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INVITED ARTICLE

DEVELOPMENTAL BIOLOGY ENRICHES PALEONTOLOGY

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ABSTRACT—Paleontology provides information about the history of morphological transformations, whereas developmental biology provides information about how such transformations happen at a mechanistic level. As such, developmental evidence enriches paleontology in formulating and assessing hypotheses of homology, character definition, and character independence, as well as providing insights into patterns of heterochrony, evolvability of features, and explanations for differential rates of evolution. The focus of this article is to review a series of case studies that illustrate how our understanding of paleontology is enriched by data generated by developmental biologists. The integration of paleontological and developmental data leads to a greater understanding of evolution than either of these sciences could have reached alone. Our case studies range from fish to mammals and involve somite and vertebral formation, limb loss, hand and foot patterning, and tooth formation.

INTRODUCTION

Evolutionary developmental biology (usually called evo-devo) is a young discipline that studies the evolution of the molecular processes that regulate embryonic development by comparing species-specific patterns and integrating them with genetics and morphology. Evolutionary developmental biologists focus less on changing morphologies and more on evolutionary changes in the working of genes and proteins that control the formation of morphologies in the embryo. As such, evo-devo is part of the discipline of developmental biology, a field that is making great contributions to furthering our understanding of molecular events shaping the body and identifying novel treatments in human health care. Unlike evo-devo, most of developmental biology is inherently very limited in scope (Raff et al., 1999), because its usefulness for medicine is based on the existence of similarities in development between its model organisms and humans.

At first glance, it would appear that evolutionary developmental biology would be a natural partner to paleontology and embryology in the study of evolution, but that is not the way in which these fields have developed. Many evolutionary developmental biologists are interested in the evolution of genes and proteins, and how they affect morphology during ontogeny. This work requires the breeding of organisms to collect living embryos at precisely timed ontogenetic ages, manipulating live embryos, and/or fixation of specimens in very precise ways. Many methods in this field cannot be applied to preserved material, such as silencing or replacing genes in a living embryo to study how development is altered. Therefore, evo-devo focuses on a few species that are well known and relatively easily and cheaply bred and where embryonic stages can be easily determined, such as the roundworm *Caenorhabditis elegans*, fruit flies, zebrafish, *Xenopus*, chickens, and mice. Some argue that evo-devo should focus on these primary model organisms only (Sommer, 2005), and not waste its time with other, less-mainstream research organisms. Such authors believe that only after the intricate interactions between control genes are worked out in the chosen models, other taxa should be studied.

Our view is that research on both primary models and other species is essential. It is important to understand the gene control of development in mice, but ultimately that only teaches us about mice. That approach leaves us in the dark about how valid it is to generalize from the mouse model to questions relating to human health care or the evolvability of the vertebrate body plan. Instead, we agree with others (Eakin and Behringer, 2004; Behringer et al., 2006; Milinkovitch and Tzika, 2007) that the study of a diversity of species is essential to progress in understanding the evolution of developmental controls, the ways in which ontogeny generates morphology in evolution, and the basis for variation in the species around us, and that the examples that follow show this. By combining paleontological and developmental data, and by analyzing them comprehensively, the study of evolution is enriched well beyond what each of those fields could have accomplished by itself.

The major aim of this article is to illustrate how paleontological evidence can be enriched by developmental data. In our view, it is obvious that paleontological data are of importance to developmental biologists in providing phylogenies, in identifying plesiomorphic states, and in identifying functional aspects of morphology. However, a discussion of this falls outside of the scope of this paper, and the point has been made eloquently by others (e.g., Raff, 2007).

We review some case studies well known to vertebrate paleontologists, synthesize evidence from developmental data, and review their implications for some concepts that are important for paleontologists, such as character definition, polarity, heterochrony, pleiotropy, and especially homology as it is a concept that is at the intersection of both fields, and in which often conflicting information from both fields is condensed. We also briefly review parts of the workings of genes and cells here, but only as far as is relevant to the examples we have chosen (Fig. 1, Table 1). For those who want a review of all of developmental biology, the excellent textbook by Gilbert (2010) is now in its ninth edition, and follows influential textbooks such as that by Hall (1998). Carroll et al. (2004) wrote an important and readable review of evolutionary developmental biology, and the textbook by Barton et al. (2007) integrates development into mostly molecular and genetic syntheses of evolution. Our final goal is to look into the future. Inspired by results from the discussed case studies, we

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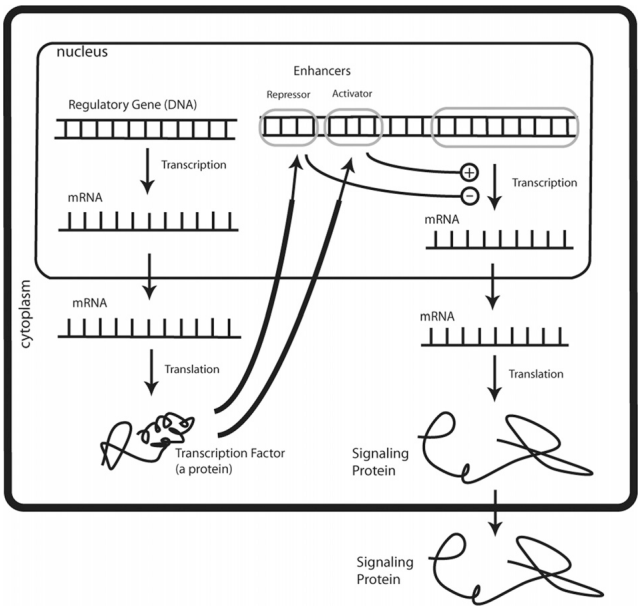

FIGURE 1. Simplified explanation of the role of DNA in making two types of proteins important in regulating development: transcription factors and signaling proteins. Transcription factors do not leave the cell; they interact with DNA segments called enhancers. Enhancers control the transcription of genes. Signaling proteins do leave the cell.

pose questions that are relevant to paleontologists and where evidence from developmental biology provides part of the answer. Most of those questions come from a clade that we are interested in: Cetacea (whales, dolphins, and porpoises). Cetaceans are the antithesis of a primary model organism, and yet, developmental and paleontological work combined can greatly enlighten the understanding of their evolution.

BODY AXIS

Early in the development of all vertebrates (week three after fertilization in humans, but much earlier in animals with a shorter gestation time), the embryo consists of three cell layers, from dorsal to ventral: ectoderm, mesoderm, and endoderm. Part of the ectoderm invaginates (rolls up), forming a hollow tube that buries itself in the dividing mesoderm underneath. This is the neural tube, which will eventually form the brain and spinal cord, and is positioned just dorsal to the notochord, a bar of cells that runs much of the length of the embryo. Then, on either side of the neural tube, the mesoderm forms a thick column of tissue. This tissue column splits into three parts: paraxial, intermediate, and lateral plate mesoderm (from medial to lateral), late in week three in humans. Subsequently, the paraxial mesoderm is segmented into a row of tissue blocks called somites (Fig. 2). Somites are part of the original segmentation of the embryo. They are involved in the development of the limb muscles and the vertebrae, and gene expression in the somites regulates much of the cranial-to-caudal organization of the body. The vertebrae form around the notochord, and a small fragment of the latter, the nucleus pulposus of the intervertebral discs, remains throughout life.

A special family of genes is expressed in the somites and the adjacent neural tube, and it regulates the basic cranial-to-caudal organization of the body. This family of (in most tetrapods) 39 *Hox* genes are part of the system that makes sure that only one forelimb forms on each side of the body and that it is placed just behind the neck, and that the last rib is cranial to the pelvis (Wellik, 2009; for italicization and capitalization of gene and protein names, see nomenclature entry in Table 1). The *Hox* genes are organized into four clusters on four different chromosomes, and these clusters are indicated by the letters a through d. In addition, different *Hox* genes are numbered, where low numbers represent genes with more cranial expression fields, and high numbers those that are expressed more caudally. *Hox* genes with the same number but different letters (usually) have similar (but not identical) expression territories and functions in axial development. Hence, *Hoxa4*, *b4*, and *d4* determine the shape of the cervical vertebrae behind the atlas, and partly complement each other's functions (Horan et al., 1995). The first thoracic vertebra forms in the area of the anterior expression of *Hoxc6* (Burke et al., 1995; Fig. 3), and *Hoxc10* and *c11* are involved in lumbar vertebra and sacrum formation (Wellik and Cappechi, 2003; Di-Poï et al., 2010).

Hox expression patterns dictate, to a large extent, where organs will form as well as which type of vertebra will develop at a particular section of the cervical, thoracic, lumbar, sacral, or

TABLE 1. Terms commonly used in embryology and developmental biology.

Downstream—In gene expression, genes that are transcribed after the gene in question, and directly or indirectly influenced by it. For instance: '*Shh* is expressed downstream from *Hand2* in the limb bud,' which means that *Shh* is expressed after *Hand2* and that *Shh* expression depends on previous *Hand2* expression in the limb bud.

Embryo—The prenatal individual during the time that most of the organ systems initially form. The embryonic period is between the zygotic and fetal period. In humans, weeks 3 to 8 constitute the embryonic period.

Exon—Discrete segment of a gene that provides the coding sequence that is transcribed into RNA and (usually) translated into a protein. Some RNAs are not translated into proteins and regulate gene expression.

Expression—The process of generating a gene product (RNA/protein) from a gene.

Fetus—The prenatal individual during the last period of gestation. In humans, the fetal period lasts from week 9 to week 38 (when birth occurs). Most mammals can be identified to order at the beginning of the fetal period.

Intron—Discrete segment of a gene (and corresponding RNA) sequence that is removed before the RNA is translated into a protein. Thus, intron sequences do not code for protein, even though they are part of the gene that codes for this protein.

Morphogen—A substance secreted by a group of cells that regulates the development of a tissue or organ. Morphogen concentration decreases with distance from the secreting cells, causing neighboring cells exposed to the morphogen to respond differently.

Nomenclature of genes and proteins—Proteins are given the same name as the genes that code for them, and are abbreviated in writing in non-standardized ways, such as FGF, fibroblast growth factor, and SHH, sonic hedgehog. Rules of nomenclature vary from taxon to taxon. Most journals use the convention that mammalian genes are italicized (*Hand2*), and also capitalized if they are human (*HAND2*), whereas mammalian proteins are in Roman script and capitalized (HAND2).

Signaling—The process by which cells in a tissue communicate with each other. Signaling molecules may be proteins or not. Signaling proteins can be secreted to reach other cells or can be present on the surface of the expressing cells to signal to adjacent cells.

Transcription factor—A protein that regulates the transcription of a gene. In animals, these proteins do not leave the cell in which they are produced.

Upstream—In gene expression, genes that are transcribed before the gene that is discussed, and partly affects that gene. For instance: '*Hand2* is expressed upstream from *Shh* in the limb bud.'

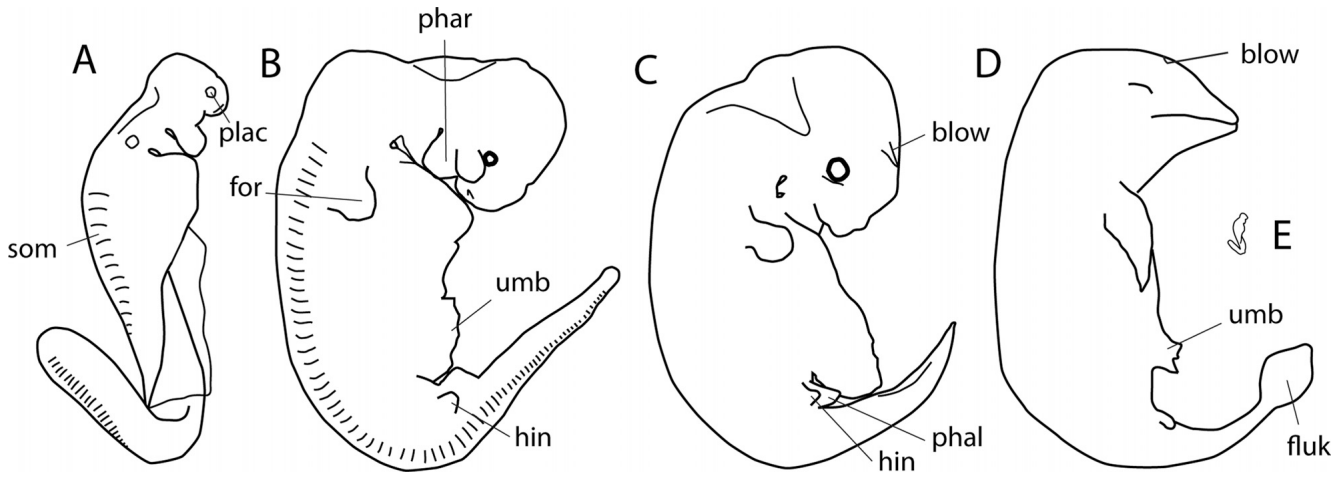

FIGURE 2. Prenatal specimens of the dolphin *Stenella* (Cetacea), illustrating anatomical structures discussed in the text and some methods of study, not to scale. **A**, lateral view of early embryo (LACM 94783; Carnegie stage 11, approximately week three after fertilization). Embryos are usually staged for study, without making reference to absolute ages. Staging systems are species specific because heterochrony is rampant. However, broad comparisons can be drawn between species. **B**, lateral view of embryo (LACM 94701; Carnegie stage 14; approximately week 4). **C**, lateral view of late embryo (LACM 94770; Carnegie stage 16; approximately week five). **D**, lateral view of early fetus stage 20 (LACM 94646). **E**, embryo of **A**, now shown at the same scale as **D**, to illustrate the enormous growth that takes place during early development. **Abbreviations**: **blow**, blowhole; **fluk**, fluke; **for**, forelimb bud; **hin**, hind limb bud; **phal**, phallus, genital tubercle; **phar**, pharyngeal arch; **plac**, nasal placode; **som**, somite; **umb**, umbilical cord.

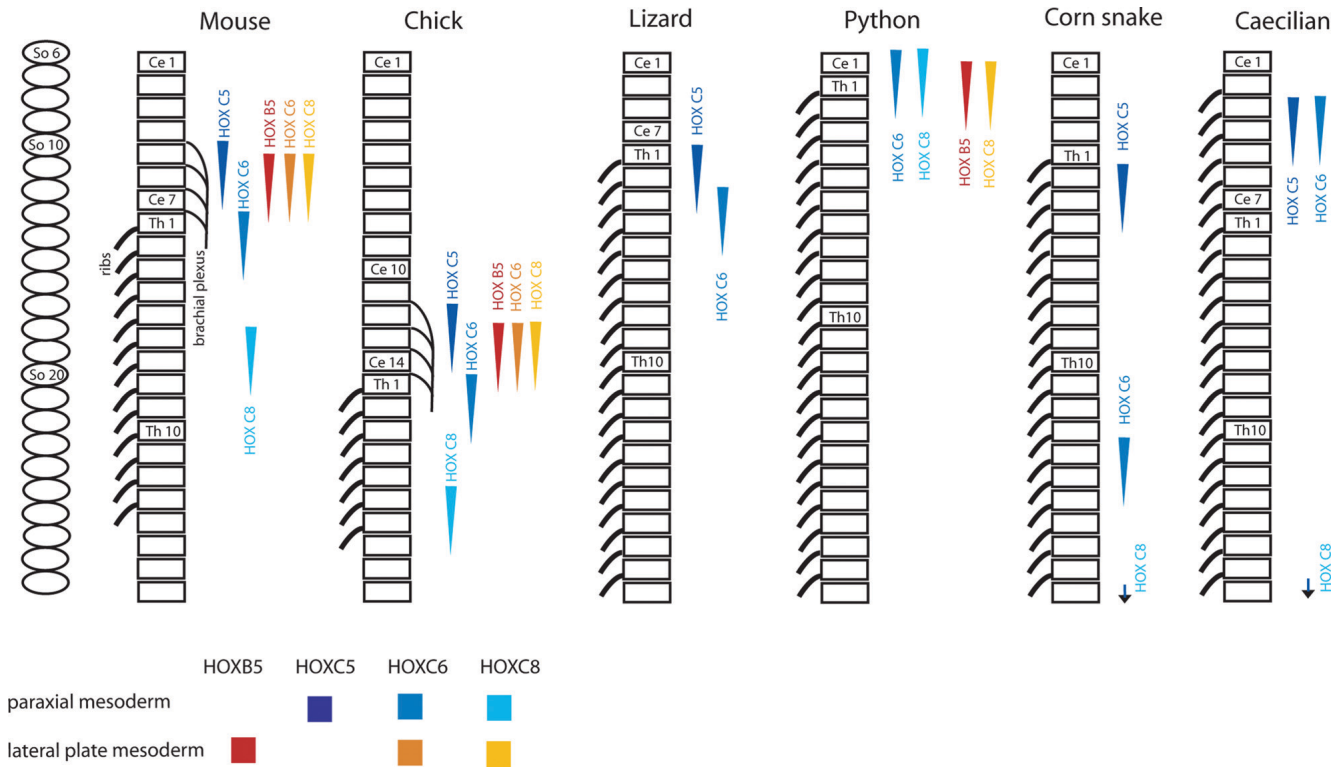

FIGURE 3. *Hox* gene expression along the developing vertebral column (cranial to top). Gene expression in the paraxial mesoderm indicated in purple and blue, and in lateral plate mesoderm in red, orange, and yellow. Diagram shows somites (So) and the cervical (Ce) and thoracic (Th) vertebrae that develop from them, and the location of ribs and brachial plexus in mouse (*Mus*), chick (*Gallus*), bearded dragon lizard (*Pogonis*), python (*Python*), corn snake (*Panterophis*), and a caecilian (*Ichthyophis*). Cranial edges of *Hox* gene expression determine morphologically important fields; caudal edges are less important and are not indicated. Summarized after Burke et al. (1995), Cohn and Tickle (1999), and Woltering et al. (2009).

caudal spine. However, their patterns of expression are not tightly linked to exact somite (and thus vertebral) levels along the spine in different species. For instance, *Hoxc6* is expressed in mice and chicks at the boundary of the cervical and thoracic vertebrae and dictates the identity of anterior thoracic vertebrae (Fig. 3). The cranial level of *Hoxc6* expression and the morphological cervical-thoracic boundary are located at the 7th vertebra in mice, and the 14th vertebra in chicks (Burke et al., 1995). As a result, chicks have many neck vertebrae, and mice have only seven. This relationship also holds for bony fishes (*Danio*, *Gasterosteus*) and amphibians (*Xenopus*), where the cervical-to-thoracic transition also coincides with the cranial limit of *Hoxc6* expression (Burke et al., 1995; Tanaka et al., 2005).

Changes in *Hox* gene expression can underlie major morphological change in evolution. In an exemplary study that combined paleontological and developmental data, Cohn and Tickle (1999) investigated the absence of forelimbs in pythons. In pythons, the cervical vertebrae (except C1) have ribs, and thus a thoracic identity. Indeed, expression of *Hoxc6* in python somites is found in all vertebrae of the neck region.

The cranially shifted *Hox* expression territory has implications for snake morphology that reach beyond vertebral identity. Somites form vertebrae and muscles of the limbs, but the skeleton of the limbs forms instead from lateral plate mesoderm. Expression of *Hoxc6* and *c8* in lateral plate mesoderm marks the cranial region of the forelimb in vertebrates that have them (Rancourt et al., 1995; Nelson et al., 1996; Cohn and Tickle, 1999). Just as in the somites, the expression of these genes in the lateral plate mesoderm is also cranially expanded, so that there is no region between the head and chest in which these genes are not expressed. Cohn and Tickle (1999) proposed that this led to the absence of forelimb development in snakes. This example shows that two seemingly unrelated morphological peculiarities of snakes, cervical ribs and absence of forelimbs, are both the result of a cranial shift in the expression territories of the *Hox* genes, and thus that these are not independent characters. Thus, pleiotropy can affect morphologically distinct organs, and this suggests that developmental data should be taken into account when assessing the independence of characters in systematic studies.

This example also elucidates the difference between morphological and developmental partitions. Organisms are integrated and continuous amalgamates of shapes, and morphologists often study these by dividing them into discrete partitions (Raff and Sly, 2000; Bolker, 2002). Gene expression studies provide insights into parts of the body that are integrated as modules, which are essentially developmentally relevant partitions. *Hox* gene expression in the neck and location and presence of the forelimb are part of the same developmental module, even though anatomically the neck and forelimb are disjunct.

Cohn and Tickle (1999) used their understanding of developmental control genes to interpret the fossil record, in particular the fossil snake *Pachyrachis*, and then they made predictions about the morphology of (as yet undiscovered) transitional fossils. Although the precise phylogenetic position of the bipedal snake *Pachyrhachis* is disputed (Caldwell and Lee, 1997; Lee and Caldwell, 1998; Zaher, 1998; Caldwell, 2000; Zaher and Rieppel, 2002), there is agreement that *Pachyrachis* is at least basal to Macrostomata, the group of snakes that includes pythons. However, the controversy does highlight the importance of a stable phylogenetic tree when interpreting biological data, both in terms of identifying a suitable outgroup and in terms of identifying convergence in the ingroup.

Expression of *Hoxc6* is associated topographically with the first rib in mouse and chick (Burke et al., 1995), as well as in python, and Woltering et al. (2009) determined that this was also the case in the lizard *Pogonis*, a member of the snake sister group. However, this association was not the case in the snake *Panterophis*, where distribution of HOXC6 was more caudal and more diffuse. This emphasizes the need for studying a greater diversity of taxa in developmental studies, well beyond established model organisms. Caecilians are amphibians that lack forelimbs but have cervical ribs, and they have lost their limbs independently from snakes. Woltering et al. (2009) found that the developmental mechanism by which caecilians (*Ichthyopsis*) arrive at a snake-like morphology may be the same as that in snakes, indicating a parallelism in developmental pathways.

VERTEBRAL NUMBERS

All the genes in the *Hox* family are characterized by a particular section of 180 nucleotides, the homeobox. The sequence of the homeobox is remarkably similar from *Hox* gene to *Hox* gene within one species and also from species to species. Most surprising, vertebrate *Hox* genes bear great similarity across their classes as well as to *Hox* genes in invertebrates (see review by Holland et al., 2008). Not only are they similar in sequence, their function is also similar: in both vertebrates and invertebrates, an important function of *Hox* genes is the cranial-to-caudal patterning of the body. This probably indicates that these genes predate the origin of the modern phyla.

As stated, *Hox* gene expression domains may include a variable number of segments (somites and thus vertebrae) in different animals, but other developmental factors constrain the number of segments. Galis (1999) proposed that, in mammals, susceptibility to cancer limited the tolerance to vary *Hox* territories in the neck, resulting in seven cervical vertebrae for most mammals. Narita and Kuratani (2005) found that, in most mammals, there are approximately 19 thoracolumbar (and thus 26 presacral) vertebrae, even though the number of thoracic and lumbar vertebrae varies greatly (but inversely). These authors considered this a developmental constraint. Müller et al. (2010) found that presacral vertebral counts are remarkably stable in amniote clades at higher systematic levels and they explored selection as a possible cause of this stability. The limited variation of presacral vertebrae is an example of a developmental constraint. This constraint has been released in clades with different numbers of vertebrae, and such unusual evolutionary events can be studied in the context of the morphology and adaptations of that clade.

Somites form from cranial to caudal in an embryo and the process is usually referred to as following a clock-and-wavefront model (reviews by Gridley, 2006, Lewis and Özbudak, 2007, and Mallo et al., 2009). In this model, a determination front moves from cranial to caudal through the paraxial mesoderm, and somites differentiate out of this mesoderm as this front passes through them. A clock-like function in the wavefront alternates between producing proteins from the NOTCH and WNT families, and each combination of NOTCH and WNT marked cells unite to form a somite. Caudal to the determination front, the signaling protein FGF8 keeps cells undifferentiated. If the front moves slower, or the clock runs faster, more somites (and thus eventually more vertebrae) will be formed. Vertebrae are complex structures, but *Hox* expression sets the boundaries for the regions of the vertebral column, determining to a large extent which kind of vertebrae are formed. These expression patterns exists before the differentiation into somites and are thus independently of the clock-and-wavefront (Gridley, 2006; Mallo, 2009; Di-Poï et al., 2010; Müller et al., 2010). This system leads to variation in the numbers of vertebrae in each part of the column. It remains largely unclear what the developmental mechanism is that constrains the number of presacral vertebrae to (approximately) 26 in most mammals.

This then poses a conundrum regarding vertebral homologies. Most humans have seven cervical, 12 thoracic, and five lumbar vertebrae, whereas in most gorillas these numbers are seven, 13, and four (McCollum et al., 2010). Is the first lumbar vertebra in

humans homologous to the first lumbar of gorillas, or is somite level the measure, and the last gorilla thoracic vertebra is homologous to the first human lumbar? Here, knowledge of developmental biology forces us to face the problem of what criteria to use to identify homology. Polly et al. (2001), for snakes, and Buchholtz (2007), for cetaceans, identified modules in the vertebral column that appear to evolve in close correlation with each other, consistent with the idea that the *Hox* code guides the regionalization of the vertebral column, and that other signaling molecules guide the number of vertebrae in each species.

Whereas the total number of presacral vertebrae is 26 in most mammals (Narita and Kuratani, 2005; Müller et al., 2010), cetaceans are an exception, having a variable number, some with but usually more than 26 presacral vertebrae (where the pudendal nerve marks the first sacral vertebra, following Sljjper, 1936, because sacral vertebrae do not fuse in mocern cetaceans). A few Eocene cetaceans have vertebral columns that are complete. In *Ambulocetus* (Madar et al., 2002), there are 31 vertebrae preceding a 4-vertebra fused sacrum. In *Rodhocetus* (Gingerich et al., 2001), there are 26 presacral vertebrae followed by a sacrum with a joint for the ilium, but the sacral vertebrae are not synostosed. In *Dorudon*, the sacrum consists of unfused vertebrae that are nevertheless recognizable by their thickened transverse processes (Uhen, 2003). This genus has 40 presacral vertebrae.

It appears than that the constraint that gives most mammals 26 presacral vertebrae was lost in Eocene cetaceans, although not in a fashion that can be interpreted easily. The loss of morphological distinction between vertebral regions suggests that central axis patterning was diminishing on a lineage segment basal to *Dorudon*, and continued to modern cetaceans (represented by *Stenella* and *Balaena* in Fig. 4). High vertebral numbers were not present in *Rodhocetus* (Gingerich et al., 1994) but they were present in *Ambulocetus*, a lower branch on the cetacean cladogram than *Rodhocetus*, implying homoplasy or an incompletely understood phylogeny. Developmental evidence implies that presacral vertebral numbers and vertebral regionalization are independent characters, and thus understanding vertebral development provides biologically relevant information for identifying characters: two independent processes shape vertebral evolution, and their morphological expression is confounded. Development does not solve questions of homology, and it does not simplify the identification of discrete characters in systematics, but it should be taken into account when making such identifications. The critical evolutionary question is whether *Hox* gene expression or the clock-and-wavefront changed and altered morphology along the vertebral column. Depending on the answer to that question, the hypotheses of homology differ, and characters as used in systematics should be defined differently.

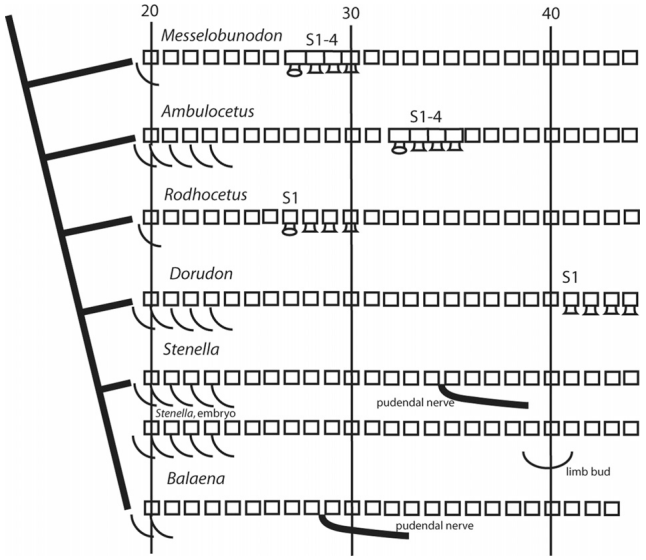

FIGURE 4. Diagram of part of the vertebral column of Eocene and modern cetaceans. Cranial is to left in these diagrams, vertical lines indicate vertebral counts starting at the atlas (vertebra 1), S1 is the first sacral vertebra, and blocks with a curved lines (ribs) indicate thoracic vertebrae. *Messelobunodon* represents the plesiomorphic artiodactyl condition with 26 presacral vertebrae, and a synostosed sacrum composed of four vertebrae, the first of which bears the sacroiliac joint (based on Franzen, 1981). *Ambulocetus* (Madar et al., 2002), *Rodhocetus* (Gingerich et al., 1994), and *Dorudon* (Uhen, 2004) are Eocene whales. *Rodhocetus* and *Dorudon* lack fused sacral vertebrae, and *Dorudon* lacks an articulation for the innominate (oval on S1). In adults of the modern dolphin *Stenella* and the bowhead whale *Balaena*, the sacrum cannot be recognized morphologically, but the position of the pudendal nerve has been used as a guide to its identification (and is here based on dissections of fetuses).

HIND LIMBS

Induced by signaling from the axis of the body, generalized vertebrate embryos develop small, paired outgrowths on the side of the body, from lateral plate mesoderm and ectodermal epithelium. These are called limb buds and they will eventually form the limbs (Fig. 2). A crest of ectodermal cells is located at the apex of the limb bud, the apical ectodermal ridge (AER). In humans, limb development starts near the end of the fourth week of development. Two separate signaling centers occur in the limb (Fig. 5): the AER and zone of polarizing activity (ZPA). In most species, the AER is morphologically distinct and produces a number of signaling proteins, including those of the fibroblast growth factor (FGF) family, which direct outgrowth of the limb bud. The ZPA is not morphologically different from the cells near it, but it comprises cells buried in the caudal part of the limb bud, and these cells produce SHH (a protein called sonic hedgehog) that plays a role in patterning the limb (e.g., number, size, and identity of digits, etc.), among other things (reviewed by Gilbert, 2010). This stage of limb development is called the bud stage, and once the limb bud has reached this point, it can grow out independently, without signals from the body. The AER and ZPA regulate each other in a feedback loop and signals from both are required to maintain outgrowth of a limb.

Evolution of limb morphology can, in part, result from differences in the signaling that occurs at the bud stage. Adult pythons, for instance, have small bony remnants of the pelvic girdle and femur immersed within the soft-tissue body walls. Python embryos do not have a forelimb bud, but they do develop a hind limb bud initially. However, after this initial formation of the hind limb bud, cessation in AER and ZPA signaling arrests limb development. Cohn and Tickle (1999) found that embryonic python hind limb buds lack expression of the gene products associated with these signaling centers (such as DLX, FGF2, MSX, and SHH). In the absence of the signaling that sustains limb outgrowth (Fig. 5), and with the body growing longer, the growth of the hind limb buds ceases and they regress. Therefore, reduction of snake hind limbs is controlled very differently from reduction of snake forelimbs. Developmental data suggest that reduction of the forelimbs and hind limbs are best considered independent characters in snakes.

Signaling downstream from *Hox* genes also plays a central role in another excellent example of evolution that combines paleontological and developmental approaches. Pelvic spines in three-spined stickleback fish (*Gasterosteus*) are paired, and are the homologue of pelvic fins in other perciforms. Evolution of pelvic spines has been studied in detail and is one of the best examples where rates of evolution can be studied on a fine timescale, sometimes at resolutions higher than centuries (Bell,

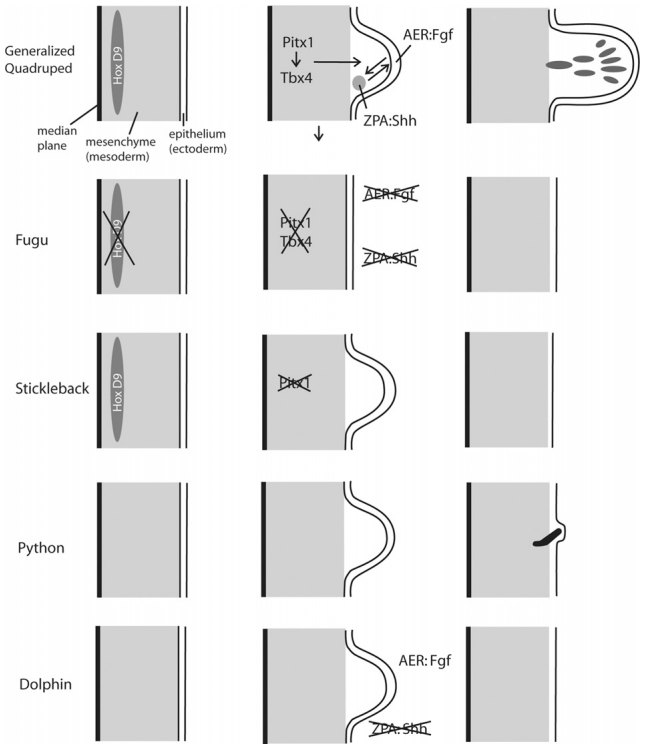

FIGURE 5. Three stages of hind limb development for a generalized quadruped (chick, mouse), two bony fish (the Fugu *Takifugu*, Tanaka et al., 2005; the stickleback *Gasterosteus*, Shapiro et al., 2004, Chan et al., 2010), a snake (*Python*, Cohn and Tickle, 1995), and a dolphin (*Stenella,* Thewissen et al., 2009). Genes/proteins investigated varied between these studies. A gene/structure that is crossed out indicates that it was positively determined that this gene/protein is not expressed/present during hind limb initiation; but if a gene/structure is not mentioned, it merely means that it was not investigated. In spite of this, it is clear that hind limb loss can follow multiple developmental paths. Anlagen for the hind limb bones are shown in the third column, but the pelvic girdle is not indicated.

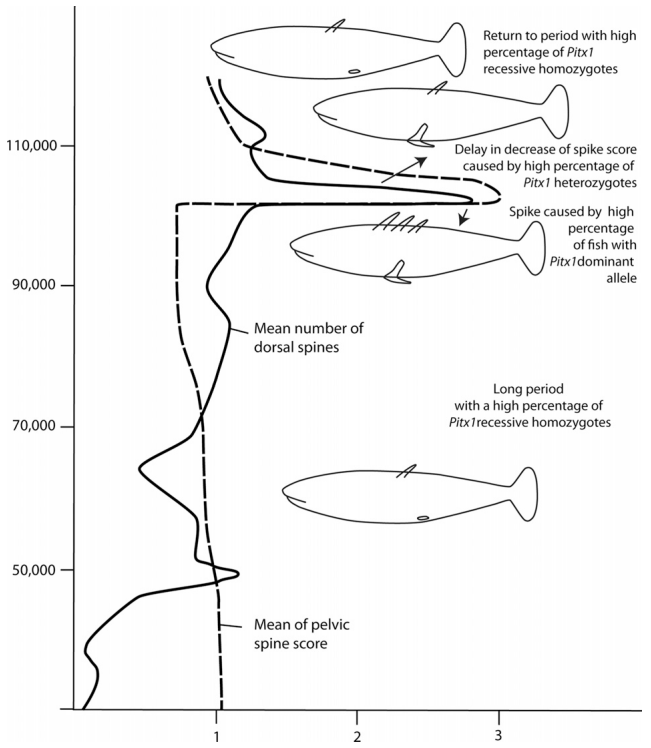

FIGURE 6. Stickleback (*Gasterosteus*) evolution in a Miocene lake in Nevada (data from Bell et al., 1985, 2006). Large sample sizes, dense sampling (years on *y*-axis), and quantitative analysis (mean number of dorsal spines, solid line, and mean score for pelvic spine size, dashed line on *x*-axis) allowed identification of detailed evolutionary pattern. Study of developmental control genes involved in spine and pelvis formation (*Pitx1*) allowed for the formulation of hypotheses that explain these patterns.

1994; Bell et al., 2006). Factors contributing to evolutionary change in pelvic spines are well understood. They include the presence or absence of predators and physiological limitations on building skeletal tissues (Bell et al., 1993; Reimchen, 1994).

Bell et al. (1985, 2006) documented stickleback evolution in a single Miocene lake deposit section in Nevada (Fig. 6). In the first 93,000 years documented by the section, a form of *Gasterosteus doryssus* with small pelvic spines evolved toward even smaller spines. It was then replaced by a form with larger spines and more armor overall, and in the subsequent 17,000 years, spines and other armor of this new form were also reduced in size. Surprisingly, in these 17,000 years, pelvic spine reduction was delayed compared with reduction of the rest of the armor by 2750 years (Bell et al., 1985, 2006).

Pelvic spine reduction in modern stickleback populations is usually caused by down-regulation of *Pitx1*, which is expressed in the flank area of the hind limb on the onset of limb development, although other mechanisms may occur too (Cresko et al., 2004; Shapiro et al., 2004). Individuals with down-regulated *Pitx1* usually have asymmetrical pelvic spines, with the left one being larger (Cresko et al., 2004; Shapiro et al., 2006). Given that this asymmetry also occurs in the Nevada fossils, Bell et al. (2006) argued that, here too, *Pitx1* played a role. In modern mutants where only one *Pitx1* allele is silent, the pelvis is not reduced, but lateral armor is reduced significantly, because the non-functioning allele only has a minor effect on pelvis morphology, but a major effect on armor size. It follows that the evolutionary change of pelvis reduction only becomes noticeable in the fossil population when frequency of the silenced allele rises and homozygotes for the recessive condition occur. Although a small morphological change in the pelvis can be the subject of selection (Shapiro et al., 2004), selection had a profound effect favoring the new morphology when homozygotes with strongly reduced pelves became common (Bell et al., 2006). This difference in gene control explains the delay in evolution between armor and pelvic reduction.

Morphological data do not indicate a priori that some features are more liable to heritable change than others, but, in the stickleback case, developmental biological data show that some features are more labile evolutionarily and prone to change. The *Pitx1* enhancer is fragile in sticklebacks and may explain differential probabilities of morphological evolution, in effect rates of evolvability. In the stickleback example, understanding of gene control offered a better understanding of the pattern of the evolutionary change by suggesting a powerful mechanistic explanation. However, the potential of developmental biological data to offer insights into paleontological analyses goes beyond this. As is common in gene regulation, mutations of the gene coding for the transcription factor are often lethal, because transcription factors are involved in many processes that contribute to formation of the body plan, and a mutation in a single gene can affect function of these processes so dramatically as to be incompatible with life. Evolution, then, is thought to not work on the genes that code for

proteins, but instead on DNA sequences that regulate the transcription, including so-called enhancers (Fig. 1). These enhancers are located on the same chromosome as the gene they act on and generally close to it (as shown in Fig. 1). Such enhancers are called *cis*-regulatory elements and, unlike the gene they regulate, they are often only involved in controlling transcription related to one or a few functions of the gene. This allows for region-specific regulation and gives evolution a tool to modify some organs affected by a gene, but not all organs affected by that gene.

For instance, the *Pitx1* gene is involved in the development of organs including the hind limb, the pituitary gland (Proszkowiec-Weglarz et al., 2010), and the retina (Markitantova et al., 2010), but some of its regulators have a much more limited function. Shapiro et al. (2004) and Chan et al. (2010) documented that an enhancer of *Pitx1* is silenced in *Gasterosteus* that lack pelves, even though the *Pitx1* gene and protein are normal. Shapiro et al. (2004, 2006) found that the same enhancer may be affected in stickleback populations that have lost their pelvis independently, such as populations in Iceland and British Colombia and even in different stickleback genera. The chromosome region where *Pixt1* and its enhancer are located is particularly fragile, leading Chan et al. (2010) to suggest that it is particularly susceptible to evolution by mutations or DNA deletions. This indicates that, from a developmental mechanistic standpoint, evolution in certain traits is more common than in other traits.

Tanaka et al. (2005) studied the absence of pelvic fins in the perciform osteichthyan *Takifugu* (Fugu), which lacks any morphological remnant of hind limbs: there are no pelvic or limb remnants at any ontogenetic stage, and embryos never have hind limb buds. Tanaka et al. (2005) compared *Takifugu* with sticklebacks with pelvic spines. These authors found that *Takifugu* lacks HOXD9 signaling in the area where the pelvic spines develop in *Gasterosteus*, and, downstream from this, also lacked expression of genes normally involved in limb development (*Tbx4*, *Pitx1*, *Shh*, *Fgf10*). They proposed that the pelvic fin was absent in *Takifugu* because of HOX signaling, a situation similar to absence of forelimb development in pythons. Consistent with this, Shapiro et al. (2009) showed that in related stickleback genera (*Gasterosteus* and *Pungitius*), morphologically similar reduction patterns of the pelvis are underlain by different developmental patterns.

Developmental evidence thus enriches the understanding of morphological traits, and can provide insights into the falsification of hypotheses of equality of character states. The absence of hind limbs can be the result of changes in several developmental pathways, indicating that the resulting similarity in morphology is induced by a different series of genes. Dissimilarity of developmental pathways is usually taken as an indication of non-homology by developmental biologists, but takes place at a level of resolution that cannot be studied by paleontologists.

Yet another example of hind limb loss is found in cetaceans. Guldberg and colleagues (e.g., Guldberg and Nansen, 1894) were the first to observe that cetacean embryos form hind limb buds and that these regress later in development. Interestingly, pelvic and hind limb remnants differ among modern cetaceans. Whereas *Balaena* males retain innominate, femur, and cartilaginous tibia (Thewissen et al., 2009), most delphinoids only have a single bone as adults (the innominate). Thewissen et al. (2006) studied protein signaling in embryos of the dolphin *Stenella* and found that the AER of the hind limb bud signaled FGF8, but there are no SHH signals, indicating a lack of a ZPA. This is in contrast to the forelimb, which expresses FGF8 and SHH normally. Thus, the mechanism of hind limb loss in *Stenella* differs from that in pythons and fugu. *Pitx1* is expressed independently from *Shh* (Fig. 5), but Thewissen et al. (2006) did not investigate whether *Pitx1* is expressed in cetacean hind limbs. Shapiro et al. (2006) made a similar argument implicating *Pitx1* in another mammal lacking hind limbs, the manatee (*Trichechus*), based on the predicted left-right asymmetry caused by this gene in other species.

Using the cetacean fossil record, Thewissen et al. (2006) were able to elucidate the evolutionary pattern of hind limb loss in cetaceans further (Fig. 7). For the initial part of their evolution, hind limbs of cetaceans were reduced in length as cetaceans become aquatic, from the pakicetid (Thewissen et al., 2007) to the ambulocetid (Thewissen et al., 1994) and to the protocetid (*Rodhocetus*, Gingerich et al., 2001) node on the cladogram. However, all bony elements of the hind limb were retained: an

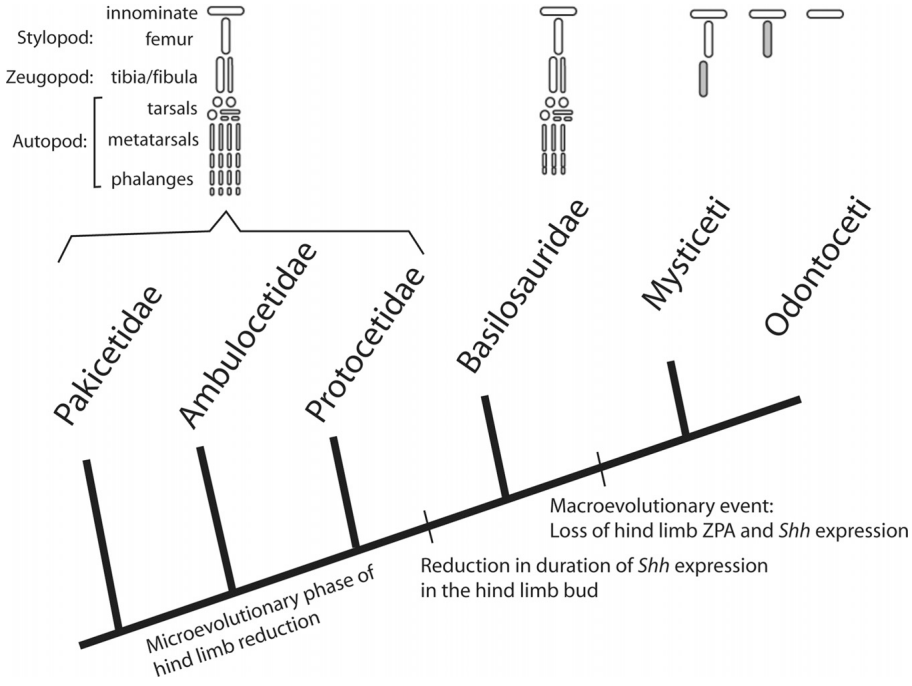

FIGURE 7. Hind limb loss in cetaceans from Eocene to modern times (based on Thewissen et al., 2006). The most basal families shown here had fully patterned hind limbs with four toes, whereas modern cetaceans may have just an innominate (*Stenella*), innominate and femur, or innominate, femur, and (cartilaginous) tibia (*Balaena*). Bone is indicated as white phantoms, cartilage as gray. The morphological evolutionary pattern combined with understanding of gene expression in modern cetacean hind limbs allowed the inferences about the evolution of developmental control along the cladogram. Cetacean origins were reviewed recently by Thewissen et al. (2009).

autopodium with four toes with three phalanges each. Hind limb patterning in the embryo was probably not different from that of their land ancestors in these early whales. The next node on the cladogram is that of basilosaurid cetaceans. These were fully aquatic and had tiny hind legs with three toes only and reduced numbers of phalanges (Gingerich et al., 1990; Uhen, 2003). This shape of autopodium (hand and foot) is very similar to that in some species of skinks. In this group, forms with more and fewer autopodial elements occur, and Shapiro et al. (2003) determined that the length of exposure to SHH in the embryo was strongly correlated to the number of digits in hand and foot, suggesting that heterochronic signaling with SHH determined foot shape. Combining the pattern of the basilosaurid foot and the developmental data on SHH in *Stenella*, Thewissen et al. (2006) proposed that *Shh* expression was reduced in the hind limb of basilosaurids, and subsequently lost completely in later whales. Thus, by combining fossil and developmental biological evidence, events in the evolution of *Shh* expression in cetaceans could be analyzed and its evolutionary timing constrained. It is likely that lack of SHH signaling is only one of several factors contributing to absence of hind limbs in cetaceans, because mice in which *Shh* is knocked out do develop deformed limbs, but these are more complete than the hind limbs of cetaceans (Charité et al., 2000; Panman and Zeller, 2003). Experimental manipulation of expression is not possible in cetaceans, limiting our ability to test hypotheses proposed on the basis of descriptive protein expression results such as those of Thewissen et al. (2006). This emphasizes the importance of studying development in model organisms.

Heterochronic expression of genes leads to differential development, and has been of great interest to developmental biologists (Smith, 2003) and paleontologists (Gould, 1977). Whereas sophisticated methods are available for the study of heterochronic patterns, the underlying processes are often not understood. Developmental biologists have found that differences in timing of interacting developmental processes cause much of the variation found among organisms (Muller, 2007; Richardson et al., 2009), and this is of importance for paleontology (Jeffery et al., 2005). The skink hand and foot example shows that heterochronic expression led to the formation of more digital elements. Such heterochronic gene expression is often caused by *cis*-regulation of genes coding for signaling proteins and is considered a major driver of evolution by developmental biologists (Carroll et al., 2004).

HANDS AND FEET

Within the tetrapod hand and foot, digit identity is partially regulated by the activity of *Hox* genes. Four *Hox* genes are expressed: *Hoxd13*, *Hoxd12*, *Hoxd11*, and *Hoxd10* (Zakany et al., 2004; Montavon et al., 2008; Wagner and Vargas, 2008), digits 2–5 express all four genes, but digit 1 (thumb and big toe) only expresses *Hoxd13* (Fromental-Ramain et al., 1996). In species where digit 1 resembles the other digits in length or shape, patterns in *Hox* gene expression in digit 1 are more similar to those of the other digits (Montavon et al., 2008; Wagner and Vargas, 2008). In some cases, the morphology of digit 1 appears to vary independently from the other digits. Most anthropoid primates display two developmental modules in the hand: a digit 1 module, and a second module for digits 2–5 and their associated metacarpals. Reno et al. (2008) found that changes in the length of digits 2–5 were correlated with each other and with changes in the radius, whereas changes in the length of digit 1 were independent in a variety of extant primates, as well as *Australopithecus*. This pattern of modular bone lengthening follows the expression pattern of *Hoxd11* in late-stage mice that express *Hoxd11* in the radius and digits 2–5 (Reno et al., 2008).

Within birds, a developmental shift in digit identity has led to controversy over digit homology that has lasted more than 150 years (Hansen, 2010; Fig. 8), and has been problematic for character polarization in phylogenetic systematics (Wagner and Gauthier, 1999; Bever et al., 2011; Young et al., 2011; Young and Wagner, 2011). The tridactyl hand of birds and theropod dinosaurs was thought to consist of digits 1–3 based on paleontological evidence (Wagner and Gauthier, 1999), but embryological evidence based on patterns of digital condensation indicated that the hand and foot of modern birds consisted of digits 2–4 (Burke and Feduccia, 1997). Recent developmental research (Fig. 7) has shown that of the five anlagen in an embryonic chick forelimb, only the middle three develop: digits 2, 3, and 4 (Harfe et al., 2004; Tamura et al., 2011; Wang et al., 2011; Welten et al., 2011). Signaling from anterior (Tamura et al., 2011; Wang et al., 2011) and posterior (Harfe et al., 2004, on mice) aspects of the developing limb affects these anlagen, causing digit 4 to assume the shape of digit 3 (due to SHH signaling) and digit 2 to assume the shape of digit 1 (Tamura et al., 2011; Wang et al., 2011, on chicks). Thus, paleontological evidence from the forelimbs of theropod dinosaurs combined with developmental and embryological data show that the developmental mechanism for digit specification was both evolutionarily and developmentally uncoupled from the progenitor identities initially formed during hand and foot development. This shift in morphological identity constitutes an apomorphic feature of avian morphogenesis (Tamura et al., 2011), and shows the complexity in distinguishing homologies, because a homologous signaling cascade is translocated on non-homologous fingers. This does not help us resolve the homologies of the fingers, but adds depth to our understanding of these homologies.

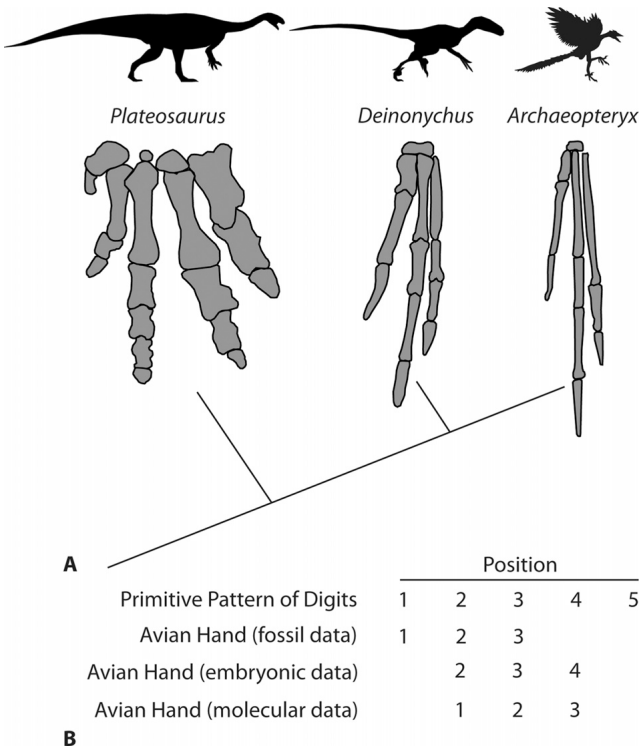

FIGURE 8. Paleontological, embryological, and developmental evidence for the digit homologies in birds. **A**, forelimb diagram and relationship of some theropods and birds (after Romer, 1956; Wagner and Gauthier, 1999). **B**, diagram indicating possible homology of digits of hand, showing different interpretation of the three fingers of the avian hand on the basis of fossil and embryological evidence. Developmental biological evidence showed that a frameshift has occurred that imposed the shape of fingers 1–3 on the anlagen of fingers 2–4.

During hand and foot development, digits can either become separated, like human fingers and toes, or the digits can stay encased in soft tissue, like the webbing in the feet of ducks, the wing membrane of bat wings, and cetacean flippers. During limb morphogenesis, the developing digits are separated through programmed cell death (apoptosis) in the interdigital tissues. A key molecular component of the interdigital apoptosis pathway is *Bmp* (Hernandez Martínez and Covarrubias, 2011). In some taxa, *Gremlin* interrupts the apoptosis pathway, causing retention of the interdigital tissues. For instance, proximal interdigital cell death is stopped by overlapping *Bmp* and *Gremlin* expression in the developing partially webbed hands and feet of mice (Pajni-Underwood et al., 2007) and completely webbed feet of ducks (Merino et al., 1999). Bats also employ *Gremlin* in the interdigital tissues, and this expression may act to inhibit interdigital apoptosis and potentially allow for maintenance of interdigital tissues that will eventually form the wing membrane (Weatherbee et al., 2006). Bats also extend *Fgf8* expression from the AER to the interdigital tissues, which may allow for proliferation and elaboration of the wing membrane tissues (Weatherbee et al., 2006). Finally, *Shh* expression in the bat ZPA extends into the interdigital tissues (Hockman et al., 2008). Taken together, overlapping *Shh-Fgf-Bmp-Gremlin* expression in the interdigital tissues may act to inhibit interdigital apoptosis, and could direct limb outgrowth from the interdigital tissues during wing formation (Hockman et al., 2008).

In this example, developmental data assist in assessing homology by showing that different developmental pathways can lead to similar morphological results. However, developmental evidence does not solve questions of homology, it only provides additional insights into the study of homology. Ultimately, in our view, that problem should be addressed by a greater understanding of what the actual evolutionary changes were that occurred in a lineage, and understanding the coherence among these changes.

TEETH

During limb formation, ectodermal epithelium and mesodermal mesenchyme signal each other, inducing growth and development of both tissues in close synchrony. The genes and proteins involved in a signaling cascade as a whole are often described as a developmental toolkit. The genes that compose the epithelial-mesenchymal toolkit are applied by the embryo time and time again in different places and at different times, whenever these tissues need to work together to produce an organ. Such organs include hairs, teeth, feathers, mammary glands, and claws. It is this toolkit that plays a special role in evolution and the organs it forms are sometimes referred to as epithelial organs (Chuong, 1998).

Interactions between epithelium and mesenchyme produce teeth. Tooth development starts after week nine in humans, early in the fetal period, when most other organs have already formed. The early development of all epithelial organs is similar. First, the epithelium thickens, forming a placode, then a clump of ectodermal epithelium sags into the underlying mesenchyme, forming tooth bud in the jaws. Hair follicles are formed similarly in other areas of the body, and the same toolkit is used. In the tooth bud, ectodermal tissue is mineralized to become enamel and mesenchymal to become dentine (Richman and Handrigan, 2011); in the hair follicle, ectodermal germinal cells produce keratin that forms the hair shaft (Paus, 1998).

Signaling proteins such as SHH, BMP, and FGF play important roles in tooth development, but the role of the protein ectodysplasin (EDA) illustrates the broad implications of developmental biological results for paleontologists (Kangas et al., 2004). The expression of *Eda* is restricted to epithelium adjacent to the tooth-forming tissues at the dental placode stage. Kangas et al. (2004) compared wild-type mice with a naturally occurring strain of mouse mutants called Tabby mice and a strain of mice that were created in the laboratory and are called K14-Eda mice. Tabby mice do not produce functional EDA, whereas K14-Eda mice produce the protein in excess. The differences in the dentition of these mice (Fig. 9) are induced by concentration differences in a single protein and show that one protein can affect multiple dental characteristics that appear to be anatomically independent both within one tooth, and across the tooth row. The mice lacking *Eda* have narrower cheek teeth, and pairs of lingual/labial cusps are fused, whereas they are separated in wild-type mice and divided by a longitudinal crest in *Eda*-overexpressing mice. The talonid of Tabby mice is shallow and does not bear distinct cusps, and an anteroconid is absent. Both cusps are present in wild-type mice, and K14-Eda mice have large hypoconunid and anteroconid. In addition, K14-Eda mice have an extra tooth, anterior to the molars.

As pointed out by Kangas et al. (2004), the differences found among these mice are reminiscent of those found in the

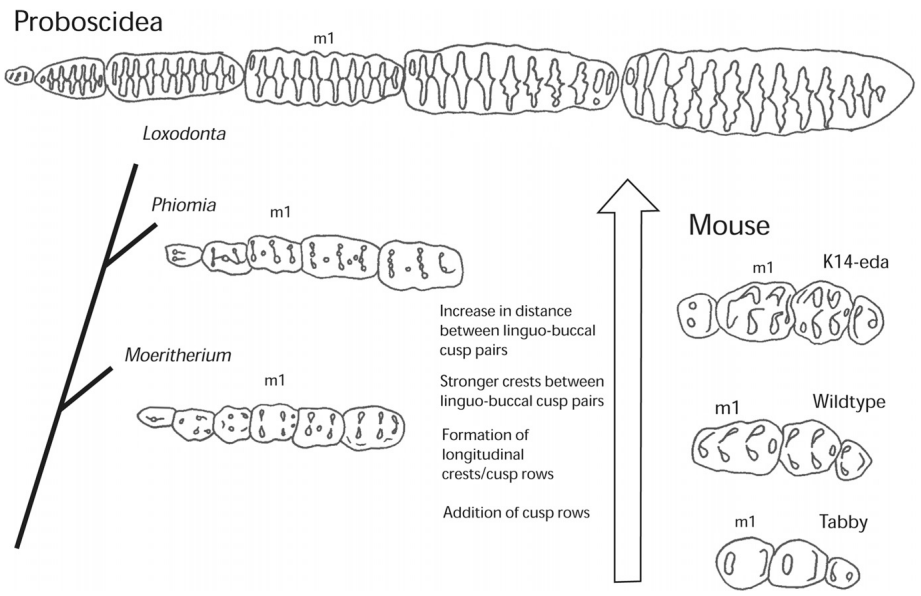

FIGURE 9. Cheek tooth morphology in three genera of proboscideans (Eocene *Moeritherium*, *Phiomia*, and modern *Loxodonta*) and three modern strains of mice (K14-Eda, Wildtype, and Tabby), showing morphological trends in the evolution of proboscideans and trends due to *Eda* expression in the mice. Conventions for figuring tooth morphology: large open figures indicate wide topographical highs (cusps or crests); lines indicate narrow topographical highs (crests). Mice data and developmental analysis is based on Kangas et al. (2004), who identified morphological trends listed (arrow). *Phiomia* and *Moeritherium* are based on Tassy (1996); *Loxodonta* is based on Laws (1966). Relation among proboscideans is indicated by cladogram. *Loxodonta* has horizontal tooth replacement and wears its cheek teeth at oblique angles to their mineralization front. Tooth row shown here is a composite of different stages, showing diagrammatic cusp and crest morphology at mineralization, and does not occur at any stage during life.

evolution of several groups of mammals. In Eocene *Moeritherium*, the distance between lingual and buccal cusps is increased and weak crests between them present. Both of these trends continue in Eocene *Phiomia* and *Loxodonta* (the modern African elephant). In *Phiomia*, m1 and m2 have an additional row of cusps, whereas in *Loxodonta* many rows are added in all cheek teeth. Finally, small cusps occur in the central axis of the tooth in *Phiomia*, and these form a constant, longitudinal feature of the teeth of *Loxodonta*, ling up to intersect the transverse lophs. These four evolutionary trends in proboscideans mirror those seen in the Eda series of mice.

Of course, the mentioned proboscideans differ in many more features than those discussed, and some are not consistent with EDA signaling (e.g., the loss of p1 at the basal node in Fig. 9). Certainly, multiple genes were involved in creating the differences among the proboscideans. We hypothesize that EDA signaling likely played a role in proboscidean evolution, but that it was modulated by the expression of other genes, and that many of the differences among proboscideans are not related to EDA signaling. Fliniaux et al. (2008) found that, in addition to the effects described above, EDA signaling also affects other developmental pathways (WNT), and may modify morphologies through these pathways in a subtle way.

Proboscidean evolution is not linear; strongly lophodont forms lived long before more bunodont forms (Sanders et al., 2010). This indicates that the evolutionary pattern is more complicated than shown in Figure 9. However, the mouse example does indicate that seemingly unrelated anatomical dental features within one tooth, and among teeth in one toothrow, are not independent.

CONCLUSION

Paleontology is the prime source of information about phenotypic evolution on a geological timescale. Developmental biology, by studying modern animals, can add information of 'how' an evolutionary change happened at a mechanistic level. Integrating paleontological evidence with that from other sciences, paleontologists are forced to challenge their assumptions. Evolutionary developmental biology research makes us examine hypotheses of homology, character definition and independence, heterochrony, and character evolution. Developmental biological data help us pinpoint distinctions among convergent morphologies. Maybe most importantly, developmental data help us realize that anatomically disjunct features may be controlled by the same gene expression pathway, whereas morphologically simple features may be determined by multiple pathways that evolve independently. This affects the evolvability of features and rates of evolution. Development biology can help paleontologists answer fundamental questions about evolution, although it does not hold all the answers. It is the synergy between all the biological and geological sciences that provides the most detail in evolution.

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