecological success [52]. On the basis of the results presented here, an evaluation of the importance of modifications in Hox gene expression patterns in relation to this neck elongation seems feasible. For the first time, the modifications in Hox gene expression in an extinct archosaur, the basal sauropodomorph Plateosaurus, were hypothesized with the aim to further our understanding of how evolutionary changes of the axial column might have occurred.

This study shows how the integration of genes, morphology and fossils can improve our understanding of the evolutionary history of modern of modern diversity [53,54]. A more holistic appreciation of vertebral development promises new insights into the evolutionary mechanisms responsible for the great morphological adaptability of the vertebrate axial column. The highly variable cervical region provides an interesting model for the study of the relationship between genomic control and phenotypic changes. The results indicate that the evolution of Hox gene expression patterns and associated changes in the axial column is likely to have mediated some of the major transitions in the archosaurian body plan.

Ethics. Nile crocodile specimens were collected under permit by the crocodile farm ‘La Ferme aux Crocodiles’ in Pierrelatte (France). All embryos come from CITES parents IAX 1119. These dead embryos can move freely in the European Union (EU) and have solely been used for scientific purposes. The study was conducted in accordance with the Institutional regulations at Ludwig-Maximilians-Universität in Munich (Germany).

Data accessibility. All novel sequences generated in this study have been deposited in the European Nucleotide Archive (http://ebi.ac.uk/ena; accession nos LN809999–LN810008). All alignments used in this study are freely available at OpenDataLMU (http://dx.doi.org/10.5282/ubm/data68).

Authors’ contributions. O.W.M.R. designed the study. C.B. conducted the analyses. G.W. contributed analytical tools and reagents. C.B., O.W.M.R. and G.W. wrote the manuscript. All authors contributed to data interpretation, manuscript editing and discussions.

Competing interests. The authors declare that no competing interests exist.

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