Title: Biological insights into the rapid tissue regeneration of freshwater crayfish and crustaceans

Running title: Tissue regeneration in freshwater crayfish

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Abstract

The freshwater crayfish is capable of regenerating limbs, following autotomy, injury and predation. In arthropod species, regeneration and moulting are two processes linked, and strongly regulated by ecdysone. The regeneration of crayfish limbs is divided into wound healing, blastema formation, cellular reprogramming and tissue patterning. Limb blastema cells undergo proliferation, dedifferentiation, and redifferentiation. A limb bud, containing folded segments of the regenerating limb, are encased within a cuticular sheath. The functional limb regenerates, in proecdysis, in 2 to 3 consecutive moults. Rapid tissue growth is regulated by hormones, limb nerves, and local cells. The TGF- β/Activin signalling pathway has been determined in the crayfish, *P. fallax f. virginalis* and is suggested as a potential regulator of tissue regeneration. In this review article, we discuss current understanding of tissue regeneration in the crayfish and various crustaceans. A thorough understanding of the cellular, genetic, and molecular pathways of these biological processes is promising for the development of therapeutic applications for a wide array of diseases in regenerative medicine.

Key words: regeneration, astacoidea, stem cells, crayfish, crustaceans, extremities, exploratory behavior, hormones

Introduction

Tissue regeneration is an important physiological process resulting in the regrowth of lost or damaged tissues (Allen, Ross et al. 2001). Several metazoan taxa are highly regenerative, for example, the hydra, can regrow entire organisms from small body fragments (Stocum 2012, Reddien 2019, Goldman and Poss 2020). In contrast, the Arthropoda phyla (subphylum crustacea and class insecta), possess limited regenerative ability, although they provide insights into conserved regenerative mechanisms and systems (Khan, Schuster et al. 2001, Lee and Walsh 2016). Freshwater crayfish (Crustacea, Decapoda, Astacida) are a diverse taxonomic group, endemic to the continents of North America, Australia-Oceania, and Europe (Lunda, Roy et al. 2020). They can regenerate various anatomical structures, such as, chelipeds, pereiopods, uropods, antennae, brain neurons, and the compound eye (Leland, Coughran et al. 2012, Ventura, Stewart et al. 2019). Regeneration in crayfish and various crustaceans differ from other species and is contingent upon their life cycle and unique structure (Khan, Schuster et al. 2001, Chang and Thiel 2015). Research in crayfish regeneration is mainly focused on limbs, which are rapidly replaced in *P. fallax f. virginalis* across two consecutive moults (Chantran 1873, Durand 1960, Shinji, Miyanishi et al. 2016, Ventura, Stewart et al. 2019). A fully functional limb is regenerated after tissue amputation of an extremity, in response to injury and/or predation, coinciding with moulting events (Bliss 1960, Weis and Mantel 1976, Juanes and Smith 1995, Goss 2013, Lee and Walsh 2016). Despite this, regeneration in crayfish remains a long-standing challenge in biological sciences (Ventura, Stewart et al. 2019). Our current knowledge of the molecular mechanisms, such as, the endocrine hormones and environmental factors underlying crayfish moulting and regeneration remains limited (Shinji, Gotoh et al. 2019). This is because there are few published studies and a large quantity of the literature is severely limited to observations published over two centuries ago (Steele 1904). In addition, there are minimal genomic resources available for decapod crustaceans, which limits

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our ability to determine regeneration-induced genes (Tan, Gan et al. 2020). Recent advances have begun to elucidate key features of both regeneration and the moult-cycle in crustacea (Chang and Mykles 2011, Das 2015, Mykles and Chang 2020). These studies indicate that limb regeneration in crayfish is possibly a blastema-mediated epimorphic process, involving wound healing, blastema formation, cell proliferation/growth and tissue patterning (Khan, Schuster et al. 2001, Bergmann and Steller 2010). The wound healing stage in limb regeneration has been investigated by using fluorescent immunohistochemistry (IHC), histology, and confocal laser scanning microscopy (Vafopoulou 2009). Mechanistic insights from in vitro and in vivo studies reveal that regulation of crustacean limb regeneration relies on ecdysone (E), fibroblast growth factors (FGF), and retinoids which act via the nuclear receptors, ecdysteroids receptor (EcR), fibroblast growth factor receptor (FGFR) and retinoid receptor (RXR) respectively (Zhang, Yang et al. 2018, Ventura, Stewart et al. 2019). Previous research also indicates that crustacean tissue regeneration could involve both conserved cell division pathways of regenerating animals and crustacean specific molting pathways (Shinji, Gotoh et al. 2019). Here, we comprehensively analyse existing scientific knowledge on pereiopod and cheliped tissue regeneration in the freshwater crayfish and identify where future research is needed. A thorough understanding of crayfish tissue regeneration is crucial for the advancement of regenerative biology and medicine, for possible future therapeutic strategies.

History of crayfish regeneration

In 1712, French Scientist René Antoine Ferchault de Réaumur conducted studies on limb regeneration in crayfish, crabs, and lobsters (DE REAUMUR 1712, Ratcliff 2005). The experiments in crayfish were successful, providing a theoretical description of limb regeneration (Steele 1904). In the scientific study, limbs of crayfish were amputated under different conditions and the cuticle surrounding the limb buds were dissected (DE REAUMUR 1712). Réaumur noticed that crayfish automise limbs at the predetermined breakage plane of

the second limb segment (DE REAUMUR 1712). Secondly, that crayfish regenerate new limbs that are identical in size and function to the original. Thirdly, that the nature of regeneration is influenced by season of the year, moulting, and age of the crayfish. Réaumur later suggested that small “eggs” located in the original limb, regenerate parts of the new limb (Maginnis 2006). The study focussed on three main focal points: (1) recording that regeneration occurred, (2) determining the abiotic and biotic effects on the rate of regeneration, and (3) determining the developmental and physiological aspects of crayfish regeneration (Maginnis 2006). The study quality is considered moderate, indicated by multiple biological and technical replicates, the large sample size, and controlled conditions. Low potential risk of error is due to limiting constraints and technical challenges associated with experiments on freshwater organisms such as, nutritional status of the crayfish, collection of crayfish, and varied temperatures. Although this study was the first reported account of crayfish regeneration, it was challenged and Reaumur’s theoretical concept was rejected, limiting the validity and reliability of the results. Other early experiments on crayfish regeneration include the observations made by Chantran in 1771. Chantran demonstrated that a fully functional appendage regenerates only after three consecutive moults (Chantran 1873, Steele 1904). Morgan in 1798 and 1901 also made observations upon crayfish limb regeneration, he found that regeneration does not occur readily or proximal to injury, and that the capacity of regeneration is independent to the liability to injury (Morgan 1901, Steele 1904). In 1882, Fredericq, introduced the term autotomy, and published an account of crustacean autotomy (Fredericq 1891, Juanes and Smith 1995). Since the 1900s, the cellular, molecular, and hormonal basis of tissue regeneration in crayfish has been well studied.

Interactions between limb regeneration and the moult-cycle

Moulting in freshwater crayfish, *Orconectes Obscurus*, is the rapid and complex developmental process essential for a diverse set of biological processes such as, growth, development, reproduction, and regeneration (Henry and Stevenson 1971, Andrews and Dillaman 1993, Chang and Mykles 2011). The moult cycle of the crayfish, *Procambarus clarkii* is further linked with changes in metabolism and behaviour (Bittner and Kopanda 1973). In the crayfishes, *Procambarus acutus* and *Procambarus clarki* the rigid and calcified exoskeleton limits continual growth (Huner and Lindqvist 1985, Andrews and Dillaman 1993). In order to increase in size, volume, and expand soft tissues, the crayfish, *Cherax destructor*, periodically replace their exoskeleton (Aiken and Waddy 1992, Verhoef, Austin et al. 1998, Hammond, Hollows et al. 2006). During the moulting process, the old calcified cuticle is separated from its hypodermis and a new, larger, and pliable exoskeleton is secreted from underneath the former (Skinner, Bliss et al. 1985, Skinner and Cook 1991, Hopkins 2001, Mykles and Chang 2020). The new exoskeleton is enlarged to allow for structural growth, and increased size and body mass of the crayfish (Chang, Bruce et al. 1993, Hopkins, Chung et al. 1999, Hopkins 2001, Goss 2013). It has been shown that mature crayfish, *Orconectes obscurus* must survive at least one or more moult cycles before the limb regenerate hypertrophies to full size (Govind and Pearce 1985). The certain stages and time courses of crayfish limb regeneration have been evaluated by careful observations, and the major stages appear to occur between moults (Bliss 1956, Durand 1960).

There are several publications that have investigated and described in detail the endocrine regulation of moulting in crustaceans (Novák 1969, Andrews and Dillaman 1993, Phlippen, Webster et al. 2000, Chang and Mykles 2011, Mykles and Chang 2020). Studies using an in vitro bioassay in crayfish, *Procambarus* clarkii have largely examined the inhibitory effects of MIH on Y-O synthesis of E during moulting (Pitts 2015). Endocrine mechanisms which

regulate the crustacean moult cycle are presumed to influence and control tissue regeneration (Ventura, Stewart et al. 2019). Moulting is regulated by several chemical factors, including steroid and neurosecretory hormones in a negative feedback loop (Chang, Bruce et al. 1993, Waterman 2012, Chang and Thiel 2015). Studies show that the Y-O glands induce moulting in the crayfish, *Procambarus clarkii* by producing and releasing the polyhydroxylated ketosteroid hormone, E (Jegla, Ruland et al. 1983, Hopkins 1992, Lachaise, Le Roux et al. 1993, Nakatsuji and Sonobe 2004, Ghanawi and Saoud 2012). This hormone is a derivative of dietary cholesterol and a versatile hormone that is exclusive to arthropods, controlling genetic expression (Lachaise, Le Roux et al. 1993, Chang and Thiel 2015, Das 2015). In the crayfish, *Procambarus clarkii* E play important roles in several biological processes, such as growth, embryogenesis and reproduction (Krishnakumaran and Schneiderman 1970, Kuballa, Holton et al. 2011, Ghanawi and Saoud 2012). Immunohistochemical studies show that ecdysone receptor signalling might be a candidate in regulation of blastemal proliferation during tissue regeneration in crustaceans, and could potentially have a significant role in wound healing, as elucidated by studies in the Fidler crab, *Uca pugilator* and crayfish, *Procambarus clarkii* (Chang 1995, Hopkins, Chung et al. 1999, Vafopoulou 2009, Das 2015).

Once synthesised and circulating in the haemolymph, E is rapidly hydroxylated by peripheral tissues to its active metabolite, 20-hydroxyecdysone (20E), known as the active moulting hormone (Skinner 1985, Chang 1995, Khan, Schuster et al. 2001, Mykles 2001, Nakagawa and Henrich 2009, Mykles 2011). In the crayfish, Orconectes limosus, titres of E in the haemolymph, during the moult cycle were determined by advanced extraction procedures (Willig and Keller 1973). Radioimmunoassay (RIA), high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) measurements indicate E titres in the haemolymph vary substantially throughout the crustacean moult cycle, affecting significant

biochemical and physiological changes (Chang and Bruce 1980, Khan, Schuster et al. 2001, Chang and Mykles 2011, Das 2015).

Implants and tissue extract experiments in the crayfishes Orconectes virlis and Orconectes immunis, show that the neurosecretory X organ (XO) and the neurohemal organ, the sinus-gland (SG), together called the XO/SG complex, within the medulla terminalis of the eyestalk ganglia, negatively regulate the moulting process (Kyer 1942, SCUDAMORE 1942, Pamuru, Rosen et al. 2012, Mykles and Chang 2020). The XO/SG complex inhibits moulting by releasing and storing neuropeptide hormones, including the crustacean hyperglycaemic hormone (CHH) and the moult-inhibiting hormone (MIH) (Chang 1995, Bulau, Okuno et al. 2005, Suneetha 2013, Mykles and Chang 2020). MIH and CHH are found only in arthropods and function by inhibiting Y-O secretion for a large proportion of the moult cycle, holding the crayfish in the intermolt stage (Lachaise, Le Roux et al. 1993, Castro, Davie et al. 2015). High throughput cDNA microarray in the crayfish, *Cherax Quadricarinatus* has identified the quantification cycle *(Cq)-MIH* gene, which appears to regulate the moult cycle (Pamuru, Rosen et al. 2012). It is known, that various environmental factors affect the XO/SG complex, in turn altering MIH biosynthesis, although the underlying mechanisms of these processes remain unclear (Shechter 2008).

The function of MIH has been reinforced by classical eyestalk ablation (ESX) experiments in the crayfish, *Orconectes limosus*, which show an instant increase in concentrations of E in the haemolymph, inducing premature moults (BROWN JR and Cunningham 1939, Smith 1940, Skinner and Graham 1972, Jegla, Ruland et al. 1983, Suneetha 2013, Mykles and Chang 2020). Removal of both eyestalks removes the XO/SG complex, and the predominant source of MIH, which places the crayfish into the proecdysis phase one day after ESA (Skinner and Graham 1972, Mykles and Chang 2020). Several studies indicate that ESA in juvenile crayfish, Cambarus clarkii Girard, although fatal, leads to an earlier moult by shortening the length of

the intermolt stage across multiple moults (Smith 1940). ESA changes in E levels provide further evidence that tissue regeneration is regulated by E (Vafopoulou 2009). In comparison, the elegant administration of exogenous MIH by injection in ESA, was shown to produce a decrease in E levels, delaying moulting (Shechter 2008, Suneetha 2013). Our current understanding of the complexities of the crustacean moulting cycle is limited, and the intricate interactions between E and functional neuropeptides remain largely unknown (Skinner 1985, Oliphant, Alexander et al. 2018). There are many additional chemical factors at the tissue level which induce the events of the moult cycle, specifically limb regeneration during the proecdysis phase (Skinner 1985). Thus, further research is necessary to elucidate the molecular mechanisms and signalling pathways regulating the moult cycle.

Following observations at the morphological and cellular levels the crustacean moult cycle has been characterised into five sequential stages, A through to E (Drach 1939, Henry and Stevenson 1971, Roer and Dillaman 1984, Skinner 1985, Burton and Mitchell 1987, Chang 1995). The exact number of substages is species specific and in the crayfish, *Cherax destructor* stages C and D are subdivided into several substages (Rao, Mohrherr et al. 1977, Aiken and Waddy 1992, Musgrove and Geddes 1995, Mykles and Chang 2020). The moult stages of *Cherax destructor*, *Cherax albidus*, and *Parastacoides tasmanicus* are based upon the hardness of the integument and development of new setae (Mills and Lake 1975, Rao, Mohrherr et al. 1977, Skinner, Bliss et al. 1985, Burton and Mitchell 1987). The various phases of the cycle mark the events occurring in preparation for and subsequent to ecdysis (Skinner, Bliss et al. 1985, Mykles and Chang 2020). In the crayfishes, *Procambarus clarkii*, *Astacus leptodactylus*, and *Orconectes virilis* (Cambaridae), these well-defined stages are metecdysis (stage A, B, C_1, C_2, C_3, or postmolt), anecdysis (stage C_4 or intermolt), proecdysis (stage D or premolt) and ecdysis (stage E) (Table 2) (Stevenson 1968, Aiken and Waddy 1992, Longshaw and Stebbing 2016, Mykles and Chang 2020). In ecdysis, the final stage of the cycle, E titre levels decrease

and the crayfish sheds its old exoskeleton (Mykles and Chang 2020). The new exoskeleton is subsequently stretched by internal hydrostatic pressure as the crayfish rapidly absorbs large volumes of water from its surroundings (Castro, Davie et al. 2015, Mykles and Chang 2020). Immediately after stage E, Metecdysis occurs, marking the beginning of the moult cycle (Skinner, Bliss et al. 1985). Throughout this period the cheliped muscles are restored, and the new exoskeleton forms before undergoing calcification (Mykles 2001, Mykles and Chang 2020). At anecdysis, the longest stage, low levels of E circulate in the hemolymph, the new exoskeleton becomes fully formed and mineralized, whilst the crayfish reproduces and consumes food (Khan, Schuster et al. 2001, Nakatsuji, Lee et al. 2009, Das and Durica 2013, Mykles and Chang 2020). Simultaneously, the basal bud/papilla of the regenerating limb forms (Mykles and Chang 2020). In proecdysis, just prior to the moult, E levels steadily rise and the muscles of the chelipeds atrophy (Dell, Sedlmeier et al. 1999, Shechter 2008, Nakatsuji, Lee et al. 2009, Mykles and Chang 2020). The epicuticle and exocuticle layers of the new exoskeleton develop, the membranous and endocuticle layers of the old cuticle deteriorate by approximately 75%, whilst growth and regeneration of lost appendages occurs (Skinner 1985, Mykles 2001, Mykles and Chang 2020). In *Cherax quadricarinatus*, calcium is also absorbed from the old exoskeleton, which is transported and stored in calcareous gastroliths or in the hepatopancreas (Skinner and Cook 1991, Mykles and Chang 2020).

A significant number of studies have examined the close relation between regeneration and the moult cycle in the crayfish and several other crustaceans (Wheatly and Hart 1995, Hopkins 2001, Minelli, Boxshall et al. 2013). In the proecdysial phase, the missing pereiopod of *Orconectes obscurus* is replaced (Henry and Stevenson 1971). Research has demonstrated that the moult cycle effects regeneration and conversely autotomised limbs and subsequent regeneration effects the length of the moult cycle and the duration of the stages (Zeleny 1905). It is reported in the crayfish, tissue regeneration might exert its affects, by accelerating or

deferring moulting (Maginnis 2006, Goss 2013, Chang and Thiel 2015). Limb autotomy could affect the moult interval, which are possibly dependent upon the incidence of autotomy and the stage of limb loss (Smith 1990). In *Procambarus clarkii*, immediately after autotomy of the pereiopods or chelipeds, moulting frequency increases and the intermoult period decreases (Bittner and Kopanda 1973). Multiple autotomised limbs (up to 5) at a given time interrupts the normal moult and accelerates the process, as a loss of several appendages impairs mobility and survival (Khan, Schuster et al. 2001, Das 2015). The timing or the stage of the moult cycle at which autotomy occurs determines the length of regeneration (Goss 2013). Specifically in the crayfish as in many other arthropods, amputation that takes place outside the 'critical window', the intermoult stage, delays regeneration as there is inadequate time for the new limb to grow (Mykles 2001, Edgecombe and Legg 2013). Autotomy that occurs late in the moult cycle causes the regenerating papilla to be undifferentiated, small and its growth continual until ecdysis (Henry and Stevenson 1971, Edgecombe and Legg 2013, Goss 2013). In nearly all arthropods, the interplay between limb regeneration and moulting is based on an all-or-nothing principle, and this concept provides an instance of physiological foresight (Goss 2013). For example, in the moult directly after amputation, the limb regenerate is either non-existent or fully grown. However, the regenerating limb in the first moult following amputation is much smaller in size and not functional (Edgecombe and Legg 2013). Following subsequent moults, the regenerate eventually attains its full morphological structure and function (Edgecombe and Legg 2013).

The presence of the limb buds of the regenerating limb in particular, might significantly affect the entire moult cycle (Edgecombe and Legg 2013). Conversely, moulting or the moult cycle, in crayfish appears to influence the rate of tissue regeneration (Adiyodi 1972, Cooper 1998, Edgecombe and Legg 2013, Goss 2013). In regenerating crayfish, moulting occurs more frequently and at a much faster rate in comparison to non-regenerating crayfish, as regeneration

shortens the intermoult stage (Stockard 1908, Bittner and Kopanda 1973, Maginnis 2006). If a growing basal bud is injured or undergoes autotomy throughout the regeneration process, moulting is postponed in order to allow time for the secondary basal bud to grow (Khan, Schuster et al. 2001). In this circumstance, the growing basal bud synthesises a peptide that inhibits MIH secretion from the X organ, whilst the second basal bud will synthesise an MIH-peptide factor which appears to function by decreasing E levels (Khan, Schuster et al. 2001). Interestingly, recent studies have found a direct association between the immune system responses during the wound healing stage of tissue regeneration and the moulting system (Vafopoulou 2009). Their findings show that these immune responses affect the moult cycle, and that the moult cycle also affects immune responses (Vafopoulou 2009). Whilst studies have uncovered several important relationships affecting moulting and regeneration, control mechanisms of these vital biological processes remain largely unknown.

Wound healing and limb regeneration in crayfish

Regeneration is present in vertebrates (amphibians) and invertebrates (e.g., planarians and cnidarians) and has been widely studied within different taxa (Giudice, Turturici et al. 2008, Seifert, Monaghan et al. 2012). In most arthropods, the freshwater crayfish, *Orconectes limosus* have the potential to regenerate appendages in moults, after the co-occurrence of amputation (Durand 1960, Mariappan, Balasundaram et al. 2000). Freshwater crayfish species thus provide a useful model system which might advance knowledge on the molecular control required for crustacean limb regeneration and wound healing (Cooper 1998, McCall and Mead 2008). Of particular interest are the morphologies and mechanisms of nerve and neural repair, and muscular tissue regeneration (Cooper 1998, McCall and Mead 2008). Moreover, studying limb regeneration in the crayfish will help us to answer active biological questions, such as, how and why regeneration occurs and whether specific mechanisms regulate cellular reprogramming during tissue renewal (Allen, Ross et al. 2001, Khan, Schuster et al. 2001). The

freshwater crayfish provide advantageous models of regeneration due to their, high abundance, low maintenance, short development time, and simple reproduction by parthenogenesis (Vafopoulou 2009, Shinji, Gotoh et al. 2019, Tan, Gan et al. 2020). Regeneration of crayfish appendages is epimorphic, involving the recruitment and proliferation of epidermal cells which generate a blastema. The blastema-mediated epimorphic process of the crayfish appendages in *Orconectes rusticus* is thought to recapitulate aspects of its ontogeny and early development, and shares similar regenerative steps, with that observed and documented in the land crab, *Gecarcinus lateralis* (Adamstone 1928, Durand 1960, Govind and Pearce 1985). For example, the reappearance of fast fibres appears to be similar in development and limb regeneration (Govind and Pearce 1985). The autotomised limbs are detrimental for crayfish survival and evolutionary studies suggest that crustacean regeneration is an important adaptive response to predation which increases organismal fitness, and some of the regenerative responses are shown to be conserved (Weismann 1893, Vermeij 1982, Gong, Yu et al. 2015, Dunoyer 2020).

Crayfish limbs are anatomically specialised and have been structurally and functionally distinguished by qualitative and quantitative observations in several crayfish species (Huxley 1896). The limbs of *Procambarus clarkii*, are structurally composed of multiple tissue types, including epidermis, muscle, blood vessels, and nerves (Nakatani and ŌTSU 1979, Shinji, Miyanishi et al. 2016). In the crayfish, *Austropotamobius pallipes* four distinct pairs of pereiopods (walking legs), extend from the thoracic and abdominal regions of the body tagmata (POND 1975, Mittenthal and Trevarrow 1984, Belanger and Moore 2013). The crayfish, *Orconectes rusticus* also use one pair of chelipeds (claws) for capture, reproduction, intra- and inter- specific manipulation, and capturing prey (Juanes and Smith 1995, Huber and Schroeder 2001, Seidel, Schaefer et al. 2007, Buřič, Kouba et al. 2009). The pereiopods and chelipeds of *Astacus fluviatilis* and *Procambarus Clarkii*, are composed of seven adjoining podomeres, in distoproximal direction these are dactylopodite, propodite, carpopodite, meropodite,

ischiopodite, basipodite and coxopodite (Huxley 1896, Reed 1904, Wood and Wood 1932). The major cheliped of *Cherax albidus* is symmetrical and every segment has an extensor and flexor muscle (Reed 1904, Dunoyer 2020). Observations show that the newly regenerated cheliped, following subsequent moults are smaller in size and are not identical in structure to the original cheliped (Dunoyer 2020). Furthermore, during the regenerative process, the regenerating cheliped might require a moult for hardening (Dunoyer 2020). However, future studies examining hardening of the cheliped during regeneration are essential.

Morphological and histological processes of regeneration have been determined in many species of crayfish, *Procambarus clarkii* and *Procambarus. fallax f. virginalis*, as described by several publications (DE REAUMUR 1712, Reed 1904, Emmel 1910, Shinji, Miyanishi et al. 2016). The morphological stages of chelae regeneration of young *Orconectes limosa* are subdivided into four stages; *lag phase, basal growth, plateau,* and *premolt growth* respectively (Durand 1960). During stage one, the lag phase, no growth occurs and this lasts for six days (Durand 1960). In the second stage, known as basal growth, there is rapid growth of the regenerating limb (Durand 1960). Stage three, known as the plateau, involves period in the intermoult period (Durand 1960). In stage four, the premolt, rapid growth occurs again 3-5 days before the moult (Durand 1960).

Tissue repair appears to be a complicated process involving different mechanisms, local recruitment of different cell types and nerve innervation at site of injury. The successive events of tissue regeneration are known to differ between the type of appendage, stage of the moult cycle, species, and level of amputation (Waterman 2012, Edgecombe and Legg 2013, Dunoyer 2020). Simultaneously, the speed of regeneration differ with type of appendage, stage of the moult cycle, and environmental factors (Waterman 2012). It is reported that crayfish show a high occurrence of regeneration in their lifespan (McCall and Mead 2008). Observational studies in species such as, *Procambarus clarkii*, clearly demonstrate that limbs regenerate

throughout both juvenile and adult life stages and the variation in age could have prominent differences on growth rates during limb regeneration (Cooper 1998). Younger crayfish exhibit rapid growth rates and the regenerating limbs are replaced within several days (Cooper 1998, Mariappan, Balasundaram et al. 2000). In comparison, adult limb regeneration takes much longer and is completed in generally several months to one year (Dunoyer 2020). The regenerating claws of adult crayfish, *Orconectes rusticus* spend a longer time in the limb bud stages and exhibit longer intermolt periods, in contrast to juvenile crayfish (Govind and Pearce 1985). It is unclear why crayfish require two moult cycles for complete limb regeneration, and we hope that future studies will investigate the precise timing and length of regeneration during the moult cycle in different species of crayfish (Shinji, Miyanishi et al. 2016). The pereiopods of the crayfish, *Cherax destructor* are relatively strong, and in comparison, to the chelipeds, are not as easily removed during autotomy (Reed 1904, West 1997, Waterman 2012).

The regeneration is preceded by limb autotomy, which has been extensively studied and described in various animal taxa from an ecological and developmental perspective (Needham 1965, McVean 1982, Fleming, Muller et al. 2007, Alupay 2013). Limb autotomy in crustaceans is a reflex severance of an appendage, used to elude capture from a predator and escape a foul moulting event (Wood and Wood 1932, Maginnis 2006, Seifert, Kiama et al. 2012). After injury to the nerves of the distal podomeres, of the crayfish, *Cherax albidus*, autotomy occurs readily along a specific performed breakage plane (Figure 1) (Dunoyer 2020). No muscles cross the breakage plane, thus limiting wounding and major hemolymph loss (Bliss 1960). The performed breakage plane is morphologically specialised and characterised by decreased thickness to enable easy fracture (FINDLAY and MCVEAN 1977, Cooper 1998). The preformed breakage plane of *Procambarus clarkii*, also called the basi-ischium segment passes between the ischiopodite and basipodite segments of the pereiopod or cheliped (Koenemann and Jenner 2005, Castro, Davie et al. 2015). Regeneration of crayfish limbs is most efficient

and occurs more rapidly when limb autotomy occurs at the breakage plane (Needham 1953). Injuries or wounds that take place external to the breakage plane are generally larger, and additional damage to the carapace and epidermis underneath leads to the formation of a new exoskeleton by inducing the moult cycle (Chang and Thiel 2015). This induced moult cycle varies in duration compared to the onset of the moult cycle in other circumstances (Hopkins 2001). Fracturing is initiated by the powerful and simultaneous contraction of the larger basi-ischiopodite anterior levator (AL) and posterior levator muscle (PL) muscle (McVEAN 1974). Although, the mechanisms of limb autotomy have been studied, not all aspects of autotomy have been determined. A more thorough description of the breakage joint in crayfish is needed to improve our understanding of the regeneration process, as the regenerated limb will grow at this region (Emmel 1910).

Morphological and histological studies indicate that tissue regeneration begins with epidermal wound healing, which are categorised into tissue regeneration, and consists of wound closure and epidermal repair (Vafopoulou 2009). Wound healing is initiated by a cascade of complex cellular events, which rapidly heal the wound and repair damaged tissue by activation of wound response genes (Vafopoulou 2009). Insects and crayfish might share similar regenerative strategies and the molecular mechanisms identified in insect wound healing are considered similar to those observed in crustaceans (Chang and Thiel 2015). Wound healing may also occur without successive regeneration, and with minimal tissue loss (Chang and Thiel 2015). The rapid rate of this process is vital as it ensures that injury to the exoskeleton is repaired quickly, reducing the risk of systemic infection (Vafopoulou 2009). The origin of the cellular cues initiating the series of cellular events remain unclear, although they are suggested to be either local or humoral (Bilandžija, Laslo et al. 2017). In arthropods, the humoral and cellular defences of the innate immune system are nodulation and encapsulation, secretion of antimicrobial peptides, phagocytosis and the intricate proteolytic cascades (Vafopoulou 2009,

Bilandžija, Laslo et al. 2017). Immune and neuroendocrine responses have critical roles in the regulation of wound healing and are activated during this process (Vafopoulou 2009). The innate immune response consists of circulating haemocytes (nucleated blood cells which coagulate), and the endocrine system controls E synthesis (Vafopoulou, Laufer et al. 2007, Vafopoulou 2009). Damage to the carapace and initiation of the wounding process results in a rapid increase in circulating E (Vafopoulou, Laufer et al. 2007).

Immediately after amputation of the injured limb, wound closure commences in the adult crayfish, *Cherax albidus* (Khan, Schuster et al. 2001). The potential role of nerves in arthropod tissue regeneration and wound healing have been intensively studied for several years, with findings to suggest they are essential for limb regrowth (Seifert, Monaghan et al. 2012, Goss 2013). For example, evidence suggests normal limb growth requires local nerve innervation, since limb denervation was shown to slow regrowth (Seifert, Monaghan et al. 2012, Goss 2013). In the crayfish, *Procambarus clarki* a nerve supply to the region of amputation was observed and is thought to be necessary for limb regeneration (Cooper 1998). Excitatory and inhibitory motor neurons are thought to innervate the limb muscles during both regeneration and original development (Cooper 1998). Successful limb regeneration is dependent upon the presence of the stump of the ganglionic root at the basis of the regenerating limb (Needham 1945). In the crayfish, O. Obscurus a single membrane known as the autotomy membrane (AM), swells, and covers the stump of the automised limb, closing off the wound (Figure 2) (Henry and Stevenson 1971, Smith 1990, Khan, Schuster et al. 2001, Castro, Davie et al. 2015). The expansion of the AM is caused by blood pressure in the main body cavity, the haemocoel (Castro, Davie et al. 2015). The AM is a special double-walled membrane which acts as a protective barrier against bacterial infection and prevents haemorrhage (Reed 1904, Chang and Thiel 2015). The AM merges inwards from the epidermis towards the pedal nerve (PN) and blood vessels, being markedly visible shortly after amputation (Reed 1904). Two blood vessels and a large PN

collectively transverse a small medial hole in the AM (Henry and Stevenson 1971, Hopkins 2001, Castro, Davie et al. 2015). During autotomy the PN becomes severed, and its distal ends pull into the stub of the coxa (Hopkins, Chung et al. 1999, Hopkins 2001). The most proximal and thickened portion of the AM remains adhered to the sheath of the cut PN (Hopkins, Chung et al. 1999, Chang and Thiel 2015). The function of the PN during limb regeneration is unclear, however it is suggested to be essential for muscle growth during arthropod limb regeneration (Hopkins, Chung et al. 1999, Hopkins 2001). Limb regrowth that follows autotomy is an efficient and rapid form of regeneration as no muscles cross the autotomy plane, therefore minimal amounts of tissue are lost besides the severance of the PN and some haemolymph sinuses (Reed 1904, Emmel 1910, Henry and Stevenson 1971, Hopkins 2001).

Wound healing is thought to be initiated by a rapid influx of recruited haemocytes that migrate to the wound site/hemococelic space of the coxopodite by chemotactic signals 30 minutes post autotomy (Chang and Thiel 2015). The haemocytes have a role in secreting the clotting and melanisation enzymes, enclosing invading bacteria, and potentially secrete growth factors and cytokines inducing wound repair (Johansson, Keyser et al. 2000, Chang and Thiel 2015). These cells originate and are stored in the enlarged space found between the double AM, and are recruited from the bloodstream (Hopkins, Chung et al. 1999). A majority of the granulocytes (characterised by their large granules) residing within this space disappear 2 to 4 days post autotomy (Hopkins, Chung et al. 1999), as their instantaneous degranulation beneath the AM rapidly produces a blood clot, which closes the wound in the AM (Khan, Schuster et al. 2001, Das 2015, Anger, Harzsch et al. 2020). During degranulation, zymogens are synthesised which facilitate the activation of the phenoloxidase (PO) enzyme via prophenoloxidase (proPO) (Vafopoulou 2009, Das 2015). The PO enzyme once activated functions by melanising the scab and engulfing pathogens at the wound site (Das 2015). Extensive clotting and melanisation occurs along the span of the AM, and together the AM and blood clot rapidly produce a dark

coloured scab within a few hours (Chang and Thiel 2015). Haemolymph clotting in crayfish is produced predominately by a system consisting of a clotting protein (CP) and transglutaminase, which is secreted by haemolymph (KOPÁČEK, HALL et al. 1993, Theopold, Schmidt et al. 2004). The dark colour of the scab is produced by the process of chemical sclerotization, and 7 to 10 days post autotomy the scab is lost (Chang and Thiel 2015).

The sheet of epithelium, known as the wound epidermis (WE) is found at the autotomy plane. The epidermal epithelium is made up of low columnar or cuboidal cells (Emmel 1910). Histological observations indicate that a few days post wound healing, hypertrophied epidermal cells present beneath the scab, migrate into the wound site, and undergo mitotic cell division and morphogenesis (Figure 2) (Chang and Thiel 2015, Das 2015, Dunoyer 2020). Beneath the specialised wound epidermis (WE) are highly distributed tiny fibroblast resembling cells known as blastocysts (Hopkins, Chung et al. 1999). The origin of these wound epidermal cells remains unknown (Das 2015). In the crayfish, *P. fallax f. virginalis*, 7 to 9 days post autotomy, continual differentiation of the epidermal cells leads to the generation of a critical structure, called the blastema, which merges from underneath the scab (Skinner, Bliss et al. 1985, Das 2015, Shinji, Miyanishi et al. 2016). In *P. fallax f. virginalis*, the blastema does not appear until the upcoming moult (Shinji, Miyanishi et al. 2016). The blastema is a restricted heterogenous mass of possibly mitotically active cells and progenitor cells, that differentiate into the multiple tissue types of the new regenerate, including connective, nerve, muscle and epidermis tissue (Khan, Schuster et al. 2001, Edgecombe and Legg 2013, Chang and Thiel 2015, Iismaa, Kaidonis et al. 2018). The blastema lengthens up until the presence of a digit-like growth (Dunoyer 2020). The wound blastema has been largely studied in the crayfish, and histology and electron microscopy have been utilised to investigate the different cell populations present in the blastema. This is particularly important, as a better understanding of the cell populations present in the blastema of regenerating limbs, is crucial to understanding

the activation and differentiation of the blastema. Based on the assumption of previous research, blastema formation could be dependent on an adequate nerve supply combined with signal interactions between the WE and the blastema. Further research is required to investigate the intricate interplay that could exist between the WE and blastema. Additionally, there remains a lack of research into the molecular and signalling pathways regulating blastema formation and this is worthy of further study (Qin, Fan et al. 2020). How does the tissue sources of the blastema regenerate into the different tissues?

Cells of the blastema reportedly undergo intense proliferation, which is succeeded by two distinct processes: dedifferentiation and redifferentiation (Tanaka 2003, Chang and Thiel 2015). Dedifferentiation is a mechanism of cellular reprogramming consisting of multiple steps, whereby a differentiated cell of the blastema loses its specification and attains an embryonic-like state (Tanaka 2003, Grafi 2004, Goss 2013, McCusker, Bryant et al. 2015, Stocum 2018). Whilst in redifferentiation, the cell reacquires this specialised function (Stocum 2018, Anger, Harzsch et al. 2020). The mechanisms by which blastemal cells have the potential to dedifferentiate is unclear, however it is thought that the growing blastema might receive positional signals regulating its development into the correct tissues (Allen, Ross et al. 2001). The cells of the blastema are readily quantified prior to extensive differentiation, as it has been reported that a limb blastema contains approximately 10, 000 cells (Tanaka 2003), however, the capacity of blastema cells for pluripotency or whether they possess a positional memory is unknown (Allen, Ross et al. 2001). There are two peaks of mitotic division in the growing blastema. The first peak was detected by labelling with tritiated thymidine incorporation into DNA, which revealed elevated mitotic activity four days post autotomy (Hopkins, Chung et al. 1999, Khan, Schuster et al. 2001, Das 2015). The second and biggest peak of mitotic activity within the blastema coincides with the presence of the papilla (Chang and Thiel 2015). After blastema formation, during the first moult cycle a protuberance of the coxa stump known as

the limb bud forms, which differentiates into a leg fragment across successive moults (Hopkins 2001, Chang and Thiel 2015, Dunoyer 2020). The limb bud of crayfish, *Orconectes Obscurus* is colourless protuberance from the tissue at the autotomy plane (Henry and Stevenson 1971). The regenerating limb bud is a small, non-functional limb and is subdivided into exopodite and endopodite (Mittenthal 1980, Dunoyer 2020). During the early of outgrowth limb bud it is exceedingly soft (Cooper 1998, Hopkins, Chung et al. 1999). The limb bud elongates into a papilla (PL). The growth of the basal bud is fast, asymmetrical, and begins with the most distal segments of the limb. The rate of regeneration of the basal bud at this stage is suggested to be regulated by the same factors controlling internal tissue growth (Henry and Stevenson 1971). The structural changes of the growing limb bud in crustaceans have been extensively studied by using histological and immunohistochemical approaches (Das 2015). There are four continuous stages of limb bud development in adult crayfish, *Cherax albidus*, during cheliped regeneration which have been characterised based on morphological changes (Dunoyer 2020). During stage 1, the basal bud is

In the crayfish, Procambarus clarkii the delicate limb bud, while confined to its cuticular sac, contains the tissues of the following pereiopod podomeres: basium-ischium, merus, carpus, propodus, and the dactylus (Cooper 1998). The continuous growth of the limb bud was either observed until ecdysis or the growth halted in short or prolonged (Henry and Stevenson 1971). The length of the regenerating limb in increases across each subsequent moult until it reaches the normal limb length (Reed 1904). The basal bud morphology differs across various decapod crustacean species (Edgecombe and Legg 2013). In the crayfish, the papilla is initially a small, externally visible and simplistic structure (Chang and Thiel 2015). It is 1-2mm in length and is comprised of only one segment with no internal components (Skinner, Bliss et al. 1985, Chang and Thiel 2015). The growth of the first podomeres occurs via the invagination of epidermal cells which are located beneath the scab (Das 2015). Other wound epidermal cells, that do not

produce the blastema, generate a pliant cuticle which develops into a crenulated sac or sheath (Edgecombe and Legg 2013, Das 2015, Anger, Harzsch et al. 2020). This thin, translucent and expanding sac is exclusive to crustaceans, encasing and protecting the growing limb bud (Edgecombe and Legg 2013, Chang and Thiel 2015, Das 2015). The regenerating tissue residing within the cuticular sac is folded until it is released from its confinement in the upcoming moult, becoming fully lengthened (Edgecombe and Legg 2013).

The regeneration and growth of the PN is simultaneous with the regrowth of the limb segments, postulated to begin regrowth six to ten days after autotomy (Hopkins, Chung et al. 1999, Chang and Thiel 2015). The severed distal ends of the pedal nerve are in close contact with the growing blastema, elongating into a conical shape (Hopkins 2001, Chang and Thiel 2015). Nerve tissue regeneration appears to occur from the proximal stumps of the coxa (Cooper 1998, Goss 2013). During autotomy the cell body of the motor neuron remains in the central nervous system (CNS) ganglia, whilst the distal processes are lost and must regrow from the proximal stub (Cooper 1998). Motor axon regeneration occurs at first through the activation of the remaining distal ends by satellite axons (Bouton and Bittner 1981). The cell bodies and the proximal axons of the sensory neurons are situated within the limb, and thus are lost during autotomy (Cooper 1998). During limb regeneration, new sensory bodies must develop and eventually regrow axonal processes (Cooper 1998). In order for the regenerated limb to attain proper functioning, new sensory neurons must grow and inaugurate a connection with the ventral nerve cord (Cooper 1998). After the limb is autotomised, the distal axon remnants of the sensory neurons and the proximal portion of the motor axons continue to reside within the nerve trunk, in close proximity to the autotomy plane (Cooper 1998).

In normal limb development, as well as in regeneration, tissue patterning and cellular fate specification are integral for the new developed tissue to acquire its proper function (Khan, Schuster et al. 2001, Goldman and Poss 2020). Tissue patterning involves the temporo-spatial

organisation and differentiation of cell subpopulations, under the regulation of specific control genes (Khan, Schuster et al. 2001). The molecular mechanisms and genetic networks of tissue patterning in crustacean regeneration are largely unknown, and further research is therefore essential to uncover these vital biological processes (Khan, Schuster et al. 2001).

Regeneration in crayfish is thought to be influenced by localised tissues, positional information, and interactions between regenerating limbs (Mittenthal, Olson et al. 1980). Transplantation of a regenerating leg or limb bud provides a technique for examining the relative roles of the host tissues on growth of the regenerating limb. The host site functions by regulating the growth of the homoeotic leg, at the growth rate of the normal leg (Mittenthal, Olson et al. 1980). Upon transplant of homoeotic legs to an ectopic host site, the pereiopod regenerating from the coxopodite of a cheliped exhibited larger dimensions, fibre length, fibre diameter and closer muscle mass (Mittenthal, Olson et al. 1980). Whilst, a cheliped regenerated from the coxopodite of the pereiopod, however, smaller in size than the normal cheliped (Mittenthal, Olson et al. 1980). The crayfish pereiopod and cheliped is surrounded by two types of "morphogenetic fields" (M-fields), a series of segmental fields, and a limb field which act as a control system (Mittenthal and Trevarrow 1983, Mittenthal and Nuelle 1988). In developmental biology, the M-field refers to a distinct region containing a clustering of cells which respond to local biochemical signalling, producing the specific organisational structure of an organ or appendage. In crayfish, grafting operations indicate segmental fields influence the normal development and growth of limbs (Mittenthal and Trevarrow 1983, Mittenthal and Nuelle 1988). The limb field appears to be essential for regulating limb morphogenesis and growth (Mittenthal and Trevarrow 1983). The limb field is organised in a proximodistal sequence, one limb field for each podomere, with each field considered to be equal (Mittenthal and Trevarrow 1983). The segmental field serves as a map between the various limb podomeres (Mittenthal and Trevarrow 1983). It is thought that the segmental morphogenetic field regulates

tissue regeneration of the 2nd, 3rd, 4th, and 5th pereiopod (Mittenthal and Trevarrow 1983). However, the link between limb development and regeneration is based on a small number of studies, and requires further research (Edgecombe and Legg 2013).

Regulation of crayfish tissue regeneration

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The molecular mechanisms and signalling pathways governing tissue regeneration in crayfish are crucial for the growth and development of a functional limb (Chang and Thiel 2015, Das 2015, Shinji, Gotoh et al. 2019). It may be presumed that the signalling pathways regulating crayfish limb regeneration are complex and conserved by nature, although our understanding of these molecular mechanisms is rudimentary (Das 2015, Shinji, Miyanishi et al. 2016). The limited knowledge means we are unable to determine the evolutionary origins of tissue regeneration in crayfish (Shi and Massagué 2003). Several animal models of regeneration, including crayfish, demonstrate successful growth of a blastema requires molecular signalling (Khan, Schuster et al. 2001). A majority of the studies examining regeneration in crayfish have focused primarily on the hormonal control of limb regrowth (Hopkins, Chung et al. 1999, Khan, Schuster et al. 2001, Das 2015). Studies quantifying changes in hormone levels by utilising RIA, high-performance liquid chromatography, and enzyme-linked immunosorbent assay, indicate the invertebrate steroid hormone E, which controls the moult cycle, is a principle regulator of crayfish tissue regeneration, limiting the regenerative potential (Table 1) (Durica and Hopkins 1996, Spaziani, Mattson et al. 1999, Khan, Schuster et al. 2001, Das and Durica 2013, Das 2015). Findings of these studies in crustaceans indicate specific E concentrations in the haemolymph are linked to particular stages of pereiopod regrowth (Das 2015). Larger dimensions of the crayfish in comparison to other phyla make them extremely beneficial for measuring haemolymph hormone levels (Das 2015). In the crayfish, considerably low levels of endogenous E circulate in the haemolymph up until the commencement of tissue

regeneration and during the basal period of limb regrowth (Smagghe 2009, Chang and Thiel 2015, Das 2015). It is thought that low E levels are essential for cellular differentiation and are enough to sustain tissue renewal, whilst not precipitating the onset of ecdysis (Chang and Thiel 2015). As the regenerating limb grows and becomes larger in size, circulating E levels in the haemolymph steadily rise (~16-17ng/ml) (Smagghe 2009). In proecdysis, E levels further increase and peak at 75-135ng/ml, during which time the basal bud ceases growth (Smagghe 2009, Das and Durica 2013). The effects of administration of exogenous E and 20E on limb regeneration was investigated across various crustaceans in vivo, appearing to differ substantially (Chang and Thiel 2015). Exogenous E can be removed very rapidly from the haemolymph, and the diverse range of its effects are thought to be due to variation in elimination rates and/or Y organ responsiveness (Chang and Thiel 2015). In crayfish, increased E levels inhibit all stages of limb regeneration including blastema formation, early, and late growth (Chang and Thiel 2015). E appears to exert its function by binding to and activating the cognate nuclear receptor (NR) heterodimer complex, which consists of the ecdysone receptor (EcR) and the retinoid receptor (RXR), both of which are located in specific tissues (Hopkins, Chung et al. 1999, Khan, Schuster et al. 2001, Smagghe 2009, Das 2015, Chen, Wang et al. 2017). These nuclear receptors are ligand-activated transcription factors which modulate genetic expression by attaching to the hormone response element (HRE) (Robinson-Rechavi, Garcia et al. 2003, Das and Durica 2013, Das 2015). Following nuclear receptor activation by E, a cascade of protein-coding genes is transcribed (Chen, Wang et al. 2017). In crustaceans, the nuclear ecdysone receptor has been isolated and cloned (Chang and Thiel 2015). Immunohistochemical evidence shows these ecdysteroid receptors are present in the nucleus of the haemocytes, muscle and epidermal cells residing within the tissues of the blastema and regenerating limb (Das and Durica 2013). Further, a profusion of ecdysone receptors are present in the limb bud tissues (Das and Durica 2013, Chang and Thiel 2015). In crustaceans,

ecdysteroid receptor signalling has been suggested to possibly regulate proliferation and differentiation of blastema cells (Chang and Thiel 2015, Shinji, Miyanishi et al. 2016). The ecdysone gene, EcR, thought to encode for the ecdysone receptor, has been obtained and sequenced in crustaceans and various insects (Durica and Hopkins 1996, Hopkins 2001, Chen, Wang et al. 2017).

Hormones, growth factors, and signalling pathways	**Role in crayfish tissue regeneration**	**Reference**
Ecdysteroids (E)	• A principle regulator of crayfish tissue regeneration, which acts by limiting regenerative potential. • Low E levels are vital for cellular differentiation and for sustaining tissue renewal • High E levels (75-135ng/ml) inhibit all stages of tissue regeneration, specifically blastema formation and its early/late growth	(Hopkins, Durica et al. 2008)
Retinoids (R)	• Might potentially upregulate the entire UpRXR mRNA expression within the blastema	(Hopkins, Durica et al. 2008)
Melatonin (MLT)	• Amplifying growth of the regenerating limb buds and ultimately increasing rates of regeneration	(Zhang, Yang et al. 2018)
TGF-β/activin signalling	• Inhibits cellular proliferation of the limb bud, slowing tissue regeneration	(Shinji, Gotoh et al. 2019)
Fibroblast growth factors (FGFs)	• Regulates blastema growth	(Hopkins 2001)

TGF-β is a superfamily of cytokines consisting of several subfamilies including bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), the TGF- β subfamily, and the activin/inhibin family (Kingsley 1994, Shi and Massagué 2003, Kubiczkova, Sedlarikova et al. 2012). Transforming growth factor -β (TGF-β) signalling is an intricate

cellular growth pathway, whose components are highly conserved across evolutionary distances (Shinji, Miyanishi et al. 2016, Shinji, Gotoh et al. 2019). It is thought this signalling diverged from a common ancestor Protostomia and Deuterostomia, during the Precambrian period approximately 4.5 billion years ago (Kingsley 1994, Konstantinides and Averof 2014, Shinji, Gotoh et al. 2019). Following research in the last decade our knowledge of the precise cellular and molecular mechanisms of the TGF-β signalling pathway has expanded (Kingsley 1994, Shi and Massagué 2003). In particular, TGF-β signalling has been extensively studied in the fruit fly, *Drosophila melanogaster*, using classical molecular and genetics approaches (Peterson and O'Connor 2014, Shinji, Miyanishi et al. 2016). However, in decapod crustaceans the structures of the ligands and receptors of this important signal transduction pathway remain to be elucidated, and thus require further research (Shinji, Miyanishi et al. 2016). In crustaceans and insects, TGF-β signalling is considered to have an upstream role in a wide range of cell behaviours, such as proliferation, differentiation, adhesion, apoptosis and migration (Shi and Massagué 2003, Huang and Chen 2012, Das 2015). TGF-β signalling is also thought to be a potential candidate for regulating arthropod pereiopod regeneration (Shinji, Miyanishi et al. 2016). The TGF-β signalling cascade appears to be triggered by the dimetric TGF-β ligands, which bind and join together the type I (TβRI) and type II (TβRI) serine-threonine kinases located on the cell membrane (Shi and Massagué 2003, Huang and Chen 2012, Peterson and O'Connor 2014). There are two type II receptors, Punt (Put) and Wishful thinking (Wit), both of which are components of the activin/BMP pathway. The type I (TβRI) receptor is distinct to the specific pathway (Shinji, Miyanishi et al. 2016). As the TGF-β ligand attaches to the receptors, the type I (TβRI) receptor is phosphorylated by the type II (TβRI) receptor (Shinji, Miyanishi et al. 2016).

Activin signalling is a branch of the TGF-β pathway thought to regulate tissue regeneration in crayfish limbs (Shinji, Gotoh et al. 2019). Activin signalling in vertebrate tissue regeneration,

for instance the zebrafish, has been examined and is thought to control cell proliferation within the blastema (Jaźwińska, Badakov et al. 2007, Shinji, Miyanishi et al. 2016). This pathway consists of ligands, a downstream transcription factor complex and a receptor complex (Shinji, Gotoh et al. 2019). The activin downstream transcription factor, Smox (R-Smad), acts through receptor type-I TGF-β, termed Baboon (Babo) (Shinji, Miyanishi et al. 2016). R-Smad has been identified and cloned in the crayfish, and its function determined in account of the other components of this pathway (Shinji, Gotoh et al. 2019). Activin signalling through R-Smad is considered to be vital for tissue regeneration in decapod crustaceans (Shinji, Gotoh et al. 2019). In crayfish, RNAi gene knockout of R-Smad resulted in delayed growth of the regenerating limb blastema, and smaller limb length, in contrast to the controls (Shinji, Gotoh et al. 2019). Despite the shorter limb length, the regenerate had the morphology and dimensions of the control crayfish limb. Thus, activin signalling via R-Smad appears to function by mediating pereiopod growth and length, independent of leg pattern, through regulating blastema cell proliferation (Shinji, Gotoh et al. 2019). RNAi gene knockout of receptor Babo elevated expression levels of R-Smad, indicating their regulatory molecular pathway (Shinji, Gotoh et al. 2019). Further research is essential in order to determine the precise interactions between TGF-β/activin signalling in relation to various other signalling pathways, such as E and BMP, including their postulated regulation of Smox transcription (Shinji, Gotoh et al. 2019).

Retinoids are derivates of vitamin A thought to have a significant role in vertebrate limb development by providing necessary signals, as well as in tissue patterning during vertebrate limb regeneration (Hopkins, Chung et al. 1999, Hopkins 2001, Das and Durica 2013). Endogenous retinoids have been detected in the growing tissues of the frog, chick, and axolotl during limb regeneration (Hopkins 2001). The retinoid hormones, all-trans retinoic acid and 9-cis retinoic acid (9cRA) were measured and detected in the blastema of a regenerating crustacean four days following autotomy (Hopkins 2001). It was reported the blastema had

small quantities of both all-trans retinoic acid and 9cRA, 19pg/µg and 83pg/µg respectively, whilst the origin of these hormones is currently unclear (Hopkins 2001). Several studies have investigated the effects of exogenous retinoids on tissue regeneration (Hopkins 2001). In vertebrates, exogenous retinoids result in respecified tissue patterning (Hopkins 2001). Administration of exogenous retinoids in crustaceans in vivo, led to an interruption of limb regeneration during blastema formation by inhibiting proliferation whilst inducing the differentiation of blastema cells (Hopkins 2001, Hopkins, Durica et al. 2008). Retinoids might further upregulate the entire UpRXR mRNA expression within the blastema (Hopkins, Durica et al. 2008).

Fibroblast growth factors (FGFs) are a family of peptide growth factors consisting of 22 members which have been isolated and identified (Hopkins, Chung et al. 1999, Eswarakumar, Lax et al. 2005, Yun, Won et al. 2010). FGF exerts its effects by attaching to and activating distinct isoforms of four cell surface receptors, namely FGFR1, FGFR2, FGFR3, and FGFR4 (Eswarakumar, Lax et al. 2005). These four receptors are part of the tyrosine kinase superfamily. FGFs were first detected in vertebrates and are thought to be crucial for the normal development of limb buds (Hopkins 2001). A disruption of FGF function may result in multiple developmental defects. FGFs have a role in cellular proliferation, differentiation, migration, and angiogenesis (Yun, Won et al. 2010). FGF homologous genes have also been detected in invertebrates (Hopkins 2001). In crustaceans, immunohistochemical evidence indicates fibroblast growth factor-2 (FGF-2) is expressed in the distal ends of the severed pedal nerve following autotomy (Hopkins 2001). FGF-2 and FGF-4-like were also stained in the cytoplasm of the epidermal cells of the basal bud (Hopkins 2001), suggesting that several FGF-like growth factors have a role in blastema growth during crustacean limb regeneration (Hopkins 2001).

MLT, N-acetyl-5-methoxy-trytamine, is a hormone produced in most organisms. In humans it is synthesised by the pineal gland (Sainath, Swetha et al. 2013, Walker 2017, Zhang, Yang et

al. 2018). MLT has been extensively studied in vertebrates, however its function in invertebrates is yet to be determined (Sainath, Swetha et al. 2013, Zhang, Yang et al. 2018). In crustaceans, MLT has been detected in several tissues and organs, such as the cranial ganglia, eye stalk ganglia, and hemolymph, utilising HPLC, ELISA and RIA (Sainath, Swetha et al. 2013, Zhang, Yang et al. 2018). MLT appears to be evolutionarily conserved in crustaceans and is considered vitally important for organism physiology (Sainath, Swetha et al. 2013). In crayfish, MLT receptors were detected in the eyestalk using the distinct melatonin receptor - agonist-8-methoxy-propianomidotetralin and antagonist, *N*-pentanoyl-2-benzyltryptamine (Mendoza-Vargas, Solís-Chagoyán et al. 2009, Sainath, Swetha et al. 2013). It has been suggested that MLT in crustaceans functions by regulating the moult cycle, controlling hemolymph glucose levels, mediating circadian rhythm, and potentially promoting tissue regeneration (Tilden, Rasmussen et al. 1997, Zhang, Yang et al. 2018). Several studies have investigated the effects of applied exogenous MLT within the eyestalks (~60µg/ml), on cheliped and pereiopod regeneration in crustaceans (Tilden, Rasmussen et al. 1997). Their findings indicate that MLT appears to exert its effects by significantly amplifying the growth of the regenerating limb buds, and ultimately increasing rates of tissue regeneration (Tilden, Rasmussen et al. 1997, Zhang, Yang et al. 2018). Furthermore, MLT appears to promote regeneration by upregulating the expression of growth specific genes (Zhang, Yang et al. 2018). Collectively these studies suggest the importance of MLT in the promotion of tissue regeneration in crustaceans, highlighting the need for further investigation of the signalling mechanisms involved.

The genetic basis of crayfish tissue regeneration

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Recent regenerative studies have predominately focused on gene regulation and its post-translational modification by utilising a variety of genetic and epigenetic approaches, including genome and transcriptome sequencing, CRISPR-Cas9, RNAi, and chromatin

immunoprecipitation-sequencing (ChIP-seq) (Chen and Poss 2017, Zhu, Xiao et al. 2018, Suzuki and Ochi 2020). Following injury or amputation the expression patterns of hundreds or even thousands of genes are thought to be altered intricately (Goldman and Poss 2020). It is these variations in gene expression levels that initiate and facilitate key regenerative events, such as wounding healing, cell proliferation, and tissue patterning (Goldman and Poss 2020). In regenerating tissues of the salamander limb, the *Prod1* gene is expressed and is considered to be regeneration specific (Kumar, Gates et al. 2007, Goldman and Poss 2020). *Prod1* has a functional role in the directional growth of the limb during regeneration (Kumar, Gates et al. 2007). Emerging evidence strongly suggests epigenetic modifications exist in the crustacean genome, of which might be integral for successful tissue regeneration (Zhu, Xiao et al. 2018). Examples of epigenetic processes include DNA methylation, histone chemical modification, non-coding RNA, chromatin remodelling complexes, and microRNAs (Zhu, Xiao et al. 2018). In crustacea, the genome of *Daphnia pulex*, a freshwater crustacean was sequenced, rigorously analysed, and it appears that the modification, 5-methyl-cytosine (5-mC) and 5-hydroxymethyl-cytosine (5-hmc), is present in its DNA, implicating epigenetic regulation (Rouhana and Tasaki 2016). To date very little is known about the genes governing the rapid tissue regeneration process in crayfish, or how epigenetics may influence its expression. This is due to the lack of available genetic and molecular tools for crustaceans, particularly for freshwater crayfish. The lack of genomic resources continues to hinder advances for the use of crayfish, as a suitable experimental model of regeneration, despite its great potential. For example, the crayfish has arguably greater regenerative potential than other crustacean species, such as *P. hawaiens*is (whose genome has recently been sequenced and intensively studied). Genes expressed in the regenerating tissues of imaginal discs and insect legs have also been suggested to have a role in crustacean limb regeneration (Chang and Thiel 2015). Similarly, it is thought that homologous genes regulating limb muscle regeneration in vertebrates could

control limb muscle regeneration in the crayfish (Shinji, Miyanishi et al. 2016). Whilst the control genes involved in other species are thought to regulate crayfish limb regeneration, further research is necessary to verify these claims. Several studies have successfully demonstrated that multiple developmental genes are reactivated and re-expressed during tissue regrowth in vertebrates and invertebrates (Suzuki and Ochi 2020). It has been well established that these developmental control genes are highly conserved, as their reuse in regeneration requires various *cis*-regulatory elements, including promoters and enhancers (Suzuki and Ochi 2020). Homeotic (*Hox*) genes (also called selector genes) are proteins which are thought to be deeply conserved by evolution in animals, plants and fungi (Krumlauf 1994, Deutsch and Mouchel-Vielh 2003, Hrycaj and Wellik 2016, Vivian, Leclerc et al. 2019). The *hox* gene was first identified in the hexapod, *Drosophila* by using mutagenesis screens (Lappin, Grier et al. 2006, Hrycaj and Wellik 2016). *Hox* genes, considered to be a subgroup of the homeobox genes, encode for transcription factors, which contain an evolutionarily conserved DNA binding protein, called the homeodomain (Lappin, Grier et al. 2006, Wang, Helms et al. 2009, Hrycaj and Wellik 2016). Earlier studies in *Drosophila* indicate *hox* gene expression is crucial for the patterning and stipulation of segmental identity adjacent to the anterior-posterior (AP) or cranio-caudal axis during embryogenesis, in a diverse range of metazoa (Averof and Akam 1995, Chang and Thiel 2015, Hrycaj and Wellik 2016). Mutations in one or many *hox* genes can cause fragmented or entire changes to the body segmentation of an organism (Myers 2005). *Hox* genes have since been cloned and investigated in several species, including the hydra (Hrycaj and Wellik 2016). In arthropods, the *hox* gene and its functional role have been extensively examined and well characterised (Chang and Thiel 2015). The arthropod *hox* complex is considered to consist of ten genes, namely: lab, pb, zen, Dfd, Scr, ftz, Antp, Ubx, abdA, and AbdB (Deutsch and Mouchel-Vielh 2003). More specifically, the crustacea phyla have very diversified and unique body plans, which are not observed in any other species

(Deutsch and Mouchel-Vielh 2003). The appendages of crustaceans develop from the abdominal, thoracic and head segments (Chang and Thiel 2015). In crustaceans, abdominal A (abdA) and ultrabithorax (*ubx*) are products of the *hox* gene, which appear to function by controlling appendage number (Deutsch and Mouchel-Vielh 2003). The *ubx* gene is thought to be expressed within the thoracic appendages of several crustaceans, as RNA gene knockout of *ubx* indicates this gene functions towards differentiating the thoracic limbs from the anterior maxillipeds (Chang and Thiel 2015). In the axolotl, data analysis revealed that homeobox-containing genes are expressed during limb development and regeneration (Gardiner and Bryant 1996). Although there is currently no direct evidence, *hox* genes are also thought to be a potential gene candidate in crayfish tissue regeneration (Chang and Thiel 2015). The biology of the *hox* genes still remains elusive, and thus further functional analysis of these genes in the developing crustacean limb is vital for determining the various other developmental genes involved in limb development, and regeneration (Chang and Thiel 2015, Hrycaj and Wellik 2016).

Summary

Commented [SB4]: Nice summary, keep going and include the important points for conclusion.

In the past decade there has been substantial progress in the field of regeneration, resulting from a global collaborative effort, and recent technological advances of molecular biology. The use of simple animal models has enabled scientists to address a plethora of unanswered questions encompassing the phenomena of tissue regeneration. Although our understanding of this process is very limited, recent cutting-edge studies have started to elucidate the cellular and molecular mechanisms underlying crayfish limb regeneration, such as the TGF β/activin signalling pathway, and MLT by utilising RNAi gene knockout and RIA respectively. However, we are yet to complete genome sequencing of crayfish and various crustaceans. The

development of new genetic tools would enable us to determine the genes controlling local factors, which consequently regulate tissue regeneration. Future experiments should explore cell populations present in the blastema at different time points and decipher the origin of the epidermal cells giving rise to the regenerating tissues. For instance, morphological, molecular, and phenotypic characterisation of the undifferentiated blastema. This may potentially be determined by utilising cell population marking and tracking approaches and lineage tracing tools. Magnetic resonance imaging (MRI) could also be applied to small crustaceans, which will enhance internal tissue visualisation by reducing loss of spatial information. This powerful tool offers us the potential to refine previous experimental designs and findings, and further advance our knowledge on the morphological complexities of tissue regeneration in crayfish and crustaceans.

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Author contributions

M.F. contributed by initial writing of the draft manuscript. S.B. and D.C edited the manuscript. J. C., and X. H. provided discussion, advice, and revision points during the process of review paper formation. D.C. X. H. and J.X. discussed and formulated the idea. J.X. coordinated this review and revised the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1. The autotomy membrane. A schematic illustration showing the autotomy membrane (AM) at the early wound healing stage of tissue regeneration and in the newly formed pereiopod. (A) In the regenerated appendage the AM is an internal structure, for which only blood vessels and nerves can pass through. (B) Following autotomy, at the early wound healing stage the PN is severed at the predetermined fracture point proximal to injury, releasing fibroblast growth factor-2 (FGF-2). The protective AM then rapidly expands and swells covering the base of the automised limb, a melanised scab forms underneath the AM.

Figure 2. Blastema development and growth in crayfish. (A) Epidermal cells migrate, mature and go through continual differentiation developing into mitotically active cells and undifferentiated lineage-confined progenitor cells. (B) The early cell mass is in direct contact with severed nerve fibres of the amputated limb and must undergo both dedifferentiation and

redifferentiation. Fibroblast growth factor, retinoids, R-Smad signalling and ecdysone signalling are considered necessary for sustaining its growth. (C) The fully developed blastema.