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The influence of fundamental traits on mechanisms controlling appendage regeneration

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ABSTRACT

One of the most compelling questions in evolutionary biology is why some animals can regenerate injured structures while others cannot. Appendage regeneration appears to be common when viewed across the metazoan phylogeny, yet this ability has been lost in many taxa to varying degrees. Within species, the capacity for regeneration also can vary ontogenetically among individuals. Here we argue that appendage regeneration along the secondary body axis may be constrained by fundamental traits such as body size, aging, life stage, and growth pattern. Studies of the molecular mechanisms affecting regeneration have been conducted primarily with small organisms at early life stages. Such investigations disregard the dramatic shifts in morphology and physiology that organisms undergo as they age, grow, and mature. To help explain interspecific and intraspecific constraints on regeneration, we link particular fundamental traits to specific molecular mechanisms that control regeneration. We present a new synthesis for how these fundamental traits may affect the molecular mechanisms of regeneration at the tissue, cellular, and genomic levels of biological organization. Future studies that explore regeneration in organisms across a broad phylogenetic scale, and within an ontogenetic framework, will help elucidate the proximate mechanisms that modulate regeneration and may reveal new biomedical applications for use in regenerative medicine.

Key words: regeneration, appendage, growth, development, metamorphosis, fundamental trait, phylogenetic constraint, regenerative medicine, body size.

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I. INTRODUCTION

(1) General introduction

The ability of organisms to regenerate tissues and structures is one of the most captivating phenomena in biology. Regeneration is a ubiquitous feature of metazoans (Brockes & Kumar, 2008), although there is substantial variation across taxa from the complete regeneration of an entire organism (e.g. hydra, cnidarians, planarians) to a restricted regeneration of certain tissues in birds and mammals. A major goal of regeneration research is to understand if the same molecular mechanisms control regeneration in distantly related taxa, or if the capacity to regenerate damaged tissue has evolved multiple times and with different control mechanisms. Additionally, there is substantial variation in regeneration among individuals within regenerating species. For example, older zebrafish (*Danio rerio*) regenerate their fins more poorly compared to young individuals (Anchelin *et al.*, 2011). What are the changes that occur throughout the lifetime of an individual to constrain regeneration? The possible discovery of conserved molecular mechanisms that control regeneration would have a profound impact on regenerative medicine. Is it possible to activate such mechanisms and reawaken latent regenerative capacity in older individuals or in mammals? Contemporary advances in molecular biology have begun to uncover the cellular, molecular, and genetic mechanisms governing regeneration, however, our understanding of what explains variation in regenerative ability within and across taxa is lacking.

One possible explanation for our limited understanding of why regenerative capacity fluctuates across taxa is the bias of regeneration research towards experiments conducted on small-sized model organisms when they are young in age, and early in life stage. This approach ignores considerable shifts in growth, development, and physiology that animals experience in their lifetime, and thus masks the influence of numerous fundamental traits on regenerative capacity. Therefore, we need to consider both the ability to regenerate across species, and alterations in regenerative capacity throughout an individual's lifetime (within species). Fundamental traits are organismal properties (e.g. life-history traits, developmental traits, physiological traits) that are inherent to individuals and species; and that influence their ecology, behaviour, and physiology. As such, fundamental traits have the potential to constrain regenerative ability or the rate of regeneration. Identifying constraints on regenerative ability that operate both within and across species will help elucidate if regeneration is inherently linked to development and growth. Exploring relationships between fundamental traits and regeneration at the mechanistic level can serve as a platform for investigating why some animals have reduced powers of regeneration.

Herein we address how a suite of fundamental traits can influence regeneration. These traits can influence the ability to regenerate, the rate of regeneration, or the quality of the regenerated appendage (i.e. heteromorphy) both within and across species. Specifically, we focus on reparative regeneration of appendages along the secondary body axis, and the following fundamental traits: (1) body size, (2) aging, (3) life stage (pre- or post-metamorphosis; larva, juvenile or adult), and (4) mode of post-embryonic growth (determinate *versus* indeterminate). We first review the literature and present published viewpoints regarding how fundamental traits affect regeneration. We then present hypotheses about how specific fundamental traits can influence the mechanisms of regeneration at the tissue, cellular, and genomic levels of biological organization. Along with these hypotheses we suggest multiple avenues for future research. In order to maximize readability, we avoid reiterating molecular mechanisms for each trait by organizing this review according to biological level, and discussing fundamental traits within each section. Our goal is to spark an interest in the underexplored relationships between fundamental traits and mechanisms of regeneration towards a better understanding of how and why regeneration is curtailed in more derived vertebrates, including humans.

(2) Types of regeneration

Regeneration is traditionally viewed in two contexts; physiological and reparative (Table 1) (Morgan, 1901). Physiological regeneration is the regular and repeated regeneration of a particular structure that is normally replaced throughout the life of an organism and examples exist in every metazoan species (Table 1). By contrast, reparative regeneration is induced by injury, and leads to replacement of the missing structure. Although this definition distinguishes repair from replacement, some traditional examples of reparative regeneration blur the lines between them because restoration of the missing part occurs with structural alterations and with

Table 1. Examples of regular and recurring regeneration (physiological) and regeneration stimulated *via* injury or damage (reparative)

Type of regeneration	Examples
Physiological	Cervid antlers, replacement of blood, epidermis, endometrium, gut lining, arthropod exoskeleton (moulting)
Reparative	Incomplete: fish barbels, lizard tails, larval urodele tails, young mammalian digit tips
	Complete: urodele limbs, adult urodele tails, some fish fins, mollusc eye stalks, arthropod and crustacean limbs, antennae

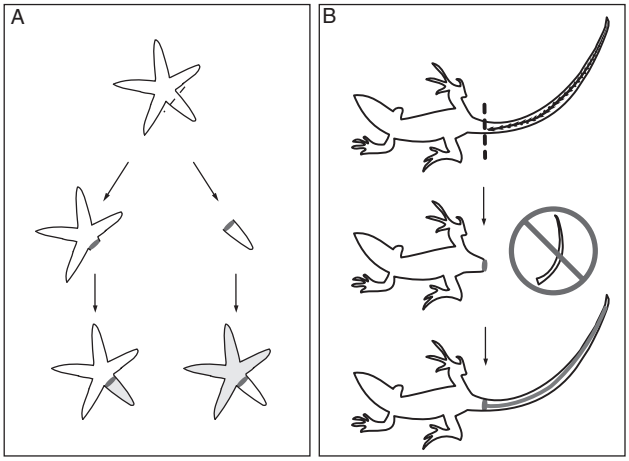

Fig. 1. Distinguishing between modes of regeneration. (A) After a seastar loses an arm, both the body (missing the arm) and the arm (apart from the body) can regenerate the missing part. (B) A lizard, following autotomy or injury, can regenerate a new tail, but the tail has no capacity to regenerate more of itself, let alone another lizard.

reductions in function. Thus, reparative regeneration can be complete or incomplete (Table 1). Examples of incomplete regeneration include tail regeneration in lizards (which do not regenerate neurons or vertebrae) (Simpson, 1964), and barbel regeneration in fish (which do not regenerate the structural mesodermal core) (LeClair & Topczewski, 2010). Interestingly, when the larval urodele tail is amputated the notochord fails to regenerate, and is replaced by cartilaginous vertebrae that will not develop until months later in the rest of the tail (Goss, 1969). In this case of regeneration the replacement is not perfect, but rather is a precursor of what will develop around the structure in the future. This fine distinction is important as it may reveal early compromises on regenerative ability in some lineages where regeneration and repair occur in tandem. Here we define regeneration as the complete replacement of the original form.

We also note a fundamental distinction between the ability to generate an entirely new organism from a severed piece (e.g. *Hydra*) and regeneration of a limb in arthropods or salamanders (Fig. 1). Whereby, if you cut a planarian in two, or sever the arm of a starfish, each piece has the ability to form a complete, autonomous animal (Fig. 1A). By contrast, the severed tail of a lizard or the limb of a cockroach cannot give rise to a new individual, and this piece fails to survive apart from the animal (Fig. 1B). Mechanistically, the ability to replace a missing structure in asexually reproducing animals stems from a continuous source of pluripotent cells with limitless proliferative potential and with the ability to form every cell type in the new animal (Wagner, Wang & Reddien, 2011). By contrast, cells involved in vertebrate, crustacean, or arthropod regeneration cannot form every part of an animal *in vivo*, and are limited in their developmental potency (Kragl *et al.*, 2009; Truby, 1983). Herein, we focus on appendage regeneration along the secondary body axis.

(3) Appendage regeneration

The early events of appendage regeneration can occur from the production of new tissue through cell proliferation (epimorphosis), or through the rearrangement of existing cells to form the new part in the initial absence of proliferation (morphallaxis) (Morgan, 1901). More recently this distinction has been argued to be purely historical, and that all instances of regeneration share the feature of re-specification of positional information at the injury site followed by intercalation and re-growth of the regenerating tissue (Agata, Saito & Nakajima, 2007). The important distinction is that in all examples of appendage regeneration along the secondary body axis cell proliferation is required to supply material for the initial regenerating appendage, and this process couples growth of the appendage with regeneration. For this reason, epimorphosis mechanistically unites leg or antennae regeneration in hemimetabolous insects, eye-stalk regeneration in snails, and limb, fin, and tail regeneration in vertebrates.

Following extirpation, epimorphic appendage regeneration proceeds through a series of similar events (Fig. 2) (Bryant, Endo & Gardiner, 2002). First, epithelial cells migrate over the wound surface (under in the case where a scab is formed) to re-epithelialize the injury site. Next, cells at the amputation plane re-enter the cell cycle and dedifferentiate. Local cell populations comprised of these newly dedifferentiated cells, progenitor cells, and fibroblasts form a regeneration blastema. The blastema proliferates, and as in embryonic development, these cells acquire spatial patterning information as they expand, ultimately re-differentiating to restore the injured structure through intercalation (French, Bryant & Bryant, 1976). An extended growth period follows regeneration once patterning is complete. Full replacement of the appendage can take years in some large terrestrial salamanders (Young, Bailey & Dalley, 1983*b*), and is restricted by moulting times in arthropods such that a small limb emerges and grows after each successive moult (Maruzzo *et al.*, 2005). That epimorphic regeneration of appendages appears highly conserved is fascinating given the great phylogenetic distance among these species, although it remains unclear if the molecular mechanisms underlying these processes are homologous across taxa.

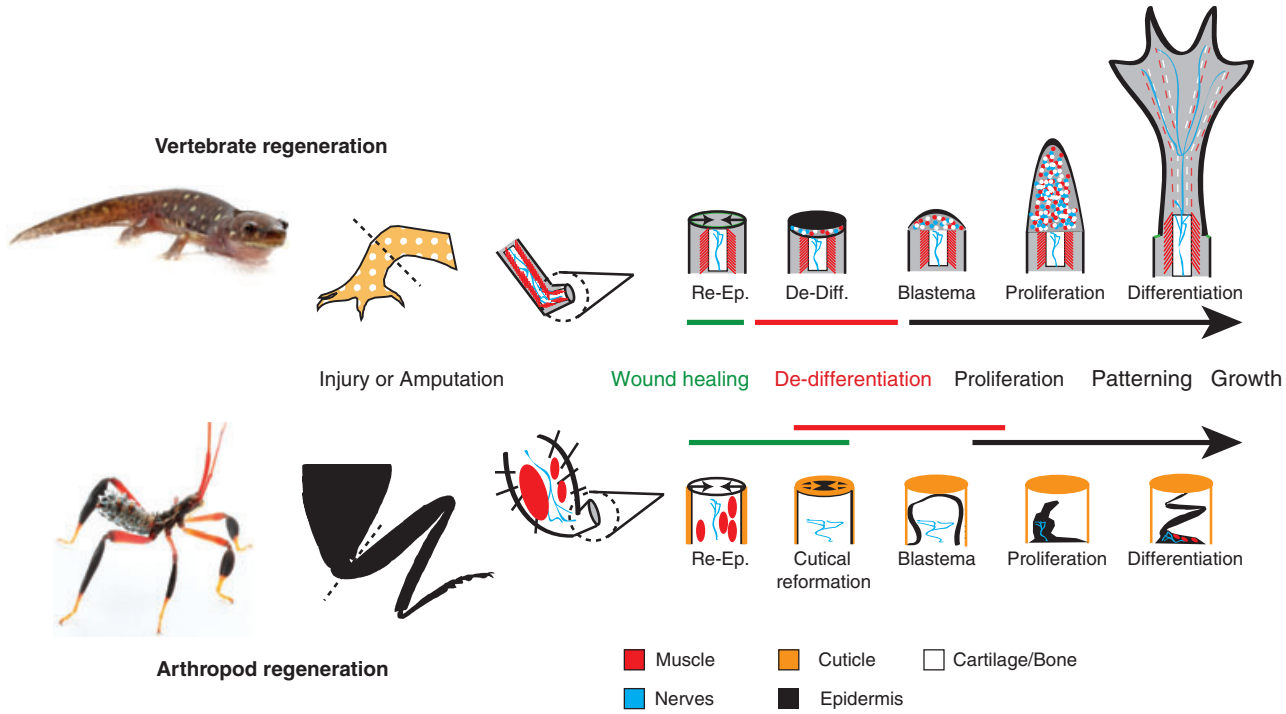

Fig. 2. Epimorphic regeneration in vertebrates and arthropods. Following injury or amputation, appendage regeneration in these taxa proceeds through a generally similar set of stages. First, re-epithelialization (Re-Ep.) occurs as the edges of the epidermis migrate to cover the wound surface. In taxa with an exoskeleton, the cuticle reforms after the epidermis has finished covering the wound. Cell cycle re-entry and de-differentiation (De-Diff.) of local cell populations leads to the formation of a regeneration blastema. Blastemal cells proliferate and acquire patterning information as they re-differentiate to replace the missing structure.

(4) Fundamental traits and regeneration: patterns and assumptions

Fundamental traits are key components of the ecology, behaviour, physiology, and life-history strategy of an organism. The critical role of fundamental traits in modifying form and function begs the question whether or not they may also influence mechanisms that control appendage regeneration. Surprisingly, the effects of such fundamental traits have been largely ignored in rigorous experimental studies, and this oversight stems partly from the difficulty in separating traits that are correlated with one another (Goss, 1969; Pritchett & Dent, 1972; Scott, 1909; Speakman, 2005; Wagner & Misof, 1992; Young *et al.*, 1983*b*).

Nonetheless, some studies have examined the influence of body size in vertebrates (but not arthropods), which has led to the perception that rate of regeneration and replacement time of a structure are negatively affected by size (Pritchett & Dent, 1972). Unfortunately, only one study has addressed body size alone (controlling for age): Pritchett & Dent (1972) found that in newts, the larger hindlimbs took longer to regenerate than did the smaller forelimbs in the same individuals. From this they inferred a negative relationship between the size of adult newts and the rate of limb regeneration (Pritchett & Dent, 1972). In practice, the study was limited by the size variation in limbs, inherent differences in the structure of hindlimbs and forelimbs, and that the patterning phase and the growth phase were analyzed together. Analyzing regeneration and growth together confounds how the size of a structure might directly influence the development and patterning phases of regeneration (Fig. 2). For instance, studies in salamanders and fish have shown that body size negatively correlates with growth of the regenerate following morphogenesis, although not necessarily for development of the regenerating anlagen (Fig. 2) (Scott, 1909; Tank, Carlson & Connelly, 1976; Young *et al.*, 1983*b*). Thus, the influence of size on regeneration remains unclear, and we can conclude only that larger organisms take longer to re-grow an appendage following regeneration of a small replacement.

Aging and life stage also strongly influence organismal performance (Lakatta, 1983; Marden *et al.*, 2003; Pough & Kamel, 1984). Among vertebrates, the general assumption is that regeneration will take longer at older ages (Wagner & Misof, 1992; Wallace, 1981). Although regeneration has been documented to occur in a few mature salamanders and fish, little information actually exists on regenerative ability or rate in aged animals. Underscoring the importance of conducting regeneration experiments in older animals, a recent study found no difference in the rate or quality of regenerating lenses in very old newts between 16 and 30 years of age (Eguchi *et al.*, 2011). Still, at a mechanistic level, there are many reasons why aging may affect regeneration. When decoupled from body size, aging becomes relevant to cell differentiation, cell cycle re-entry (de-differentiation), metabolic stress, and the capacity for cell proliferation.

Similarly, metamorphosis represents a major transition in life where structure, function, and physiology change dramatically. Life-stage-limited regeneration has been demonstrated in anurans (Dent, 1962) and holometabolous insects (see references in Maginnis, 2006; Maruzzo *et al.*, 2005) where the capacity to regenerate is lost after metamorphosis. Regenerative capacity in hemimetabolous insects, crustaceans, and some chelicerates is dependent on remaining moulting periods (see references in Goss, 1969; Maginnis, 2006; Maruzzo *et al.*, 2005); whether or not these organisms can regenerate an appendage beneath the cuticle following their final instar remains unknown. Assuming that the cellular and genetic structure of these animals does not change at metamorphosis (or moult), then why there is a change in their regenerative ability is puzzling, and warrants further inquiry in a variety of pre- and post-metamorphic animals.

While the relationship between regeneration and growth has long been appreciated (Goss, 1965; Voit, Anton & Blecker, 1995), the interaction between regeneration and mode of growth (determinate *versus* indeterminate) remains poorly studied. Some evidence suggests that species which continue to grow after reaching sexual maturity (indeterminate growth) may be more likely to retain the capacity to regenerate throughout their life (Kara, 1994; Klapper *et al.*, 1998), whereas this capacity may be limited to juvenile growth periods in species that attain maximum size at maturity (determinate growth). In newts and other amphibians, the yearly addition of skeletal bone (measured as growth rings) is a key factor in attributing indeterminate growth to these animals (Homan, Reed & Windmiller, 2003; Jakob *et al.*, 2002). Despite continued skeletal addition, overall growth rates for many species of indeterminate growers often decrease with age, metamorphosis, or sexual maturity, and this may greatly reduce regenerative capacity. For example, some effects of aging that may inhibit regeneration have been observed in mammalian cells, but appear to be absent from fishes and amphibians with indeterminate growth. Interestingly, arthropods that do not exhibit post-larval cell division (e.g. mites) are incapable of regeneration because they are incapable of further growth (Rockett & Woodring, 1972). While regeneration in determinate growers remains to be tested at stages where growth plateaus, the current evidence supports a relationship between growth potential and the capacity for regeneration.

After considering previous attempts to explain how fundamental traits can affect appendage regeneration, we are left with conflicting results, and with no clear set of hypotheses for how these traits may affect the specific mechanisms that control regeneration. Below we discuss how fundamental traits can modulate mechanisms of regeneration at three levels of biological organization: tissue, cellular, and genomic. Some traits may have diverse effects at multiple levels, while others (e.g. size) may have effects at only one level (Fig. 3). Because of the complex interaction between traits and mechanisms, we present hypotheses for each level of organization and reference traits where applicable.

II. TISSUE-LEVEL EFFECTS

An appendage is comprised of multiple tissues that can include combinations of nerves, muscle, cartilage, and bone surrounded by epidermis. At the tissue level, fundamental traits of an organism may limit regeneration through effects on these tissue components alone or in combination (Fig. 3). Appendage size, which is correlated with body size, dictates the area of the injury plane. In large-bodied animals, the area of this surface may delay re-epithelialization to such a degree that it prevents the formation of a blastema. Also, injury above a critical size could lead to lethal loss of vital fluids (e.g. blood, haemolymph, water). The inability to recover from such a large injury would negate the need for regenerating

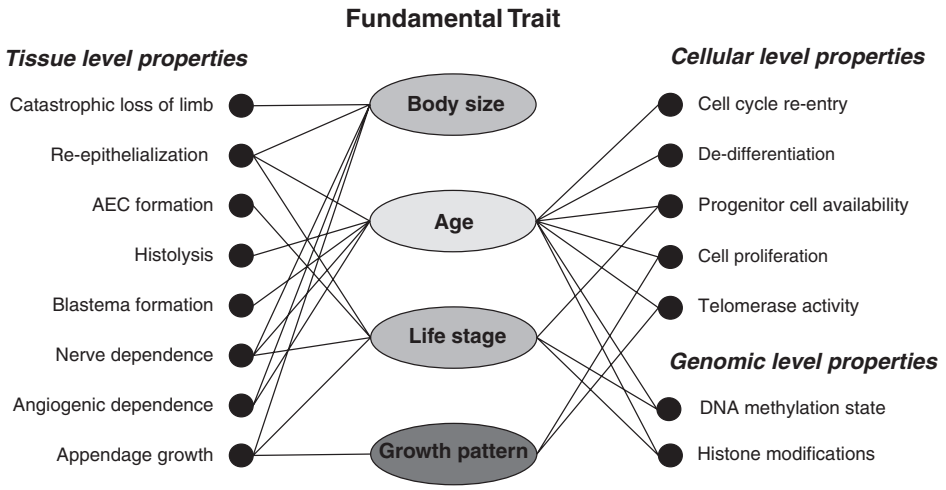

Fig. 3. Fundamental traits can exert their effect on regenerative processes at multiple levels of biological organization. Biological processes are listed for the level of biological organization at which they can exert their effect on regenerative capacity. Lines connecting a fundamental trait to a biological process indicate a hypothesized interaction that can affect regeneration. These interactions are discussed in the text. AEC, apical epithelial cap.

the appendage, and would provide a natural limit for the injury size an animal could sustain. Provided the injury is not life threatening, how does the size of the appendage, or its developmental stage impact particular requirements to mount a successful regenerative response?

(1) Wound healing and re-epithelialization

In order to stabilize an injury, animals need to close the wound surface to protect against infection, desiccation (terrestrial animals), or osmotic imbalance (aquatic animals). Both initiation and the rate of re-epithelialization can regulate the regenerative response, and may be dependent on size, aging, or life stage. All animals capable of appendage regeneration completely re-epithelialize the wound surface. A survey of limb regeneration among amphibians showed that species exhibiting some form of regenerative outgrowth have rapid epithelial migration, whereas species with delayed migration exhibit no regeneration (Scadding, 1981). Moreover, the largest individuals in this survey showed a reduced regenerative capacity (i.e. slower rates, incomplete patterning, incomplete regeneration); but variation in size among species, and variation in age within species were not rigorously controlled for. This suggests that size may have increased the time needed to re-epithelialize the wound, which in turn inhibited a successful regenerative response. Delayed re-epithelialization contributes to scarring and can lead to chronic, non-healing wounds (Sivamani, Garcia & Isseroff, 2007). By contrast, rapid re-epithelialization occurs during scar-free healing in fetal mammals and an increased rate of re-epithelialization can reduce scarring (Whitby *et al.*, 1991).

Among arthropods, re-epithelialization follows haemolymph coagulation which initially protects the amputation plane by forming a scab (similar to mammals) (Adidoyi, 1972; Rockett & Woodring, 1972). The rate of re-epithelialization in arthropods is much slower compared to amphibians, but has not been examined across an age or size gradient; and whether or not re-epithelialization occurs in non-moulting adults following amputation is not known.

The neo-epidermis also plays an important role in directing regeneration. Following re-epithelialization, the new epidermal layer becomes a specialized signaling centre called the apical epithelial cap (AEC). The AEC directs growth of the underlying tissue and is necessary for successful appendage regeneration (Wallace, 1981). Formation of this structure appears to depend partially on the life stage when the amputation occurs. The inability to regenerate a tail during frog metamorphosis is correlated with a lack of AEC formation, and post-metamorphic anuran species that show heteromorphic regeneration do not form an AEC as thick as that seen in urodeles capable of complete limb regeneration (Wolfe, Nye & Cameron, 2000).

The loss of limb regeneration ability in anurans also may be due to a loss of competency in the mesenchyme as opposed to the epidermis. Yokoyama *et al.* (2000) created recombinant limbs with epidermis collected from regeneration-incompetent limbs and mesenchyme from regeneration-competent limbs (and *vice-versa*). They showed that only the regeneration-competent mesenchyme can rescue a regeneration-incompetent epidermis (i.e. regeneration-competent epidermis did not rescue regeneration-incompetent mesenchyme). Thus, some controversy remains about whether changes in the epidermis are the cause of regenerative loss. However, complete formation of the AEC is required for complete limb regeneration in salamanders; and available evidence shows a correlation between life stage and AEC formation.

Life stage may affect the regenerative response due to alterations in tissue physiology of the epidermis. During regeneration, changes in ion flux across the wound bed cause a shift in electric potential that correlates with a regeneration-permissive environment (Borgens, Vanable & Jaffe, 1977). *Xenopus laevis* tadpoles are normally capable of tail regeneration but undergo a short refractory period (stages 45-47) preceding metamorphosis where they become regeneration incompetent (Beck, Christen & Slack, 2003). Regeneration prior to this phase is partly due to the movement of protons (*via* an ion pump) across the new epithelium and to the subsequent rapid physiological restoration of a polarized plasma membrane that covers the wound site (Adams, Masi & Levin, 2007). An inability to rapidly re-polarize during the refractory period leads to loss of regeneration while mis-expression of a heterologous cell membrane H^+ pump in refractory epidermis can rescue tail regeneration in tadpoles (Adams *et al.*, 2007). Though previous research has been conducted only on tail regeneration, appendage regeneration following metamorphosis may be constrained in a similar manner. The involvement of tissue-specific changes that alter ion movements underscores the role that life stage may play in the control of regeneration through changes in tissue physiology.

(2) Histolysis and blastema formation

Following re-epithelialization, histolysis of underlying stump tissue takes place to break down injured tissue and the surrounding extracellular matrix (ECM). This, in turn, frees up cells to migrate and form a blastema (Fig. 2). The process of histolysis and blastema formation may be influenced by structural changes that occur to the ECM during aging. Collagen is the major component of the ECM in skin, and must be degraded for cell migration to proceed. As vertebrates age, cross-linking of collagen increases tensile strength and decreases solubility of the ECM (Baum, Faris & Franzblau, 1975; Kara, 1994). Thus, aged animals likely have a more difficult time breaking down the ECM, which would consequently delay or inhibit blastema formation. In addition to the ECM, bone and cartilage at the amputation plane must undergo histolysis prior to replacement during regeneration (Wallace, 1981). While the ratio of bone to cartilage increases with age, the effects of these ratios on regeneration rates are not known. Further testing of these observations is necessary to understand if adult appendages with mineralized skeletons exhibit reduced regenerative capacity.

(3) Nerve dependence

One of the most extensively studied phenomena in regeneration is the dependence on an intact nerve supply as observed in hydra, echinoderms, planarians, annelids (reviewed in Brockes & Kumar, 2008), teleost fins (Geraudie & Singer, 1985), *Xenopus laevis* froglet limbs (Suzuki *et al.*, 2005), urodele limbs (Singer, 1952), lizard tails (Simpson, 1970), and fetal wound healing in chickens and mammals (Harsum, Clarke & Martin, 2001; Stelnicki *et al.*, 2000). Nerves play a supportive (rather than instructive) role by secreting nerve-derived factors that facilitate appendage regeneration (Carlson, 2007). Body size, aging, and life stage each may alter the nerve-limb relationship and lead to the loss of regenerative capacity.

In vertebrates, the number of nerve fibres or the cross-sectional area of nerve bundles (expressed as a fraction of the cross-sectional area of the limb) is often negatively correlated with increasing body size (Peadon & Singer, 1965; Wallace, 1981). In a large animal, an insufficient concentration of nerves may constrain regeneration in a large appendage that would normally be able to regenerate if it was smaller. The hypothesis that nerve concentration constrains regenerative capacity was championed by Marcus Singer, based primarily on experiments showing that newt limbs must have a minimum number of nerve fibres in order to regenerate (Singer, 1952). Also, there may be an interspecific relationship between regeneration rate and degree of innervation. However, the evidence for this is equivocal: nerve abundance appears to be positively correlated with the rate of regeneration across *Ambystoma* species (Young *et al.*, 1983*b*), but other studies have shown that regenerative capacity among species does not correlate with innervation (Kurabuchi, 1990; Scadding, 1982; Van Stone, 1964) or with regeneration rate within the same species (Scadding, 1983). Furthermore, the Mexican axolotl has a relatively fast rate of regeneration, despite having one of the lowest nerve densities among amphibians (Scadding, 1982). More experiments that compare innervation parameters across larger interspecific ranges in appendage size are necessary to better resolve the connection between nerve fibre abundance and regenerative ability or rate.

In addition to body size, aging and metamorphosis may constrain regenerative capacity by inhibiting the ability of a nerve to produce the vital nerve-derived factor(s). Compared to old individuals, spinal cord extracts from young axolotls are more mitogenic than extracts from old axolotls when added to blastemal cells (*in vitro*) from a regenerating limb (Boilly & Albert, 1988). This suggests that there is some decrease in the neurotrophic influence of spinal cords across an age gradient in salamanders. Nerves may also lose the ability to re-grow over ontological time, making them unable to provide support for regeneration. The ability of mammals to regenerate peripheral nerves decreases with age, and all vertebrates regenerate nerves better when they are younger (Tanaka & Ferretti, 2009; Verdu *et al.*, 2000). Additionally, some forms of axon re-growth are lost at metamorphosis (Gibbs, Chittur & Szaro, 2010). For example, *X. laevis* axons can regenerate across a spinal cord lesion when metamorphosis is pharmacologically inhibited, but this ability is lost when metamorphosis is precociously forced. Thus, during aging and after metamorphosis, there is a clear decrease in the regenerative capacity of the nervous system and this likely contributes to the reduced ability of an animal to support peripheral tissue regeneration.

An exception to the nerve-dependency phenomenon is that larval salamander limbs can regenerate without innervation if the limb was never innervated during development (termed aneurogenic) (Yntema, 1959). During development, limb buds are formed before nerve fibres enter the limb, meaning that early limb development is nerve independent. Limb buds and surgically created aneurogenic limbs may produce their own neurotrophic-like factors (Filoni *et al.*, 1999), or they may not need the neurotrophic factor for growth (Tassava & Olsen-Winner, 2003). Regardless of the mechanism, innervation of developing or aneurogenic limbs leads to nerve dependency (Thornton & Thornton, 1970). Surgically creating aneurogenic limbs may simply extend the time in which the limb can produce its own neurotrophic-like factors, or the time in which the limb does not need the neurotrophic-like factors for growth. Recent studies have shown that denervated limbs can regenerate (albeit without muscle) if a single molecule, anterior gradient 2, is introduced into the denervated limb blastema (Kumar *et al.*, 2007). An explanation for why aneurogenic limbs can regenerate may be provided through functional studies during limb development and in aneurogenic limbs. The key point is that once limbs are innervated for some time, they become nerve dependent for the life of the animal, and thus may be affected by fundamental traits later in life.

The role of the nervous system during arthropod regeneration has received little attention, partly due to difficulties associated with denervating arthropod appendages. Some studies have established that muscle regeneration is dependent upon innervation after moulting (Consoulas & Levine, 1997), while others suggest that innervation plays only a partial role in the support of appendage regeneration (Nuesch, 1968). Although the available evidence supports a role for nerves, the conflicting results and experimental hurdles from various arthropod studies make it difficult to test hypotheses about how changes in the nervous system across ontogeny may influence regeneration (Goss, 1969; Nuesch, 1968).

(4) Angiogenesis

Similar to nerve requirements, vascular support is also a likely requisite for proper appendage regeneration. The formation of new blood vessels (angiogenesis) is necessary for organ development and tissue homeostasis in adulthood. Upon injury, hypoxic conditions produced by the local destruction of tissue are thought to induce the formation of angiogenic factors including vascular endothelial growth factor (VEGF) (Ferrara, 2002). During fin regeneration in zebrafish, blocking angiogenesis by inhibiting VEGF signaling does not affect blastema formation or early tissue outgrowth (~800 μm), but does block further growth of the regenerate (Bayliss *et al.*, 2006). This suggests that while vascularization does not play

a role in blastema formation or early cell proliferation, it is necessary for growth and morphogenesis of the regenerating appendage (Fig. 2). The age and size of a regenerating structure likely affects angiogenesis in a similar manner to nerve regeneration, because growth of the two tissues is intimately connected. Angiogenic potential decreases with age in mammals (that do not regenerate), and affects the injury response (Edelberg & Reed, 2003), but whether animals with regenerative abilities also have decreased angiogenesis with increased age is unknown.

(5) Growth and differentiation

The previous sections discussed influences on the ability of an organism to mount a regenerative response (i.e. prior to differentiation of the regenerate; Fig. 2). Alternatively, fundamental traits may affect the growth phase of regeneration. Intriguingly, the rate of regeneration prior to differentiation may be relatively constant across body size, and only vary as the newly regenerated appendage grows (which would be a function of growth rate). Observations from regenerating limbs of large and old *Ambystoma mexicanum* demonstrate that the digit-stage regenerate is actually a small limb in the centre of the amputation plane, surrounded by a collar of tissue (unpublished data). In contrast to previous research (Scadding, 1977), a study on large adult *Ambystoma annulatum*, *A. maculatum*, *A. texanum*, and *A. tigranum* found they were capable of regenerating their limbs, although in some cases complete replacement took nearly two years (Young, Bailey & Dalley, 1983a; Young *et al.*, 1983b). In hemimetabolous insects and crustaceans, regeneration is wholly dependent on moulting (and thus on growth). For many arthropods and crustaceans, the regenerate following the first moult is always a small version of the missing appendage, and subsequent moults are necessary in order to completely replace the missing part (Maruzzo *et al.*, 2005). The formation of a miniature replacement suggests that fundamental traits may constrain regeneration after patterning, and that given enough time and energy, a small appendage will eventually regenerate to the normal size. Hence, claims that larger and older animals regenerate more slowly may have nothing to do with regeneration *per se*, but instead be a consequence of growth of the replacement part. To understand more fully how growth and regeneration are coupled, future studies examining this relationship should consider: (1) if factors permit a regenerative response but preclude growth to fully restore the missing tissue; and (2) how various factors affect the rate of regeneration during the development phase and growth phase independently.

III. CELLULAR EFFECTS

Next, we address how fundamental traits may affect regeneration at the cellular level. During embryonic development of an appendage, the proliferation, patterning, and differentiation of dividing cells are integrated to produce the final structure. Appendage regeneration following injury appears to recapitulate the developmental process. But in contrast to development, differentiated cells must re-enter the cell cycle during regeneration, and are induced to proliferate amongst populations of cells that necessarily must remain in the differentiated state. The delicate regulation of de-differentiation, proliferation, and re-differentiation is critical to the regeneration process. The effects of aging and growth on the ability of cells to de-differentiate and proliferate likely contribute to constraints on regeneration. Aging in some animals is intimately tied to cellular senescence and a decrease in telomere size. The role of cellular senescence and telomere length in relation to regeneration has been almost completely unexplored outside of mammals and a few fish. A lack of cellular senescence in cold-blooded vertebrates and some crustaceans may provide for prolonged regenerative capacity throughout life (Elmore *et al.*, 2008; Gomes, Shay & Wright, 2010). Here we explore how these cellular mechanisms can be affected during the aging process, and how indeterminate growers might escape the effects of senescence, thereby exhibiting near-limitless regenerative potential.

(1) Cell cycle re-entry and de-differentiation

Following appendage amputation in vertebrates and arthropods, local signals at the site of injury stimulate differentiated cells to "rewind" their cellular history and re-enter the cell cycle (Echeverri, Clarke & Tanaka, 2001; Hay & Fischman, 1961; Lentz, 1969; Truby, 1983). Simultaneously, progenitor cells are mobilized and contribute to regenerating tissue (Morrison *et al.*, 2006). Both differentiated cells and local progenitor cells participate in appendage regeneration, although the relative contribution of these cells to the subsequent tissue components remains unclear (Morrison *et al.*, 2006). Recent research examining axolotl limb regeneration confirmed that blastemal cells respect strict cell lineages, suggesting that de-differentiation does not produce a blastema of pluripotent stem cells, but rather a blastema of lineage-committed cells that respect their embryonic origin (Kragl *et al.*, 2009). Following activation or de-differentiation, both cell types must proliferate, acquire positional identity, and then differentiate to populate the regenerated tissue (Fig. 2). Subsequent regeneration will not proceed correctly if cells at the injury site are unable to complete all of these processes. Research on the cellular mechanisms of de-differentiation has mainly focused on lens regeneration in newts (Eguchi & Shingai, 1971; Imokawa & Brockes, 2003), muscle regeneration in amphibians and mammals (Carlson, 2007; Slack, 2006), and heart regeneration in the zebrafish (Jopling *et al.*, 2010; Kikuchi *et al.*, 2010). The most well-studied example of de-differentiation is the re-entry of myonuclei into the cell cycle during urodele appendage regeneration (Fig. 4). Following amputation, muscle fibres located near the amputation plane fragment into mononucleate cells, divide, and contribute to new muscle in the regenerate (Calve & Simon, 2011; Echeverri *et al.*, 2001; Hay & Fischman, 1961; Kumar *et al.*, 2004; Lentz, 1969; Morrison *et al.*, 2006; Thornton, 1938).

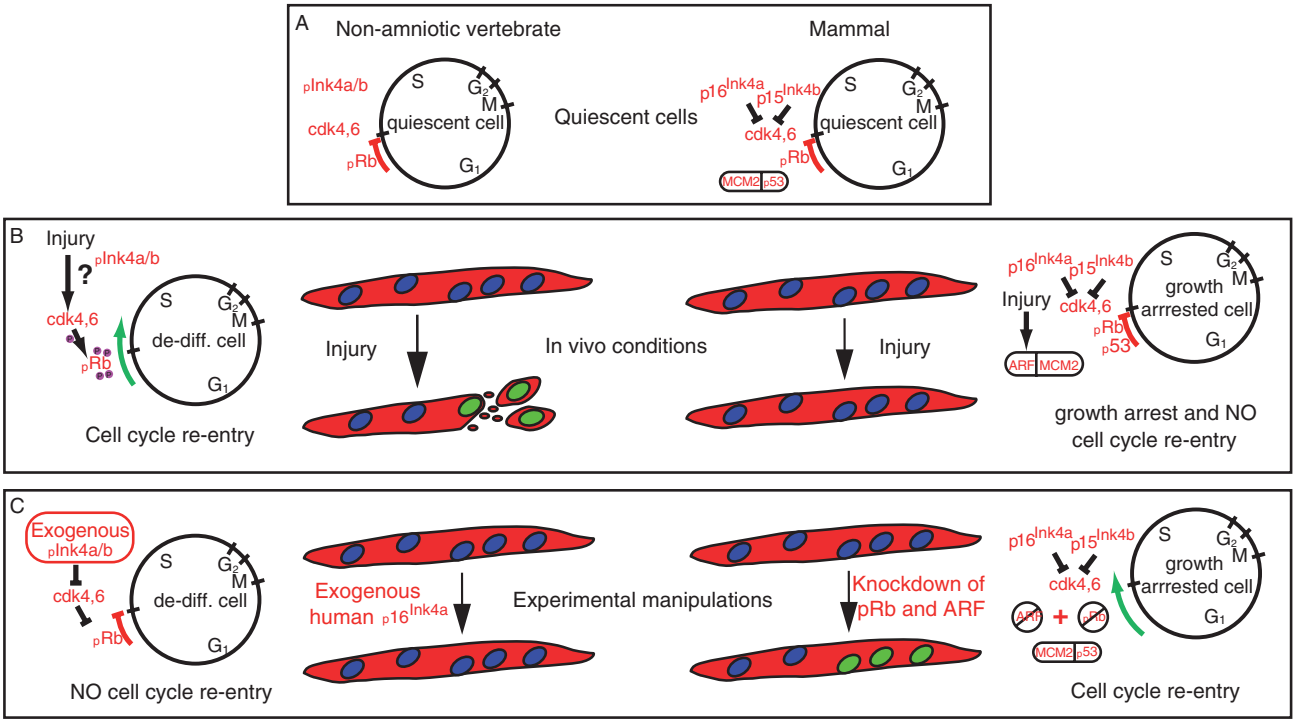

Fig. 4. Myofibre cell cycle re-entry in non-amniotic vertebrates *versus* mammals. (A) Schematic representation of the inhibitory mechanisms present in quiescent cells that block myofibre cell cycle re-entry in vertebrates. Non-amniotic vertebrates possess one *INK4* gene that produces cyclin-dependent kinase inhibitor 2 ($p16^{Ink4}$) while mammals possess two *Ink4* genes (*Ink4a* which produces $p16^{INK4a}$ and ARF and *Ink4b* which produces $p15^{INK4b}$). $p16^{INK4a}$ and $p15^{INK4b}$ block cyclin-dependent kinases 4 and 6 (cdk4,6) activity under normal conditions. Mammals possess an extra level of cell cycle re-entry inhibition by alternate open reading frame (ARF) through tumor protein p53. Under normal conditions, maintenance of chromosomes 2 (MCM2) ubiquitinates p53 and targets it for destruction. pRb, retinoblastoma protein. Black circles depict complete cell cycle and individual phases (G1, S, G2, M). (B) The vertebrate response to muscle injury. Left: the non-amniotic myofibre response to appendage amputation. pInk4a/b is either not produced or blocked upon injury, allowing cdk4,6 to inhibit pRb by phosphorylation (purple circles) thus permitting cell cycle re-entry. de-diff., de-differentiated. Right: the known cell cycle re-entry inhibitor pathways in mammals. ARF is produced and binds directly to MCM2, inhibiting its activity on p53 and allowing p53 to block cell cycle re-entry. (C) Left: red text indicates experimental manipulations performed in urodele myotubes which block cell cycle re-entry. Over-expression of a human form of $p16^{INK4a}$ blocks cell cycle re-entry by inhibiting pRb phosphorylation. Right: red text indicates experimental manipulations performed in mammalian myotubes which allow cells to re-enter the cell cycle. Only when both pRb and ARF are knocked down can primary mammalian myotubes re-enter the cell cycle and mimic the urodele response to injury.

Vertebrate muscle development is partly regulated by retinoblastoma protein (pRb) that initiates and maintains cell cycle withdrawal during the fusion of myoblasts into myotubes (Zacksenhaus *et al.*, 1996) (Fig. 4A). Newt myofibers circumvent mitotic block following injury by phosphorylation of pRb, which allows the formation of a myoblast pool that regenerates into new muscle (Calve & Simon, 2011; Tanaka *et al.*, 1997) (Fig. 4B). By contrast, mammalian myofibres cannot be induced to re-enter the cell cycle upon injury or serum stimulation (Huh *et al.*, 2004; Tanaka *et al.*, 1997) (Fig. 4B). The inhibition of cell-cycle re-entry may be due to mammals possessing an additional level of protection caused by gene duplication at the *Ink4* locus such that two proteins, cyclin-dependent kinase inhibitor 2 ($p16^{INK4a}$) and alternate open reading frame (ARF), are produced from alternative splicing of *INK4a* (Gilley & Fried, 2001; Kim *et al.*, 2003). In mammals, $p16^{INK4a}$ and $p15^{INK4b}$ prevent cell cycle re-entry through regulation of pRb, whereas ARF regulates tumour protein p53 activity, thus adding an additional layer of protection (Pajcini *et al.*, 2010) (Fig. 4A, C). Subsequent knockdown of both pRb and ARF allow mammalian myotubes to re-enter the cell cycle (Pajcini *et al.*, 2010) (Fig. 4C).

Interestingly, $p16^{INK4a}$ is a potent tumour suppressor that is undetectable in young mammals but accumulates in older individuals. Experimentally reducing $p16^{INK4a}$ levels in old individuals leads to a substantial increase in regenerative ability (Krishnamurthy *et al.*, 2006). Furthermore, when a plasmid encoding human $p16^{INK4a}$ is injected into newt myotubes, their ability to re-enter the cell cycle (and thus de-differentiate) is blocked (Tanaka *et al.*, 1997) (Fig. 4C). Homologs of $p16^{INK4a}$ and $p15^{INK4b}$ are present as one gene product (pInk4a/b) in amphibians (*X. tropicalis*; Gene ID 448767) and fish (Gilley & Fried, 2001), suggesting potential conservation of this mechanism of pRb control (Pajcini *et al.*, 2010). Whether or not pInk4a/b is inhibited following injury in salamanders, fish and arthropods to control pRb, or

operates in aged individuals, awaits detailed investigation of pInk4a/b to shed light on this mechanism of cellular control.

An open question is whether de-differentiation in other cell types may be controlled *via* regulation of alternate cell cycle control genes that permit entry into S-phase. The nucleolar protein nucleostemin can regulate stem cell self-renewal and proliferation in a p53-dependent manner, and regulates cell-cycle re-entry during regeneration (Ma & Pederson, 2007; Maki *et al.*, 2007; Tsai, Kittappa & McKay, 2002). In newts, upregulation of nucleostemin precedes cell-cycle re-entry and it co-localizes with de-differentiating pigmented retinal epithelium during lens regeneration, and with blastema cells during limb regeneration (Maki *et al.*, 2007). Interestingly, ARF expression (from the *Ink4a* locus) downregulates nucleostemin which leads to p53-mediated cell cycle arrest (Ma & Pederson, 2007). These findings suggest that nucleostemin may contribute to cell cycle re-entry in other cell types, and supports a role for the *Ink4a* locus in restricting de-differentiation in mammalian cells through multiple pathways. In cockroaches, epidermal cells re-enter the cell cycle following amputation, although the mechanisms of this action are unclear (Truby, 1983). Hypothesizing that similar events occur in these and other arthropods capable of regeneration is reasonable and warrants further investigation into the control of cell cycle re-entry during appendage regeneration. Future research should address: (1) whether or not de-differentiation in other organs and appendages involves removing similar inhibition of cell cycle re-entry; (2) if this blockade is maintained in an age-dependent fashion; and (3) whether other metazoans also have such mechanisms.

(2) Progenitor cells

Progenitor cells are lineage-restricted stem cells that can differentiate into specific cell types a limited number of times, and serve to replenish various cell types throughout the body. Along with de-differentiating cells, progenitor cells account for an unknown fraction of regenerating tissue. Data from mammalian and avian taxa indicate that the number and proliferative ability of progenitor cells declines with age (Carlson, 1995; Renault *et al.*, 2000). Furthermore, evidence from both newts and mammals has demonstrated the involvement of resident progenitor cells from a variety of tissues during regeneration (Carlson, 2007). Progenitor cells are thought to re-enter the cell cycle following injury in a similar manner to de-differentiating cells (Dhawan & Rando, 2005; Morrison *et al.*, 2006). Evidence in favour of a repressive role for progenitor cell cycle re-entry as cells age comes from investigations examining the Notch/Delta signaling pathway and transforming growth factor-ß induced phosphorylation of Smad3 (Tgf-ß/pSmad3) in satellite cells during muscle regeneration (Carlson, Hsu & Conboy, 2008; Hjiantoniou *et al.*, 2008; Odelberg, 2002). Inhibiting the Notch pathway in young muscle inhibits regeneration, while forced activation of Notch in old muscle rejuvenates repair (Conboy *et al.*, 2003). Additionally, heterochronic parabiotic pairings between old and young mice have shown that systemic factors are capable of rescuing the proliferative and regenerative ability of aged satellite cells through activation of the Notch pathway (Conboy *et al.*, 2005). Conversely, young regenerative muscle has low levels of Tgf-ß/pSmad3, and this balance shifts towards higher levels in old, non-regenerative muscle (Carlson *et al.*, 2008). Whether or not these pathways interact directly to convey regenerative capacity remains unclear. At least in mammalian cells, aging does change the way that these progenitor cells respond to injury cues through these and other cell cycle modulators. Because these pathways are involved in mammalian muscle regeneration, they are intriguing candidates to explore in non-mammalian vertebrates and arthropods (especially in cases where regeneration is curtailed because of metamorphosis or life stage).

(3) Cell proliferation and growth

Telomeres are regions of repetitive DNA at the ends of chromosomes that function to prevent cellular degradation. Telomeres progressively shorten during successive cell divisions, eventually leading to cellular senescence and the cessation of cell proliferation (Lee *et al.*, 1998). The enzyme telomerase acts to combat this shortening. Thus, the ability of cells to remain in the cell cycle and proliferate is partially due to telomerase (Bousman, Schneider & Shampay, 2003; Greider, 1998; Klapper *et al.*, 1998; Lee *et al.*, 1998). Telomerase activity also appears to function independently of telomere maintenance to maintain cell proliferation *via* an unknown mechanism (Smith, Coller & Roberts, 2003). Telomerase expression and subsequent activity may be influenced during aging, following metamorphosis, or by growth patterns.

Following de-differentiation and blastema formation, cells must proliferate for morphogenesis to proceed. The molecular pathways described above confer the ability of cells to re-enter the cell cycle primarily through a loss of repression on cell cycle control genes (or signaling pathways that interact with them). As regenerative capacity declines, either with age or following metamorphosis, these repressive states become harder to overcome. Following de-differentiation, mitotic division follows and proliferation must be maintained for regeneration to proceed. While maintaining cells in a proliferative state is clearly a nerve-dependent process (see Section II.3), blastemal cells must also retain the ability to divide repeatedly (instead of differentiating) in order to produce enough cells to replace the missing tissue. Examining the role of telomerase during appendage regeneration seems appropriate given the importance for maintaining cell proliferation in regenerating tissues. In fact, an examination of telomerase activity across taxa suggests a strong correlation between regeneration, aging, and growth pattern (Gomes *et al.*, 2010).

In addition to being used as a marker of cellular age, telomerase activity also can be used as an indicator for growth capacity (Klapper *et al.*, 1998). Telomerase activity increases to restore telomere length and remains high during proliferation in regenerating tissues (Elmore *et al.*, 2008; Klapper *et al.*, 1998). Although not examined in the context of metamorphosis, evidence from *Xenopus laevis* suggests that telomerase activity is highest in embryonic tissue and in

adult tissues with high regenerative capacity (e.g. testis, liver, spleen) (Bousman *et al.*, 2003). Telomerase activity is high in both larval tissue and in fully differentiated adult tissue of arthropods that are capable of appendage regeneration (e.g. lobsters) (Gomes *et al.*, 2010; Klapper *et al.*, 1998). The same pattern holds for a diverse array of fish, and an upregulation of telomerase activity has been detected in fin tissue during regeneration (Elmore *et al.*, 2008). In birds and mammals that exhibit cellular aging and senescence, telomerase activity is predictably high in embryonic tissues and germ cells, but low in fully differentiated tissues incapable of regeneration. Taken together, these studies demonstrate a correlation between regenerative capacity, high telomerase activity, "young" tissue, and high growth capacity. This correlation suggests that telomerase activity might also predict the ability to regenerate both intraspecifically (as an animal ages) and interspecifically. In addition, cells capable of indefinite growth have high telomerase activity, but there is little to no activity in terminally differentiated cells from animals that age (Gomes *et al.*, 2010; Greider, 1998). Some urodeles, like many fish, are indeterminate growers whose cells may not senesce (Goss, 1994; Kara, 1994). Thus, indeterminate growth may confer a high degree of regenerative ability, and this can be tested in a diverse array of animals (Klapper *et al.*, 1998).

IV. GENOMIC-LEVEL EFFECTS

Lastly, we examine the involvement of a single gene family that may permit regeneration regardless of fundamental traits; and then address how fundamental traits may affect regenerative capacity at the level of the epigenome. Genetic tools (e.g. microarrays, high-throughput sequencing) and proteomics have identified a number of key signals that act in time and space to coordinate a regenerative response, yet the factors that govern activation of these genes remain unclear (Monaghan *et al.*, 2009; Rao *et al.*, 2009; Whitehead *et al.*, 2005).

At the genomic level, the ability to respond to injury by expressing key genes could be affected by fundamental traits if such traits alter the ability of genes to become activated (either directly or through a loss of inhibition) in response to injury. Access to promoter or enhancer sites (and thus the ability to activate or repress transcription) can be maintained through epigenetic mechanisms. Epigenetic control over chromatin structure could in turn govern the ability to mount a regenerative response. Thus, it seems plausible to ask if regenerative capacity is ultimately controlled by cellular changes that are directed by epigenetics, and if these changes could be influenced by fundamental traits of an organism.

(1) Regeneration-specific genes

As discussed throughout this review, several key processes are necessary for complete appendage regeneration to occur. Some researchers have proposed the involvement of regeneration-specific genes that coordinate these processes and ultimately permit appendage regeneration (Garza-Garcia *et al.*, 2009; Garza-Garcia, Driscoll & Brockes, 2010). The existence of regeneration-specific genes was based on the discovery of Prod1, a cell surface protein of the CD59/Ly6 protein family that regulates proximodistal cell identity (Blassberg *et al.*, 2011). Furthermore, the subsequent discovery that the Prod1 ligand, newt anterior gradient protein (nAG), was sufficient to partly rescue limb regeneration in denervated newt limbs, suggested that Prod1 coordinated both growth and patterning to regulate the regenerative response (Kumar *et al.*, 2007). That Prod1 is found only in salamanders led to the suggestion that the lack of regenerative ability in certain vertebrate lineages stems partly from a lack of this gene (Garza-Garcia *et al.*, 2010).

Subsequent research has identified axolotl Prod1, although it is a secreted molecule instead of being linked to the cell membrane as in newts (Blassberg *et al.*, 2011). A different member of the CD59/Ly6 protein family, CD59, was also found to play a role in proximodistal cell identity during gecko tail regeneration, suggesting that the CD59/Ly6 family of proteins may have been adapted by different vertebrate lineages to regulate patterning during regeneration (Wang *et al.*, 2011). It will be interesting to see if the CD59/Ly6 protein family plays a similar role in all regenerating species, and if the activity of these proteins is lost in non-regenerating species. Ultimately, the existence of mammalian CD59 and additional CD59/Ly6 family members, along with the role of CD59 during gecko tail regeneration, suggests that it is not the presence or absence of a particular gene that controls regenerative ability, but rather an inability to coordinate gene expression to induce a regenerative response.

(2) Epigenetic control of regeneration

During the regenerative response, both the ability of cells to re-enter the cell cycle, and the ability to activate specific genetic networks can be controlled through cell-cycle control genes and transcription factors. Epigenetic modification refers to changes in non-sequence DNA that alters transcription and gene function, resulting in phenotypic changes at the cellular level (Fraga, 2009). Various epigenetic mechanisms can regulate gene expression, and changes in these mechanisms due to age or metamorphosis may influence the regenerative ability of an organism.

A number of potential epigenetic alterations can affect gene expression. First, a change in methylation state at CpG islands (genomic regions of >50% same strand CG associations) or in promoter and enhancer regions can affect the chromatin state of DNA. Alterations to chromatin state can subsequently render genes in a transcriptionally silent or active state (reviewed in Bird & Wolffe, 1999). Second, modifications to histones (through selective methylation, acetylation, sumolation, or phosphorylation at specific amino acid residues) can directly affect the ability of genes to be actively transcribed either directly or through higher order chromatin rearrangement (reviewed in Jenuwein & Allis, 2001). Both of these modifications to DNA architecture are governed through the enzymatic activity of various proteins

such as the polycomb-group (PcG), trithorax-group (TcG), histone methyltranferases (HMTases), histone deacetylases (HDACs), and lysine demethylases. Each of these proteins has a high specificity for particular modifications that alone or in combination can regulate cell behaviour (reviewed in Berger, 2007). Clearly epigenetic alterations that affect cell cycle re-entry and proliferation (de-differentiation), or that affect the transcriptional networks underlying positional information would have profound effects on regenerative ability (Yakushiji, Yokoyama & Tamura, 2009*b*). Although few studies examining structural regeneration have accounted for the possibility of epigenetic modifications, recent work has just begun to address this issue. Moreover, we understand little about how fundamental traits may influence epigenetic modifications.

Can life stage (i.e. metamorphosis) influence the modification of DNA architecture? In anurans, pre-metamorphic tadpoles are capable of limb regeneration, whereas post-metamorphic froglets are not (Dent, 1962; Polezhayev, 1946). Using *Xenopus laevis,* Yakushiji *et al.*, (2007) examined methylation states at CpG islands of an enhancer region for the *Sonic hedgehog* (*Shh*) gene that plays an important role during embryonic limb development and limb regeneration. They found low levels of methylation in the *Shh* enhancer region, and correspondingly high expression of *Shh* in the limb cells of tadpoles. By contrast, they found a higher degree of methylation in froglets, correlating with a failure to activate *Shh* and incomplete regeneration. Further, when *Shh* expression was experimentally activated (exogenously, using small molecules) following amputation in froglets, there was an increase in cell proliferation and regenerative capacity (Yakushiji *et al.*, 2009*a*). These findings suggest an increased methylation in control regions of genes that participate in regeneration, and therefore may help explain the loss of regenerative ability following metamorphosis in *X. laevis*. Interestingly, axolotls and newts show relatively low levels of methylation in the *Shh* enhancer in both intact and regenerating limbs (Yakushiji *et al.*, 2007). This suggests a species-specific component to methylation at this enhancer, which might contribute to retention of regenerative ability in urodeles. These examples suggest a strong correlation between regeneration and growth that may be dependent on the drastic physiological and morphological changes that occur during metamorphosis. They also underscore the importance of multiple genetic inputs for successfully rescuing regeneration, and cast doubt on a "magic bullet" in the form of one lone gene or pathway. These studies offer tantalizing clues to how life stage might alter methylation states of key regenerative genes to suppress transcription, and imply that methylation may also be regulated at the species level in the context of regeneration.

While methylation state at CpG islands is most often associated with gene silencing, histone modifications provide an attractive model for how a regeneration program can be controlled to affect regeneration. In another study examining caudal fin regeneration in zebrafish, (Stewart, Tsun & Izpisua Belmonte, 2009) the "bivalent loci" control of gene expression through selective trimethylation of lysine 27 histone 3 (me^3K27 H3) by polycomb group proteins (PcGs) and trimethylation of lysine 4 histone 3 (me^3K4 H3) by trithorax group proteins (TcGs) was examined. Based on previous work in embryonic stem cells, the authors proposed that maintenance of both histone marks act to prime genes for activation, and that subsequent loss of the repressive me^3K27 H3 mark can lead to gene activation. When they examined genes involved in fin regeneration they observed a significant decrease in the me^3K27 H3 mark, but not in the me^3K4 H3 mark that also correlated with a significant increase in expression of some genes involved in the regenerative response. Underscoring the complexity of these modifications, this pattern does not hold for many of the genes known to be important for regeneration, and the authors suggest that alternative control mechanisms might have confounded their results. Nonetheless, their data suggests the potential for these types of epigenetic modifications to affect limb regeneration and should spur future experiments in more relevant systems, particularly after metamorphosis in anurans.

V. FUTURE PERSPECTIVES

With the re-emergence of regeneration research in the context of regenerative medicine, the prospects for discovery have never been richer. The last twenty years has seen huge progress in our ability to replace failing organs with bioengineered replacements based on biological scaffolds and autologous human cells. Damaged blood vessels, heart valves, tracheas, bone fragments, and even bladders can all now be replaced through advances in regenerative medicine. Despite these successes, bioengineering approaches have failed to find a way to regenerate skin, appendages, or parts of complex organs containing specialized cell types. The future lies in the ability of science to coax damaged tissue to repair itself, thus regenerating a perfect replacement *in situ*. If we are to succeed in this task, researchers must look towards organisms that regenerate damaged tissues in order to understand the mechanisms that naturally regulate and constrain regeneration at various levels of biological organization.

Underlying the hypotheses presented above is an implicit suggestion that regenerative ability is fundamentally coupled to development and growth. Although not a new idea, discovering how these processes interact is tantamount to understanding how and why some animals can regenerate but others cannot. Traditional evolutionary comparisons of regenerative ability across metazoans have used simple presence or absence (of regeneration) when comparing species, rather than comparing whether regenerative capacity changes across life stage. This has led to the misconception that adult appendage regeneration is widespread. In fact, when life stage is considered, the available evidence suggests otherwise. Very few species that reproduce sexually are capable of appendage regeneration along the secondary

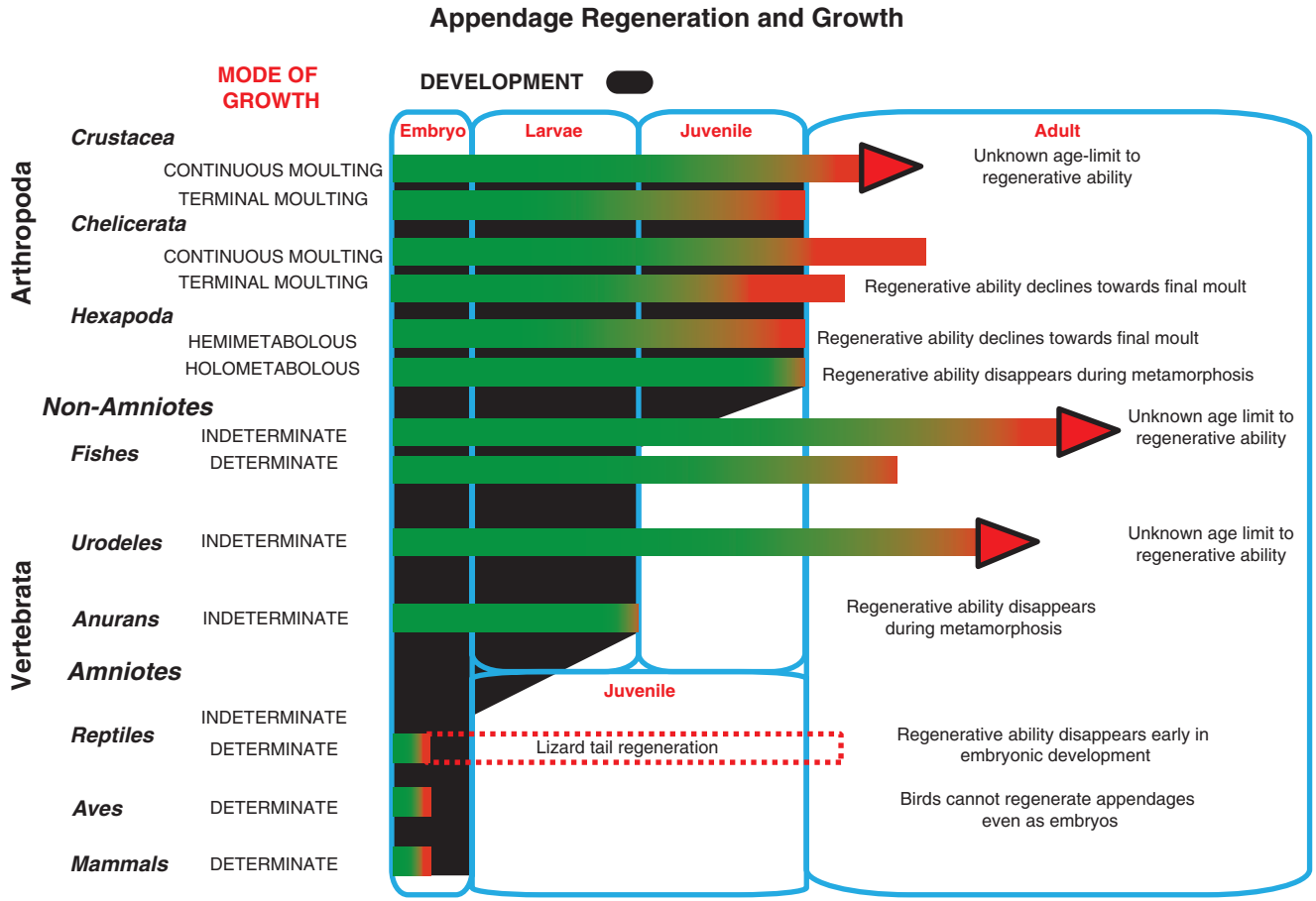

Fig. 5. Appendage regeneration is restricted among adult forms of Arthropoda and Vertebrata. Regenerative ability appears highly correlated with development and growth capacity. Shaded bars represent regenerative ability. Green represents complete regeneration and red, no or partial regeneration. Species from each phyla are grouped according to their mode of growth (growth patterns for many species are unknown). All amphibians are considered indeterminate growers (see Section I.4). Within amniotes, appendage regeneration either does not occur (birds) or is lost during embryogenesis. Tail regeneration in certain reptiles is the exception and is incomplete as the spinal cord is not regenerated.

body axis as adults (Fig. 5). Instead, the data suggest a near-ubiquity of appendage regeneration in embryos and larvae, with a general decline as juveniles move towards adulthood (Fig. 5). Exceptions occur in some salamanders, fish, and crustaceans that exhibit seemingly boundless regenerative ability, even as adults. Interestingly, indeterminate growth and neoteny (some salamanders) occur in conjunction with this extended regenerative capacity, suggesting a mechanism that hijacks aspects of juvenile development to block cellular senescence or to provide a constant source of progenitor cells to the continually growing organism. An important advance in understanding the relationship between indeterminate growth (or neoteny) and extended regeneration will be to find the upper age boundaries at which regeneration fails (if at all). This relationship also makes revisiting regeneration across metamorphosis appealing as it provides an experimental system where regenerative capacity is naturally lost in the same tissue over a short time period. Future research attempting to identify the cellular and molecular mechanisms underlying regeneration through comparative studies will benefit from experiments conducted across various developmental stages, and from species with different modes of growth.

The discussion and hypotheses in this paper have aimed to frame future research by emphasizing the importance of fundamental traits in the context of appendage regeneration. This stems partly from a need to understand if regeneration fluctuates within a species, or is an all-or-nothing process within taxa. Researchers must continue to explore new species and gather data on the presence and absence of regeneration. Simultaneously, they will need to uncover how (if at all) regenerative capacity changes over variable body sizes, during aging within species, across metamorphosis, and with respect to growth patterns. While time consuming, this approach is vital to elucidating mechanisms that disrupt regeneration, and will provide insight into mammalian repair processes. We conclude that increasing phylogenetic sampling and exploring these ideas from multiple levels of biological organization is vital to finding an answer to the question that continues to elude science: why can some animals regenerate but others cannot?

VI. CONCLUSIONS

(1) This review summarizes our current knowledge of how certain fundamental traits (e.g. body size, aging, life stage, and growth pattern) affect appendage regeneration in metazoans. We highlight the need for future experiments to decouple these traits to examine if they constrain regeneration within and across species.

(2) We posit specific ways in which fundamental traits can modify mechanisms controlling regeneration at the tissue, cellular, and genomic levels. These include: age-dependent loss of telomerase expression and activity (which restricts proliferative capacity and thus regeneration); prolonged regenerative capacity with indeterminate growth (through the lack of cellular senescence, sustained access to progenitor cells, and maintenance of high proliferative capacity); and life-stage-associated changes in the epigenetic control of gene expression (which can restrict the ability to mount a complete regenerative response).

(3) We review possible taxon-specific differences over the molecular control of cell-cycle re-entry (e.g. *Ink4a/b* locus genes, nucleostemin) and highlight the need to investigate these interactions across an array of regenerating species and tissue types.

(4) Appendage regeneration in metazoans is strongly coupled to developmental stage. When viewed across taxa, regeneration appears widespread among larval and juvenile states but becomes restricted among adult forms. Limited data suggest that some taxa may escape this developmental constraint through extended growth capacity, although more information on the presence and absence of regeneration in larger and older organisms is necessary.

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