

Title: (Non)Parallel developmental pathways to vertebrate appendage reduction

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Abstract

Appendages have been reduced or lost hundreds of times independently during vertebrate evolution. This suggests that selection routinely favors appendage reduction. How often are the same developmental and genetic pathways used during loss by independent lineages? We reviewed the developmental and evolutionary literatures of appendage reduction in 12 genera spanning fish, reptiles, birds, and mammals. We found that appendage reduction and loss resulted from modified gene expression in each case but one. However, the genes for which expression was modified were rarely shared. Our findings suggest that adaptive loss of complex traits might proceed relatively easily through changes in gene expression along multiple developmental pathways.

19 **Introduction**

20 Though vertebrate appendages have evolved into fins, wings, flippers, claws, hooves, and
21 myriad other structures, they have also been reduced or lost repeatedly across the vertebrate
22 phylogeny. This repeated, independent reduction offers the potential to deduce general
23 mechanisms of appendage evolution. That is, to what extent are the developmental bases of
24 appendage loss shared across lineages, and to what extent is loss idiosyncratic (i.e., non-parallel
25 (Bolnick et al., 2018))?

26 Here, we introduce some significant molecular pathways involved in appendage
27 development and loss across major vertebrate clades to ask whether same or different pathways
28 are involved in appendage reduction and loss. This question requires that we find taxa that (i)
29 show appendage loss or reduction, and (ii) have data on the molecular and developmental
30 components driving reduction. At this time, the union of these two conditions is quite small and
31 biased to relatively few clades. Though there are hundreds of independent instances of lost or
32 reduced appendage elements reported in the literature, we found only a handful for which the
33 pathways involved are described even in part, likely limited by the difficulty of studying
34 developmental and molecular pathways in non-model organisms.

35 Despite limited data, we found literature investigating development across the vertebrate
36 phylogeny, representing about 450 million years of vertebrate evolution (López et al., 2016).
37 Teleost fish comprise roughly half of all vertebrate species (Weitzman, 2015), so in our search
38 for generality in appendage loss across vertebrates, we must first discuss homology, or the lack
39 thereof, between rayed fins and limbs. Teleosts and tetrapods have paired appendages
40 superficially similar in position and function (Drucker and Lauder, 2003; Fish et al., 2003;
41 Standen, 2008), but is it fair to compare fish fin elements to tetrapod limb elements?

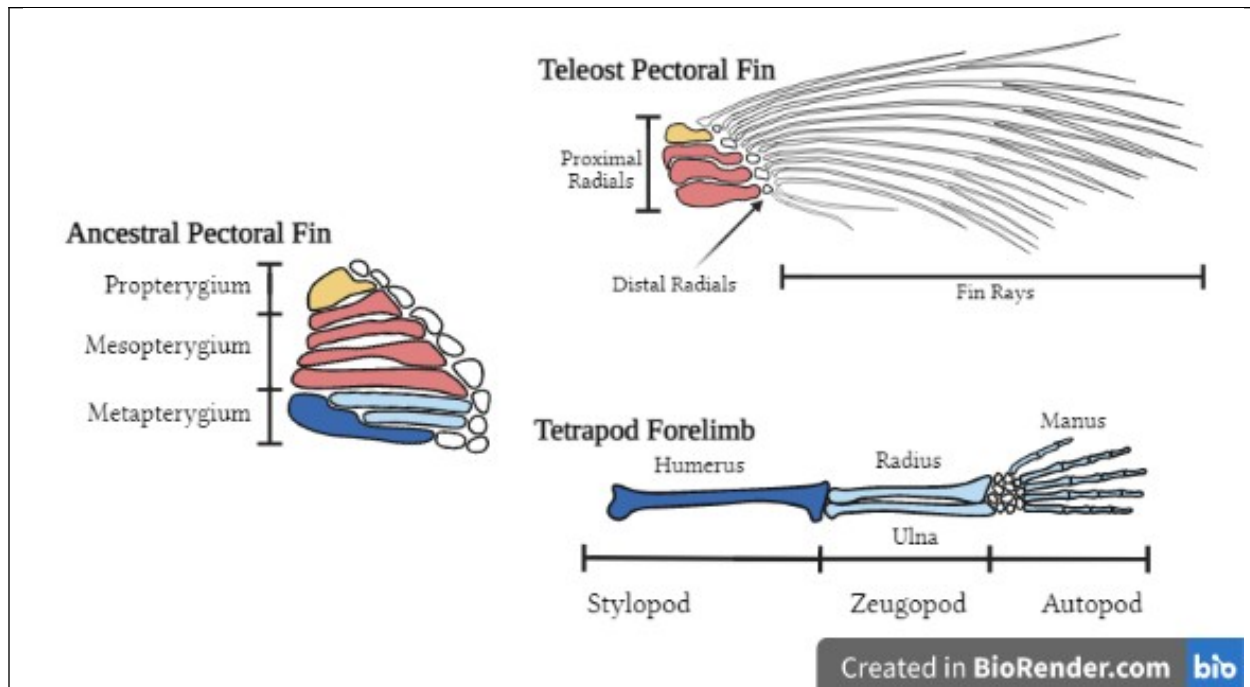


Figure 1. The teleost pectoral fin is based on zebrafish fin morphology while the tetrapod forelimb is based on human anatomy. Elements of the ancestral pectoral fin are retained and modified in extant vertebrates: appendage structures are colored to reflect their evolutionary origins. The propterygium (yellow) and mesopterygium (red) were retained and modified in teleost evolution while the metapterygium (dark and light blues) makes up the tetrapod limb. The distal portion of the metapterygium (light blue) was likely elaborated to form the tetrapod zeugopod and autopod, while its more proximal region (dark blue) contributed to the stylopod (Ahn and Ho, 2008; Don et al., 2013; Freitas et al., 2007; Hawkins et al., 2021).

Teleost fins and tetrapod limbs arose by modifications to the paired fins of their last common ancestor. Ancestral gnathostome fins were composed of long-bone segments arranged into three structures along the anteroposterior axis: the propterygium, the mesopterygium, and the metapterygium (Coates, 1994; Don et al., 2013; Hawkins et al., 2021) (**Fig. 1**). In teleosts, the propterygium and mesopterygium form the fins whereas the metapterygium is lost (Coates, 1994; Don et al., 2013; Hawkins et al., 2021) (**Fig 1**). In contrast, the metapterygium is the only element retained in lobe-finned fishes and was modified in the evolution of the tetrapod limb (Coates, 1994; Don et al., 2013; Hawkins et al., 2021). Thus, the teleost fin and the tetrapod limb are derived from distinct tissues of the ancestral vertebrate appendage and therefore are not developmentally homologous.

52 However, much of the genetic architecture controlling this non-homologous development
53 is shared (Hall, 2007). For example, the Hedgehog pathway plays a role in anteroposterior
54 appendage patterning and maintaining downstream gene expression (Chiang et al., 2001; Lettice
55 et al., 2003; Ros et al., 2003; Sagai et al., 2005; Tickle and Towers, 2017) in both fish and
56 tetrapods. Alterations to this signaling pathway result in aberrant appendage development and
57 morphology in both clades. For example, experimental loss of *Sonic hedgehog* (*Shh*) expression
58 results in truncated mouse limbs (Chiang et al., 1996; Sagai et al., 2005) and in fin-absence in the
59 medaka, a teleost (Letelier et al., 2018). Similarly, the expression and function of *Gli3*, a *Shh*
60 antagonist, is conserved from fish to tetrapods (Letelier et al., 2020), restricting cellular
61 proliferation and *Shh* expression. *Gli3*-knockout medaka grow extra distal fin elements; *Gli3*-
62 deficient mice develop a similar polydactyl phenotype (Letelier et al., 2020; Litingtung et al.,
63 2002; Welscher et al., 2002b).

64 Teleosts and tetrapods also share Hox gene regulation and function in their appendages
65 (Ahn and Ho, 2008; Hall, 2007; Tanaka et al., 2005). Hox genes are a group of transcription
66 factors that are essential for animal embryo patterning (Cohn and Tickle, 1999; DuBuc et al.,
67 2018; Parrish et al., 2009; Ryan et al., 2007; Scott, 1993). Hox genes were likely present in the
68 last common animal ancestor, though they have been lost in some lineages (Ramos et al., 2012).
69 Despite dramatic differences in adult morphology, there are three phases of Hox gene expression
70 in teleost and tetrapod pectoral appendages; orthologous genes in chick and zebrafish are
71 expressed in similar regions of the appendage during each phase (Ahn and Ho, 2008).

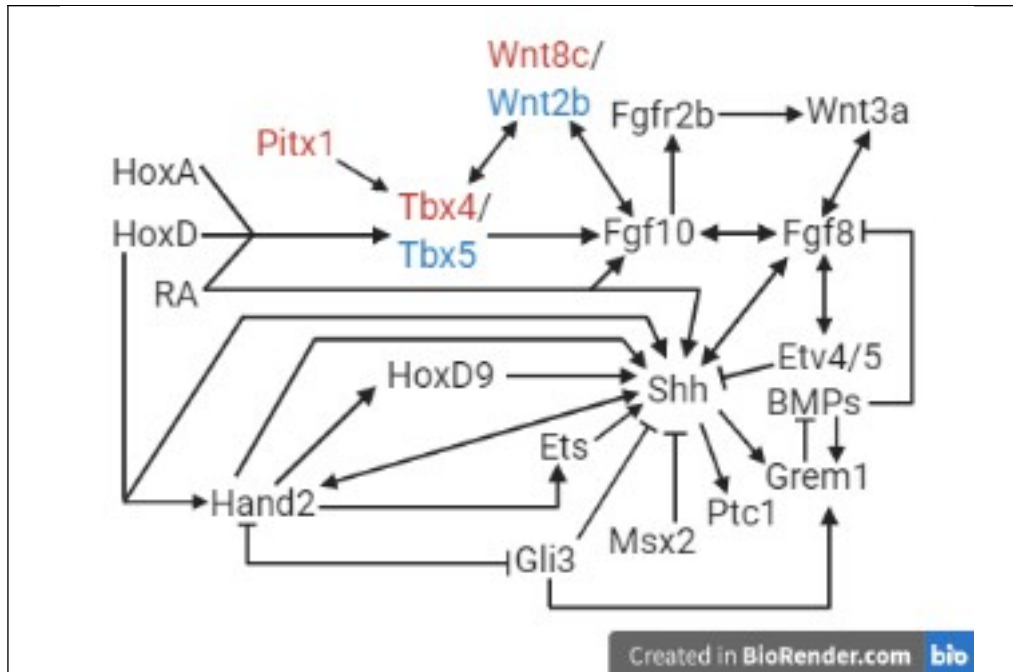


Figure 2. A simplified gene regulatory network implicated in vertebrate appendage development. Genes coded in red are unique to the hindlimb and those in blue are unique to the forelimb (Butterfield et al., 2009; Charité et al., 2000; Delgado and Torres, 2015; Fernandez-Teran et al., 2000; Hockman et al., 2008; Jin et al., 2019; Lafage-Proust, 2015; Ng et al., 2002; Nishimoto et al., 2015; Tanaka et al., 2005; Welscher et al., 2002a; Zúñiga, 2015).

Additional genes perform similar roles across vertebrate taxa. For example, orthologs of

Tbx5 and *Tbx4* are required for anterior (Ahn and Ho, 2008; Bickley and Logan, 2014; Don et al., 2016; Garrity et al., 2002; Minguillon et al., 2005; Rallis et al., 2003) and posterior (Ahn and Ho, 2008; Don et al., 2016; Minguillon et al., 2005; Naiche and Papaioannou, 2007, 2003; Takeuchi et al., 2003) appendage formation in both teleosts and tetrapods. *Pitx1* expression is similar in the developing posterior appendage bud of teleosts and tetrapods (Lanctôt et al., 1999; Logan and Tabin, 1999; Marcil et al., 2003; Shapiro et al., 2006; Szeto et al., 1999; Thompson et al., 2018), thereby inducing similar *Tbx4* expression and subsequent appendage development (Duboc and Logan, 2011; Infante et al., 2013; Logan and Tabin, 1999) (**Fig. 2**). Altogether, we suggest that there is sufficient homology between fins and limbs to assess parallelism in the

genetic basis of appendage loss and reduction across all vertebrates. Are the same genes used for appendage reduction and loss by distantly related vertebrate clades?

Another challenge in such studies is definitional. Defining appendage “loss” is straightforward: the absence of appendage elements, from the pelvic or pectoral girdle to the most distal elements (e.g., lepidotrichia or digits). “Reduction” is more difficult to define because of the continuous allometric relationship between appendage size and body size. Some studies have taken a categorical approach; for example, in squamates, researchers have defined limb reduction as the loss of at least one bone (Brandley et al., 2008; Greer, 1991; Wiens et al., 2006). In contrast, other researchers define reduction as a deviation from a standard allometric relationship measured in adult (Chiang et al., 2001; Klepaker et al., 2013; Kragestein et al., 2018; Thompson et al., 2018) or embryological (Bickley and Logan, 2014) specimens of interest. For our review, however, because there are so few molecular studies of appendage reduction in non-model organisms, we consider “reduction” to be a diminishment in bone number, relative bone size, or both.

Having established homology and defined reduction, we now divide the rest of our discussion by clade and appendage type to allow for comparisons within and between appendage-reduced taxa.

Teleost Pelvic Fin Reduction

Threespine stickleback (*Gasterosteus aculeatus*) are small fish with populations in saltwater ocean and estuarine habitats, as well as freshwater lake and stream habitats (Bell and Foster, 1994; Schluter and McPhail, 1992). Marine threespine sticklebacks have robust bony armor that includes lateral plates, dorsal spines, and a pelvic girdle with spines. However, likely

105 due to differences in water chemistry and predation regimes, freshwater stickleback usually
106 evolve armor reduction, including loss of pelvic elements (Bell et al., 1993; Colosimo et al.,
107 2005; Giles, 1983; Grant and Grant, 2010; Hoogland et al., 1957; Marchinko, 2009; Mobley et
108 al., 2012; Reimchen, 2000, 1992, 1983, 1980; Shapiro et al., 2006, 2004; Smith et al., 2014;
109 Spence et al., 2013, 2012; Tanaka et al., 2005; Zeller et al., 2012; Ziuganov and Zotin, 1995).

110 The pelvic girdle is a modified pelvic fin comprised of two articulated spines and a bony
111 plate that extends along the belly and up the sides of the fish. Over 100 geographically distinct
112 freshwater stickleback populations have evolved pelvic-reduction or loss (Chan et al., 2010;
113 Coyle et al., 2007; Klepaker et al., 2013; Shapiro et al., 2009, 2006; Shikano et al., 2013;
114 Thompson et al., 2018). Because these freshwater populations were independently colonized by
115 oceanic ancestors at the end of the last glacial maximum (Schluter and McPhail, 1992), these
116 losses represent repeated instances of evolution and provide a good system in which to ask about
117 the genetic parallelism of appendage reduction (Bolnick et al., 2018).

118 Many instances of pelvic reduction have been linked to the gene *Pitx1* (Bell et al., 2006;
119 Klepaker et al., 2013; Shapiro et al., 2006; Thompson et al., 2018). Pelvic-reduced populations
120 of *G. aculeatus* show no variation in their *Pitx1* amino acid sequences, relative to the pelvic
121 complete form (Chan et al., 2010; Shapiro et al., 2006). Instead, pelvic-complete and reduced
122 morphs vary in *Pitx1* expression. *Pitx1* is expressed in the mouth, jaw, and pelvis of pelvic-
123 complete larvae, but is missing from the corresponding region of pelvic-absent fish (Chan et al.,
124 2010; Shapiro et al., 2006; Thompson et al., 2018). Differential expression is governed by
125 mutations to two pelvic-specific *Pitx1* enhancer elements—*PelA* and *PelB* (Chan et al., 2010;
126 Coyle et al., 2007; Kragestein et al., 2018; Thompson et al., 2018; Xie et al., 2019). Genomic
127 studies have shown that mutations to *PelA* arise *de novo*, likely because the enhancer is in a

128 region is prone to double strand breakages (Xie et al., 2019). The *PelA* enhancer region is subject
129 to strong positive selection that drives the null allele to fixation within a population (Chan et al.,
130 2010; Xie et al., 2019). That *de novo* mutations arise frequently in and are acted on by positive
131 selection at this enhancer locus suggests that the *Pitx1* regulatory region is an unconstrained
132 locus that could underlie posterior appendage reduction in other taxa.

133 For example, more than thirty populations of the ninespine stickleback (*Pungitius*
134 *pungitius*) have also evolved pelvic reduction (Klepaker et al., 2013). There are also no
135 differences in the *Pitx1* amino acid sequence between pelvic-complete and pelvic-absent fish of
136 either species (Shapiro et al., 2006, 2004), despite their 26-million-year divergence
137 (Varadharajan et al., 2019). Rather, as in *G. aculeatus*, *Pitx1* expression in *P. pungitius* is
138 missing in pelvic tissue in pelvic-absent fish (Shapiro et al., 2006, 2004). Hybrids of three- and
139 ninespine stickleback with one pelvic-complete parent and one pelvic-reduced parent have a full
140 pelvis, while hybrids with two pelvic-reduced parents demonstrate pelvic girdle reduction
141 (Shapiro et al., 2006). These results further indicate that pelvic reduction is controlled by the
142 same locus, *Pitx1*, in threespine and ninespine sticklebacks (Shapiro et al., 2006). Moreover,
143 *Pitx1* has been implicated in pelvic reduction of a fossil sequence of *G. doryssus*, a threespine
144 stickleback from the Miocene (Stuart et al., 2020). This study used indirect, phenotypic evidence
145 to infer modified *Pitx1* expression: pelvic asymmetry in which left vestiges are larger than right
146 (Chan et al., 2010; Gurnett et al., 2008; Kragestein et al., 2018; Lanctôt et al., 1999; Marcil et
147 al., 2003; Nelson, 1971; Shapiro et al., 2006, 2004; Shiratori et al., 2014; Szeto et al., 1999;
148 Thompson et al., 2018; Xie et al., 2019). Pelvic-reduced *G. doryssus* fossils show this same left-
149 larger bias. As such, it appears that pelvic reduction in more than 100 populations across at least
150 three stickleback species proceeds by the same genetic pathway.

However, *Pitx1* expression does not drive pelvic reduction in a different teleost, the fugu (or pufferfish) *Takifugu rubripes*. Fugu also has a reduced pelvic girdle, perhaps because pelvic structures would interfere with its defensive “puffing” mechanism (Tanaka et al., 2005). Pelvic reduction is likely due to the absence of *HoxD* gene expression (Tanaka et al., 2005). Stickleback embryos express *HoxD9* in pectoral and pelvic buds to initiate and position fin buds (Tanaka et al., 2005). In contrast, the fugu ortholog, *HoxD9a*, is not expressed in the pelvic region of fugu embryos, preventing pelvic development (Tanaka et al., 2005) (**Fig. 2**).

Squamate Limb Reduction

Squamate reptiles have evolved reduced limbs dozens of times (Brandley et al., 2008; Greer, 1991), most notably in snakes. All snakes have lost forelimb elements, and most have no hindlimb or pelvic elements (Bellairs and Underwood, 1951; Cohn and Tickle, 1999). However, basal snakes like the python (*Python regius*) possess vestiges of the pelvis and femur (Cohn and Tickle, 1999; Leal and Cohn, 2016; Vitt and Caldwell, 2013).

In *P. regius*, early embryos develop hindlimb buds that then regress (Bellairs and Underwood, 1951; Leal and Cohn, 2016). Hindlimb development arrests because the feedback loop involving *Shh* and fibroblast growth factors (Fgfs) is attenuated in the limb bud. In typical tetrapods, *Fgf4* and *Fgf8* are signals essential for distal growth of the limb bud (Boulet et al., 2004; Cohn and Tickle, 1999; Hockman et al., 2008; Laufer et al., 1994; Leal and Cohn, 2016; Neubüser et al., 1997; Nissim et al., 2006; Ohuchi et al., 1997; Provot et al., 2008; Zúñiga et al., 1999) while *Shh* controls development along the anteroposterior axis of the limb bud (Chang et al., 1994; Cohn and Tickle, 1999; Fernandez-Teran et al., 2000; Leal and Cohn, 2016; López-Martínez et al., 1995; Riddle et al., 1993) and specifies bud width and the presence and identity

of digits (Chiang et al., 2001; Ros et al., 2003; Tickle and Towers, 2017). Reciprocal interactions between *Shh* and *Fgfs* is important for maintenance of their expression and limb outgrowth in the developing limb (Boulet et al., 2004; Cohn and Tickle, 1999; Leal and Cohn, 2016).

Shh expression in the tetrapod limb is controlled by an enhancer called the ZRS (Galli et al., 2010; Leal and Cohn, 2016; Lettice et al., 2003; Park et al., 2008; Riddle et al., 1993; Young and Tabin, 2017). The python ZRS has three large deletion mutations relative to *Anolis sagrei*, a lizard with fully developed hindlimbs (Leal and Cohn, 2016). These mutations result in reduced *Shh* expression in pythons. Though initially expressed in the python hindlimb bud, *Fgf8* levels decrease following loss of *Shh* signaling (**Fig. 2**), preventing distal limb growth (Leal and Cohn, 2016). The ZRS sequences are even more poorly conserved in advanced snakes, likely causing complete pelvic loss (Kvon et al., 2016; Leal and Cohn, 2016).

While less striking than that of snakes, limb reduction has evolved independently over a dozen times (Brandley et al., 2008; Greer, 1991) in Scincidae, a squamate family of over 1,700 described species (Uetz et al., n.d.) characterized by varying degrees of forelimb and hindlimb reduction (Greer, 1990). For example, fore- and hindlimb digit number varies between and within the seven species of the Australian genus *Hemiergis* (Shapiro et al., 2003; Uetz et al., n.d.). This variation is correlated with variable duration of expression of *Shh* in the limb bud: shorter expression corresponds to fewer digits (Shapiro et al., 2003). Though a specific mechanism has not yet been identified in *Hemiergis*, changes to *cis*- and/or *trans*-regulation may underlie the attenuation of *Shh* expression (Shapiro et al., 2003; Young and Tabin, 2017).

Mammal hindlimb reduction

Sirenians (manatees and dugongs) and cetaceans (dolphins, porpoises, and whales) are aquatic mammals that have converged independently on a phenotype that retains a vestigial pelvis and no external hind limb elements (Adam, 2009; Bejder and Hall, 2002; Cooper, 2009; Foote et al., 2015; Geisler and Uhen, 2005; Senter and Moch, 2015; Shapiro et al., 2006; Springer et al., 2004; Thewissen et al., 2001, 2006). Cetacean vestigial pelvic elements likely help support genitalia and musculature (Dines et al., 2014; Senter and Moch, 2015). Hand2, an activator of *Shh* (Charité et al., 2000; Fernandez-Teran et al., 2000; Galli et al., 2010; Leal and Cohn, 2016; Ros et al., 2003), is not expressed in the hindlimb bud of the spotted dolphin (*Stenella attenuata*) (Thewissen et al., 2006), preventing *Shh* initiation (Ros et al., 2003; Thewissen et al., 2006), and diminishing *Fgf8* (Leal and Cohn, 2016; Thewissen et al., 2006) expression in turn (**Fig. 2**). Like the python (Leal and Cohn, 2016), *Fgf8* is initially present in the cetacean hindlimb bud (Richardson and Oelschläger, 2002; Sedmera et al., 1997; Thewissen et al., 2006), but is not sustained without *Shh* expression (Leal and Cohn, 2016; Thewissen et al., 2006; Zhu et al., 2008). This results in the attenuation of limb outgrowth, regression of the limb bud, and a vestigial pelvis (Bejder and Hall, 2002; Cooper, 2009; Leal and Cohn, 2016; Sedmera et al., 1997; Thewissen et al., 2006; Zhu et al., 2008).

The molecular origins of sirenian limb reduction have yet to be explored, but their pelvic morphology offers some insight. As in *G. doryssus* fossils and extant populations of *G. aculeatus* and *P. pungitius* (Bell et al., 2006; Chan et al., 2010; Nelson, 1971; Shapiro et al., 2006, 2004; Thompson et al., 2018; Xie et al., 2019), the manatee *Trichechus manatus latirostris* has asymmetrical pelvic vestiges: out of 114 skeletal specimens, 93 had larger left-side than right side pelvic vestiges, indicative of modified *Pitx1* expression in pelvic tissue (Shapiro et al., 2006).

219

220 **Mammal Forelimb Reduction**

221 The monophyletic order Chiroptera contains over 1400 species of bats (Database, 2021;
222 Lei and Dong, 2016; Simmons et al., 2008). They are the only mammals capable of powered
223 flight. Flight evolved early in the bat lineage and was facilitated by structural changes to the
224 forelimb and pectoral girdle (Simmons et al., 2008). As in volant birds, the bat sternum has
225 evolved a keel to which large pectoral muscles attach (Simmons et al., 2008). The hand bones
226 are thin and elongated (Hockman et al., 2008; Simmons et al., 2008), and the length and width of
227 the ulna is reduced relative to the radius, and its distal tip is fused to the radius (Sears, 2007),
228 reducing weight without compromising wing function.

229 In the Natal long-fingered bat (*Miniopterus natalensis*) *Shh* expression is spatially
230 expanded while its initiation is delayed (Hockman et al., 2008) relative to mouse. In
231 experimental studies, *Shh*-knockout mice demonstrate reduced cell proliferation and increased
232 cell death in forelimb buds (Chiang et al., 2001), resulting in a mutant phenotype similar the bat
233 wing—a normal radius and a reduced ulna (Ahn and Joyner, 2004; Chiang et al., 2001; Hockman
234 et al., 2008; Sears, 2007).

235 This change in *Shh* expression may contribute to expanded Hox expression that further
236 shrinks the ulna (Chiang et al., 2001; Hockman et al., 2008; San-Ezquerro and Tickle, 2000).
237 The Global Control Regions (GCRs), a Hox regulatory sequence, of the little brown bat (*Myotis*
238 *lucifugus*) and the greater horseshoe bat (*Rhinolophus ferrumequinum*) show conserved changes
239 to the GCR relative to other mammals (Ray and Capecchi, 2008). Transgenic mice possessing
240 the *R. ferrumequinum* GCR demonstrate a proximal expansion of enhancer activity relative to
241 wildtype mice (Ray and Capecchi, 2008). This increased expression of *HoxD12* and *HoxD13* in

proximal tissue is linked to mouse ulnar reduction through suppression of *HoxA11* and *HoxD11* (Boulet and Capecchi, 2004; Hérault et al., 1997; Peichel et al., 1997; Sears, 2007). In *HoxA11/HoxD11* double knockout mice, the outgrowth of the zeugopod is disrupted, resulting in shorter, abnormal forelimbs (Boulet and Capecchi, 2004). This suggests that changes to Hox gene regulation and expression impact the morphology of the bat ulna (Boulet and Capecchi, 2004; Sears, 2007).

Bird Forelimb and Sternal Reduction

The emu, *Dromaius novaehollandiae*, is a flightless bird with a reduced sternum, humerus, radius, ulna, and autopodial elements (Bickley and Logan, 2014; Farlie et al., 2017; Kawahata et al., 2019; Maxwell and Larsson, 2007; Smith et al., 2016; Vokes et al., 2008). Wing morphology is highly variable between and even within individuals (Kawahata et al., 2019; Maxwell and Larsson, 2007), suggesting relaxed selective constraint. For example, vestigial digits II and/or digit IV are commonly but not always fused to digit III, while digit three is the only digit retained across individuals (Farlie et al., 2017; Kawahata et al., 2019; Maxwell and Larsson, 2007; Vokes et al., 2008).

The emu wing bud has fewer progenitor cells than the chick wing bud and is about half of its relative size (Bickley and Logan, 2014). Emu expression of *Tbx5* is delayed relative to chick (Bickley and Logan, 2014; Minguillon et al., 2005) contra Farlie et al., 2017), reducing recruitment of progenitor cells in sternal and forelimb tissues (Bickley and Logan, 2014). With a restricted progenitor population, rates of proliferation and outgrowth are reduced (Bickley and Logan, 2014; Farlie et al., 2017; Smith et al., 2016) and the emu wings grow 64% slower than in

264 chicken (Faux and Field, 2017). Also, unlike in chick, the emu wing bud emerges after and
265 develops more slowly than the hindlimb bud^{35,128,130}.

266 *Shh* expression is also delayed and decreased in the emu forelimb relative to its hindlimb
267 and relative to chick (Smith et al., 2016). *Msx2* and *Gli3*, two *Shh* repressors, are upregulated in
268 the emu forelimb relative to its hindlimb (Bakker et al., 2013; Smith et al., 2016). Experimental
269 expression of *Msx2* in the chick wing bud led to a reduction in length and number of wing
270 elements and produces an emu-like wing (Ferrari et al., 1998; Smith et al., 2016). In normal
271 chick and mouse development, *Gli3* restricts the expression domain of *Hand2* (Welscher et al.,
272 2002a, 2002b), indirectly restricting that of *Shh* (Smith et al., 2016; Welscher et al., 2002a) (**Fig.**
273 **2**). Elevated (Bakker et al., 2013) and expanded (Smith et al., 2016) expression of *Gli3* in the
274 emu wing further restricts *Shh* relative to the chick. *Gremlin1*, a gene important for digit
275 patterning, is repressed by *Gli3* (Kawahata et al., 2019; Vokes et al., 2008) but upregulated and
276 maintained by *Shh* (Litington et al., 2002; Panman and Zeller, 2003; Vokes et al., 2008;
277 Welscher et al., 2002b; Zúñiga et al., 1999) and *Hand2* (Welscher et al., 2002a) (**Fig. 2**).
278 Restriction of *Shh* and upregulation of *Gli3* in the emu wing likely alters *Gremlin1* expression
279 relative to chick (Farlie et al., 2017; Kawahata et al., 2019; Smith et al., 2016; Vokes et al.,
280 2008), decreasing digit number in the emu forelimb (Kawahata et al., 2019; Lopez-Rios et al.,
281 2012; Smith et al., 2016).

282 A transcription factor associated with cardiac tissue, *Nkx2.5*, is found in the forelimb of
283 early emu embryos (Farlie et al., 2017) but not in the wing buds of chicken, zebra finch, or
284 ostrich embryos that possess the typical three-digit wing pattern (Farlie et al., 2017).
285 Experimental expression of *Nkx2.5* in the chick wing bud results in reduced distal wing elements
286 and emu-like wings (Farlie et al., 2017). Therefore, *Nkx2.5* likely plays a role in emu forelimb

reduction and may be involved in reduction in the kiwi and cassowary (Farlie et al., 2017), two species closely related to the emu (Farlie et al., 2017; Faux and Field, 2017; Harshman et al., 2008; Mitchell et al., 2014; Phillips et al., 2009; Sackton et al., 2019).

The flightless Galápagos cormorant (*Phalacrocorax harrisi*) has a radius and ulna shortened relative to the humerus, perhaps to improve its diving efficiency (Burga et al., 2017; Elliott et al., 2013; Halsey et al., 2006; Watanabe et al., 2011). Compared to volant cormorant species, the Galápagos cormorant has a deletion of four amino acids in the coding sequence of the gene *Cux1* (Burga et al., 2017)—the only coding variant revealed in our review. In experiments with mouse cell lines, the resultant gene product is less effective in activating *Indian Hedgehog (Ihh)* (Burga et al., 2017), the expression of which is needed for the proliferation and differentiation of cartilage producing cells (Burga et al., 2017; Kronenberg, 2003; Peckham et al., 2003).

Conclusion

Appendage reduction is a major mode of morphological diversification in vertebrates. Convergence on this phenotype across vertebrate clades suggests that natural selection repeatedly favored appendage reduction and loss. We found that appendage reduction is underlain by a mix of shared and unique molecular pathways, depending on taxon and limb position. For example, *Pitx1* expression is repeatedly modified within and among stickleback species, suggesting parallel evolution within that lineage. On the other hand, altered regulation of *Pitx1* does influence pelvic reduction in the other teleost for which we found data, nor in any of the other vertebrate groups surveyed here (except potentially manatee).

310 Unlike *Pitx1*, altered expression of *Shh* is correlated with limb reduction across vertebrate
311 clades, including squamates, cetaceans, and emu, suggesting parallel use of the pathway. This
312 may because of the central role of *Shh* in the outgrowth and patterning of the limb. However, the
313 specific molecular mechanisms by which *Shh* is altered vary by taxon and limb type. For
314 example, altered *Shh* expression in pythons results from deletions to the ZRS, whereas attenuated
315 *Shh* expression in the cetacean hindlimb is altered by changes to *Hand2* expression and the *Shh*
316 expression pattern of the emu forelimb is controlled by changes in *Msx2* and *Gli3* expression.
317 Hox genes were also implicated multiple times, though again the underlying mechanisms
318 differed by taxon and limb. For example, in fugu, pelvic reduction stemmed from a lack of
319 *HoxD9a* expression in the pelvic region. In bats, ulnar reductions resulted from alterations in
320 *HoxA11*, *HoxD11*, *HoxD12*, and *HoxD13* expression.

321 Altogether, mixed of shared and unique solutions to the same selective problem is
322 perhaps not surprising. Appendage development requires spatially and temporally regulated
323 expression of dozens of interacting genes—complexity that creates numerous pathways to
324 appendage reduction and therefore non-parallel, many-to-one solutions. However, many of these
325 key developmental genes have pleiotropic effects, thereby constraining their evolution, and
326 forcing clades into the use of only a handful of labile pathways (i.e., parallel change). Such
327 constraint may explain the most salient result in our review: in all cases but one, appendage loss
328 resulted not from changes in protein coding DNA but rather changes to enhancer sequences and
329 gene expression specific to the limb tissues. The evolutionary importance of regulatory
330 mutations is contentious, especially for gain-of-function adaptations (Hoekstra and Coyne,
331 2007). However, our findings support the assertion that regulatory changes play a repeated role

332 in loss-of-of-function phenotypes (Hoekstra and Coyne, 2007), in ways that are likely adaptive
333 (e.g., Chan et al., 2010).

334

335 **Data Accessibility Statement**

336 No datasets were generated or analyzed in production of this review.

337

338 **Conflicts of Interest**

339 Authors involved in preparation of this manuscript have no conflicts of interest to declare.

340

341 **Author Contributions**

342 Conceptualization - Swank, Stuart

343 Data curation - NA

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345 Funding acquisition - Stuart

346 Investigation - Swank

347 Methodology - Swank, Stuart

348 Project administration - Swank

349 Resources - NA

350 Software - NA

351 Supervision - Stuart

352 Validation - Sanger, Stuart

353 Visualization - Swank

354 Writing – original draft - Swank

355 Writing – review & editing - Jointly, all

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