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REVIEW

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Unraveling the heritage of lost traits

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Abstract

We synthesize ontogenetic work spanning the past century that show evolutionarily lost structures are rarely entirely absent from earlier developmental stages. We discuss morphological and genetic insights from developmental studies reveal about the evolution of trait loss and regain.

KEYWORDS
embryology, evo-devo, trait loss, vestigial organs

"Loss is nothing else but change, and change is Nature's delight — Marcus Aurelius, AD 121–180"

1 | INTRODUCTION

Organ loss or regression is a repeated theme in the history of life on Earth. However, complete structure loss is rare and many organisms retain vestigial structures (retained body parts or organs that have lost part or the totality of their ancestral function; Müller, 2002) that can be present transiently during development or retained in the adult. While not explicitly looking at vestigial structures, Ernst Haeckel's iconic and controversial drawings of phylotypic stages and biogenetic law of "ontogeny recapitulates phylogeny" highlighted the importance of looking at developmental form to understand the evolution of species (Anon). However, after the golden age of embryology in the XIX century, these anatomical observations were largely excluded from the evolutionary synthesis and explanations for the process of evolution (reviewed in Hall, 2012).

This view changed, at least in part, when Stephen J. Gould revisited these concepts in the 1970s (Gould, 1977) and with the subsequent re-emergence of evo-devo and emergence of genomics (see, Hall, 2012 for a beautiful synthesis). The discovery of the genetic toolkit changed views of development (Carroll, 2008; Hall, 2012) and paved the way for many gene regulation studies that tried to explain morphological evolution and find rules for the evolution of organisms that explicitly incorporated development (reviewed in Hall, 2012). For example, attention was brought to heterochrony as a developmental mechanism that could be selected for (Smith, 2003), or lately, (Carroll, 2008; Müller, 2002), by linking adult morphology with developmental modules and their respective gene regulatory networks (Davidson and Erwin, 2006; Erwin & Davidson, 2009; Kuratani, 2009; Klingenberg, 2010; Uller et al., 2018). Importantly, these studies revealed gaps in our understanding of the genotype to phenotype map (Orgogozo et al., 2015), and highlighted the importance of including developmental processes in the explanations of evolution. However, while genetic studies of developmental processes have increased, detailed anatomical studies that link developmental changes in specific structures to genetic mechanisms remain largely unexplored.

In the last 10 years, the emergence of new techniques and the reexamination of previous work have reinforced Haeckel's vision that anatomical development can reveal important information about the evolution of traits (Moczek, 2006; Metscher, 2009; Wang et al., 2017). These integrINTRODUCTIONative approaches have led to the reexamination of old questions about trait losses and the significance of vestigial rudiment in our understanding of organismal evolution, as well as a better understanding of the constraints that direct the evolution of phenotypes.

In this review, we discuss how developmental studies examining trait loss connect ontogeny and phylogeny and enhance our understanding of evolutionary developmental biology. We synthesize morphological ontogenetic work spanning the past century to show evolutionarily lost structures are rarely completely lost from earlier

developmental stages, revealing a trait loss continuum. We explain this continuum with developmental constraints that direct and, in certain cases, limit the evolution of phenotypes. To better understand the mechanisms behind these constraints, we integrate recent genomic, transcriptomic, and protein expression data with morphological work. Finally, we discuss what these morphological and genetic insights from developmental studies reveal about the evolution of trait loss and regain.

2 | TRAIT LOSS IS NOT BINARY, BUT SPANS A CONTINUUM THAT REVEAL UNDERLYING CONSTRAINTS

From the beginning, developmental studies revealed a trait loss continuum. This continuum spans from traits that never appear throughout ontogeny, to traits that begin developing but are completely resorbed before sexual maturity, to traits that begin developing and leave behind vestigial traits in adults (Figure 1). In this section, we show that trait losses have always been viewed as a developmental continuum and bridge these early observations to recent work, revealing the importance of constraints (as defined by Smith et al., 1985: biases on the production of variant phenotypes of limitations on phenotypic variability caused by the structure, character, composition, or dynamics of the developmental system) and new potential rules for organismal evolution.

2.1 | Vestigial structures, in building a theory of evolution/an historical perspective

Study of organ loss and regression has deep roots in biological studies and has always been linked to developmental processes. In his book, History of Animals, in the 4th century BC, Aristotle reasoned that moles could barely see because their eyes were "stunted in development" (Aristotle n.d.). Lamarck compared the mole rat and the mole, explaining that both have lost foresight because of their common habitat, leaving a "vestige" of an organ. Saint-Hilaire wrote that "Nature never works by rapid jumps and always leaves a vestige of an organ" (Lamarck, 1809; St. Hilaire & Geoffroy, 1798). Later, Darwin proposed two other interesting hypotheses, introducing a putative function of vestigial structures for the organisms: first, he explained the regression of certain characters by the fact that structures can be "disused" (Darwin, 1859, Barrett P). Second, he suggested that some of these structures could retain secondary roles and be used for "a distinct object." Finally, the term "vestigial" appeared for the first time in 1893 in *The Structure of a Man* by Robert Wiedersheim who also questioned the function of residual organs (Wiedersheim, 1893). He explained that some vestigial organs are present in the adults but "wholly or part functionless" while others are transient structures that are only present during development. Thus, from the very beginning, the idea of homology and regression has been evoked to explain their presence. At the same time, Haeckel was investigating links between ontogeny and phylogeny and using embryology to reconstruct the ancestral relationships between species. Together, all these observations reveal the importance of morphological losses in our historical understanding of development and evolution, and suggest that losses are best viewed as a continuum ranging from total loss to vestigiality. In the next two sections, we explore different levels of the loss continuum.

2.2 | The rarity of traits lost without a trace (no morphological evidence)

Given the regularity of vestigial structures, how many structures are truly lost, that is, are not even induced during development and what are the evolutionary consequences of these losses? Few examples can be confirmed, because detailed developmental investigations of lost adult traits remain rare. But some cases of complete developmental program loss, even before genetic induction (see mechanisms section), have been discovered. The case of the teeth loss in birds is a textbook example of a complete structure loss. While all living birds are toothless, many transitional fossil forms document the progressive loss of teeth in various fossil bird taxa (Zhou & Li, 2010; reviewed Louchart & Viriot, 2011), and establish that the ancestor of modern birds, *Archaeopteryx lithographica*, had teeth that were then lost approximately 100 million years ago (Davit-Béal et al., 2009). However, the question remains, are the teeth of modern birds completely lost or do some rudiments persist during development? Observations of XIX century naturalists revealed the existence of a transient epithelium thickening at embryonic Day 5 (E5) in the

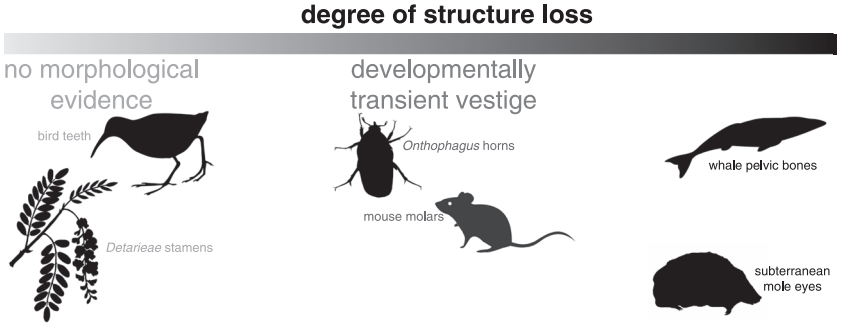

FIGURE 1 Traits are lost to varying degrees along a trait loss continuum. Although we depict trait loss examples that correspond with three separate levels, in the main text we describe additional trait losses and inter- and intraspecific variation that span these levels of loss

chicken, similar to the very first step of tooth formation in other vertebrates (Blanchard, 1860; Carlsson, 1896; Gardiner, 1884; Geoffroy Saint-Hilaire, 1820; Röse, 1892, reviewed in Davit-Béal et al., 2009). However, later (see genetic and developmental mechanisms) studies of the developmental program suggested that bird teeth are lost even before the induction stage. Thus, it appears that bird teeth represent a trait that is completely lost, despite other studies confirming that the tissues in the bird jaw remain capable of generating teeth if they receive the correct stimulation (Harris et al., 2006). Therefore, even in the case of a "complete loss," there remains potential for organ development, probably because of the pleiotropy of pathways used during organ development, to re-evolve some structures in certain conditions that might imply GRN rewiring (see Mechanisms). Another example of a lost trait that is absent throughout ontogeny is stamens in Detarieae (Tucker, 2001), but further examples of true loss are hard to find. Together, these results suggest that true losses are rare and that most structures are not completely lost.

2.3 | Developmentally transient traits are the rule rather than the exception

If structures are rarely lost, then how widespread are transient structures in the tree of life? And if they are a regularity, then why are they maintained? The existence of transient structures has led early anatomists to speculate about the existence of constraints that might prevent the total disappearance of organs. The most well-studied level is developmental vestigiality, that is, traits that are completely absent in adults but are present at early development stages before disappearing (e.g., via fusion or resorption) or being strongly reduced. Examples of developmentally transient structures are numerous and have paved our understanding of organ evolution. Early studies of hind limb reduction in cetaceans (HoweLL, 1930), and digit reduction in birds (Heilmann, 1927; Witschi, 1956), various artiodactyls (Mettam, 1895), and horse (Rensch, 1959) revealed that early development of some organs is maintained before being arrested at various time points. Later, developmental studies of squamate species that completely lack or have severely reduced fore- and hind-limbs (Lande, 1978; Raynaud, 1962; Raynaud & Van den Elzen, 1976) or resorption of tooth bud in baleen whale (Ishikawa & Amasaki, 1995) showed similar transient appearances of lost structures during early ontogeny.

More recently, studies that have incorporated genetic and molecular work have identified more transient structures and the underlying mechanisms behind losses, refining our vision of the continuum. In dolphins that have lost hindlimbs (Thewissen et al., 2006), molecular examination have revealed that the two developmental regions, the AER and ZPA, that are crucial for the development of the hindlimb are not maintained or failed to develop: the AER is initiated but not maintained, and the ZPA failed to establish, leading to developmental arrest at an early stage. Comparative studies of eye degeneration between surface and underground animals that integrated anatomical and genetic data have revealed different degrees of losses in the eye components and linked them to underlying developmental mechanisms. In subterranean salamanders, (Tovar et al., 2018) the eye lens starts to form during development but regresses and is absent in adulthood, seemingly linked to changes in the *Pax 6* gene expression. While in the cave salamander *P. anguinus*, the cornea involute and the lens undergoes a lytic process resulting in a reduced eye (Durand, 1976). In the cavefish *A. mexicanus*, while some eye structures start to develop and differentiate (such as the lens and the retina), others, like cornea or the iris, fail to develop, revealing that structure loss can be unequal among organ tissue types. Both cellular and molecular mechanisms have been linked to cavefish eye degeneration, such as apoptosis, differential neural crest migration, and gene expression variation, showing that complex organ regression is not simple and involves variation at multiple levels (Jeffery, 2009; Yamamoto & Jeffery, 2000; Yoshizawa et al., 2018). In other cavefish species, we see different degrees of loss corresponding to different timepoints of developmental arrest. In *P. andruzzii* eyes develop normally with a lens, a retina, and a cornea before degrading via apoptosis within a month (Berti et al., 2011). This contrasts with *A. mexicanus* in which eye loss fate is decided earlier, during the eye field patterning (Stemmer et al., 2015). *S. anophtalmus* shows even later timing of eye developmental arrest, the eyes are internal and extremely reduced which has been linked to reduced proliferation and downregulation of some transcription factors involved in retinal development and maintenance (Meng et al., 2013). Together, these results exemplify gradation in the evolution of eye loss in cave animals.

The retention of structures that differ in developmental arrest timing—at the induction stage, at the beginning of structure formation, or post morphogenesis followed—over long stretches of evolutionary time pose a conundrum. If developing transient traits are costly, energetically, spatially, or otherwise, we would expect selection to eradicate these ontogenetic remnants. To reconcile the observation of such structures with hypotheses behind evolutionary cost, it was hypothesized their presence is the result of developmental constraints that cannot be overcome or because they retain a certain function. In the next parts, we will explore these hypotheses and show how they can uncover rules influencing the evolution of organisms.

3 | EXAMINING THE DEVELOPMENTAL CONTINUUM OF INTER- AND INTRASPECIFIC TRAIT LOSSES REVEALS EVOLUTIONARY RULES/AXES OF VARIATION

3.1 | Organ retention and functional role during development

One of the first explanations for the maintenance of transient structures is the retention of some function for the organism or its

development. Examples of such functions are numerous and often involve interdependencies in organ development, suggesting that certain structures cannot be entirely lost because they initiate or share important developmental mechanisms needed for the development of one or other organs. In elephants, the development the adult tusks depends on the development of a first vestigial tooth, the tush, that is later resorbed by the surrounding tissues (Raubenheimer, 2000; Raubenheimer et al., 1995). In beetle horns of the genus *Onthophagus*, we see a different example of transient function. Beetle horns have been evolutionarily lost many times and are sexually dimorphic. However, the developmental examination of many species has revealed developmental transient beetle horns play a crucial role in the ecdysis of the larval head capsule in all species and sexes examined (Moczek, 2006). As discussed before, in cave animals, eyes are often see as transient structures that almost undergo complete development before being lost. This observation brought the idea that these structures might be useful for the development of other organs (Rétaux & Casane, 2013). In particular, it has been suggested some changed expression pattern of key genes such as *Shh* and *Fgf8* could amplify the development of other sensory structures such as taste buds or olfactory pits that are enhance in cavefish populations (Bibliowicz et al., 2013; Varatharasan et al., 2009; Yoshizawa et al., 2012; see Rétaux & Casane, 2013; and Krishnan & Rohner, 2017 for comprehensive reviews). These observations suggest the existence of a strong developmental constraint at the early stages of forebrain development in vertebrates that prevent the complete disappearance of the eye despite the cost of its development. Finally, another great example of the role of a vestigial organ in a species is the maintenance of vestigial imaginal discs in the worker caste of the ant genus *Pheiole*. In these ants, the queen and the male castes possess wings whereas they are lost in worker castes such as the large-head soldiers and the small-headed minor workers. As a result, the role of the maintenance of such discs has been questioned. Elegantly, it was shown that the growth of rudimentary wing discs is necessary to regulate allometry between the head and the body size in soldiers and this mechanism is also used to control the proportion of soldiers and minor-workers to adjust the worker caste needs in the colony (Rajakumar et al., 2018). These examples, along with many other relevant studies (see (Prochazka et al., 2010) and (Sadier et al., 2019) for further examples), reveal how a structure can be lost in the adult but maintained during development because of an ontogenetic role and highlight the importance of developmental constraints in the maintenance of vestigial and/or transient organs.

Finally, other studies revealed vestigial organs can play a critical role in the evolution of morphological variation and/or novelty. In rodents, development of the two lost premolars, called MS and R2 in mice, is arrested respectively at the epithelium thickening and bud stages, which represent the very early stages of tooth formation (see also Box 1). However, one of the lost premolars, R2, seems to play a role in the morphology of another tooth, participating in M1 formation in the lower jaw (Prochazka et al., 2010; Sadier et al., 2019). The growth regulation of wing imaginal discs (mentioned earlier) has also been shown important in the evolution of novel phenotypes: by investigating allometry variation in *P. hyatti*, intermediate variants that mimic the worker caste systems of other species of ants can be produced, revealing that the maintenance of vestigial organ can result in the emergence of dramatic new phenotypes (Rajakumar et al., 2018). Finally, the study of the staminode of Penstemon flowers provide another good example of how novelty can emerge from vestigial structures. In these plants, a structure, the staminode, develop transiently during development, representing the stamen that has been lost and reacquired in this clade. This reappearance has been followed the acquisition of new functions: depending on the species, the stamen can enhance visit duration and contact with sexual organs in some bee pollinated species while its presence has no effect on pollination by other species (Walker-Larsen & Harder, 2001).

Together, these examples reveal how transient structures could retain some function during development and/or can act as driver of the evolution of others.

Box 1 Repeated organs: A particular case for gains, losses and regains

Repeated structures or organs are serial homologs that are present all over the body. Some of the best examples of repeated structures are external features such as mammal hair and bird feathers, and internal structures such as ribs, vertebrae, digits and branchial arches. While the development of repeated structures varies from organ to organ, the evolutionary addition or removal of a repeated structure is controlled by conserved mechanisms that adjust their number and size during pattern formation. An example of such mechanisms are reaction/diffusion or Turing processes (Kondo & Miura, 2010), positional information involving tightly regulated gene expression in space and time (Jaeger et al., 2004), or a combination of both and other factors (Green & Sharpe, 2015). As a result, the variation in the number of units is frequent and a unit lost can be considered a complete loss of the organ or its developmental program.

Examples are numerous and one of the most iconic might be vertebrate ectodermal appendages that comprise external glands, hair, feather, scales, nails, claws, teeth, and among others traits whose unit number is highly variable and that are considered evolutionary hot spots (Sadier et al., 2014). The mechanisms behind unit development and variation have been intensively studied and have revealed how the reiteration of the same developmental program triggers unit number variation. For example, feather location, number, and loss have been linked to variation in the signaling molecules, which triggers Turing mechanisms (Mou et al., 2011), primary hair density in mice is dependent on the intensity of the molecular signal

(Mou et al., 2006), and tooth and cusp number can be modulated by activators and inhibitors (Cai et al., 2007; Cooper et al., 2018; Sadier et al., 2019).

The evolution of digit numbers provides another well-known example of evolutionary flexibility in repeated structures. Digits are thought to develop through both activation/inhibition mechanisms and morphogen gradients (Cooper, 2015; Onimaru et al., 2016; Zuniga & Zeller, 2014) and digit reduction and/or loss has been a frequent phenomenon over tetrapod evolution. Study of this phenomenon suggests that, rather than resulting from loss of entire developmental programs, digit loss is associated with changes in activation/inhibition mechanisms and/or in specific enhancer modifications that change the expression domain of morphogens. These examples suggest that the gains/losses of single components of repeated structures are directed by the existence of more specific domains during development rather than a modification of the overall program and pathways associated with their development (discussed in Seher et al., 2012). This could explain why additional components of repeated structures can be so easily lost and regained during evolution in response to environmental pressures, and are often fine-tuned during evolution.

3.2 | Heterochronies as evolutionary rules

Incorporating ontogenetic data can reveal variation in the degree of trait loss among clades that have lost the same structure and point out some potential evolutionary rules for the evolution of traits. Many structures show inter- or intraspecific variation in their place on the trait loss continuum. As one example, frog and toad (anuran) middle ear ears have been lost in at least 32 clades (Pereyra et al., 2016). Numerous anuran clades have vestigial middle structures, in which some earlier developing structures of the middle ear remain (Pereyra et al., 2016) but seemingly lack any hearing-related function (Womack et al., 2018). Other middle ear loss clades have transient middle ear structures during development but completely lack middle ear structures as adults (Stynoski et al., 2020). Even within a population, some individuals show no trace of the middle ear as adults and others have small cartilaginous chunks of middle ear bone as adults (Stynoski et al., 2020). Similarly, forelimbs, hind limbs, and pelvic bones have been convergently lost to varying degrees among skink species with additional intraspecific variation in the degree of loss noted in some lineages (Moch & Senter, 2011). Together, these results show that lost structures display developmental variation in the degree of loss among closely related species or populations. Because of this, it has been proposed that heterochrony, that is, varying developmental time by accelerating or slowing down the development of an organism or some of its traits, is critical to this trait loss lability (see examples in Buendía-Monreal & Gillmor, 2018; McNamara, 2012). Heterochrony was introduced by Haeckel as deviations from its "Biogenetic Law," uncoupled from the recapitulation theory by De Beer (1951, embryos and ancestors), and popularized by Gould in "Ontogeny and Phylogeny" (Gould, 1977). Examples of heterochrony are numerous. In dolphins, the formation of finger bones is likely to have arisen from an extension of the growth period of finger formation (Richardson & Oelschläger, 2002). In bats, recent research combining morphological and molecular developmental work has revealed bat face length variation can be explained by heterochrony (Camacho et al., 2020). To conclude, the trait loss continuum can be considered a line of least resistance for evolution via heterochrony. Can dissection of the genetic and cellular mechanisms more clearly define this line of least resistance?

4 | GENETIC AND DEVELOPMENTAL MECHANISMS BEHIND TRAIT LOSS REVEAL ADDITIONAL RULES OF MORPHOLOGICAL EVOLUTION

Recent research has enhanced our understanding of the mechanisms driving trait losses across the continuum and in doing so, has revealed more general concepts of genetic and developmental evolution. Traits are formed during embryonic development, ergo, losing a trait involves loss, or modification of the developmental program responsible for formation of these traits. Organismal development is controlled by gene regulatory networks (GRNs) comprised of developmental genes/pathways that control cell fate, and transcription factors (TFs) that act as switches to activate them through cis-regulatory elements (CREs or enhancers; Erwin & Davidson, 2009). Any change in these players or in their interactions can result in morphological modification or loss. In this section, we will review some key examples and propose some new evolutionary rules for the loss of organs, from the loss of terminal genes to GRN rewiring.

4.1 | Gene losses linked with structure losses

The most extreme way to lose a trait is to lose the genes essential to its generation. Without terminal or essential genes necessary to its development, a trait would not be able to form and/or be fully functional. Research in the last 15 years has revealed interesting features about gene losses and their role in trait loss, from the genome to protein expression (Albalat & Cañestro, 2016; Sharma et al., 2018). This study has revealed two main ways to lose a gene: (i) an abrupt, disruption or removal of a gene following the insertion of a repeated element or a deleterious crossing over, (ii) the gradual accumulation of mutations during the pseudogenization of the gene after a loss-of-function mutation. In this later case, gene loss is gradual, from loss of function, pseudogenization to complete gene loss, i.e. the gene sequence cannot be found in the genome. Except when mentioned, gene loss will be used at its broader sense, that is, from loss of function to

complete absence. The description of all such cases is beyond the scope of this review, but we highlight a few iconic examples that explain the link between gene loss and trait loss. One of the most well-studied examples is the repeated, independent loss of pigmentation in the cavefish *Astyanax* in different cave populations, compared to surface relatives. The genetic basis at the origin of albinism in cavefish have been intensively studied (Protas et al., 2006, reviewed in (Jeffery, 2009). Using crosses, QTL and more recently, CRISPR, these studies have revealed that pigmentation loss is due to the loss of function of a terminal gene, *Oca2*, involved in the first steps of the melanin synthesis pathway (Klaassen et al., 2018; Protas et al., 2006), causing a complete loss of cell pigmentation due to the disruption of the gene coding sequence. In another example, (Sharma et al., 2018) cetaceans lost genes associated with hair and epidermis related functions as an adaptation to ocean conditions. In birds, the loss of teeth is followed by both the loss of the developmental program (GRN, see below) and the associated terminal genes necessary for teeth differentiation (Sire et al., 2008).

If a gene is lost, are there intermediate processes leading to this that would eventually influence potential regains? Other examples have unraveled some of the processes behind gene loss, showing gradation in processes even before pseudogenization. In bats (Sadier et al., 2018), the S opsin gene (and associated cone cells) is frequently lost, which led to UV/blue vision loss, in association with diet (Sadier et al., 2018) or roosting (Gutierrez Eduardo de et al., 2018; Kries et al., 2018; Li et al., 2018; Simões et al., 2019; Wu et al., 2018). However, the examination of the opsin gene and its conserved expression reveals that independent losses of UV vision occur at different levels of gene expression (e.g., genomic sequence changes, RNA expression loss, or protein localization loss) depending on the species (i.e., from the loss of protein expression to the pseudogenization of the opsin gene; Sadier et al., 2018). This reveals a gradual mechanism behind rapid trait loss and regain evolution. Other cases have revealed another fine way to tinker trait loss, by losing gene isoforms. In mammals, the A isoform of the *Edaradd* gene has been convergently lost among different lineages (Sadier et al., 2015), leaving the other isoform, which is supposed to maintain core functions, intact. In potatoes, the evolution of a new gene isoform I in the BRC genes allows potatoes to modulate gain/loss of lateral shoot branches in response to environmental cues (Nicolas et al., 2015).

Together, these examples show that gene loss is an important mechanism behind trait loss and has led many to research the prevalence of gene loss in the tree of life. Recent analyses of 102 genomes covering the breadth of the animal kingdom (Guijarro-Clarke et al., 2020) revealed widespread gene losses during the evolution of animals. Analyses further suggest that these losses (some of them being complete) helped shape the distinctive biological characters of the Ecdysozoa, Lophotrochozoa, and Deuterostomia clades. In sum, these studies suggest gene loss is a regularity in animal evolution (Albalat & Cañestro, 2016). Regarding loss of traits, our examples highlight some mechanisms behind trait loss that could also favor non-reversibility of losses.

4.2 | Loss of Cis-regulatory regions

Changes in Cis-regulatory regions (CREs), as opposed to coding changes, have long been suspected drivers of morphological evolution since modification of CRE activity often has fewer pleiotropic effects (Carroll, 2008; Wray, 2007). While both coding and cis-regulatory changes are now acknowledged to be important in morphological evolution (Cheatle Jarvela & Hinman, 2015; Martin & Orgogozo, 2013; Orgogozo et al., 2015; Sadier, 2016), loss of CREs are a particularly important trait loss mechanism. In the last 20 years, many studies have associated partial or total trait losses to regulatory changes, uncovering numerous mechanisms for the gradual reduction or loss of a trait. The loss of armor plates in sticklebacks, *Gasterosteus aculeatus*, exemplifies how changes in gene regulation can result in varying degrees of structure loss. While most three-spined sticklebacks live in the ocean, several freshwater populations were isolated during the last glacial retreat (Bell & Foster, 1994). In contrast to marine sticklebacks, which harbor a full skeletal pelvic structure, some freshwater populations exhibit a reduction or complete loss of this structure in association with reduced calcium and fewer predators (Shapiro et al., 2004). The locus driving the trait reduction was mapped by crossing marine and freshwater populations (Shapiro et al., 2004) as well as populations that exhibit partial or total pelvic reduction (Chan et al., 2010). These last experiments identified that gradual losses of an enhancer associated with the gradual losses of the phenotype (Figure 2). Similar work has been done in snakes, in which researchers have identified several CREs linked with snake limb loss by combining genome comparisons among several snakes, limbed reptiles, and other limbed vertebrates ATAC-seq (Roscito et al., 2018).

Using a similar approach, Roscito et al., 2018 investigated the evolution of CREs implicated in eye loss and other visual system changes (i.e., disorganized lenses, reduced eye size, thinner retinas, and loss of neural connections) found in subterranean mammals, such as the blind mole rat, the naked mole rat, the star-nosed mole, and the cape golden mole. Following-up on previous results (Partha et al., 2017) that demonstrated convergence in the mechanisms behind eye loss in these species, Roscito et al. (2018) found hundreds of CREs located near genes implicated in eye development whose function have diverged in subterranean mammals, elegantly elucidating mechanisms behind Lamarck's observations in the XIX century.

These results suggest that CRE changes are a hallmark of trait loss. In addition, they further support the hypothesis that specific phenotype associated CREs are often targeted multiple times during parallel evolutionary losses, and that CRE modulation can generate the degrees of structure loss described in previous sections.

4.3 | Progressive loss of the developmental GRN/pathways

A third driver of vestigiality and organ loss is modification of gene regulatory networks or (GRNs) that control organ development.

However, GRNs contain numerous, interacting developmental circuits that are often pleiotropic, participating in the development of many structures (Erwin & Davidson, 2009). Perhaps one of the most iconic is the hedgehog pathway which is implicated in the developmental patterning of: the central nervous system, ectodermal appendages, somites, middle ears, limbs, and other structures. Because of this pleiotropy, changes at the most peripheral branches of the GRN or in modifiers of the GRN are more likely associated with trait loss. The loss of teeth in vertebrates, and in particular in birds, sheds light on how GRNs can be maintained despite modification to produce a trait loss. Over the past 40 years researchers have investigated the GRN underlying tooth development in birds through experiments mixing induction of genes and in vitro and in vivo tissue recombinations in mice and chicken. These findings revealed that tooth induction is possible in birds, suggesting that the early phases of the developmental program of teeth, which involve common developmental pathways (Catón & Tucker, 2009), can be reactivated in birds (reviewed in Davit-Béal et al., 2009; Louchart & Viriot, 2011 and papers therein). However, essential genes required to form dental specific proteins (such as ameloblastin or enamelin) have been pseudogenized in birds (Sire et al., 2008), revealing that while the odontogenic capacity of birds' dental epithelium can be reactivated, this is insufficient to form fully functional teeth. Another example of phenotypic loss linked to underlying changes in a GRN is hind limb loss in Cetaceans. Like limbed mammals, the hind limbs of Cetaceans grow out from the body during development. However, this outgrowth is halted around the bud stage of development when the limb is as long as it is wide, and the limbs regress. Studies investigating the molecular basis of hind limb loss in the tropical spotted dolphin revealed that this cessation of outgrowth and subsequent limb regression likely occur because of a failure to activate the pleiotropic hedgehog pathway (i.e., *Shh*; Thewissen et al., 2006), while other circuits of the GRN (in particular in early phases) are maintained.

These examples reveal intriguing and consistent properties of GRNs that can be viewed as evolutionary rules of trait loss. First, they suggest that early steps of organ formation are more buffered and robust than late terminal steps because of their central importance to the development of the organism, due to the hierarchical organization of GRNs. Second, they provide a comprehensive framework for understanding the pleiotropic nature of developmental pathways in organ loss and regression: organ loss rarely involves loss of a pleiotropic gene, but instead more commonly involves a circuit loss of the GRN at the origin of organ development. GRNs can thus be viewed as modular, sharing pathways and branches for the development of various organs. As a result, in instances of complete phenotypic trait loss, the underlying gene loss and resulting pseudogenization tend to occur in peripheral and terminal genes (see gene losses). An intriguing consequence of this phenomenon is that lost organs can be regained by re-evolving these peripheral parts of the developmental circuits, as the overall network is maintained.

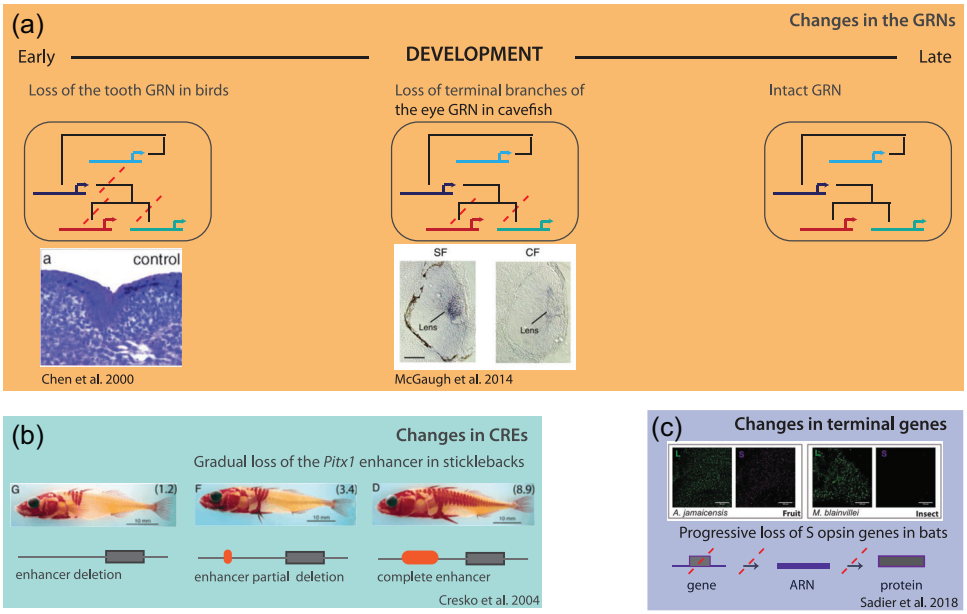

FIGURE 2 GRN, CRE, and terminal gene modification parallel developmental trait offset. (a) Trait loss can result from some branches or sub-circuits of the GRN that control their development while the pleiotropic developmental pathways are conserved. The degree of loss can be linked to how early and peripheral the branches are. For example, tooth loss in birds is thought to the result of the loss of the tooth GRN whereas the loss of the eye in cavefish is supposed to be more peripheral. (b) Trait loss can result from deletion of a single and/or multiple CRE in a given GRN. An iconic example is the loss of armor plates in sticklebacks in which the degree of the phenotype depends on the partial/complete deletion of the *pitx1* enhancer. (c) Trait loss can result from loss of terminal-specific genes, generally active at late phases of development. The loss of UV vision is bats is a good example of the loss of terminal genes. While photoreceptors form normally in these bats, the S-opsin gene, that produce the S-opsin pigment, is lost. CRE, Cis-regulatory region; GRN, gene regulatory network [Color figure can be viewed at wileyonlinelibrary.com]

5 | RULES OF LOSS HELP EXPLAIN REGAINS AND LACK OF SUPPORT FOR DOLLO'S LAW

The historically known continuum of trait loss and more recently revealed genetic and developmental pathways provide critical data for understanding the evolutionary history of traits. Dollo's Law of irreversibility, which states that an organism never returns exactly to a former state (Dollo, 1893), has been evoked repeatedly to explain lack of structure regains after evolutionary loss (Gould, 1977; Moch & Senter, 2011). However, ancestral reconstructions of trait loss have provided evidence for a number of morphological trait regain (second molar in lynx—Werdelin, 1987; stick insect wings—Whiting et al., 2003; squamate limb elements—Kohlsdorf & Wagner, 2006; Brandley et al., 2008; mandibular teeth in frogs—Wiens, 2011; sex combs in drosophila—Seher et al., 2012; third molar in marmosets—Scott, 2015; middle ear structure in toads—Pereyra et al., 2016), prompting many to question the validity of Dollo's Law in regard to the regain of morphological structures (for teeth and other serial organs, see Box 1). Although ancestral reconstructions can generate regain hypotheses, they leave as many questions as they answer. Inference errors due to phylogenetic uncertainty and incomplete sampling make it impossible to ever know the true ancestral state of traits and the developmental pathways that generate them (Cronk, 2009; Cunningham, 1999; Goldberg & Igić, 2008). Furthermore, many studies discuss the difficulty in knowing whether the "same" genetic pathways and developmental mechanisms are responsible for the re-emerged trait, leaving questions regarding whether re-evolved traits are actually identical to their lost predecessors (Church & Extavour, 2020; Gould, 1970; Marshall et al., 1994; McIntyre, 1997). These questions resemble those found in discussions of deep homology. Deep homology refers to independent traits that share the same developmental network and program (reviewed in Tschopp & Tabin, 2017 and references therein), for example, ectodermal appendages, hind and fore limbs, or bilaterian body appendages. These difficulties leave trait regains controversial, but, acknowledging that trait losses are less binary at the developmental stage provides a clearer roadmap for how traits could be regained using preserved genetic and developmental pathways, such as the GRNs described above. In addition, the possibility of regain could also affected by the degree of trait lost.

Beyond increasing the plausibility of trait regains, developmentally transient structures reveal hidden genetic and developmental variation that selection could act on to generate traits distinct from the originally lost trait. Even if structures begin with the same developmental underpinnings, later developmental stages could be modified to generate "cryptic innovations" or completely novel structures. Cronk (2009) describes "cryptic innovation" as an apparent reversal that is actually an innovation caused by a gain of gene function. However, developmentally transient structures could also produce a truly novel structure that is completely distinct from the originally lost structure in form and function. Vestigial structures have been discussed as evolutionary intermediates and opportunities for novelty and exaptation (Brandley et al., 2008, Walker-Larsen & Harder, 2001) and developmentally transient structures similarly provide opportunities for the evolution of modified or novel structures (see rodent teeth in previous paragraphs).

6 | CONCLUSION

Cumulative evidence from studies over the past century clearly demonstrates trait loss is not as binary or simple as sometimes presented and often involves many layered modifications of genetic, cellular and other developmental processes. These comparisons also reveal the crucial importance of studying comparative morphology across development to understand trait evolution and constraint. Integration of modern genetic techniques within this comparative morphological framework has the potential to reveal mechanisms that facilitate and constrain the parameters regulating this evolutionary tinkering. Here we identify numerous "rules" of trait loss, which are simply common themes that have accumulated from developmental investigations of lost structures.

First, they suggested that constraint conserves the developmental pathways of lost traits, often leaving developmental or adult vestiges. Second, the examples provided here showed gradual losses dictated by heterochrony are more of a rule than an exception, as spotted by Haeckel and that vestigial structures can retain a certain function as spotted by early naturalists. Third, studies of underlying genetic mechanisms organ/structure losses involved losses at the gene and CRE level as well as loss of developmental GRN circuits. Fourth, GRN modifications allow for repeated organ evolution despite developmental pleiotropy and can facilitate regains and/or the evolution of new structures from pre-existing circuits. Integrating morphological, genetic and modeling studies has enhanced our understanding of evolution but we echo other studies in underscoring the importance of integrating development into our understanding of evolution (Alberch & Blanco, 1996; Müller, 2002). Incorporating anatomical development into this integrative framework has expanded our definition and understanding of evolutionary loss highlighted patterns of evolution that parallel Haeckel's invalid biogenetic law "ontogeny recapitulate phylogeny", and uncovered tweakable rules for the organismal evolution.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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