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RESEARCH ARTICLE

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Review and experimental evaluation of the embryonic development and evolutionary history of flipper development and hyperphalangy in dolphins (Cetacea: Mammalia)

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Summary

Cetaceans are the only mammals to have evolved hyperphalangy, an increase in the number of phalanges beyond the mammalian plesiomorphic condition of three phalanges per digit. In this study, cetaceans were used as a novel model to review previous studies of mammalian hyperphalangy and contribute new experimental evidence as to the molecular origins of this phenotype in embryos of the pantropical spotted dolphin (*Stenella attenuata*). Results show embryos of dolphins, mice, and pigs share similar spatiotemporal patterns of signaling proteins known to shape limbs of mammals (e.g., FGF8, BMP2/4, WNT, GREM). However, fetal dolphins differ in that their interdigital tissues are retained, instead of undergoing apoptosis, and that multiple waves of interdigital signals likely contribute to the patterning of supernumerary joints and phalanges in adjacent digits. Integration of fossil and experimental evidence suggests that the presence of interdigital webbing within the fossils of semi-aquatic cetaceans, recovered from the Eocene Epoch (49Ma), was probably the result of BMP-antagonists counteracting interdigital apoptosis during embryonic limb development. Modifications to signals originating in these interdigital tissues likely contributed to the origin of an incipient form of hyperphalangy in obligatorily aquatic cetaceans about 35Ma. Finally, an extreme form of hyperphalangy, with six or more phalanges per digit, evolved independently in rorqual whales (Balaenopteridae) and delphinids, and was probably associated with a wave of signaling within the interdigital tissues.

KEYWORDS
cetacea, FGF, flipper, WNT

1 | REVIEW OF MAMMALIAN LIMB DEVELOPMENT AND HYPERPHALANGY

The field of limb development continues to elucidate the complex processes underlying human limb malformations (Little & Cornwall, 2016) and offers insights into the developmental plasticity that creates novel and complex phenotypes (Zuniga, 2015). Some recent advances in mammalian evolutionary developmental (evo-devo) biology compared the molecular mechanisms shaping the limbs of terrestrial taxa and bats (Cooper et al., 2014; Sears, 2011). However, the molecular mechanisms shaping the limbs of aquatic mammals remain poorly understood. This paucity of data is unfortunate as the limbs of aquatic mammals display several critical adaptations that presumably aid in aquatic locomotion. For example, cetaceans (whales, dolphins, and porpoises) possess digits with supernumerary phalanges and joints (hyperphalangy, Figure 1), as well as soft tissue flippers encasing these digits (Cooper, Berta, Dawson, & Reidenberg, 2007a; Fedak & Hall, 2004; Richardson & Oelschläger, 2002). Although the anatomy and performance of cetacean limbs have been well-studied, the study of cetacean limbs at the molecular level has the potential to produce a greater understanding of the mechanisms underlying the phenotypic diversity among mammalian limbs.

1.1 | Morphological innovations of the cetacean manus and their hydrodynamic consequences

The manus of cetaceans displays soft-tissue and bony innovations including a hydrofoil-shaped, soft-tissue flipper that encases hyperphalangeous digits. Most mammalian limbs (Figure 1b,c) display the

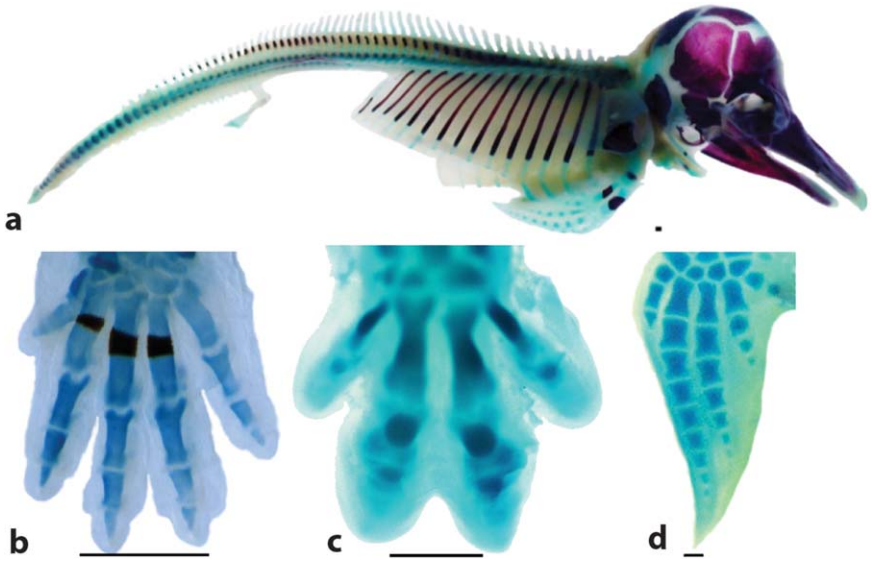

FIGURE 1 Cetaceans are the only mammals that evolved a soft-tissue flipper with digits made of supernumerary phalanges (hyperphalangy). Within the pantropical dolphin (**a**,**d** ~110 day old *Stenella attenuata*, LACM 94285), hyperphalangy is present in digits II and III, contributing to a phalangeal formula of 1/9/6/2–3/1. Mice (**b**, 15.5 dpc) display the plesiomorphic mammalian condition of having separated digits consisting of 2/3/3/3/3 phalanges. Pigs (**c**, 33 dpc) share this typical mammalian phalangeal count but lack digit I. No experimental evidence has yet shown how dolphins develop a soft-tissue flipper and hyperphalangy (**a**, **d**). This study utilizes *Stenella* as a model for the evaluation of the evolution and development of hyperphalangy. All images show fetal specimens with soft tissues removed and connective tissues stained such that bone is red and cartilage is dark blue. Number of phalanges per digit are separated by/. Scale bars are 1 cm in length

plesiomorphic morphology of five metacarpals and digits, with each digit consisting of a maximum of three phalanges each (2/3/3/3/3) (Fedak & Hall, 2004). This formula is remarkably conserved and variation is typically limited to a reduction in the number of elements. Bats both reduced the number (Wang et al., 2014) and extended the length of their phalanges to support a wing membrane, and are the only mammals to have evolved powered flight. Several terrestrial ungulates and two families of cetaceans (Cooper et al., 2007a; Flower, 1885; Kunze, 1912; Kükenthal, 1893; Turner, 1892) lack metapodials and phalanges (Figure 1c), potentially as a result of modified sequences of *Hoxd12* genes (Wang et al., 2009). This reduction potentially increases locomotor efficiency (Clifford, 2005; Cooper et al., 2014; Sears et al., 2011).

Within mammals, only cetaceans display supernumerary phalanges (Cooper et al., 2007a; Howell, 1930; Richardson & Oelschläger, 2002; Sedmera, Misek, & Klima, 1997). Although most cetaceans display 4–5 phalanges in a digit (Figure 2a), rorqual whales and delphinids (Figures 1d and 2b) independently evolved an extreme form of hyperphalangy, with a minimum of six phalanges in some digits (Cooper et al., 2007a). For example, a member of the dolphin family, the pilot whale (*Globicephala melaena*), can display 11–17 phalanges in a single digit (Cooper et al., 2007a; Howell, 1930; Kükenthal, 1889). These hyperphalangeous digits typically lie adjacent to the leading edge of a flipper (Figure 3). Forces acting on the leading edge are probably transmitted to the underlying phalanges and joints. While most mammals only display two joints within a digit, dolphins distribute loadings along their supernumerary joints, and thereby presumably minimize loading on an individual joint. Within these joint tissues, loading probably manifests as tensile and compressional forces on the leading and trailing edges of joints, respectively.

Both the presence of a flipper and elongation of the digits via hyperphalangy effectively increase surface area of the cetacean manus. Flippers are shaped as hydrofoils with a smooth and cambered contour along the chord (distance between the leading and trailing edges of the flipper, Figure 3). This shape allows the limb to act as a control surfaces that aids in steering, counteracts body pitch, and increases drag when attempting to slow forward rectilinear locomotion (Cooper et al., 2008; Fish & Rohr, 1999; Fish, 2002; Miklosovic, Murray, Howle, & Fish, 2004; Weber, Howle, Murray, Reidenberg, & Fish, 2014; Woodward, Winn, & Fish, 2006). Flippers in general do not generate propulsion or thrust, although occasionally humpback whales can flap their forelimbs like penguins and sea-lions to produce forward motion (Segre, Seakamela, Meÿer, Findlay, & Goldbogen, 2017). Movements of the cetacean flipper are generated by muscles associated with the glenohumeral joint and are therefore mostly housed within the body wall. The manus is typically stiff and no evidence suggests cetaceans can actively control digit movements. Muscles that could allow for digit flexion and extension are absent, reduced, or impeded by the presence of dense connective tissues encasing these digits (Cooper et al., 2007b). The flippers of humpback whales and some dolphins bend and flex along their length, but this movement is probably the result of deformation, rather than the result of controlled skeletal muscle contractions.

The stiffness of cetacean flippers is unusual compared to other taxa that also evolved webbing as a response to life in a fluid habitat. Pinnipeds (seals, sea lions, and walruses) generate thrust via flippers that act as flexible, oscillating propulsors (Feldkamp, 1987), and display

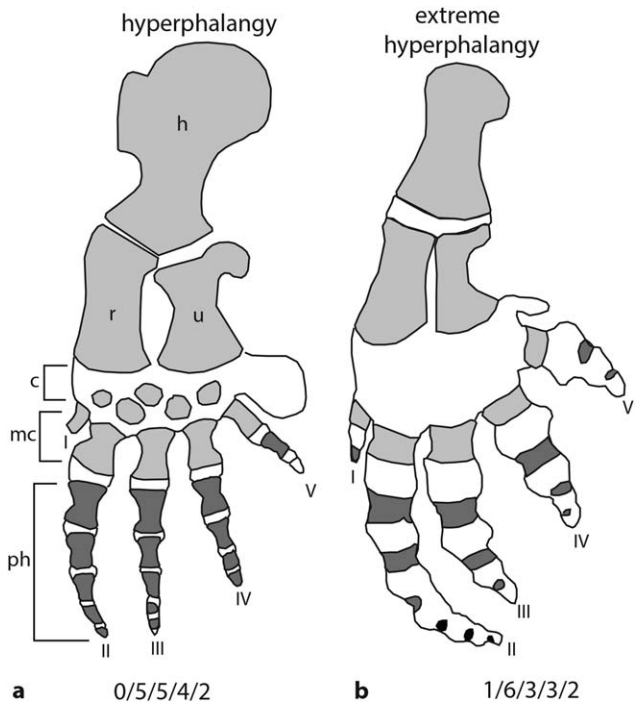

FIGURE 2 Most cetaceans display a minor form of hyperphalangy (A, *Physeter*) with five or fewer phalanges in a single digit. In contrast, delphinids (B, *Orcinus*) and rorqual whales (balaenopterids) display an extreme form of hyperphalangy, characterized by at least six phalanges in a single digit. Roman numerals (I-IV) indicate digit number. Light gray indicates bones not part of the digits (e.g., h, mc). White shapes indicate cartilage. Hyperphalangy is shown in dark gray while extreme hyperphalangy is shown in black. h, humerus, r, radius, u, ulna, c, carpals, mc, metacarpals, ph, phalanges. Number of phalanges per digit are separated by/. Not to scale

dexterity (Howell, 1930; Levermann, Galatius, Ehlme, Rysgaard, & Born, 2003). Within the aerial habitat, the thin wings of bats act as control surfaces to generate lift and balancing forces. Bats are unique in that they actively controlling wing camber and displacement via contractions of muscles embedded within their soft-tissue wing membrane (Cheney et al., 2014; Hedenström & Johansson, 2015). Moreover, the bones of bat wings are also compliant as they bend with wingbeats (Swartz & Middleton, 2008; Swartz et al., 2005, 2012).

1.2 | Embryonic limb development and digit patterning

Within the developing limbs of amniotes, two signaling centers control early limb development: (1) the apical ectodermal ridge (AER), which is a specialized region of cells at the tip of limbs that controls outgrowth and patterning along a proximodistal axis, and (2) the zone of polarizing activity, which regulates patterning along the anterior-posterior axis. Within vertebrates, activity of the AER ensures limb outgrowth and skeletal elements are deposited in a proximodistal sequence in underlying mesenchyme, beginning with the humerus and ending with phalanges. The AER is shaped like a ridge in cross-section and consists of a pseudostratified layer of ectodermal cells topped with additional non-stratified cells (Christensen & Tassava, 2000). This morphology is consistent among mammals including pigs and mice, but differs in dolphins as they lack a ridge, and instead display a thickened ectoderm with a smooth contour (Cooper et al., 2011b; Richardson & Oelschläger, 2002). Bats and dolphins also undergo a biphasic pattern of AER morphology during forelimb development. Initially, the AER forms along the distal end of the developing limb bud, and during the fetal period the AER becomes restricted to the tips of digits II and III (Richardson & Oelschläger, 2002; Weatherbee et al., 2006).

Among other genes, the AER expresses fibroblast growth factor (*Fgf*) genes 4, 8, 9, and 17 to promote cell survival and proliferation of underlying undifferentiated mesenchymal cells (Cooper et al., 2011a; Niswander, 2002; Niswander, Jeffrey, Martin, & Tickle, 1994a; Niswander, Tickle, Vogel, & Martin, 1994b; Weatherbee et al., 2006). Regulation of the duration and spatial area of *Fgf8* expression influences which parts of the limb experience outgrowth. For instance, the domain of *Fgf8* expression of embryonic bats is approximately three times wider than that of mice (Cretekos, Deng, Green, Rasweiler, & Behringer, 2007), resulting in an anterior-posteriorly expanded pool of mesenchymal cells at the distal ends of developing wings. This relatively

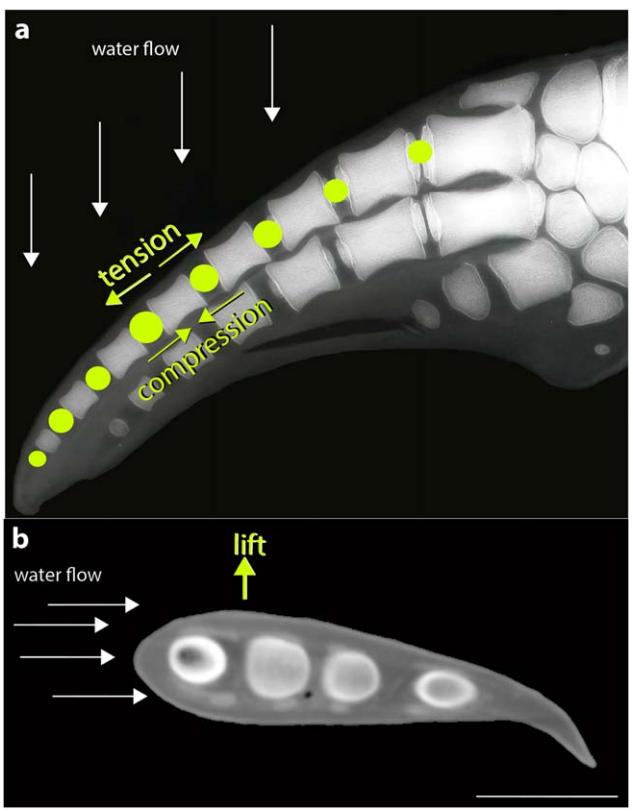

FIGURE 3 Hyperphalangy streamlines the flipper and dissipates joint loadings. (**a**) Each interphalangeal joint probably undergoes tensile forces on its leading edge and compressional forces on the trailing edge. (**b**) Flippers are hydrofoils that act as control surfaces to steer and counteract body pitch. Their cross-sectional shape generates lift and torque. (**a**) X-ray of the flipper of *Tursiops* (USNM 484931), and (**b**) CT-scan of a *Balaenoptera acutorostrata* (modified from Cooper et al., 2008). Scale bar is 5 cm in length

wide pattern of *Fgf8* expression is then followed by a restriction of *Fgf8* to the tips of digits II and III in bats (Weatherbee et al., 2006). Conversely, early termination of *Fgf8* activity results in truncated limbs and partially explains why adult dolphins (Thewissen et al., 2006) and snakes (Cohn & Tickle, 1999) lack hindlimbs. Within the pantropical spotted dolphin (*Stenella attenuata*), FGF8 protein signals are initially detected in the AER of hind limb buds, but this signaling fails and is followed by regression of the AER, and subsequent cell necrosis. This cascade of events culminates in the truncation and eventual absorption of the hind limb, leading to an absence of hind limbs within adult dolphins (Thewissen et al., 2006). In the python (*Python morulus*), early hind limbs fail to develop due to weak and transient *Shh* transcription and subsequent termination of *Fgf8* activity (Leal & Cohn, 2016), but can be rescued via transplantation of a functional AER from the limb of an embryonic chicken with successful *Fgf8* expression (Cohn & Tickle, 1999). For further discussion on this topic, see also reviews by Leal and Cohn, and by Infante and Menke in this issue.

Beyond basic limb patterning, mesenchymal cells that lie between developing digits (interdigits) act as molecular signaling centers (interdigital signaling centers) that are responsible for regulating the periodicity of phalanx and joint formation, and thereby digit phenotypes. These signaling centers, in coordination with AER-ZPA activity, are critical for establishing developmental and evolutionary patterns of phalangeal formulae within adult amniotes (e.g., 2/3/3/3/3 in mice, 2/3/4/5 in chick limbs) (Huang et al., 2016; Suzuki, Hasso, & Fallon, 2008). By modulating levels of *Bmp* agonists and antagonists (under control of *Gli3*, *Hoxd*), interdigital signaling centers set net *Bmp* levels that act on adjacent developing digits to regulate the timing of phalanx and joint formation. Evidence from developmental assays in mice and chicks show that relative levels of *Hoxd11* and *Hoxd12* expression within interdigits are associated with phalanx/joint counts. Specifically, interdigits displaying the greatest relative concentrations of *Hoxd11* and *Hoxd12* flank those digits that ultimately develop the greatest number of phalanges (Huang et al., 2016). In contrast, these interdigits display uniform levels of *Hoxd13*, suggesting this gene may not play a great role in influencing digit phenotype.

During digit outgrowth, the AER and interdigital signaling centers coordinate with one another. *Fgf*s within the AER maintain a sub-AER pool of uncommitted mesenchymal cells. As outgrowth continues, cells become responsive to BMP and condense as phalangeal precursors. As these precursors mature, cells along their proximal border (opposite from the side closest to the AER), experience a loss of BMP by the presence of the *Bmp*-antagonist *Noggin*. In the presence of *Noggin*, these proximally-located cells enter a joint-forming pathway and express joint-specific signals (e.g., *Gdf5*, *Wnt9a*). This periodic process of phalanx/joint formation repeats as *Fgf*-directed outgrowth continues, and finally arrests after formation of the distal-most phalangeal precursor. Integrated efforts of the AER and interdigits therefore synchronize the periodicity of signaling required to pattern mesenchymal cells into phalanges and joints as consecutive and discrete structures at the digit tips (Huang et al., 2016; Suzuki et al., 2008).

As phalanx/joint anlagen mature, they have opposing fates. Phalangeal precursors undergo endochondral ossification by first following a chondrogenic pathway to form a hyaline cartilage template that is ultimately replaced by bone, and this process is not controlled by the interdigital region. In contrast, formation of interphalangeal joints is largely regulated by *Wnt9a* expressed within the joints and interdigital tissues adjacent to the developing digits (Hartmann & Tabin, 2001). *Wnt9a* suppresses the chondrogenic potential of a developing joint and instead encourages differentiation into synovial connective tissues (Guo et al., 2004; Hartmann & Tabin, 2001; Koyama et al., 2008; Später, Hill, Gruber, & Hartmann, 2006a; Später et al., 2006b).

After completion of outgrowth and patterning of digits, the interdigits of most taxa enter an apoptotic pathway, resulting in separated digits. *Bmp* plays a critical role in the interdigital apoptosis pathway, but its effects can be functionally antagonized by *Gremlin* and *Fgf*s. For instance, amniotes that display varying degrees of webbing as adults [ducks (Merino et al., 1999), camels (Cooper et al., 2014), bats (Hockman et al., 2008; Weatherbee et al., 2006), and mutant mice (Pajni-Underwood, Wilson, Elder, Mishina, & Lewandoski, 2007)] display *Bmp*, *Gremlin*, and occasionally *Fgf* expression within their developing interdigits. By antagonizing *Bmp* and maintaining interdigital tissues, these taxa presumably have the potential to modify the rate and duration of digit patterning. In addition, maintenance of these tissues allows their proliferation and elaboration into structures essential for life in fluid habitats including webbing, wings, or flippers.

1.3 | Development of hyperphalangy within cetaceans

Although scientists have speculated about the cellular processes driving the development of hyperphalangy within cetaceans for over 120 years (Fedak & Hall, 2004; Howell, 1930; Kükenthal, 1889; Richardson & Oelschläger, 2002), a lack of fresh embryonic tissues created a major obstacle in experimentally testing molecular hypotheses (Fedak & Hall, 2004). Nevertheless, considerable advances were made by documenting patterns of phalangeal ossification (Benke, 1993; Calzada & Aguilar, 1996; de Carvalho et al., 2015; Fedak & Hall, 2004; Gihr et al., 1982; Mellor, Cooper, Torre, & Brownell, 2009; Ortega-Ortiz, Villa-Ramirez, & Gersenowies, 2000; Richardson & Oelschläger, 2002; Sterba, Klima, & Schlidger, 2000). Morphology-based hypotheses were then augmented by a mechanistic understanding of limb development, leading to detailed hypotheses of the developmental prerequisites for hyperphalangy (Fedak & Hall, 2004). Developing flippers of dolphins were hypothesized to (a) lack apoptosis within interdigital tissues, (b) maintain an AER at the tips of hyperphalangeous digits, (c) experience prolonged digit elongation, and (d) undergo extra joint formation via maintenance of pattern-forming mechanisms (Fedak & Hall, 2004; Richardson & Oelschläger, 2002).

Studies of the pantropical spotted dolphin (*Stenella attenuata*) supported these hypotheses. The forelimb of *Stenella* progresses through a standard bud-to-paddle progression (Figure 4), with a flipper developing during the mid-to-late fetal stages of prenatal development (Sterba et al., 2000). The AER is present over the developing hand plate and is later isolated to the tips of digits II and III where additional phalanges are added to the terminus of these digits during late fetal development. Researchers accordingly hypothesized *Stenella* embryos undergo a

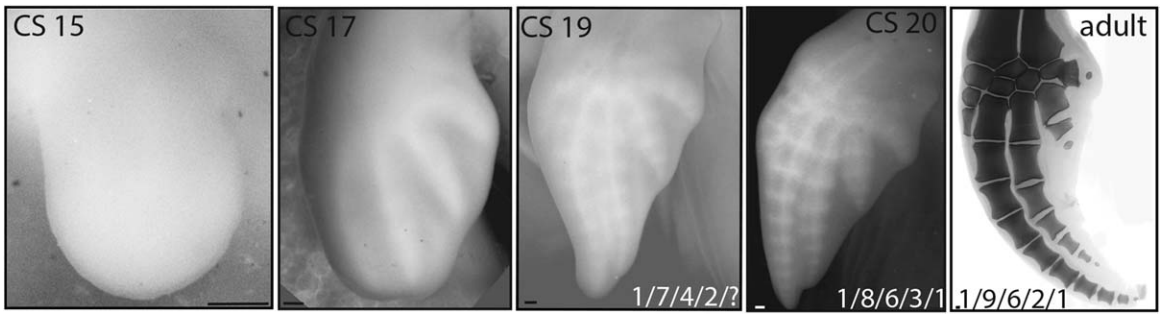

FIGURE 4 Forelimb development in *Stenella* begins by the formation of a hand plate. Interdigital tissues fail to undergo apoptosis, leading to the formation of a sickle-shaped flipper. Extreme hyperphalangy develops first in digit II (CS 17) and is followed by digit III (CS 20). Digits II and III act as bony supports for the flipper. Number of phalanges per digit are separated by/. CS = Carnegie Stage. Scale bar is 200 μm in length

biphasic pattern of *Fgf* expression during digit development. As in amniotes, *Fgf* expression was hypothesized to be first located within the AER in early limb development, however, only cetaceans would display a second period of *Fgf* expression isolated to the tips of hyperphalangeous digits within the fetal period (Richardson & Oelschläger, 2002). These developmental anatomical studies also led to the hypothesis that dolphins experience a novel heterochronic shift in the duration of molecular and developmental processes regulating digit elongation and patterning, and this extended duration of activity is not seen in terrestrial mammals (Fedak & Hall, 2004; Richardson & Oelschläger, 2002).

Only in the last decade have a small number of embryonic tissues become available (Thewissen & Heyning, 2007) for experimental evaluation of the molecular signals driving the cetacean body plan. For instance, tissues of *Stenella* were used to pinpoint at least some of the protein signaling events leading to hind limb loss (Thewissen et al., 2006) and the generation of supernumerary teeth (Armfield, Zheng, Bajpai, Vinyard, & Thewissen, 2013). Similarly, tissues of bowhead whales (*Balaena glacialis*) were used to trace protein signals associated with the development of baleen (Thewissen et al., 2017). The present study is the first to trace the derived molecular signals governing the formation of the pectoral flipper of cetaceans.

This study quantifies the spatiotemporal pattern of protein signals within the developing manus of the pantropical spotted dolphins (*Stenella attenuata*), and tests the following hypotheses. First, dolphins retain interdigital tissues by altering the duration and location of BMP functional antagonists (GREMLIN, FGF8) that are known to counteract interdigital apoptosis. Second, FGF8 protein signals are maintained well into the late fetal period at the tips of digits II and III of *Stenella*. Finally, the duration of joint patterning *Stenella* is extended and therefore protein signals of WNT9a, a key factor in joint formation, are also extended. Results are compared with equivalent assays in pigs, which are a closely-related terrestrial ungulate, as well as laboratory mice. This study therefore integrates anatomical and molecular data to evaluate the potential functional role of proteins that contribute to the novel and complex phenotype of dolphin digits.

Results of this study also offer additional molecular insights into the limb-to-fin transition that took place as cetaceans invaded the seas over 49 Ma (Cooper et al., 2007a; Gingerich, 2003; Gingerich, Haq, Zalmout, Khan, & Malkani, 2001; Madar, Thewissen, & Hussain, 2002; Thewissen, 1994; Thewissen & Bajpai, 2001; Thewissen, Cooper, George, & Bajpai, 2009; Thewissen & Fish, 1997; Thewissen, Madar, & Hussain, 1996, 1998; Uhen, 2007). Results inform hypotheses as to the evolutionary origins of a soft tissue flipper in semi-aquatic cetaceans (Madar, 2007; Thewissen et al., 1996; Uhen, 2004), and subsequent timing of the multiple origins of an extreme form of hyperphalangy in cetaceans (Cooper et al., 2007a).

2 | RESULTS AND DISCUSSION

2.1 | Suppression of interdigital apoptosis was a critical event leading to the origin and evolution of the cetacean flipper

Variable efficacy of the apoptotic pathway within the developing interdigits results in a continuum of adult limb phenotypes, including our own hands with separated digits, the webbed feet of ducks, and the wings of bats, among others. With BMP as a key player, the apoptotic pathway proceeds normally in amniotes with separated digits, including mice and humans. However, in the presence of direct (GREMLIN) or functional (FGF) antagonism of BMP, mesenchymal tissues may survive and elaborate into webbing or wings. Accordingly, the soft tissue flippers that encase the digits of dolphins are also likely the result of ineffective interdigital apoptosis during limb development. To experimentally evaluate for activity of direct or functional BMP-antagonists within the developing limbs of dolphins, this study used immunohistochemical assays to retrieve evidence of protein signals at various stages of limb development within the pantropical spotted dolphin (*Stenella attenuata*), compared to pigs and mice.

Results of this study show interdigital apoptosis effectively separates the digits of pigs and mice (Figure 1), but the interdigits of dolphins survive and display protein signals of BMP and GREMLIN (Carnegie Stage (CS) 17-19), and FGF8 (CS 19+) (Figures 5 and 6). Staining of these proteins was present in the middle and distal aspects of the interdigits. These findings are in strong contrast to the signaling patterns found in our sample of terrestrial taxa. Within our sample of pigs ranging from CS 16-22, FGF8 staining was present in the AER

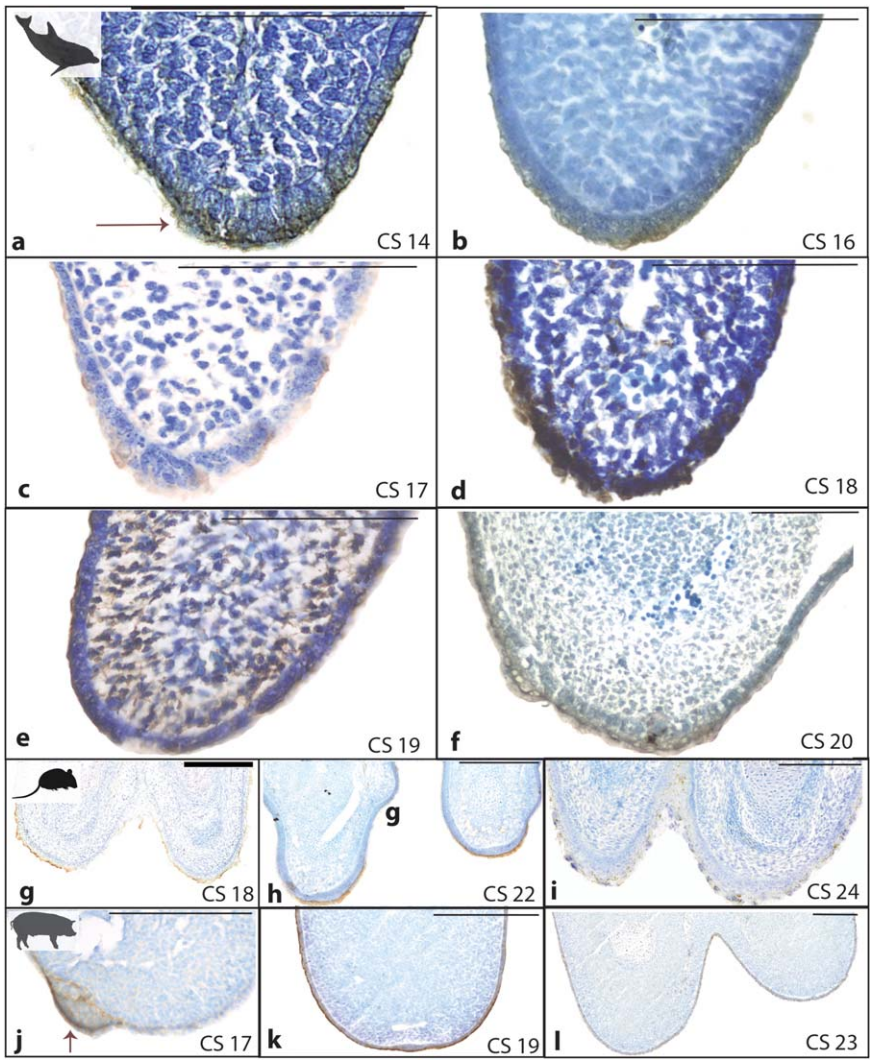

FIGURE 5 *Stenella* display FGF8 protein signals (brown) within the ectoderm and apical ectodermal ridge (AER) through CS 18 (**a-d**). At CS 19–20 (**e-f**), signaling is found throughout the ectoderm and underlying mesenchyme, including the interdigital tissues. Pigs and mice differ in that their FGF8 signals are localized to the ectoderm only (**g-l**). Images show sections along the longitudinal axis of the digits. Apical surface of the limb is oriented to the bottom of each image. A brown arrow indicates the apical ectodermal ridge (AER). Protein signals are stained brown, the remaining cells are stained blue with a thionin counter-stain. CS = Carnegie Stage. Scale bar is 100 μm in length (**a-f**) and 200 μm in length (**g-l**)

over the developing digits at CS 16, 18.5, and 19 (Figure 5). These results are consistent with previous reports of FGF8 protein signals within the AER of pigs at CS 16-17, 19 (Cooper et al., 2011b) and mRNA of the *Fgf8* gene within the AER at CS 18.5 and 19 (Cooper et al., 2014). Although FGF8 protein signals were present in the ectoderm overlying the digits at CS 22, this study found that pigs lacked a morphological AER at this stage (Figure 5k). Within our sample of mice ranging from CS 17-24, FGF8 protein signals were found within the AER and the ectoderm overlying the interdigital tissues at CS 20, 22, and 24 (Figure 5g–i). FGF8 proteins were absent from the interdigital tissues at all sampled stages of pig and mouse limb development.

Presence of FGF8 within the interdigits of late-fetal dolphins, but not pigs and mice, suggests dolphins experience a novel phase of interdigital signaling, while also extending duration of FGF8 signaling at the digit tips. In addition, presence of both GREMLIN and FGF8 signals within the interdigits of dolphins suggest efficacy of BMP-related interdigital apoptosis is likely diminished at these developmental stages, and this pattern probably contributes to mesenchymal cell survival, proliferation, and elaboration into a soft-tissue flipper. This prolonged maintenance of the interdigits, and their signaling centers, probably creates a unique developmental opportunity for an extended period of digit patterning. Provided that AER-directed outgrowth of the limb continues, the interdigital signaling centers of dolphins could thereby extend the duration of joint and phalanx patterning. Taken together, results suggest that maintenance of the interdigits and their signaling centers may have been a critical step in allowing for the development of a soft tissue flipper, as well as the development of hyperphalangy within mammals.

Among the rare number of mammals that occupy fluid habitats, experimental evaluations of interdigit survival are known for only bats

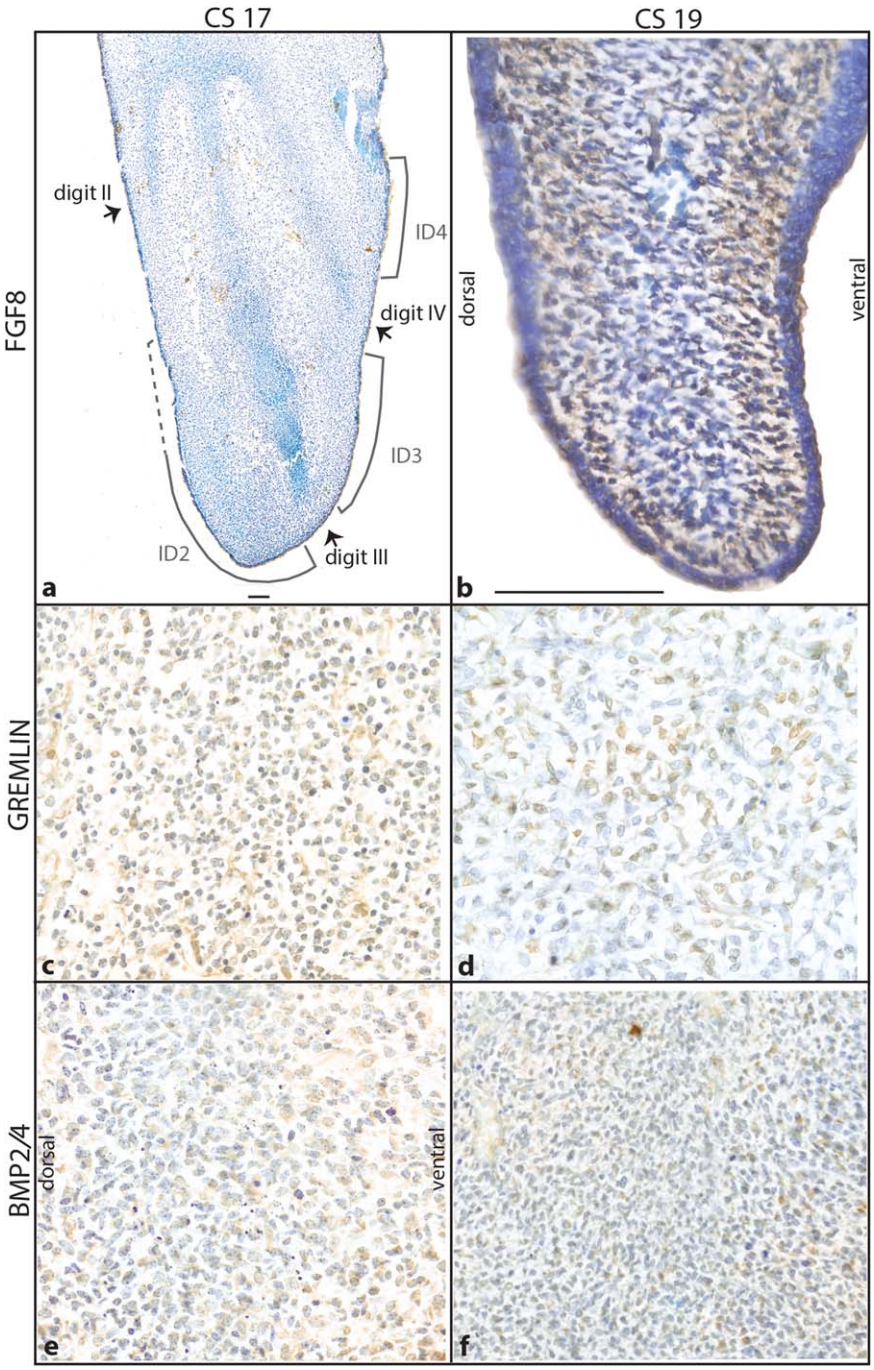

FIGURE 6 The interdigital tissues of *Stenella* display signals of FGF8 (**b**), GREMLIN (**c-d**), and BMP (**e-f**). BMP plays a role in the apoptosis of interdigital tissues that separate and sculpt the digits. *Stenella* displays BMP signals within these tissues, but also displays signals of the BMP-inhibitor GREMLIN. FGF8 is present within these tissues only from CS 19+ (**b**). Images show sections along the longitudinal axis of the limb with the tip of the flipper oriented toward the bottom of the figure. Staining within interdigit 2 (ID 2) is shown in **c-f.** Tissue is counterstained blue via thionin in **a-d**. CS= Carnegie Stage. Scale bar in **a** is 100 μm in length. Scale bar in b represents 100 μm for **b-f**

(Hockman et al., 2008; Weatherbee et al., 2006) and dolphins (this study). Studies of gene expression within the developing interdigits of bats showed that they too display *Gremlin* and *Fgf8* signals, and also fail to undergo apoptosis. Fetal bats augment *Bmp* gene expression within the interdigits 2-4 with *Gremlin* and second wave of interdigital *Fgf8* signaling (Hockman et al., 2008; Weatherbee et al., 2006). Results of this study show that although bats and dolphins are not closely related, they independently evolved and utilize similar molecular mechanisms to avoid interdigital apoptosis (Figure 8), and ultimately achieve limb phenotypes essential for life in fluid habitats.

Although results of this study identify spatiotemporal shifts in FGF8 protein signals, as well as timing of GREMLIN signaling during interdigit development, other studies shed light on the genetic and evolutionary origins of a soft-tissue flipper in cetaceans. At the genetic

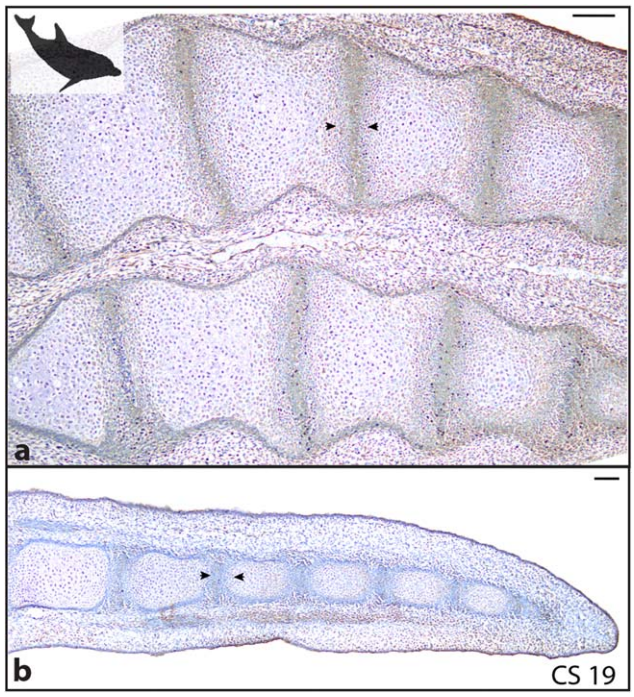

FIGURE 7 Hyperphalangy requires the formation of supernumerary interphalangeal joints. WNT-9a acts as a major controller of joint formation as it assists in the differentiation and maintenance of joint connective tissues. WNT-9a signaling is found in all developing interphalangeal joints (arrowheads) of *Stenella*. Although bats and dolphins share similar patterns of FGF signaling associated with limb elongation, only dolphins increase the number of joints. Wnt-9a signals are shown in brown. Tissue is counterstained blue via thionin. CS= Carnegie Stage. Scale bar is 100 μm in length

level, presence of a soft-tissue flipper in cetaceans is thought to be at least partially the result of a novel expansion of a polyalanine tract at the 5′ –end of the *Hoxd13* gene. Specifically, some cetaceans display 2–3 additional alanine residues within the middle of the polyalanine stretch, and similar modifications in other taxa are associated with webbing, or syndactyly (Wang et al., 2009). At the molecular level, evolution of the sequence of the *Hoxd13* gene may therefore represent an important addition to the growing body of evidence as to the molecular origins of a soft-tissue flipper in cetaceans.

Integration of our developmental data with evidence of phenotype from the fossil record provides a limited but useful understanding of the sequence of morphological and molecular innovations (Organ, Cooper, & Hieronymus, 2015) that shaped cetacean flippers over deep time (Figure 9). Skeletons of the earliest fossil cetaceans were recovered from sediments in Pakistan dating about 49Ma. Pakicetid cetaceans recovered from these beds were quadrupedal, lacked hyperphalangy, and displayed digit morphologies leading to the hypothesis that the presence of interdigital webbing potentially supported locomotion in soft, muddy substrates (Madar, 2007). Development of this webbing probably required activity of a *Bmp*-antagonist (e.g., *Gremlin*) to suppress interdigital apoptosis, as seen in modern taxa with webbing (Cooper et al., 2014; Merino et al., 1999; Pajni-Underwood et al., 2007), including fetal dolphin used in this study (CS17, Figure 6). Activity of at least the *Gremlin* gene within the interdigits of most cetaceans was probably maintained for the next 30Ma while the cetacean body plan underwent remarkable changes to support life in an aquatic habitat. During this time, transitional fossils document the hypothesized retention of webbing, lack of hyperphalangy (Bejder & Hall, 2002) and gradual reduction of hind limbs to mere vestiges that eventually were encased within the body wall (Thewissen et al., 2009).

In contrast to hindlimbs, forelimbs of extant cetaceans diversified into a variety of shapes. Forelimbs can be broad and paddle-shaped (right and sperm whales), sickle-shaped (pilot whales), and elongate with tubercles (humpback whales) (Cooper, 2009; Sanchez & Berta, 2010; Weber et al., 2014). The molecular events underlying this phenotypic diversity remain unknown, but future evaluation of activity of the *Hoxd* genes, may further our understanding of the evolutionary mechanisms driving flipper shape. Some of these phenotypes have known biomechanical and performance consequences (Cooper et al., 2008; Segre et al., 2016; Weber et al., 2014), and are of interest for applications using bio-inspired technology (Fish, Weber, Murray, & Howle, 2011). Augmentation of these phenotypic and biomechanical data sets with evidence of their molecular underpinnings may further the field of evolutionary developmental biology and enhance the ever-expanding bridge between the fields of biology and engineering.

2.2 | The development and evolution of minor and extreme forms of hyperphalangy

In all of mammals, only cetaceans display hyperphalangy and evidence of its developmental origins has the potential to expand our current understanding the field of limb developmental biology. While mammals as a group display little variation in phalanx counts, cetaceans evolved a rich continuum of counts (Cooper et al., 2007a) that can be broadly divided into two categories of hyperphalangy: an incipient, or minor, phenotype consisting of 4–5 phalanges, and an extreme phenotype with individual digits displaying at least 6 phalanges (Fedak & Hall, 2004). Results of this study show that *Stenella* displays this minor form of hyperphalangy in digits II and III around CS 16, indicated by the presence of 4-5 phalanges per digit. The extreme form of hyperphalangy originates at CS 19-20 as 6-8 phalanges were present in digits II and III. Digit outgrowth and patterning continues throughout prenatal development and adult specimens of *Stenella* ultimately achieve a phalangeal formula of 1/9/6/2-3/1. By associating phalanx counts and shifts in protein signals throughout digit development, results of this study allow additional insights into how minor and extreme forms of hyperphalangy are associated with a different suite of protein signals within the interdigits.

Only specimens of *Stenella* near CS 16 display the minor form of hyperphalangy (∼CS 16), consisting of 1-2 supernumerary phalanges. At this stage, signaling results show that the interdigits are rich in GREMLIN and BMP, but lack FGF8 signals (Figures 5 and 6). Because GREMLIN is a known BMP-antagonist, it presumably functions to counteract apoptosis of the interdigits. Taken together, these results suggest dolphins achieve a minor phenotype of hyperphalangy even in

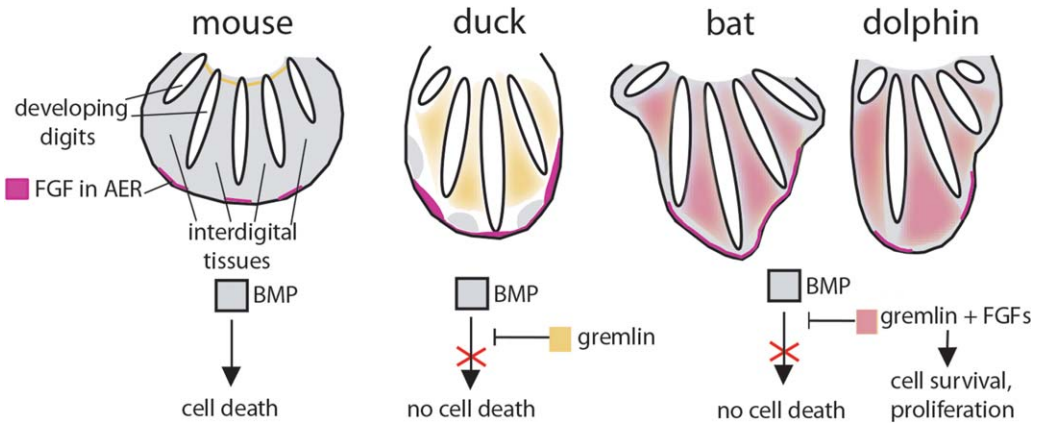

FIGURE 8 Bats and dolphins independently evolved similar molecular strategies to maintain and elaborate interdigital tissues into wing membranes and flippers. In the developing interdigital tissues of mice, *Bmp* acts within the apoptosis pathway to separate and sculpt adjacent digits. In contrast, ducks (Merino et al., 1999), bats (Weatherbee et al., 2006, Hockman et al., 2008), and dolphins (this study) co-localize signals of *Gremlin*, a *Bmp* antagonist, within these interdigital tissues and probably experience reduced efficacy of interdigital apoptosis. Bats (CS 15+) and dolphins (CS 19+) differ from ducks in that they also extend *Fgf* signaling from the ectoderm into the interdigital tissues. Data for dolphins came from protein assays, whereas data for other taxa come from gene expression assays. Bright pink indicates *Fgf* within the ectoderm. Gray indicates *Bmp*, yellow indicates *Gremlin*, and pink-yellow indicates co-localized *Fgf* and *Gremlin* within the interdigits. CS = Carnegie Stage. Not to scale

the absence of FGF8 from the interdigits, and instead may increase the rate of phalanx/joint patterning and/or AER-directed outgrowth relative to pigs and mice. This pattern of interdigital signaling differs from late-stage dolphins displaying the extreme form of hyperphalangy.

Stenella display an extreme form of hyperphalangy at CS 19 and 20 in digits II and III. During these stages, BMP, GREMLIN, and FGF8 signals are present within the interdigits (Figures 5 and 6), suggesting that an additional phase of FGF8 signaling is associated with the origins of extreme hyperphalangy. In addition, WNT9a signaling within the interphalangeal joints indicates effective joint patterning at CS 19 (Figure 7). In this case, augmentation of typical AER-focused FGF8 signaling may allow for expansion and outgrowth of the interdigits 1-3, and thus lay a critical foundation for the extended patterning of the digits of dolphins.

Integration of these developmental data with fossil evidence of digit evolution allow for the reconstruction of molecular events that potentially drove the evolution of cetacean hyperphalangy (Figure 9). Direct evidence of hyperphalangy is rare within the fossil record, however it is possible to observe the diversity of digit phenotypes within extant cetaceans and infer their evolutionary history. For instance, all modern cetaceans, with the exception of dolphins and rorqual whales, display a minor form of hyperphalangy, suggesting this phenotype was shared by their common ancestor. This group of modern cetaceans and their common ancestor make up the Neoceti, which evolved about 35Ma. As such, hyperphalangy probably first appeared within the fossil record about this time. The first fossil evidence of this minor form of hyperphalangy was recovered from sediments deposited during the Oligocene Epoch. The ancient whale *Imerocetus* (Mchedlidze, 1988) displayed a single additional phalanx in digit III. However, the presence of hyperphalangy in *Imerocetus* is questionable as the skeleton lacks several phalangeal elements and it is not clear if the limb was articulated.

Extreme hyperphalangy [at least six phalanges in a digit (Fedak & Hall, 2004)] evolved independently in modern rorqual whales (Balaenopteridae: fin, blue, minke, humpbacks) and dolphins (Delphinidae: orcas, pilot whales, dolphins) (Cooper et al., 2007a). Taxa within these families share a common morphology of narrow and elongated flippers that are probably advantageous for swimming at high speeds associated with foraging (Cooper, 2009; Cooper et al., 2008; Goldbogen et al., 2006; Weber et al., 2014). The first fossil evidence of this extreme form of hyperphalangy is found in fossil rorqual whales. *Balaenoptera siberi* (Pilleri, 1989, 1990), a fossil rorqual recovered from the Pisco Formation of Peru (7-8 Ma sediments), displayed extreme hyperphalangy in digit III while coeval *Incakujira anillodefuego* displayed digit morphologies consistent with the presence of extreme hyperphalangy (Marx & Kohno, 2016). No fossils indicate the origin of hyperphalangy in extinct dolphins. Based on our assays of the developing digits of *Stenella*, these taxa likely required a second phase of digit outgrowth coordinated by the AER and interdigital signaling center. Specifically, we hypothesize that this phase of development resulted in a heterochronic shift in the duration of digit outgrowth and patterning specifically focused on those digits that ultimately evolved at least six phalanges (Figure 9).

2.3 | Conclusions and future work

Results of this study augment the exciting and expanding field of evolutionary developmental biology by elucidating some of the molecular signals contributing to the extreme phenotype of hyperphalangy in mammals. Our studies illustrate how classically canalized developmental pathways could be repeatedly exploited to generated webbing, wings, and flippers in mammals.

Future comparative developmental studies may elucidate the mechanisms driving the formation of both minor and extreme forms of

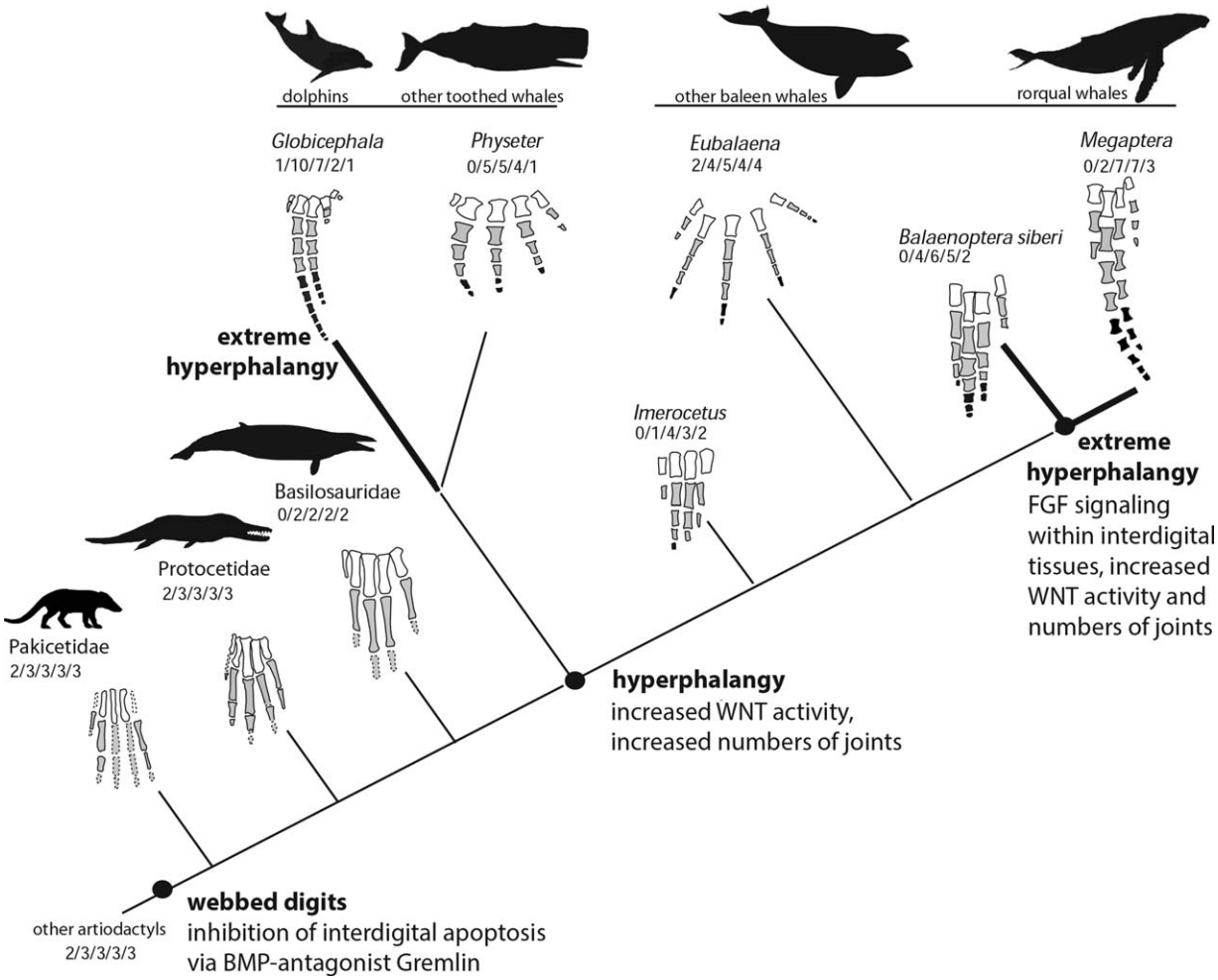

FIGURE 9 Both developmental and evolutionary evidence suggest interdigital tissues were likely prerequisite for the eventual development of hyperphalangy. The earliest fossil whales probably altered signals of the BMP-antagonist GREMLIN to counteract interdigital apoptosis, resulting in webbed digits [pakicetids (Madar, 2007), protocetids (Gingerich et al., 2009), basilosaurids (Uhen, 2004)]. By modifying signals associated with joint patterning within these tissues, a minor form of hyperphalangy probably emerged in the common ancestor of modern cetaceans about 35Ma. A fossil cetotheriid *Imerocetus* (Mchedlidze, 1988) perhaps had an additional phalanx, indicating the presence of a minor form of hyperphalangy with the Oligocene Epoch. Finally, two groups of cetaceans, rorqual whales and dolphins, independently evolved an extreme form of hyperphalangy (dark black lines). The fossil rorqual whale *Balaenoptera siberi* (Pilleri, 1989, 1990) displayed morphologies consistent with an extreme form of hyperphalangy was recovered from sediments dated to 7Ma. Findings of this study suggest this extreme phenotype requires a second wave of FGF signals within the interdigital tissues to generate six or more phalanges in a digit. Number of phalanges per digit are separated by/. White bones are metacarpals, gray bones are phalanges, bones with dashed lines are not known, and black bones indicate extreme hyperphalangy

hyperphalangy. Because modifications of *Hoxd* genes are known to shape digit phenotypes, it could be that exploration of interdigital signaling regulated by *Hoxd11* and *Hoxd12*, in addition to *Gli3*, could offer insight into how the rate and/or duration of digit patterning of cetaceans differ between taxa displaying hyperphalangy. Because of the lack of fresh and sustainable sources of tissues of cetaceans that display the extreme phenotype of hyperphalangy, it may only be feasible for future exploration to identify the mechanism driving this minor form of hyperphalangy. Ongoing work could utilize tissues of *Delphinapterus* and *Balaena* as they display this minor form of hyperphalangy and are currently used to study molecular and anatomical adaptations associated with bone development (Thewissen et al., 2017), sensory biology (Drake, Crish, George, Stimmelmayr, & Thewissen, 2015; Sensor et al., 2015), and physiology (Ball et al., 2015, 2017; George et al., 2016). Furthermore, augmentation of these developmental data with direct evidence of their biomechanical consequences would link gene function to phenotype and add insight into how a given morphological trait can influence performance within a complex, three-dimensional environment.

3 | MATERIALS AND METHODS

3.1 | Materials

Pigs (*Sus scrofa*) and cetaceans are members of the mammalian Order Cetartiodactyla, in which cetaceans are the only obligatory aquatic forms. Laboratory mice are a standard for studies of limb development and represent a generalized quadrupedal mammal (Bandyopadhyay

et al., 2006; Cretekos et al., 2008; Hockman et al., 2008; Moon, Boulet, & Capecchi, 2000; Sears, Behringer, Rasweiler, & Niswander, 2006; Sun, Mariani, & Martin, 2002). Embryos and fetuses of the spotted dolphin (Thewissen & Heyning, 2007) were used for immunohistochemical assays that indicate the timing and location of proteins known to play a role in limb development and patterning (i.e., FGF8 (N-19) SC-6958, BMP-2/4 (A-20) SC-6267, WNT9a (WNT14) (N-13) SC-20265, Gremlin-1 (N-20) SC-18274, Santa Cruz Biotechnology, Inc., Dallas, Texas, U.S.A.).

Ontogenetic series of embryonic and fetal specimens of pantropical spotted dolphins (*Stenella attenuata*), pigs (*Sus scrofa*), and laboratory mice (CD-1) were immersion-fixed and stored for histological processing and analysis. Stages of fetal development were assigned based on the Carnegie Staging (CS) system (see (Thewissen & Heyning, 2007)) in which pigs ranged from E20.5 (CS 15) – E32 (CS 22), and mice ranged from E13.0 (CS 17) – E17.5 (CS 24). Dolphins were supplied by the Los Angeles Museum of Natural History (LACM). Dolphin samples ($n = 11$), ranged from Carnegie Stages (CS) 13–20 (CS 13 (LACM 94815); CS 14 (LACM 94594); CS 16 (LACM 94747, 94770); CS 17 (LACM 94650, 94670, 94742); CS 18 (LACM 94634); CS 19 (LACM 94817, 94818); CS 20 (LACM 94679)]. Embryonic pig tissues ($n = 14$, Large-White breed) that ranged from 25 through 32 days post fertilization (CS 18–23) were obtained at an animal processing plant (Chuck's Meat Locker, Ivesdale, IL). Pig samples are as follows [CS 15 ($n = 1$), CS 16 ($n = 1$), CS 17 ($n = 1$), CS 18.5 ($n = 2$), CS 19 ($n = 2$), CS 19.5 ($n = 2$), CS 22+ ($n = 5$)]. Embryonic laboratory mouse tissues ($n = 13$, CD-1 strain) were obtained from breeding colonies maintained by the Cooper Lab and Sears Lab. Female mated mice were checked for sperm plugs and embryos harvested at the desired stages of development. Mice were euthanized via carbon dioxide inhalation and cervical dislocation. Mouse samples are as follows [CS 16 ($n = 1$), CS 17 ($n = 3$), CS 18 ($n = 3$), CS 19 ($n = 2$), CS 20 ($n = 1$), CS 22 ($n = 2$), CS 25 ($n = 1$)].

3.2 | Tissue fixation and storage

Dolphin tissues were originally immersion fixed and stored without refrigeration for up to 35 years, making nucleic acid retrieval unreliable. Pigs and mice were fixed in 4% paraformaldehyde (PFA) and then dehydrated into 100% methanol for storage at −20°C until use.

3.3 | Immunohistochemistry

Immunohistochemistry was used to document protein localization as RNA was not preserved in the dolphin embryos. Furthermore, legal and ethical issues preclude the acquisition of fresh dolphin embryos.

To determine protein signals during digit development, we assessed the location of FGF8, GREM, BMP2/4, and WNT9a in the developing metacarpals, phalanges and associated joints via immunohistochemistry. For a detailed protocol, see Armfield et al. (2013). Briefly, embryos were dehydrated, cleared, and embedded in paraffin wax. Tissues section at 7 μm thickness underwent heat-induced antigen retrieval using 0.01 M sodium. Hydrogen peroxide (3%) was used to prevent enzyme activity naturally present in the tissues from reacting with the DAB (Diaminobenzidine). Proteins of interests were detected using the avidin-biotin complex following the protocol of the goat ABC kit (Santa Cruz-2023). Sections were counterstained with 0.01% thionin. Two control methods were used to ensure positive staining of DAB in the tissue. Using a protocol provided by the ABC kit, the first control lacked the primary antibody. The second was a positive control. We compared protein signals to literature sources that indicate the protein of interest should be present in certain tissues during the developmental stages analyzed.

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