2020

FROM ORGANISMS TO ECOSYSTEMS: IMPACTS OF LIMB LOSS AND REGENERATION ON CRAYFISH BEHAVIOR

Luc Arnaud Dunoyer
University of Kentucky, lucdunoyer@gmail.com
Author ORCID Identifier:
https://orcid.org/0000-0001-6573-3844

Digital Object Identifier: https://doi.org/10.13023/etd.2020.244

Recommended Citation

Dunoyer, Luc Arnaud, "FROM ORGANISMS TO ECOSYSTEMS: IMPACTS OF LIMB LOSS AND REGENERATION ON CRAYFISH BEHAVIOR" (2020). *Theses and Dissertations--Biology*. 64.
https://uknowledge.uky.edu/biology_etds/64

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Luc Arnaud Dunoyer, Student

Dr. Ashley Seifert, Major Professor

Dr. Dave Weisrock, Director of Graduate Studies

FROM ORGANISMS TO ECOSYSTEMS: IMPACTS OF LIMB LOSS AND REGENERATION ON CRAYFISH BEHAVIOR

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
at the University of Kentucky

By

Luc Arnaud Dunoyer

Lexington, Kentucky

Co- Directors: Dr. Ashley Seifert, Professor of Biology

and Dr. Jeremy Van Cleve, Professor of Biology

Lexington, Kentucky

2020

https://orcid.org/0000-0001-6573-3844

ABSTRACT OF DISSERTATION

FROM ORGANISMS TO ECOSYSTEMS:
IMPACTS OF LIMB LOSS AND REGENERATION
ON CRAYFISH BEHAVIOR

The ability of some organisms to regenerate tissues and organs has fascinated naturalists since antiquity, dating back to the earliest accounts of scientific inquiry with Aristotle in Ancient Greece. Then, Darwin's theory invigorated some scientists' dream of stimulating (or reactivating) regenerative capacities in human beings by showing that we are related to highly regenerative organisms. More recently, a renewed interest in discovering the molecular and genetic basis for organ and tissue regeneration has led biologists to focus more specifically on a restrictive set of model organisms.

Although the process of limb regeneration is different between invertebrate and vertebrate organisms, it follows the same general principles and steps. The presence or absence of regenerative ability appears to broadly correlate with the incidence of autotomy (reflex severance of a limb). Autotomy allows individuals to reduce costs associated with damaged structures, often limbs, by severing the appendage at a predetermined breakage plane, thus limiting wounding and helping the regeneration process. Individuals capable of rebuilding damaged structures during their lifespan may have higher fitness than individuals without this. This may help to explain how selective pressures for regenerative capacities could be maintained or acquired. The regenerative process, induced by autotomy, may also have high physiological costs and this comes with its own set of challenges in turn affecting how regenerating individuals interact with and change their environment.

Organisms capable of regeneration can also have impacts on their environment through their behavior. Therefore, regeneration of structures important for behavioral effects on the environment can mediate organisms' environmental impacts. Recently, these reciprocal impacts have been the focus of new theory conceptualizing the ecological implications of organismal habitat, namely, ecosystem engineering (defined as the process by which organisms modify their physical environment from one physical state to another).

Furthermore, regeneration creates periods of time during which regenerating organisms face newfound challenges mediated by the way those organisms interact with their environments. Once again, regenerating crayfish might not be able to dig a burrow when needed (during drought, reproduction, etc.). Thus, they could find themselves competing for existing burrows or facing a now necessary migration to find water all of which non-regenerating crayfish may not experience. Therefore, ecosystem engineers capable of regeneration represent a powerful system allowing us to develop a clearer understanding of how regeneration might evolve as well as persist through its effects on organismal fitness.

KEYWORDS: autotomy, regeneration, crayfish, *Procambarus clarkii*, ancestral state reconstruction, animal behavior

Luc Arnaud Dunoyer

04/25/2020

Date

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Dr. Ashley Seifert
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Dr. Dave Weisrock
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Date

DEDICATION

To my family in France who never stopped believing in me even when I stopped believing in myself. I love you all and missed you dearly over the past six year and a half.

ACKNOWLEDGMENTS

I wish to thank all the people who helped me complete this work over the last six and a half years of grueling mental labor. I will only name the most important people here; however, if you helped me during my time at UK, even a little as it may have been, you have my deepest thanks. First and foremost, I wish to thank Jacqueline Rae Dillard, my ex-wife without whom I would not have been able to complete this PhD. You have been with me during the ups and downs of this journey and you never faltered and for that I thank you. I also would like to thank my family in law, Karen and Jack, who have been very supportive of both Jacqueline and me during those years. Big thanks to the crayfish international community in which I found countless support and ideas (International Association of Astacology). Finally, I'd like to thank Ashley and Jeremy, my dissertation co-advisers and my committee members, Dave and Chuck, for continuous advice all along the way.

This dissertation work raised more questions than it initially set out to answer on the interactions between autotomy, regeneration, organisms, and their environments. The next steps in my exploration of these problems is to continue to scale up the approach I have followed here by building a mathematical model of ecosystem engineer populations to investigate large scale consequences of autotomy and regeneration on life history traits. Additionally, I intend to investigate further the behavioral consequences of autotomy and regeneration on physiology and behavior of ecosystem engineers in the laboratory and in the field. The new setting where I find myself today (as a full time faculty member teaching biology mainly to students intending to transfer from Wake Technical Community College where I teach to North Carolina State University) provides me with great opportunities to continue my basic research on the impacts of autotomy and regeneration mediated by crayfish ecosystem engineering. I am excited for the next chapter of my scientific life and cannot wait to tackle the challenges awaiting me along the way as I know the support network I have developed over the years will be there for me.

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CHAPTER 1. DISSERTATION INTRODUCTION

1.1 Background and scientific blind spot

The ability of some organisms to regenerate tissues and organs has fascinated naturalists since antiquity, dating back to the earliest accounts of scientific inquiry with Aristotle in Ancient Greece (Aristotle 1965). Not without selfish interests, human beings have tried to harness the power of regeneration exhibited by animals and plants (Réaumur 1712, Lenhoff and Lenhoff 1986a, Dinsmore 1996, Hijmans and Elith 2013). Scientists first focused on specific organisms such as *Hydra* and described their extensive regenerative abilities during the 18th century (Réaumur 1712, Dinsmore 1996). With the emergence of Darwin's theory of natural selection (Darwin 1859a), scientists expanded their inquiry to explore regenerative capacities over a wider taxonomic breadth. Indeed, Darwin's theory invigorated some scientists' dream of stimulating (or reactivating) regenerative capacities in human beings by showing that we are related to highly regenerative organisms (rev. in Carlson, 2007; Goss, 1969; Morgan, 1901; Needham, 1953). More recently, a renewed interest in discovering the molecular and genetic basis for organ and tissue regeneration has led biologists to focus more specifically on a restrictive set of model organisms. A byproduct of this focus has been that evolutionary studies investigating the ecological and evolutionary aspects of regeneration have fallen out of fashion (Maginnis 2006). In this dissertation, I integrate evolutionary analysis, tissue regeneration, behavior, and ecology to understand ecological and evolutionary aspects of regeneration.

Indeed, with much effort spent inquiring about *how regeneration functions* with a heavy focus on cells and molecules, researchers have created a blind spot in scientific inquiry regarding how regeneration can impact organisms and in turn the environment. By focusing the study of regeneration around the goal of transferring such abilities to humans, we have ceased to investigate how regeneration might differently impact organisms or the ecosystems those organisms inhabit or both (Maginnis 2006, Bely 2010). Not all regenerative organisms will be important at larger ecological scales; however, specific organisms may have ecological effects due to the level of their ecological importance (e.g., ecosystem engineers, see below). Investigating the consequences of regeneration for organisms also capable of wide ecological impacts is an understudied area of research, and studying these interactions between organisms' ecological impacts and regeneration places my research at the intersection of physiology, regeneration, behavior, and ecology.

1.2 Regeneration

Although the process of limb regeneration is different between invertebrate and vertebrate organisms, it follows the same general principles and steps (Seifert et al. 2012b). For example, following injury in vertebrates, wound healing first takes place via re-epithelization of the wound surface by keratinocyte migration. Then, local cells beneath the epidermis re-enter the cell cycle and de-differentiate under the influence of the Apical Epidermal Cap (AEC, layers of cells "hormonally directing" differentiation of the newly formed pluripotent cells; (Seifert et al. 2012b). As local cells in the injury area induced to proliferate, a blastema forms. The blastema is a heterogeneous mass of lineage-restricted progenitor cells that will experience proliferative expansion while acquiring spatial information before re-differentiation replaces the missing structure. Finally, a period of growth ensues to completely recover the missing limb. Invertebrate appendage regeneration differs at least slightly because their bodies are contained within an exoskeleton and growth is constrained by molting. Following re-epithelization, invertebrates reform a layer of protective cuticle under which a blastema forms and cell proliferation, re-differentiation, and patterning take place (Truby 1983, Seifert et al. 2012b). Only following the first molt post injury will the newly formed limb emerge from the old cuticle to follow normal growth with the rest of the organism (but see Reyes et al. 2017 and Chapter 4 for a slightly different account of invertebrate regeneration without molting).

Research on regeneration has emphasized the ontogeny of this process by focusing on the origin of the blastema (Goss 1969). Seminal work on salamanders and newts distinguished between epidermal and connective tissue cells contributions to the blastema confirming the importance of connective tissues for the blastemal (Hay and Fischman 1961). Similarly, researchers focused heavily on understanding mechanistic patterns of regeneration across taxa by working on supernumerary limbs and skin grafts to explore the development of new limbs (Bryant et al. 1981). Following extensive research in several regenerative taxa, the evolutionary origins of regeneration have been investigated with a focus on linking mechanisms across phylogeny (Sánchez Alvarado 2000, Brockes and Kumar 2008, Bely and Nyberg 2010, Seifert et al. 2012b). Although more recent studies have expanded their focus to comparing gene expression during regeneration across taxa (Fumagalli et al. 2018), most research still heavily focuses on shared mechanistic pathways and not on how the environment may influence regenerative ability. This attention on animal regeneration stemmed from identifying the pathways expressed in regenerative animals like amphibians yet not expressed in more recent taxonomic groups like bird and mammals with the objective to unlock regeneration in the latter (Galis et al. 2003, Seifert et al. 2012b).

1.3 Autotomy, environmental impacts, and selection pressures

The presence or absence of regenerative ability appears to broadly correlate with the incidence of autotomy (reflex severance of a limb; Bliss, 1960; McVean, 1982; Wood & Wood, 1932). Autotomy allows individuals to reduce costs associated with damaged structures, often limbs, by severing the appendage at a predetermined breakage plane, thus limiting wounding and helping the regeneration process. Individuals capable of rebuilding damaged structures during their lifespan may have higher fitness than individuals without this capacity (Reichman 1984, Maginnis 2006). This may help to explain how selective pressures for regenerative capacities could be maintained or acquired (Goss 1992, Bely 1999, Seifert et al. 2012b, Zattara and Bely

2016, Emberts et al. 2019). The regenerative process, induced by autotomy, may also have high physiological costs and this comes with its own set of challenges in turn affecting how regenerating individuals interact with and change their environment.

As mentioned above, organisms capable of regeneration can also have impacts on their environment through their behavior. Therefore, regeneration of structures important for behavioral effects on the environment can mediate organisms' environmental impacts. Scientists have long recognized the importance of reciprocal impacts between an organism and its environment as evidenced by Darwin's work on earthworms and their impact on soil formation (Darwin 1881) and development of the extended phenotype theory (Dawkins 1976). Recently, these reciprocal impacts have been the focus of new theory conceptualizing the ecological implications of organismal habitat, namely, ecosystem engineering (Jones et al. 1994, Lawton 1994). Ecosystem engineering is generally defined as the process by which organisms modify their physical environment from one physical state to another (Jones et al. 1994, 1997a). Although almost every organism on Earth engineers its' environment to some degree (Jones et al. 1997b), not all engineers will have substantial effects on the environment, and the magnitude of these effects can depend on the environment itself or on traits of the engineer such as the capacity to autotomize or regenerate (or both) appendages. For example, crayfish use their first pair of legs, their chelipeds, to dig and build extensive burrows, thus, heavily impacting their environment (ecosystem engineering). However, crayfish can also autotomize and regenerate their chelipeds; consequently, regenerating crayfish might not be able to dig and impact their environment as compared to non-wounded crayfish.

Furthermore, regeneration creates periods of time during which regenerating organisms face newfound challenges mediated by the way those organisms interact with their environments. Once again, regenerating crayfish might not be able to dig a burrow when needed (during drought, reproduction, *etc.*). Thus, they could find themselves competing for existing burrows or facing a now necessary migration to find water all of which non-regenerating crayfish may not experience. Therefore, ecosystem engineers capable of regeneration represent a powerful system allowing us to develop a clearer understanding of how regeneration might evolve as well as persist through its effects on organismal fitness. What type of selective pressures have crayfish faced while evolving regenerative capacities? Or did regeneration evolve allowing crayfish to maintain or recover (or both) their ecosystem engineering capacities? In other words, does regenerative capacity influence crayfish behavior and does crayfish behavior influence regenerative crayfish capacity?

Finally, studying the evolutionary significance of regeneration has to be carefully thought out in order to compare scenarios where animals autotomize and then regenerate from scenarios where animals autotomize without regenerating (see Figure 1 in Maginnis 2006). Although such comparisons are not always possible (i.e., absence of autotomizing but not regenerating individuals), approximating this comparison framework as much as possible will guide effective research into the feedback loop between individual regeneration and evolutionary consequences at the environmental scale (Maginnis 2006).

1.4 The impact of regeneration on organismal traits

Behavior has been documented to affect behavior in multiple organisms (rev. in Maginnis 2006) and those effects likely have consequences for fitness. For example, foraging in shore crabs is negatively impacted by autotomy and regeneration as crabs having regenerated one of their claws were found to forage on smaller prey compared to individuals showing no signs of prior regeneration (Elner 1980). Interestingly, it is unclear if those impacts are truly due regeneration and not autotomy and limb loss as those two are extremely hard to detangle. Nonetheless, the foraging capacities of some spiders are affected by autotomy and regeneration as spiders having lost a leg build less efficient webs for retaining prey (Weissmann and Vollrath 1999). Although spider webs are an extended phenotype (Dawkins 1982) and represent an example of ecosystem engineering (Jones et al. 1994), no study to my knowledge has investigated the impact of regeneration on spider-influenced ecosystems (but see Lill and Marquis 2007 for some examples of spider shelter-building and its impacts on their microhabitat). In vertebrates, regeneration has been shown to directly impact reproduction while indirectly affecting home ranges in lizards (Salvador et al. 1995). Inducing autotomy and subsequent regeneration in large dominant *Psammodromus algirus* males led to a reduction of their home range and their access to females (Salvador et al. 1995). Thus, if male behavior reduces home range size, cascading ecological effects could exist in the lost home range areas as males do not affect them anymore. Fitness impacts of autotomy and regeneration have been found in crayfish where autotomized males remove less competitor sperm during mating compared to their non-autotomized and non-regenerating counterparts (Galeotti et al. 2008). In a European crayfish family having a different mating sequence than their North-American counterparts (Astacidae and Cambaridae respectively), crayfish missing a cheliped were able to compensate by depositing more sperm than non-regenerating crayfish resulting in similar reproductive successes (Galeotti et al. 2008, 2012). These studies suggest other potential unforeseen effects of crayfish autotomy and regeneration on their environment when accounting for crayfish burrowing behavior (Nystrom and Strand 1996, Statzner et al. 2003, Albertson and Daniels 2016) as well as their essential place in freshwater food webs (Huryn and Wallace 1987, DiStefano 2005, Mueller et al. 2006). Regeneration also affects development by negatively impacting growth rate in vertebrates (Ballinger and Tinkle 1979). Hatchling *Sceloporus undulates* and *S. scalaris* exhibited slower growth during tail regeneration (Ballinger and Tinkle 1979) and those effects could potentially trickle up all the way to the ecosystem scale when accounting for the lizards' life history and the potential cascading impacts on their environment (e.g., slower lizard growth exposes them more to predators). Moreover, the intermolt period of littoral crab juveniles (*Crytograpsus angulatus*) was shortened as the number of regenerating limbs increased (Spivak 1990). Slower growth leads to impacts on life history and population growth rate then cascading into ecological consequences. Finally, though fiddler crabs have also been a major focus of regenerative studies, little is still known about the evolutionary and environmental impacts of their regenerative abilities (Skinner and Graham 1972, Hopkins 1982, Mohrherr 1987, Sekkelsten 1988). Although research on fiddler crab regeneration branched off to include how they interact with their environment (Kristensen 2008, Mokhtari et al. 2016, El-Hacen et al. 2018), a missing piece of regeneration research in this and other flagship species is how regeneration mediates the important ecological impacts of those species through regeneration impacts on species life history.

Research on the ecological context of regenerative ability in fiddler crabs and other arthropods has been mostly an effort to understand how regeneration influences individual-scale consequences (Juanes and Smith 1995, Mariappan et al. 2000, Maruzzo et al. 2005a, Maginnis 2006). However, disregarding how regeneration can impact organisms' immediate environment creates an opportunity for future investigation. Indeed, we now stand on the shoulder of giants ready to integrate autotomy, regeneration, behavior, and ecology into a comprehensive understanding of evolutionary ecology.

1.5 Aquatic ecosystems and ecosystem engineering

Aquatic ecosystems provide an excellent opportunity to investigate how organismal traits create feedback loops between an individual and its environment. One of the most important physical process taking place in aquatic environments is bioturbation, the increase of turbidity in an aquatic system through biological activity (Meysman et al. 2006). Organisms living in both marine and freshwater environments are affected by bioturbation (Fager 1964, Heinzelmann and Wallisch 1991, Ciutat et al. 2005, Mermillod-Blondin and Rosenberg 2006). In freshwater streams specifically, turbidity is affected both by environmental (watershed size, soil composition, precipitation regime, *etc.*) and organismal variables (*i.e.*, bioturbation). Ecosystem engineering is the causal agent of several processes involved in bioturbation in those environments including burrowing, walking (Statzner 2012), structure building, and even providing structure in the case of autogenic engineers such as plants (Jones et al. 1994). Within freshwater stream ecosystems, crayfish act as major engineers of their environment and they exhibit persistent regenerative ability throughout their lifespan (Nystrom and Strand 1996).

Crayfish tie the freshwater stream environment together by connecting the aforementioned concepts of bioturbation, engineering, autotomy, and regeneration. Indeed, crayfish have important roles as bioturbators in streams putting sediments in suspension in the water column by burrowing and walking along the streambed (Nystrom and Strand 1996, Statzner et al. 2000, 2003, Albertson and Daniels 2016). More importantly, crayfish burrowing activities represent a perfect example of engineering by mixing soil nutrients and altering substrate surface (Statzner et al. 2003), increasing turbidity (Statzner et al. 2000, Angeler et al. 2001, Creed and Reed 2004, Usio and Townsend 2004), providing shelter for themselves and other animals during droughts (Semlitsch 1981, Trenham 2001), or being used by countless other organisms as physical resources (damselflies lay eggs in crayfish burrows, Pintor & Soluk, 2006, and crawfish frogs live exclusively within crayfish burrows, Heemeyer et al. 2012). Furthermore, their roles as engineers of freshwater ecosystems is coupled to the central place they take in the ecosystem food web as keystone species (Paine 1966) preyed upon by numerous species (Goddard 1988, Momot 1995, Whitledge and Rabeni 1997, DiStefano 2005) and preying upon a variety of species (Mueller et al. 2006). Their importance in the food web also includes their essential role as detritivores accelerating the transformation of raw and coarse vegetal matter entering the food web into processed fine particles relied upon by all other aquatic organisms (Huryn and Wallace 1987). Finally, all of those essential freshwater processes with crayfish as central actors are mediated by crayfish physiology, notably their capacity to autotomize and regenerate their first pair of chelea

(Wood and Wood 1932b, Robinson et al. 1970, McVean 1982, Maginnis 2006). The the fact that crayfish can regenerate autotomized limbs that are important for their burrowing activities (McClain et al. 2007) leads to inevitable interactions between their regenerative ability and their environmental manipulations which can feedback to affect crayfish behavior.

1.6 Dissertation overview

The second chapter of my dissertation investigates the phylogenetic relationship between regeneration and autotomy in arthropods and reptiles. This large-scale exploratory effort allows me to draw inferences about the intertwined evolutionary history of these two traits within and across two major phylogenetic groups. Moreover, this chapter puts in perspective our understanding of how autotomy and regeneration are or are not maintained in specific lineages. One potential way to detangle regeneration and autotomy is to compare closely related species that do or do not express these traits (Maginnis 2006). Because all crayfish can autotomize and regenerate their appendages, the third and fourth chapters of my dissertation rely on the next best approach of contrasting autotomized crayfish with non-autotomized crayfish. The third chapter studies the behavioral impacts of autotomy and regeneration on crayfish burrowing, their main ecosystem engineering behavior. This study reveals unexpected consequences of autotomy and regeneration on crayfish ecosystem engineering and speculates about how these effects might impact the environment the far-reaching consequences for the environments in which crayfish live. The fourth chapter of my dissertation explores the fitness consequences of autotomy and regeneration in adult crayfish and shows how measuring potential fitness costs of regeneration can be complicated by likely compensatory changes that buffer these costs. Furthermore, this chapter also details the process of chelae regeneration in juvenile crayfish and reveals that molting and limb regeneration can be de-coupled; an unexpected finding. Finally, the fifth chapter of my dissertation places our understanding of autotomy and regeneration in the ecosystem by exploring bioturbation by regenerating as well as non-regenerating crayfish in freshwater streams. This last chapter truly places the investigation of autotomy and regeneration in an environmental context where these animals evolved and still heavily influence selective pressures for other organisms. This study reveals important and unexpected interactions between autotomy, regeneration, and the environment.

CHAPTER 2. EVOLUTIONARY BEDFELLOWS: RECONSTRUCTING THE ANCESTRAL STATE OF AUTOTOMY AND REGENERATION

Luc A. Dunoyer, Ashley W. Seifert, and Jeremy Van Cleve

2.1 Introduction

Long before *On the Origin of Species* (Darwin 1859b) introduced the world to the theory of natural selection, naturalists were busy exploring regenerative phenomena in animals and plants (Réaumur 1712, Lenhoff and Lenhoff 1986b, Dinsmore 1996, Hijmans and Elith 2016). As Darwin's ideas took root among different scientists, evolutionary thinking came to influence regenerative biologists who began offering explanations for the persistence or loss of regenerative ability across the tree of life (Cravens and Allen 1980). In parallel with the influence of evolutionary thinking, a shifting trend in embryological research catalyzed a more rigorous experimental approach to the study of embryonic development and regeneration. At its height, the dichotomy between naturalists and experimentalists embodied a key argument concerning the distribution of regenerative abilities among animals. The argument centered on whether regeneration was an adaptive trait or instead, an irreducible and fundamental property of life. During this period, August Weismann and Thomas Hunt Morgan embodied opposing viewpoints in this debate: *"…whereas for Weismann, animal regeneration was a contingent, naturally selected adaptation, Morgan insisted that animal regeneration was neither contingent nor a selected adaptation but rather a general property of the organism as a whole: a universal organic quality characterizing any organism occurring in different degrees*" (page 516, Esposito 2013).

Evidence in favor of tissue regeneration as a fundamental organismal property comes from the fact that on its surface, regeneration has close links to embryogenesis (Sánchez Alvarado 2000) and asexual reproduction (Bely and Nyberg 2010); these links suggest regeneration has deep evolutionary origins dating back to the advent of multicellularity or even earlier. Given that pattern restoration extends to the scale of single-cell organisms and their internal structures (Needham 1952, Tartar 1961), regeneration may simply be the expression or augmentation of a more basic process of pattern formation and elaboration. This idea rests with regeneration predating the evolution of the embryo where basic cellular-level regenerative processes are amplified in postembryonic animals as tissue (multi-cell) regeneration. Regardless of whether regenerative processes originated before or after the evolution of multicellular organisms, it likely emerged early in the evolution of complex life on earth. Thus, the distribution of regenerative ability in different lineages is likely shaped by phylogenetic constraint or inertia where the physical and genetic mechanisms underlying early regenerative processes constrained the evolution of later regenerative mechanisms. Arguments for regeneration as an adaptive trait rely on fitness advantages gained by regenerating a whole organism or a part thereof. For example, if a piece of a planaria or *Hydra* is severed, regrowth via regeneration can be so substantial that it can create new progeny where the parent organism regenerates and the severed piece regenerates into a daughter organism. Agonistic interactions with predators or with conspecifics can cause tissue damage which regeneration can serve to mitigate, thus minimizing fitness declines (Morgan 1901b, Maginnis 2006, Slack 2010). In the context of appendages, the loss of limbs or tails is so common, and minimizing the damage so important, that in some species these tissues are preferentially lost through autotomy. During autotomy, the structure is removed at a weak point or breakage plane allowing a more limited wound which facilitates regeneration of the structure. In the absence of direct testing, there is a potential role for both selection and phylogenetic inertia in the evolution of regeneration.

How can we determine the relative strength of selection and constraint in the evolution of regeneration? One tool is the comparative method from evolutionary biology where we look for patterns that emerge from analyzing the presence and extent of tissue regeneration among different taxa across the tree of life. When considering multicellular animals, regenerative ability varies substantially (Needham 1952, Bely and Nyberg 2010). For instance, some cnidarians show near limitless regenerative ability with the power to regenerate entire individuals from dissociated cells (Goss 1969). This stands in contrast to animals that can only regenerate specific structures, such as the tail but not limbs in lizards (Vitt et al. 1977), and further still to animals like mites that appear to lack any appreciable regenerative capacity whatsoever (Maruzzo et al., 2005). This variation suggests that either the adaptive value of regeneration varies across taxa, resulting in its selective maintenance in some taxa and selective elimination in others, or the developmental and pattern formation processes that produce regeneration in some taxa evolve complexities that lead to its elimination in other groups. Thus, distinguishing the relative roles of adaptation and constraint remains a crucial task in understanding the evolution of regeneration (Sánchez Alvarado 2000, Bely and Nyberg 2010, Tiozzo and Copley 2015).

Here we attempt to address this issue by looking at the phylogenetic history of regeneration and its co-occurrence with autotomy. Studying autotomy is crucial for understanding if (or how) the fitness costs and benefits of regeneration vary across different species because it is necessary to separate the potential costs and benefits of regeneration from the costs of autotomy that may precede regeneration in many species. Numerous arthropods are capable of autotomizing their limbs following damage or predation attempts (Fleming et al. 2007). Since the growth of many arthropods is tied to molting, many of them develop a regenerated limb in a papilla sac that later unfolds after molting (e.g., fiddler crab, Hopkins 1993; cockroach, Truby 1983). Moreover, autotomy can be beneficial in arthropods since molting can lead to appendages becoming stuck in the exuviate (i.e., shed exoskeleton) and autotomy ensures that the exoskeleton is shed even in these cases. Reptiles are another group of organisms heavily studied for their regenerative capacities and a group in which autotomy is widespread (Bellairs and Bryant 1985). Notably, lizards are well-known to autotomize their tail when attacked by predators and a number of scincid and gekkonid lizards can shed parts of their integument in response to physical contact (Bauer et al. 1989, Zani 1996, Scherz et al. 2017). Although lizards can regenerate their autotomized tail, the replacement is not a perfect facsimile since the missing vertebrae and spinal cord are replaced by a cartilaginous rod and ependymal tube respectively (Simpson 1968, Bateman and Fleming 2009, Gilbert et al. 2013, Lozito and Tuan 2016). While not perfect, autotomy followed by regeneration would seem to offer an advantage as tail autotomy alone causes diminished locomotor performances (McElroy and Bergmann 2013). However, other reptiles, such as chameleons, are neither capable of tail autotomy nor tail regeneration, which highlights the phylogenetic variation in both of these traits (Bellairs and Bryant 1985, Anderson and Higham 2014).

Here we use this variation in regenerative ability and autotomy to reconstruct the ancestral states of these two traits across arthropods and reptiles. Knowing these ancestral states is crucial for determining the evolutionary timing for the origin of autotomy relative to regeneration, and this timing in turn provides evidence for the value of regeneration in rebuilding structures lost by autotomy. For example, if autotomy evolved first, this could have set the stage for the adaptive evolution of regeneration. On the other hand, if regeneration evolved first, this would suggest the function of regenerative abilities must be broader than simply appendage replacement. In fact, our results show that autotomy and regeneration were present at the base of the arthropod and reptile trees. While this result suggests that untangling the relative role of adaptation and constraint in the evolution of tissue regeneration will require a more sophisticated approach than the one used here, our reconstructions provide important groundwork to explore how selection may play a role in the loss of regeneration in particular lineages.

2.2 Methods

2.2.1 Definitions

2.2.1.1 Regeneration

The term regeneration refers to the functional replacement of tissue that is lost or damaged. Importantly, we consider reparative regeneration in this paper as it occurs in response to injury or autotomy, either self-induced or caused by an external agent. More precisely, we refer to regeneration as the restoration of a pattern that occurs when the organism's body system is disrupted. This definition separates regeneration from scar formation that occurs in some animals (e.g., replacement of lost tissue with dissimilar, non-functional tissue). This definition also applies across multiple levels of biological organization and unites regeneration in single-cell organisms with the higher-level combination of processes that occurs during replacement of an appendage. As a singular event, regeneration encompasses individual processes at different levels of biological organization. For example, barrier restoration, homeostasis, and morphogenesis broadly describe regeneration from beginning to end. Thus, for the purposes of identifying regeneration as a trait, this necessitates considering these processes together.

2.2.1.2 Autotomy

Autotomy has been broadly defined as the loss of tissue induced directly by an individual itself (as a reflex severance – autotomy – or by pulling on the appendage – autotilly) or indirectly by the action of outside agent (autospasy) (Wood and Wood 1932). In this paper we refer to autotomy broadly, including all of the aforementioned definitions. Autotomy can occur voluntarily or as the result of extensive damage to a limb or appendage. In either case, an autotomy plane prevents hemolymph or blood loss and facilitates regeneration of the missing appendage (Bellairs and Bryant 1985, Maginnis 2006, Fleming et al. 2007). Following autotomy, tissue either regenerates or heals with a scar. Regeneration in arthropods and reptiles generally proceeds via hemostasis, inflammation, re-epithelialization, blastema formation, and morphogenesis (Seifert et al., 2012).

2.2.2 Ancestral State Reconstruction

Ancestral state or trait reconstruction in a phylogenetic context was proposed as early as 1938 (Dobzhansky and Sturtevant 1938) and is an approach that improves on similar methodologies appropriated from cladistics. What distinguishes ancestral state reconstruction is how it considers shared evolutionary history between taxa. Character mapping traits on a cladogram using parsimony to manually reconstruct ancestral states ignores shared evolutionary history and favors a mild view of a traits' history because it ignores the effect of branch lengths in shaping the topology of the tree. Formal ancestral state reconstruction uses a more standardized approach in that it applies a hypothetical model of evolution to a phylogeny and in doing so takes into account evolutionary time.

2.2.2.1 Character Coding

In our data gathering, we code each experimental or observational report of bonafide regeneration, or its absence, with a value of 1 or 0, respectively; autotomy was similarly recorded. In the absence of experimental or observational information explicitly specifying trait presence of absence, we attributed a value of NA. If genera or species within a higher taxon level scored differently (e.g., some regenerate, others do not) then a branch tip on the tree would receive a 1/0 as was the case for Pterygota (see Figure 2.1). Although some authors use regeneration in referring to embryos, for the purposes of our reconstructions we only considered regeneration for post-embryonic stages of development. Most embryos can regenerate parts and tissue regeneration in embryos can be influenced by the degree of autonomous or conditional development present in a given taxa. Because life stage, tissue type, and organismal traits can influence regenerative ability (Seifert et al. 2012b), we considered data for presence or absence of autotomy and regeneration when it was reported for (1) any post-embryonic life stage and (2) for any appendage or body part. An exception to these criteria applied to the Pterygota where we only considered regeneration if reported for adults due to the role of regenerative ability in early development for these insects, which undergo partial or complete metamorphosis. While regenerative ability can vary across life-stage, it should be noted that the majority of data we found scored the presence or absence of these traits in adults. Even when life stages clearly separate regenerative capacities in some clades (as in holometabolous insects), we decided to embrace this fact and explore the differences between those groups and other closely related groups (see below). Since many species within a clade lack direct information on whether they autotomize or regenerate, we assigned presence or absence values for autotomy and regeneration to a tip of a clade even when only one or two species in the clade had been investigated. A potential bias in our dataset is that studies are more likely to report presence rather than absence of regeneration and autotomy. This particular limitation cannot be avoided until more research is conducted as advocated for below.

In surveying the existing literature for robust information about autotomy and regeneration in arthropods, we relied on comprehensive reviews for arthropods (Maruzzo et al. 2005b), invertebrates (Fleming et al. 2007), and extensive compendiums of these traits (Morgan 1901a, McVean 1982). Similarly, in order to determine the ancestral state for autotomy and regeneration in squamate reptiles, we surveyed the existing literature and relied heavily on a monograph that compiled information on these traits (Bellairs and Bryant 1985) which we supplemented with species-level studies when possible (see below).

2.2.2.2 Phylogenies

In order to map both autotomy and regeneration in arthropods, we selected one of the most recent arthropod phylogenies (Regier et al. 2010). Although this phylogeny provides the most complete coverage of arthropod families and is congruent with recent discussions of arthropod relationships, it is not a chronogram whose branch lengths are proportional to time, which are preferred when reconstructing ancestral states using maximum likelihood (Litsios and Salamin 2012). Thus, for the ancestral state reconstruction, we used the most recent arthropod phylogeny with calibrated divergence times (Rehm et al. 2011). Ultimately, this phylogeny was trimmed to include only species for which we had regeneration and autotomy data from the literature search before running the ancestral state reconstruction. However, this new tree did not include any species where regeneration was absent rendering it impossible for us to run a formal ancestral state reconstruction for regeneration. Nevertheless, this analysis might be unnecessary in view of the lack of variation in regeneration capacities across the arthropod tree.

For squamate reptiles, we used a recent phylogeny based on morphological and genetic data as our backbone for character mapping and for the ancestral state reconstruction of autotomy and regeneration (Pyron 2017). Due to a lack of data on regeneration and autotomy for the Serpentes, we omitted this infra-order from our analysis. When focusing on Iguanidae, we used a less recent, but more specific phylogeny to manually map autotomy and regeneration in this group (Wiens and Hollingsworth 2000).

2.2.3 Analyses

To examine the evolutionary history of autotomy and regeneration in arthropods and reptiles, ancestral state reconstruction analyses were done using R version 3.0.1 (R Core Team 2013) and the ape (Paradis et al. 2004), caper (Orme et al. 2012), geiger (Harmon et al. 2008), phytools (Revell 2010), phangorn (Schliep 2011), and xlsx (Dragulescu 2014) packages. Each ancestral reconstruction figure depicts a density map of 100 stochastic trait histories generated from our dataset using an MCMC approach (stochastic character mapping; Huelsenbeck et al. 2003). The reconstructed ancestral state at an internal node of the tree for autotomy and regeneration is the probability of that traits occurring among the 100 stochastic trait histories.

2.3 Results

2.3.1 Ancestral state for autotomy and regeneration in arthropods

Mapping regeneration and autotomy for all arthropods revealed that regeneration, while nearly ubiquitous across arthropod orders, appears to have been independently lost in three orders; in the *Opiliones* (harvestmen), *Geophilomorpha* (soil centipedes) and then again within the *Endopterygota* comprising all the holometabolous insects (Figure 2.1 and Table 2.1). However, based on the phylogenetic resolution of our branches, there are a few additional fine scale losses (i.e., genus and species) that are not present on the tree and represent isolated losses of regenerative ability (e.g., mites). In contrast with regeneration, autotomy was lost in ten major lineages containing approximately 23,000 species (Figure 2.1 and Table 2.1; Resh and Cardé 2009).

Table 2.1: Data for autotomy and regeneration in arthropods associated with Figure 1. Each tree branch is presented with the corresponding order and class. Autotomy and regeneration are characterized as present (1), absent (0), or unknown (NA). Unless indicated by other superscripts, the reference for the character state is [1]Maruzzo et al., 2005; [2]Wood and Wood, 1932; [3]Korschelt, 1907; [4]McVean, 1982 and [5]Fleming et al., 2007. [*]Velvet worms and water bears are outgroups in this phylogeny.

Tree branch	Class	Subclass or Order or Infraorder	Genus	Rege	Autoto
Winged insects	Insecta	Pterygota, Paleoptera	NA	1	1
		Pterygota, Neoptera	NA	0/1	0/1
Silverfish &	Insecta	Zygentoma	NA	1	1[5]
Jumping	Insecta	Archaeognatha	Machilis,	1	1[5]
Springtails	Entognatha	Entomobryomorpha	Orchesell	1	0
Two-pronged	Entognatha	Diplura	Campode	1	1[5]
Horseshoe	Cephalocari	Brachypoda	NA	NA	NA
Barnacles	Maxillopoda	Thecostraca	Lepas	1	NA
Crabs	Malacostrac	Brachyura	Uca	1[3]	1[2]
Crayfish &	Malacostrac	Pleocyemata	Cambaru	1[3]	1[2]
Isopods	Malacostrac	Peracarida	Asellus &	1[5]	1[4]
Mantis shrimps	Malacostrac	Stomatopoda	Squilla	1	0
Nebalia	Malacostrac	Leptostraca	Nebalia	1	0
Plankton	Hexanauplia	Cyclopoida	NA	1	0
Water fleas	Branchiopod	Cladocera	Triops	1	NA
Fairy shrimps	Branchiopod	Anostraca	Branchip	1	NA
Fish lices	Maxillopoda	Arguloida	Argulus	1	0
Seed shrimps	Ostracoda	Halocyprida	NA	1[2]	0[4]
Millipedes	Diplopoda	Spirostreptida	Julus &	1[5]	NA
Garden centipedes	Symphyla	Scolopendrellidae	NA	NA	NA
Soil centipedes	Chilopoda	Geophilomorpha	NA	0[3/4]	0[4]
Other centipedes	Chilopoda	Scutigeromorpha	Scutigera	1	1[4]
Spiders &	Arachnida	Amblypygi	Centauru	1	1
False scorpions	Arachnida	Pseudoscorpiones	NA	NA	NA
Harvestmen	Arachnida	Opiliones	Leiobunu	0	1
Mites & Ticks	Arachnida	Opilioacarida	Opilioaca	1	0
Horseshoe crabs	Merostomat	Xiphosura	Limulus	1	NA
Sea spiders	Pycnogonida	Pantopoda	Phoxichil	1	1
Velvet worms[*]	Udeonychop	Peripatopsidae	NA	NA	NA
Velvet worms[*]	Udeonychop	Peripatidae	NA	NA	NA
Water bears[*]	Eutardigrada	Parachaela	NA	NA	NA
	Eutardigrada	Apochela	NA	NA	NA

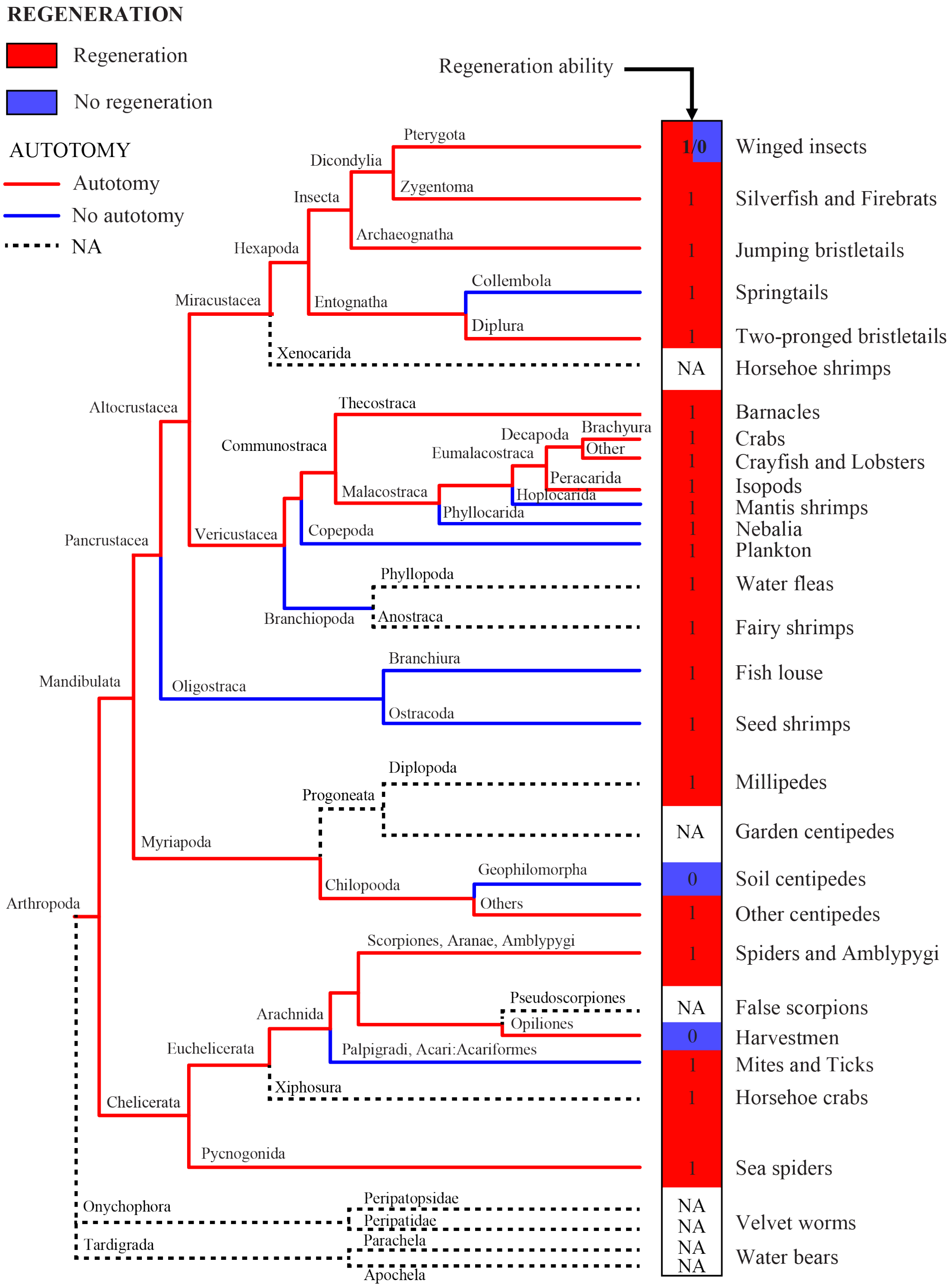

Figure 2.1: Evidence of regeneration and autotomy manually mapped onto an arthropod phylogeny (Regier et al., 2010). Common names of organisms at each branch tip are on the right-hand side for convenience (see Table 1 for data). Red and blue boxes at the tips of the tree indicate presence or absence, respectively, for regeneration. Red and blue branches on the phylogeny indicate the presence or absence, respectively, of autotomy. Dashed branches indicate data are not available (NA).

We next used ancestral state reconstruction to resolve the phylogenetic history of autotomy. In the case of regeneration, there was no variation in regenerative ability (all remaining taxa were capable of regeneration) using the Rehm et al. (2011) tree; thus, the basal condition was presence of regeneration. Even in the Regier et al. (2010) tree, regeneration is the most parsimonious ancestral state for arthropods because only three derived groups showed a lack of regenerative ability (Figure 2.1 and Table 2.1). We determined that autotomy was the ancestral state in arthropods based on the available data (scaled likelihood of autotomy at the root: presence > 0.99, absence = 5.9×10^{-4}; Figure 2.2).

Next, we sought to investigate two arthropod groups where autotomy and regeneration appeared to be more evolutionarily labile. First, we examined the insects (class Insecta) in more detail (Figure 2.3 and 2.4). Despite limited data given the number of species within this class, the available data for adult Odonata (Figure 2.3; Table 2.2) strongly suggests that most, if not all, hemimetabolous insects (incomplete metamorphosis) are capable of autotomy and regeneration. In contrast, examining holometabolous insects (complete metamorphosis), we found no instances of regeneration in adults (note that *Frankliniella* and *Dictyoptera* are hemimetabolous and outgroups in this tree; Figure 2.4; Table 2.3). As for autotomy, although the data are incomplete, they support that all holometabolous insects conserve their capacity to autotomize limbs (Figure 2.4; Table 2.3). That some holometabolous insects exhibit regenerative ability as larvae suggests that the radical developmental shift to complete metamorphosis created a negative trade-off with regenerative ability.

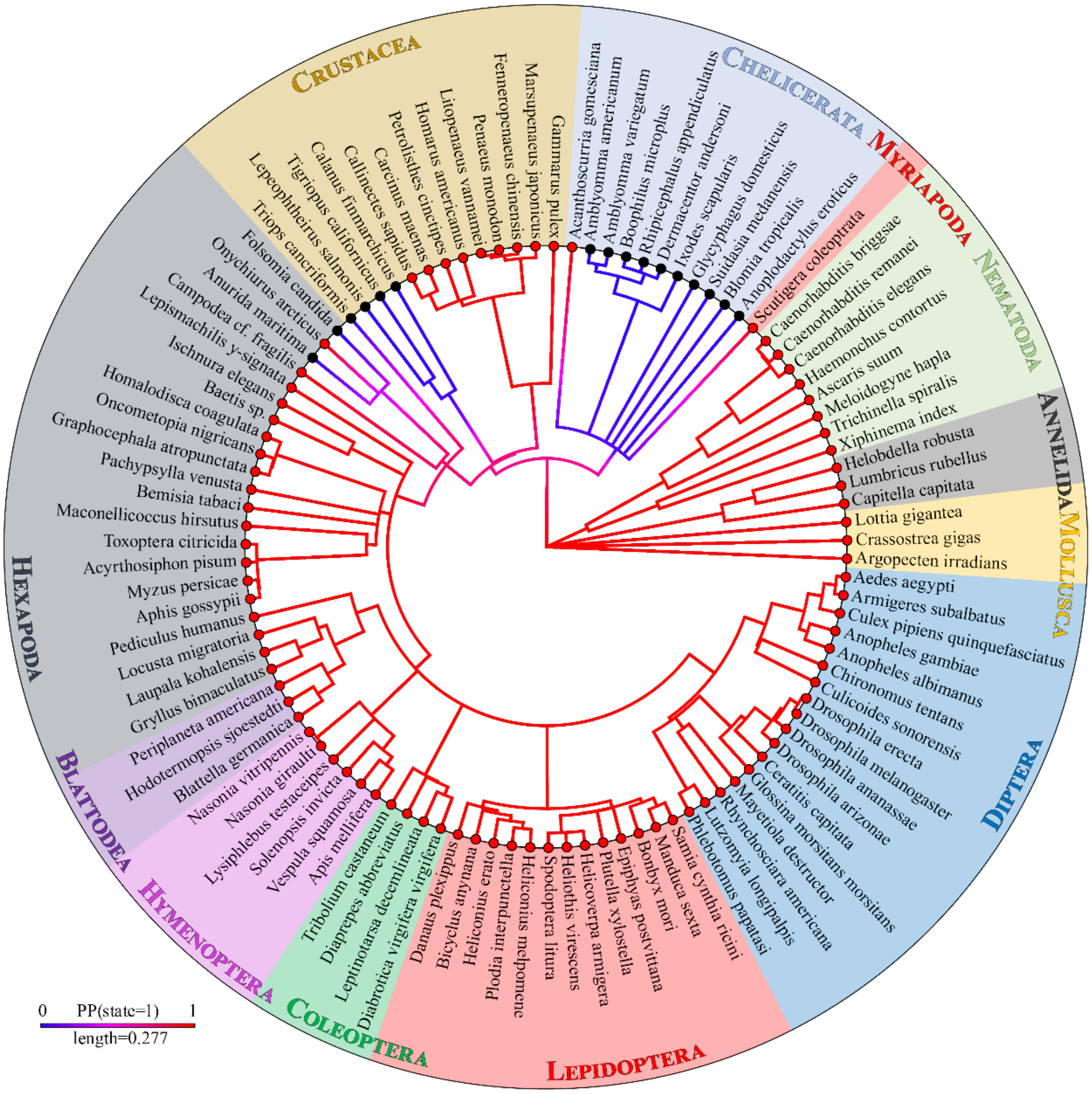

Figure 2.2: Ancestral state reconstruction of autotomy (see text for methodological details) using a recent arthropod phylogenetic tree (Rehm et al. 2011 based on Meusemann et al. 2010). Red and black circles at the tips of the tree indicate autotomy or no autotomy, respectively (see Table 1 for data). The ancestral reconstruction recovers probabilities for the presence or absence of autotomy along the tree branches showing an ancestral state of autotomy in the present phylogeny. Along those tree branches, absence of autotomy is represented in blue and presence in red. Major arthropods' phyla or orders have been layered on top of the phylogeny for convenience.

Table 2.2: Autotomy and regeneration data for adult Odonata associated with Figure 3. Each taxon is presented with the associated literature reference used for characterization of autotomy and regeneration as present (1), absent (0), or unknown (NA). [1]Child and Young, 1903; [2]Robinson et al., 2006; [3]Maruzzo et al., 2005; [4]Fleming et al., 2007; [5]Stoks, 2003; [6]Tennessen, 2009; [7]Madhavan and Schneiderman, 2007; [8]Moore and Tabashnik, 1989; [9]French, 1976 and [10]Maginnis, 2006. Outgroups used for Figure 3 are: Pyralidae, Curculionidae, Trichogrammatidae, Ectobiidae, Acrididae, Sminthuridae and Baetidae.

Genus or Species name	**Suborder**	**Family**	**Regeneration**	**Autotomy**
Argia moesta	Zygoptera	Coenagrionidae	1[1/10]	1[2]
NA	Zygoptera	Calopterygidae	NA	1[3]
Burmargiolestes sp.	Zygoptera	Megapodagrionidae	NA	1[3]
Noguchiphaea sp.	Zygoptera	Calopterygidae	NA	1[3]
Pseudolestes sp.	Zygoptera	Pseudolestidae	NA	1[3]
NA	Zygoptera	Hetaerinidae	NA	1[3]
NA	Zygoptera	Rimanellidae	NA	1[3]
NA	Zygoptera	Heliocharitidae	NA	1[3]
NA	Zygoptera	Protoneuridae	NA	1[3]
NA	Zygoptera	Platycnemididae	NA	1[3]
NA	Zygoptera	Philogeniidae	NA	1[3]
Stenocnemis sp.	Zygoptera	Platycnemididae	NA	1[3]
Ceriagrion sp.	Zygoptera	Coenagrionidae	1[1/10]	1[4]
NA	Zygoptera	Pseudostigmatidae	NA	1[3]
Nephallenia sp.	Zygoptera	Coenagrionidae	1[1/10]	1[4]
Devadatta sp.	Zygoptera	Amphipterygidae	NA	1[3]
Rhinagrion sp.	Zygoptera	Megapodagrionidae	NA	1[3]
Coeliccia sp.	Zygoptera	Platycnemididae	1[10]	1[3]
Philoganga sp.	Zygoptera	Amphipterygidae	NA	1[3]
NA	Zygoptera	Euphaeidae	NA	1[3]
NA	Zygoptera	Chlorocyphidae	NA	1[3]
NA	Zygoptera	Megapodagrionidae	NA	1[3]
NA	Zygoptera	Megapodagrionidae	NA	1[3]
NA	Zygoptera	Plastystictidae	NA	1[3]
Lestes viridis	Zygoptera	Lestidae	1[5]	1[4]
NA	Epiprocta	Libellulidae	NA	1[6]
NA	Epiprocta	Macromiidae	NA	1[6]
Macromidia sp.	Epiprocta	Corduliidae	NA	1[6]
NA	Epiprocta	Corduliidae	NA	1[6]
NA	Epiprocta	Gomphidae	NA	1[6]
Anax imperator / A. cyanea	Epiprocta	Aeshnidae	1[3]	1[4]
NA	Epiprocta	Petaluridae	NA	1[6]
NA	Epiprocta	Chlorogomphidae	NA	1[6]
NA	Epiprocta	Neopetaliidae	NA	1[6]
NA	Epiprocta	Cordulegastridae	NA	1[6]
NA	Epiprocta	Epiophlebiidae	NA	1[6]
Galleria mellonella	Heteroneura	Pyralidae	1[7]	1[8]
Sitophilus zeamais	Polyphaga	Curculionidae	NA	NA
Trichogramma minutum	Apocrita	Trichogrammatidae	NA	NA
Blattella germanica	Cockroaches	Ectobiidae	1[9]	1[9]
Oxya chinensis	Caelifera	Acrididae	1[3]	1[3]
Sminthurus viridis	Collembola (order)	Sminthuridae	1[3]	0[3]
Callibaetis ferrugineus	Pisciforma	Baetidae	1[3]	1[3]

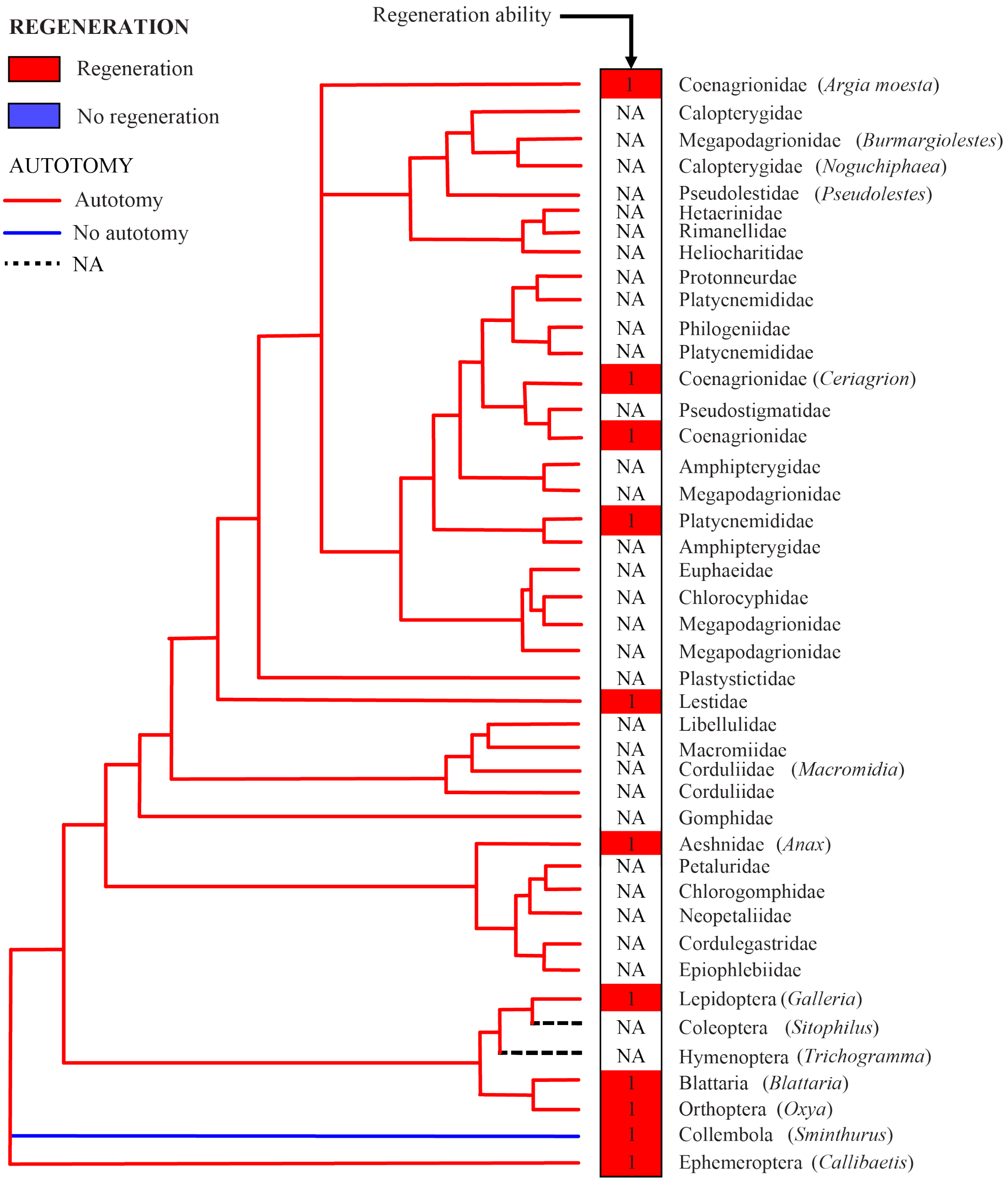

Figure 2.3: Modified phylogeny of adult Odonata (hemimetabolous insects; from Figure 2 in Dumont et al., 2010) onto which both regeneration and autotomy have been mapped (see text for methodological details and Table 2 for data). Taxonomic groups are on the right-hand side for convenience. Red and blue boxes at the tips of the tree indicate presence or absence, respectively, for regeneration. Red and blue branches on the phylogeny indicate the presence or absence, respectively, of autotomy. Dashed branches indicate data are not available (NA).

Table 2.3: Autotomy and regeneration data for adult holometabolous insects associated with Figure 4. Each taxon is presented with the associated literature reference used for characterization of autotomy and regeneration as present (1), absent (0), or unknown (NA). [1]Wigglesworth, 1944; [2]Maginnis, 2006; [3]Fleming et al., 2007; [4]Haynie and Bryant, 1976; [5]Marchand, 1917; [6]Shcherbakov et al., 1995; [7] Yang et al., 2016 and [8]Hermann, 1971. [9]Frankliniella and Dictyoptera are hemimetabolous insects and outgroups in this phylogeny.

Genus or Species name	Order or Suborder	Family	Regeneration	Autotomy
Pterostichus	Adephaga	Carabidae	NA	NA
Bembidion	Adephaga	Carabidae	NA	NA
Laccophilus	Adephaga	Dytiscidae	NA	NA
Hydroscapha	Myxophaga	Hydroscaphidae	NA	NA
Tenomerga	Archostemata	Cupedidae	NA	NA
Strangalia	Polyphaga	Cerambycidae	0[1]	NA
Tribolium	Polyphaga	Tenebrionidae	0[2]	1[3]
Chauliognathus	Polyphaga	Cantharidae	0[1]	NA
Halictophagus sp.	Strepsiptera	Halictophagidae	NA	NA
Mengenilla	Strepsiptera	Mengenillidae	NA	NA
Kempynus	Hemerobiiformia	Osmylidae	NA	NA
Austroneurorthus	Neuroptera	Nevrorthidae	NA	NA
Platystoechotes	Neuroptera	Polystoechotidae	NA	NA
NA	Megaloptera	NA	NA	NA
Mongoloraphidia	Raphidioptera	Raphidiidae	NA	NA
Microchorista	Mecoptera	Nannochoristidae	NA	NA
Nannochorista	Mecoptera	Nannochoristidae	NA	NA
Panorpa	Mecoptera	Panorpidae	NA	NA
Bittacidae	Raptipeda	Bittacidae	NA	NA
Boreus	Mecoptera	Boreidae	NA	NA
Neotyphloceras	Hystrichopsyllomorpha	Hystrichopsyllidae	NA	NA
Ctenocephalides	Pulicomorpha	Pulicidae	NA	1[5]
Drosophila	Brachycera	Drosophilidae	0[4]	1[3]
Musca	Brachycera	Muscidae	NA	NA
Tipulidae	Nematocera	Tipulidae	NA	1[3]
Anopheles	Nematocera	Culicidae	NA	1[6]
Noctuidae	Glossata	Noctuidae	0[7]	1[3]
Bombyx	Glossata	Bombycidae	0[2]	1[3]
Hydropsyche	Annulipalpia	Hydropsychidae	NA	NA
Pteromalidae	Apocrita	Pteromalidae	NA	1[8]
Tenthredinidae	Symphyta	Tenthredinidae	NA	1[8]
Apis	Apocrita	Apidae	NA	1[8]
Frankliniella[9]	Terebrantia	Thripidae	NA	NA
NA	Dictyoptera[9]	NA	1[3]	1[2]

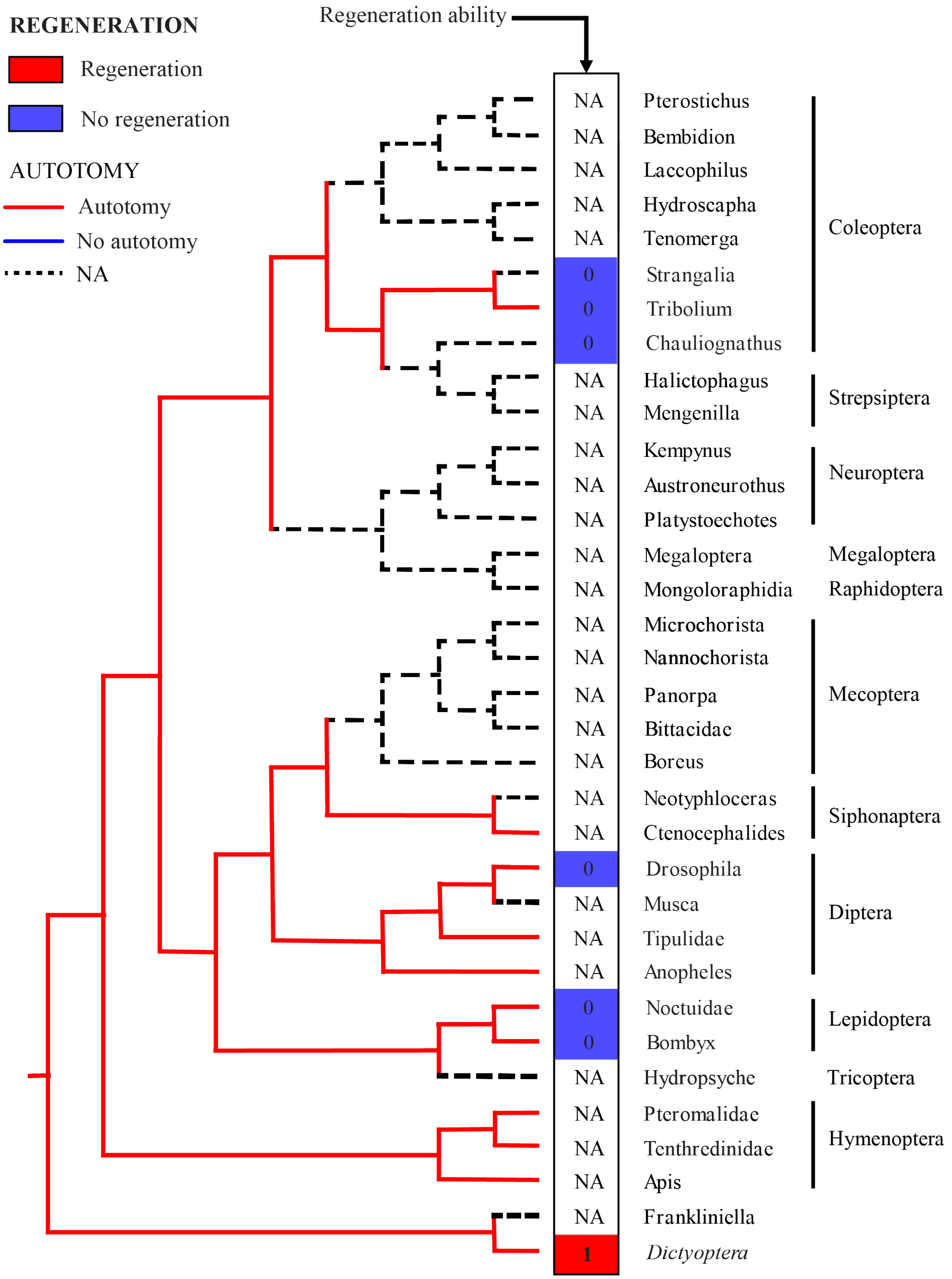

Figure 2.4: Modified phylogeny of adult holometabolus insects (from Figure 2 in McKenna and Farrell, 2010) onto which both regeneration and autotomy has been mapped (see text for methodological details and Table 3 for data). Taxonomic groups are on the right-hand side for convenience and genera are included where known. Red and blue boxes at the tips of the tree indicate presence or absence, respectively, for regeneration. Red and blue branches on the phylogeny indicate the presence or absence, respectively, of autotomy. Dashed branches indicate data are not available (NA).

2.3.2 Ancestral state for autotomy and regeneration in reptiles

We next investigated the evolutionary history of these two traits in reptiles and assigned each tip of the phylogeny with a value for regeneration and autotomy based on family and infra-order information (Figure 2.5; Table 2.4). Character mapping of both traits revealed that they were widespread across reptiles (Figure 2.5; Table 2.4). Similar to arthropods, we determined autotomy and regeneration as the ancestral state for squamate reptiles based on our data (autotomy: scaled likelihood of autotomy at the root: presence = 0.9747, absence = 0.0253, standard error = $7.74\text{x}10^{-4}$; regeneration: Scaled likelihood of regeneration at the root: presence = 0.9942, absence = 0.0058, standard error = $4.940\text{x}10^{-4}$; Figure 2.6 and Figure 2.7). To investigate the association of regeneration and autotomy in more detail, we examined the Iguanidae family where both traits showed independent losses (Figure 2.8; Table 2.5). Our analysis revealed that loss of one trait did not necessarily predict the other. For example, *Iguana delicatissima* is capable of regeneration but not autotomy, whereas a sister species *Iguana iguana* is capable of autotomy but not regeneration (Figure 2.8). Together, our results show that regeneration and autotomy are the basal condition in squamate reptiles and that tail regeneration, albeit imperfect, has persisted in most major reptilian lineages.

Table 2.4: Autotomy and regeneration data for squamate reptiles associated with Figures 5, 6, and 7. Each taxon is presented with the associated literature reference used for characterization of autotomy and regeneration as present (1), absent (0), or unknown (NA). [1]The reptile database (http://www.reptile-database.org/). Unless indicated by other superscripts, the reference for the character state is from [2]Bellairs and Bryant 1985; [3]Arnold 1994; [4]Anderson and Higham 2014; [5]Cope 1967; [6]Zug et al. 2006; [7]Wiens and Etheridge 2006; [8]Zani 1996; [9]Laspiur et al. 2007 and [10]Poe 2006.

Species name	**Suborder[1]**	**Family[1]**	**Regeneration[2]**	**Autotomy[2]**
Acontias percivali	Scincomorpha	Scincidae	1	1
Aeluroscalabotes felinus	Gekkota	Eublepharidae	1	1
Agama agama	Iguania	Agamidae	1	1
Amphiglossus splendidus	Scincidae	Amphiglossus	1	1
Amphisbaena fuliginosa	Lacertoidea	Amphisbaenidae	1	1
Anniella pulchra	Anguimorpha	Anniellidae	1	1
Anolis carolinensis	Iguania	Dactyloidae	1	1
Aspidoscelis tigris	Sauria	Teiidae	1	1
Basiliscus basiliscus	Iguania	Corytophanidae	1	1
Bipes biporus	Lacertoidea	Bipedidae	1	1
Bipes canaliculatus	Lacertoidea	Bipedidae	1	1
Brachylophus fasciatus	Iguania	Iguanidae	1	0[3]
Brachymeles gracilis	Scincidae	Scincidae	1	1
Brookesia brygooi	Iguania	Chamaeleonidae	0	0[4]
Callopistes maculatus	Sauria	Teiidae	1	1
Calotes emma	Iguania	Agamidae	1[6]	1
Celestus enneagrammus	Anguimorpha	Diploglossidae	1	1
Chalarodon madagascariensis	Iguania	Opluridae	1	1
Chamaeleo laevigatus	Iguania	Chamaeleonidae	0	0[4]
Coleonyx variegatus	Gekkota	Eublepharidae	1	1
Corytophanes cristatus	Iguania	Corytophanidae	0	0[8]
Cricosaura typica	Scincoidea	Xantusiidae	1	1
Crotaphytus collaris	Iguania	Crotaphytidae	0	0[3]
Delma borea	Gekkota	Pygopodidae	1	1
Diplometopon zarudnyi	Amphisbaenia	Trogonophidae	1	1
Dipsosaurus dorsalis	Iguania	Iguanidae	1	1
Elgaria multicarinata	Anguimorpha	Anguidae	1	1
Enyalioides laticeps	Iguania	Hoplocercidae	1	1[7]
Eublepharis macularius	Gekkota	Eublepharidae	1	1
Eugongylus rufescens	Scincoidea	Scincidae	1	1
Feylinia polylepis	Scincoidea	Scincidae	1	1
Gambelia wislizenii	Iguania	Crotaphytidae	1	1
Gekko gecko	Gekkota	Gekkonidae	1	1
Geocalamus acutus	Lacertoidea	Amphisbaenidae	1	1
Gonatodes albogularis	Gekkota	Sphaerodactylidae	1	1
Heloderma horridum	Anguimorpha	Helodermatidae	1	0[3]
Heloderma suspectum	Anguimorpha	Helodermatidae	1	0[3]
Lacerta viridis	Lacertoidea	Lacertidae	1	1
Lanthanotus borneensis	Anguimorpha	Lanthanotidae	1	1
Leiocephalus barahonensis	Iguania	Leiocephalidae	1	1
Leiolepis belliana	Iguania	Agamidae	0	0[7]
Leiosaurus catamarcensis	Iguania	Leiosauridae	0	0[9]
Lepidophyma flavimaculatum	Scincoidea	Xantusiidae	1	1
Lialis burtonis	Gekkota	Pygopodidae	1	1
Liolaemus bellii	Iguania	Liolaemidae	1	1
Morunasaurus annularis	Iguania	Hoplocercidae	1	1
Oplurus cyclurus	Iguania	Opluridae	1	1
Petrosaurus mearnsi	Iguania	Phrynosomatidae	1	1
Phelsuma lineata	Gekkota	Gekkonidae	1	1
Phrynosoma platyrhinos	Iguania	Phrynosomatidae	0	0

Table 2.4 (continued)

Species name	Suborder[1]	Family[1]	Regeneration	Autotomy
Phymaturus palluma	Iguania	Liolaemidae	1	1
Physignathus cocincinus	Iguania	Agamidae	1	0[7]
Platysaurus pungweensis	Scincoidea	Cordylidae	1	1
Plestiodon fasciatus	Scincoidea	Scincidae	1	1
Plica plica	Iguania	Tropiduridae	1	1
Pogona vitticeps	Iguania	Agamidae	1	1
Polychrus marmoratus	Iguania	Polychrotidae	0	0[7]
Pristidactylus torquatus	Iguania	Leiosauridae	1	1
Pseudopus apodus	Anguimorpha	Anguidae	1	1
Rhacodactylus auriculatus	Gekkota	Diplodactylidae	1	1
Rhineura floridana	Amphisbaenia	Rhineuridae	1	0[5]
Saltuarius cornutus	Gekkota	Carphodactylidae	1	1
Sauromalus ater	Iguania	Iguanidae	1	1
Sceloporus variabilis	Iguania	Phrynosomatidae	1	1
Scincus scincus	Scincoidea	Scincidae	1	1
Shinisaurus crocodilurus	Anguimorpha	Shinisauridae	1	1
Smaug mossambicus	Scincoidea	Cordylidae	1	1
Sphenodon punctatus	Rhynchocephalia	Sphenodontidae	1	1
Sphenomorphus solomonis	Scincoidea	Scincidae	1	1
Stenocercus guentheri	Iguania	Tropiduridae	1	1
Strophurus ciliaris	Gekkota	Diplodactylidae	1	1
Takydromus sexlineatus	Lacertoidea	Lacertidae	1	1
Teius teyou	Gymnophthalmoide	Teiidae	1	1
Teratoscincus przewalskii	Gekkota	Sphaerodactylida	1	1
Tiliqua scincoides	Scincoidea	Scincidae	1	1
Trachylepis quinquetaeniat	Scincoidea	Scincidae	1	1
Trogonophis wiegmanni	Lacertoidea	Trogonophidae	1	1
Tupinambis teguixin	Gymnophthalmoide	Teiidae	1	1
Uma scoparia	Iguania	Phrynosomatidae	1	1
Uranoscodon superciliosus	Iguania	Tropiduridae	1	1
Uromastyx aegyptia	Iguania	Agamidae	1	1
Urostrophus vautieri	Iguania	Leiosauridae	0	0[10]
Uta stansburiana	Iguania	Phrynosomatidae	1	1
Varanus acanthurus	Anguimorpha	Varanidae	1	0[3]
Varanus exanthematicus	Anguimorpha	Varanidae	1	0[3]
Varanus salvator	Anguimorpha	Varanidae	1	0[3]
Xantusia vigilis	Scincoidea	Xantusiidae	1	1
Xenosaurus grandis	Anguimorpha	Xenosauridae	1	0[3]
Xenosaurus platyceps	Anguimorpha	Xenosauridae	1	0[3]

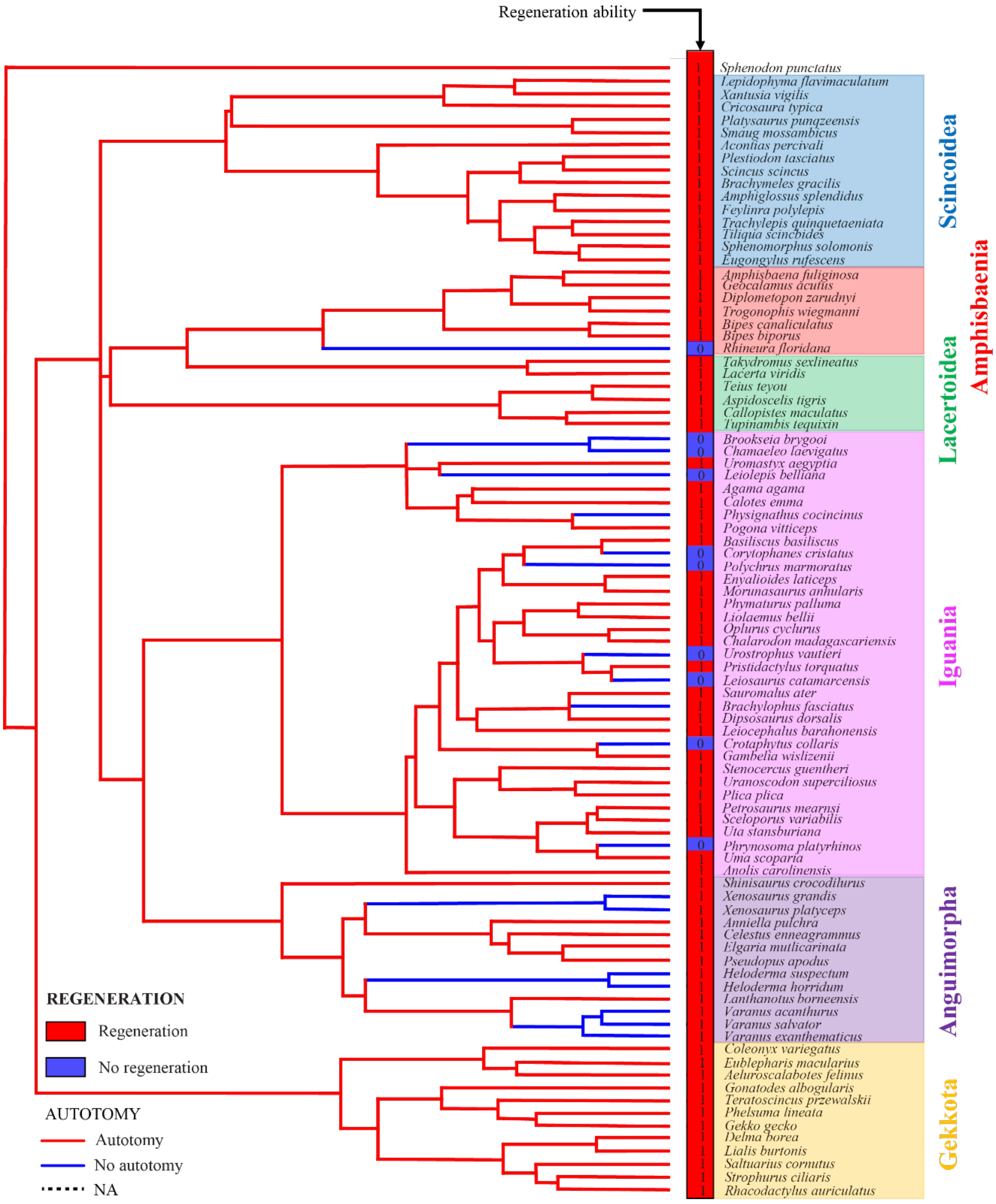

Figure 2.5: Modified phylogeny of squamate reptiles (Pyron, 2017) onto which regeneration and autotomy have been mapped (see text for methodological details and Table 4 for data). Full names of the taxa are on the right-hand side with family color coded for convenience. Red and blue boxes at the tips of the tree indicate presence or absence, respectively, for regeneration. Red and blue branches on the phylogeny indicate the presence or absence, respectively, of autotomy. Dashed branches indicate data are not available (NA).

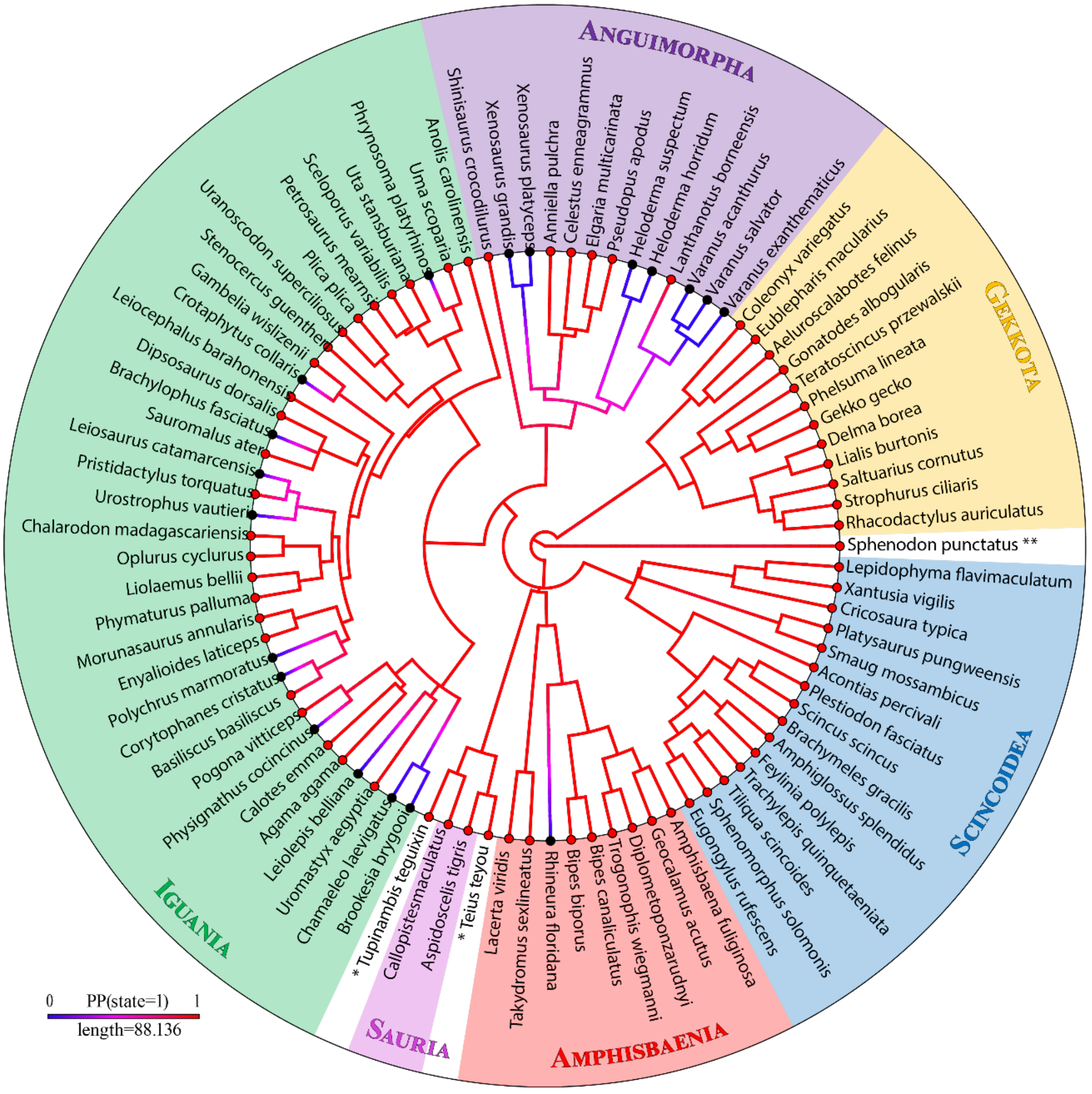

Figure 2.6: Ancestral state reconstruction of autotomy (see text for methodological details and Table 4 for data) using a recent reptile phylogenetic tree (Pyron, 2017). Colors are the same as Figure 2 in terms of presence and absence of the trait. The ancestral reconstruction shows an ancestral state of autotomy for the whole phylogeny. Major squamate reptile families have been layered on top of the phylogeny for convenience; (*) denotes Lacertoidae and (**) denotes Rhynchocephalia.

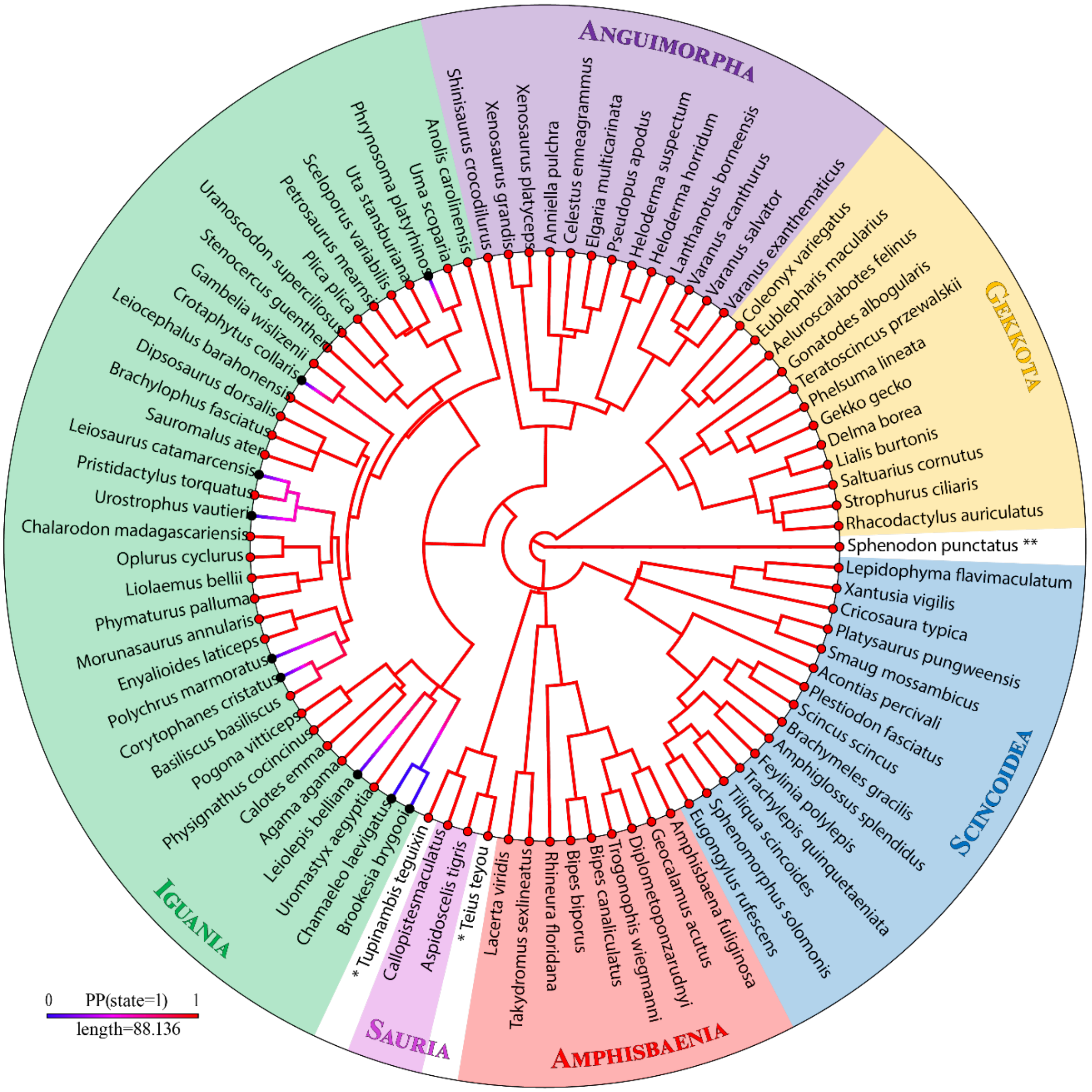

Figure 2.7: Ancestral state reconstruction of regeneration (see text for methodological details and Table 2.4 for data) using the reptile phylogenetic tree from Figure 6 (Pyron 2017). Colors are the same as Figure 2 in terms of presence and absence of the trait. The ancestral reconstruction shows an ancestral state of regeneration for the whole phylogeny. Major squamate reptiles families have been layered on top of the phylogeny for convenience; (*) denotes Lacertoidae and (**) denotes Rhynchocephalia.

Table 2.5: Autotomy and regeneration data for Iguanidae associated with Figure 8. Each tree tip is presented with the associated literature reference used for characterization of autotomy and regeneration as present (1), absent (0), or unknown (NA). [1]The reptile database (http://www.reptile-database.org/); [2]Bellairs and Bryant 1985; [3]Arnold 1994; [4]Smith 2016; [5]Burger and Gochfeld 2006; [6]de Queiroz 1987; [7]Robyn 2013; [8]Ariano-Sanchez and Gil-Escobedo 2016; [9]Koleska and Jablonski 2018; [10]Wu et al. 2014 [11]Koleska et al. 2017 and [12]Carter and Hayes 2004.

Species name	**Suborder[1]**	**Family[1]**	**Regeneration**	**Autotomy**
Dipsosaurus dorsalis	Iguania	Iguanidae	1[2]	1[2]
Brachylophus fasciatus	Iguania	Iguanidae	1[2]	0[3]
Amblyrhynchus cristatus	Iguania	Iguanidae	1[2]	0[3/6]
Conolophus pallidus	Iguania	Iguanidae	0[2]	0[3/6]
Conolophus subcristatus	Iguania	Iguanidae	0[2]	0[6]
Ctenosaura hemilopha	Iguania	Iguanidae	1[4]	0[2]
Ctenosaura palearis	Iguania	Iguanidae	1[7]	1[6]
Ctenosaura quinquecarinata	Iguania	Iguanidae	1[8]	1[6]
Ctenosaura similis	Iguania	Iguanidae	1[5]	1[5]
Iguana delicatissima	Iguania	Iguanidae	1[9]	0[3/6]
Iguana iguana	Iguania	Iguanidae	0[10]	1[2/3]
Sauromalus ater	Iguania	Iguanidae	1[11]	1[6]
Sauromalus varius	Iguania	Iguanidae	NA	1[6]
Cyclura cychlura	Iguania	Iguanidae	1[12]	1[6]
Cyclura nubila	Iguania	Iguanidae	1[12]	1[6]
Cyclura ricordii	Iguania	Iguanidae	1[12]	1[6]

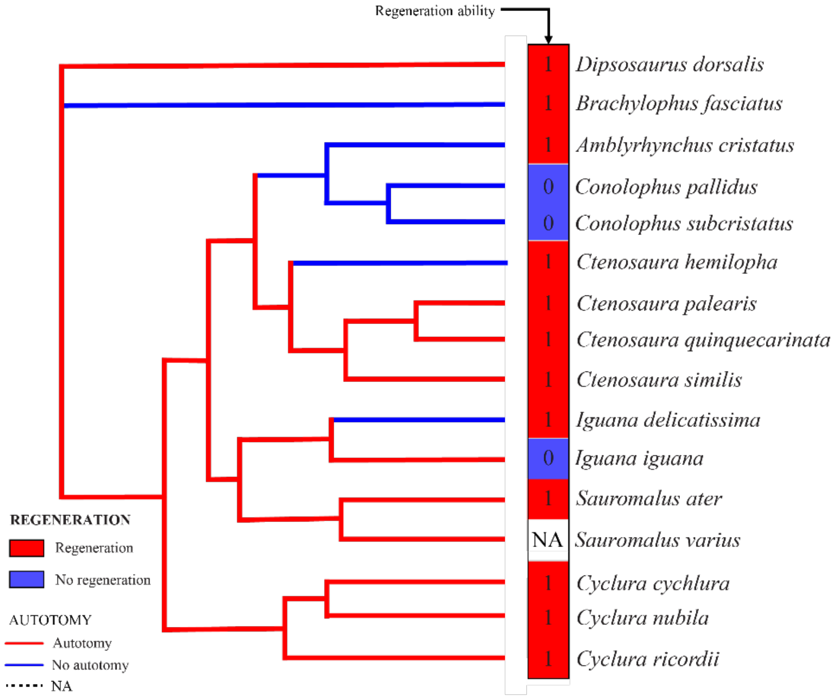

Figure 2.8: Modified phylogeny of Iguanidae (from "combined data" in Figure 1 in Wiens and Hollingsworth, 2000) onto which both regeneration and autotomy has been mapped (see text for methodological details and Table 5 for data). Full names of the taxa are on the right-hand side for convenience. Red and blue boxes at the tips of the tree indicate presence or absence, respectively, for regeneration. Red and blue branches on the phylogeny indicate the presence or absence, respectively, of autotomy. Dashed branches indicate data are not available (NA).

2.4 Discussion

With few exceptions, biologists continue to debate the adaptive nature of regeneration in the absence of formal evolutionary tests or experiments to directly assess the fitness consequences when regeneration is lost (Sánchez Alvarado 2000; Brockes and Kumar 2008; Bely and Nyberg 2010; Lai and Aboobaker 2018; Maden 2018). Directly measuring fitness can be difficult in regenerative species where lifespans extend for many years (with reproduction occurring over a large portion of the lifespan) and reproductive output can be large. Analyzing specific traits using ancestral state reconstruction provides a rigorous test to assess the evolutionary history for a particular trait. Strong inference is possible when species relationships are supported by robust phylogenies and sampling for a particular character is represented widely across the tree branches. In an effort to begin addressing whether the presence or absence of regenerative ability might be under selection, we endeavored to perform an ancestral state reconstruction of appendage regeneration in two groups where it has been relatively well-studied (arthropods and reptiles). We also reconstructed the evolutionary history of autotomy in order to ascertain whether regenerative ability might be constrained or influenced by this trait since the two are often associated for any given species in these taxa (Tiozzo and Copley 2015).

First, our data demonstrated that regeneration and autotomy were present in the basal arthropod ancestor and that these traits have persisted during the evolution of most arthropod lineages. However, the data also showed that forces, known and unknown, can lead to loss of regenerative ability across entire clades. For example, a major shift in life-history strategy from incomplete to complete metamorphosis was accompanied by a complete loss of regenerative ability among adult holometabolous insects. In the case of arthropods, we could not find an instance where regeneration was lost, only to later remerge in a more recent lineage. Compared to regeneration, our data also showed that autotomy has been lost more frequently and that once lost within a lineage this change tends to persist in all descendent lineages. Although autotomy and regeneration are present in many arthropod lineages, the data suggest that the association of these traits has relaxed during the evolution of more recent taxa.

In contrast, regeneration and autotomy appear tightly linked in reptiles and our ancestral state reconstructions show that autotomy and regeneration were present in the common ancestor of all reptiles. Thus, when autotomy is lost in a particular reptilian lineage, regenerative ability is often also lost and vice versa. We did find exceptions to this general trend within the Iguanidae and Anguimorpha. The Anguimorpha were the only group in which autotomy was repeatedly lost but where regeneration remained. Similar to our finding in arthropods, when autotomy was lost at deep nodes it did not appear to re-evolve. It is important to note that conflicting molecular and morphological information exists concerning the phylogenetic relationships among reptiles and thus it is possible that topological inaccuracies within the tree may ultimately explain observed discrepancies (Wiens and Hollingsworth 2000). Nevertheless, that these patterns were present in both trees adds strong support to our general conclusions. We did find intriguing counterexamples to the tight linkage between these traits, and investigating these counterexamples may help disentangle the evolution of autotomy and regeneration.

Broadly examining when autotomy and regeneration were lost in certain lineages, we find that autotomy is generally lost in more basal lineages, whereas loss of regeneration appears to be a more recent phenomenon. Furthermore, when autotomy is lost it appears very difficult to regain in descendant lineages (with two exceptions in each tree). This suggests that loss of autotomy is relatively permanent and does not leave organisms with dormant processes that are easily re-activated under the right conditions. This may reflect how autotomy is dependent on the presence of complex morphological and physiological components (e.g., precisely located fracture plane, contractile mechanism of muscle, nervous induction, etc.) that may be constrained by similar evolutionary pressures.

Interestingly, while autotomy could be uncoupled from regeneration, loss of regenerative ability appears strongly linked to autotomy in both groups. In general, in every reptilian clade where regeneration was lost, so too was autotomy. Although the converse pattern, loss of autotomy leading to loss of regeneration, was common, Iguanas are an exception where autotomy has been lost several times but regeneration persists (Arnold 1994, Bateman and Fleming 2009, Gilbert et al. 2013). Aside from these losses, autotomy is widespread among iguana species, and these losses provide fertile ground for more empirical studies focused on why species have lost the ability to autotomize but are still able to regenerate. Similarly, the persistence or loss of regeneration in Pterygota (winged insects), while not necessarily connected to autotomy, was highly correlated with another trait: incomplete or complete metamorphosis (Maruzzo et al. 2005b, Seifert et al. 2012b). In this case, the association between metamorphosis and regeneration suggests that the selective pressures associated with the radical life history change to complete metamorphosis imposed alterations that were antagonistic to regeneration in adult holometabolous insects.

The discrepancy between the timing observed for autotomy and regeneration losses could be explained in several different ways. First, it is possible that in those taxa that regenerate ancestrally, regeneration provides significant fitness benefits and thus those taxa that lose it are more likely to go extinct. In this case, only species who have lost regeneration relatively recently will be present in a particular phylogenetic tree. This is difficult to test unless a phylogeny contains closely related species with and without regeneration where fitness consequences can be directly measured (Maginnis 2006). That autotomy is lost more frequently in the trees may reflect a lower cost associated with its loss and thus, taxa that lose it persist for longer and are represented in the trees. It is also possible that the more frequent loss of autotomy reflects different rates of gain and loss between autotomy and regeneration. In this scenario regeneration is more easily gained and lost, whereas autotomy may be lost more slowly but is rarely re-gained. As a complex trait with many interacting processes, regeneration can be easily lost and gained in some taxa when mutations inactivate a signaling pathway required for regeneration. Examples include loss (and gain) of head regeneration in annelids and some flatworms (Bely and Sikes

2010). However, it is also possible that mutations which restore regeneration may be likely if the inactivated process can be replaced by the augmented function of a different but related process. Moreover, such mutations might be likely to sweep through populations if regeneration is under strong positive selection. Thus, for those species that lose and regain regeneration, the gain might occur before the species that lost it diversifies, which would make inferring the gain difficult using contemporary data. The loss of regenerative ability has also been associated with the evolution of increased cellular diversification, specific life-histories, differences in growth mode, and alterations in adaptive immunity (rev. in Needham 1953; Sánchez Alvarado 2000; Harty et al. 2003; Mescher and Neff 2005). In contrast, autotomy may be a trait more susceptible to complete loss without easy re-enabling options besides evolving the trait altogether anew. Coupled with weaker selection for mutations that do cause gain of autotomy, this could result in slower rates for gaining autotomy and thus losses would persist in the phylogeny when they do occur.

When considering inferences derived from data presented in the current paper, several cautionary notes are appropriate. One problem concerning comparative regeneration studies has always been a paucity of data from non-model species. Data concerning the absence or presence of autotomy and regeneration are lacking for many species, which underscores the need for more empirical studies collecting data for both traits. Data indicating *true absences* of autotomy or regeneration are particularly important. Indeed, inconspicuous appendages might be capable of autotomy or regeneration or both despite reports to the contrary for a more prominent appendage. Thus, only a systemic approach testing several appendages and organs can truly reject the presence of autotomy or regeneration or both in one species compared to another. For these reasons, we caution against strong inference based on the data presented here and instead present our conclusions as testable hypotheses in need of more systematic data.

To conclude, we found convincing support for the hypothesis that regeneration and autotomy were present in the common ancestors of arthropods and squamate reptiles and neither likely re-evolved in lineages where they were lost. In addition, our work supports the hypothesis that regeneration is lost relatively quickly possibly due to trade-offs with other costly capacities (Giangrande and Licciano 2014). Recalling the views of Weismann and Morgan who believed, respectively, that regeneration is adaptive or that it is an ancestral and fundamental property, we suggest that our work finds evidence for both of these views. Finally, we emphasize the need for extensive empirical studies that collect data on regenerative ability and autotomy such that stronger inferences can be drawn from phylogenetic reconstructions. Similarly, we encourage researchers to expand exploration of the ancestral states of autotomy and regeneration in other clades such as birds, amphibians, and mammals. For instance, we know that complex tissue regeneration has evolved independently within mammals at least twice (rabbits and spiny mice) (Vorontsova and Liosner 1960, Joseph and Dyson 1966, Seifert et al. 2012a). With these data in hand, we will be better equipped to answer a myriad of questions including: have cases of appendage regeneration across vertebrate lineages evolved independently; does autotomy drive selection for regeneration; does the loss of regeneration always lead to the loss of autotomy; and why is regeneration seemingly more essential to maintaining autotomy than autotomy is to maintaining regeneration? Only with a broader understanding of the origins of regeneration and autotomy will we be able to enhance our understanding of those traits and one day harness the power of regeneration for the benefits of human beings.

CHAPTER 3. LIMB LOSS, HABITAT STRUCTURE AND PREDATION SHAPE BEHAVIOR IN RED SWAMP CRAYFISH

Luc A. Dunoyer, Makayla Dean, Stephanie Hacker, Jeremy Van Cleve, and Ashley W. Seifert

3.1 Introduction

The diversity of animal regenerative ability has been a subject of modern scientific study ever since René-Antoine Ferchault de Réaumur and Abraham Trembley described the phenomena in insects and *Hydra,* respectively (Réaumur 1712, Dinsmore 1996). Over the ensuing centuries, biologists have focused on trying to understand the mechanistic basis for how organisms regenerate lost structures and what forces shape the distribution of regenerative ability across animals (rev. in Morgan 1901, Needham 1953, Goss 1969, Carlson 2007). What has received far less attention, however, is how the regenerative process impacts organismal behavior and physiology and how these interactions generate ecological costs/benefits that may result in natural selection (Maginnis 2006, Bely 2010). Although regenerating individuals with a missing appendage may ultimately regrow it, the period preceding tissue recovery leaves an organism facing new and important challenges.

Among arthropods, damage to an appendage is often accompanied by autotomy. Autotomy is the induced release of an appendage or tissue that most often occurs at a pre-determined breakage point (Wood and Wood 1932b, Bliss 1960, McVean 1982). Although the evolutionary linkage between regeneration and autotomy is complex (Goss 1992; Bely 1999; Seifert et al. 2012; Zattara and Bely 2016; Emberts et al. 2019; Chapter 1), it is clear that autotomy can be used to escape predation (Mariappan et al. 2000) and that most animals capable of autotomy also regenerate the missing tissue (Maginnis 2006, Dunoyer et al. 2019; Chapter 2). Although poorly understood, it is likely that regeneration imposes physiological and behavioral costs that may impact fitness. Because some regenerating invertebrates fulfill important ecological roles as keystone species (Paine 1966, Power et al. 1996) or as ecosystem engineers (Jones et al. 1994, 1997b), autotomy and regeneration may affect ecosystems in previously unknown ways.

A well-known marine ecosystem engineer, the fiddler crab (*Uca* spp.), has also been used to study how autotomy and regeneration can affect organismal fitness (Mohrherr 1987, Hopkins 1993, 2001). Fiddler crabs use their chelipeds (first modified pair of leg bearing large chelae) to create burrows which serve as shelters protecting them from predation or desiccation or both (Bildstein et al. 1989). Fiddler crabs also build chimneys on top of their burrows (Gusmao-Junior et al. 2012) and their burrowing can differentially affect their environment based on species, crab size, and soil properties (Mokhtari et al. 2016). Finally, interactive effects of ecosystem engineering between fiddler crabs and flamingos promote food resources for both species by maintaining a spatially complex mosaic of habitats (depressions and mounds respectively; El-Hacen et al. 2018). In freshwater ecosystems, numerous studies have investigated how habitat modification by invertebrates impact local ecology (Mermillod-Blondin and Rosenberg 2006, Statzner 2012, Mancinelli et al. 2013) but none have focused on how the effect of autotomy and regeneration on these behavior might cascade into ecological perturbations.

Crayfish serve as keystone species in freshwater streams where they occupy a central position within the food web by predating upon small invertebrates and fish (Mueller et al. 2006) and simultaneously acting as an important foodstuff for mammals, birds, and fish (DiStefano 2005). Moreover, by shredding abundant leaf litter, they are partly responsible for the transformation of particulate organic matter from course to fine allowing numerous other organisms to access this important food resource (Huryn and Wallace 1987). Crayfish also modify their environment as ecosystem engineers (Jones et al. 1994; Jones et al. 1997) where they use their claws to create burrows that support complex communities (Nystrom and Strand 1996, Statzner et al. 2000, 2003, Albertson and Daniels 2016). For example, damselflies lay eggs in crayfish burrows (Pintor and Soluk 2006), crawfish frogs live exclusively within crayfish burrows (Heemeyer et al. 2012), burrows act as refugia from drought for fish and other aquatic organisms (Semlitsch 1981, Madison 1997, Trenham 2001), and crayfish burrowing influences primary production by affecting light penetration (Creed and Reed 2004, Dunoyer et al. 2019).

Crayfish have a complex relationship with the burrows they create. For example, previous ownership of a shelter provides an advantage to all life stages during intraspecific interactions and especially so for females (prior residence effect, Peeke et al. 1995, Figler et al. 1999). Female *Procambarus clarkii* in berry (*i.e.*, bearing eggs) have high aggression levels allowing them to win fights over shelters (Figler et al. 1995); this may be useful for survival since the *P. clarkii* life cycle requires females to remain in the burrow until craylings (young of the year crayfish; Jones and Colin 2018) are ready to be independent (usually after their fifth molt following hatching; Huner and Barr 1991; McClain et al. 2007). Interestingly, *P. clarkii* have a low fidelity to burrowed shelter, uses natural shelter readily, and usually only burrow when trying to avoid desiccation (Huner and Barr 1991, Ilheu et al. 2003).

In response to damage, crayfish autotomize their limbs and this event is followed by regeneration (Wood and Wood 1932b). While the impact of limb loss on normal behavior and fitness is not usually considered, several studies suggest the presence of indirect and direct effects. For instance, in *Astacidae,* the diminished capacity to remove competitor sperm during copulation (due to regenerative status; Galeotti et al. 2008) is compensated by bigger sperm ejaculate in regenerating crayfish (Galeotti et al. 2012). In addition, diminished competitive capacities for shelter and food (Mariappan et al. 2000) have also been tied to regenerative status. Nonetheless, the impacts of autotomy and regeneration on crayfish burrowing behavior have not been directly investigated even though these traits may have important consequences for freshwater stream ecosystems due to the central place of crayfish burrows in those environments.

Red swamp crayfish (*Procambarus clarkii*; Girard 1852) burrows are always connected to the water flow when found in streams (tertiary burrower; Hobbs and Horton H. Hobbs 1974).

However, when the water level lowers or in case of drought, burrow entrances are found outside the water where they are topped with a chimney and reach down to the water table (Ilheu et al. 2003). Females can carry up to 500 eggs when in berry (clutch size highly correlated with female size), reach sexual maturity between 6-12 months, and live for 2-3 years on average (McClain et al. 2007). Females seek shelter in burrows during berry where they remain until their eggs hatch and the juveniles (crayling; Jones and Colin 2018) are ready to swim on their own (typically after the fifth molt after hatching while still staying under the mother's tail; McClain et al. 2007). Life-cycle timing are highly dependent on burrow availability and fluctuating water levels.

In this study we investigated the potential for behavioral changes imposed by autotomy and regeneration in the Red Swamp crayfish (*P. clarkii*, Girard 1852). Specifically, we asked if limb loss and subsequent regeneration affected burrow construction and shelter seeking. First, we observed a gradual restoration in cheliped size during regeneration and growth after each molting event following autotomy (see Figure 2 for details). Because regenerating crayfish possess only one fully functional cheliped, we predicted that cheliped autotomy would immediately hinder crayfish burrowing behavior, and that crayfish would complete less-complex burrows (without a chimney or secondary tunnel) following autotomy. Alternatively, autotomized crayfish could be incapable of burrowing altogether. We also predicted that *P. clarkii* would recover burrowing ability as a function of molt number, with increasing molts leading to more complex burrows. Because burrows serve as predator refuge, we hypothesized that regenerating crayfish would minimize predator exposure while undergoing tissue regeneration and thus would spend more time hiding in shelters or burrows (when available). Finally, we exposed crayfish to predator cues and predicted this would modify the interaction between limb loss and burrowing behavior; regenerating *P. clarkii* would burrow more and spend more time outside of the water (exposed on the mud) in the presence of conspecific predation cues.

3.2 Material and methods

3.2.1 Crayfish cheliped anatomy and regeneration

Crayfish chelipeds are composed of seven different segments (Figure 3.1). Cheliped autotomy prevents major hemolymph loss and takes place at the preferred breakage plane between the ischium and basis segments. Following autotomy, wound healing occurs, which facilitates subsequent regeneration. Cell proliferation ensues during regeneration and the epidermal cells (i.e., blastema) undergo morphogenesis (Truby 1983, Seifert et al. 2012b). The blastema elongates until the digit phase is reached (presence of a digit-like growth) and this event is followed by patterning of different cheliped segments. Although all of these stages can take place between molts, it varies depending on crayfish age and molting is necessary for hardening the new limb (Chapter 4). Once the cheliped segments are regenerated, growth continues, though the regenerated cheliped does not reach the same size as the unwounded cheliped. The entire regenerative process may take several days when it occurs during early life stages (right after hatching) or several months to a year in adults. Individuals used in our experiments underwent segment regeneration in approximately one month and completed cheliped growth over a period of six months (Figure 3-2).

3.2.2 Crayfish maintenance and autotomy procedure

Crayfish were maintained in a 100-gallon tub with a water pump (for water circulation) and filter under a controlled day/night light cycle (12/12 hours). The environment was enhanced with orange mesh and PVC pipes of different sizes to provide shelter and reduce cannibalism. Individuals were obtained from laboratory stocks at the University of Kentucky and purchased via Carolina Biological Supply (https://www.carolina.com/). Animals were fed weekly *ad libitum* with salmon pellets (soft, moist salmon feed pellets, size 5/32", from Rangen Inc.).

Autotomy of one cheliped was induced by cutting the limb at the merus/ischium articulation (Figure 3.1A) using a sharp blade or scalpel (Dryad, https://doi.org/10.5061/dryad.h70rxwdf2).

The remaining limb part was either readily autotomized during or after the cut and autotomy effectively prevented hemolymph loss. All experiments were conducted in our laboratory at the University of Kentucky. All crayfish were measured to the nearest tenth of millimeter using digital calipers from the tip of the rostre to the tip of the tail (total body length; Figure 3.1B).

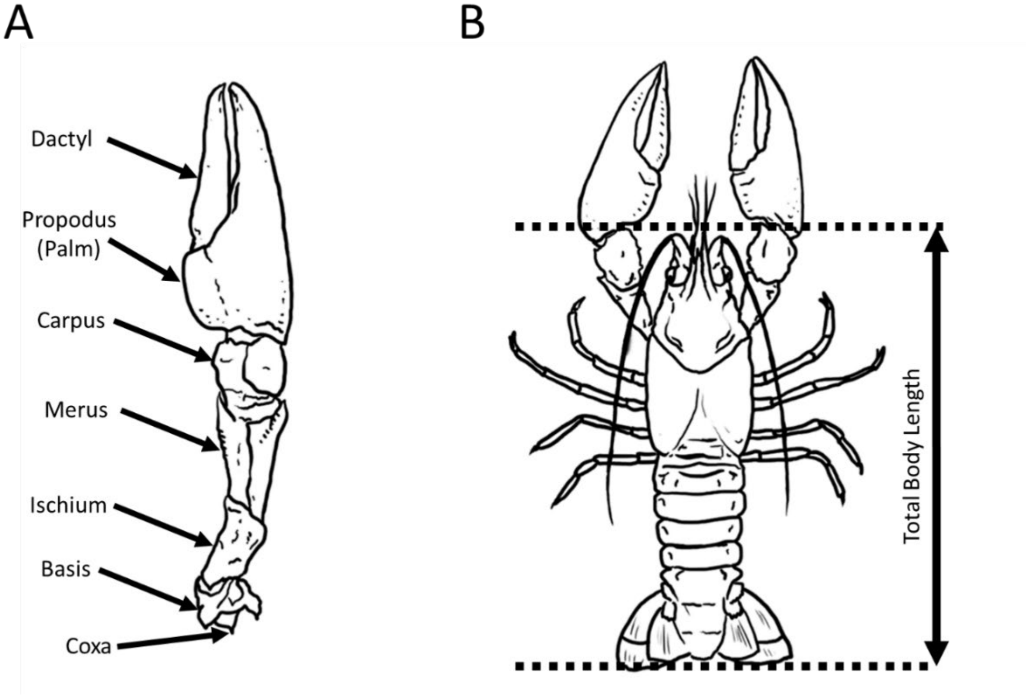

Figure 3.1: Body and cheliped dimensions for *P. clarkii* used in this study. A) Drawing of the left cheliped with anatomical sections. The preferred breakage plan is at the articulation of the basis and ischium. B) Drawing of a typical crayfish body with total body length measurement shown.

3.2.3 Quantifying the effect of limb loss on crayfish burrowing capacities

Fourteen crayfish were individually maintained in 15-gallon glass aquaria under a 12/12 day/night light cycle at 65˚F over a period of nine months. Autotomy was induced in ten crayfish (autotomized) and four crayfish were used as controls (unmanipulated). Then, each crayfish was individually positioned in a meshed arena containing mud placed in a 15-gallon aquarium filled with gravel for water table control. The water level was kept two inches below the mud surface throughout the experiment. Each crayfish was put into the arena at the start of the experiment as well as after each molting event over the experimental period. A period of two days post molting was given to each crayfish allowing them to harden their exoskeleton before they were put in the experimental setting. Each trial was recorded for two consecutive nights using security cameras equipped with infrared lights were. Videos were scored for (1) time until burrowing (burrowing latency in minutes), (2) capacity to build a chimney (presence or absence), and (3) proportion of time spent exposed on the surface. Repeating measurements of burrowing behavior over several molts for the same crayfish allowed us to characterize the recovery (if any) of crayfish burrowing abilities after autotomy and regeneration. Seven males and seven females were used in this experiment and they were all of a similar size (mean ± SE = 6.8 ± 0.2 cm).

3.2.4 Determining the effect of shelter availability on crayfish behavior

Two inches of wet mud were placed in ten, 15-gallon glass aquaria separated by black fabric to prevent social interactions between crayfish. One randomly chosen control crayfish (unmanipulated) was placed in each tank at the start of the experiment with time to acclimate before dark onset (~10 hours). The experiment was recorded for 12 hours each night. After 12 hours, individual crayfish were removed to a new tank with water and an air stone until the next night trial. Before starting the second and fourth nights, one shelter made of three clay bricks (two parallel and two inches apart on the mud and one on top forming a roof) was placed in the test aquarium. Autotomy was induced in one randomly chosen cheliped of each crayfish prior to the third night of recording. Overnight recordings were then made for control and autotomized crayfish with and without shelter. The proportion of time spent standing, walking, burrowing, or in the shelter were scored from the videos. Finally, crayfish burrowing capacities were assessed using a burrowing score index (BSI; 0 = no burrow, 1 = depression, 2 = burrow, 3 = partial chimney, 4 = chimney; see Figure 3.4 for drawings of the different BSIs). Using the same crayfish for two and one clawed treatments allowed us to control for any individual behavioral differences, hence, isolating the effect of shelter presence on crayfish behavior mediated by limb loss. Eight males were used in this experiment. Each individual was first run through the two-claw treatment (control), then autotomized for the remainder of the experiment as explained above. Individuals were of similar sizes (mean ± SE = 9.8 ± 0.3 cm).

3.2.5 Determining the effect of predatory cues on crayfish undergoing regeneration

15-gallon glass aquaria were kept under a 12/12 day/night light cycle. A bank of mud was created on one side of each tank to mimic a stream bank, then each tank was filled with water two inches lower than the mud bank. This created an artificial mud bank exposed to air and a water pool. Automatic infrared cameras were used to record the experiment in light and darkness. Videos were then downsampled post-production before scoring to reduce the frame rate and speed up scoring (crayfish move slowly enough to allow scoring at one frame per second and sped up to 32x). Fifteen adult male crayfish of similar size (total body length: mean ± SE = 94.2 ± 2.5 cm) were randomly selected from our colony. Each crayfish was then placed in a tank and left to acclimate until dark onset (between 6 and 10 hours). The first recording cycle comprised two days and two nights. At the end of the second day, conspecific predation cues were added to the water in each tank. These cues consisted of fresh crunched crayfish in a one-gallon bucket filled with water (crayfish perceive predation risk from conspecific cues rather than predator cues; Gherardi et al. 2011b); 40 ml of this water was then added to each experimental tank. The second recording cycle comprised another two days and two nights. Similar addition of predation cues occurred, but autotomy of a randomly chosen cheliped was induced in each crayfish prior to this second cycle. Crayfish were kept individually in well-oxygenated water inside holding tanks between the two cycles for 24 hours while the mud dried. Drying effectively removed all traces of conspecific predation cues. Finally, crayfish burrowing capacity using BSI and proportion of time spent standing, walking, burrowing, outside of water, or unseen (in the water or burrow) were scored during each night trial.

3.2.6 Statistics

All results are presented with averages and standard errors in parentheses. Proportions of all activities recorded (standing, walking, burrowing, outside, or hidden) were calculated from each experimental time due to small discrepancies in total experiment times. The data were analyzed using generalized linear models (quasi-binomial or Gaussian families; R Core Team 2017) and general linear hypotheses (Tukey tests; Hothorn 2006). Graphs and analyses were generated using the R-packages ‘ggplot2’ (Wickham 2009a), ‘reshape2’ (Wickham 2007), ‘cowplot’ (Wilke 2017), ‘multcomp’ (Hothorn et al. 2008), ‘psych’ (Revelle 2017), and ‘stats’ (Team 2017). All the data and R script with packages used in this work are available on Dryad (https://doi.org/10.5061/dryad.h70rxwdf2).

3.3 Results

3.3.1 Cheliped regeneration in adult crayfish occurs over several molts

We observed cheliped regeneration over a period of six months during which crayfish replaced the lost appendage across successive molts (Figure 3.2A-E). Although only a small and non-functional limb bud is regrown after the first molt following autotomy (Figure 3.2C), a substantial, yet not totally functional, appendage is regenerated after the second molt (Figure 3.2D). Finally, by the third molt post autotomy, crayfish have regenerated a complete and functional cheliped which is only smaller in proportion compared to the original cheliped (Figure 3.2E). After subsequent molts, we observed the regenerated limb growing to approximate the contralateral limb in size. However, it never completely matched the contralateral, non-autotomized, limb.

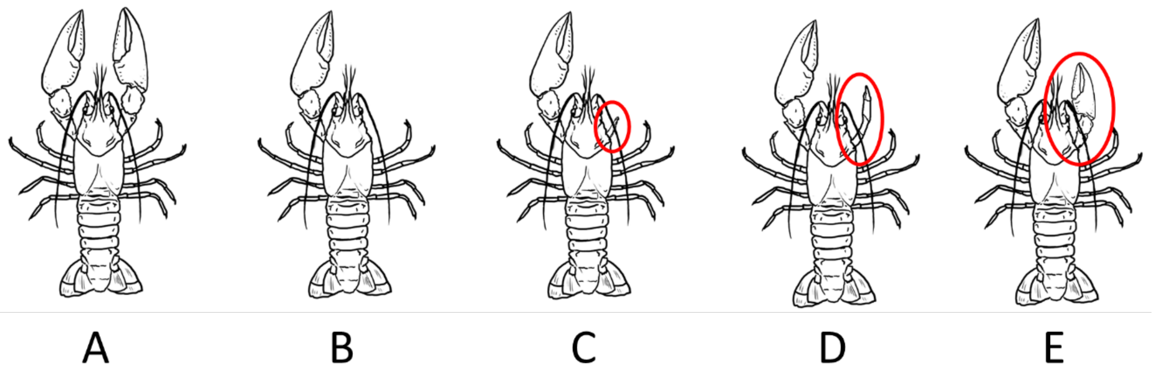

Figure 3.2: Brief overview of the regeneration process showing recovery (red circles) of cheliped size and ultimately function over several molts. A) Before autotomy and limb loss. B) Right after autotomy of the right cheliped. C) After first molt post autotomy and limb loss, the adult crayfish has regenerated a limb bud not yet functional. D) After the second molt post autotomy and limb loss, the regenerated limb has started rudimentary patterning but is not yet functional because it is not hardy enough to burrow effectively. E) Final molt of the experiment and third molt post autotomy and limb loss. The regenerated limb is everything like the original cheliped only proportionally smaller.

3.3.2 Burrowing ability is reduced immediately after limb autotomy but recovers during regeneration

Knowing the general time frame of cheliped regeneration, we first sought to explore if limb autotomy and subsequent regeneration impacted crayfish burrowing ability. Control crayfish (no autotomy) showed almost no burrowing latency (BL) with burrowing beginning soon after the experiment was initiated ($\text{BL}_{\text{CONTROL}}$ = 48 ± 9 minutes; Figure 3.3). In contrast, autotomized crayfish showed a significantly longer latency to burrowing compared to control crayfish ($\text{BL}_{\text{AUTOTOMY}}$ = 905 ± 180 minutes; t = 2.6, P = 0.016; Figure 3.3) and this latency continued after the first molt ($\text{BL}_{\text{FIRSTMOLT}}$ = 815 ± 230 minutes; t = 2.2, P = 0.035; Figure 3.3). Although non-significantly different from the control, a similar latency was present after the second molt ($\text{BL}_{\text{SECONDMOLT}}$ = 594 ± 230 minutes; t = 1.5, P = 0.146; Figure 3.3). After the third molt, autotomized crayfish latency was undistinguishable from control crayfish showing a total recovery ($\text{BL}_{\text{THIRDMOLT}}$ = 40 ± 1 minutes; t = -0.02, P = 0.986; Figure 3.3).

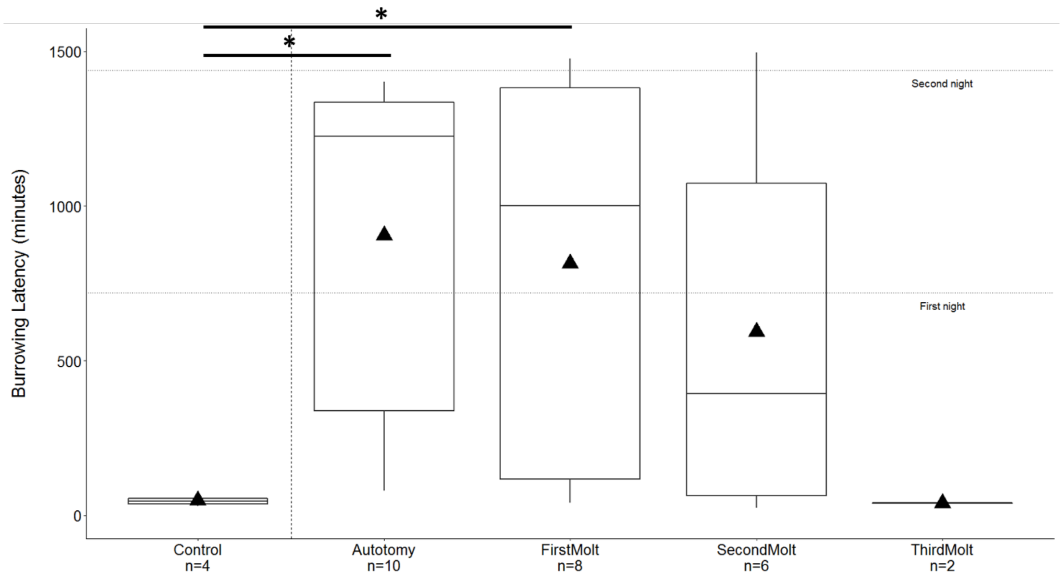

Figure 3.3: Autotomized crayfish initiate burrowing significantly later than two-clawed counterparts, but recover burrowing ability over the course of regeneration. Figure depicts time before first burrowing activity (burrowing latency – BL) in control (two chelipeds) and autotomized (one claw) crayfish. Autotomized crayfish were followed after each post-autotomy molt. Box plots with quantiles, median, and outliers are plotted. Large triangles represent averages. Asterisks represent significant differences between groups (see text). Dotted lines represent the length of the first and second night (note that each recorded night was "roughly" 12 hours long). The dash line symbolizes the fact that different individuals are used for the control treatments. Sample sizes are given under each treatment.

Next we asked how limb loss and regeneration might affect chimney building by measuring the proportion of animals that engaged in chimney building (PCB) and the extent to which chimneys were built. All control crayfish built a complete chimney during the experiment while none of the autotomized crayfish did after the induced autotomy ($\text{PCB}_{\text{CONTROL}} = 1.0$; $\text{PCB}_{\text{AUTOTOMY}} = 0.0$; Table 3.1; Figure 3.4). The proportion of burrows with some degree of chimney construction then gradually increased after each molt until complete recovery after the third molt ($\text{PCB}_{\text{FIRSTMOLT}} = 0.13$; $\text{PCB}_{\text{SECONDMOLT}} = 0.67$; $\text{PCB}_{\text{THIRDMOLT}} = 1.0$; Table 3.1). Similarly, the crayfish burrowing score index (BSI) followed the same trend with control crayfish constructing their burrow with a complete chimney ($\text{BSI}_{\text{CONTROL}} = 4.0 \pm 0.0$; Figure 3.4). This ability was slowly recovered after the induced autotomy until complete recovery after the third molt ($\text{BSI}_{\text{AUTOTOMY}} = 0.70 \pm 0.26$, $z = -6.9$, $P < 0.001$; $\text{BSI}_{\text{FIRSTMOLT}} = 1.33 \pm 0.42$, $z = -5.1$, $P < 0.001$; $\text{BSI}_{\text{SECONDMOLT}} = 2.50 \pm 0.34$, $z = -2.9$, $P = 0.031$; $\text{BSI}_{\text{THIRDMOLT}} = 3.0 \pm 0.0$, $z = -1.43$, $P = 0.597$; Figure 3.4).

Table 3.1: Sample sizes and proportions of chimney built by control (two chelipeds) or autotomized (one cheliped) crayfish. Autotomized crayfish were observed after each molt.

	Control	Autotomy	First Molt	Second Molt	Third Molt
Proportion of Chimney built	1.00	0.00	0.13	0.67	1.00
Sample size	4	10	8	6	2

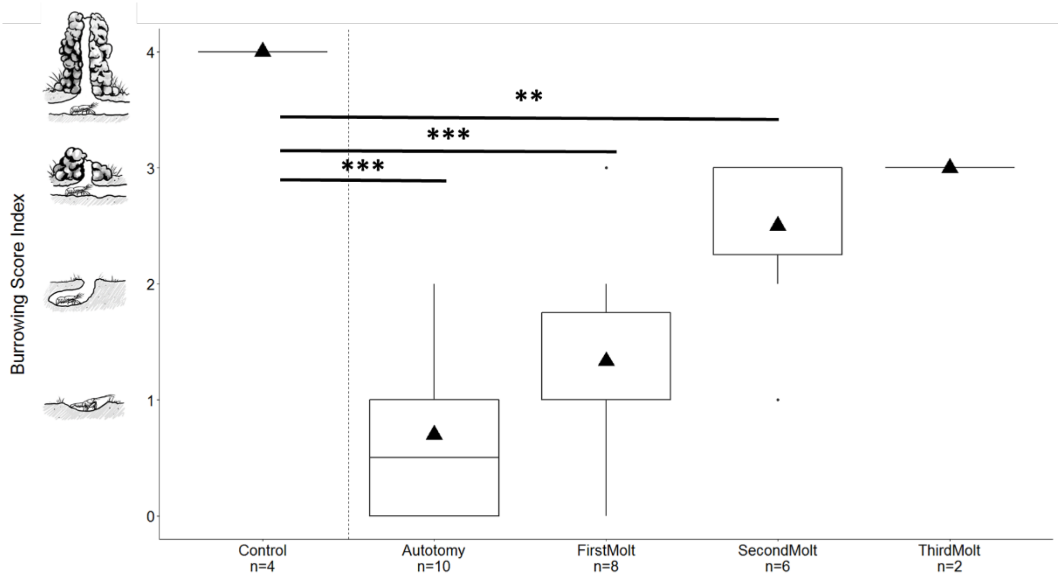

Figure 3.4: Burrowing ability is severely reduced after autotomy but recovers during regeneration. Drawings represent qualitative burrowing output (0 = no burrow, 1 = depression, 2 = burrow, 3 = partial chimney, 4 = chimney) of control (two chelipeds) and autotomized (one cheliped) crayfish. Box plots with quantiles, median, and outliers are plotted, and large triangles represent averages. Asterisks represent significant differences between groups (see text). The dash line symbolizes the fact that different individuals are used for the autotomized treatments. Sample sizes are given under each treatment.

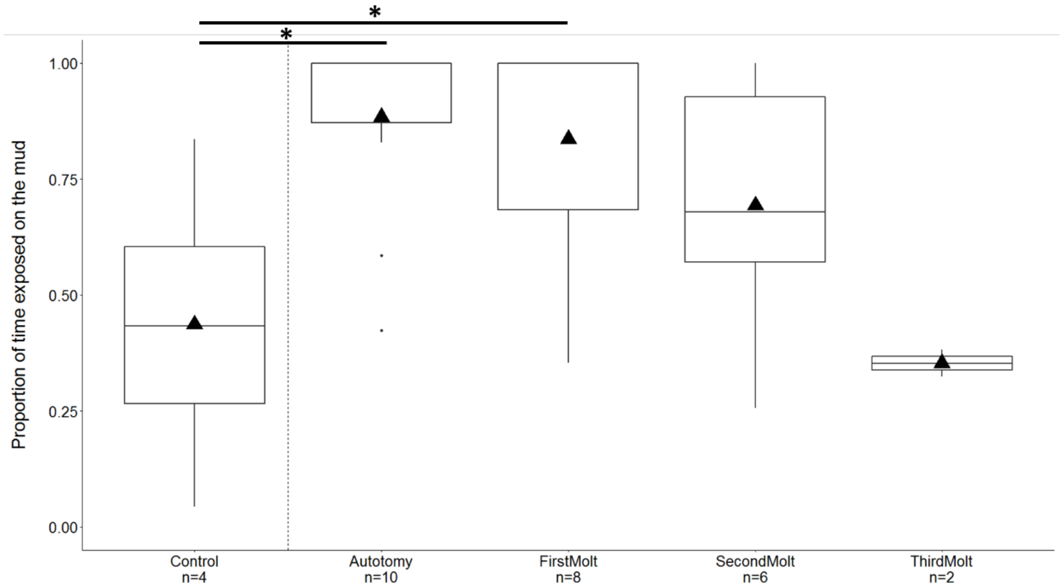

Figure 3.5: Regenerating crayfish significantly more time exposed to predation compared to their unmanipulated counterparts. Proportion of time spent outside on the mud (exposed) by control (two chelipeds) or autotomized (one cheliped) crayfish. Crayfish were followed after induced autotomy of one cheliped for three molts. Box plots with quantiles, median, and outliers are plotted, and large triangles represent averages. Asterisks represent significant differences between groups (see text). The dash line symbolizes the fact that different individuals are used for the autotomized treatments. Sample sizes are given under each treatment.

The proportion of time spent out or exposed to predation (PTE) showed a similar pattern as burrowing latency. Control crayfish spent on average less than half of the experimental time exposed on the mud surface ($PTE_{CONTROL} = 0.44 \pm 0.17$; Figure 3.5); whereas, autotomized crayfish spent significantly more time exposed on the mud after the induced autotomy ($PTE_{AUTOTOMY} = 0.88 \pm 0.07$; $t = 2.6$, $P = 0.016$; Figure 3.5). Similarly, after their first molt autotomized crayfish still spent more time exposed on the mud surface compared to control crayfish ($PTE_{FIRSTMOT} = 0.84 \pm 0.09$, $t = 2.3$, $P = 0.034$; Figure 3.5). Autotomized crayfish showed signs of recovery only after their second molt while still spending more time exposed on the mud although this time was non-significantly different from control crayfish ($PTE_{SECONDMOLT} = 0.69 \pm 0.12$, $t = 1.3$, $P = 0.208$; Figure 3.5). Finally, autotomized crayfish spent similar amounts of time on the mud surface compared to control crayfish after their third molt exhibiting a total recovery with less than half of the experimental time spent exposed on the mud ($PTE_{THIRDMOLT}$ = 0.35 ± 0.03, $t = -0.3$, $P = 0.765$; Figure 3.5). Together, these data demonstrate that limb loss negatively impacts time to burrowing and burrow construction. This behavioral alteration is recovered as a function of regenerative ability. In addition, losing a limb causes crayfish to spend more time exposed to predation when away from water.

3.3.3 Shelter presence diminishes regeneration effect on burrowing and changes crayfish activity

Given our observation that crayfish undergoing limb regeneration exhibit reduced burrowing ability, and thus are more exposed to predation, we asked if limb loss and regeneration affected decision making to seek shelter when a shelter was already present. This is in opposition to crayfish having to dig out their own shelter in the previous experiment. We used two inches of mud in this experimental setup to prevent crayfish from digging very deep burrows. Thus, under this paradigm crayfish could score a maximum of two on the burrowing score index (BSI). Nonetheless, we confirmed our previously observed difference in burrowing score index between control and autotomized crayfish with the former still achieving more complex burrows compared to the latter ($BSI_{CONTROL}$ = 0.94 ± 0.22, $BSI_{AUTOTOMIZED}$ = 0.25 ± 0.11, $z = 3.2$, $P = 0.001$; Figure 3.6). Crayfish regenerative status only affected the time spent inside the shelter with control crayfish spending more time hidden, but had no effect on the time spent burrowing or walking when exposed on the mud (Proportions of time spent; $Inside_{CONTROL}$ = 0.43 ± 0.11, $Inside_{AUTOTOMIZED}$ = 0.37 ± 0.10, $z = 2.0$, $P = 0.046$; $Burrowing_{CONTROL}$ = 0.12 ± 0.05, $Burrowing_{AUTOTOMIZED}$ = 0.14 ± 0.06, $z = -0.4$, $P = 0.722$; $Walking_{CONTROL}$ = 0.40 ± 0.07, $Walking_{AUTOTOMIZED}$ = 0.42 ± 0.06, $z = -0.3$, $P = 0.775$; Figure 3.7).

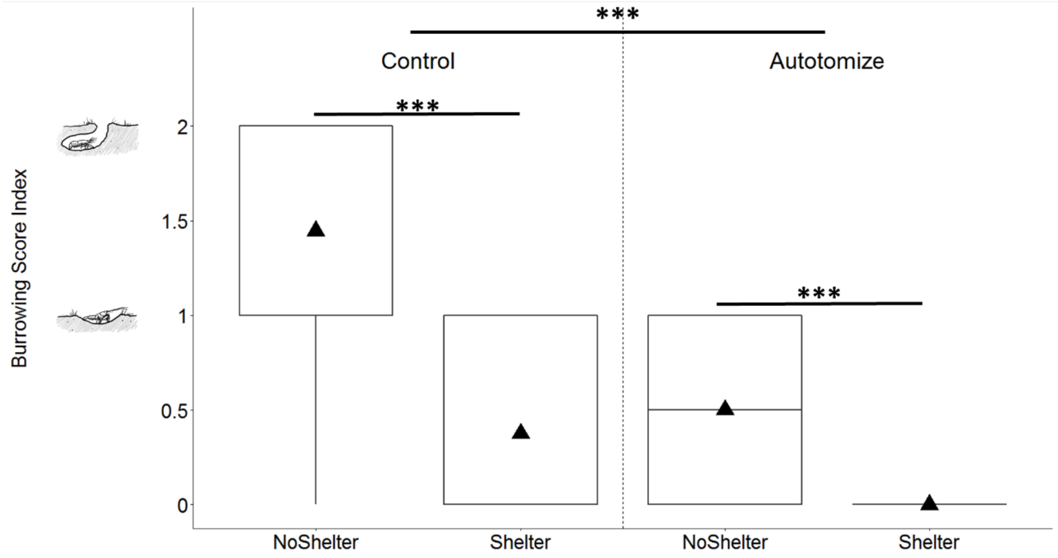

Figure 3.6: Shelter reduces crayfish burrowing output. Qualitative burrowing output (0 = no burrow, 1 = depression, 2 = burrow) of control (two chelipeds – first and second nights) and autotomized (one cheliped – third and fourth nights) crayfish when shelter is absent (NoShelter) or present (Shelter). Boxplots with quantiles, median, and outliers are plotted and large triangles represent averages. Asterisks represent significant differences between groups (see text). n=8 across the experiment.

As predicted, the presence of a shelter influenced the burrowing score index (BSI) with more complex burrows achieved in the absence of shelter ($BSI_{NOSHELTER} = 1.0 \pm 0.21$, $BSI_{SHELTER} = 0.19 \pm 0.10$, $z = -3.8$, $P < 0.001$; Figure 3.6). Moreover, the presence of shelter changed the kind of activity crayfish engage in when exposed on the mud; when a shelter was absent crayfish burrow more and walk less (Proportions of time spent; $Burrowing_{NOSHELTER} = 0.23 \pm 0.06$, $Burrowing_{SHELTER} = 0.04 \pm 0.03$, $z = -2.3$, $P = 0.023$; $Walking_{NOSHELTER} = 0.22 \pm 0.03$, $Walking_{SHELTER} = 0.60 \pm 0.06$, $z = 5.5$, $P < 0.001$; Figure 3.7). Using the presence or absence of a shelter and measuring burrowing index, we found that when a shelter is available crayfish burrow significantly less regardless of their regenerative status and engage in more exploratory behaviors when regenerating.

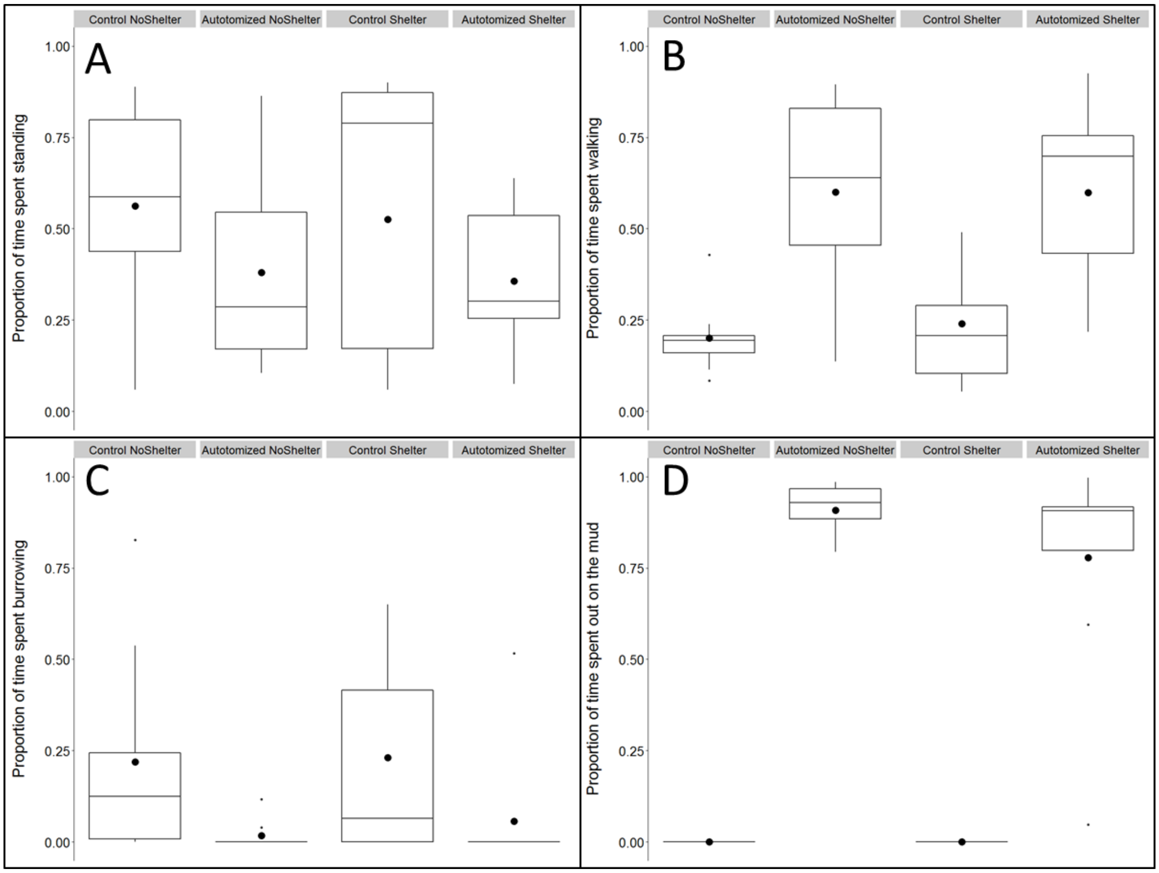

Figure 3.7: Regenerating crayfish access shelter more compared to their unmanipulated counterparts. Given an available shelter, crayfish were scored for the proportion of time spent standing (A), walking (B), burrowing (C), and time spent inside the shelter (D) while exposed to predation on the mud overnight (12 hours). Crayfish possessed either two chelipeds (control – first and second nights, see Methods) or one cheliped (autotomized – third and fourth nights, see Methods). Shelter was absent (NoShelter) or present (Shelter). Boxplots with quantiles, median, and outliers are plotted and symbols represent averages. n=8 across the experiment.

3.3.4 Regenerative status offsets effect of predation cues on burrowing output

Finally, we asked if predatory cues (see methods) would affect crayfish burrowing output and behavior as measured by the burrowing score index (BSI) and proportion of time spent engaged in different activities, respectively. Our results confirmed the impact of limb loss on crayfish burrowing with control individuals achieving higher BSI than autotomized individuals ($BSI_{CONTROL} = 0.89 \pm 0.15$, $BSI_{AUTOTOMIZED} = 0.14 \pm 0.08$, $z = 3.8$, $P < 0.001$; Figure 3.8). However, only control crayfish burrowing output was affected by the addition of predatory cues

($BSI_{CONTROL\_PreCue} = 1.32 \pm 0.23$, $BSI_{CONTROL\_PostCue} = 0.47 \pm 0.16$, $z = -3.6$, $P = 0.002$; $BSI_{AUTOTOMIZED\_PreCue} = 0.21 \pm 0.15$, $BSI_{AUTOTOMIZED\_PostCue} = 0.07 \pm 0.07$, $z = -0.5$, $P = 0.949$; $BSI_{INTERACTION}$, $z = -4.3$, $P < 0.001$; Figure 3.8).

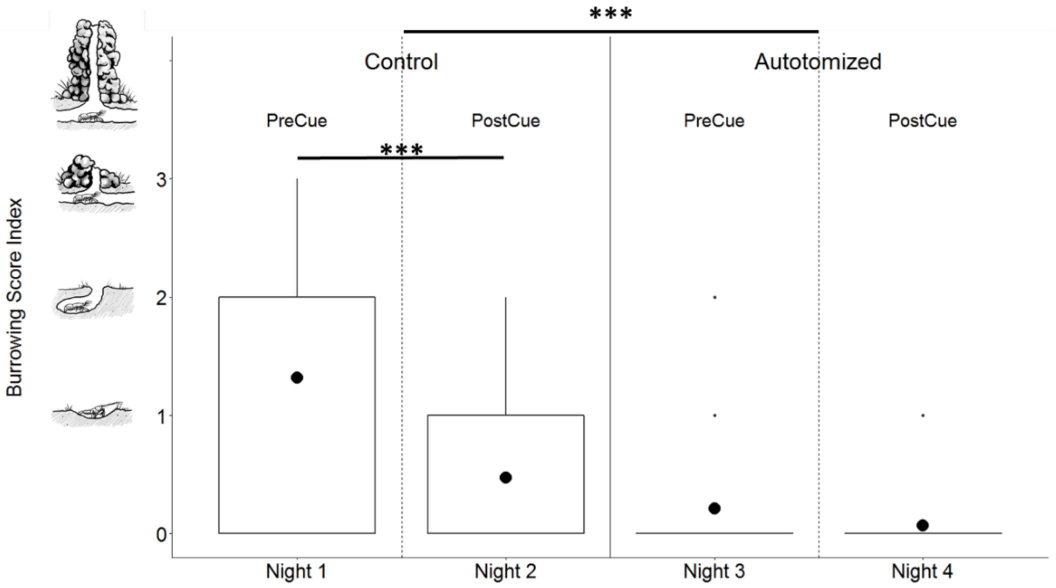

Figure 3.8: Predator cues reduce burrowing output in unmanipulated crayfish. Qualitative burrowing output (0 = no burrow, 1 = depression, 2 = burrow, 3 = partial chimney) by control (two chelipeds – left of the solid line) and autotomized (one cheliped – right of the solid line) crayfish before and after conspecific predatory cues have been added (dash lines). Boxplots with quantiles, median, and outliers are plotted and large dots represent averages. Asterisks represent significant differences between groups (see text). n=15 across the experiment.

Unexpectedly, regenerative status did not change the kind of activity crayfish engage in when exposed on the mud in this experimental context (Proportions of time spent; $Inside_{CONTROL} = 0.62 \pm 0.04$, $Inside_{AUTOTOMIZED} = 0.66 \pm 0.05$, $z = -0.7$, $P = 0.482$; $Burrowing_{CONTROL} = 0.03 \pm 0.01$, $Burrowing_{AUTOTOMIZED} = 0.03 \pm 0.1$, $z = 0.4$, $P = 0.723$; $Walking_{CONTROL} = 0.03 \pm 0.01$, $Walking_{AUTOTOMIZED} = 0.02 \pm 0.1$, $z = 0.2$, $P = 0.809$; Figure 3.9). Although regenerative crayfish had the opportunity to adjust their behavior (Figure 3.9D), the addition of predatory cues did not significantly change the kind of activity crayfish engage in when exposed on the mud in this experimental context (Proportions of time spent; $Inside_{CONTROL\_PreCue} = 0.60 \pm 0.05$, Inside$_{\text{CONTROL\_PostCue}}$ = 0.64 ± 0.06, z = 0.4, P = 0.971; Inside$_{\text{AUTOTOMIZED\_PreCue}}$ = 0.56 ± 0.07, Inside$_{\text{AUTOTOMIZED\_PostCue}}$ = 0.75 ± 0.06, z = 2.0, P = 0.192; Burrowing$_{\text{CONTROL\_PreCue}}$ = 0.04 ± 0.01, Burrowing$_{\text{CONTROL\_PostCue}}$ = 0.03 ± 0.02, z = -0.3, P = 0.988; Burrowing$_{\text{AUTOTOMIZED\_PreCue}}$ = 0.04 ± 0.03, Burrowing$_{\text{AUTOTOMIZED\_PostCue}}$ = 0.01 ± 0.01, z = -1.4, P = 0.508; Walking$_{\text{CONTROL\_PreCue}}$ = 0.04 ± 0.01, Walking$_{\text{CONTROL\_PostCue}}$ = 0.02 ± 0.01, z = -1.4, P = 0.464; Walking$_{\text{AUTOTOMIZED\_PreCue}}$ = 0.04 ± 0.02, Walking$_{\text{AUTOTOMIZEDPostCue}}$ = 0.01 ± 0.01, z = 1.9, P = 0.199; Figure 3.9). Together, this data shows that when crayfish sense predator cues they reduce burrowing. However, regenerative status has a far stronger effect on crayfish behavior than does predation pressure.

3.4 Discussion

Autotomy and regeneration are important components of crayfish biology that have the potential to impact local ecosystem dynamics. In this study, we sought to understand how autotomy and regeneration might affect ecologically relevant crayfish behaviors. In support of our first hypothesis, we demonstrated how the presence or absence of the first leg pair (chelipeds) in *Procambarus clarkii* directly mediated burrowing capacity. Additionally, we found that regenerative status (i.e., stage of cheliped regeneration) further mediated this behavior. *P. clarkii* missing one cheliped exhibited poor burrowing ability and were unable to build a chimney, behaviors which potentially leave them more exposed to predation compared to their two-clawed counterparts.

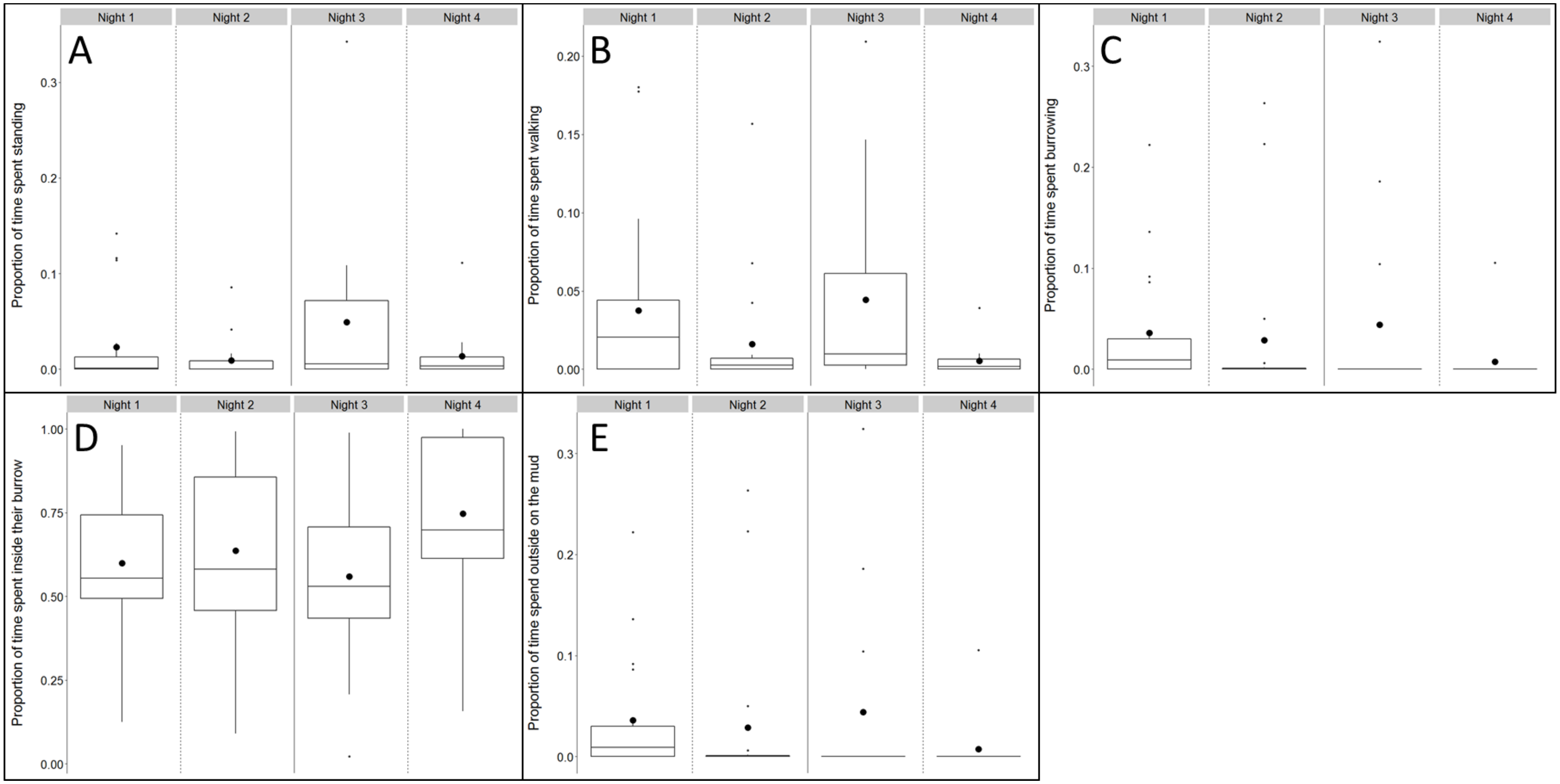

Figure 3.9: Crayfish behaviors are not significantly impacted by the addition of predator cues. Proportion of time spent standing (A), walking (B), burrowing (C), inside their burrow (D), and outside on the mud (E) overnight (12 hours) by control (two chelipeds, nights 1 and 2 – left of the solid line) and autotomized (one cheliped, nights 3 and 4 – right of the solid line) crayfish before (nights 1 and 3) and after (nights 2 and 4) conspecific predatory cues have been added (dash lines). Boxplots with quantiles, median, and outliers are plotted and different symbols represent averages. n=15 across the experiment.

Although crayfish might simply seek shelter when missing a cheliped (instead of creating a burrow), we found that individuals with two claws spent significantly more time in the open compared to individuals missing a cheliped that spent more time hiding in shelter. Because control crayfish burrow with greater efficiency, they could gain shelter in their burrow, instead of that provided in the experimental setup. Indeed, even if control and autotomized crayfish spent a similar amount of time burrowing in the presence of a shelter, crayfish missing a cheliped were unable to construct a burrow (BSI $\leqq 1$) compared to their two clawed counterparts. Furthermore, when a shelter was present, crayfish (regardless of their regenerative status) spent less time burrowing, but interestingly more time walking, perhaps relying on the shelter for a quick escape if endangered. Finally, supporting our hypothesis, the presence of predatory cues affected only unmanipulated crayfish burrowing output but did not affect autotomized crayfish burrowing outcome (already diminished by limb loss) activity regardless of how much they had regenerated. This trend is suggestive of a weak effect of predatory cues, particularly for autotomized crayfish, and we encourage larger studies investigating the effects of predatory cues on crayfish missing a cheliped to zero-in on the exact impacts of the former on the latter.

Crayfish burrowing behavior has been extensively described and crayfish can be classified based on burrowing tendency as primary, secondary or tertiary burrowers (Hobbs 1942, Grow and Merchant 1980, Grow 1981, Berrill and Chenoweth 1982, Trépanier and Dunham 1999, Welch and Eversole 2006, Thoma and Armitage 2008). Among all three types, the first pair of chelipeds are necessary for burrowing. However, chelae morphology and function vary among the three crayfish burrowing types suggesting autotomy and regeneration might differentially affect burrowing capacities among these types. Tertiary burrowers such as *P. clarkii* are capable of burrowing when faced with desiccation risks, although their burrowing abilities are less developed compared to primary and secondary burrowers (Hobbs 1981, Berrill and Chenoweth 1982). The signal crayfish (*Pacifastacus leniusculus*; Dana 1852) is also a tertiary burrower and uses its chelae in a scissor like action together or alternatively to excavate mud pellets from the mud substrate (Hobbs 1981, Guan 1994). Indeed, chelae orientation is more vertical (compare to the ground) for primary compared to secondary or tertiary burrowers allowing primary burrowers to completely fill their burrows with their body from cephalothorax to the tip of the chelipeds. In contrast, tertiary burrowers tend to have horizontally oriented chelae to aid in squeezing under rocks when facing strong water currents (Loughman, Schuster, pers. comm.). Primary burrowers perhaps rely more on their second pair of chelipeds for burrowing complex structures essential to their survival (reaching the water table far from running water); thus, they might not exhibit any loss of burrowing capacity after losing or while regenerating (or both) one or even both of their chelae. Although crayfish also use their second and third pair of legs for burrowing (Guan 1994), we found that the absence of the first pair of legs (chelipeds) is sufficient to considerably hinder burrowing ability of a tertiary burrower. While our observation likely extends to primary and secondary burrowing types, further studies with a focus on the first two pairs of chelipeds are necessary to directly assess the impact of limb loss and regeneration on burrowing behavior in these other types.

In freshwater ecosystems, crayfish serve as ecosystem engineers through their effects on bioturbation. Bioturbation is an increase in an aquatic system turbidity through biological activity (Meysman et al. 2006) and is known for diminishing light penetration and primary production in aquatic ecosystems (Fager 1964, Heinzelmann and Wallisch 1991, Ciutat et al. 2005, Mermillod-Blondin and Rosenberg 2006). In turn, bioturbation also influences organic resource availability in stream communities contributing to species biodiversity and ultimately to ecosystem services. Crayfish engineering is known for greatly influencing the availability of organic resources directly (Momot 1995) or indirectly via bioturbation during burrow construction or sediments displacement (Angeler et al. 2001, Usio and Townsend 2004, Yamamoto 2010). As we show in this study, the ability to produce burrows is severely impacted by autotomy and can be restored through regeneration. Hence, factors changing incidence of autotomy or the efficiency of crayfish regeneration are likely to impact water quality and nutrient availability in freshwater ecosystems. Such impact may be particularly important as ecosystems face amplified stress due to climate change and increased anthropogenic land-use change. For example, crayfish invasions commonly result from anthropogenic impact and are a known factor increasing predation pressure on native organisms (Phillips et al. 2009). Thus, when native crayfish experience higher predation pressure, we can expect to see higher regeneration rates in those populations in turn diminishing the induced bioturbation (invasive species might not be functionally redundant to their native counterparts; *i.e.*, of a different burrowing type). Lower bioturbation leads to increase light penetration and primary production could potentially lead to eutrophication. If the invasive crayfish population induces more bioturbation due to its burrowing activities, we can expect light penetration as well as primary productivity to diminish affecting the entire community.

Chelae have diverse functions and it is likely that their autotomy and regeneration affect other behaviors. In addition to burrowing, chelipeds are important for aggressive or display behaviors or both; e.g., mantis shrimp bluff aggressive displays when freshly molted and fiddler crabs attract females by displaying enlarged cheliped despite being more exposed to predation by doing so (Bildstein et al. 1989, Adams and Caldwell 1990). Some species rely heavily on their chelipeds either for retention or acquisition of shelter (O'Neill and Cobb 1979). In hermit crab, regenerative status is highly predictive of who initiates and wins intraspecific fights (Neil 1985). Cheliped regenerative status was found to influence territory size in a prawn species where individuals missing one or two chelipeds secured smaller territories than their two clawed counterparts (Seidel et al. 2007). Given broad cheliped utilities across crustaceans, we can expect similar behavioral shifts in crayfish with respect to cheliped usage between individuals possessing both chelipeds compared to those who have autotomized or are regenerating one cheliped.

Crayfish interspecific competition for shelter usually leads the native crayfish to be displaced by the invader (Usio et al. 2001, Gherardi and Daniels 2004). Similarly, several experiments showed a size dependent displacement of fish species when competing for shelter with crayfish (Rahel and Stein 1988, Guan and Wiles 1997, Light 2005, Hirsch and Fischer 2008). Nonetheless, none of the studies investigating crayfish interspecific interactions accounts for the regenerative status of the chelipeds despite the fact that we know numerous crayfish populations have a significant proportion of individuals regenerating limbs (*i.e.*, up to 30%, meaning almost every individual is going to regenerate at some point over its lifetime; Powell et al. 1998). Thus, regenerative status is likely to play an important role during interspecific competition for shelter and our study underlines the importance of future research investigating the impacts of limb loss and regeneration in intraspecific competition for shelter (especially during drought events).

The impacts of predation risk on crayfish behavior and shelter usage have been previously hypothesized (Willman et al. 1994, Nyström 2005). Previous research has shown that crayfish key on conspecific cues (released by crushed conspecifics during successful or unsuccessful predation events for example) for assessing predation risks (Gherardi et al. 2011). Interestingly, our results show that *P. clarkii* are less sensitive to predation while undergoing regeneration. We found that crayfish missing a cheliped were unable to react to the addition of predatory cues potentially because the loss of a limb already led crayfish to reduce their burrowing output significantly. Note that we did not eliminate the possibility of predatory cue habituation due to the nature of the predatory cues used in the experiment (crushed conspecifics) potentially leading regenerative crayfish to habituate to the cue coming from their own missing limb in this experiment. Nonetheless, crayfish unable to burrow during drought are exposed to predation by avian and terrestrial predators more so than crayfish able to hide in their burrows or elsewhere. Moreover, crayfish missing a limb may be unable to secure a refuge when most needed (e.g., during drought or following reproduction for females only, see Introduction). Because of the differential susceptibility to predation between one and two clawed crayfish showed in this study, our understanding of crayfish population dynamics might be incomplete especially given the increasing pressures on crayfish population (pollution, invasions, global climate change; Taylor et al. 2007, DiStefano et al. 2009).

To conclude, the consequences of limb loss and regeneration on crayfish behavioral ecology represent an important avenue for future research. Although ecosystems are resilient, ecosystem-wide changes could result from behavioral shifts, as mentioned above, given the prominence of crayfish for freshwater food-webs and habitat characteristics. Our study highlighted several avenues for future research in this regard. First, more research is needed on behavioral impacts (if any) of limb loss and cheliped regeneration in secondary and primary crayfish burrowers. Similarly, all crustacean species can potentially exhibit similar behavioral changes due to autotomy and regeneration. Second, more research is needed to uncover the impact of crayfish cheliped loss and regeneration on water quality as well as nutrient availability in freshwater ecosystems. Third, research investigating crayfish competition for shelter usually ignores the impact of limb loss and regeneration on intra- and interspecific competitive interactions. Future work investigating how cheliped loss and regeneration affect the impact of competition on crayfish population dynamics will be an important step in unraveling the ultimate impact of crayfish behavior on ecosystem stability.

CHAPTER 4. AUTOTOMY DOES NOT AFFECT REPRODUCTIVE SUCCESS OF ADULT RED SWAMP CRAYFISH AND REGENERATION CAN BE A CONTINUOUS PROCESS IN JUVENILE CRAYFISH

Luc A. Dunoyer, Zoe Dapore, Jeremy Van Cleve, and Ashley Seifert

4.1 Introduction

Only six phyla possess the ability to regenerate large sections of the primary or secondary body axes (Hughes 1989, Sánchez Alvarado 2000). In crustaceans, regeneration occurs in response to the reflex severance (autotomy) of one or more limbs after injury or threat, and autotomy always occurs at a predetermined breakage plane (Wood and Wood 1932b, Robinson et al. 1970, McVean 1982). The occurrence of limb autotomy and subsequent regeneration are thought to be tightly linked to molting (ecdysis) as the former could not proceed without the latter (Bliss 1960, Hopkins 1982, 1993, Cooper 1998, Shinji et al. 2019). This vision is not shared by all biologists, as reported by some early regeneration researchers (Réaumur 1712) or by more recent research showing eight stages of regeneration before ecdysis in male shrimp, *Cryphiops caementarius* (Reyes et al. 2017). Furthermore, although autotomy and regeneration have been extensively studied for more than a century, their impact on reproductive success is poorly understood (Maginnis 2006; but see below).

The red swamp crayfish (*Procambarus clarkii*, Girard 1865) is native from the Gulf of Mexico from Texas to Florida and is found along the Mississippi river basin from Louisiana up to Kentucky (U.S. Department of the Interior | U.S. Geological Survey 2018). *P. clarkii* is the most translocated invertebrate in the world, mainly through pet-trade or as fishing bait (DiStefano et al. 2008). Moreover, the red swamp crayfish is responsible for extensive ecological damage; perhaps best documented in Europe (Barbaresi et al. 2004, Gherardi and Aquiloni 2011), this includes outcompeting native crayfish species due to the crayfish plague (*Aphanomyces astaci*; Schikora 1906; Provenzano 1985), which is deadly to non-American crayfish species (Alderman et al. 1990, Holdfch and Rogers 1997, Lozán 2000). *P. clarkii* is commercially important throughout the world but mainly in the United States of America where the industry totals $45 Million each year (McClain et al. 2007). This species typically reproduces twice a year; in the spring and fall. Females can carry up to 500 eggs when in berry (clutch size highly correlated with female size) and reach sexual maturity between six months and a year old while living for two to three years in average (McClain et al. 2007). When in berry, female seek shelter in a burrow in which they stay until after the eggs hatch and the juveniles (craylings; Jones and Colin 2018) are ready to swim on their own (typically after the fifth molt after hatching while still staying under the mother's tail; McClain et al. 2007). Their economic importance renders this species ideal for the investigation of the impacts of limb loss via autotomy and regeneration on reproductive success and, ultimately, on commercial harvest sizes.

Although, survival is reduced for autotomized *Procambarus clarkii,* individuals in high versus low density ponds as well as compared to non-regenerating individuals in those same ponds (Figiel and Miller 1995), autotomy may provide an obvious immediate advantage as a strategy to avoid predation or to limit wounds (Wood and Wood 1932b, Bliss 1960, McVean 1982)., Autotomized individuals can also experience reduced access to shelter (due to lack of burrowing capacities, Chapter 3) or food in addition to a reduced ability to find a mate compared to their non-autotimized counterparts (Kuris and Mager 1975, Sekkelsten 1988, Davenport et al. 1992, Abelló et al. 1994, Juanes and Smith 1995, Smith 1995). These costs might affect the reproductive output of autotomized individuals (Maginnis 2006 and references therein). Similarly, regenerating individuals continue to face costs associated with their missing appendage such as physiological allocation costs (Maginnis 2006 and references therein). Oftentimes, allocation costs can be affected by several factors including species lifespan, time of the autotomy (*e.g.*, during the breeding season or not), and food availability (Maginnis 2006 and references therein). Although we have a good understanding of crustacean regeneration at the genetic, cellular, tissue, organ, and organismic levels (Bliss 1960, Skinner 1985, Juanes and Smith 1995, Hopkins 2001, Vafopoulou 2009, Shinji et al. 2016), there are very few studies assessing the impact of regeneration on reproductive output generally and specifically in crayfish (Galeotti et al. 2008, 2012). Moreover, these studies focus solely on the impact of limb loss on male sperm deposit size in Astacidae (European crayfish; Galeotti et al. 2008, 2012). However, in the main North American crayfish family, Cambaridae, males' spermatophores do not remain on females' annulus ventralis and are instead, stored internally by females until oviposition (Taylor and Schuster 2004). However, those measures of fitness are indirect and may miss the important costs of autotomy right after it takes place (e.g., reproductive or growth costs). These costs could generate a trade-off between reproduction (egg production) and regeneration (lost appendage regrowth) in females that could be key to understanding how autotomy and regeneration affect reproductive success in crayfish.

Crayfish are one of the few invertebrates capable of regenerating lost appendages while having large environmental impacts via complex interactions such as ecosystem engineering (environmental modification by organisms from one physical state to another; Jones et al. 1994, 1997). Crayfish are well-known ecosystem engineers; modifying freshwater stream ecosystems while foraging and burrowing (Nystrom and Strand 1996, Statzner et al. 2000, 2003, Albertson and Daniels 2016). Such behaviors profoundly affect the ecosystem, altering water properties (e.g., turbidity; Mermillod-Blondin and Rosenberg 2006; Meysman et al. 2006), the physical state of the sediment (Usio and Townsend 2004), and the survival and reproduction of other organisms (Abrahams and Kattenfeld 1997, Meager et al. 2006, Kimbell and Morrell 2016). Indeed, many permanent residents of stream communities rely on crayfish burrows to cope with seasonal stressors, such as low water flow or drought (salamanders, Semlitsch 1981; Madison 1997; Trenham 2001; dragonflies, Pintor and Soluk 2006; crawfish frogs, Heemeyer et al. 2012). Finally, crayfish are also essential food source for numerous inhabitants of freshwater streams (over 200 species from insects to mammals and including many important sport fishes; DiStefano 2005).

These community and ecosystem effects are highly dependent on crayfish demography; thus, understanding how autotomy and regeneration affect crayfish reproductive success will help us start to understand how ensued crayfish demographic changes will affect the freshwater stream ecosystem. The aim of the present study was to investigate the process of regeneration and its link to molting as well as to investigate the costs of autotomy and regeneration on female reproductive success in a widely known arthropod (crustacean), the red swamp crayfish (*Procambarus clarkii*, Girard 1865). We first examined the process of regeneration by documenting papilla (the regenerate) development in juvenile crayfish over a period of 20 days while monitoring for ecdysis. Then, we assessed the impact of limb loss via autotomy and subsequent regeneration on crayfish reproductive success in female adult red swamp crayfish. We predicted that papilla stages would not require molting to produce a regenerate and that regenerative female crayfish (missing one of their chelipeds) would have a lower reproductive success as measured by egg number and average size.

4.2 Material and methods

4.2.1 Adult crayfish maintenance, autotomy, and mating procedures

4.2.1.1 Colony

Crayfish (Red swamp crayfish, *Procambarus clarkii*, Girard 1852) were maintained in a 100-gallon tub in the laboratory with a circulatory water pump and filter under a controlled day/night light cycle (12/12 hours). The environment was enhanced with orange mesh and PVC pipes of different sizes to provide shelter and reduce cannibalism. Individuals were obtained from laboratory stocks at the University of Kentucky and purchased via Carolina (https://www.carolina.com/). Animals were fed weekly ad libitum with salmon pellets (soft, moist salmon feed pellets, size 5/32", from Rangen Inc.).

4.2.1.2 Autotomy

Autotomy of one cheliped was induced by cutting the limb at the merus/ischium articulation (Chapter 3, Figure 4.1A) using a sharp blade or scalpel. The remaining limb part is either readily autotomized during or after the cut effectively preventing hemolymph loss. All experiments were conducted in laboratory at the University of Kentucky. All crayfish were measured to the nearest tenth of millimeter using digital calipers along seven measurements: Total Body Length (TBL), Cephalothorax Total Length (CeTL), Cephalothorax Width (CW), Cheliped Total Length (ChTL), Propodus Length (PL), Palm Width (PW), and Dactyl Length (DL; Figure 3.1).

4.2.1.3 Mating

Adult female crayfish (Red swamp crayfish, *P. clarkii*) were selected from our colony and isolated in individual tanks (20x15x10 cm) as part of a flow-through system under a controlled day/night light cycle (12/12 hours). Their environment was enriched with one PVC pipe providing shelter and mimicking the natural behavior of this species when in berry. After producing eggs, a female *P. clarkii* retreats to a burrow and stays there until her crayling reached their fifth molt and are ready to live on their own (McClain et al. 2007).

Each mature female was individually presented to three random males at a time in a mating arena (circle, radius = 25 cm). The water temperature was kept between 23-25˚C using a water heater (300W submersible fish tank water heater adjustable temperature) prior to adding the crayfish to improve mating success. Mating was attempted for a period of 30 minutes after which the female was removed from the mating arena; attempts were repeated with different males until a successful mating was observed or after a month passed, whichever came first. The mating was successful if females were seen held by one male in the mating position (chelae locked over her head by the male chelae and abdomens facing each other) for at least 10 consecutive minutes.

4.2.2 Juvenile rearing, maintenance, measurements, and papilla stage histology

Female adult crayfish were removed from our colony and individually kept when in berry (*i.e.*, with visible eggs on the underside of the tail; McClain *et al.*, 2007). Once hatched, we separated the craylings and raised them individually in cups from which the bottom had been replaced with a fine nylon mesh and placed in a plastic tub with all other juveniles and air pumps under a controlled day/night light cycle (12/12 hours). Juveniles were monitored and fed salmon pellets (soft, moist salmon feed pellets, size 5/32", from Rangen Inc.) daily. Once juveniles reached at least 3 cm (body size) autotomy was induced similarly as in adults (see above). After autotomy individuals were checked twice daily to assure accurate molt detection since crayfish eat their ecdysis to recover calcium bicarbonate (McClain et al. 2007). We measured cheliped sizes prior to autotomy and regenerate length and papilla stage every day following autotomy (by taking a picture of the individual underside and using ImageJ; Schneider, Rasband & Eliceiri, 2012). The only exception to this process was when a molt was seen in the cup (because the individual was too soft to handle).

4.2.3 The effect of limb loss and regeneration on adult female crayfish reproductive success

After inducing autotomy of one randomly chosen cheliped, each female was mated and then isolated (see above). When females were observed in berry the eggs were gently removed using a dull blade or pen cap onto a paper towel for counting. The width of twenty randomly chosen eggs were then measured to the nearest tenth of millimeter using electronic calipers and compiled to determine the average egg size of the clutch. Control individuals went through the same procedure minus the induced autotomy.

4.2.4 Data analysis

A Principal Component Analysis (PCA) was run with the seven body measurements (see above and Figure 4.1 and 4.2). Furthermore, Welsh two-sample t-test were run for each of the seven body measurement variables to statistically compare regenerating and unmanipulated crayfish size.

The effect of limb loss on crayfish fitness was analyzed using linear mixed models following a Gaussian distribution with the number of eggs or the average egg size per female as the dependent variable and the regenerative status (yes/regenerating or no/unmanipulated) as the independent variable. Because no size difference was found between regenerating and unmanipulated crayfish (see results), we used Total Body Length (TBL, in mm) as a fixed factor in our models to control for the effect of female size (clutch size being highly correlated with female size; McClain et al. 2007). Finally, p-values were calculated using the Satterthwaite's method (Kuznetsova et al. 2017).

All analyses were conducted in R (Team 2017) using the following packages: ggplot2 (Wickham 2009b), reshape2 (Wickham 2007), cowplot (Wilke 2017), multcomp (Hothorn et al. 2008), psych (Revelle 2017), nlme (Pinheiro et al. 2017), betareg (Cribari-Neto and Zeileis 2010), gridExtra (Anguie 2017), lme4 (Unknown 2010), lmerTest (Kuznetsova et al. 2017), and factoextra (Kuznetsova et al. 2017). All the data and R script with packages used in this work are available on Dryad (https://doi.org/10.5061/dryad.8kprr4xj8).

4.3 Results

4.3.1 Adults

4.3.1.1 Body measurements

A Principal Component Analysis (PCA) ran with the seven body measurements did not reveal any major differences between regenerating and unmanipulated crayfish (not presented here). Furthermore, no significant differences were found between regenerating and unmanipulated crayfish with respect to: Total Body Length ($TBL_{Regenerating} = 98.8 \pm 1.29$ mm, $TBL_{Unmanipulated} = 97.9 \pm 2.5$ mm, $t = -0.31$, $P = 0.77$), Cephalothorax Total Length ($CTL_{Regenerating} = 50.6 \pm 0.8$ mm,

$\mathrm{CBL}_{\mathrm{Unmanipulated}}$ = 50.4 ± 1.5 mm, t = -0.12, *P* = 0.91), Cephalothorax Width ($\mathrm{CW}_{\mathrm{Regenerating}}$ = 22.8 ± 0.4 mm, $\mathrm{CW}_{\mathrm{Unmanipulated}}$ = 21.7 ± 0.6 mm, t = -1.42, *P* = 0.19), Cheliped Total Length ($\mathrm{CTL}_{\mathrm{Regenerating}}$ = 70.1 ± 2.8 mm, $\mathrm{CTL}_{\mathrm{Unmanipulated}}$ = 68.0 ± 2.5 mm, t = -0.56, *P* = 0.59), Propodus Length ($\mathrm{PL}_{\mathrm{Regenerating}}$ = 34.0 ± 1.7 mm, $\mathrm{PL}_{\mathrm{Unmanipulated}}$ = 36.6 ± 1.8 mm, t = 1.04, *P* = 0.32), Propodus Width ($\mathrm{PW}_{\mathrm{Regenerating}}$ = 12.1 ± 0.2 mm, $\mathrm{PW}_{\mathrm{Unmanipulated}}$ = 12.2 ± 0.6 mm, t = 0.04, *P* = 0.97), Dactyl Length ($\mathrm{DL}_{\mathrm{Regenerating}}$ = 20.6 ± 1.6 mm, $\mathrm{DL}_{\mathrm{Unmanipulated}}$ = 21.9 ± 0.9 mm, t = 0.72, *P* = 0.49) (mean ± standard error; Figure 4.1).

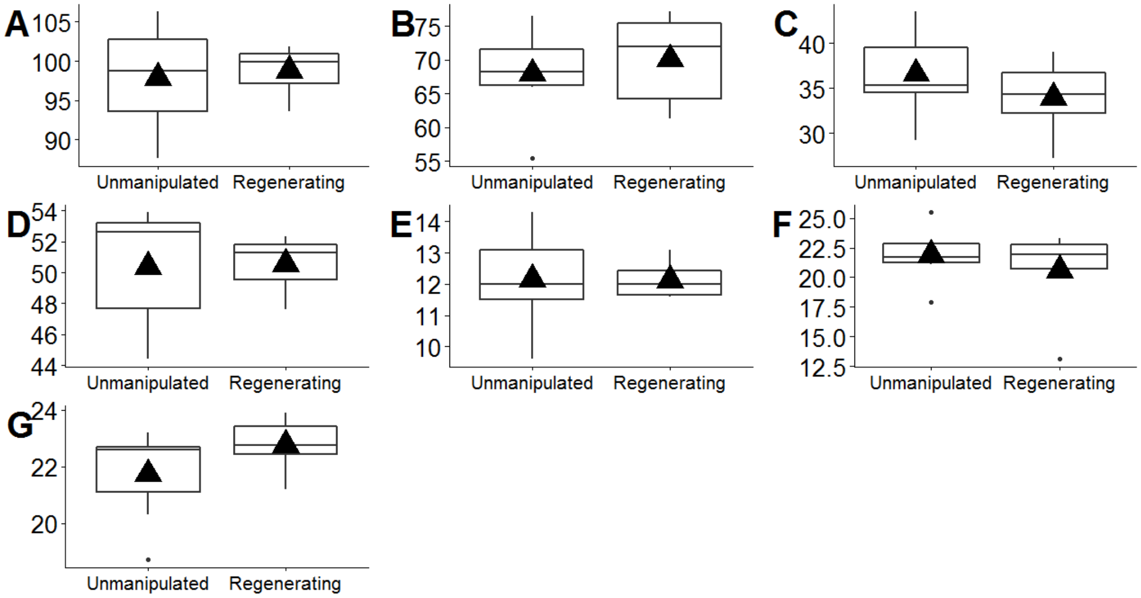

Figure 4.1: There is no difference between the different body measurements of unmanipulated crayfish (two-clawed) and manipulated crayfish (one-claw). See Figure 3.1 for measurements description and text for statistical values. Dependent variables on each boxplot panel (all in mm): (A) Total Body Length, (B) Total Cheliped Length, (C) Propodus Length, (D) Cephalothorax Total Length, (E) Palm Width, (F) Dactyl Length, and (G) Cephalothorax Width. Triangles symbols represent averages.

4.3.1.2 Limb loss and regeneration does not affect adult female crayfish reproductive success

Adult crayfish regenerating status did not affect the number of eggs produced (mean ± standard error; $\mathrm{Egg\#}_{\mathrm{Unmanipulated}}$ = 268 ± 58 mm, $\mathrm{Egg\#}_{\mathrm{Regenerating}}$ = 323 ± 80 mm, *t* =0.31, *P* = 0.76;

Figure 4.2A) or the average egg size per female (mean ± standard error; $EggSize_{Unmanipulated}$ = 1.4 ± 0.1 mm, $EggSize_{Regenerating}$ = 1.5 ± 0.1 mm, $t = 0.02$, $P = 0.98$; Figure 4.2B).

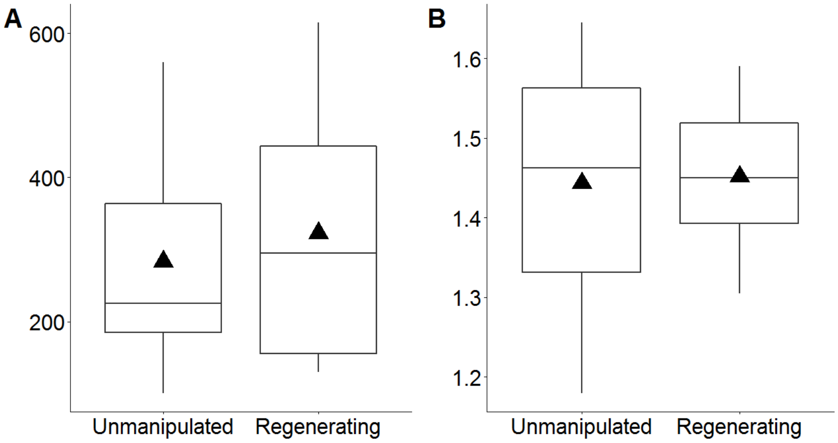

Figure 4.2: Regenerating status does not affect adult female crayfish reproductive success. Dependent variables on each boxplot panel: (A) Egg number (B) Egg size (in mm). Triangles symbols represent averages.

4.3.1.3 Juvenile papilla stages of chelae regeneration

Thirty-six juveniles were successfully individualized after separating from their mother (crayling stage). Only 14 juveniles reached at least three centimeter in body size allowing autotomy to be performed. Contrary to previous reports (Cooper 1998) we observed papilla regeneration prior to molting in 10/14 juveniles in our experiment (Table 1, Figure 4.3). After 20 days, cheliped regenerates had reached a third of their original size (Figure 4.3A-B). Notably, growth accelerated substantially after reaching the third stage of papilla regeneration (digit, Figure 4.3C). We provide some visual context to the reader with pictures paired with line drawings of each stage of chelae regeneration (Figure 4.4). Six papilla stages have been observed during juvenile chelae regeneration including: Blastema, elongation, digit, segments, dactyl, shaped (Figure 4.4). The first stage, blastema, represents the bulging of tissues at the preferred breakage plane. Elongation, the second stage, is characterized by the growth of tissues beyond the blastema as well as the formation of a slit on top of the blastemal. The third stage, digit, represents the growth of a digit through the blastemal slit. Segments, the fourth stage, is characterized by the definition of segments along the regenerate starting to delineate the different parts of the chelae (see Figure 3.1 and 4.4 for details). The fifth stage, dactyl, represents the apparent segmentation of the last chelae segment forming the propodus and dactyl of the chelae (see Figure 3.1 and 4.6 for details). Shaped, the sixth and last stage, is characterized by a fully segmented and shaped chelae, which is a perfect replicate of the original claw only smaller in size.

Table 4.1: Papilla regeneration does not require molt in juvenile crayfish. Regenerate stage determination for all 14 juvenile crayfish over 20 consecutive days. A = Autotomy; B = Blastema; E = Elongation; Di = Digit; Se = Segments; Da = Dactyl; Sh = Shaped. Notice that only four individual molted during the observation period as indicated by the mention "Molt" in the table; thus, no measurement were taken on those days for those individuals. Finally, two individuals died on day three.

ID #	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**	**Day 15**	**Day 16**	**Day 17**	**Day 18**	**Day 19**	**Day 20**
	4/16	*4/17*	*4/18*	*4/19*	*4/20*	*4/21*	*4/22*	*4/23*	*4/24*	*4/25*	*4/26*	*4/27*	*4/28*	*4/29*	*4/30*	*5/01*	*5/02*	*5/03*	*5/04*	*5/05*
1	A		B	*Molt*	B	E				Di		Se		Da	Sh					
2	A	B	Dead																	
3	A	B							Di				Se			Da			Sh	
4	A	B			*Molt*	E		Di		Se		Da				Sh				
5	A		B			E			Di			Se		Da			Sh			
6	A		B						E			Di						Se		Da
7	A				B		E			Di		Se			Da		Sh			
8	A			B			E													
9	A		B					E												
10	A	B					E											*Molt*	Di	Se
13	A		B			E	*Molt*	Di		Se			Da			Sh				
14	A	B	Dead																	
16	A	B		E					Di									Se		
17	A	B						E					Di		Se	Da		Sh		

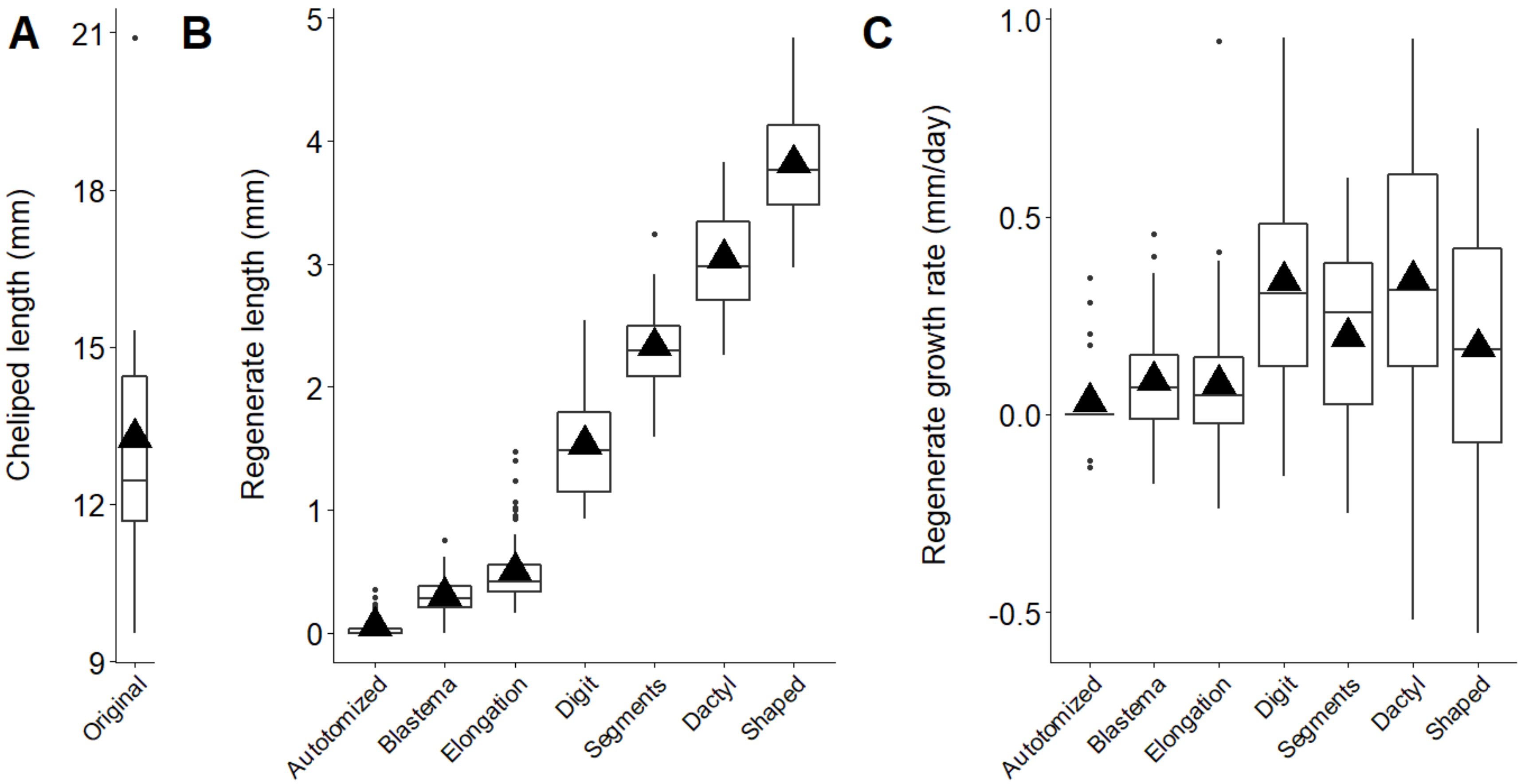

Figure 4.3: Regenerate grows along the 20 consecutive day period, maximum growth took place after the digit stage, and regenerates recovered a third of the original cheliped size in only 20 days. (A) Original cheliped size as measured before autotomy. (B) Regenerate lengths over each papilla stage as measured every day. (C) Regenerate growth per day over the different papilla stages.

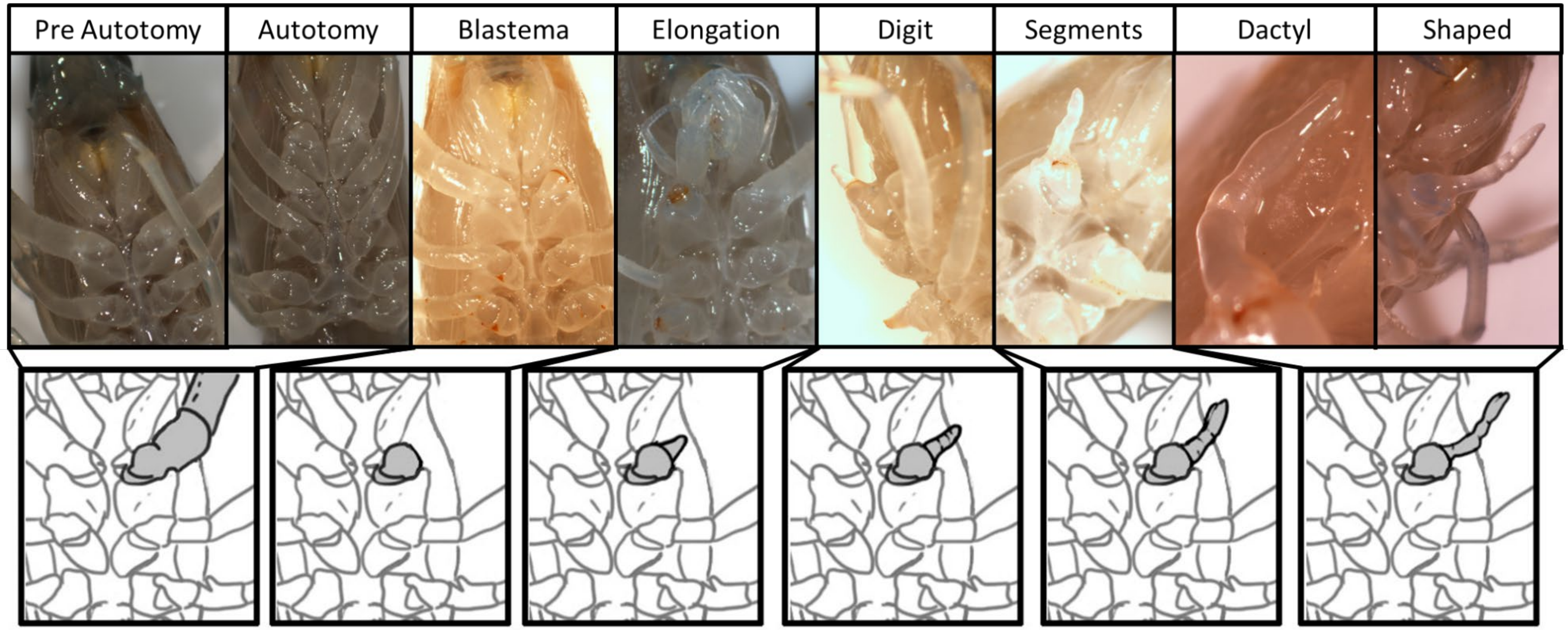

Figure 4.4: Papilla stage regeneration pictures and drawings providing context for the reader. The stages are arranged temporally from pre-autotomy on the left to the shaped stage on the right. Each picture is a different crayfish and line drawings provide details for readers. Drawings by Jacqueline Rae Dillard.

4.4 Discussion

Although we predicted that female crayfish missing a cheliped would have lower reproductive success than their two-claw counterparts, we found no impact of limb autotomy and regeneration on egg number or average egg size while controlling for female size. Furthermore, we observed that ecdysis (molt) was not required for juvenile chelae regeneration in crayfish. Six stages of papilla regeneration were described, and maximum growth was observed from the third (digit) to last (shaped) stage of regeneration. All of our adult crayfish had similar body measurements regardless of their experimental group. This seems counterintuitive since regeneration is assumed to be physiologically “costly” in a way that affects fitness as discussed below.

Five stages have been previously established for crayfish cheliped regeneration including two molting events during the process (in *Procambarus clarkii* as well; Cooper, 1998). Although previous research shows post-blastema and pre-ecdysis growth (Cooper 1998), our results clearly show that progress through regenerative stages and growth can be separated during crayfish chelae regeneration. This link had been established in crabs early on (Hopkins 1982) and then assumed to be similar in other crustaceans (e.g., Shinji *et al.*, 2019; but see Reyes *et al.*, 2017 for a study of continuous regeneration in male shrimp prior to ecdysis). By partially detangling molting and regeneration processes in crayfish we allow for further investigations of the exact role of ecdysis for cheliped regeneration. Indeed, molt might still be essential for hardening the regenerated chelae and future experiments testing for chelae hardness throughout the process of regeneration will be needed to determine this.

In general, researchers have assumed costs associated with regeneration due to physiologic/energetic demands. Therefore, it is often believed that regeneration impacts fitness. Nonetheless, only one body of work focuses on fitness costs of autotomy in crayfish and regeneration costs have not been explored yet. In some male crayfish (Astacidae), the capacity to remove conspecific sperm during mating was diminished by cheliped autotomy; yet, this cost was fully compensated by a bigger ejaculate size of autotomized compare to two-clawed males (Galeotti et al. 2008). However, the same male crayfish do experience reduced reproductive output with males missing a cheliped producing sperm with shorter longevity than their unmanipulated counterparts (Galeotti et al. 2012). Importantly, as noted before, reproduction is different in Astacidae compared to Cambaridae (focus group of our study) since sperm is stored internally by Cambaridae females until oviposition (Taylor and Schuster 2004) effectively preventing Cambaridae males to remove competitors' sperm prior to depositing theirs. Although those effects cannot be directly attributed to autotomy due to the nature of the study (field survey and it is unclear if autotomy or density was responsible for lower survival of regenerating crayfish), it seems that crayfish autotomy impacts some measures of fitness. Our study did not show an impact of autotomy and regeneration on female *P. clarkii* crayfish reproductive success suggesting three different possible explanations. First, one-clawed *P. clarkii* adult females may be capable of delaying regeneration energy allocation to maintain similar reproductive success compared to their two-claw counterparts. Second, regenerative *P. clarkii* adult females could be reallocating resources from other parts of their bodies locally or more distally to the autotomy plane while maintaining optimal investment in their reproductive parts. Finally, regeneration might not be that costly at least in the initial steps of the process (time when we conducted matings). Hence, similarly to the aforementioned study on Astacidae, our study emphasizes the importance of individual behavior in mitigating the negative effects of autotomy. However, our experiment did not explore interactions between regeneration and developmental stages in crayfish energy allocation responses. For example, we cannot extend the results of the present study on adult crayfish to juvenile crayfish: young crayfish might be able to delay reproduction in favor of regeneration due to their greater future fitness prospects compared to adult crayfish, or early regeneration investment could be more costly to future reproduction than later investment in juvenile crayfish.

Our results would appear to build on studies only examining the impact of autotomy in other arthropods. Male blue crabs (*Callinectes sapidus*) were negatively affected by cheliped autotomy in their capacity to defend mates from unmanipulated intruders (Url and Smith 2012). Similarly, a snowball effect of limb autotomy was found in crab spiders (*Misumena vatia*) with individuals losing limbs repetitively after the first loss potentially compounding long term fitness impact of autotomy (as measured by body mass; Morse 2016). Furthermore, growth and inter-molt time were negatively impacted by cheliped autotomy in a shrimp (*Cryphiops caementarius*; Terrones & Baltaodano, 2017) potentially impacting fitness especially in females where size is highly correlated with number of eggs produced (McClain et al. 2007). Finally, crushing force of either intact or regenerated cheliped are lower in autotomized compared to intact red rock crabs (*Cancer productus*) indicating substantial foraging disadvantage for injured individuals (Brock and Smith 1998). Potential fitness effects of leg autotomy have been proposed in harvestmen (Arachnida, opiliones) because autotomized individuals avoid terminal costs (death; Guffey, 1998). Leg autotomy was found to have no impact on competitive abilities and minimal impacts on development (increased intermolt time) in non-regenerating spiders, *Holocnemus pluchei* (Aranea, Pholcidae; Johnson & Jakob, 1999). Male competitive abilities were not impacted by leg autotomy in field crickets (*Gryllus bimaculatus*); however, autotomized females had difficulties maintaining mount during the ampulla exchange leading to failed mating and a reduced longevity as measured on autotomized males (Bateman and Fleming 2005). Furthermore, autotomy was found to significantly decrease burst sprint speed in wolf spiders (*Pirata sendentarius*) potentially affecting prey capture and predation escape (Apontes and Brown 2005). Indeed, leg loss via autotomy was effectively linked to lower foraging success in another wolf spider (*Rabidosa santrita*; Steffenson, Formanowicz & Brown, 2014). Finally, predator detection using chemical cues as well as female wolf spider (*Pardosa valens*) survivorship were unaffected by leg autotomy (Brown and Steffenson 2018).

However, a focus on indirect impacts of autotomy on fitness (as discussed in the studies presented above) ignores internal energetic trade-offs between regeneration and reproductive success potentially mitigating the negative effects measured in those studies. We suggest future studies to focus more on direct measures of fitness rather than relying on indirect measurements far removed from reproductive success. Although more challenging, only direct fitness measurements can be used to wholly interpret the evolutionary importance of autotomy and regeneration.

To conclude, chelipeds are the limbs most often injured in arthropods, but no effects beyond individual impacts have been investigated so far (Juanes and Smith 1995). Indeed, we do not know if individual fitness costs of autotomy, assuming those costs are real (but see above), have compounded effects at the population level. Moreover, to our knowledge, only one study suggests a positive effect of autotomy on fitness due to an energy allocation trade-off between costly sexual weapons and testes (Joseph et al. 2018). Indeed, in a non-regenerating leaf-footed cactus bug (*Narnia femorata Stal* – Hemiptera, Coreidae), males that autotomized their hind legs (this species weapons) during development were able to produce up to 42% more offspring than intact males due to enlarged testes (Joseph et al. 2018). That is why, beyond the investigation of direct impacts of autotomy on individual fitness, we emphasize the importance of investigating the impacts of autotomy on fitness in regenerating as well as non-regenerating species. The former would allow us to focus on evolutionary relevant effects, while the latter would allow us to decouple the effects of autotomy from those of regeneration.

CHAPTER 5. NON-CONSUMPTIVE PREDATOR EFFECTS MODIFY CRAYFISH INDUCED BIOTURBATION AS MEDIATED BY LIMB LOSS: FIELD AND MESOCOSM EXPERIMENTS

Luc A. Dunoyer, Dakota Coomes, and Philip H. Crowley

5.1 Introduction

Ecosystem engineering is the modification of the physical environment from one state to another by organisms (Jones et al. 1994, 1997b). Almost every organism on Earth engineers its environment to some degree (Jones et al. 1997a). The importance of ecosystem engineering in each case depends on its intensity and potential for cascading effects (similar to trophic interactions; Wilby 2002). The effects of ecosystem engineering have been extensively investigated, including biodiversity effects (Caliman et al. 2013), management options (Byers et al. 2006), and implications for ecosystem services (Daily 1997). However, little is known about other ecological and physiological processes may influence the effect of ecosystem engineering within the ecosystem (Folgarait 1998, Rietkerk et al. 2004). Environmental impacts of ecosystem engineers mediated by non-consumptive effects of the engineers' predators is one of those interactions. Previous studies have focused on density-mediated interaction (Wilby et al. 2001, Sanders and van Veen 2011, Sanders et al. 2014, Nishijima et al. 2016); whereas, we focus here on trait-mediated interaction.

We develop a case study of such effects by looking at the non-consumptive impact of fish on crayfish engineering in freshwater streams, combining in- and ex-situ experimental approaches. Organic resource availability in stream communities contributes to species biodiversity (Vannote et al. 1980) and ultimately to ecosystem services, though often in complex ways. These resources are influenced by bioturbation, a form of ecosystem engineering based on increased turbidity in an aquatic system resulting from biological activity (Meysman et al. 2006). Bioturbation is known to diminish light penetration in the water column, leading to a reduction of primary production in marine and freshwater systems (Fager 1964, Heinzelmann and Wallisch 1991, Ciutat et al. 2005, Mermillod-Blondin and Rosenberg 2006). Previous research has explored this process by investigating single macroinvertebrate taxa in isolation, most commonly crayfish (Creed and Reed 2004) or micro-invertebrates (Duarte et al. 2012). To our knowledge, however, the effects of interactions between taxa have not been shown to influence bioturbation (Usio and Townsend 2001).

Crayfish are well-known ecosystem engineers that greatly influence the availability of organic resources directly by shredding leaf litter and consuming micro-invertebrates (Momot 1995, Dunoyer et al. 2014) or indirectly via bioturbation during burrow construction or sediment displacement (Angeler et al. 2001, Usio and Townsend 2004, Yamamoto 2010). Crayfish induce bioturbation simply by walking on the substrate (Statzner and Sagnes 2008), anchoring themselves to the stream bed in fast flowing current areas (Maude and Williams 1983), tail-flipping when evading predators, and especially by burrowing (Statzner 2012). These behaviors result in long term effects on bed stream composition (Statzner 2012).

Like most arthropods, crayfish are capable of regeneration following the loss of a limb or other appendages via autotomy (Wood and Wood 1932b). Large fish, birds, raccoons, and other predators of crayfish can have direct impacts on crayfish through predation and indirect non-lethal effects by inducing limb loss. Natural populations of crustaceans and crayfish have up to 30% of individuals regenerating a missing limb at any given time (Juanes and Smith 1995, Powell et al. 1998, Kouba et al. 2011). Because regeneration is a slow and costly process that inhibits burrow construction (one clawed-crayfish are incapable of burrowing; Dunoyer et al., in prep), predator-induced injuries substantially influence bioturbation at least until regeneration is completed and burrowing capacities are fully recovered (Chapter 3).

Crayfish induced turbidity (Maude and Williams 1983, Statzner and Sagnes 2008) reduces light penetration and primary production in wetland habitat characterized by low water flow (Anastacio et al. 2005). This reduction in primary production can in turn affect diversity in those environments (Rodríguez et al. 2005). Turbidity also changes fish predator-avoidance by crayfish.

The skew of mortality pattern toward small individuals in clear water is eliminated in turbid water (Abrahams and Kattenfeld 1997, Kimbell and Morrell 2016). Furthermore, fish are less able to escape rapid attacks from other fish in turbid water, while the opposite is true when facing a slow predator (Meager et al. 2006). Those studies underscore the importance of water turbidity in shaping habitat uses and predation patterns by fish. For crayfish, conspecific chemical alarm cues are more important than odor cues from fish for predator avoidance (Gherardi et al. 2011). Regenerative crayfish may induce higher turbidity through inefficient burrowing (since crayfish rely on their cheliped to burrow; pers. comm.; Berrill and Chenoweth 1982; Helms et al. 2013) and thereby impede detection by predators.

We aim to assess the consequences of non-consumptive predator effects on crayfish-induced bioturbation. First, we predict that regenerating crayfish will have an increased bioturbidity impact compared to their unmanipulated counterparts due to their reduced burrowing capacity (they will struggle more to accomplish similar burrowing output). Second, if predators influence this ecosystem engineering process, we predict that crayfish-induced bioturbation will be further increased in the presence of a predatory fish, in an attempt to provide more protection against predation.

5.2 Material and methods

5.2.1 Study sites

Our field experiment was conducted in the Green River drainage of the Ohio River watershed in Kentucky, USA (Subregion Hydrologic Unit Code 4-digit: 0511). This area is located at the convergence of the Cincinnati Arch with the Appalachian Basin, resulting in a highly diverse assemblage of freshwater species (103 of the 297 species of North American mussel species, 248 species of freshwater fish, 57 amphibian species, and 54 out of the 360 North American crayfish species; Kentucky Department of Fish and Wildlife Resources, 2013). We used three stream sites in nearby but separate creeks for our experiments (GPS coordinates): site 1 (37.333699, -85.420170); site 2 (37.342768, -85.458752); site 3 (37.384984, -85.463014).

In addition to our field experiment, we also conducted a mesocosm experiment using artificial pools outside at a field station to determine the specific effects of predation by fish (and subsequent regeneration by crayfish) on the crayfish bioturbation process. Mesocosm experiments allow us more control of environmental variation. The site was the University of Kentucky's Ecological Research and Education Center (EREC) field station in Lexington, Kentucky. The same completely randomized design was used for a single block (see below), with fifteen 40 gallon plastic tanks placed under shade-cloth mesh and half dug into the ground to mimic stream conditions in slow moving water flow under canopy. The substrate in each tank was a mix of gravel and sand similar to what was found in the streams in the field.

5.2.2 Study species

We used kick-sampling (Mather and Stein 1993) to capture native crayfish (*Faxonius rusticus*, Girard 1852; Crandall & De Grave, 2017) and non-lethal electro-fishing methodology (Cowx and Lamarque 1990) to capture crayfish predators (fish – rock bass, *Ambloplites rupestris*, a known predator of crayfish), then used the fish and crayfish to establish a predator enclosure-exclosure experiment in the field. Before addition to the enclosures, crayfish were measured from the back of the orbit to the center of the dorso-posterior margin of the carapace (within 0.1 mm, OCL; size range = 3.5 – 9 cm; average size +/- standard deviation = 5.56 +/- 1.27 cm). Fish were measured from the tip of the snout to the tip of the longer lobe of the caudal fin (Total Length, TL; size range = 7 – 12 cm; average size +/- standard deviation = 9.83 cm +/- 1.34 cm). All fish behaved normally in the enclosures and were all healthy at the end of the experiment.

5.2.3 Experimental design and methods

Each enclosure-exclosure was a 3D rectangle of 90 x 30 x 30 cm made out of a frame of PVC-pipes (drilled to prevent floating) and garden stakes (Figure 5.1). The structure was then covered with 0.6 cm plastic mesh attached with zip locks. Finally, a door was made atop the structure in the plastic mesh to allow addition of the animals.

The study design was a randomized complete block, with three replicates of five treatments within each stream location for each of the three remaining stream locations. Each enclosure-exclosure in each block of 15 was placed at least five meters from each other and never directly downstream from one another (staggered placement); thus, each replicate and treatment was not influenced by other replicates and treatments. Treatments were as follows: 1) fish and crayfish excluded – control (C); 2) fish excluded, unmanipulated crayfish added (UM); 3) fish excluded, regenerating crayfish (limb autotomized) added (R); 4) fish added, unmanipulated crayfish added (FUM); and 5) fish added, regenerating crayfish added (FR). Treatments were added into the enclosure-exclosure placed in a slow water flow area. We shoveled 5 cm of substrate over the bottom surface of each, effectively sealing them to the streambed by embedding the bottom mesh into the sediment.

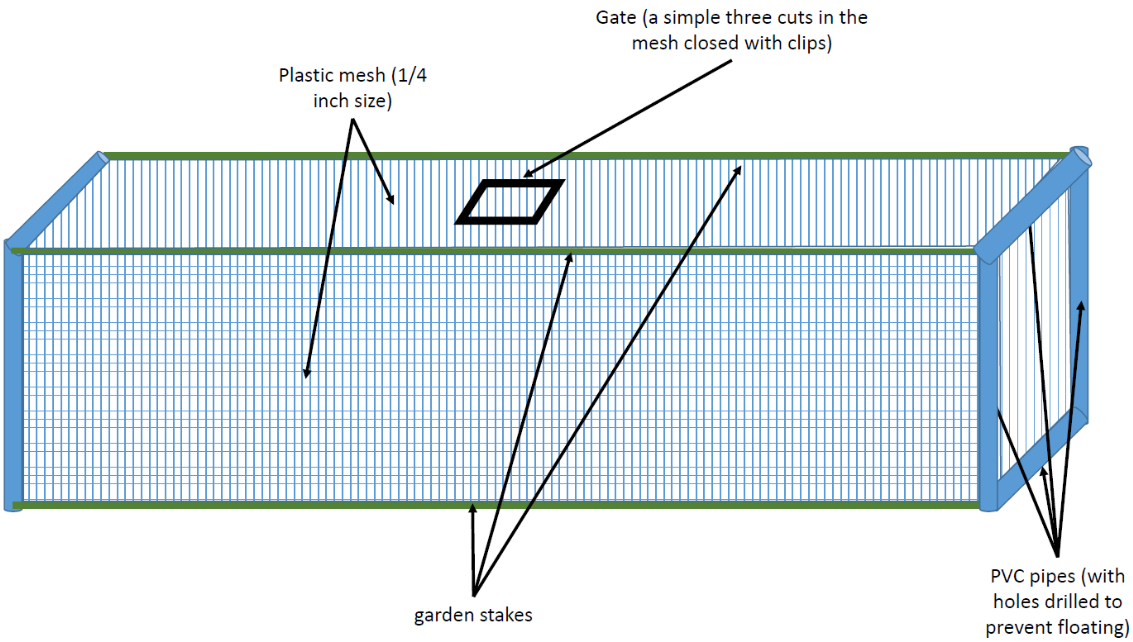

Figure 5.1: Diagram representing the enclosure-exclosure design.

We quantified the crayfish bioturbation process by sampling turbidity (in NTU using a LaMotte 2020we Turbidity Meter) directly downstream of the enclosure-exclosure channels (to prevent the influence of nearby/upstream outside factors) every week, starting a week after setting-up the experiment. The turbidity meter processed a small water sample at the field site. Moreover, we gathered water samples from the downstream end of the enclosures to prevent influences on subsequent measurements. This experiment ran from September 12th to October 13th, 2015. Both field and mesocosm experiments lasted four weeks, yielding 48 temporal data points per treatment (4 locations (3 field sites and 1 mesocosms site) * 3 replicates * 4 weeks). We also assessed how body size affects regeneration and bioturbation processes. Fish slightly larger than the crayfish were chosen when paired in the enclosure-exclosure in the field experiment or in the artificial pools in the mesocosms experiment. This size pairing included fish large enough to be perceived as a predation threat by crayfish, while not large enough to actually consume the crayfish. Finally, neither fish nor crayfish were expected to grow significantly during the experiment duration.

5.2.4 Data analyses

All analyses were conducted in R (Team 2017) using several additional packages (ggplot2, Wickham 2009; cowplot, Wilke 2017; nlme, Pinheiro et al. 2017; sjstats, Lüdecke 2018; MuMin, Barton 2017; dplyr, Wickham et al. 2017; gridExtra, Anguie 2017; psych, Revelle 2017; car, Fox and Weisberg 2011).

Field and mesocosm data were analyzed separately using information theory (Burnham and Anderson 2002). This approach ranks several models in a set and allows multimodel inferences using evidence of statistical support from the given dataset based on each model fit. First, we determined the best fit for our complete mixed effects model, addressing our hypothesis about the consequences of non-consumptive predator effects on crayfish-induced bioturbation in stream and pond, respectively. The dependent variable was turbidity induced (in NTU) following a normal distribution. The different variables used in the mixed effects models were time, treatment (see above), their interaction, and fish as well as crayfish length at the start of the experiment (TL and OCL respectively). Site was chosen as a random factor for the field experiment to allow for unknown differences between sites influencing turbidity measurements. Furthermore, a specific variance structure was incorporated to improve model fit as assessed graphically. This structure accounts for the variance of the covariate crayfish length per treatment level while allowing for each stratum of fish length to have different variances (Zuur et al. 2009).

Table 5.1: Information theory output of the field models.

Model[†]	**Treatment**[§]	**Date**[¶]	**Crayfish length**[◊]	**Fish length**[↓]	**Treatment * Date**[↕]	**df**	**AIC_c**	**ΔAIC_c**	**ω_i (%)**
I[‡]		-0.06803				16	890.9	-	32.70
G	+	-0.06923				20	891.3	0.39	26.90
F	+	-0.06865	-0.3021			21	891.8	0.94	20.40
H	+					19	894.0	3.08	7.00
E	+	-0.06965		-0.005515		21	894.1	3.16	6.60
B	+	0.06923	-0.3014	0.039080		22	894.7	3.82	4.80
D	+		-0.3007	-0.125000		21	897.5	6.61	1.20
C		-0.06747	-0.1836	0.048820		18	900.1	9.16	0.30
A	+	-0.09152	-0.03030	-0.042720	+	26	920.5	29.57	0.00

[†] A particular model (row) contained a particular variable either if there is a "+" (categorical variable) or if there is a coefficient (continuous variable) in the respective variable column. Site was chosen as a random factor to control for any unmeasured differences between sites impacting our turbidity measurements. Finally, a variance structure was implemented to improve model fit (following crayfish length per treatment level and fish length, see methods).

[‡] For example, the model I is: Turbidity ~ Date.

[§] Treatment variable Akaike weight = 0.67 (appeared in seven models).

[¶] Date variable Akaike weight = 0.92 (appeared in seven models).

[◊] Crayfish length variable Akaike weight = 0.27 (appeared in five models).

[↓] Fish length variable Akaike weight = 0.13 (appeared in five models).

[↕] Treatment and Date interaction variable Akaike weight < 0.01 (appeared in one model).

Subsequently, based on our complete model, we compared all nested models and determined the best models from this set using information theory (see above). All models derived from the parent model were compared using the corrected Akaike Information Criterion (AIC_c) and Akaike weights (ω_i) (Anderson et al. 2001, Anderson and Burnham 2002, Burnham et al. 2011; Table 5.1). AICc represents model fit with smaller values being better fits, and the best-supported models include all models with $\Delta AIC_c < 2$ (Burnham and Anderson 2002). Akaike weights represent strength of evidence for each model in a given data set (Burnham and Anderson 2002). Additionally, alternate Akaike weights were computed for each variable to assess their individual importance (as the sum of the ω_i of the models in which the variable is present). Although the Akaike weights of all models add up to 1, alternate Akaike weights for individual variables do not generally sum to 1; these individual-variable sums are between 0 and 1, where 0 denotes a lack of importance and 1 a high importance of the considered variable in the model set (Burnham and Anderson 2002). Finally, Cohen's d values were computed to report the effect sizes associated with variables having large Akaike weights. These effect sizes were calculated on non-standardized data for repeatability and comparability of our results with future research (Morris and DeShon 2002, Bakeman 2005). However, unstandardized effect sizes ignore the structure of the data set (i.e., here repeated measurements and stream identities). Hence, we consider all effect sizes with confidence intervals not at all or slightly overlapping the null value as well as effect sizes from relevant comparisons (involving controls for example). In doing so, we address both the amount of overlap between effect-size confidence intervals and zero and the magnitudes of the effect sizes themselves. By presenting the entire set of effect sizes with their confidence intervals (for the treatment covariate), we are allowing readers to interpret the results for themselves and reach their own conclusions, while providing our own interpretation, hoping that these two are ultimately in agreement.

Finally and *a posteriori*, we took advantage of the whole data set by analyzing the data using the last data recorded at each stream. We regressed the final number of crayfish found both per channel and per stream against the final turbidity measurement per channel and per stream (averaged) respectively.

Our study protocol and procedures were ethically reviewed and approved by the Institutional Animal Care and Use Committee at the University of Kentucky (protocol #2015-2068). All the data and R script with packages used in this work are available on Dryad (https://doi.org/10.5061/dryad.58k2h35).

5.3 Results

5.3.1 Consequences of non-consumptive predator effects on crayfish-induced bioturbation in the field experiment

The best-supported models identified from the set of nested models using the Information Theory approach were models "I" (Turbidity ~ Date), "G" (Turbidity ~ Treatment + Date), and "F" (Turbidity ~ Treatment + Date + Crayfish Length; Table 5.1). Among the set of models run through the Information Theory approach, neither fish nor crayfish size affected turbidity; likewise, the interaction between time since the start of the experiment and treatments did not affect turbidity (Table 1). Turbidity decreased with time since the start of the experiment ($\text{Coefficient}_{\text{time}} = -0.07$, $\text{CI95\%}_{\text{time}} = [-0.10;-0.03]$; Figure 5.2). Since turbidity over time does not seem to affect control and treatment condition differently (non-significant interaction; Figure 5.3), this impact is likely due to the stream background turbidity. Furthermore, the treatment covariate significantly influenced turbidity; specifically, unmanipulated crayfish treatment created less turbidity than regenerating crayfish or fish with regenerating crayfish treatments (Cohen's $d_{\text{UMvsR}} = -0.60$, $\text{CI95\%}_{\text{UMvsR}} = [-1.08; -0.13]$; Cohen's $d_{\text{FRvsUM}} = 0.46$, $\text{CI95\%}_{\text{FRvsUM}} = [0.01; 0.87]$; Figure 2; Table 5.1).

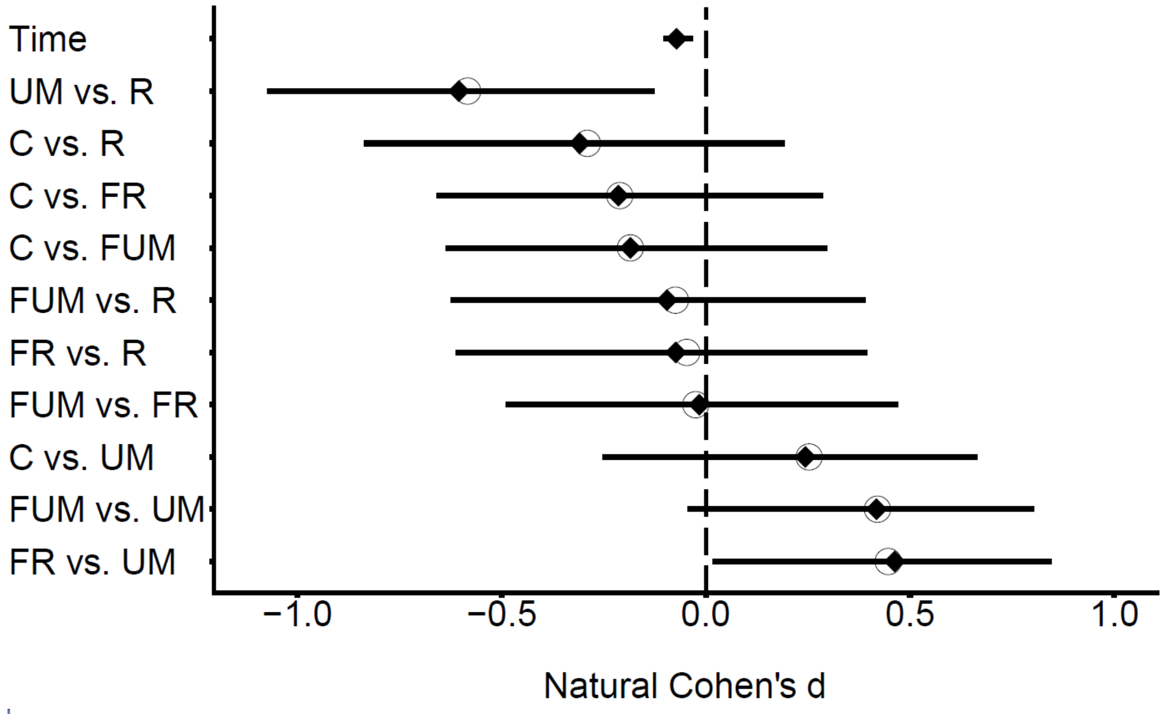

Figure 5.2: Effect sizes associated with the variables “Treatment” and “Date” in the field part of the experiment. The diamonds and lines are the average Cohen’s d values with 95% confidence intervals after 10,000 bootstraps except for the variable “Date” for which the mean value is simply the average of its coefficients in all the considered models (see Table 5.1). Open circles represent the Cohen’s d value calculated on the experimental data rather than from the 10,000 bootstraps. A variable has a significant effect on turbidity if its 95% confidence interval does not overlap with 0 (the dashed line). C = control, UM = unmanipulated crayfish, R = regenerating crayfish, FUM = fish with unmanipulated crayfish, FR = fish with regenerating crayfish.

Furthermore, there was a non-significant trend toward higher turbidity for treatments with fish and unmanipulated crayfish than for those with unmanipulated crayfish alone (Cohen’s $d_{FUMvsUM}$ = 0.42, CI95%$_{FUMvsUM}$ = [-0.03; 0.81]; Figure 5.2; Table 5.1). Finally, while statistically undistinguishable from controls, each treatment condition except unmanipulated crayfish induced more turbidity than the control treatment (Cohen’s d_{CvsR} = -0.32, CI95%$_{CvsR}$ = [-0.86; 0.18]; Cohen’s d_{CvsFR} = -0.21, CI95%$_{CvsFR}$ = [-0.67; 0.28]; Cohen’s d_{CvsFUM} = -0.19, CI95%$_{CvsFUM}$ = [-0.65; 0.32]; Cohen’s d_{CvsUM} = 0.25, CI95%$_{CvsUM}$ = [-0.24; 0.68]; Figure 5.2; Table 5.1). Overall, in the field experiment, regenerating crayfish induced more turbidity than their unmanipulated counterparts while predatory fish presence always enhanced turbidity.

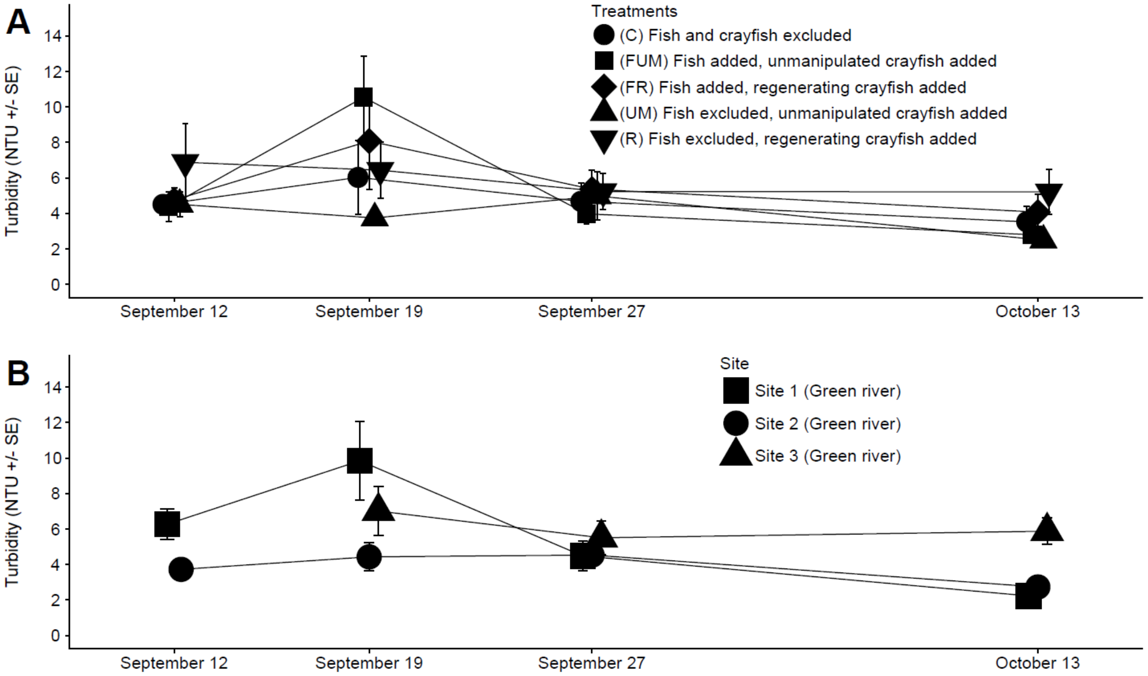

Figure 5.3: The evolution of turbidity over time in the field experiment. A – Breakdown of turbidity by treatment. B – Breakdown of turbidity by field site. See Methods for details.

5.3.2 Consequences of non-consumptive predator effects on crayfish-induced bioturbation in the Mesocosm experiment

The best-supported models identified from the set of nested models using the Information Theory approach was model “D” (Turbidity ~ Treatment + Date + Crayfish Length + Fish Length; Table 5.2). Neither time since the start of the experiment nor its interaction with treatments affected turbidity (Figure 5.4).

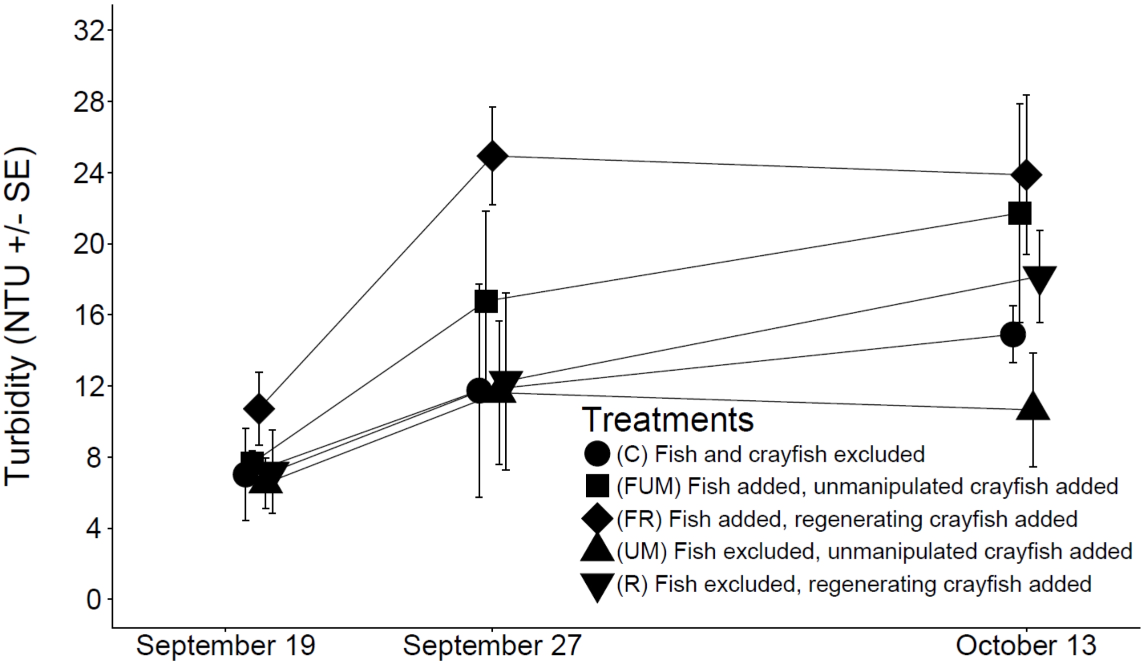

Figure 5.4: The evolution of turbidity over time in the mesocosms experiment with a breakdown of turbidity by treatment. See Methods for details.

However, crayfish and fish sizes significantly increased turbidity, while the treatment covariate also significantly influenced turbidity (Coefficient$_{\text{crayfish\_length}}$ = 2.75, CI95%$_{\text{crayfish\_length}}$ = [1.74; 5.41]; Coefficient$_{\text{fish\_length}}$ = 0.92, CI95%$_{\text{fish\_length}}$ = [-0.21; 2.62]; $\omega_{\text{treatment}} = 1$; Figure 5.5; Table 5.2). Specifically, fish with unmanipulated crayfish treatment created more turbidity than controls or fish with regenerating crayfish or unmanipulated crayfish treatments, while unmanipulated crayfish treatment created less turbidity than fish with regenerating crayfish treatment (Cohen's d$_{\text{CvsFUM}}$ = -0.79, CI95%$_{\text{CvsFUM}}$ = [-1.49; -0.19]; Cohen's d$_{\text{FUMvsFR}}$ = 0.64; CI95%$_{\text{FUMvsFR}}$ = [0.05; 1.30]; Cohen's d$_{\text{FUMvsUM}}$ = 1.21; CI95%$_{\text{FUMvsUM}}$ = [0.59; 1.93]; Cohen's d$_{\text{FRvsUM}}$ = 0.59; CI95%$_{\text{FRvsUM}}$ = [0.04; 1.14]; Figure 5.5; Table 5.2).

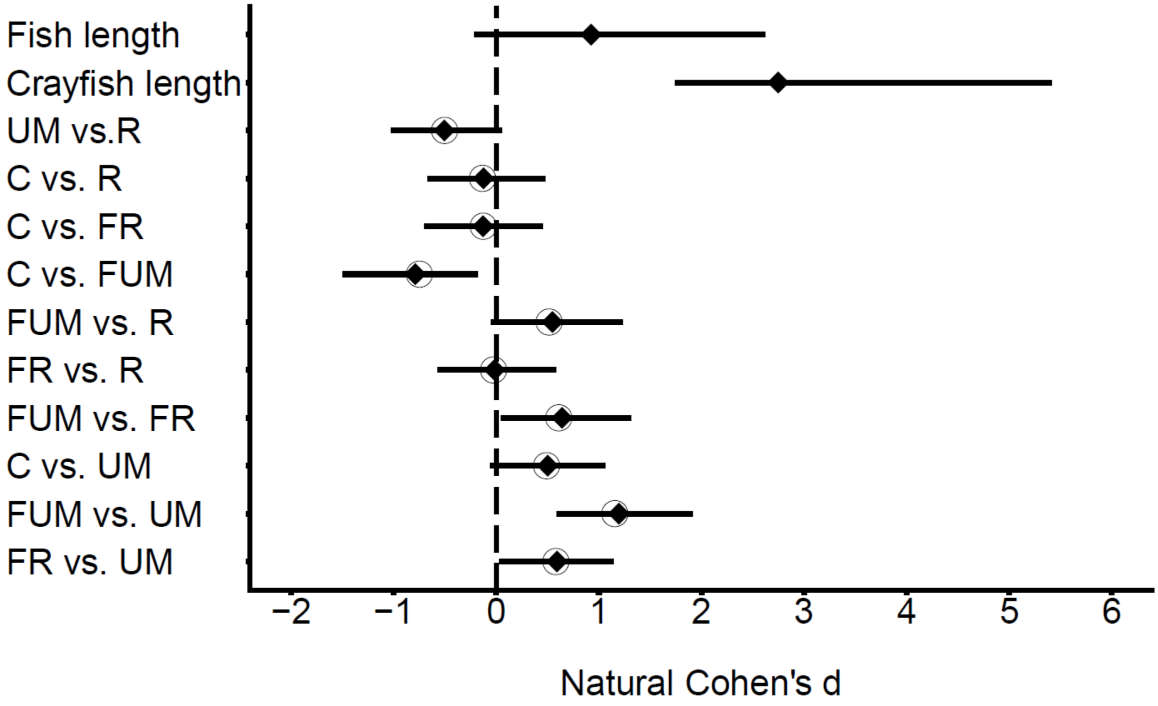

Figure 5.5: Effect sizes associated with the variables “Treatment”, “Crayfish length”, and “Fish length” in the mesocosms part of the experiment. The diamonds and lines are the average Cohen’s d values with 95% confidence intervals after 10,000 bootstraps except for the variables “Crayfish length” and “Fish length”, for which the mean values are simply the average of their coefficients in all the considered models (see Table 2). Open circles represent the Cohen’s d value calculated on the experimental data rather than from the 10,000 bootstraps. A variable has a significant effect on turbidity if its 95% confidence interval does not overlap with 0 (the dashed line). C = control, UM = unmanipulated crayfish, R = regenerating crayfish, FUM = fish with unmanipulated crayfish, FR = fish with regenerating crayfish. The treatment factor contrasts have been ordered similarly to Figure 1 to facilitate visual comparison.

Finally, there was a non-significant trend suggesting that unmanipulated crayfish tend to induce less turbidity than regenerating crayfish or control, while fish with unmanipulated crayfish tended toward inducing more turbidity than regenerating crayfish (Cohen’s d_{UMvsR} = -0.50, $CI95\%_{UMvsR}$ = [-1.03; 0.05]; Cohen’s d_{CvsUM} = 0.50, $CI95\%_{CvsUM}$ = [-0.06; 1.06]; Cohen’s d_{FUMvsR} = 0.55, $CI95\%_{FUMvsR}$ = [-0.05; 1.23]; Figure 5.5; Table 5.2). Overall, in the mesocosm experiment, once again predatory fish presence always increased turbidity, while regenerating crayfish induced more turbidity than their unmanipulated counterparts. However, this last result did not hold true when regenerating crayfish were paired with a predatory fish, seemingly hindering regenerating-crayfish-induced turbidity in the mesocosm experiment.

Table 5.2: Information theory output of the mesocosms models.

Model[†]	**Treatment**[§]	**Date**[¶]	**Crayfish length**[◊]	**Fish length**[↓]	**Treatment * Date** [↕]	**df**	**AIC_c**	**ΔAIC_c**	**ω_i (%)**
D[‡]	+		3.5830	1.2030		15	810.0		96.3
B	+	0.01103	3.6050	1.2020		16	818.3	8.22	1.6
H	+					13	818.8	8.78	1.2
F	+	0.01245	2.9690			15	819.3	9.25	0.9
G	+	0.01104				14	826.9	16.87	0
E	+	0.01134		0.3245		15	828.1	18.08	0
A	+	0.10160	3.8730	1.2670	+	20	831.1	21.09	0
C		0.01006	-0.2935	0.6230		12	844.3	34.22	0
I		0.01363				10	849.7	39.66	0

[†] A particular model (row) contained a particular variable either if there is a "+" (categorical variable) or if there is a coefficient (continuous variable) in the respective variable column. Site was chosen as a random factor to control for any unmeasured differences between sites impacting our turbidity measurements. Finally, a variance structure was implemented to improve model fit (following crayfish length per treatment level and fish length, see methods).

[‡] For example, the model D is: Turbidity ~ Treatment + Crayfish length + Fish length.

[§] Treatment variable Akaike weight = 1 (appeared in seven models).

[¶] Date variable Akaike weight = 0.03 (appeared in seven models).

[◊] Crayfish length variable Akaike weight = 0.99 (appeared in five models).

[↓] Fish length variable Akaike weight = 0.98 (appeared in five models).

[↕] Treatment and Date interaction variable Akaike weight < 0.01 (appeared in one model).

5.3.3 Testing the impact of crayfish on turbidity – An a posteriori analysis

We found a positive relationship between the turbidity and the number of crayfish at the end of the experiment in each channel (m = 0.22; df = 32; F-statistic = 8.802; $P = 0.006$; $R^2_{adj.} = 0.19$; Figure 5.6). However, we found no relationship between the turbidity and the number of crayfish at the end of the experiment per stream (m = 0.39; df = 1; F-statistic = 3.393; $P = 0.32$; Figure 5.6). This lack of significance, despite a positive trend ($R^2_{adj.} = 0.55$), may reflect the relatively small sample size. Finally, we found no relationships between the turbidity and the number of crayfish at the end of the experiment at each individual stream (Site 3: m = 0.15, df = 12, F-statistic = 1.582, $P = 0.23$; Site 4: m = 0.14, df = 3, F-statistic = 0.2387, $P = 0.66$; Site 5: m = -0.22, df = 13, F-statistic = 0.5498, $P = 0.47$; Figure 5.6). Overall, more crayfish induced more turbidity at the relevant scale in the field experiment.

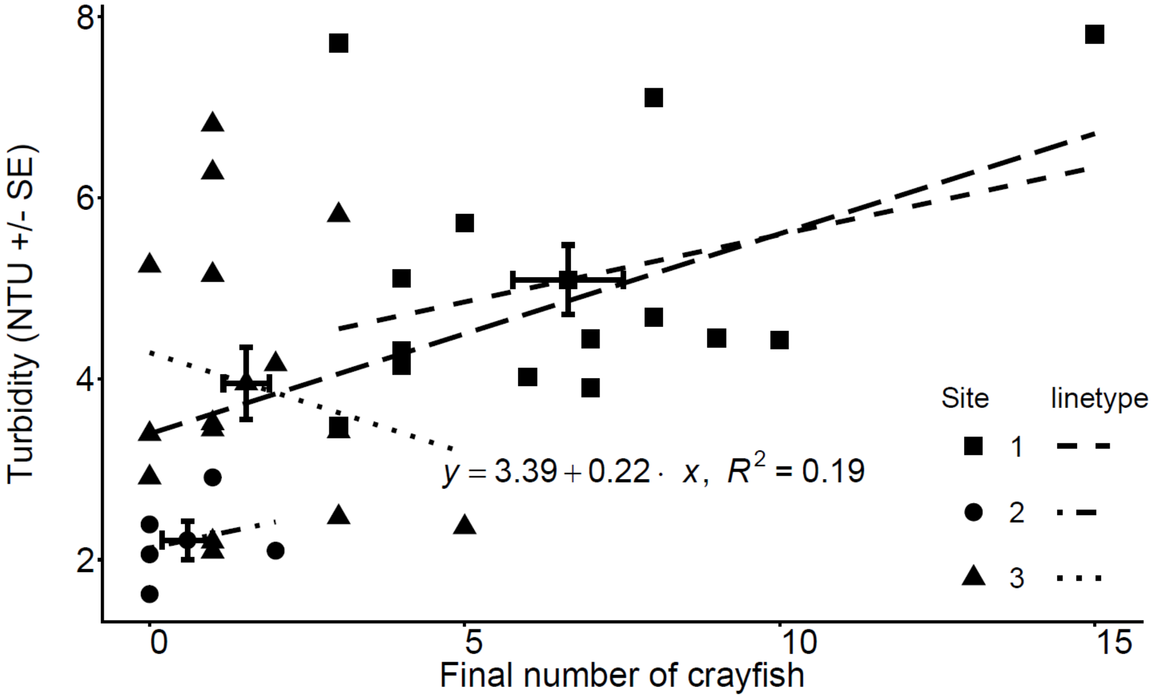

Figure 5.6: A-posteriori regressions. Scatter plots of the numbers of crayfish against turbidity at the end of the experiment in each remaining channels with site averages. The long-dash regression line, equation, and R^2 value correspond to channels' turbidity and not site averaged turbidity (see text for details about each regression per stream). Note that the analysis has already gone through outlier assessment procedures. Finally, if the seemingly extreme data point (x=15, y~8) is removed, model fit is greatly impeded as assessed graphically (Normal Q-Q, Residuals vs Fitted, Scale-Location, Residual vs Leverage, and cook's distances plots).

5.4 Discussion

Our results demonstrated that greater crayfish abundance in a location induces higher turbidity at a small spatial scale (between channels irrespective of location) compared to larger scale (between streams). We also showed that crayfish limb loss and predation risk lead to more turbidity in field and mesocosm conditions. Furthermore, larger crayfish induce more turbidity than smaller crayfish under mesocosm conditions. Finally, experimental removal of a crayfish chela did increase turbidity. However, we did not find an effect of fish size on turbidity in the field or mesocosm experiments. Nonetheless, fish presence seems to hinder crayfish turbidity inducing behaviors in the mesocosm experiment, despite an overall turbidity increase. The direct influence of crayfish number and size on turbidity confirms the importance of crayfish as turbidity-inducing organisms in freshwater streams. Nonetheless, several results were unexpected and deserve closer attention.

Crayfish burrowing behaviors have been linked to increased soil respiration (Richardson 1983) and phosphorus mixing in soil (Stone 1993). In freshwater systems, fish and crayfish have been shown to increase bioturbation (Table 5.1 and 5.2; Statzner 2012). Here we provided a case study of the interactive effect of fish and crayfish on crayfish bioturbation in streams. Our study suggests that predation avoidance by crayfish increased turbidity when predatory fish were present. This turbidity can be induced either by walking on the streambed (Statzner 2012), tail-flipping, or burrowing for shelter (Dorn and Mittelbach 1999, Ilheu et al. 2003).

We also demonstrated that limb loss can enhance crayfish-induced turbidity, which may account for a substantial proportion of natural turbidity. Crayfish use their two chelipeds to excavate and mold mud pellets during burrowing (Berrill & Chenoweth, 1982; Helms *et al.*, 2013; LAD, personal observations), often forming chimneys on top of their burrows. One-clawed crayfish can only burrow a depression in the ground rather than a functioning burrow (Dunoyer et al. in prep). Nonetheless, regenerating crayfish may avoid exposure as much as possible while undergoing limb regeneration, having only one fully functional cheliped for protection from predators. Since burrowing is inefficient for autotomized crayfish, they avoid predation in turbidity-enhancing ways by walking on the streambed or tail-flipping when evading predators or both (Statzner 2012). Moreover, when trying to burrow for shelter, autotomized crayfish are less efficient than their unmanipulated counterparts, generating more turbidity in the process.

Intrinsic differences between experimental streams and mesocosms could have caused the lack of increased turbidity for regenerating crayfish when paired with predatory fish. Flowing water might rapidly dilute and remove chemical cues of fish predators. Thus, crayfish in streams might have been less aware of fish presence than their conspecifics in the mesocosm experiment, where predatory may last longer. This reduced predator awareness could have allowed exploratory and burrowing behavior to continue in the presence of a predator, resulting in greater turbidity in stream environments. Alternatively, the control treatment in the field might have included exposure to predator cues, unlike the control treatments in the mesocosm experiment. It is likely that only fish were generating turbidity when paired with regenerating crayfish in the mesocosms, since autotomize crayfish must reduce exploratory and burrowing behavior to avoid predation. In contrast, control crayfish possessing both chelipeds were less likely to adjust their behavior, having both double cheliped defense and cheliped autotomy available in the presence of the predator.

Increased energy requirements due to regeneration following autotomy may also contribute to increased foraging activity by regenerating crayfish. However, this explanation relies on two assumptions empirically unsupported. First, limb loss and regeneration are assumed to induce an increase in crayfish energy budget. Second, if indeed limb loss and regeneration do induce an increased energy requirement, crayfish are then assumed to fulfill this increase by foraging more. Thus, increased foraging implies more movement on the stream bed leading to more turbidity (Statzner 2012). Alternatively, crayfish might be able to regenerate using energy reserves or otherwise adjust energy utilization without the need to increase their energy uptake.

Alternatively, crayfish-induced turbidity following limb loss may be attributed to predation avoidance. Indeed, fish predation efficiency is reduced in turbid water (Abrahams and Kattenfeld 1997, Meager et al. 2006, Kimbell and Morrell 2016). However, it is unclear if the observed range of turbidity in this experiment (between 0 and 8 NTU) affects crayfish predation by fish. Studies of fish predation on crayfish at different turbidity levels are needed to resolve this.

Our work emphasizes the role of crayfish behavior and autotomy, depending on the level of predation risk, in determining turbidity levels in freshwater streams. Because species live in a community context and by uncovering the complexity of crayfish-fish interactions, we raised questions about the ways that crayfish induce turbidity as well as about the explanation for crayfish behavioral changes following cheliped autotomy. It is our hope that future research will uncover both mechanisms and causes of crayfish behavioral change induced by autotomy.

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VITA

Education

2013 – 2020 (expected)	Ph.D.	University of Kentucky, Biology, Lexington, KY. Dissertation Advisors: Drs. Jeremy Van Cleve & Ashley W. Seifert.
2010 – 2012	M.S.	University of Burgundy, Biology, Dijon, France. Thesis Advisors: Pr. Loic Bollache & Dr. Clement Lagrue.
2006 – 2009	B.S.	University of Savoie Technolac, Biology, Chambery, France.

Professional positions

2019 – Present	Instructor 9M Biology in the Life Sciences department at Wake Technical Community College in Raleigh, NC.

Scholastic and professional honors

2019	University of Kentucky Outstanding Teaching Award, University of Kentucky.
2019	Arts & Sciences' Outstanding Teaching Assistant, University of Kentucky.
2019	Biology's Outstanding Teaching Assistant Award, University of Kentucky.
2019	GradTeach Live! competition (3rd place), University of Kentucky.

Professional publication

Luc A. Dunoyer, Zoe Dapore*, Jeremy Van Cleve, and Ashley W. Seifert (**in prep**) Effects of limb loss via autotomy and regeneration on crayfish reproductive success in the red swamp crayfish. *Functional Ecology*.

Luc A. Dunoyer, Makayla Dean*, Stephanie Hacker*, Jeremy Van Cleve, and Ashley W. Seifert (**in prep**) Shelter availability and predation cues drive behavioral decisions following limb loss in the red swamp crawfish. *Animal Behavior*.

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