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Running head: regeneration versus scarring in animals

ABSTRACT

The present paper summarizes previous theoretical studies that have suggested why broad regeneration is present only in some animals. It is speculated that the evolution of regenerative versus non-regenerative species is linked to specific environments where genes involved in regeneration can or cannot be expressed. While the marine conditions allow regeneration since PreCambrian and Paleozoic, the conquest of the mainland from invertebrates and vertebrates met conditions un-compatible with broad organ regeneration, such as drying, higher oxygen, nitrogen and UV radiation. It is speculated that adaptation to the life on land changed numerous anatomical and physiological characteristics of marine animals, also increasing the immune competence, which often negatively affected the process of organ regeneration. The loss of regenerative abilities was an unavoidable consequence, as extensively discussed in previous studies. The ability to regenerate organs is linked to developmental gene pathways, only partially known, that evolved in harmony with the remaining anatomical-physiological characteristics of these species. These genes or gene-modules cannot be simply transferred into non-regenerative species, with the likely effect to disrupt their genomes and transcriptomes with lethal or teratogenic consequences. Animals are not jigsaw puzzles where a genetic engineer can shift a gene module of a puzzle (from a regenerative-competent animal) into another puzzle (non-regenerating animal). Present knowledge indicates that, while minor injuries could be improved using genetic technologies by transferring genetically engineered cells into damaged organs, the replacement and not a real organ regeneration will be feasible by the combination of future biotechnological and bioengineering interventions.

KEYWORDS: regeneration, environment, evolution, genetic manipulations, regenerative medicine

Healing multicellular bodies after injury is a fundamental but variable life property

The present text summarizes several theoretical studies and publications that in the last years have proposed an *Evolutionary Environmental Theory* to explain not *how* but *why/where* a different distribution of the regenerative ability is present among invertebrates and vertebrates (Alibardi, 2022, 2023a,b, 2024a,b,c,d, 2025; Fig.1). The present essay supports new ideas and hypotheses but cannot be exhaustive in the attempt to explain the distribution of animal regeneration. The main scope of the present manuscript is to unify and summarize the broad speculative discussions reported in the previous papers, with some other additions related to evolutionary adaptations to the terrestrial conditions. Here I re-state my hypothesis that the *loss of regenerative capability among animals derives from their terrestrial adaptation*, like for other physiological-anatomical traits of terrestrial animals. It is very important that next generation of developmental biologists realize the inseparable relationships of animal functions as a terrestrial adaptation, including the distribution of healing abilities and organ regeneration.

In fact, after over 100 years of modern cellular-molecular scientific investigation on animal regeneration, the quest for explaining differences in the regeneration ability among animals is essentially dealt from a still largely empirical cellular and molecular, namely reductionist, point of view (Brookes and Kumar, 2008; Katsuyama and Paro, 2011; Tanaka and Reddien, 2011; Stocum, 2017; Lai *et al.*, 2018; Goldman and Poss, 2020; Bideau *et al.*, 2021; Khyeam *et al.*, 2021; Lubetsky *et al.*, 2023; Huang *et al.*, 2024). Most of these studies come up with important new discoveries, about new genes and gene modules activated in regenerative competent species of invertebrates and vertebrates. Attempts to understand differences in regeneration among animals, based on evolutionary considerations, have been recently submitted (Bely and Nyberg, 2009; Bely, 2010; Tiozzo and Copley, 2015; Elchaninov *et al.*, 2021; Srivastava, 2021; Zarsisky *et al.*, 2024). However, these studies also essentially rely on relatively poorly known molecular mechanisms and genes present in invertebrates and vertebrates. Since regeneration involves a large number of genes, it is the lack of one or few of these genes, or the deactivation by natural processes such as mutation or by experimental manipulations, that determine loss of regeneration. Molecular papers report lists of genes necessary in different animal models to elicit organ regeneration, which experimental deactivation precludes regeneration. However, the presence of these genes in both regenerating and non-regenerating animals does not explain the failure of regeneration in the latter. I have hypothesized that this is related to the natural environment and to evolution of the model species.

Most of past and recent studies indicate the utility of this new information for improving human regeneration. The latter biomedical attitude however neglects the natural meaning of animal regeneration as part of the specific biological characteristics present in each animal species. While animals *evolved regenerative mechanisms together their other body functions*, step by step during million years, following new processes and erasing those unfit with the previous ones, human science discovers the mechanisms of regeneration *by a still largely empiric research that slowly fills the puzzle of knowledge*. The search for a biological sense for the disparity in the distribution of regeneration among invertebrates and vertebrates is complex, but the only sense that I found is *considering animal evolution in changing environments to explain animal regeneration* (Alibardi, 2023a,b, 2024a-d). I believe that the biological context (ecology and evolution) where regeneration occurs leads toward a synthetic biological understanding of the phenomenon. Molecular studies extract important information from simple invertebrate models (hydra, flatworms) to more complex invertebrate models (starfish, sea cucumbers, sea squirts) to anamniotes (urodeles and some fish). They try to find common genes and basal cellular process but they devote scarce consideration on the completely different anatomical-physiological nature of these animals, their environment and their specific evolutionary history, in particular aquatic versus terrestrial (Fig. 1).

Here, I re-state *my addition* to the famous Theodore Dobzansky sentence, “nothing in biology makes sense except in the light of evolution, *including regeneration*” (Alibardi, 2023a). Therefore, only evolutionary considerations can offer a rational explanation (*why? /where?*) for the distribution of regeneration among extant animals (like for other life properties). Molecular information aims to explain *how* regeneration occurs and *are the necessary details to grasp the process* but, let alone, this information is insufficient to understand this post-developmental morphogenetic phenomenon, related to injuries or to the physiological renewing that occurs during an animal life. Molecular information alone cannot answer *why* the process takes place today in some and not in other animals, since this depends from the environment where each species evolved, by itself an almost unsolvable bio-historical problem.

Molecules are the basic stuff from which any progressive level of biological complexity derives, so any biological process has its origin and its basal explanation from genes and bio-molecular interactions. The genomic-DNA stores all the information that will be utilized during the lifetime of an animal, comprising development, growth, reproduction, aging, and healing ability after injury, often referred, with no distinction, as “regeneration” (Alibardi, 2025). However, neglecting zoological considerations cannot explain macroscopic phenomena present among different animals, including organ regeneration. We should be aware that the DNA program for regeneration in extant animals can only be expressed in relation to the environment where any species live, and that this genetic program has a long evolutionary history.

If we believe in biological evolution, we should consider that life was born in the sea over 4 billion years ago, and that *unicellular and later multicellular regeneration was born in ancient times*, as a basal property of life (Carlson, 2007; Alibardi, 2023a,b, 2024a,d; Fig. 2). During the evolution of cells and multicellularity, regeneration initially involved metabolites in proto-cells (metabolite turnover), then informational replicating macromolecules (RNA and DNA), then cells and simple multicellular organisms, all living in the sea. It is here submitted that cell regeneration in unicellular protozoans and later regeneration of simple or more complex cell aggregates, like in *Volvox* sp. or placozoans like *Trichoplax adhaerens*, were common processes in the PreCambrian and Paleozoic Eras, when life became multicellular. It is likely that also these simple ancient animals were naturally endowed with broad regeneration, as it is testimonial in the similar extant simpler metazoans, placozoans, sponges, cnidarians, flatworms and ribbon worms. Later, a broad regeneration was maintained but also reduced in more complex metazoans such as sea worms, echinoderms, bryozoans, acorn worms, sea squirts, lancelets etc. As a result from the process of evolution in different animals, present-day regenerative abilities in extant animals vary from “whole body regeneration” (here considered the primitive condition), to “epimorphic regeneration” (here considered as a derived conditions with ample fluctuations among species), to poor or no regeneration (here considered as a derived and mainly a terrestrial-adaptive process) (Fig. 2). The above proposed evolutionary sequence derives from the following considerations, since we can assume that regenerative processes present in extant metazoan today were also present in their forbears of PreCambrian periods (earlier than 550-570 million years ago).

Whole body or large epimorphic regeneration after injury or loss of body parts is only present in the simpler extant invertebrates (hydras, flatworms, some file worms and sea squirts), because it is part of their physiological activity as asexual reproduction in the marine environment (Bely and Nyberg, 2009; Rinkevich *et al.*, 2022; Alibardi, 2023a,d; Fig. 2). These clades are the most ancient metazoans, present since the Pre-Cambrian (sometimes before 550-570 million years ago). As in the extant forms, also the ancient forms likely underwent whole body regeneration, often indicated as morphallaxis. Epimorphic regeneration, a polarized regeneration that occurs from injured areas of the body with intense cell proliferation, shows the progressive restriction of regenerative abilities in more complex invertebrates (annelid sea worms, echinoderms, chordates), and particularly in vertebrates (some fish and amphibians). These invertebrates, in most cases cannot regenerate entire new bodies from minute fragments of their injured body, but only from variable larger regions or from body appendages. Epimorphic regeneration therefore, based on the present hypothesis, was likely also present in protostome invertebrates that evolved

after the simplest metazoans listed above (Fig. 2). In addition, in basal deuterostome invertebrates (tunicates, cephalochordates) epimorphosis is frequent, and even whole-body regeneration is possible in some ascidians, all marine (Rinkevich et al., 2022). In the more complex aquatic vertebrates, epimorphic regeneration of some appendages at least, was also present in ancient fish and amphibians, as the fossil record indicates (van Der Voss et al., 2018; Darnet et al., 2019). Finally, in terrestrial conditions also epimorphic regeneration be-came/-comes more restricted, from a “*variable recovering healing*” (variable tissues recovery) to “*regengrow*” (regeneration during somatic growth), but epimorphic regeneration mainly vanish-ed/-es and remain-ed/-es as scarring in most land invertebrates and vertebrates (Alibardi, 2025). These extant animals with no/poor regeneration are phylogenetically more recent than the previous marine forms indicated above, and they initiated their evolution from the Devonian-Carboniferous, during land colonization of invertebrates and later, also from amphibious vertebrates (sarcopterygians and amphibians). Like their ancestor chordates, these amniotes included larval stages and metamorphosis in their aquatic biological cycle, so preserving developmental programs also in part re-usable for organ regeneration (Alibardi, 2019, 2020). This “educated speculation” indicates that metamorphosis and larvae were lost during amniote evolution, together broad capabilities also for organ regeneration.

Broad regeneration, like development, can only occur in very soft and hydrated tissues, as those of most marine invertebrates and vertebrates (80-97% of water), and this process likely holds since the origin of life on earth. *High regeneration has been maintained during the life history* and is still present in marine invertebrates today, while regeneration is variably lost or completely absent in terrestrial species. When organisms begun to colonize the emerged lands in Paleozoic times, this ecological transition probably also influenced numerous anatomical-physiological-developmental characteristics of transiting animals, including their regenerative abilities. This consideration suggests that it is *life on land and its evolutionary conquest that determined the loss of broad organ regeneration* among invertebrates and vertebrates (Figs. 1, 2, Alibardi, 2024a-d, 2025).

Problems for regenerating organs on land

When we speak about animals not capable of broad regeneration, *we generally and unwittingly refer to terrestrial species* (Alibardi, 2023a,b, 2025). Exceptions to this generalization, also present in some aquatic species (Bely, 2010; Lai et al., 2018), are still not explained, but are possibly related to the loss of regeneration due to changes during the evolution of their new traits for adapting to a changing life cycle, even in watery conditions. Here I further hypothesize that some changes derived from adopting a parasitic life, e.g. in leeches, with reproductive adaptations to hosts living in water and inland. Another change was the elimination of soft larvae and the indirect form of development in aquatic animals, since these larvae could not withstand terrestrial desiccation and also collapsed under land gravity on a solid substrate (Figs. 2, 3). Therefore, transitional animals had to evolve direct forms of development and also terrestrial-adapted larval forms (e.g. in insects) or intermediate larval forms adapted to colonize aquatic or terrestrial hosts (flukes, tapeworms, leeches; Fig. 2). The latter invertebrates show variable but specialized types of epimorphic regeneration, based on the different localization of species-specific stem cell niches (see later). In some species of invertebrates (e.g. some echinoderms and arthropods) and vertebrates (e.g. few amphibians and numerous lizards), broad regeneration follows the self-amputation of a body appendage (autotomy). Various interpretations that attempt to explain the presence and evolution of these two processes in different animals have been presented (Maginnis, 2006; Embert et al., 2019).

In which conditions is broad regeneration possible? Regeneration of injured organs is their return to an embryonic condition of tissue softness, a phenomenon only possible in the water conditions of aquatic animals, many of which undergo an almost complete anatomical and functional recovery (Figs. 1, 2). As opposed, only in few cases terrestrial animals, however spending most or part of their life in water or in very humid conditions such as terrestrial flatworms, earthworms, some slugs, fish and amphibians, can still

regenerate parts of their lost organs. Regeneration is however more limited in these animals, in comparison to marine species, and is feasible if they evolved mechanisms to maintain highly hydrated their tissues, over 80%, by hyaluronate or hyaluronate-like polymers and water in a soft connective tissue. A morphogenetic process such as organ regeneration activates a massive cell proliferation and differentiation that is only possible in highly hydrated, embryonic-like conditions, including immuno-suppression or immuno-evasion (see later). In a *dry environment, no regeneration can occur*, and this simple and obvious consideration is generally neglected in molecular studies that otherwise report complex mechanisms of cellular activities, including absence or presence of developmental genes involved in regeneration in some but not in other animals. These studies focus on genes and cell processes, neglecting the environment where these genes can or cannot be expressed (Alibardi, 2023b, 2024b,c).

Considering that all the main pathways involved in regeneration, Wnt, Notch, MAPK, FGFs, Hh, BMP etc., are conserved and present in most animals (Stoick-Cooper *et al.*, 2007; Taghiyar *et al.*, 2018; Suzuki and Ochi, 2020), it is unexplained *why terrestrial animals cannot use these gene pathways for regeneration*. However, even if terrestrial animals possess the same genes that in aquatic animals are involved in regeneration, at least some of *these genes or gene activation pathways are useless* and cannot be expressed in the dry, UV, Oxygen- and Nitrogen-rich terrestrial environment. It has been suggested that at least some of these genes, even if still present in non-regenerative competent animals, were likely *epigenetically turned off or detoured into other developmental processes* during invertebrate and vertebrate evolution to terrestriality (Alibardi, 2023, 2024b; Figs. 2, 3). Animals keep their genes functional if they respond to the changing environment, as it is now supported from the knowledge of epigenetic mechanisms (Turner, 2009; Katsuyama and Paro, 2011; Chen *et al.*, 2018; Sato, 2018).

With the emergence of invertebrates and vertebrates from the sea during terrestrial adaptation in the Paleozoic, broad regeneration present in marine animals encountered the obstacles of the terrestrial environment, as well as higher levels of unscreened and mutagenic UV solar rays. Without a high hydrated condition, absent in animals living on land, the genetic program utilized for regeneration in aquatic animals was useless. Therefore, it has been suggested that regeneration *was likely progressively altered by epigenetic processes for the adaptation to a land life* during the evolution of these pioneer animals (Alibardi, 2023b, 2024a; Fig. 3). The previous UV protection underwater in marine animals, likely determined a lower number of deleterious mutations, including those related to genes involved in regeneration. The hypothesized epigenetic changes are hard to be detected today, but some experiments or deductive future studies have been previously suggested and could be done to confirm or deny this hypothesis (Alibardi, 2023b, 2024b).

Furthermore, the higher ROS (Reactive Oxygen Species) production derived from the highly oxygenated terrestrial environment, from about 1-10% during Precambrian to Devonian, and rising to above 21% estimated in the Carboniferous-Permian to reach the present concentration, much higher than oxygen dissolved in saltwater (0.001%). Too much oxygen was not favoring broad organ regeneration since it generated higher levels of ROS such as H_2O_2 (hydrogen peroxide), $O_2^{\cdot-}$ (superoxide anion), and $\cdot OH$ (hydroxyl radical), that determin-ed/-es DNA damages and mutations, protein alteration and deactivation, cell membrane disruption. In addition, ROS determine *damage and deactivation of telomerases*, therefore limiting cell and tissue renewal in terrestrial animals and accelerating aging (Fernandes *et al.*, 2021). Aging is evident in terrestrial animals but is not or however less evident in aquatic animals, invertebrates and vertebrates. In addition, living in a fluid medium with 78% concentration of nitrogen, likely present on Earth since 2-3 billion years, much higher than nitrogen dissolved in water (0.00005%), determin-ed/-es increase of nitrosation with the production of dinitrogen trioxide (N_2O_3) and peroxyxynitrite ($OONO\cdot$), or NO^+ (nitrosonium ion), molecules that also damage DNA and proteins, including shortening of telomeres and damaging of telomerases (Li *et al.*, 2011; Parrish *et al.*, 2018).

Another radical change was the higher microbial contact/pollution and tissue exposition, since washout of the outer and inner surfaces of the body is much lower on the ground than in flushing water or under water

currents. In addition, this condition *favoured the evolution of a stronger and more elaborated immune system in terrestrial species* to defend from microorganisms and tumors, but often with a negative side-back for regeneration (Harty *et al.*, 2003; Godwin *et al.*, 2017; Mescher *et al.*, 2017; Adhikary and Hui, 2021). The evolution of an intense inflammatory reaction with a sophisticated immune control, the adaptive immunity, is generally considered negatively correlated to regeneration, although recent discoveries have indicated qualitative differences between healing and scarring (Godwin *et al.*, 2017; Adhikary and Hui, 2021). As the sophisticated adaptive immunological protection is one of the last evolutionary achievements in vertebrates, like complex behaviors and intelligence, contrasting effects of these newer organic systems that have probably co-evolved (Castillo-Morales *et al.*, 2014), have originated un-compatibility with the more primitive regenerative abilities of animals (Alibardi, 2020, 2024a,b, 2025, see later).

Hypothetic epigenetic mechanisms and loss of regeneration during land transition

This chapter extends previous hypotheses (Alibardi, 2023b, 2024b), and is only meant to provide some indications, based on present information, on possible gene mechanisms present in marine invertebrates and vertebrates that were primarily involved in sensing the new land environment. During long periods, it is submitted that in the genomes of these pioneer species, mainly ancient arthropods and sarcopterygians-amphibians, some “new sensorial inputs” gradually determined epigenetic changes in their development for the adaptation to a fully terrestrial life. We can make hypotheses on the ability of animals of the past to sense drying, higher levels of UV-light, oxygen and nitrogen, and encounters with new microbes, based on what is known on extant invertebrates and vertebrates.

Some extant vertebrates can sense dryness or desiccation, and activate various physiological responses that eventually also influence genes such as RAAS (Renin-Angiotensin System, including *REN1*), *AGT* (Angiotensinogens), *AGTR1* (Angiotensin II Receptor), aquaporin (*AQP2*), *ADH*, *Atg1,6, 8, 9, 13,18* (Autophagy-Related genes), *epi74EF*, *epi75EF1*, *co-but*, *maf-s* (transcription factors), but in particular various heat shock protein (HSP) genes such as *HSP70*, *HSP90AB1*, *HSP90AA1* (Chen *et al.*, 2018; Storey and Storey, 2023). In invertebrates, desiccation sensing occurs by the activation of Mn-superoxide dismutase against oxidative stress derived from dehydration, TPS (threulose-6 phosphate synthase), TPP (threulose 6 phosphate phosphatase), cyp40 (cyclophilin 40, a chaperone), HSF (heat shock factor), LEA (Late Embryogenesis Abundant) proteins, IRS (a ionotropic receptor), neuropeptides such as CAPA, ITC and ITP, DAF-12 (Dauer Formation Abnormal-12), desaturases and fatty acid synthases (Watanabe, 2006; Knecht *et al.*, 2017; Tang *et al.*, 2018).

Sensors for oxygenation or hyperoxygenation (Wilson *et al.*, 2020) activate *Sesn1-3* genes (sestrins, an antioxidant), PHDs (Prolyl hydroxylase) sensors that modify and degrade HIF (hypoxia inducible factor). In addition, the activation of VHL (Von Hippel Lindau) proteins with high oxygen exposure, determines the labeling of HIF α , a sub-unit of HIF, for its destruction. Sensing high Nitrogen levels, N₂, or oxidative compounds such as NO₂, peroxynitrite or NO⁺ (nitrosonium ion) is poorly known in animals in comparison to plants or lower metazoans (Luter *et al.*, 2020). It is however indicated that long exposure to these reactive nitrogen molecules can determined respiratory problems, signaling alteration, and also breakage of DNA double filament, cell and tissue death (Ji *et al.*, 2015; Parrish *et al.*, 2018).

UV-irradiation induces gene expression (McKay *et al.*, 2004; Laughery *et al.*, 2024), and can activate innate immune sensing cellular mechanisms such as the inflammasome including NLRP3 (NOD-like receptor family pyrin domain containing 3), NLRC4 (NLR family CARD domain-containing protein 4), and AIM2 (Absent in Melanoma 2). Other genes activated from microbial invasion (Bauernfeind and Hornung, 2023) are PRRs (Pattern Recognition Receptors, Toll-like receptors such as TLR2 and TLR4, the cGAS-STING pathway sensing bacteria DNA penetration, a host ubiquitin ligase for destruction of microbial altered

proteins, MHC (Major Histocompatibility Complex), RAF1 (c-RAF), RGS12 (Regulator of G protein signaling 12).

Although we do not know whether/which of these/other gene-sensing pathways were present in ancient animals colonizing the mainland in the Paleozoic, early sensory mechanisms for the above external stimuli might have influenced and gradually changed the epigenome during thousands and million years of evolution for land colonization. This hypothesis suggests that these changes might have also influenced developmental programs unfit with the terrestrial conditions, contributing to originate terrestrial-adapted animals but un-capable to regenerate organs in the new environment. This is because these genes were useless for regeneration that require continuous wetting conditions, absent on land.

Also, possible phenomena of DNA base changes, DNA elimination (Wang and Davis, 2014; Smith *et al.*, 2021) and pseudogenization derived from base mutations, favored by the higher UV-exposition, might have been tolerated or even positively selected in case these genes were not useful in a terrestrial environment. As a final comment, although the loss of broad regeneration was a consequence of terrestrialization, a counterbalanced acquisition derived from the evolution of the complex neuro-muscle coordination necessary to move on land, the derived evolution of complex brains and behaviors, and eventually of intelligence. The latter, also allows much more efficiently avoiding injuries and organ-loss in comparison to invertebrates and vertebrates with lower behaviors and intellectual capacity. Despite octopod mollusks and some fish exhibit some complex behaviors, the *evolution of higher levels of intelligence appears a terrestrial acquisition* in our planet, also in animals returning to the sea (e.g. cetaceans among mammals).

Main terrestrial animal groups where regeneration is limited or absent

Three main evolutionary lineages of animals that lost the initially high regenerative capacity during their evolution from an initial marine condition are shown in Fig. 4 (Alibardi, 2023b,c, 2024c). Nematodes evolved *eutelia*, generating a fixed number of cells forming their adult body that cannot be replaced after loss or injury. Arthropods evolved *terrestrial-adapted larvae for dissemination*, eliminating the plastic aquatic larval forms (trochophora-like), and reached adulthood by direct developmental growth or through repetitive activation of imaginal disks during molting. The latter, is the only periods in which they can regenerate their appendages before die out after reaching adulthood, in particular in insects (the adults in most species has a short life). In addition, amniotes lost the larval forms and metamorphic transformations in the Carboniferous Period, still present only in extant amphibians, and evolved direct development, originating terrestrial adults but lost broad regenerative ability.

Among vertebrates, while numerous urodele amphibians and their tadpoles still regenerate various organs, the terrestrial transition from larvae to adults in anurans, determines the inhibition of the larval potential for broad regeneration (Harty *et al.*, 2003; Mescher *et al.*, 2017). Without bathing in water or in high humidity conditions, no terrestrial animals can regenerate lost organs, not even numerous earthworms or urodele amphibians. Actually, only some species of urodele amphibians, salamanders and neotenic amphibians (axolotl, newts, etc.) can still regenerate numerous organs. Many other post-metamorphic terrestrial salamanders and all the anurans do not regenerate their appendages or form short heteromorphic outgrowths, *showing a gradation of healing capabilities loss* (Scadding 1979, 1981, 1983; Harty *et al.*, 2003; Dwaraka and Voss, 2021; Alibardi, unpublished observations on *Salamandra salamandra* that does not regenerate limbs). In numerous species of urodeles, heteromorphic limb regeneration occurs, evidencing the difference between limb development and regeneration. In his studies, Scadding (1979, 1981), also pointed out the importance of the size of the studied amphibians and their limb regeneration, indicating that even numerous species of urodeles larger than 10 cm snout-vent, often shows a limited and heteromorphic limb regeneration or even do not regenerate. In addition, urodele tadpoles regenerate faster than juveniles or adults, likely in

relation to the different status of tissue differentiation and of the size of the regenerating limb (Goodwin, 1946; Dwaraka and Voss, 2021).

In drier but still humid conditions, also the axolotl after metamorphosis decreases or even stops its regenerative ability or regenerates incomplete, heteromorphic organs (Monaghan *et al.*, 2014; Demircan *et al.*, 2018). Plethodont urodeles, living in high humidity conditions, regenerate very slowly (Arenas-Gomez *et al.*, 2017), and they possibly mix their ongoing somatic growth with regeneration in a process of *regengrow* (Alibardi, 2024a,b; Fig. 5). Even more surprising is the lack of regeneration in several limb bones after removal in salamanders, *Ambystoma* in particular, and also in newts that usually regenerate complete limbs (Goss, 1958). Anurans are an extant example of regeneration loss after the transition to land life, the testimonials of the modifications that occur-ed/-s from water to land. These changes rapidly stop or strongly down-regulate the expression of some genes (Mukhi *et al.*, 2010; Raj *et al.*, 2023), as they are no longer useful or appropriate for functioning on land, probably including genes also involved in regeneration.

The study on the modification of genetic networks determining this loss in anurans might be discovered (epigenetic methylations of DNA/histones?, gene silencing?, DNA elimination? pseudogenization of some genes? ; Wang and Davis, 2014; Reverdatto *et al.*, 2022; Fig. 3). As previously indicated (Alibardi, 2023b, 2024b), the study on these likely epigenetic modifications and the comparison of transcriptomes between developing and regenerating organs should be pursued in future research. The genetic program allowing organ regeneration was/is conserved and utilized only in extant amphibious animals (some earthworms, snails, fish and urodele amphibians) that also present aquatic or humid-adapted larval stages and metamorphosis in their life cycle. These animals are capable to re-utilize their genetic programs also for the regeneration of their damaged organs, since these programs were used for their metamorphic transformations to reach adulthood (Alibardi, 2020). Consequently, regeneration, as is the case with other biological characteristics that changed during invertebrate and vertebrate terrestrialization, was replaced with a *variable recovery healing, regengrow* but mainly through scarring, the more derived healing ability evolved among terrestrial animals (Alibardi, 2025; Figs. 2, 5). The variable recovery healing is still indicated with the general term of “regeneration” in terrestrial animals, but this “land regeneration” is only a partial tissue recovery or physiological regeneration, a very different process from the broad organ regeneration present in aquatic animals. Land “regeneration” is very limited in size and anatomical-physiological recovery (0.5 mm-some mm), does not utilize signaling gradients, often takes long time (*regengrow*), and cannot be repeated since a second injury only give rise to scars (Alibardi, 2024a,b).

The term animal regeneration still generates confusion

Since the beginning of most regeneration studies, the animal model under study was evaluated as an isolated organism from its past evolutionary history and also from the present ecological context (Sanchez-Alvarado and Tsonis, 2006; Farah *et al.*, 2016; Mehta and Sing, 2019). Unfortunately, most researchers still consider, for instance, appendage regeneration related to starfish, insects, crustaceans, newts, mouse or humans, as a basically similar problem like “if they can why we can’t?”, without considering that *these appendages with a similar name and function* have different evolution, development, anatomical-histological structure and environmental utilization. The knowledge or the non-knowledge of the evolution in these different animals is however the basic condition that has determined how much an animal, today, can re-activate the developmental programs accumulated and deposited in the genome from long time, including the program for a variable healing (regenerative) capability.

These species-specific genetic programs, still largely unknown, can be invested in different extant animals for their own regeneration when an organ injury occurs during their lifetime. Most of tissue-organ healing/reparative processes that occur in different animals, invertebrates or vertebrates, are still indicated as “regeneration”, assuming the significance of the restitution of large or complete tissue or organ, despite

its size and the tissue composition. What is a “variable recovering healing”, meaning *a spectrum of variations in the reparative capabilities among animals*, from none (scars) to broad (e.g. limb or spinal cord regeneration in newts), is still termed “regeneration”. Therefore, even a small injury of the tip of a tiny digit in mammals or in children, less than 0.5-1 mm in rodents or 2-5 mm in children, the closure of a few mm ear hole in some mammals, the injury of an organ such as the skin or the spinal cord in a still developing/growing embryos/fetuses or in growing juveniles, is considered as “regeneration”, implying a broad *de novo* recovery. In these cases, no consideration is given to the fact that *during this healing period*, sometimes taking over 4-5% of the entire lifetime span of an animal, *the animals are also growing* (Alibardi, 2018, 2024a,b, 2025; Fig. 5). Therefore, it is unknown what can be “dissected” from the indicated process of “regeneration”, what is truly regenerated (actually healed) from what instead derived from the natural tissue growth (cell proliferation, hyperplastic and hypertrophic cellular increase) that took place during this same period of somatic growth, and still indicated as “regeneration”. The cells produced during somatic growth, together those generated from healing (regeneration), are simply neglected and this type of recovering healing should be instead indicated as “*regengrow*” (*regeneration+growth*). Probably, numerous processes indicated as regeneration among invertebrates and vertebrates in reality represent *regengrows*, since healing occurs during the somatic growth/restructuring of the body in these animals. Again, specific zoological information on the life cycle of these animals is required for a proper evaluation of their regeneration abilities.

Looking carefully, regeneration even in the most capable animals *cannot reconstitute an organ exactly as it was formed during embryonic development* where the starting point was different. In fact, while an injured organ begins a process of regeneration from residual cells located among millions of somatic cells, during embryonic development the same organ instead derived from the progressive multiplication of a single cell, the zygote. The two developmental programs cannot be equal, and therefore *regeneration cannot be like development*, except perhaps in simple marine or aquatic animals such as sponges, flatworms, file worms, sea squirts where asexual reproduction, from one or few cells, also naturally occurs in these organisms in a watery environment (Rinkevich *et al.*, 2022). As previously hypothesized, regeneration has decreased its potential and mechanisms, with respect to development, from whole body regeneration in the simplest metazoan (sponges, cnidarians, flatworms) to epimorphosis (regeneration by cell proliferation from specific body sites) in more complex metazoans (annelid sea worms, starfish, sea cucumbers, acorn worms, lancelets), and from epimorphosis to scarring mainly in terrestrial animals (Fig. 2).

In complex vertebrates, such as in fish and amphibians, organ regeneration is even more different from organ development than in invertebrates. For instance, spinal cord regeneration in newts or axolotl does not take place exactly like during embryogenesis, although the final result is truly outstanding (Clarke *et al.*, 1988; Tanaka and Ferretti, 2009; Freitas *et al.*, 2019). In fact, a closer analysis reveals that the regenerated spinal cord is not anatomically and even functionally complete as it was originally (Stensaas, 1983; Clarke *et al.*, 1988; Davis *et al.*, 1990; Freitas *et al.*, 2019). In addition, the regenerating limb or tail does not repeat the same processes of development (Stocum, 2017; Khan and Crawford, 2021; Bothe *et al.*, 2025), and the *full size* of a regenerated limb is only reached in a long period, one year or longer (Young *et al.*, 1983). During these long periods 1-2 years, also somatic growth/remodeling occurs in the spinal cord and in the skeletal bones of these animals, and what is referred as regeneration is actually a process of *regengrow* (Alibardi, 2018, 2022; Fig. 5).

Regengrow or a variable recovering healing are reparative processes that allow a good to partial recovery of the initial anatomical and physiological functions, but do not completely restore those of the original lost organ. In some invertebrates (arthropods mainly), *regengrow* is associated with molting for growing in the terrestrial-adapted larvae or juveniles (e.g. insects) to the adults (Figs. 4, 5). This is due to imaginal disks or equivalent “adult primordia”, small internal sacs or external sacs/organs made of stem cells, a terrestrial adaptation of insects and other arthropods, not from a diffuse stem cells distribution across the entire body

like in aquatic species (Rinkevich *et al.*, 2022). Imaginal disks can regulate the number of cell divisions according to environmental stimulations (Stanger, 2008), for instance the loss of a leg, and increase the number of cells until a new leg is formed with the appropriate size *during successive phases of molts*. In many arthropod species, not only the adult cannot regenerate organs or appendages after the end of molting, but often the adult rapidly dies, the end of its biological cycle (e.g., in many insects, by far the prevalent forms of terrestrial animals).

In vertebrate amniotes, a limited *regengrow* occurs in some organs, sometimes reaching a voluminous but heteromorphic recovering like in the liver, the tail of lizards, or broad areas of the skin in rare rodents. Although these organs can reach a considerable dimension and histological complexity of their tissues, they cannot reconstitute the anatomy and physiology of the original organ as this was developed in an embryo. Other injured or lost organs are incompletely repaired, but they form scars with no function and anatomical recovering but rapidly protect the animals from dehydration and microbial infection in the terrestrial environment. With no useful genes sustaining regeneration because of water shortage etc., scarring probably evolved by still unknown epigenetic processes as a rapid mechanism for wall-off small or large injuries, mainly preventing dehydration and microbial penetration (Ferguson and O’Kane, 2004).

The vastly utilized term “regeneration” should be limited to large, morphogenetic, reconstructive organ processes such as those present in marine invertebrates and in some anamniotes among fish and amphibians. In contrast, the remaining reparative tissue processes present in arthropods-amniotes should be indicated as “*variable recovery healings or regengrow*”, including the physiological regeneration of epidermis, blood and epithelial intestine tissues, that should be indicated as “*tissue renewal*”. This concept is broadly explained in previous studies carried out for vertebrates and invertebrates “regeneration” (Alibardi, 2022a, 2023a,b,c, 2024a,b, 2025). In conclusion, a variable recovering healing but not true and broad regeneration occurs in terrestrial animals since it *was the evolution of terrestriality that determined which invertebrate or vertebrate can broadly regenerate or not*.

Can medical therapies improve human healing or broad regeneration?

Based on the previous biological premises, what can Regenerative Medicine therapies really expect to achieve in the future? Comparative genomic-transcriptomic-epigenomic studies, after determining successive gene activations between the same developing and regenerating organs in different animals, will eventually reveal the differences generated inside a mature adult body after injury with respect to those of its embryo. Discovering and tracing the evolutionary changes in developmental gene networks that occurred in each evolutionary lineage of animals, regenerative versus non-regenerative, will be a hard challenge for future researches. However, this knowledge will indicate whether safe translational genetic medical applications can be attempted. The cooperation among laboratories working on different regenerative species, and the utilization of super-computers capable to translate into a visual image how gene activation pathways give rise to organ morphogenesis in these species, *a virtual vision*, will make a true progress in this direction. This essential information and these predictions will allow us to envision whether or not broad regeneration can be induced in animals that are unfit to regenerate, such as humans.

It is usually stated in papers dealing with regeneration that “the information gained in the molecular process leading to regeneration in amphibians (axolotl, newt etc.) will improve human regenerative ability”, maybe designing gene therapies for human “regeneration” (Stoick-Cooper *et al.*, 2007; Stocum, 2017; Taghiyar *et al.*, 2018; Hosseinkhani *et al.*, 2023; Huang *et al.*, 2024; Inagaki, 2024). These constantly repeated, hopeful statements should be, at the present time of molecular and genetic knowledge, specified. What are we really expecting from these studies, are they referring to precise ideas or just a hopeful belief that are repeated from over 80 years in the modern biomedical literature? Based on gene information acquired from numerous regenerative organisms, the experimental application of genetic manipulations to non-regenerative animals

or humans might end up in unpredictable and even risky medical applications at the present time of knowledge (Alibardi, 2023a,b).

As previously discussed, we have three options to apply genetic therapies for improving human regeneration: 1) a theoretical, but hope un-feasible project, would be to modify the human genome introducing in the human egg/early embryos genes that in regenerative animals allow regeneration; 2) a feasible project would be implanting genetically transformed cells with inserted genes determinant of regeneration, in damaged or lost organs, such as limbs, heart, kidneys, eyes, spinal cord etc.; 3) another but also un-certain project, would be to deliver through vectors specific mRNAs coding for different signaling proteins to cells of the damaged organs. In the first case, the resulting embryo would *very unlikely be viable*, but even if viable this intervention *would lead to another species*, however not human, a kind of chimera with unpredictable other effects. In the second and third cases, they are presently already conducted in various trials (Taghiyar *et al.*, 2018; Hosseinkhani *et al.*, 2023; Huang *et al.*, 2024; Inagaki, 2024). However, the possible effects of genetically modified cells inserted in a human body or in a damaged organ should be evaluated by long-term studies, monitoring the health of the person receiving the modified cells and whether they have a lasting effect. As regards RNAs administration, the health effects of different vectors utilized (viruses, liposomes, electroporation etc.) should also be carefully tested.

What we learn about the cellular and molecular processes that occur in highly regenerative models of regeneration, with a completely different evolutionary history from species un-capable of regeneration, should be more carefully and realistically evaluated. While general molecular and cellular aspects of regeneration are common among different animals, the biological complexity of invertebrates and vertebrates should be also considered. I believe that the essential molecular and genetic information on highly regenerative animals *cannot be readily* utilized to improve human regeneration abilities. In fact, the information on the molecular complexity of developmental mechanisms that have evolved in different species, regenerating vs non-regenerating, gained in the last 80 years of research, indicates enormous problems generated for the translational application of this knowledge. At the present time, it should be evident that we cannot really expect that the regeneration of a limb, an arm or an inner organ in a terrestrial animal, including humans, will be feasible. As discussed in details for the limb (Alibardi, 2018), inducing the regeneration of a voluminous human limb is not only a problem of cell survival, growth and its control, but is also a problem of limb mass. Furthermore, a continuous medical treatments and time are necessary to grow the lost organ to the previous size since there are biological limits of growth speed (Stranger, 2008; Alibardi, 2018). The regenerating limb should start from a tiny, induced blastema that gradually expands into a voluminous new limb. Furthermore, while the size of an adult human limb usually requires 18-20 years of growth from the newborn, the hypothetical regeneration of the limb to the same size *would however involve years of treatments*, 2.5 to over 5.2 years in the best-discussed hypotheses (Alibardi, 2018).

It is important to realize that inducing limb regeneration in humans by experimental interventions cannot occur like that of an axolotl, newt or an amphibian tadpole that regenerate their much smaller and softer appendages in submerged conditions. Although numerous basic cellular developmental mechanisms are shared between axolotl and humans, what specifically drives regeneration in an axolotl is partially valid for a human limb after amputation. Different animals are not like a puzzle where one takes out some parts (genes or gene modules) and insert them into the puzzle of a different animals, neglecting the interaction that this operation can produce on the remaining parts of the puzzle (Alibardi, 2023a; Fig. 3 C). This is a type of reductionist vision that involves complex genetic interpretations, but ignores simpler zoological explanations. These vague statements should be corrected and replaced with more solid scientific and realistic arguments, considering that animals live in environments after million years of adaptations and that their regeneration is linked and derived from this evolution. During this time, within their changing environmental adaptation, these regenerative species likely evolved not only a new specific anatomy and physiology, different from those of their ancestors, but also new developmental gene pathways. The latter

allow a small or high healing (regenerative) ability, in harmony with their other anatomical-physiological characteristics. *A long and specific evolution also selected regeneration abilities present in some extant animals.*

Cell and molecular biologists should be aware and reflect that only an environment where some developmental genes can be expressed, allows a broad, a limited or no regeneration. Observing the terrestrial species, generally limited or not capable to regenerate (Figs. 1, 4), it is likely that when regeneration was/is non compatible with the new conditions of terrestrial environments, *regeneration became more and more limited and was eventually replaced by scarring*. In fact, the first priority was the immediate survival on land. In simple terms, can an axolotl or a newt limb or tail, which is experimentally maintained in an exposed air environment, regenerate? For sure all the involved genes are there, ready to build a blastema but with no wetting, the blastema would dry out and die, and no regeneration would occur. Therefore, making an axolotl terrestrial would stop regeneration, an interesting experiment that could be made. Maybe also the reversal would be testable, keeping bathed the limb or the tail stump of an amniote under appropriate conditions and treatments may improve healing (Kovalev and Ivanishchuk, 1999). The benefit of a fluid environment on healing is known in cetaceans that can heal efficiently even large and deep skin wounds (Corkeron *et al.*, 1987; Zasloff, 2011; Su *et al.*, 2022).

The fixed idea or paradigm of belief, “if an invertebrate or an axolotl can regenerate why another animal (humans particularly) cannot?” should be replaced with “if an animal *living in watery conditions*, a simple invertebrate or more complex anamniote (axolotl, newt, fish etc.), can regenerate its organs, how could a terrestrial animal improve its poor regenerative capabilities?” Can a land vertebrate really be turned into a condition where broad regeneration is feasible only using genetic and medical engineering? Are we really convinced that this is feasible and is not a naïve and hazy hope?

Personally, I still believe that some healing abilities will be ameliorated for tissues and some organs, but for large organs or appendages I presently sustain that only the combination with biocompatible, laboratory bioreactors generated bio-prostheses will be feasible (Mao and Mooney, 2015; Stocum, 2017; Taghiyar *et al.*, 2018; Hosseinkhani *et al.*, 2023; Huang *et al.*, 2024). This means that future humans with replaced (not really regenerated) injured organs, arms, legs, inner organs, will be, by definition “*bionic people but still humans*”, with perfectly functioning organs that were lost by trauma or disease during their lives and that have been replaced by bio-engineering procedures. *What can we hope more than that in 2026?*

Acknowledgments: study self-supported. Part of the content of the present paper was presented during the SCRM-2024 (Stem Cell and Regenerative Medicine), held in Berlin on 7 and 8 October 2024. Mr. Dario Alibardi helped in making Fig. 5.

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Figures

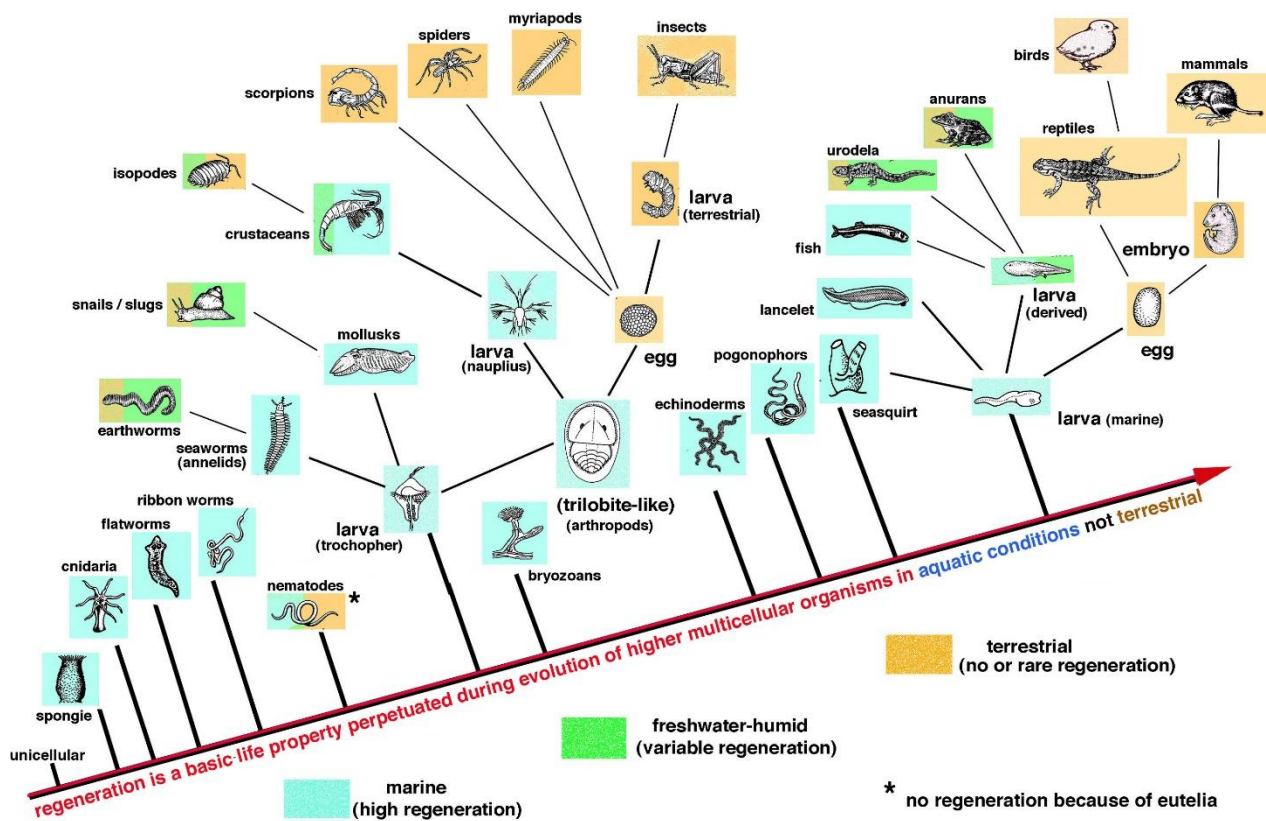


Fig. 1. Schematic and simplified Cladogram (modified from Alibardi, 2023a,b) showing the main animal groups (Phyla), featuring differences in regeneration abilities. This ability is high in marine invertebrates, fish and some amphibians (light blue background), high to low in amphibious invertebrates and vertebrates living in aquatic and in terrestrial environment (green and brown background), and little to none in some nematodes, most arthropods and vertebrate amniotes living on land (brown background). Nematodes evolved eutelia (bodies made from fix number of cells) and cannot regenerate. Terrestrial animals eliminated larvae and metamorphosis (indirect development) for the terrestrial egg (direct development) during land colonization. Insects evolved terrestrial-adapted larvae that metamorphose inside enveloping membranes from localized "imaginal disks". During molting, arthropod appendages *regengrow* (regeneration during growth and molting), and after molting they stop healing and, often, the adults rapidly die. Erasing larval stages and metamorphosis determined the loss or changes of genes involved in metamorphosis and regeneration in aquatic and amphibious animals that was replaced by a limited healing ability, *regengrow*, but mainly scarring in terrestrial animals. See explanation in the text.

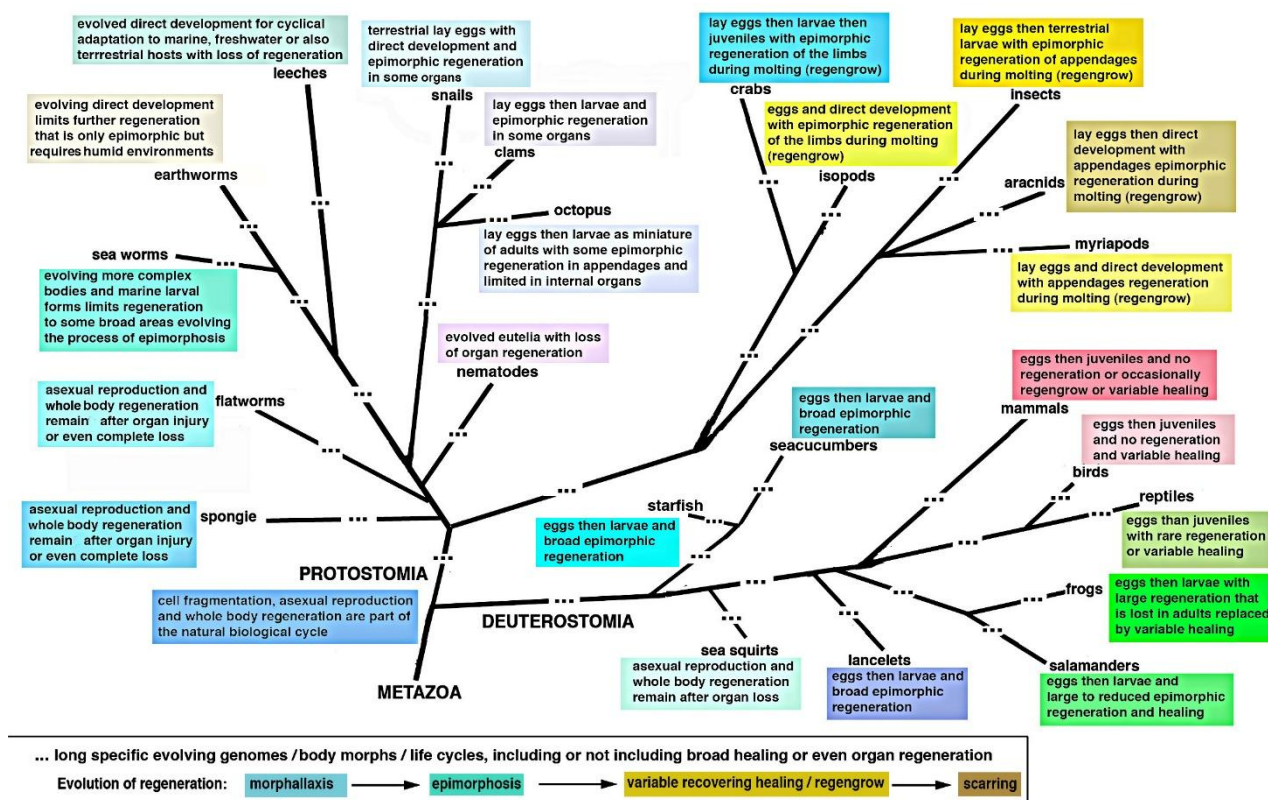


Fig. 2. Schematic evolutionary tree showing the distribution of the main groups of invertebrates and vertebrates that have followed specific pathways of evolution (indicated by dots, ...) into present-day, extant animals. During their specific evolution, each type of animal has adapted the previous genome, development, anatomy, physiology and biological cycle, to its changing environment and the prize was survival and later fitness in the new environment. Both primitive protostomes (spongie, flatworms) and deuterostomes (echinoderms and seasquirts) might undergo whole body regeneration (morphallaxis). With increasing biological complexity, regeneration became/becomes more localized as epimorphosis in some marine or humid dweller animals (seaworms, earthworms). In other invertebrates (snails, clams, octopods) epimorphosis only occurs in a limited number of appendages or organs. No regeneration is present in leeches and nematodes. The drawing shows that the pathways undertaken for optimal terrestrial adaptation lead to a lack of broad regenerative capability (arthropods and amniotes). Crustaceans regenerate only appendages during molting (regengrow). Broad regeneration only remains in extant marine species.

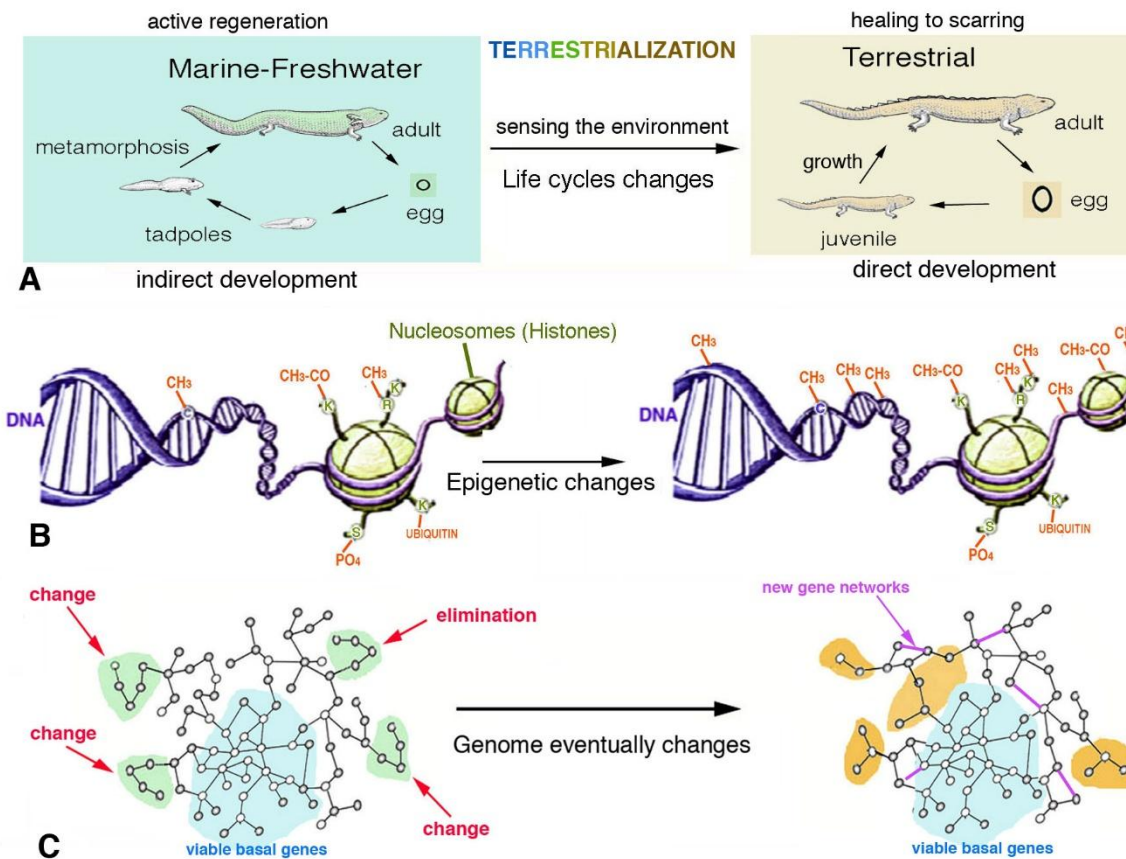


Fig. 3. General hypothetical changes first in the epigenome and later in the genome during mainland conquest by marine vertebrates (original figure composition, and also modified images from Alibardi, 2019, 2023a,b). During terrestrialization, sensing the changing environment might have epigenetically modified genomes for adaptation during long geological periods. **(A)** Change in the life cycle with loss of the larval stage and metamorphosis in land vertebrates. Sensing the changing environment, the soft larval stage was replaced by direct development with genome changes, un-compatible with broad regeneration. **(B)** Methylation, acetylation and other modifications of the DNA and/or histones of aquatic animals determined a different gene expression that was passed on to new generations of offspring during evolution. **(C)** Eventually genomes changed in transitional and land species through gene loss (DNA elimination?), deactivation (pseudogenization/bases change?), or by forming new gene networks in land vertebrates, including those orchestrating development and healing capabilities. The latter, complex development programs might have also influenced genes involved in regeneration, lowering or erasing the latter process (see text and cited literature for more details). The light blue and central area of the schematic genome representation includes essential cellular vital genes that remain largely unchanged. The peripheral, smaller areas colored in light green only indicate regions of the genome that could change without compromising/threatening life during long evolutionary periods.

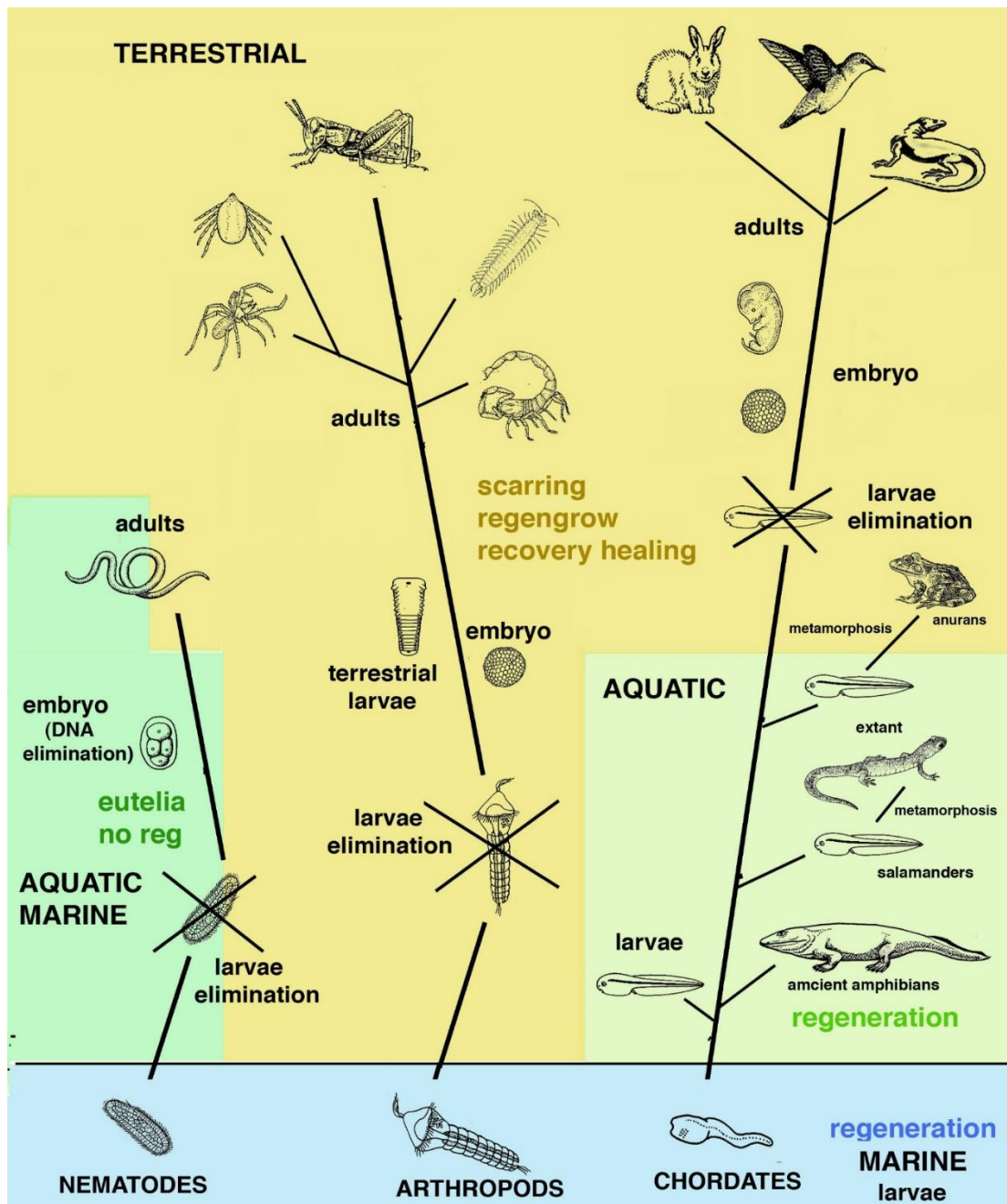


Fig. 4. Simplified drawing (modified from Alibardi, 2024c) illustrating the three main groups of animals un-capable of organ regeneration: nematodes, arthropods (insects, arachnids, myriapods) and amniotes (reptiles, birds and mammals). Nematodes, derived from marine larvae (planulae-like) evolved eutelia (left) in both aquatic (green) and terrestrial (yellow) environments. Arthropods originated from trochophore-like larvae that cannot survive on land; they evolved direct development and terrestrial-adapted larvae that undergo molting by the expansion of inner small stem cell niche pouches indicated as imaginal disks. Amniotes also lost the larvae and metamorphosis that was/is present in ancient and extant amphibians. In the latter, some urodeles are still able of a broad regeneration during their entire life, but not in anurans after metamorphosis that take a prevalent land adaptation. The loss of a larval stage and metamorphic transformations from larvae into adults occur-ed/-es by genomic modifications that also involve the ability to regenerate, while a rapid process of scarring ensued in both anurans and amniotes (reptiles, birds and mammals).

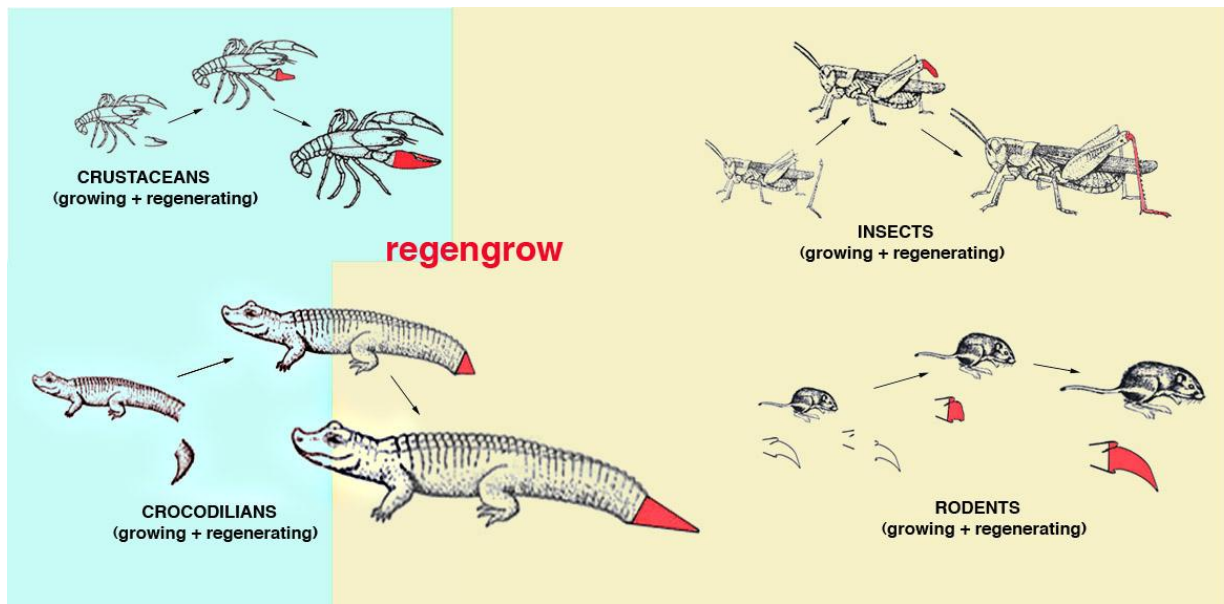


Fig. 5. Four examples of *regengrow* (red color) in water (light blue background) or land (light brown background) (modified from Alibardi, 2023b). A lost claw in a crustacean (a lobster or a shrimp) undergoes to regengrow as also the body grows. A lost insect leg undergoes to regengrow during molting and somatic growth. A crocodilian tail after loss of the tip undergoes a regengrow process during body growth. After loss of the apical part of the digit (associated to the stem cell niche of the nail bed), the rodent claw/digit undergoes to regengrow forming tiny claws during somatic growth or continuous nailbed renewal in rodents (see text for more explanation).