ABSTRACT

Title of Dissertation:	PHYSIOLOGICAL DYNAMICS OF INJURY AND REGENERATION IN THE CLONAL FRESHWATER ANNELID *PRISTINA LEIDYI*
	Corey W. Rennolds, Doctor of Philosophy, 2022
Dissertation directed by:	Professor Alexandra E. Bely, Department of Biology

The threat that mechanical injury poses to homeostasis and survival has spurred the evolution of diverse processes to mitigate these effects. The most dramatic of these is regeneration, a process that restores the form and function of lost body parts. The apparent benefits of regeneration may come at considerable cost, however, and these may substantially diminish regeneration's adaptive value in certain contexts, potentially contributing to evolutionary losses of regeneration. The costs and benefits of regeneration are poorly understood in most animals, precluding more than speculation of the evolutionary drivers of regeneration. Naids are a group of small, clonally reproducing freshwater annelids that feature great diversity of regenerative ability and are well suited to experimental studies. I used the species *Pristina leidyi* to determine how injury and regeneration affect organismal function and fitness, integrating physiological and molecular approaches. I first investigated how injury and regeneration differentially affect an individual's ability to tolerate environmental

stress, an ecologically relevant and energetically demanding task. I found that stress tolerance is reduced by regeneration in a stressor- and tissue-specific manner while, unexpectedly, tolerance is temporarily improved shortly after injury. These effects are unrelated to whole-organism metabolic rate, which surprisingly does not differ between early and late injury recovery. Using 3' TagSeq, I found that, while injury and heat stress elicit largely distinct responses, both upregulate certain shared damage control pathways. I then tested whether the physiological cost of regeneration has potential to translate into fitness costs by examining the interaction between regeneration and reproduction, which occurs by asexual fission in this species. By modulating resource availability, I found evidence for an energetic trade-off between regeneration and reproduction that is masked when food is abundant. This tradeoff is manifested through a reduction in per-offspring allocation rather than reproductive rate. Overall, my results demonstrate that injury and regeneration costs are highly context dependent in *P. leidyi*. More broadly, these findings contrast in key ways from evolutionarily distant animals with very different life history traits, illustrating the importance of investigating the physiological mechanisms that may mediate selection on regeneration in diverse lineages.

PHYSIOLOGICAL DYNAMICS OF INJURY AND REGENERATION IN THE CLONAL FRESHWATER ANNELID *PRISTINA LEIDYI*

by

Corey William Rennolds

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Advisory Committee:
Professor Alexandra E. Bely, Chair
Professor Eric S. Haag
Professor Maile C. Neel, Dean's Representative
Dr. Christopher L. Rowe
Associate Professor Jeffrey W. Shultz

Preface

This dissertation contains a brief introduction (Chapter 1), one literature review chapter in manuscript form (Chapter 2), two research chapters in manuscript form (Chapters 3-4), and appendices to the research chapters (Appendices 1-3). A single bibliography is provided at the end for literature cited throughout the dissertation.

Dedication

To John Rennolds, who I'm not sure could say exactly what I did with my life but was proud of me anyway.

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General acknowledgements:

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Chapter 1: Introduction

Evolutionary and physiological patterns of regeneration

Regeneration is one of the most remarkable manners by which animals address the traumatic loss of body parts. In contrast to mere wound healing or scarring, reparative regeneration (as distinct from physiological regeneration, such as the routine shedding and regrowth of deer antlers (Elchaninov, Sukhikh, & Fatkhudinov, 2021; Seifert & Muneoka, 2018)) not only seals the wound from further loss of body fluids or pathogen entry but also restores the original form and function of the lost body part with at least partial and often flawless fidelity (Arenas Gómez, Sabin, & Echeverri, 2020; Bely & Nyberg, 2010). The advantage such an ability confers to an animal is unmistakable, which may lead one to reasonably predict strong positive selection upon the ability to regenerate where- and whenever it arises. The phylogenetic pattern of regeneration in extant lineages supports an early origin of regeneration, likely not far removed from the emergence of multicellularity itself (Bely & Nyberg, 2010; Elchaninov et al., 2021). Yet, while indeed ubiquitous in the animal kingdom, it is equally unmistakable that many animals, including ourselves, are frustratingly incapable of regeneration to any significant degree. The same phylogenetic pattern in fact indicates numerous losses of regeneration in diverse taxa, including endothermic vertebrates (i.e., birds and mammals), nematodes, rotifers, leeches, and others (Bely, 2010). From the earliest days of formal biological study through today, great effort has and continues to be expended in discovery of the factors that govern regeneration, and how those might one day be applied to humankind, through research using a variety of animal systems. Several of these have now become

established as classic regeneration models, such as hydra, planarian flatworms, and amphibians (Sánchez Alvarado & Tsonis, 2006).

Recent years have witnessed a revolution in evolutionary-developmental biological research, a large and admirably productive portion of which attempts to reveal the cellular and molecular mechanisms that are involved in regeneration and may have served as proximate targets of regeneration reduction or loss (Lai & Aboobaker, 2018; Sánchez Alvarado & Tsonis, 2006; Tiozzo & Copley, 2015). However, the whole organism is the fundamental biological unit in which these mechanisms operate and that directly experiences the biotic and abiotic forces driving evolutionary change. The robust output of work on the processes governing regeneration at lower biological scales must therefore be matched by an equally vigorous dissection of the context in which these processes occur at organismal and ecological scales. Additionally, work is necessary that addresses integrative hypotheses across biological scales within single species, a criterion that unfortunately narrows the number of those regenerating species that we possess a holistic understanding of at a high level. For example, what is the adaptive value of regeneration in a species, and what factors differentiate the utility of regeneration to one species versus another or between different environmental circumstances? Addressing such questions will not only help with understanding how and why known mechanisms of regeneration emerged in different lineages, including why mechanistic differences exist between lineages, but also facilitate the discovery of new mechanisms in more, diverse species.

Two hypotheses as to why regeneration has been lost in some lineages are, first, that the cost of regeneration outweighs its benefits in certain circumstances and is

selected against, or second, that regeneration does not impose much of a cost but simply fails to offer enough of a fitness benefit to be actively maintained and is subsequently lost due to drift (Bely, 2010; Goss, 1969; Reichman, 1984). However, there remains a notable lack of investigation into the potential costs of regeneration, including how they compare to the costs of injury itself explicitly, in most regenerating animals. As a result, evolutionary-developmental biologists are limited in their ability to explain patterns of change in regenerative ability, including between even relatively closely related species. Yet broad patterns exist that suggest relationships between regenerative ability and a number of physiological traits. One example is endothermy, which is correlated with reduced regenerative ability between species, spurring the hypothesis that perhaps the cost of maintaining a high baseline metabolic rate precludes extensive regeneration (Goss, 1969; Hirose et al., 2019; Reichman, 1984). Another is ontogeny, which within species is characterized by a general reduction in the rate, extent, or fidelity of regeneration as an organism ages or progresses through distinct developmental stages (Bely, 2010; Seifert, Monaghan et al., 2012). Thus, one could predict that developmental mechanisms that are engaged earlier in life and critical to regeneration become inactivated later on, perhaps because they no longer offer any use or conflict with physiological processes that occur in maturity. Yet another is immunity, which both within and between species is associated with decreased regenerative potential as immune function becomes more complex and incorporates more adaptive components, as occurs from tadpoles to adult frogs and from basal to more derived vertebrates (Elchaninov et al., 2021; Godwin, Pinto, & Rosenthal, 2017; Tiozzo & Copley, 2015). One last trend worth mentioning is general anatomical and physiological complexity,

which is very broadly negatively correlated with regeneration between species, across ontogeny, and even between structures in the same organism (Elchaninov et al., 2021; Giangrande & Licciano, 2014; Reichman, 1984; Tiozzo & Copley, 2015), although great differences in regenerative ability often exist between species of similar complexity, such as within the naids (Bely & Sikes, 2010). For example, some relatively simple planarians (Bely, Zattara, & Sikes, 2014; Reddien & Sánchez Alvarado, 2004) and acoels (Srivastava et al., 2014) are capable of regenerating entire bodies from just a few cells, whereas no vertebrates and only a few invertebrate lineages, such as some annelids (Bely et al., 2014), nemerteans (Zattara et al., 2019), and hemichordates (Luttrell et al., 2016), can regenerate their heads to any extent. In animals with more complex body plans, relatively simple, redundant, or nonvital structures are most commonly regenerable, such as spider legs (Vollrath, 1990), lizard tails (but never other limbs) (Jacyniak, McDonald, & Vickaryous, 2017), or mammalian digit tips (Seifert & Muneoka, 2018). Thus, as structures grow more elaborate or vital to survival, one might hypothesize that they become inordinately costly, or encounter too many molecular hurdles produced as a byproduct of increasing complexity, to regenerate quickly or at high enough fidelity for regenerative capability to endure.

Each of the aforementioned correlations hints towards physiological factors underlying the loss of regeneration in animals. Yet direct investigation of these factors and the immediate costs of regeneration remains at times scattershot, of secondary importance, or basic in scope. However, researchers have contributed valuable knowledge on the costs of regeneration in some animals. Restoring a lost body part requires energy and materials that are limited in the environment and that organisms have

limited capacity to assimilate and store. Work to date indicates that the required investment can be considerable: both head and tail regeneration deplete body lipids and triglycerides in the fireworm *Eurythoe* (Yáñez-Rivera & Méndez, 2014), and central disc regeneration significantly reduces total body mass and caloric content, including protein, carbohydrates, and lipids, in brittlestars (Dobson et al., 1991). A lost body part itself may also have included resources that then become unavailable to other physiological processes, as in the case of lizards, some of which maintain substantial caudal fat reserves that may be lost with the tail (Chapple & Swain, 2002a; Smyth, 1974) or in some sea stars which may lose their arms containing nutrient reserves stored in pyloric caeca (Lawrence, J. M. & Larrain, 1994). These energetic costs of loss and investment may impose constraints on organismal function that manifest in myriad ways during or after regeneration, including loss of fecundity (Maiorana, 1977), reduced body size (Ballinger & Tinkle, 1979), altered development (Holland & Skinner, 1976), or reduced locomotory performance (Maginnis, 2006a). Regenerated parts are not always as useful as the original, either: regenerated lizard tails feature a less-flexible cartilaginous tube to provide support rather than vertebrae, and lizards with regenerated tails exhibit changes in gait (Jagnandan, Russell, & Higham, 2014), less effective anti-predator tail function (Naya et al., 2007), and changes in biochemical activity that may indicate poorer metabolic capacity in the tail (Meyer, V., Preest, & Lochetto, 2006); and spiders with regenerated legs construct differently-designed webs, which may reduce prey capture efficacy (Vollrath, 1987), and lack structures that are important visual cues for mating success (Uetz et al., 1996). Many animals besides those listed here can regenerate various body parts, but the proximate or ultimate consequences of regeneration have been

described to any appreciable extent in few of them, especially across scales within the same species.

Naid annelids are useful study organisms for regeneration physiology research

The naid annelids provide many advantages for studying regeneration broadly. Naids, an informal grouping of the subfamilies Pristininae and Naidinae of the annelid family Naididae, are small (several mm long), typically infaunal worms abundant throughout freshwater habitats, although some brackish species are known, with a global distribution (Bely, 2022; Brinkhurst, 1986). The rocky or sandy streams and rivers in which naids are often found may subject them frequently to physical forces that can inflict bodily damage in aquatic animals, including hydraulic forces (Nietzel et al., 2000) and sediment flow (Newcombe & Macdonald, 1991). Additionally, naids are likely to be common prey targets for insects, larval fish, and other small predators, making them both susceptible to sublethal predation injury and ecologically relevant (Kaliszewicz, 2003). Naids are morphologically diverse but generally reflect the standard annelid body plan of a series of largely iterative segments (Bely, 2022). Naids vary in their regeneration ability (Fig. 1.1), but posterior-end regeneration is common. Anterior regeneration is somewhat less common, but no species is capable of anterior regeneration without also being able to regenerate posteriorly (Bely & Sikes, 2010). Asexual reproduction is the norm for naids, usually through paratomic fission, in which a new head and tail are intercalated along the body and fully develop before division occurs (Bely, 1999; Zattara & Bely, 2016), although sexual reproduction occurs seasonally in the wild (Loden, 1981). Recent work supports the hypothesis that regeneration is ancestral in the naids and served as an evolutionary pre-requisite for asexual reproduction, which developed multiple times

independently via the likely co-optation of regeneration pathways (Zattara & Bely, 2016). Subsequent recent losses of regeneration, leading to notable inter- and even intrageneric variation in regenerative ability, make for a valuable system in which to study the potential links between physiological factors and regeneration.

Of the naids, *Pristina leidyi* offers several particular advantages as a regeneration model species (Bely, 2022). It is an especially proficient regenerator, able to restore substantial portions (a third or more) of the body from anterior, posterior, or middle fragments with high fidelity, such that new segments are often indistinguishable from old tissue, within five days. Fission is similarly rapid when food is available, with typically just a few days elapsing between the appearance of a fission zone typically at segments 15 to 17 and the separation of a fully-formed, genetically identical "daughter" worm (zooid) (Zattara & Bely, 2011). *P. leidyi* is small (2-6 mm long on average) and anatomically relatively simple, comprising a tube-within-tube body construction containing a regionalized through gut, long hairlike dorsal bristles (chaetae) (including particularly long ones on the second segment), and an elongated anterior proboscis (Fig. 1.2). The body is mostly transparent, allowing for the easy identification of anatomical landmarks, such as fission zones, sections of the alimentary canal, and motile coelomic cells. *P. leidyi* are easy to maintain both in bulk cultures and singly, permitting use in a wide range of experimental studies, including physiological work. These features have contributed to a burst in molecular and developmental research focused on *P. leidyi* (Bely, 2022), including detailed descriptions of the regeneration and fission processes (Zattara & Bely, 2011, 2013). Altogether, it is an excellent species for conducting work

to test hypotheses concerning the physiological effects of injury and regeneration, which may then be applied to other naids in future comparative studies.

Dissertation overview

The objective of this dissertation is to characterize the physiological responses to tissue loss (injury) and regeneration at the level of the organism, and link these to possible underlying mechanisms and fitness consequences, in the naid *Pristina leidyi*. Although I have provided an evolutionary context for this work to this point, the generally sparse understanding of regeneration physiology in naids, and annelids more broadly to some extent, presents both challenges and opportunities. By focusing my investigation on one species, I can more closely relate my findings with previous work in the species and use similar techniques (e.g., how and where to injure animals), which would not necessarily be appropriate in other species in which the details of regeneration are not well known and would thus necessitate such preliminary research to make fair comparisons with *P. leidyi*. Additionally, the use of one representative species allows for a deeper examination of mechanisms underlying the regeneration process. While I therefore sacrifice some breadth for depth, I use an integrative approach that can be easily adapted to other naid species as basic knowledge and resources permit.

Understanding how regeneration affects organismal function requires consideration of the impacts that mechanical injury alone can have across biological scales and how these vary with intrinsic and extrinsic contexts. Chapter 2 is presented as a thorough literature review and synthesis of the integrative biology of injury in animals. By providing examples from throughout the kingdom, I showcase the range of factors influencing how injury affects animals over the short to long term and how responses at

lower levels of organization, such as molecular cascades, connect with high order phenomena, such as changes in ecological interactions. I highlight the diversity of injury effects with respect to life history, phylogeny, and environment, providing a helpful context and foundation for generating specific hypotheses and designing studies to test these effects, and how they might be distinguished from those of diverse recovery processes, in animals including the naids and other lineages that have received little attention in injury research.

Chapter 3 addresses how injury and regeneration affect environmental stress tolerance, an ecologically relevant indicator of physiological performance, in *P. leidyi* and how these effects differ between the anterior and posterior body ends. I used multiple experimental techniques, including survival assays, microplate respirometry, and next generation RNA sequencing, to characterize the functional impact of tissue loss in comparison to regeneration, by applying treatments at different time points post-injury. My findings can be used to make predictions about the types of conditions that may affect the relative benefit of regenerating body parts in the short term as compared to when those parts are absent. With respect to the naids as a group, these results can be used to develop hypotheses regarding physiological factors driving the variation in anterior or posterior regeneration. Additionally, I present a selection of gene candidates that may represent shared components of the injury and general stress response, which could have significant implications for understanding the evolutionary origins of regeneration and include targets for natural selection.

Chapter 4 applies life history theory to test how resource supply affects the interaction between regeneration and fission. I predicted that, if the cost of regeneration is

primarily an elevation of resource demand, then that would result in an energetic trade-off with reproduction leading to fewer or poorer quality offspring, but the extent of this trade-off would vary with the amount of available food. This study took advantage of *P. leidyi*'s rapid, sequential, and clonal propagation to test the longitudinal effects of discrete numbers of injury and regeneration events on both discrete and continuous measures of reproduction without the confounding influence of genetic variation. I measured reproductive effects as a function not only of regeneration frequency but also time, which allowed me to draw inferences concerning the general allocation strategy involving these two evolutionarily and mechanistically related processes. These findings also offer an example of regeneration effects on reproduction in an animal markedly distinct from better studied, predominantly sexual species with very different evolutionary histories whilst allowing for interesting and useful comparisons on the basis of life history traits.

Figures

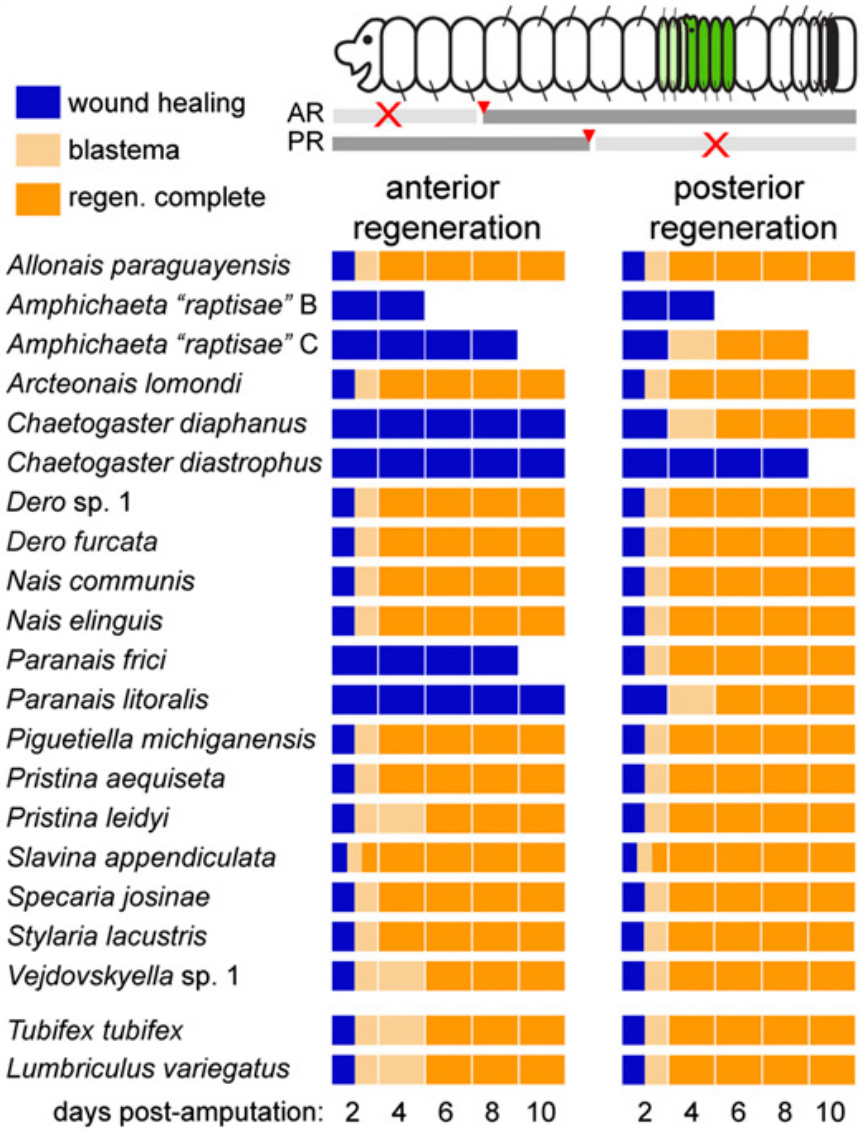

Fig. 1.1. Regeneration ability in 19 naid species and 2 outgroup species (bottom) from comparative regeneration experiments. Cartoon shows standardized amputation injury locations in worms; segments highlighted green indicate a paratomic fission zone. "Blastema" refers to the undifferentiated mass of cells that form at the beginning of regeneration. Spaces with no scoring indicate that worms do not survive beyond that point following amputation injury. Figure from Bely & Sikes (2010).

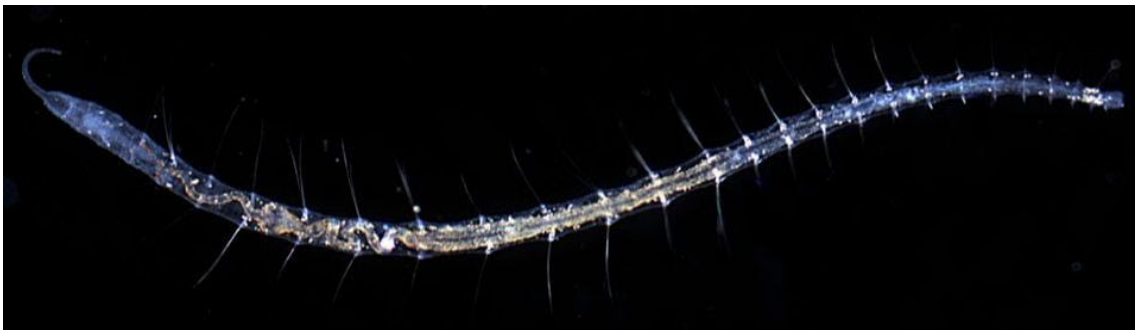

Fig. 1.2. Photograph of *Pristina leidyi*. Dorsal view, with anterior to the left. (Photo credit: Eduardo Zattara)

Chapter 2: Integrative biology of injury in animals

Abstract

Mechanical injury is a prevalent challenge in the lives of animals with myriad potential consequences for organisms, including reduced fitness and death. Research on animal injury has focused on many aspects, including the frequency and severity of wounding in wild populations, the short- and long-term consequences of injury at different biological scales, and the variation in the response to injury within or between individuals, species, ontogenies, and environmental contexts. However, relevant research is scattered across diverse biological subdisciplines, and the study of the effects of injury has lacked synthesis and coherence. Furthermore, the depth of knowledge across injury biology is highly uneven in terms of scope and taxonomic coverage: much injury research is biomedical in focus, using mammalian model systems and investigating cellular and molecular processes, while research at organismal and higher scales, research that is explicitly comparative, and research on invertebrate and non-mammalian vertebrate species is less common and often less well integrated into the core body of knowledge about injury. The current state of injury research presents an opportunity to conceptually unify work focusing on a range of relevant questions, to synthesize progress to date, and to identify fruitful avenues for future research. The central aim of this paper is to review and synthesize research concerning the broad range of effects of mechanical injury in animals. We organize reviewed work by four broad and loosely-defined levels of biological organization: molecular and cellular effects, physiological and organismal effects, behavioral effects, and ecological and evolutionary effects of injury. Throughout,

we highlight the diversity of injury consequences within and between taxonomic groups while emphasizing the gaps in taxonomic coverage, causal understanding, and biological endpoints considered. We additionally discuss the importance of integrating knowledge within and across biological levels, including how initial, localized responses to injury can lead to long term consequences at the scale of the individual animal and beyond. We also suggest important avenues for future injury biology research, including better distinguishing between related yet distinct injury phenomena, expanding the subjects of injury research to include a greater variety of species, and testing how intrinsic and extrinsic conditions affect the scope and sensitivity of injury responses. It is our hope that this review will not only strengthen understanding of animal injury but will contribute to building a foundation for a more cohesive field of "injury biology".

Introduction

Injury is a common challenge that animals encounter in nature. Mechanical injury—damage to anatomical structure that results from direct contact (hereafter simply referred to as "injury")—can be caused by a variety of factors, including predatory interactions, non-predatory biotic interactions (e.g., territorial encounters, mating rituals), damaging movements (e.g., falls, impacts), and damaging abiotic forces (e.g., crushing or shearing by physical substrates) (Archie, 2013; Crook et al., 2011; Feder, J. A. et al., 2019; Figiel & Semlitsch, 1991; Juanes & Smith, 1995; Meszaros & Bigger, 1999; Mukherjee & Heithaus, 2013; Palmer et al., 2011). Injuries themselves also vary greatly in severity, from minor nicks, bumps, and abrasions to complete destruction or amputation of large body portions.

The sources and prevalence of injury vary greatly across animals for numerous reasons, and assessing injury rates in the wild remains challenging. Studies which have attempted to estimate injury rates reveal often striking findings, indicating that an injured state may be the norm for many animals. For example, an average of roughly one-third to one-half of marine benthic invertebrate populations are visibly injured at any given time, and in some populations, over 70% of individuals may be injured (Lindsay, 2010); an average of about one-quarter, and up to 80%, of decapod crustacean populations have been reported as suffering limb damage (Juanes & Smith, 1995); over one-quarter of pygmy octopuses (*Octopus digueti*) may show signs of injury to one or more arms at a time (Voight, 1992); rates of tail damage in many lizard species are often over 50% (Arnold, 1984; Fleming, Muller, & Bateman, 2007); up to 100% of sabellid polychaetes (e.g., *Schizobranchia insignis*) may exhibit damage to feeding and respiratory structures (Brown, S. D. & Emlet, 2020); and close to half of anuran tadpoles in a number of species—and, in some populations, almost 90% of individuals—may show signs of tail damage (Blair & Wassersug, 2000). Animal fossil records indicate that sublethal injury was prevalent in the past, with some of the best evidence coming from Paleozoic invertebrates like crinoid echinoderms, trilobites, and molluscs (Baumiller & Gahn, 2004, 2013; Bicknell & Holland, 2020; Ebbestad & Peel, 1997). Injury is so pervasive that, for many species, every individual can be expected to sustain some kind of injury in its lifetime, and, in some species, individuals will likely experience frequent, repeated injury (Juanes & Smith, 1995; Lindsay, 2010). Furthermore, sublethal injury rates are likely underestimated in animals capable of regeneration, the process by which new tissue replaces that which is damaged or lost, resulting in new tissue which is often visually

indistinguishable from the original (Bernardo & Agosta, 2005; Juanes & Smith, 1995; Lindsay, 2010). Many animals can even lose and regenerate the same body parts multiple times throughout their lives, such as clam siphons (Sasaki et al., 2002; Tomiyama & Omori, 2007), polychaete palps (Zajac, 1985), hydra tentacles (Wenger et al., 2014), and lizard tails (Barr et al., 2019; Jacyniak et al., 2017).

Injury threatens organismal function, homeostasis, and survival, and animals have evolved diverse responses to mitigate these effects of injury, and these responses manifest across levels of biological organization. At the lowest level, focused on the activities of individual cells and molecules, injuries induce complex pathways that serve to seal wounds; prevent the loss of circulatory fluid; combat infection; direct cells to move, divide, and differentiate (or de-differentiate); and govern expression of genes that regulate these pathways. At the physiological and organismal level, injuries may lead to changes in organismal function over the short or long term, either directly or as a compensatory response. At the level of behavior, injuries may change the way animals interact with one another or with their environment in order to avoid further injury or mitigate the effects of injuries already suffered. Consequences of injury to organisms can collectively produce effects discernible at the ecological level, affecting population or community dynamics and composition. Responses to injury are ultimately shaped by evolutionary history and also mediate ongoing selection on the injury response. These effects will be discussed in detail in the following sections of this review.

Although it is convenient to discuss injury effects within the loose bounds of these levels, these responses are complex and expected to involve feedbacks, linkages, and interrelated effects across these levels (Fig. 2.1). For example, an injury can induce

molecular and metabolic changes that affect an animal's behavior, and at a large scale, injury in natural populations can lead to natural selection favoring traits that minimize the harm done by injuries or reduce the risk of receiving them altogether. There is evidence, particularly at lower levels of biological organization, that injuries have long played a significant role in evolution, as many injury responses are conserved across metazoans (Galko & Krasnow, 2004; Lockwood, Sanders, & Somero, 2010; Martin & Nunan, 2015; Niethammer, 2016; Palmer et al., 2011; Wenger et al., 2014). However, there is also a great diversity of responses to mechanical injury at higher orders of biological organization, and even similar processes, such as regeneration, may represent convergent evolution (Bely et al., 2014; Lai & Aboobaker, 2018; Zattara et al., 2019). Importantly, in any given species, specific responses to injury also depend on a broad range of factors (Fig. 2.2), including characteristics of the injury, the context in which injury occurs, and any recovery processes an animal might be capable of for repairing the damage (such as regeneration).

Injury responses have been studied from many perspectives and in a broad diversity of species, but knowledge is uneven across focal areas and animal groups. Importantly, despite clear linkages between effects at different levels of biological organization, the literature on injury lacks broad synthesis and cohesion. Much current knowledge of injury responses is derived from studies focusing on a handful of species and taxa, particularly in well-established model organisms, with little known from many animal groups. Research on injury spans disparate subdisciplines within biology, and study systems used to investigate lower- and higher-level responses are often different, hampering dialogue between different research communities working on injury. Many

studies use injury as an intervention to address hypotheses not directly concerned with the effects of injury themselves, and few studies attempt to explicitly link complex injury responses across levels of organization, such as from changes in gene expression to whole-organism responses, or from physiology to population biology. Establishing these connections and developing an integrative view of "injury biology" is challenging but necessary in order to understand animals in all their functional complexity.

In this review, we synthesize current knowledge of the effects of mechanical injury on animal biology. We review information from across the animal kingdom and across levels of biological organization, focusing specifically on molecular and cellular responses, physiological and organismal responses, behavioral responses, and ecological and evolutionary consequences of injury. Where possible, we highlight links between these levels and indicate taxonomic patterns in the information available. Finally, we discuss the importance of stronger integration across injury biology and identify important gaps in current knowledge about the effects of mechanical injury in animals.

Effects of injury across levels of biological organization

In this section, we summarize knowledge on the effects of injury across animals. We organize information by broad levels of organization, focusing on *molecular and cellular*, *physiological and organismal*, *behavioral*, and *ecological and evolutionary* effects of injury. These categorizations are subjective, but we employ them to simplify and focus our discussion.

Molecular and cellular effects of injury

Molecular and cellular processes are involved in wound detection, pathogen defense, hemostasis, gene expression, inflammation, cell proliferation, and wound healing end states (e.g., scarring and regeneration). Much of the available information on wound healing at the molecular and cellular level comes from vertebrates, particularly model systems such as zebrafish (*Danio rerio*) and rodents (e.g.: Bielefeld, Amini-Nik, & Alman, 2013; Desmouliere, Chaponnier, & Gabbiani, 2005; Godwin & Brockes, 2006; Gurtner et al., 2008; Levesque, Villiard, & Roy, 2010; Martin & Nunan, 2015; Niethammer, 2016; Velnar, Bailey, & Smrkolj, 2009), in addition to a handful of invertebrate systems, especially *Drosophila* (Antunes et al., 2013; Belacortu & Paricio, 2011; Razzell, Wood, & Martin, 2011; Repiso et al., 2011). As wound healing in these systems has been reviewed in depth, here we provide only an overview of the key processes involved and emphasize information from outside the major model systems. Readers may additionally gain broader perspective on eukaryotic wound responses through review of the considerable work concerning injury signaling and repair mechanisms in plants (e.g.: Asahina & Satoh, 2015; León, Rojo, & Sánchez-Serrano, 2001; Savatin et al., 2014; Schilmiller & Howe, 2005; Vasyukova et al., 2011).

(a) Wound detection and pathogen defense

When a mechanical injury is sustained, the first step in the wound response is detection. Early wound detection involves processes largely conserved among metazoans and overlap substantially with damage and pathogen detection pathways in plants and unicellular eukaryotes, including choanoflagellates (the closest relatives of animals); this suggests that basic wound healing responses have ancient origins (Archie, 2013; Wenger

et al., 2014). These organismal groups all respond in some manner to the suite of molecules released by cellular lysis, collectively referred to as a damage associated molecular pattern (DAMP). These DAMPs include formylated peptides, adenosine triphosphate (ATP), free fatty acids, and calcium ions, among other molecules. In metazoans, many of these molecules induce transcription of pro-inflammatory gene products via various receptors; DAMPs also serve as chemoattractants for leukocytes in vertebrates (Chisholm, 2014; Niethammer, 2016; Wenger et al., 2014). Upon cellular recognition of DAMPs, various pathways activate that primarily function to eliminate or repair damaged cells and subcellular components, mitigate pathogenic threats, and rebuild damaged tissue; these pathways comprise animal innate immunity. In addition to DAMPs, there has been intriguing recent work implicating the role of bioelectrical gradients (Levin, 2009; Levin et al., 2019) and mechanical forces (Abrams et al., 2015) in both early injury signaling and subsequent coordination of wound healing and extensive structural repair.

Preventing infection by foreign organisms is one of the primary functions of the injury response. Wounding typically includes a breach of physical barriers to the external environment, such as the skin, cuticle, or outer epithelium, which increases the risk of entry by harmful bacteria, viruses, or other invaders (Archie, 2013; Velnar et al., 2009). Humoral defense mechanisms triggered by wounding have been documented in diverse animals including cnidarians, arthropods, molluscs, and vertebrates (the term "humoral" here encompassing a diverse number of body fluids, such as blood or hemolymph (Monahan-Earley, Dvorak, & Aird, 2013)). Antimicrobial peptides (AMPs), structurally and functionally diverse molecules that protect injured animals against pathogens (Wang,

G., 2010), are known to be upregulated early on following tissue damage in diverse animals including cnidarians, molluscs, annelids, nematodes, arthropods, and vertebrates (Bodó et al., 2021; Chisholm, 2014; Romo, Pérez-Martínez, & Ferrer, 2016; Vafopoulou, 2009; van de Water et al., 2015; Wenger et al., 2014). AMP expression may be complemented by expression of other molecules with antimicrobial function, such as lysozymes and lectins, as in arthropods (Liu, F., Ling, & Wu, 2009; Rowley & Powell, 2014; von Wyschetzki, Lowack, & Heinze, 2016). The presence of pathogens during wounding can increase AMP expression further, as demonstrated in bees (Erler, Popp, & Lattorff, 2011; Koleoglu et al., 2017). Nonsterile wounding may compromise other components of immunity. For example, Liu et al. (2009) found in silkworm (*Bombyx mori*) larvae that sterile wounding elicits more serine proteases, serpins, lectins, and other genes with non-pathogen-specific immune functions (e.g., which may be involved in clotting pathways) than nonsterile wounding, which the authors suggest may be a strategy to conserve energy and other resources in the latter case in order to invest more heavily in defending against the introduced pathogens. Some animal lineages have evolved an additional anti-pathogenic pathway induced by wounding known as melanization. For example, in arthropods, the pigment melanin has evolved a pleiotropic role in wound healing and anti-microbial defense (Bilandžija et al., 2017; González-Santoyo & Córdoba-Aguilar, 2012; Palmer et al., 2011; Rowley, 1996; Theopold et al., 2002). Wounding has been shown to upregulate phenoloxidase, which catalyzes melanin synthesis (Bilandžija et al., 2017), in several insects (Bidla et al., 2009; Reavey et al., 2014) and crayfish (Vafopoulou, 2009), as well as in *Acropora* corals (van de Water et al., 2015).

(b) Hemostasis

Open wounds may leak fluids such as blood or hemolymph, which must be stopped quickly to prevent severe homeostatic disruption or fatality. The process of fluid leak cessation, known as hemostasis, is common in animals but differs with respect to the mechanisms, cellular components, and level of complexity involved (Archie, 2013; Galko & Krasnow, 2004; Godwin & Brockes, 2006; Grdisa, 2010; Soslau, 2020). Contraction of tissue (e.g., skin, muscle) surrounding the wound and of damaged proximal vasculature, if present, can occur reflexively (via e.g., altered calcium flux (Chisholm, 2014; Niethammer, 2016)) to reduce wound diameter; such contractions have been described in diverse taxa including annelids (Bely & Özpolat, 2016), octopuses (e.g., *Octopus vulgaris*, *Eledone cirrhosa*) (Andrews et al., 2016; Polglase, Bullock, & Roberts, 1983), asteroid echinoderms (Pinsino, Thorndyke, & Matranga, 2007), and vertebrates (Desmouliere et al., 2005; Levesque et al., 2010; Velnar et al., 2009). Cnidarians are known to use a combination of cell "crawling" and contraction of actin filaments to close wounds depending upon the degree of damage (Kamran et al., 2017), and a similar "purse-string" process occurs in wounded *Drosophila* embryos (Wood et al., 2002). Snakes, unique among vertebrates for routinely shedding their entire skin at once, do not exhibit regular cutaneous wound contraction as mammals do but rather form a crust over the wound area prior to re-epithelialization (Smith, D. A. & Barker, 1988). Following reflexive wound contraction, cellular plugs or clots often form at the wound site through a process called coagulation: migratory epithelial cells cover wound openings in octopuses (Andrews et al., 2016; Polglase et al., 1983), coelomocytes form clots in common sea stars (*Asteria rubens*) (Pinsino et al., 2007), platelet plugs precede

the formation of a fibrin mesh containing blood cells in mammals (Brockes & Kumar, 2008), and other (not necessarily homologous) variants of coagulation occur throughout other invertebrates (Bely, 2014; Galko & Krasnow, 2004; Palmer et al., 2011; Razzell et al., 2011; Theopold et al., 2002, 2004). In some groups, such as nematodes (Chisholm, 2014) and crayfish (Vafopoulou, 2009), the immediate mechanisms or signals of hemostasis are not well understood despite the common use of these animals as model systems. For very large wounds, especially in endotherms or other animals with high-pressure circulatory systems, hemostasis may not occur quickly enough to prevent fatal fluid loss (Soslau, 2020).

(c) Gene expression

Wounding and the subsequent activation of wound healing pathways elicit significant changes in gene expression. Injury has been shown to induce differential expression of up to 9% of the transcriptome in *Cardiocondyla obscurior* ant queens (von Wyschetzki et al., 2016), up to 21% in two-spotted crickets (*Gryllus bimaculatus*), up to 15% in the sea cucumber *Apostichopus japonicus* (Sun et al., 2013), hundreds of genes in the sea anemone *Calliactis polypus* (Stewart et al., 2017), thousands of genes in the earthworm *Eisenia fetida* (Bhambri et al., 2017), hundreds of genes in the hemichordate *Ptychodera flava* (Luttrell et al., 2016), and hundreds to thousands of genes in fish (Sveen et al., 2019; Wang, W. et al., 2020). Transcriptomic responses are complex and highly variable, differing not only between species, wound location, regenerative potential, and time points but also with respect to environmental conditions and individual factors like body size (Husmann et al., 2014) and ontogenetic stage (Husmann et al., 2014; Koleoglu et al., 2017). Diverse genes and pathways are induced by

wounding, typically including ones with functions in signaling, cell-to-cell communication, immunity, structural composition, adhesion, cell motility, tissue growth, metabolism, and molecular synthesis, among others (Belacortu & Paricio, 2011; Erler et al., 2011; Galko & Krasnow, 2004; Gurtner et al., 2008; Lõhelaid et al., 2014; Sveen et al., 2019; von Wyschetzki et al., 2016; Wenger et al., 2014).

One key aspect of the wounding response that is particularly important and generally consistent across animals is the minimal stress proteome, or cellular stress response (CSR). The CSR is a well-conserved, nonspecific expression network that serves to repair cellular, protein, and nucleic acid damage, prevent further damage, regulate the cell cycle, and mobilize and reallocate energy for maintaining biological system integrity (Kültz, 2004, 2020b; Milisav, 2011; Sulmon et al., 2015). Protein damage, resulting ultimately from the lysing of cells and the release of molecules such as reactive oxygen species (ROS) and cytokines (Basu et al., 2002), serves as the primary signal for many CSR components. Following wounding, markers of oxidative stress, such as antioxidants that mitigate ROS damage, are elevated in the orb weaver spider *Larinia jeskovi* (Mouginot et al., 2020) and side-blotched lizards (*Uta stansburiana*) (Hudson et al., 2021), with levels of expression varying depending on wound location or severity, respectively. Heat shock proteins (HSPs) are also induced by ROS and have perhaps received the most attention among CSR components in studies of wounding. HSPs, a diverse family of proteins including both common and taxon-specific members with a range of cytoprotective functions (Richter, Haslbeck, & Buchner, 2010; Sørensen et al., 2005), are upregulated following wounding in diverse animals including cnidarians (Stewart et al., 2017; Wenger et al., 2014), planarians (Sánchez Navarro et al., 2009),

bivalves (e.g., *Laternula elliptica*) (Husmann et al., 2014), echinoderms (Matranga et al., 2000; Pinsino et al., 2007), and fish (Li et al., 2014; Sveen et al., 2019). While many other genes comprise the CSR (Imada & Leonard, 2000; Kassahn et al., 2007; Kültz, 2003, 2020b; Milisav, 2011; Roelofs et al., 2008; Shaughnessy et al., 2015; Sulmon et al., 2015), many have not been examined in relation to wounding directly or have only been studied in a limited number of animal groups. Separate sets of adaptive, stressor-specific responses for restoring homeostasis often complement the CSR (Kültz, 2003), but wounding-specific stress responses distinct from the CSR have not been well characterized. Additionally, much of the information on expression-level injury responses are derived from organism-wide sequencing studies, leaving a great deal to be learned regarding spatially localized expression patterns.

(d) Inflammation and cellular activity

Inflammation plays a major role in early wound healing in many animals, serving to clear out debris, pathogens, and other cells from the wound area. The role of inflammation during wound healing has been well studied in a variety of vertebrate and invertebrate systems, as discussed in several excellent reviews (Bielefeld et al., 2013; Chisholm, 2014; Godwin & Brockes, 2006; Levesque et al., 2010; Martin, P. & Leibovich, 2005; Martin, P. & Nunan, 2015; Razzell et al., 2011; Velnar et al., 2009). ROS can act as a direct signal for inflammatory activation by stimulating immune cells like phagocytes (Martin & Nunan, 2015; Niethammer, 2016) and promoting transcription of inflammatory cytokines (Niethammer, 2016), which further attract inflammatory cells and perpetuate the process of inflammation (Archie, 2013; Martin & Nunan, 2015; Palmer et al., 2011; Rowley, 1996; Velnar et al., 2009). While best characterized in

mammalian systems, inflammatory responses and the cells involved have been described in many animals, including cnidarians (Palmer et al., 2011; Robb et al., 2014), octopuses (Andrews, 2016), arthropods (González-Santoyo & Córdoba-Aguilar, 2012; Liu, H. et al., 2007; Reavey et al., 2014), sea cucumbers (e.g., *Apostichopus japonicus*, *Thyone briareus*) (Lv et al., 2017; Menton & Eisen, 1973), salps (e.g., *Thalia democratica*) (Cima et al., 2018), and snakes (Smith, D. A. & Barker, 1988). However, some groups, including members of the cnidaria (Rodríguez-Villalobos, Work, & Calderon-Aguilera, 2016), axolotls (*Ambystoma* spp.) (Levesque et al., 2010), and both mammalian and *Drosophila* embryos (Galko & Krasnow, 2004), exhibit little to no inflammation during wound repair. These animals or life stages are all known for their scarless wound healing ability, and inflammatory activity during wound repair is known to contribute to fibrosis and scarring in some species (Bielefeld et al., 2013; Levesque et al., 2010). Studies in mice (*Mus musculus*) even suggest that inhibition of inflammation may not be entirely detrimental, or may even provide some benefits, to wound repair in adults, as the activity of various immune cells are either unnecessary under sterile conditions or their absence can be compensated for (Martin & Leibovich, 2005).

Diverse cell types may contribute to wound repair, and these engage in the wound response through a range of cellular activities. In addition to hemostasis and, if present, inflammation, as discussed above, cell functions may include wound sealing, debris removal, and tissue reconstruction (Archie, 2013; Brockes & Kumar, 2008; Velnar et al., 2009). Individual cellular activities vary considerably across species and depend on the nature of the damage being repaired and the extent of tissue reconstruction (e.g., wound healing only, complete regeneration); processes that are commonly involved in repair

include cell proliferation (division), cell death, cell migration, cell shape and adhesion changes, dedifferentiation, transdifferentiation, and redifferentiation (Brockes & Kumar, 2008; Carlson, 2007; Ricci & Srivastava, 2018; Sánchez Alvarado & Tsonis, 2006; Tanaka & Reddien, 2011; Velnar et al., 2009). Although cell proliferation is reported to be minimal during repair in a few species and contexts (e.g.: Abrams et al., 2015; Galko & Krasnow, 2004; Razzell et al., 2011; Tseng & Levin, 2008), injury induces significant proliferation in most species that have been investigated, including a wide diversity of animals (Archie, 2013; Ricci & Srivastava, 2018). Cells may also be removed during the wound response through apoptosis (programmed cell death) or autolysis. Apoptosis removes cells to reshape remaining tissues and possibly recycle resources (Greenhalgh, 1998; Palmer et al., 2011; Velnar et al., 2009) and may be induced by the CSR (Kültz, 2020a). Apoptosis also appears to be an important regulator of injury-induced proliferation in diverse animals, including *Hydra*, planarians, insects, frogs, and lizards (Delorme, Lungu, & Vickaryous, 2012; Ricci & Srivastava, 2018; Tseng & Levin, 2008). Cell migration is common during wound repair, having been well characterized in several vertebrate and invertebrate wounding and regeneration model systems (Bielefeld et al., 2013; Levesque et al., 2010; McCusker et al., 2015; Ricci & Srivastava, 2018) and described or inferred in many other animals including corals (e.g., *Plexaurella fusifera*) (Meszaros & Bigger, 1999), molluscs (Husmann et al., 2014; Polglase et al., 1983), sipunculids (D'Ancona Lunetta, 2005), annelids (Bely, 2014; Tweeten & Anderson, 2008; Zattara, Turlington, & Bely, 2016), arthropods (Vafopoulou, 2009), and echinoderms (Pinsino et al., 2007), among many others. In species that regenerate, the cellular sources of regenerated structures can differ widely (Brockes & Kumar, 2008;

Tanaka & Reddien, 2011); for example, in groups such as planarians and acoels, resident stem cells appear to be the sole source of regenerated structures, while in many other groups, including such disparate animals as vertebrates and annelids, regenerated structures appear to have major contributions from heterogeneous populations of previously differentiated cells, primarily derived from tissues close to the wound site (Bely, 2014; Gehrke & Srivastava, 2016; McCusker et al., 2015).

(e) Wound end states: degrees of regeneration, degrees of scarring

Wound repair concludes within two end state gradients: from complete regeneration to no regeneration, and from extensive scarring to scar-free healing. The end state of a wound depends on factors including animal lineage, location of damage, life stage, individual condition, and other variables. Complete regeneration rebuilds lost tissue with high fidelity to the original and is documented for a broad range of structures across animal phylogeny (Bely & Nyberg, 2010). Partial or imperfect regeneration occurs in some lineages, as in reptiles which regenerate tails that are not structurally identical to the original (Jacyniak et al., 2017). Absence of regeneration has also been documented for body parts in numerous animal groups. Comparative analysis of the presence and absence of regenerative abilities suggests that there have been both losses and gains of regeneration over evolutionary time (Bely & Nyberg, 2010; Bely & Sikes, 2010; Zattara & Bely, 2016; Zattara et al., 2019). Regeneration processes and end states have been studied in a large number of animals and reviewed extensively (e.g.: Alvarado & Tsonis, 2006; Bely et al., 2014; Brockes & Kumar, 2008; Goss, 1969; Imperadore & Fiorito, 2018; Murawala, Tanaka, & Currie, 2012; Özpolat & Bely, 2016; Seifert, Monaghan et al., 2012). Scarring may also occur to varying extents after wound healing and is

commonly (but not exclusively) found in animals with poor or no regeneration ability. Scar tissue permanently seals a wound but does not restore the original tissue structure and is instead fibrous, relatively inflexible, and generally less functional (Levesque et al., 2010; Martin & Nunan, 2015; Murawala et al., 2012). Wounds may also seal without scarring, as commonly occurs in animals that regenerate well. However, scar-free healing can occur even in non-regenerative contexts, as has been documented in groups such as annelids (Bely, 2010; Bely & Sikes, 2010), nemerteans (Zattara et al., 2019), adult *Drosophila* (Razzell et al., 2011), harvestmen (Opiliones) (Townsend et al., 2017), and geckos (Gekkota) (Subramaniam, Petrik, & Vickaryous, 2018). In a number of groups, including invertebrate and vertebrate models, the occurrence and extent of scarring is associated with inadequate remodeling of the extracellular matrix (Archie, 2013; Grdisa, 2010; Levesque et al., 2010; Martin & Nunan, 2015; Miguel-Ruiz & García-Arrarás, 2007; Murawala et al., 2012; Velnar et al., 2009; Yokoyama, 2008) and the presence (or absence) and activity of certain cell types, such as macrophages (Godwin, Pinto, & Rosenthal, 2013; Murawala et al., 2012). Animals with atypical regenerative and scarring abilities by comparison with their close relatives, such as spiny mice (*Acomys*) which exhibit scar-free healing and regeneration of multiple tissues to an extent not found in other mammals (Brant et al., 2016; Seifert, Kiama et al., 2012), offer particularly useful systems for studying regeneration and scarring end-points and their evolution.

Physiological and organismal effects of injury

Injury often causes significant changes in physiology and can impair whole-organism function. Some of the best-characterized physiological effects of injury are

shifts in metabolism and body condition, altered investment in growth, and modified reproductive investment and output. Additionally, injury can directly impair organismal functions by compromising critical body parts, such as those responsible for feeding, locomotion, and gas exchange.

(a) Metabolism

Injury can directly or indirectly alter metabolism. Wound healing and any subsequent repair processes require the mobilization of energy reserves (Archie, 2013; Bely & Nyberg, 2010; Bernardo & Agosta, 2005; Henry & Hart, 2005; Hu et al., 2014; Lawrence, John M., 2010; Maginnis, 2006b; Starostová, Gvoždík, & Kratochvíl, 2017), and, if energetic demands are high, these processes (such as large-scale regeneration) may even require assimilation of additional energy (Bernardo & Agosta, 2005; Henry & Hart, 2005; Lawrence, John M., 2010; Maginnis, 2006b; Starostová et al., 2017). Furthermore, wounding often compromises barriers to infection, and even mild immune challenges can be quite costly calorically and metabolically (Lochmiller & Deerenberg, 2000). Therefore, injury is expected to increase metabolic rate and lead to a concomitant increase in free glucose levels (e.g., derived from stored glycogen or body fat). Studies on a range of animal groups have found direct evidence of these expected changes. Increased resting metabolic rate has been observed in the annelid *Tubifex tubifex* within two weeks following amputation of posterior segments (Collier, 1947), the planarian *Schmidtea mediterranea* within hours following amputation (Lewallen & Burggren, 2022), multiple species of insects within hours following piercing injury (Ardia et al., 2012), and the brittlestar *Amphiura filiformis* within days following arm amputation (Hu et al., 2014). In the former study, researchers also detected decreasing lysozyme and increasing

phenoloxidase activity, indicating an interaction between metabolic and immune responses (Ardia et al., 2012). Injury induces a rapid reduction and subsequent prolonged elevation of nitrogen product excretion—indicative of depressed and accelerated metabolism, respectively—in a number of animals including crabs (e.g., *Carcinides*) (Needham, 1955) and earthworms (e.g., *Eisenia foetida*, *Lumbricus terrestris*) (Needham, 1958). In homeothermic vertebrates such as rats (*Rattus norvegicus*), body temperature drops and then rises accordingly (Stoner, 1970). Active metabolic rate (i.e., the metabolic rate during activity, such as swimming) may also increase following injury, as shown in common carp (*Cyprinus carpio*) following caudal fin amputation (Fu, Cao, & Fu, 2013). Increases in body glucose have also been documented following injury and can occur very rapidly. Body glucose increases are detectable within minutes following limb removal in decapod crustaceans (Manush et al., 2005; Patterson, Dick, & Elwood, 2007) and within hours after surgery (to insert radio transmitters) in bighead carp (*Hypophthalmichthys nobilis*) (Luo et al., 2014); in the latter study, other molecules were also elevated, including cortisol, total blood protein, globulin, and tissue damage and nutritional status markers, indicating physiological stress and increased catabolic demand. Accelerated metabolic rate may not be a universal response to injury, however: in one study, injured *Nerodia rhombifer* watersnakes did not exhibit any significant difference in standard metabolic rate versus controls (Korfel, Chamberlain, & Gifford, 2015). More studies directly assessing metabolic rate over the course of injury recovery in a variety of animal taxa are warranted.

Increased metabolic rate following injury solicits the mobilization and breakdown of energy stores, which are necessary to fuel the healing process. Glycogen is expected to

serve as at least an initial major energy source, and this expectation is well supported in amphibians. In newts (Pleurodelinae) and tadpoles, total glycogen declines rapidly (over 1-2 days) following tail loss and remains depressed for weeks as the tail regenerates (Alibardi, 2014). Energy for recovery may also be derived from stored fat. Mule deer (*Odocoileus hemionus*) that have had radio collar surgery experience reductions in body fat and total body weight, with these reductions persisting for months (Bleich et al., 2007), and lizards that are regenerating their tails exhibit a reduced respiratory quotient indicative of lipid metabolism (Alibardi, 2014). Injury mobilizes both fat and carbohydrate stores in rats via activation of the sympathetic nervous system (Stoner, 1970). Although perhaps best studied in vertebrate systems, the energy sources that fuel wound recovery have also been investigated to varying extents in invertebrates, such as corals (in which lipids, glucose, proteins, and free amino acids have been implicated) (Henry & Hart, 2005), annelids (in which lipids have been implicated) (Yáñez-Rivera & Méndez, 2014), and brittlestars (in which protein and carbohydrates have been implicated) (Dobson et al., 1991). Injury can deplete energy stores not only indirectly, by drawing on these to fuel the wound healing and recovery processes, but also directly, if significant energy stores reside in tissues that are lost. This scenario is perhaps best characterized in some species of lizards that store significant amounts of lipid in their tails, which are prone to being lost to sublethal predation (Bernardo & Agosta, 2005; Starostová et al., 2017). In such species, tail loss may directly lead to a significant loss of energy stores. Furthermore, because lipids are heterogeneously distributed along the tail, the energy dynamics of tail injury and recovery may depend on the extent of tail loss (Chapple & Swain, 2002a; Dial & Fitzpatrick, 1981; Starostová et al., 2017). Asteroid sea

star arms are also often used as storage organs, and loss of these would similarly lead to direct loss of key nutrient reserves that may affect biological processes (Lawrence, John M & Vasquez, 1996). In contrast, their cousins the brittlestars, which have no dedicated storage organs and possess thin, delicate arms, regenerate at a rate largely independent of nutritional status. Instead, these animals suffer loss of organic matter based on the availability of food and amount of the central disc, which houses the digestive organs, that remains intact or has been regenerated (Dobson et al., 1991; Fielman et al., 1991).

Some animals may compensate for the increased demands of wound recovery by increasing resource assimilation through a handful of mechanisms. One mechanism is physiological plasticity, as in *Podarcis erhardii* lizards, which alter their digestive performance by reducing gut passage time and increasing uptake of protein after tail loss (Sagonas et al., 2017). Another mechanism is increasing the frequency and amount of feeding following injury, but direct measurement of the capacity for animals to do so remains sparse. Tailless *Coleonyx* lizards increase their caloric intake relative to controls, but locomotory inefficiency due to the lack of the tail may lead to additional energetic demands, potentially diminishing the compensatory ability of this response (Dial & Fitzpatrick, 1981). Similarly, multiple polychaete species are unable to compensate for palp loss regarding food intake (Lindsay & Woodin, 1992). More broadly, foraging behavior often changes in response to injury, as discussed further below.

(b) Growth and reproduction

The loss and consumption of energetic resources associated with injury and repair often impact somatic growth and reproduction. Wound healing, and regeneration when it occurs, solicit energy and molecular building blocks (e.g., proteins, carbohydrates) to

repair and rebuild damaged tissue. As these resources are limited, they must be strategically allocated between processes, leading to frequent trade-offs (Archie, 2013; Hudson et al., 2021; Lochmiller & Deerenberg, 2000; Maginnis, 2006b), such as between injury recovery (e.g., regeneration), growth, and reproduction (Heino & Kaitala, 1999). The demands of injury recovery may not only reduce investment in other processes but potentially alter relative apportioning between them (Aira et al., 2007; von Wyschetzki et al., 2016).

Injury often decreases somatic growth, at least in the short term. This relationship may manifest as a reduction in overall body growth rate, as has been shown in clams following siphon loss (Coen & Heck, 1991; Kamermans & Huitema, 1994; Tomiyama, 2016), in polychaetes following posterior segment loss (Campbell & Lindsay, 2014), in lizards following tail loss (Ballinger & Tinkle, 1979), and in watersnakes following cutaneous wounding (Korfel et al., 2015). Injury can also decrease growth by disrupting development, as shown in tadpoles that develop more slowly following tail injury (Blair & Wassersug, 2000) and in some decapod crustaceans that experience either prolonged or accelerated intermolt periods and limited post-molt size increases following appendage loss (Juanes & Smith, 1995). Negative effects of injury on somatic growth can also occur through reapportioning of investment to non-injured body parts, as in stick insects, where leg loss causes reduced wing growth (Maginnis, 2006a). In annelids, amputation injury elicits rapid shutdown of cell proliferation in wound-adjacent segments in the polychaete *Capitella teleta* (de Jong, D. M. & Seaver, 2016) and across the body in the clitellate *Pristina leidyi* (Zattara & Bely, 2013), largely halting somatic growth in the latter for several days as the animal begins regenerating. The direct loss of energy reserves, such as

the fat stored in lizard tails, may also exacerbate resource restrictions and thus growth rates. Indirect correlations between increasing number of injuries and reduced body size have also been observed, without clear demonstrated effects on growth rate, in animals such as crab spiders (Thomisidae) (Lutzy & Morse, 2008; Morse, 2016), starfish (Marrs et al., 2000), and larval *Ambystoma* salamanders (Mott & Steffen, 2014). However, growth is not always inhibited following injury. Some studies on lizards have found no effect of injury on growth rate or body mass (Althoff & Thompson, 1994; Hudson et al., 2021; Starostová et al., 2017); in bivalves the effect of siphon injury on growth rate can depend upon species, habitat, or degree of damage (Peterson & Quammen, 1982; Sasaki et al., 2002; Trevallion, 1971); and in sponges and corals, injury may increase, decrease, or not affect growth (Henry & Hart, 2005), to list just a few examples. This variability suggests that simple energetic trade-offs are not sufficiently explanatory, and other mechanisms may be the cause of unexpected relationships between injury and growth.

Growth may also be reduced due to damage to structures used in feeding or foraging, thereby decreasing resource intake. In *Ananteris* scorpions, loss of the tail, which is used to subdue prey, results in reduced ability to capture larger prey items (Mattoni et al., 2015) and may subsequently lead to growth reductions. In decapods, when limb loss reduces foraging efficiency, reductions in growth increment can be magnified (Fleming et al., 2007; Juanes & Smith, 1995). Following siphon injury in clams, loss of foraging efficiency combined with increased energetic demands of regeneration are hypothesized to lead to reduced growth rates (Coen & Heck, 1991; Kamermans & Huitema, 1994; Peterson & Skilleter, 1994), and both factors likely contribute to reduced growth in spionid polychaetes following palp amputation

(Matthews & Hentschel, 2012). Weakened body condition resulting from crushing injury in the soft coral *Gersemia rubiformis* was hypothesized to similarly impair feeding, leading to energetic limitations and subsequent reduced growth rates (Henry et al., 2003). In some cases, as in side-blotched lizards suffering cutaneous wounds, wounding that has no direct impact on feeding structures can still lead to reduced food consumption (Hudson et al., 2021), possibly also due to a general reduction in physiological condition.

Effects of injury on sexual reproduction are variable across animals, likely reflecting the diversity of life history strategies. Sexual reproduction is commonly suppressed following injury in a range of taxa. Wounding reduces reproductive rate in polychaetes (Zajac, 1985, 1995), six-rayed sea stars (*Leptasterias hexactis*) (Bingham, Burr, & Head, 2000), ants (von Wyschetzki et al., 2016), and burying beetles (*Nicrophorus vespilloides*), although the effect in the latter was dependent on the timing of injury with respect to breeding (Reavey et al., 2014). In sponges and corals, sexual reproduction is commonly reduced, in favor of regeneration, in the form of lower fecundity, fertility, and offspring viability (Henry & Hart, 2005). Injury-induced decreases in reproductive rate and total fecundity can result from a variety of underlying effects, including reductions to the rate or success of mating as in *Drosophila melanogaster* (Sepulveda et al., 2008), extended brooding time as in a *Polydora* polychaete (Zajac, 1985), slowed maturation as in a *Capitella* polychaete (Hill, Grassle, & Mills, 1982), or reduced gonad mass as in the purple sea urchin (*Strongylocentrotus purpuratus*) (Haag, Russell, & Hernandez, 2016). Severity of injury may be linked to the degree of reproductive impact, as in a study of female lynx spiders (*Peucetia viridans*), where the loss of two legs reduced the number of eggs produced, but the loss of one leg

had no significant impact (Ramirez, Takemoto, & Oliveri, 2017). Offspring quality may also be affected by injury, possibly through reductions in parental investment resulting from trade-offs with other processes. For example, in *Desmognathus* salamanders, a continuous negative relationship was found between maternal injury severity and egg size (Bernardo & Agosta, 2005). Injury effects on reproduction may also be revealed or exacerbated by simultaneous limiting factors, such as food availability. For example, in a study of female *Urosaurus* lizards, minor cutaneous wounding reduced the mass of vitellogenic follicles when individuals were on a restricted diet but had no effect when they had unlimited access to food (French, Johnston, & Moore, 2007). Lizards and salamander species that store proportionally more caudal versus abdominal fat typically show greater reductions in clutch size following tail loss, suggesting an energetic restriction due to the proportionally greater amount of lipid stores lost along with the tail (Bernardo & Agosta, 2005). However, as in the case of growth, evidence for resource limitations of reproduction following injury is not always observed. In the same study by French et al., there was no significant difference in follicle mass between lizards with unlimited access to food and lizards that were not fed at all; the authors hypothesize this may be due to starvation inducing a trade-off with the immune system, redirecting resources from (and thus suppressing) immunity in order to survive food scarcity (2007). Zajac (1985) noted that the polychaete *Polydora cornuta* (formerly *ligni*) continued to reproduce while regenerating lost segments, indicating that a total diversion of resources from reproduction to recovery does not occur. In other cases, injury can actually enhance reproduction, as in pea aphids (*Acyrthosiphon pisum*) by accelerating reproductive rate (Altincicek, Gross, & Vilcinskas, 2008) and anoles (*Anolis*) by increasing egg and

hatchling size (Beatty, Mote, & Schwartz, 2021). The particular strategy employed by injured animals is likely to be strongly shaped by life history. For example, in salamanders and lizards, relative investment into injury repair (tail regeneration) and reproduction is predicted to depend on lifespan, with reproductive output relatively favored over regeneration in shorter-lived species and the opposite in longer-lived species. However, these predictions still need to be tested explicitly (Bernardo & Agosta, 2005).

Some, but comparatively less, is known about the impact of injury on asexual agametic reproduction (e.g., fission, budding). Although these effects are expected to be comparable in many ways to those on sexual reproduction, offspring produced by asexual agametic reproduction are genetic clones of the parent, typically develop more quickly, and are substantially larger than sexually produced offspring, leading to potentially distinct effects. In forms of asexual reproduction like fission, where much or all of the offspring tissue is directly derived from the parental soma, significant tissue loss from injury would be expected to negatively affect asexual reproduction because that tissue and the resources it contains are no longer available to be allocated to viable offspring, but studies that explicitly address this expectation are needed. However, injury can also have the opposite effect on asexual reproduction. For example, injury may actually facilitate asexual reproduction if the injury severs the original individual into two or more fragments that are each capable of fully regenerating. In such a scenario, injury actually causes the asexual propagation. Animals for which this kind of injury-induced asexual propagation has been suggested include members of the sponges (Padua et al., 2016; Wulff, 1991), nemerteans (Coe, 1929), annelids (Martinez-Acosta & Zoran, 2015),

bryozoans (O'Dea, 2006), planarians (Bely et al., 2014; Carter et al., 2015), and echinoderms (Mladenov, 1996). This phenomenon has even been exploited for easy culture of ornamental sabellid worms for commercial purposes (Murray et al., 2013). In two asexually-reproducing clitellate worms (*Paranais*, *Pristina*), injury (decapitation) of fissioning individuals often leads to accelerated fission (Bely, 1999; Zattara & Bely, 2013). Interestingly, the two clitellate species investigated represent independent origins of asexual reproduction, indicating that this injury effect is repeated across evolutionary lineages, possibly reflecting that it is adaptive to accelerate the release of a clonal offspring when the parent worm has been damaged. In *P. leidyi*, fission acceleration following injury is common but the opposite response—fission deceleration and even resorption —can also occur; the specific injury response is dependent on both the stage of fission as well as the site of injury (Zattara & Bely, 2013). These findings suggest that optimal resource allocation between the asexual parent and offspring can depend on the nature of the injury. In organisms capable of switching between sexual and asexual modes, injury could promote one reproductive mode over the other. In octocorals (Octocorallia), injury has been shown to favor asexual over sexual propagation, a shift which is hypothesized to be partly a consequence of resource reallocation towards repair and regeneration (Henry et al., 2003). Prevalence of sexual versus asexual reproduction may reflect cost advantages in certain environments, including those subject to disturbances (Meirmans, Meirmans, & Kirkendall, 2012), which may include injury. Given the similarities and probable shared evolutionary history between asexual agametic reproduction and regeneration in many animal groups (Kostyuchenko & Kozin, 2020; Martinez, V. G., Menger, & Zoran, 2005; Zattara & Bely, 2011), the effects of injury on

asexual reproduction warrant special attention for their potential mechanistic and evolutionary insights.

(c) Organismal function

Beyond physiological effects, injury can directly impact organismal function through the removal or damage of structures involved in key body functions. For example, injury to structures involved in feeding, locomotion, or gas exchange are likely to impact these associated functions. Injury to feeding structures impair energy assimilation and can impact growth, as described in the previous section. Effects of injury to locomotion and gas exchange structures are two additional organismal-level effects that have been relatively well characterized.

Damage to or loss of locomotory structures, such as tails or limbs, can have potentially large consequences for animals. Such injuries can impair not only the ability of an animal to move about its environment but also important processes that depend on locomotion, such as feeding and reproduction. Locomotory disruption often results from directly altered biomechanics and gait following injury, as has been well described in crabs (Pfeiffenberger & Hsieh, 2021), lizards (Jagnandan et al., 2014), and dogs (*Canis familiaris*) (Fuchs et al., 2015). These mechanical changes are the likely culprit of detrimental impacts to a range of motor functions, as has been documented in numerous groups. Motor endpoints that suffer from appendage injury include reduced movement speed and/or acceleration, as in damselflies (Zygoptera) (Robinson, J. V., Hayworth, & Harvey, 1991), arachnids (Amaya, Klawinski, & Formanowicz, 2001; Domínguez et al., 2016; Houghton, Townsend, & Proud, 2011; Townsend et al., 2017), crabs (Pfeiffenberger & Hsieh, 2021), fish (Fu et al., 2013; Krause et al., 2017; Sinclair, E. L.

E., Ward, & Seebacher, 2011), tadpoles (Figiel & Semlitsch, 1991), and lizards (Chapple & Swain, 2002b; Martín & Avery, 1998); reduced sprint distance or stamina, as in wolf spiders (Lycosidae) (Brown, C. A. & Formanowicz, 2012), tadpoles (Figiel & Semlitsch, 1991), and lizards (Chapple & Swain, 2002b; Martín & Avery, 1998); and destabilized or eliminated ability to perform certain types of movements, as in crabs (Pfeiffenberger & Hsieh, 2021) and lizards (Fleming & Bateman, 2012; Gillis, Kuo, & Irschick, 2013; Savvides et al., 2017). Although injury to locomotory structures often affects animal movement, in some cases locomotory function is not disrupted, as has been shown for limb damage in a range of animals including wolf spiders (Brueseke et al., 2001), brittlestars (Price et al., 2014), and plethodontid salamanders (Hessel, Ryerson, & Whitenack, 2017), or varies in a manner dependent on factors like sex (Chapple & Swain, 2002b). The magnitude of functional impact can depend on features such as the physiological costs of damage, the importance of the structure to locomotion (Chapple & Swain, 2002b), limb redundancy (Brautigam & Persons, 2003; Pfeiffenberger & Hsieh, 2021), acclimatory ability (Fuchs et al., 2015), or allometry (e.g., different impacts of comparable injury in similar species of different sizes), as noted in wolf spiders (Brueseke et al., 2001).

Injury to gas exchange organs may have considerable consequences for respiration and subsequent downstream effects on animal physiology and behavior. External structures, such as gills, that extend from the body to increase surface area exposure to the environment are particularly prone to damage, but many animals can regenerate these structures (Cadiz & Jonz, 2020), including annelids (Bely & Sikes, 2010; Brown, S. D. & Emlet, 2020; Drewes & Zoran, 1989; Wells, 1952), damselflies

(Robinson, J. V., Shaffer et al., 1991), amphibians (Eycleshvmer, 1906; Goss, 1969; Saito et al., 2019), and fish (Mierzwa et al., 2020). Siphons, which are used for pumping external water to the gills in bivalves and thus are important in respiratory function, can also often be regenerated (de Vlas, 1985; Meyer, J. J. & Byers, 2005; Tomiyama, 2016). Although loss of these organs in the wild has been documented (Brown, S. D. & Emlet, 2020; de Vlas, 1985; Drewes & Zoran, 1989; Robinson, Shaffer et al., 1991; Wells, 1952), few studies have investigated the functional consequences, and these often establish only loose or indirect relationships between structure damage and effects. For example, clams with cropped siphons reduce their burrowing depth (Meyer, J. J. & Byers, 2005; Zwarts, 1986). However, it is not clear whether the ability to efficiently inhale oxygenated water through the siphon is directly impeded by siphon injury or if clams reduce burrowing depth solely to compensate for reduced siphon length and maintain exposure to the overlaying water. The contribution of many respiratory structures to total gas exchange is not well known under even routine conditions in many animals, and so the consequences of damage to these structures are also not well understood. Some existing data do indicate compromised respiration following damage to gas exchange organs, particularly in the annelids. External respiratory structures in these animals are diverse, and many can be lost and regenerated (Bely, 2006), including paired lateral gill filaments along the body, as in *Branchiura sowerbyi* (Spencer, 1932) and the lugworm (*Arenicola*), in which gills also serve as accessory hearts (Jouin & Toulmond, 1989); ciliated terminal (tail) gills, as in *Dero* (Drewes & Fourtner, 1993); and anterior "crowns" of tentacles with both respiratory and feeding function, as in sabellids (Dales, 1961; Wells, 1952). Amputation of posterior segments in *Branchiura* induces compensatory

elongation of remaining filaments and formation of new filaments (Drewes & Zoran, 1989), and crown amputation leads to an 80% reduction in total respiration in a sabellid (Giangrande, 1991). Crown damage is hypothesized to differentially impact sabellid respiration based on allometry and compensatory capacity (e.g., through cutaneous or enteric gas exchange) (Wells, 1952). Other than annelids, indirect evidence of physiological impacts of respiratory appendage damage comes from larval damselflies, which reduce their habitat breadth to highly oxygenated waters following loss of lamellae (Robinson, J. V., Shaffer et al., 1991).

Behavioral effects of injury

Injury can alter a range of animal behaviors. Changes in behavior are often dependent upon the body part injured and the degree to which function is compromised due to damage. Among the best studied behavioral consequences of injury are impacts on foraging behavior, social behavior, and sensitization.

(a) Foraging behavior

The impacts of injury on foraging behavior are among the most well-documented, and such impacts can have important secondary effects on the injured animal. Injury that affects mouthparts or limbs used for foraging often directly reduces feeding efficiency and overall food intake, as discussed previously. However, injury can also have significant effects on foraging behavior, whether or not the injury is to structures directly involved in feeding. Both decreases in feeding efficiency and changes to foraging behavior can have the downstream consequence of reduced energy assimilation, leading to reduced growth or reproductive output of injured animals, as discussed previously.

Injury can lead to a variety of changes in foraging behavior, such as in foraging strategy, habitat utilization, and feeding mode.

Injury-induced shifts in foraging strategy have been documented in a number of animal groups. In several species of decapod crustaceans, damage to or loss of claws can lead to animals becoming more herbivorous (reducing predatory foraging) or taking fewer risks in predation, such as choosing softer invertebrate prey (Juanes & Smith, 1995). Wolf spiders missing legs are poorer at capturing larger prey (Brueseke et al., 2001) and at foraging in complex environments (Wrinn & Uetz, 2008). Yet in some cases, injury can actually increase foraging. In several lizard species, for example, injury leads to an elevated rate of foraging, apparently to compensate for the energetic costs of regeneration (Sousa et al., 2016; Webb, 2006), and salamanders make more exploratory movements, presumably in part to assess foraging opportunities, following tail injury (Bliss & Cecala, 2017).

Even if behavioral changes can compensate for functional impairment in foraging, such behavioral shifts may still come at a cost, such as increased predation risk. For example, spionid annelids with lost palps occasionally expose themselves by emerging from the sediment at higher frequency to compensate for reduced feeding efficacy, which leaves them at higher risk of predation (Lindsay & Woodin, 1992). Similarly, bivalves with damaged siphons, which are used to pull food particles from the surrounding water, burrow less deeply in the sediment, increasing their exposure risk to predators (de Goeij et al., 2001; Meyer, J. J. & Byers, 2005). Alternatively, injured animals may adopt foraging strategies that reduce the risk of further injury, such as by spending more time foraging in safer habitats or reducing activity levels. For example, lizards without tails

alter their habitat occupation to keep out of sight of predators (Martin & Salvador, 1992, 1993) and injured salamanders that increase occupation of benthic microhabitats do not suffer any loss of foraging efficiency (Mott & Steffen, 2014). Shifts in microhabitat selection following injury may also be driven by physiological needs, such as temperature (Bliss & Cecala, 2017).

Injury may also elicit a change in feeding mode itself. In spionid polychaetes, damage to feeding palps induces a switch from suspension feeding to mouth feeding. The magnitude and efficacy of this behavioral shift in feeding was found to be influenced by how many palps were damaged, corresponding to the degree of impaired function (Lindsay & Woodin, 1995). Bivalves also exhibit a switch in feeding mode from risky but rewarding deposit feeding to safer but less profitable suspension feeding following siphon damage (Peterson & Skilleter, 1994); however, the prevalence of such a shift may be dependent upon both bivalve and potential predator density (Skilleter & Peterson, 1994).

(b) Social behavior

In social animals, injury has been shown to alter a range of social behaviors, particularly those relating to mating and parental care. Courtship and mating behaviors can be altered by damage to or loss of body structures that are used in such behaviors. For example, male wolf spiders alter the frequency and intensity of a variety of courtship and mating behaviors after leg loss (Brautigam & Persons, 2003; Taylor, Roberts, & Uetz, 2006) and experience reduced mating success (Brautigam & Persons, 2003). In octopuses, arm loss is posited to alter mating strategy by inhibiting the locomotory ability of males, leading to indirect female mate choice of uninjured males and shifts to

"sneaking" behavior in injured males (Wada, 2017). In species with parental care, offspring may suffer not only from reduced direct investment of resources when their parents are injured, as discussed previously, but also from behavioral adjustments in their injured parents. For example, in Hawaiian monk seals (*Monachus schauindslandi*), mothers that are injured express reduced offspring care behaviors and decreased lactation, which likely contributes to greater pup mortality (Becker et al., 2008).

(c) Sensitization

Behavior may also change following injury simply as a way to avoid further noxious stimulation. Nociceptive plasticity is a sensitization response that has been demonstrated following injury in vertebrates, including humans (Woolf & Walters, 1991), as well as several invertebrates, including tobacco hornworm (*Manduca sexta*) (Walters et al., 2001), *Drosophila* larvae (Babcock, Landry, & Galko, 2009), medicinal leech (*Hirudo medicinalis*) (Sahley, 1995), and molluscs such as *Aplysia* sea slugs (Walters, 1987) and longfin squid (*Loligo pealeii*) (Crook et al., 2011). Nociceptive strategies that may be elicited include being more prone to flee from stimulus, to engage in defensive stances or actions (e.g., hissing; displaying warning coloration, teeth, claws, spines; increased aggressive behaviors), and to hiding. In longfin squid, individuals are not only more likely to escape when presented with visual stimuli in the hours following injury, but they also employ crypsis more readily very shortly after an injury is inflicted (Crook et al., 2011). The molecular and physiological mechanisms by which nociception affects animal behavior have been studied in a range of animals, especially cephalopods, established invertebrate models such as *Drosophila*, and vertebrate models such as zebrafish and mice (Alupay, Hadjisolomou, & Crook, 2014; Malafoglia et al., 2013;

Oshima et al., 2016; Tobin & Bargmann, 2004; Tracey, 2017; Walters & Williams, 2019).

Ecological and evolutionary effects of injury

Injury can have consequences above the organismal level as well, impacting ecological and evolutionary processes. At ecological scales, injury can affect predator-prey dynamics, competitive interactions, and, when injury is particularly prevalent, population and trophic dynamics. Injury can also affect evolutionary processes, by impacting the fitness of injured animals as well as the fitness of conspecifics and heterospecifics (such as predators) with which injured individuals interact. When injury has large individual effects or is especially common, it can be an important driver of ecological processes and influence evolutionary trajectories.

(a) Predator-prey dynamics

Injury often increases the susceptibility of prey animals to subsequent predation. For example, tail loss in tadpoles increases predation by crayfish (Figiel & Semlitsch, 1991), *Ambystoma* salamanders are more likely to be the targets of intraspecific aggression after injury (Mott & Steffen, 2014), loss of lamellae in larval damselflies increases the likelihood of being cannibalized (Robinson, J. V., Shaffer et al., 1991), and male wolf spiders with many lost legs are more frequently cannibalized by females (Brautigam & Persons, 2003). A variety of factors can be responsible for increased predation susceptibility of injured individuals. One of the most obvious is that injury can impair anti-predator defenses or escape ability. For example, injury-induced impairment of locomotory ability has been shown to directly increase susceptibility to predation in a number of animal groups (Figiel & Semlitsch, 1991; Martín & Avery, 1998; Zamora-

Camacho & Aragón, 2019); leg loss in *Acheta domesticus* crickets even elevates the risk of capture by mucilaginous plants (Cross & Bateman, 2018). Predator defenses, especially physical defenses, may also be impaired through physiological trade-offs with other processes. For example, soft corals produce shorter defensive sclerites after suffering damage to other tissues, possibly as an energetic cost of regenerating those tissues, and this can lead to increased predation (Bythell, Gladfelter, & Bythell, 1993; West, 1997); and in the land snail *Satsuma caliginosa,* shell growth is delayed after foot autotomy (Hoso, 2012). In addition, injured individuals may be more easily detected or actively preferred by predators. Several studies have shown that crabs with missing chelipeds or claws are preferred by predators relative to uninjured crabs (Juanes & Smith, 1995 and studies cited within). However, although the idea that predators pick out vulnerable—including wounded—prey is commonplace in ecological literature and popular accounts of animal behavior, this hypothesis has been formally tested only rarely (Krumm et al., 2010).

As discussed previously, injury can cause important changes to an individual's behavior, and these changes can have complex effects on subsequent predation risk. The altered behavior of injured animals may make individuals either more likely or less likely to be preyed upon subsequently. In marine clams, siphon cropping, which can be very common, forces clams to bury themselves at shallower depths in order to avoid suffocation (de Vlas, 1985; Zwarts, 1986), which in turn increases their risk of predation, primarily by decapods (Meyer, J. J. & Byers, 2005; Zwarts & Wanink, 1989). Similarly, a study of harvestmen found that individuals that had lost legs (possibly by autotomy as a predator-evasive tactic) climbed slower and occupied lower perches, effects that are

expected to raise future predation risk (Houghton et al., 2011). And in sardines (*Sardinella aurita*), injuries sustained by predators reduce swimming performance and drive spatial sorting in schools, such that injured individuals are more exposed to possible further predation (Krause et al., 2017). However, injury can also lead to behavioral changes that decrease predation risk, likely as adaptive behavioral responses to mitigate the heightened vulnerability of animals when in an injured state. These changes often take the form of decreased activity levels, heightened predator sensitization, and increased time in habitats with lower predation risk. For example, lizards that have lost their tails reduce the length of their daily active periods (Martín & Salvador, 1995), flee more readily from predator cues despite having impaired movement (Downes & Shine, 2001), and spend more time in habitat types that offer more opportunities to hide, perhaps as compensation for reduced locomotory performance (Martín & Salvador, 1992, 1993). Injured individuals spending more time in certain habitats may then indirectly impose increased predation pressure upon other prey individuals in those or other habitats, potentially affecting community ecological interactions. Behavioral sensitization may also heighten predator avoidance following injury, as with injured longfin squid, which become hyper-responsive to visual stimuli, although this may lead to the respective animals making themselves more conspicuous (Crook et al., 2011). Injury may also affect the specific anti-predation strategies used by animals. For example, crayfish with missing limbs switch from burrowing to tail-flipping behavior to avoid predators, which has the added effect of increasing water turbidity (Dunoyer, Coomes, & Crowley, 2020).

Particularly in aquatic habitats, chemical cues emitted by injured individuals can be important in mediating injury effects on predator-prey dynamics. For example, injury

cues can serve as a warning signal to other individuals, eliciting antipredator or avoidance behaviors and even inducible physical defenses in prey species. Chemically-mediated injury signaling to conspecifics has been documented in a variety of animals including clams, gastropods, annelids, flatworms, crustaceans, aquatic insects, and fish (Alemadi & Wisenden, 2002; Gall & Brodie, 2009; Kaliszewicz, 2015; McCarthy & Dickey, 2002; Smee & Weissburg, 2006; Wasserman et al., 2014; Wisenden, Chivers, & Smith, 1997; Wisenden, Pohlman, & Watkin, 2001; Wisenden & Millard, 2001). Some animals can even learn to respond to injury signals released by heterospecifics subject to a similar class of predators. For example, tadpoles can learn to respond to chemical cues released by injured *Hyalella patagonica* amphipods, apparently because these cues indicate a potential predation risk to the tadpoles themselves (Pueta & Perotti, 2016). Inducible anti-predator defenses are known to exist in a variety of invertebrate taxa, and the cues for these inducible defenses include injury cues. For example, blue mussels (*Mytilus edulis*) exposed to chemical cues from wounded conspecifics develop thicker, stronger shells (Leonard, Bertness, & Yund, 1999). A wholly different and unusual strategy for responding to conspecific injury cues occurs in some meiofaunal annelids (e.g., *Stylaria lacustris, Nais christinae*), which lack obvious antipredator physical defenses and instead accelerate rates of asexual fission while also increasing the size of both parent and offspring worms when exposed to such cues (Kaliszewicz, 2015). Injury signals from a wounded animal can also attract opportunistic conspecific or heterospecific predators, further compounding the detrimental effects of injury. For example, starved blue crabs (*Callinectes sapidus*) are more likely than non-starved crabs to track olfactory injury cues emitted by conspecifics, presumably to prey upon them (Moir & Weissburg, 2009), and

crayfish respond to injured snail prey cues by increasing their activity, although this does not seem to improve their success of prey capture (McCarthy & Dickey, 2002).

(b) Competitive interactions

Relatively few studies have directly investigated how injury modulates inter- or intraspecific competitive interactions, but based on the known effect of injury on organismal physiology, function, and behavior, it is likely that injury effects on competition are common and substantial. As discussed earlier, functional impairment from wounding can impact foraging strategies, prey preference, habitat occupation, mating success, growth, and other factors, and such impacts are thus likely to bring injured animals into more frequent or contextually altered contact with one another in competition for food, physical space, mates, or other resources. This remains an open area of investigation, but a handful of studies have examined the relationship between injury and competitive ability or its likely correlates. For example, in *Ambystoma* salamanders, Mott and Steffen (2014) found that sublethal injuries were correlated with reduced body size, passive behaviors, and more cryptic habitat usage, all of which are likely to reduce intraspecific competitive success. Injury thus may be a significant factor in niche partitioning and hierarchical structure (i.e., the distribution of individuals within and across habitats) within populations. A number of studies in corals suggest that wounding may reduce inter- or intraspecific competitive ability as a result of increased fouling of lesions, increased risk of sublethal predation, and impaired growth, which reduces occupation of habitat space (see numerous studies cited in Henry & Hart, 2005). To give one specific example, damaged corals are significantly more susceptible to being overgrown, and ultimately killed, by certain sponge species as opposed to maintaining

"standoff" interactions (Aerts, 2000). Studies that experimentally assess competitive impacts of wounding are challenging to do in the wild, but one such study in edible crabs (*Cancer pagurus*) has found that induced claw injury reduces competitive ability among conspecifics (McCambridge, Dick, & Elwood, 2016). Another found that the presence of siphon-nipping fishes forced greater intraspecific competition in clams by reducing the number of viable feeding modes to those with less siphon exposure (Skilleter & Peterson, 1994). A noteworthy pool of studies has concerned the direct and indirect effects of sublethal predation upon infaunal polychaetes in soft-sediment ecosystems, which are of particular importance due to their role as sedimentary engineers. As these animals are capable of rapidly and substantially altering physical properties of their environment, their activity has a considerable effect on the frequency and nature of sediment-mediated interactions, and injury especially of parts subject to cropping by browsing predators (e.g.: fish, crustaceans) can modify activity patterns and thus competitive interactions within the sediment (Wilson, 1991). Experimental (Woodin, 1984), observational (Lindsay & Woodin, 1996), and modeling work (Lindsay, Wethey, & Woodin, 1996) suggest that sublethal predation injury, via its impacts on sediment-engineering behaviors, indeed has the capability of shaping community dynamics within these vast and abundant ecosystems. More research would be of great value to parsing the ecological dynamics of wounding, especially concerning non-predatory interactions within and between species, in other types of ecosystems, especially terrestrial ones.

(c) Population dynamics

Injury is very common in wild populations, as detailed previously, and when sufficiently prevalent can affect population dynamics. In most cases, injury is expected to

decrease population growth rates, as negative impacts on reproduction are already well evidenced in many species (Bernardo & Agosta, 2005; Henry & Hart, 2005; Ramirez et al., 2017; Reavey et al., 2014; Sepulveda et al., 2008; von Wyschetzki et al., 2016; Zajac, 1985, 1995), as discussed above. For example, models of population dynamics in mudflats suggest that sublethal cropping of polychaetes can reduce their population growth rates (though less than if the predation was lethal) (Zajac, 1995), and in some clams, siphon cropping, which is a common occurrence, is often so effective at facilitating subsequent lethal predation that cropped clams are considered "as good as dead" (Meyer, J. J. & Byers, 2005). Mortality risk likewise increases in amphibians with sublethal predation: tail injury by predators in salamander larvae reduces survival prior to metamorphosis (Segev, Mangel, & Blaustein, 2009), and predation-driven missing-limb abnormalities increase mortality rates in adult *Rana cascadae* frogs, which are expected to have significant ecological consequences (Bowerman, Johnsonfi, & Bowerman, 2010). In environments where injury is common, repeated injury in the same individual is likely also common, with potentially important implications for population dynamics. Modeling of these effects is especially needed, as suggested by Lindsay (2010). Although injury is generally expected to decrease population growth, its role in governing population size is likely determined by many complex factors. For instance, injury can increase population growth rate if it causes individuals to be fragmented into multiple viable pieces of which two or more can subsequently regenerate to complete individuals, as discussed above. This situation is most likely in highly regenerative animals, such as sponges and annelids, and colonial animals, such as cnidarians and bryozoans, and has been documented in a number of aquatic animals in response to abiotic forces, such as wave action and storms.

Modeling by Wethey et al. (2001) shows that intermediate levels of sublethal browsing predation on adults of the clam *Macoma balthica* may actually be necessary for maximizing equilibrium population density by promoting a balance between adult occupancy and larval recruitment.

(d) Trophic transfer

Sublethal predation, in which a predator consumes part of a prey individual's body, is a form of injury that can be very common in some habitats and be of considerable consequence for trophic energy transfer and community dynamics. The cropping of body parts by predators is particularly common among benthic invertebrates in soft-bottomed aquatic environments such as mudflats and sandflats. It is well demonstrated that bivalve siphons and annelid tails, heads, and palps can be routinely cropped by predators like fish and decapods (de Vlas, 1985; Lindsay, 2010; Meyer, J. J. & Byers, 2005; Peterson & Quammen, 1982; Skilleter & Peterson, 1994; Tomiyama, Omori, & Minami, 2007). The injured animals often regenerate tissues lost to cropping and may do so many times over their lifespans. For example, Sasaki et al. (2002) estimated that the bivalve *Nuttalia olivacea* may regenerate their siphons an average of twenty-six times in a single season, and another study found that clams are estimated to suffer cropping damage to their siphon tips several times a day during summer (de Vlas, 1985). Tissue cropping and subsequent regeneration can be so prevalent that cropped tissues can serve as major sources of secondary production (de Vlas, 1979b, 1985; Henry & Hart, 2005; Lindsay, 2010; Sasaki et al., 2002; Tomiyama et al., 2007; Zajac, 1995). For example, juvenile stone flounder (*Platichthys bicoloratus*) are able to meet the majority of their nutritional needs by cropping the siphons of clams (Sasaki et al., 2002),

one study found that up to 70% of the diet of plaice (*Pleuronectes platessa*) in tidal flats consisted solely of siphon tips (de Vlas, 1979a), and another study found that the trophic transfer of cropped brittlestar arms alone in one community accounted for secondary production on a scale comparable to that of other communities in their entirety (Pape-Lindstrom et al., 1997).

(e) Evolutionary consequences

As reviewed in previous sections, injury can have substantial effects on many components of fitness, including an individual's growth, mating success, reproductive output, and survival, and can thus ultimately have evolutionary consequences. If injury frequency and magnitude of effect are sufficiently large as to represent a significant selection pressure, and if there is heritable variation in injury responses among individuals, organismal responses to injury will evolve over time. The costs of injury (or autotomy), for example, may drive adaptive switches towards better morphological defenses that reduce the chance of injury (Hoso, 2012) or towards more effective recovery pathways, such as compensation or regeneration (Bely & Nyberg, 2010). Although variation in injury responses between species is extensive and well-described, variation in injury responses within species has not been well documented, and this area represents a significant knowledge gap to fill for understanding the evolutionary consequences of injury. There is also a need to better understand the frequency of injury in the wild, which is challenging for many reasons, including cryptic or absent indicators of past injury, particularly in regenerating species (Lindsay, 2010). However, acknowledging these gaps in understanding, many injury responses in animals can be reasonably interpreted as adaptations either to avoid injury or to reduce the negative

fitness consequences of injury. Thus, wound healing, injury-induced immune responses, organismal-level compensatory responses to injury, injury-induced behaviors, physical defenses, predator avoidance behaviors, autotomy, and regeneration abilities may all be evolved responses to injury, at least in some contexts and taxa. Sublethal predation pressures could even have stimulated major transitions in animal mobility, as proposed for Paleozoic crinoids (Baumiller et al., 2010). Collectively, the widespread presence of injury-reducing or injury-responsive phenomena suggest that injury has imposed strong and taxonomically widespread selection pressures that have impacted the evolution of animals.

As yet, limited work has focused on understanding the evolutionary forces that have shaped injury responses, but available data suggest that many possible factors merit consideration. For instance, autotomy has evolved many times across animals (Bateman & Fleming, 2009; Cromie & Chapple, 2013; Fleming et al., 2007; Maginnis, 2006b), but the factors driving its evolution are likely multi-faceted. Experimental evidence in insects suggests that autotomizing damaged limbs significantly reduces the potential costs of injury by minimizing negative consequences such as fluid loss or infection risk (Emberts et al., 2017). Thus, autotomy may be beneficial not only as a way to avoid full predation but also as a way to decrease injury costs. Disentangling the relative importance of these two effects will be important for understanding the evolution of this injury response. Organismal features such as size, rate of aging, and life history strategy will also affect how organisms respond to injury and the likelihood of survival following injury, thus affecting the evolutionary consequences of injury (Bely & Nyberg, 2010; Seifert, Monaghan et al., 2012; Webb, 2006). For example, the fitness cost of losing a head is

dramatically higher in a species that reproduces exclusively sexually and requires head structures to survive and reproduce than in a species that reproduces asexually by fission, in which the loss of the head may not preclude the production of offspring by fission. This scenario has been proposed as a possible evolutionary explanation for the loss of head regeneration ability in a group of fissioning annelids: several such species have been shown to be able to reproduce asexually even if anteriorly amputated, suggesting a very low cost to anterior amputation that would decrease selection for maintaining anterior regeneration ability following injury (Bely, 1999; Bely & Sikes, 2010). Among lizards and salamanders, short-lived species tend to have poorer regenerative ability than species with greater longevity while exhibiting fewer negative consequences for reproduction; this may similarly represent a scenario in which life history modulates investment trade-offs related to injury (Bernardo & Agosta, 2005). Which traits are selected for under injury pressure may thus vary or even conflict with one another in a number of ways, such as trade-offs between evolving greater ability to escape injury versus evolving cumbersome but effective defensive structures. Vermeij (1982) argued that, in the case of injuries caused by sublethal predation, selection for traits involved with injury recovery or defense ought to be stronger than for avoidance when the incidence of sublethal predation is high, that is, when rates of detection and capture are also high. Selection may even act on diverging strategies within the same structure or trait. For example, studies in damselfly larvae indicate a link between lamellar joint allometry and environmental predation risk, where smaller, weaker joints are correlated with increasing predation risk (Gleason, Fudge, & Robinson, 2014), presumably reflecting past and ongoing selection to facilitate autotomy or breakage by direct predation, whereas larger, stronger joints

enhance swimming in low-predation risk environments (Bose & Robinson, 2013). Injury-related phenomena that have evolved repeatedly across animals, such as autotomy and changes in regenerative ability, provide particularly powerful frameworks for disentangling the many factors that shape the evolution of injury responses.

Integration and future research

Data on injury biology are broad and deep, spanning animal phylogeny and the spectrum of biological levels of organization (Fig. 2.3). As highlighted throughout this review, this breadth and depth enables some integration to begin to understand the multi-level effects of injury as well as how injury effects at one level can affect other levels. Integration is strongest where the effects of injury have been studied across multiple levels of organization in the same taxon. Lizards, dipteran insects, decapod crustaceans, and bivalve molluscs stand out among the groups best studied across levels of biological organization, providing the clearest pictures thus far of the multi-level effects of injury. The body of work in lizards is particularly large and diverse in scope. Collectively, studies in anoles, skinks (Scincidae), and geckos, focused predominantly on tail loss but also on cutaneous injury, have revealed the cellular and immune dynamics involved in wound healing, the developmental processes of tail regeneration and scarring, the impacts of tail loss or other injury on physiology (including relative investment in various organismal processes), the consequences of injury for locomotion and several types of behavior, and the influence of injury on intra- and interspecific interactions, as well as some cross-talk between these levels. In insects, the primary emphasis has been on the molecular, cellular, and developmental responses to injury, which have been studied with a high level of detail owing primarily to work in *Drosophila*. Studies at other levels in

insects, especially ecological scales, are sparse or lacking, precluding broad integration. Both decapod crustaceans and bivalve molluscs are common subjects of work at the levels of behavior and population and community ecology, in part due to their commercial and ecological relevance. In these groups, lower-level responses to injury are understood to some extent, but genetic and organismal knowledge particular to pathways and processes involved in injury and regeneration, including especially how these relate to higher-order phenomena, is still developing.

Although better-studied groups such as lizards, arthropods, and molluscs provide information on the multi-level effects of injury, most studies still focus predominantly on endpoints at narrow biological scopes. Very little work has investigated correlative or causal links between injury effects at multiple levels of organization within species despite the critical value of understanding such relationships. Commonalities between taxonomic groups in stress responses at lower levels become less apparent at higher levels due to environmental complexity, evolutionary distance, and true divergence in responses as a result of many other potential factors (Sulmon et al., 2015). Understanding, for example, why injury accelerates reproductive output in one species but inhibits it in another requires clarifying the links between the genetics, development, physiology, life history, and evolution of these processes. The few examples of such directly integrative research reveal important connections between injury effects at different levels. For example, injury-induced shifts in gene expression are associated with altered reproductive output in ants (von Wyschetzki et al., 2016) as well as sensitization behavior in *Drosophila* (Babcock et al., 2009) (although the latter study used UV light rather than mechanical injury), and injury-induced post-embryonic developmental effects

have been shown to affect predator-prey interactions in *Pelobates cultripes* toads (Zamora-Camacho & Aragón, 2019). These are examples of research to guide future integrative work in the area of injury biology. Ecological modeling offers a generative direction for improving quantitative understanding of injury on population and community dynamics. Such models would require quality datasets of injury frequencies and characteristics, which are currently lacking for most groups, but could particularly enhance our ability to assess the effects of sublethal predation or other sources of injury on recruitment, environmental stress tolerance, or species interactions, to list a few possibilities. Work by Lindsay and colleagues (1996) offers an example of the capability for models that incorporate injury to illustrate their influence on ecosystems.

A critical task for injury researchers going forward is to clearly demarcate several similar yet distinct phenomena, in particular endogenously versus exogenously induced injury, and wound healing versus regeneration. Endogenously induced injury, namely autotomy, differs from exogenously induced injury in that it is induced by the animal upon stimulation at pre-existing fracture planes, which serves partly to reduce damage and fluid loss. While it might be hypothesized that the negative effects of autotomy would be diminished compared to typical injury, only scant attention has been devoted to investigating these differences. By comparison with autotomy, exogenously induced injury has been found to reduce intraspecific competitive ability in crabs (McCambridge et al., 2016) and increase blood loss in lizards (Delorme et al., 2012); more investigations such as these, comparing exogenously and endogenously induced injuries, are needed. A complicating issue is that many studies claim to assess the effects of "injury" when they actually focus specifically on induced autotomy, which may have different consequences

at one or more biological levels; it will be beneficial to improve clarity of language and avoid using these terms interchangeably. As with exogenous injury and autotomy, the effects of wound healing and regeneration are often conflated with one another; these are more appropriately considered separate but partly overlapping processes (Brockes & Kumar, 2008; DuBuc, Traylor-Knowles, & Martindale, 2014; Jacyniak et al., 2017), ones that may even exhibit trade-offs with one another in some contexts, such as mammals (Wang, W. et al., 2020). Not only are wound healing and regeneration often not well delineated, but our knowledge of injury responses in general is strongly skewed toward species that can regenerate well, including many which also autotomize their body parts, such as crabs, salamanders, and lizards (Fleming et al., 2007; Juanes & Smith, 1995; Maginnis, 2006b), potentially introducing significant biases. Increasing the diversity of species in injury research to include those that cannot autotomize or regenerate, or that exhibit a gradient of injury responses, is necessary to clarify the origins—both proximate and ultimate—and mechanistic underpinnings of these responses. Important insights are likely to come from research on species in which often-confounded processes can be dissociated, such as the insect *Narnia femorata*, which can either automize or regenerate its limbs but cannot do both (Emberts et al., 2017), or certain members of the Naididae, such as *Chaetogaster* or *Paranais*, which often fully regenerate at one end of the body but only wound heal at the other (Bely & Sikes, 2010). Drugs or other molecular disruptions that selectively inhibit certain processes (e.g., inhibiting only autotomy or regeneration (Arnoult & Vernet, 1995; Coomber, Davidson, & Scadding, 1983)) may also prove useful.

Two additional important factors remaining underexamined in injury research are injury history and the nature of the injury itself. Although it is common for animals to sustain multiple injuries either at once or over time, most experimental work concerns the effects of single discrete injuries. The cumulative effects of repeated injury and regeneration on animal physiology, fitness, or subsequent rate or success of repair are not well known, a point also made by Lindsay (2010). A small number of studies have reported such effects, including increased susceptibility to further damage and potential resource limitation in corals (Henry & Hart, 2005), reduced body growth in bivalves (Tomiyama & Omori, 2007) and, alongside reduced activity levels, in polychaetes (Campbell & Lindsay, 2014), and the regeneration of smaller limbs with reduced innervation or failure to regenerate in axolotl (Bryant et al., 2017); however, a lack of effects has also been reported following repeated lens regeneration in newts (Eguchi et al., 2011), and repeated spinal cord transection has virtually identical outcomes for animal functional recovery and tissue repair in sea lamprey (*Petromyzon marinus*) (Hanslik et al., 2019). Repeated injury may even be beneficial, as suggested by a study showing that repeated injury and regeneration can extend lifespan in the clitellate annelid *Paranais litoralis*, possibly as a consequence of inducing repair mechanisms conferring longevity (Martinez, D. E., 1996). Given the often substantial (and likely underestimated) rates of injury documented in the wild, it is important to expand experimental treatments to ecologically relevant frequencies of injury. More work is also needed to assess the impact of the nature of injury on the injury response; most research on injury employs controlled, “clean” injuries, such as total amputation of appendages or body extremities, standardized cutaneous incisions, or piercing wounds. Wound types beyond these, such

as crushing, abrasion, or partial amputation, are uncommon in the literature, but may be highly relevant in the wild. Wound severity, including lesion size or degree of amputation, is also rarely manipulated experimentally, despite these characteristics being far from consistent in natural injuries. Injuries of different sizes or qualities may require different amounts of investment in repair, may vary in the amount of time that repair takes, and may elicit quantitatively and qualitatively different responses and compensatory changes, potentially leading to variable downstream impacts of injury. For example, a study in the planarian *Schmidtea meditteranea* found different spatial and temporal patterns of stem cell proliferation between puncture and amputation wounds (Wenemoser & Reddien, 2010). Therefore, future research should consider incorporating gradients of damage whenever possible rather than single, homogeneous injuries. More generally, expanding the design scope of experimental work on injury will benefit our understanding of its biological consequences.

Although injury represents a special type of insult to the body, it is theoretically and practically useful to recognize the ways in which injury affects animal biology as a stressor. Like other typical stressors, such as thermal stress or pollutant stress (Kassahn et al., 2009; Killen et al., 2013; Sulmon et al., 2015), sublethal injury often disturbs homeostasis, reduces fitness, and induces the CSR (Kassahn et al., 2009; Killen et al., 2013; Makrinos & Bowden, 2016; Matranga et al., 2000; Mydlarz et al., 2008; Sulmon et al., 2015). And as is the case with other physical stressors (Gianguzza et al., 2014; Sulmon et al., 2015; Vasquez et al., 2015), non-summative effects result from combinations of injury and other stressors, including osmotic (Hickey, 1979; Stueckle, Shock, & Foran, 2009), thermal (Henry & Hart, 2005; Jensen et al., 2015), nutritional

(Hickey, 1979; Jensen et al., 2015), sedimentation (Henry & Hart, 2005), pollution (Grdisa, 2010), and parasitic stress (Johnson et al., 2006). Trade-offs between wound healing and environmental stress resistance have been documented, and these trade-offs can be mediated by factors like nutrition, social status, and seasonality (Archie, 2013; Juanes & Smith, 1995; Maginnis, 2006b), underlining the importance of not only studying injury at a mechanistic level but also within the broader ecological context of particular animals. Additionally, as injury is known to stimulate the CSR, it will be valuable to assess whether mild injury could have beneficial hormetic effects or confer cross-tolerance, as evidence suggests can occur with other stressors (Costantini, Metcalfe, & Monaghan, 2010; Kültz, 2003; McClure et al., 2014). Given the frequency of injury in nature and the likelihood of more extreme environmental stress scenarios in the future because of anthropogenic impacts on biological systems, it will be important to understand the interactions between injury and these stressors across levels of organization in diverse species.

Figures

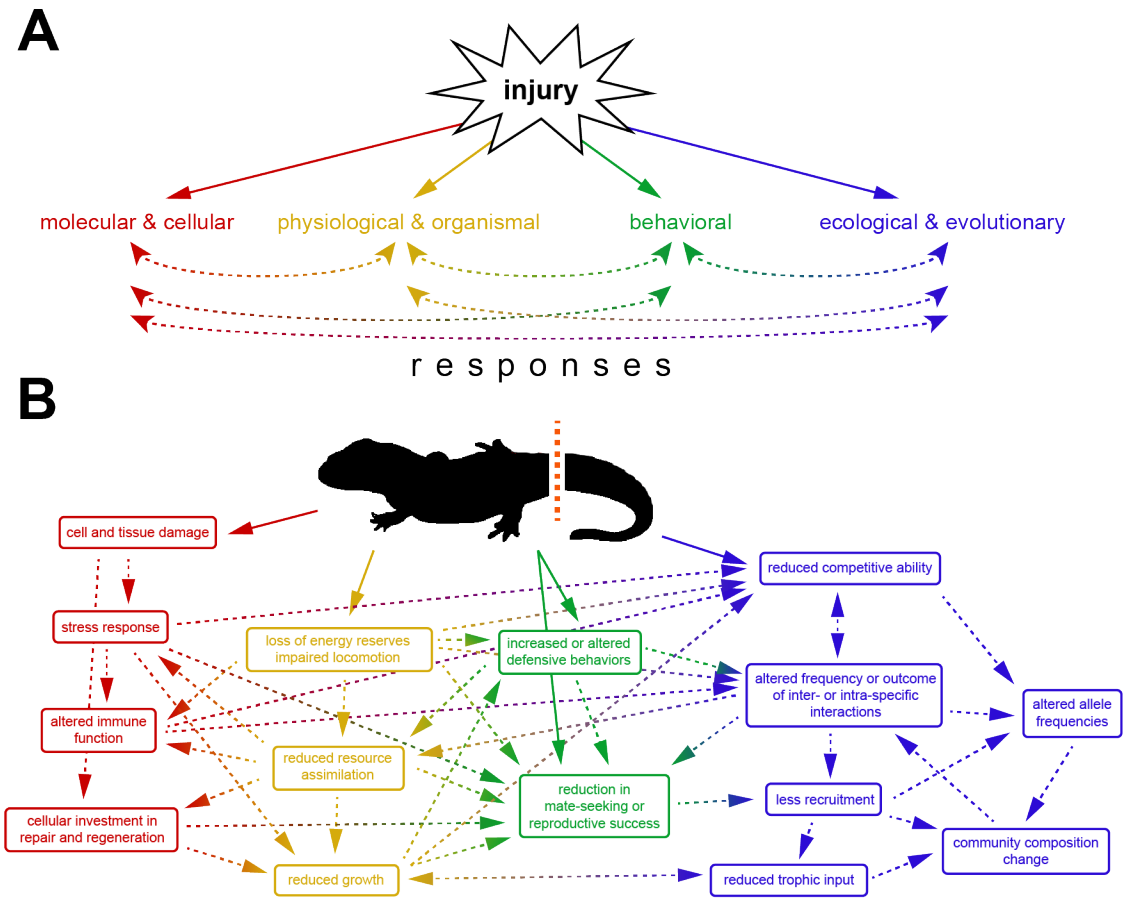

Figure 2.1. Consequences of sublethal mechanical injury and interactions between effects within and between levels of biological organization. Solid arrows indicate possible immediate or direct effects of injury; dashed arrows indicate possible interactions between these effects. (a) Conceptual schematic of the biological scales at which injury may lead to effects or responses and the potential linkages between responses at these levels. (b) Hypothetical example of direct and indirect consequences of injury in an animal (e.g., tail amputation in a tetrapod), based broadly on conjectured and demonstrated relationships in multiple species.

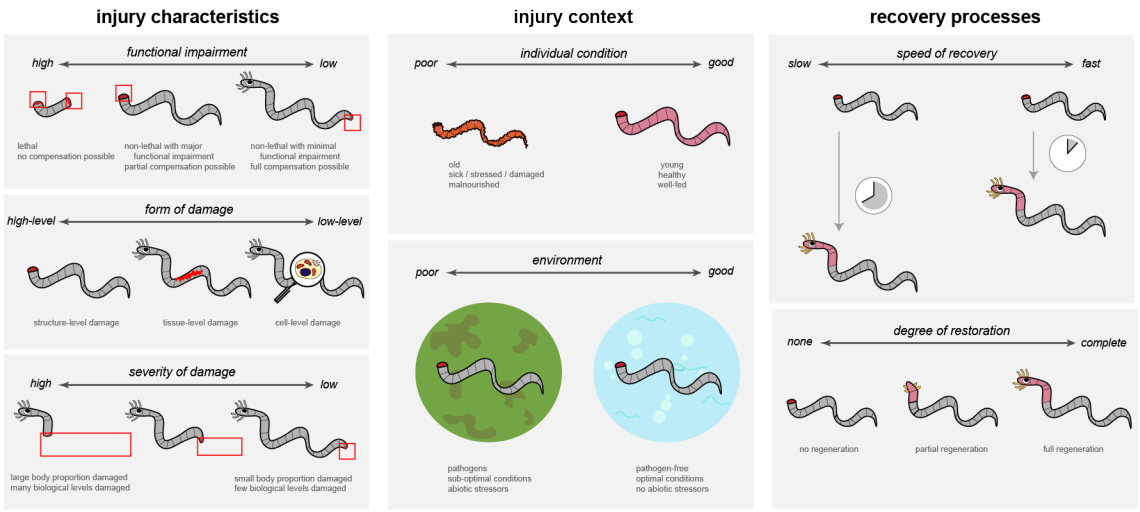

Figure 2.2. Intrinsic and extrinsic factors that influence the nature and severity of injury consequences in animals. Factors may be categorized into three broad categories: (i) immediate injury characteristics, which includes the degree of direct functional impairment resulting from the injury, the form or level of damage inflicted (e.g., scales of biological structures damaged, such as whole-appendage vs cellular-level damage), and the severity or degree of damage (e.g., relative proportion of a given body part damaged); (ii) injury context, which includes the individual condition of the animal (e.g., nutritional status, age, parasite load, disease, pre-existing un- or partially/imperfectly-repaired damage) and characteristics of the environment which may be optimal or physiologically stressful (e.g., temperature, oxygen availability, humidity, salinity, microbiota); and (iii) recovery processes, which includes the speed at which recovery occurs and the degree of restoration that takes place, including the potential regeneration of lost structures. Factors presented here are not exhaustive.

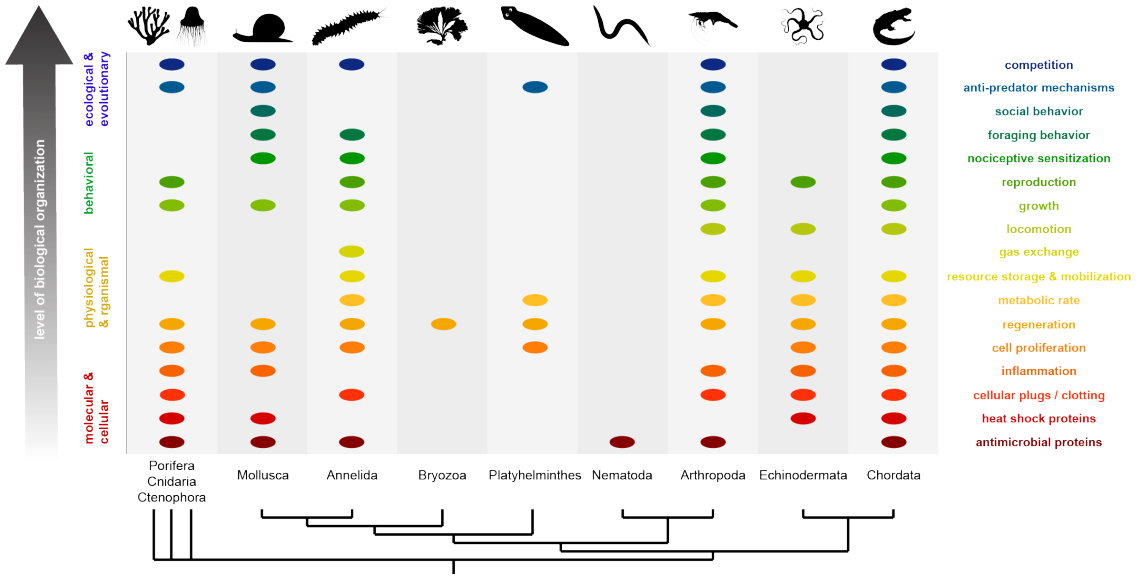

Figure 2.3. Graphical representation of research findings on the effects of injury across levels of biological organization and across animal phylogeny. Color scheme used indicates where various endpoints studied (right) roughly fall within broader levels of organization (left). Relative ordering of endpoints is arbitrary. Colored ovals indicate the existence of literature cited in this review that reports direct or suggested effects, either positive or negative, resulting from mechanical injury on the corresponding endpoint (row) within the corresponding animal phylum or group of phyla (column). Absence of an oval does not mean the absence of an effect in nature, only the absence of effects reported in the reviewed literature, indicating areas of research opportunity. Phylogenetic relationships are based on Giribet et al. (2007), but we acknowledge that the placement of basal taxa remains a subject of debate. Silhouette attributions are provided in Appendix 1. See text for literature references and additional detail.

Chapter 3: Physiological responses to injury, regeneration, and environmental stress in a freshwater annelid

Abstract

Evolutionary patterns of regenerative ability may reflect the relative costs of injury versus regeneration. While the costs of injury seem straightforward, the resource investment required for regeneration may itself have considerable short- or long-term costs. Additionally, the costs of injury and regeneration can each vary with myriad factors, producing complex effects. However, these effects and their variation are not well understood in most species. Distinguishing the physiological impacts of injury and regeneration can help clarify the potential ecological and evolutionary role of these processes. We tested how injury and regeneration affect the annelid *Pristina leidyi* by measuring several physiological and molecular endpoints, utilizing environmental stress as a simultaneous physiological challenge to clarify these responses. While regeneration of anterior segments reduced survival under heat stress, injury unexpectedly improved survival under both heat and salinity stress. Survival patterns were not related to metabolic rate. We used TagSeq to assess how injury and heat stress affect gene expression and to identify candidate genes and pathways implicated in injury-induced stress tolerance. Genes differentially expressed in response to both injury and heat stress include several members of the heat shock protein family and others involved in pathways related to inflammation, metabolism, and development. Combined injury and heat stress induced a transcriptomic response that is synergistic in degree but not in kind, with many more genes differentially expressed but representing similar biological

functions. Our work demonstrates that the cost of regeneration is conditional and suggests that injury can produce unexpected beneficial effects via general stress pathways.

Introduction

Mechanical injury is a relevant threat to the survival and function of almost any animal in nature. Injury damages tissue and organs, severing physical links and communication channels between cells, disrupting function in myriad ways; injury breaks the barrier between internal and external environments, allowing circulatory fluids to escape and pathogens to enter; direct loss of tissue may involve the loss of biological materials and energy reserves; and severe, unmitigated injury may lead to death of the organism (Archie, 2013; Niethammer, 2016). Injury can also lead to losses in function associated with the particular tissue or appendage damaged and a variety of secondary effects (Bernardo & Agosta, 2005; Juanes & Smith, 1995). The homeostatic challenge that injury can impose on animals at cellular or higher levels of biological organization is comparable in many ways to forms of environmental stress (Kassahn et al., 2009; Kültz, 2020a; Sulmon et al., 2015), such as extreme temperature, radiation, pH, pollution, or salinity (Gunderson et al., 2015; Holmstrup et al., 2010; Todgham & Stillman, 2013; Vinebrooke et al., 2004). The effects of stress have the potential to shape the structure of entire biological communities and influence the course of evolution (Kassahn et al., 2009; Kültz, 2020b; Sulmon et al., 2015; Vinebrooke et al., 2004), and injury can likewise be expected to have similar potential on biological processes that extend beyond the scope of individual organisms if sufficiently prevalent in the wild. In fact, injury is quite common in many natural populations (Lindsay, 2010), whether the source is predatory

attacks, nonpredatory biotic interactions, or environmental accidents. The considerable consequences that may result from injury makes it a significant stressor itself that animals are likely to avoid or mitigate whenever possible.

As with other forms of stress, animals have evolved a range of responses to injury that serve to reduce its negative effects on function. Among the most dramatic of these responses is regeneration, the process by which damaged or lost tissue is restored, either partially or totally, to its previous form and functional capacity. Regeneration within animals involves a range of physiological and genetic mechanisms, many of which may be convergent and only some of which have been identified or characterized to a substantial degree (Bely & Nyberg, 2010; Lai & Aboobaker, 2018). These processes are united, however, in their requirement for energy, material resources, and time. Since these variables are limited in individual organisms, regeneration often proceeds at a cost to other biological functions, although trade-offs may manifest adaptively or due to other constraints. The effects of regeneration on reproduction and non-regenerative growth have perhaps been best described in animals broadly (Archie, 2013; Bernardo, 2005; Henry & Hart, 2005; Maginnis, 2006; Sepulveda et al., 2008). However, there are more species that can regenerate, with a great diversity in physiology, life history, and environmental contexts, than those in which the costs of regeneration have been studied. As such costs might hinder animal function and subsequently reduce fitness, the gap in knowledge of these costs and their variation limits our ability to understand the broader ecological and evolutionary relevance of regeneration, including upon what physiological mechanisms underlying regeneration might be subject to selection.

The impacts of injury are often not so easily distinguished from those of regeneration itself, nor are these impacts necessarily straightforward or consistent. Myriad factors such as life history, nutritional status, or environmental parameters can modulate an animal's response to injury, such as the rate or fidelity of regeneration and the magnitude or nature of trade-offs with other biological processes (Maginnis, 2006b). The nature of the injury itself may also lead to a range of effects, particularly if the damaged structure is of functional importance or included the loss of energy reserves, such as in lizard tails where fat storage can vary significantly in amount or distribution along the tail between species (Bernardo & Agosta, 2005; Smyth, 1974). Injury does not occur in static conditions in nature, and thus testing the effects of injury alone without introducing variation, such as in the abiotic environment or in where injury is inflicted, can mask the complexity in animal responses to injury and thus distort our understanding of its role in shaping biological processes. Failing to adequately distinguish these effects, and factors driving their variation, from those induced by the resource consumption or other shifts resulting from regeneration similarly precludes a greater understanding of why regeneration evolved in the diverse manner that it has.

For this study, we investigated how mechanical injury and regeneration affect the physiology of the naid annelid *Pristina leidyi* in an attempt to distinguish the effects of each and to better understand how these effects vary between environmental conditions and which structures are damaged. *P. leidyi* is an emerging model for studying post-embryonic development due to its frequent agametic propagation, rapid and high fidelity regenerative ability, small size, iteratively structured and largely transparent body, and ease of culture (Bely, 2022). These features make *P. leidyi* a great candidate for this type

of work, as many genetically identical individuals can be generated quickly and easily manipulated. Injuries can be inflicted at standardized locations across the body, the amount of tissue removed can vary without greatly differing in the types of structures that are removed, and regeneration is easily observed and consistent between individuals. How *P. leidyi* responds physiologically to injury and regeneration is not well understood overall but is known to vary in some respects with timing and injury location (Zattara & Bely, 2013), presenting an opportunity to build on these findings and facilitate future work in the species. *P. leidyi* regenerates large portions of its body following transverse amputation within an average span of five days, including tissues at both the anterior and posterior ends of the body (Zattara & Bely, 2011). Regeneration of posterior segments is always epimorphic, while loss of more than four anterior segments induces epimorphic regeneration of up to four segments coupled with morphallactic remodeling of existing segments into new segmental identities (Özpolat & Bely, 2016; Zattara & Bely, 2011). Anterior and posterior ends possess different complements of organs and associated functions; therefore, the physiological costs of losing these tissues and the costs of restoring them are likely to differ from one another.

We assessed the physiological impact of injury and regeneration in *P. leidyi* at multiple levels, including whole organism function (survival and metabolism) and molecular responses (gene expression). Specifically, we wanted to address the following questions: how do the effects of injury differ from those resulting from investment in regeneration, and how are these effects modulated by the presence of simultaneous physiologically challenging conditions? To do so, we used environmental stress tolerance, including thermal and salinity tolerance, as a measure of physiological

performance. By applying environmental stress, which is expected to be physiologically demanding itself, we sought to clarify the ways in which injury and regeneration affect organismal physiology that might otherwise be subtle. If injury and regeneration significantly reduce energy availability, for example, we expected to see considerable reductions in environmental stress tolerance. We measured how tolerance to stress (extreme temperature and high salinity) was affected by both the location of injury (anterior vs. posterior) and the stage of injury recovery (immediate post-injury vs. post-regeneration) at which a thermal or salinity shock was applied. We measured short-term survival and metabolic rate via microplate respirometry to assess how injury and regeneration affect physiological performance at both lethal and sublethal levels. Additionally, we quantified gene expression via 3' tag-based RNA-seq (TagSeq) to identify candidate genes and broad molecular pathways shared between the response to injury and environmental stress that may be involved in the performance differences that we observed.

Methods

Culturing and obtaining experimental animals

Pristina leidyi originally purchased from Carolina Biological Supply (sold as *Stylaria)* were cultured at room temperature (23 ºC) in glass bowls (12 cm diameter) filled approximately halfway (~150 mL) with artificial spring water (1% artificial seawater) (ASpW). Strips of brown paper towels were provided as substrate. Cultures were fed once weekly with 10 mg powdered Spirulina. Half-volume water changes were administered weekly.

To obtain animals for experiments, worms that appeared healthy were washed several times in clean ASpW and held together in a glass bowl for three days, allowing worms to clear their gut contents. Worms that appeared damaged, showed signs of aging (e.g., shrunken, dense chloragogenous pigmentation), or had visible fission zones were removed after three days. Worms were randomly selected from this remaining pool of animals for use in the following experiments.

Amputation

Individuals were first anesthetized in 0.05 mM nicotine and then pipetted onto glass slides, where either the anterior or posterior end was removed with a scalpel. Uninjured worms were anesthetized for approximately equivalent times. Worms were amputated between segments 6 and 7 (anterior injury), between segments 16 and 17 (posterior injury), or not injured (controls). Amputated sections (the shorter piece following amputation) were discarded.

P. leidyi forms a wound epithelium over the site of injury and begins construction of the blastema, the undifferentiated cell mass that develops into regenerated tissue, by approximately 24 hours post-injury (Zattara & Bely, 2011). At roughly five days, regeneration of either anterior or posterior tissue is typically complete. We therefore used one and five days post amputation (dpa) as measurement time points throughout the study.

Thermal stress experiments – acute thermal tolerance range-finding

In order to determine appropriate stress temperatures, we first found the thermal tolerance range of *P. leidyi*. After amputation, worms were placed singly in 0.5 mL

microcentrifuge tubes filled completely with fresh ASpW. Tubes were placed in low light at room temperature (23 ºC) for 1 dpa or 5 dpa without disturbance. The 1 dpa treatment was intended to measure changes in thermal tolerance resulting from injury and the loss of tissue prior to any significant regeneration and, presumably, resource and energetic investment in the regeneration process. The 5 dpa treatment, on the other hand, was intended to measure the impact of regeneration on thermal tolerance. Uninjured controls in the 5 dpa treatment group additionally allowed us to determine the relative impact of fasting on thermal tolerance, as worms had no access to food during any point of the experiment including the regeneration period.

At either 1 or 5 days, worms were checked for mortality (i.e., resulting from injury) (>95% of worms survived initial injury). Their tubes were then randomly arranged in a water bath inside an adjustable low temperature incubator (for temperatures below 23 ºC) (146E, Fisher Scientific, Waltham, MA) or in flooded wells of a thermal incubator (for temperatures above 23 ºC) (Isotemp 125D, Fisher Scientific, Waltham, MA). A range of temperatures (5, 7, 15, 22, 35, 39, 40 ºC) were tested to determine *P. leidyi*'s acute tolerance range. Worm tubes were also placed in a water bath on a benchtop in low light at room temperature (23 ºC) as a thermal control. Temperatures were regularly verified by an analog thermometer to be ±0.5 ºC from the set point. Tubes remained undisturbed in these conditions for 48 continuous hours.

At 48 hours, tubes were removed from experimental temperature and left to sit at room temperature for roughly 20 minutes. Mortality was then assessed by looking for body movement or gut peristalsis within fifteen seconds of observation. Worms that exhibited neither, or exhibited any clear decomposition, were considered dead. Most dead

worms were in various degrees of decomposition at the time of assessment. Survival was scored as a percentage of the number of worms alive at this check point (e.g., 7 of 10 worms alive = 70% survival). This experiment was replicated $n = 5$ times per treatment combination, using groups of ten worms per treatment combination per replicate.

Thermal stress experiments – survival assay at thermal extremes

Worms were amputated, placed in tubes, and allowed to sit for 1 (1 dpa) or 5 days (5 dpa) as described previously. All other procedures up to and including moving tubes to incubators were also identical. However, only two temperatures were tested, representing the lowest observed high (39 ºC) and highest observed low (5 ºC) temperatures at which worm death was significant (at or near 0% survival) at 48 hours on average across all injury treatments. Mortality of all worms was scored approximately every 3 hours until all worms in each run ($n = 15$ worms per treatment combination) were dead.

Salinity stress experiments – acute salinity tolerance range-finding and survival assay

Salinity tolerance was used to assess whether effects of injury and regeneration on thermal tolerance was attributable to general mechanisms involved in the stress response. Amputations and pre-stress incubation periods were conducted as described previously, but worms were placed singly in filled (~1 mL fresh ASpW) wells of 24-well tissue culture polystyrene plates. At 1 dpa or 5 dpa, worms were moved to wells filled with water of varying salinities (1-10 ppt, in increments of 1 ppt) and placed in low light. Survival was scored at 48 hours. Survival was 100% at 9 ppt and 0% at 10 ppt, so we

repeated this assay with a narrower range of salinities (9.1-9.9 ppt, in increments of 0.1 ppt).

The lowest observed salinity at which mortality was substantial (<50% survival) at 48 hours across all injury treatments was determined to be 9.5 ppt. To determine the difference in survival between injury treatments at a finer temporal scale, an identical experimental design was used as described above for survival at thermal extremes, but at this elevated salinity rather than extreme temperatures, and scoring of survival was identical (n = 15 worms per treatment combination).

Respirometry

Worms were amputated, placed singly in filled wells of 24-well plates, and allowed to sit for 1 (1 dpa) or 5 days (5 dpa) as described previously.

To measure how injury at different body sites at different stages of recovery affected thermal tolerance at a sublethal level, we measured oxygen uptake (assumed hereafter to be equivalent to consumption) under exposure to 35 ºC, the highest temperature at which no mortality occurred in our range-finding experiment, and 10 ºC, which we found to be nonlethal (approximately 100% survival) to worms for at least several days when testing culture temperatures for an unrelated study (unpublished data). We used a complete optical microplate system with an 80 µL well volume (Loligo Systems, Viborg, Denmark). The microplate was prepared for each experimental run by first immersing the wells in fresh ASpW for >20 minutes. The ASpW was then removed, and the system was calibrated using fresh ASpW that was first allowed to equilibrate to the relevant trial temperature and bubbled with air for >20 min (100% O_2) or a solution of 20 g/L Na_2SO_3 in fresh ASpW (0% O_2), according to the manufacturer's instructions.

The plate was then rinsed, and wells were filled with fresh ASpW. Worms were randomly assigned to wells and transferred, removing any air bubbles formed during the transfer. Each run with the respirometer included eighteen worms (six worms per injury location (anterior, posterior, or uninjured)) and six wells used as blanks, and only included worms from the same recovery stage and processed batch (i.e., injured at the same time). After removal of bubbles, the plate was sealed with a sheet of translucent adhesive PCR film (Thermo Fisher Scientific, Waltham, MA) and placed on the microplate reader. A silicone gasket and compression block were placed on top of the plate to ensure a continuous seal. The microplate apparatus was then moved to either a low-temperature incubator at 10 ºC (146E, Fisher Scientific, Waltham, MA), an oven at 35 ºC (6241, Fisher Scientific, Waltham, MA), or in low light at room temperature (23 ºC) as a thermal control. Recording was then initiated, and oxygen measurements were taken every minute until all samples reached 70% air saturation. Data were recorded in the included MicroResp™ software (v1). Worms were then removed and checked for survival; runs with more than one worm death were not used in analysis. Surviving worms were removed and imaged for body volume measurements, as described below.

Respirometry of worms under differing salinities (0.35 “ASpW” versus 6 ppt) was identical to the procedure described above with the following exceptions: all respirometry was conducted exclusively at room temperature; calibration used either ASpW or 6 ppt water as appropriate to the trial; and worms were first rinsed in 6 ppt before transfer to the respirometer to minimize dilution for all 6 ppt trials.

Imaging and calculations of mass and oxygen consumption rate

Due to the logistical constraints of directly measuring individual body mass in *P. leidyi*, mass was approximated through measurements of individual volume. Individual worms were pipetted onto glass slides and anesthetized with 0.05 mM nicotine until movements ceased, and then worms were imaged under a 2.5x objective with a Zeiss Axioplan 2 microscope and AxioVision 4.8 image processing software. Measurements were done in ImageJ using the Fiji package. Individual length and width were measured in Fiji. Body length (*L,* mm) spanned from the posterior tip to just prior to the beginning of the anterior proboscis. Body width (*W,* mm) was determined by averaging three width measurements, drawn just prior to where the anterior and posterior ends began to taper, and across the approximate center of the worm. Volume was calculated using the equation of a cylinder ($V=\pi r^2 h$), substituting $W/2$ for r and L for h. Body mass was then calculated by multiplying volume (L) by the density of water (ρ) at room temperature (ρ = 0.997773 kg/L) (Colt, 2012).

Calculation of absolute oxygen consumption rates was performed in the MicroResp™ software. Phase values in each well were converted to oxygen concentration in mmol/L. Data from the first 30 minutes of each run were not included in calculation of oxygen consumption rate. Only oxygen data between 90% and 70% air saturation were used in the calculation of the slope of oxygen concentration, deemed the oxygen consumption rate (MO_2) (nmol/hr). Linear regression of oxygen concentration within these limits was performed for each worm, and those with R^2 <0.95 (23 ºC and 35 ºC; ASpW and 6 ppt) or <0.90 (10 ºC) were not used in analysis. MO_2 of the blank well means were subtracted from those of the experimental wells for each run in order to

account for background factors, yielding absolute MO_2 for each worm. These values were divided by calculated body mass of each worm to obtain the relative metabolic rate (nmol/hr*mg^{-1}). Q_{10} coefficients for each recovery stage (1 dpa, 5 dpa) and injury condition (anterior, posterior, uninjured) combination across the range of temperatures (10, 23, 35 ºC) or salinities tested (0.35, 6 ppt) were calculated using $Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)}$ where R_x is MO_2 at temperature or salinity T_x as appropriate.

Thermal stress experiments – gene expression

We performed a gene expression study using TagSeq to assess the transcriptomic response to injury and thermal stress at 1 dpa following anterior or posterior amputation. TagSeq differs from standard RNASeq by focusing sequencing on the 3' end of each transcript, producing one read per transcript. This serves as a cost-effective methodology for differential gene expression studies when a reference genome or transcriptome is available (Lohman, Weber, & Bolnick, 2016). Our experiment included 4 injury treatments, including 2 amputations and 2 tissue controls (see below), replicated across 2 temperature conditions (23, 35 °C) for a total of 8 treatment combinations. This matrix was replicated $n = 6$ times.

Worms were amputated and placed in tubes as described previously. At 1 dpa, worms were either left at room temperature (23 °C) or moved to a thermal incubator at 35 °C for six hours. Tubes were then removed, worms were checked visually to confirm survival and then pooled in a single tube in groups of 10-12 worms of the same injury type per tube. Uninjured worms were amputated as described previously in groups of 10-12 per injury location (anterior or posterior) immediately following removal from their

individual tubes and then quickly (< 5 min) collected in single tubes. These worms served as tissue loss controls, in order to account for potential differential gene expression in body segments not present in amputated worms that is unrelated to injury or thermal stress, such that each injury group had its own control group. Following transfer of worms to single tubes, these tubes were then snap frozen in liquid nitrogen and moved to -80 °C until RNA extraction.

RNA extraction

Tubes containing worms were administered 50 µL TRIzol (Thermo Fisher Scientific, Waltham, MA), vortexed, frozen at -80 °C for approximately 15-30 minutes, and thawed. 1 µL polyacryl carrier and 150 µL TRIzol was added to each sample, which was then mixed and incubated a few minutes at room temperature. Then, 40 µL chloroform was added, tubes were incubated 15 minutes at room temperature, and samples were then centrifuged at maximum speed at 4 °C for 15 minutes. The upper aqueous phase of each tube was carefully removed and mixed with 100 µL isopropanol, incubated for 30 minutes, and centrifuged at maximum speed at 4 °C for 15 minutes. Supernatant was removed and precipitated RNA was washed with 75% ethanol and centrifuged at room temperature for 5 minutes. RNA pellets were air dried and then resuspended in 30 µL of RNase-free water. Total RNA was analyzed on a NanoDrop 8000 (Thermo Fisher) spectrophotometer for yield and purity. Yield was confirmed and integrity assessed by running RNA on a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA). RNA was kept frozen at -80 °C until library preparation.

TagSeq library construction and sequencing

All library construction and sequencing was carried out at the Genomic Sequencing and Analysis Facility at the University of Texas at Austin. Total RNA for all samples was used to construct TagSeq libraries according to the protocol described in Lohman, Weber, & Bolnick (2016). In brief, RNA was heat-fragmented at 95 °C for 2.5 minutes and transcribed into first-strand cDNA using SMARTScribe™ reverse transcriptase (Takara Bio Inc., Mountain View, CA) and template switching oligos (Integrated DNA Technologies, Coralville, IA). cDNA was purified using AMPure XP beads (Beckman Coulter, Brea, CA) and PCR amplified for 18 cycles. PCR product was further purified, indexed (Integrated DNA Technologies), purified once more, quantified using PicoGreen (Thermo Fisher), pooled equally, and size-selected to 350-550 base pairs (bp) with a BluePippin electrophoresis system (Sage Science, Beverly, MA). Libraries were sequenced in two separate batches of three replicates per experimental treatment combination each on a NovaSeq 6000 SR100 lane (Illumina, San Diego, CA).

Gene expression analysis

Sequenced libraries ranged from approximately 4.7 to 22.4 million raw reads per sample. Custom scripts (https://github.com/z0on/tag-based_RNAseq) were used to collapse duplicate reads and trim Illumina TagSeq adapter contaminants (one of four possible adapters + 24 downstream bp) and 3' poly(A) tails (8 or more bp). Reads less than 20 bases long after trimming were discarded. After deduplication and trimming, libraries ranged from approximately 1.1 to 5.5 million reads per sample. Trimmed and filtered reads were mapped and quantified using 'Salmon' v1.1 (Patro et al., 2017) with a *k*-mer size of 19. Reads were mapped to an unpublished *P. leidyi* IsoSeq reference

transcriptome (Solana & Kenny et al., unpublished data) prepared using a mixture of fissioning and non-fissioning worms (see Table 3.1). Mapping rates ranged between approximately 83-89%.

Differential expression analyses were performed using 'edgeR' v3.34.1 (Robinson, M. D., McCarthy, & Smyth, 2009) in the R computing environment (R Development Core Team, 2019). Low abundance transcripts were filtered using the filterByExpr function to maximize DEG discovery, leaving 34,351 transcripts included in subsequent analysis. Library sizes were normalized using the calcNormFactors function and sample read quantifications were fitted to a generalized linear model using a combined injury and temperature condition factor and incorporating a batch effect to account for differences between the two three-replicate groups sequenced separately. A multidimensional scaling plot of the Euclidean distances between samples showed strong clustering based on batch; we therefore decided to exclude the earlier batch from all downstream analysis and only include the later batch (n = 3 per treatment combination), which cluster more obviously by treatment factors (Fig. A2.1).

Functional annotation and gene ontology (GO) enrichment analysis

The Trinotate v3.2.2 pipeline (Bryant, Johnson et al., 2017) was used to functionally annotate the *P. leidyi* IsoSeq transcriptome. TransDecoder v5.5.0 (https://github.com/TransDecoder) was first used to predict open reading frames (ORFs) and protein coding regions using the default minimum length of 100 amino acids. Homology to known proteins was used as ORF retention criteria by performing a BLASTp search against the UniRef90 database (Suzek et al., 2015) with BLAST v2.12.0 (Altschul et al., 1990). Assembled transcripts and predicted proteins were then used to

perform homology searches against the UniProtKB/Swiss-Prot database (The Uniprot Consortium, 2019) with BLASTx and BLASTp, respectively, using the default *E*-value cutoff of 0.001. Predicted proteins were also used to search for protein domains with HMMER v3.3.2 (Finn, Clements, & Eddy, 2011) against the Pfam database (Mistry et al., 2021). Results were loaded into a Trinotate SQLite Database, and all hits that did not meet the E-value threshold of 0.001 were removed. For transcripts with multiple valid hits, only the top hit was retained for annotation of gene expression data. If there were discrepancies between BLASTx and BLASTp hits, the one with the lower E-value was used. Pfam hits, if available, were used if none were returned by BLASTx or BLASTp.

GO enrichment analysis was performed on differentially expressed transcripts using 'topGO' v3.14 (Alexa & Rahnenfuhrer, 2021) in R with GO term assignments retrieved from Trinotate output. The analysis was conducted with a Kolmogorov-Smirnov like test and a minimum of 10 genes in the input list for a given pairwise comparison present in a particular GO term. Of 34,351 transcripts, 22,147 (64.47%) had GO annotations and were used for enrichment tests.

Statistical analysis

We performed all statistical analysis in R. We tested differences in thermal tolerance and salinity tolerance after fixed 48-hour exposure across a range of temperatures or salinities, between injury recovery stages and injury location via multiple factorial ANOVA and Tukey's-adjusted post-hoc comparisons. A QQ plot of survival was symmetrical with minor heteroscedasticity in the residuals, and these were not corrected by transformation and did not affect analysis. To compare survival within these groups at thermal extremes or high salinity at higher temporal resolution, we used

Kaplan-Meier survival analysis using Cox proportional hazards models (Cox, 1972) on interval-censored data with the 'icenReg' package (Anderson-Bergman, 2017) and specifying 100 bootstrapped samples. We used Type III SS analysis-of-covariance to test the effects of injury location, recovery stage, and temperature or salinity on MO_2, setting calculated worm mass as the covariate, using the 'car' package (Firth et al., 2009). To perform Tukey's-adjusted post-hoc comparisons, we used the 'lsmeans' package (Lenth, 2016). As the raw temperature assay MO_2 data violated normality (Shapiro-Wilk test $P < 0.001$) and heteroscedasticity (increasing residuals) assumptions, we log-transformed MO_2, which improved each. This led to a significant ($P < 0.05$) interaction between injury and timepoint. However, we detected no significant differences in Tukey's post-hoc comparisons between injury × timepoint treatments, and all other ANCOVA terms remained significant or nonsignificant as prior to transformation. Because of this, we concluded that transformation did not substantially alter our analysis, and therefore the following results are discussed in reference to the raw MO_2 data. Salinity assay MO_2 data did not violate the heteroscedasticity assumption and were only slightly right-skewed, which was not improved by transformation, and so we ran our analysis with raw values.

Results

Thermal tolerance

We found that both uninjured and injured *P. leidyi* can survive 48-hour exposure to a broad range of temperatures. Survival was above 96% for temperatures from 15 and 35 °C, then dropped rapidly as temperatures decreased to 5 or increased to 40 °C, at either of which there was no survival (Fig. A2.2). Neither injury condition (uninjured,

anteriorly amputated, posteriorly amputated) ($P = 0.6296$) nor recovery timepoint (1 dpa, 5 dpa) ($P = 0.84727$) affected thermal tolerance according to multiple ANOVA, with no significant interactions. Both the upper and lower median lethal temperatures were comparable across all treatments. Despite a lack of statistical significance, we noted some slight differences in survival between injury conditions towards the lower and upper acute thermal limits. We therefore tested whether these represented meaningful biological differences in tolerance by performing a more sensitive survival assay with frequent survival checks at or near these lower and upper thermal limits. These studies were performed at 5 and 39 °C, at which mortality in our thermal tolerance study was substantial (<25% survival) after 48 hours.

By increasing the temporal resolution during the thermal stress assay, we were able to detect significant differences in thermal tolerance between injury conditions and recovery timepoints. We originally predicted that injured animals would uniformly have a decreased thermal tolerance. Unexpectedly, however, at 1 dpa, we found that injury improved heat tolerance relative to controls: both anteriorly cut and posteriorly cut worms survived longer at 39 °C than did uninjured worms ($P < 0.05$) (Fig. 3.1). However, at 5 dpa, posteriorly-regenerated worms did not differ in cold tolerance relative to controls ($P = 0.7586$), while anteriorly-regenerated worms exhibited poorer heat tolerance than both controls and posteriorly-regenerated worms ($P < 0.05$). Interestingly, the median time to 50% mortality was approximately the same (~50 h) for control worms between 1 and 5 dpa at 39 °C, suggesting that food restriction over this duration does not substantially affect heat tolerance. Results near the lower thermal limit differed from those near the upper thermal limit. At 5 °C, 1 dpa injured worms exhibited slightly poorer

cold tolerance than controls, but this difference was not significant, while at 5 dpa, posteriorly-regenerated showed a slightly elevated thermal tolerance, but no differences were statistically significant. Overall, worms did not live as long at 5 °C as they did at 39 °C, with all worms being dead by roughly half the time under cold at both 1 dpa and 5 dpa as under heat. Follow-up trials (not shown) confirmed the effect of injury-induced heat tolerance.

Our finding that thermal tolerance was improved in recently injured worms led us to hypothesize that injury induces a broad stress-protective response. To test this, we stressed worms in a similar manner using a different stressor, extreme salinity. *P. leidyi* dwell in freshwater, which is normally < 0.5 ppt, and are reared in the lab at ~0.35 ppt. We tested *P. leidyi* survival at salinities from 0.35 ppt to 10 ppt and found that 9.5 ppt was the point at which survival was substantially reduced (<50%). Under this high salinity stress, we found that worms also had a short-term improved stress tolerance when injured, comparable to findings for thermal tolerance. At 9.5 ppt, both anteriorly and posteriorly injured worms exhibited greater tolerance than controls at 1 dpa (both $P < 0.05$) (Fig. 3.2), but there were no differences at 5 dpa. Curiously, a fraction of individuals in each injury condition group at 1 dpa survived for a very long time under continuous high salinity and remained alive when the experiment was ended after about 220 hours. In contrast, most worms died rapidly at 5 dpa, with no significant differences detected between treatments, although anteriorly-regenerated worms died off slightly more quickly. We attribute this rapid death across all injury treatments in comparison to 1 dpa worms to the effects of more prolonged food restriction.

Respirometry

The oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis predicts that temperature-driven changes in standard metabolic rate (SMR) are responsible for decreases in performance under thermal stress, as oxygen availability and delivery, including effects on solubility, is unable to match respiratory demands (Schulte, 2015). We used single-worm microplate respirometry to assess how injury affects MO_2 at rest, a common proxy for SMR (Chabot, Steffensen, & Farrell, 2016; Tomlinson et al., 2018), under a range of nonlethal temperatures and salinity. We predicted that injury would initially suppress respiration after injury but that at later time points, regenerated worms would exhibit elevated respiration, a pattern demonstrated in a variety of animals (Collier, 1947; Hu et al., 2014; Needham, 1955, 1958; Stoner, 1970). If so, this would serve as evidence that, based on an application of the OCLTT framework, changes in organismal performance (i.e., survival) are based on changes in SMR after injury and regeneration. Contrary to our hypothesis, respirometry data indicate generally no effect of injury (at 1 dpa) or regeneration (at 5 dpa) on oxygen consumption under a range of temperatures or salinities (Figs. 3, 4). MO_2 increased predictably from colder to hotter temperatures (Fig. 3.3). However, while the interaction term between timepoint and temperature was significant ($P < 0.05$), injury condition was not, suggesting only an age or feeding restriction effect. There was a slight elevation in mass-specific MO_2 for posteriorly injured worms at 1 dpa and 23 °C compared to the other two injury conditions, comparable to MO_2 of posteriorly-injured worms at 35 °C, but this difference was statistically insignificant. Q_{10}, a measure of the sensitivity of biological processes to changes in environmental parameters (Mundim et al., 2020), of each injury condition at

both recovery timepoints was generally larger across intervals between lower temperatures, but there were no unusually large differences in Q_{10} between injury conditions or recovery timepoints. In the salinity assay, MO_2 increased from 0 to 6 ppt, but the timepoint and salinity interaction was just short of statistically significant (P = 0.0663) (Fig. 3.4). Q_{10} varied somewhat between injury conditions in the salinity assay but was not associated with statistical differences in MO_2, as mentioned. Ultimately, we found that the effects of injury and regeneration on oxygen consumption under a range of temperatures and salinity were ambiguous and statistically nonsignificant.

We noted in our thermal respirometry trials that, for all injury conditions, the estimated individual mass of worms was lower at 5 dpa than at 1 dpa ($P < 0.005$) (Fig. A2.3), a likely effect of feeding restriction. Allometric scaling of MO_2 was generally similar between injury conditions, temperatures, and recovery timepoints with some variation but no consistent relationships beyond an increase in individual MO_2 with increasing total mass (Fig. A2.4).

Gene expression

The absence of a relationship between survival and SMR led us to hypothesize that injury induces stress response pathways at the molecular level, effectively "frontloading" worms to tolerate additional stressors of differing nature in a manner similar to that hypothesized to underlie cross-tolerance between heat and hypoxia in amphipods (Collins et al., 2021). We used 3' TagSeq to test for differential expression of transcripts (hereafter "genes", differentially expressed genes = DEGs) in injured and heat-stressed worms at 1 dpa and identify overlapping features of these separate responses. We generated mRNA libraries from worms injured anteriorly (A) and

posteriorly (P) at 1 dpa, along with tissue controls to account for localized gene expression differences for each body region (CA and CP, respectively). Worms were subjected to nonlethal heat stress (35 °C) or held at room temperature (23 °C) within each injury group. For the remainder of this manuscript, we refer to these treatment combinations using the following shorthand: A23, P23, CA23, CP23, A35, P35, CA35, and CP35.

The size of the transcriptomic response to injury, heat, and these two factors combined may serve as an indicator of the relative physiological impact of each treatment, and how they differ when experienced simultaneously. We identified DEGs between a selected subset of comparisons of interest (Table 3.2; Fig. A2.5). In general, heat stress alone (CA35 x CA23 and CP35 x CP23) induced the greater number of DEGs of the two treatment factors when controlling for the other, although the most DEGs were detected within anteriorly injured worms after heat stress (A35 x A23), with 200 upregulated (UR) genes and 263 downregulated (DR) genes. The fewest total DEGs were induced by posterior injury under heat stress (P35 x CP35), with 80 UR and 16 DR genes, suggesting a marked difference in how different body fragments contribute to the overall stress response. The highest degree of differential expression overall was induced by combined injury and heat stress versus the respective control group: 1,650 total DEGs in the anterior-less group (A35 x CA23) and 1,296 total DEGs in the posterior-less group (P35 x CP23), numbers substantially higher than the combined DEGs induced by the factors separately, indicating a synergistic response to these two sources of stress. We also found 65 UR and 97 DR genes between worms without heads and worms without tails (CA23 x CP23), indicating spatially localized gene expression. Tables of the most

highly significant DEGs for each of these comparisons can be found in Appendix 2. Roughly one-third of the genes included in these analyses were unannotated (see Methods), which suggests that a considerable fraction of the *P. leidyi* transcriptome includes taxon-specific or otherwise undescribed genes.

If injury-induced molecular frontloading underlies improvements in stress tolerance, then this should be detectable as a molecular signature. Specifically, we expected to see the same genes, or genes with similar function, differentially expressed by both injury and thermal stress separately. We identified overlapping DEGs between selected combinations of our focal comparisons to identify candidate genes, focusing especially on those that may either confer a stress-protective effect or that may distinguish responses to stress between posterior and anterior tissue (Table 3.3). Lists of these overlapping DEGs (those differentially expressed in the same direction) for those combinations of comparisons that are of greatest interest can be found in Tables 3.4-7. Top DEGs broadly include a number of molecular chaperones, metabolic enzymes, genes involved in extracellular matrix composition, genes with immune function, genes with transport function, and genes involved with the regulation of cellular proliferation and development.

We then looked for overlap in the shared DEGs list to identify commonalities in the response to both injury and heat stress irrespective of which tissue was present. From this comparison, we identified only two genes: mitochondrial *hsp10* and mitochondrial *hsp70*, both of which were upregulated. Heat stress alone consistently upregulated other members of the heat shock protein (HSP) family, including alpha-crystallin, heat shock cognate 71, and several co-chaperones. Injury notably upregulated several histone

proteins, suggestive of elevated DNA replication during wound healing and early regeneration, and downregulated a number of membrane transporters, suggestive of changes in metabolic activity and cellular homeostasis. We noted that heat stress elicited a larger shared response between tissue fragments (160 total DEGs) than injury elicited between fragments (37 total DEGs), suggesting that localized expression may be a relatively greater proportion of the transcriptomic response to injury than to heat stress, the latter of which is applied to the entire animal rather than to a specific point on the body.

GO enrichment analyses of selected comparisons restricted to the anterior tissue-less group (for brevity, as we noted these did not differ substantially from posterior tissue-less comparisons) show consistent enrichment of genes involved in biological regulation, signal transduction, localization/transport, and response to stimulus across treatment factors (Fig. 3.5). Although broad, these process categories are expected to be affected by both injury and heat stress, and we find that little differentiates the most significantly enriched processes between the two factors. Similar GO categories are enriched under combined injury and heat stress, and we did not note major processes uniquely enriched by one factor and not the other. Interestingly, heat stress alone includes the enrichment of both UR and DR genes associated with cell, tissue, and organ system development. These and terms related to cellular differentiation and migration are predictably enriched in injured worms.

Discussion

In this study, we used a combination of experimental endpoints to broadly characterize how injury and regeneration affect the organismal response to abiotic

environmental stressors. By doing so, we attempt to provide a more integrative picture of these responses in a single organism than more traditional studies that examine one endpoint closely at a time. In particular, we controlled our experimental designs to align closely in timing and conditions such that we can draw links between endpoints, including survival, respiration rates, and gene expression. While many factors will influence the effects of any given injury and aspects of its recovery (Rennolds and Bely, submitted), we intend for our work to act as an example for controlling these factors through our choice of a relatively simple, clonally propagating, quickly regenerating organism and a multilevel approach that takes advantage of these qualities. Through this approach, we found that the effects of injury and regeneration on *P. leidyi* physiological condition are likely brief and subtle. Most notably, we discovered an unexpected synergy between the early post-injury response and environmental stress tolerance, highlighting the importance of studying the effects of injury under a variety of conditions and adding to the complex picture of how animal injury responses may have evolved.

That amputation injury improves short-term resistance to multiple forms of subsequent environmental stress is unexpected but may be a result of both the overlapping qualities of diverse, unrelated stressors and the conserved roots of the biological stress response at lower levels of organization. Although the origins of mechanical injury and thermal or osmotic stressors in nature are likely to be wholly separate and independent, these disparate sources of stress threaten biological function in at least some similar ways, including membrane or matrix disruption, generation of radical oxygen species, and the induction of molecular and cellular pathways that mitigate damage (Cossins & Prosser, 1978; Hazel & Williams, 1990; Somero, 2020).

These pathways include, for example, the heat shock protein family at the molecular level and endocrine pathways, respiratory and circulatory responses, and other feedback-driven systems at organismal levels (Barton, 2006; Calow, 1989; Evans & Kültz, 2020; Feder, M. E. & Hofmann, 1999; Kassahn et al., 2009; Kültz, 2004; Milisav, 2011; Shaughnessy et al., 2015; Todgham & Stillman, 2013; Yancey, 2020). Responses at higher levels are likely to diverge, as they involve great complexity that is dependent on the specific physiological traits and evolutionary history of a species (Sulmon et al., 2015). Yet many components of lower-level stress responses, collectively the CSR, are evidently ancestral and so may respond in a less divergent manner to diverse forms of homeostatic challenge between species. These may together partly explain why we found similarities in the transcriptomic response to injury and environmental stress that were not evident at the level of the whole organism.

In the present study, tissue, cell, and protein damage sustained as a result of amputation injury likely serve as signals to pathways that stimulate wound healing and various changes to physiological and cellular activity throughout the body of *P. leidyi*, as evidenced in prior studies (Zattara & Bely, 2011, 2013; Zattara et al., 2016). Injury thus may act to frontload protein expression and prime various pathways to provide elevated resistance to subsequent forms of stress that may similarly induce these responses, which may act on an insufficient time scale to prevent mortality under sufficiently extreme environmental stress. Such phenomena have been described in a vast assortment of studies and organisms under the umbrella of "hormesis" (Calabrese & Baldwin, 1998; Costantini et al., 2010). Nonlethal injury may ultimately act in a hormetic manner, altering the physiological status of the worm sufficiently for a short period to withstand

additional threats to biological integrity of a low enough magnitude or duration of exposure. However, the survival data presented were recorded under continuous exposure to a stressor, so whether the observed mortality is irreversible after a certain degree of exposure, and worms simply delayed death long enough to be distinguished statistically, cannot be discounted. Follow-up work should vary the intensity and duration of stress to discern whether improved thermal resistance is of any meaningful benefit.

Stress responses are theorized to manifest in a hierarchical manner, with whole-organism systemic adjustments preceding cellular-level responses. Therefore, potentially costly responses at the cellular level may be avoided under sufficiently low intensity or short duration stress, such as by modifying respiratory and cardiovascular function (Kassahn et al., 2009; Pörtner, 2002). Thus, the mechanism behind improved stress tolerance in recently injured worms and reduced heat tolerance in anteriorly-regenerated worms may be indicated by one or a combination of whole-organism and lower-order responses.

We first employed optical microplate respirometry to investigate metabolic responses after injury prior to regeneration as compared to after regeneration. According to OCLTT predictions, reductions in thermal tolerance (which may reasonably be generalized to any stressor, including salinity, that drives an increase in standard metabolic rate with increasing level of the stressor) result from reductions in aerobic scope, defined as the difference between maximum and standard metabolic rate at a given temperature (or respective level of other environmental factor), essentially the case of energy (ATP) demand exceeding that supplied by aerobic metabolism (Kassahn et al., 2009; Pörtner, 2010; Schulte, 2015; Sokolova, 2013; Sokolova et al., 2012). We

hypothesized that reduced heat tolerance in anteriorly-regenerated worms at 5 dpa may coincide with increased SMR when measured under elevated but nonlethal temperature, at which oxygen availability (solubility) is also expected to be reduced for *P. leidyi*'s most likely diffusion-dependent respiration. Improved heat and salinity tolerance could be attributed to metabolic quiescence in the immediate post-injury period, a pattern commonly observed in other animals and possibly indicated by a body-wide decrease in cellular activity in *P. leidyi* (Zattara & Bely, 2013). However, we did not note any clear relationship between respiration rates and patterns of survival under stress beyond the predictable increase in respiration as temperature and salinity increase. Due to *P. leidyi*'s small size and presumably diffusion-dependent respiration, OCLTT predictions may not be applicable to this organism in explanation of stress tolerance. We concluded that injury and regeneration do not cause major changes in metabolic rate at the timepoints tested, and thus metabolic rate changes do not explain differences in thermal tolerance between injury and regeneration treatments.

In a variety of animals, wound healing, including regeneration if present, is often characterized by a brief post-injury decrease of metabolic rate, which may be minimal, and a subsequent prolonged increase of metabolic rate as recovery progresses (Hu et al., 2014; Needham, 1955, 1958; Stoner, 1970). However, we did not observe this pattern, instead finding largely similar metabolic rates across recovery time periods. With respect to the absence of any detected metabolic depression, it is possible that an initial metabolic depression does occur in *P. leidyi*, but that it occurred and passed before our earliest measurement time at 1 dpa. Prior work in this species has shown that cellular proliferation remains reduced for several days following amputation (Zattara & Bely,

2013), therefore whole-body respiration rates may not be significantly affected by proliferation patterns. We also did not observe any regeneration-associated metabolic elevation. Again, it is possible that a metabolic increase does occur in *P. leidyi*, but that it occurred prior to (or possibly following) 5 dpa. Studies in *Tubifex*, another annelid in the same family as *P. leidyi*, found an elevated metabolic rate only during the later differentiation phase of regeneration (Collier, 1947) and that the oxygen dependence of this stage contrasts with oxygen-independent regeneration initiation, suggesting largely different metabolic pathways (Anderson, 1956). Later differentiation concludes just before 5 dpa in *P. leidyi* on average (Zattara & Bely, 2011), and so we may have missed a similar response in our experiment. Recent work in planarians, however, complicates understanding of post-injury metabolic responses: in contrast to other animals, injury induces a spike in oxygen uptake just hours following amputation in *Schmidtea mediterranea*, followed by a depression at or below resting metabolism throughout the remainder of regeneration in a manner dependent on sexual mode and which body fragment is regenerating (Lewallen & Burggren, 2022). This together with our own findings suggests that clonally reproducing animals may respond to injury in a quite distinct manner from sexual reproducers and that some body structures may be substantially more expensive to regenerate than others even without differing much in mass. An alternative possibility is simply that neither injury nor regeneration elicit detectable changes in metabolic rate in *P. leidyi*, which if true has important implications for our understanding of comparative regeneration physiology. In a study by Starostová et al. (2017), tail regeneration elicited no appreciable effect on metabolic rate in juvenile geckos with unlimited food access, suggesting that metabolic changes during

regeneration are likely to be conditional on factors like nutritional status, life history stage, and species-specific physiological traits.

Differences in the functional contributions of body parts and in the physiological changes required to regenerate them, if possible, may underlie the costs of injury and regeneration of those body parts, respectively. The anterior and posterior segments of *P. leidyi* possess a high degree of metameric anatomical and functional redundancy that characterizes the annelid body plan broadly (Balavoine, 2014) yet are distinguished in a number of key features. Both ends of the body include metabolically active growth zones in which cellular proliferation and differentiation proceed at relatively constant rates (Zattara & Bely, 2013); the anterior segments include the mouth, brain, and proboscis; and the posterior segments include the hindgut. Had there been differences in survival or oxygen uptake between anteriorly- and posteriorly-amputated worms, we may have speculated on the contributions of, for example, the brain in neural and endocrine inputs on the physiological response to stress, or posterior modifications to improve oxygen uptake as observed in other annelids (admittedly much larger and less effective at meeting oxygen needs through simple diffusion) (Glasby, Erséus, & Martin, 2021; Julian, Passman, & Arp, 1996). That these organs play no role cannot be discounted but were not detected at our experimental resolution or metrics. However, reduced heat tolerance in anteriorly- but not posteriorly-regenerated worms could be associated with elevated resource demands of anterior regeneration, which involves both robust epimorphic regeneration and morphallactic remodeling of downstream segments when more than four segments are removed (Zattara & Bely, 2011). By contrast, posterior regeneration does not involve morphallaxis in *P. leidyi* and investment is often more variable and

sensitive to feeding (Zattara & Bely, 2011; pers. obs.). Future work should make use of calorimetry or other assays to deduce the relative energetic costs of anterior versus posterior regeneration as well as the responsiveness of these processes to nutrition.

Our findings at the transcriptomic level provide some intriguing insights that may explain injury-induced improvement in stress tolerance. Differences in expression between injured and heat-stressed worms separately involve highly divergent groups of specific genes overall, with, for example, only 2.94% of the total DEG pool (18 of 630 total DEGs) being shared genes significantly expressed in a similar manner between factors among anterior-less worms and 4.94% (28 of 567) among posterior-less worms. However, enriched GO categories are highly comparable between both separate and combined treatments, suggesting that different molecular players are utilized in nevertheless, to some extent, broadly similar processes. We acknowledge and appreciate that these very general biological processes do not capture the diversity of genes that are involved in the transcriptomic responses to our experimental treatments. We additionally found that even the shared responses to our two experimental factors differed markedly based on which tissue was present in our samples, as only 2 of a combined 46 DEGs common to injury and heat stress were shared between anterior- and posterior-less worms.

We discovered that two genes were consistently upregulated by both injury and heat stress regardless of where amputation occurred: mitochondrial variants of members of the heat shock family of proteins, *hsp10* and *hsp70*. Heat shock proteins are upregulated following injury in myriad diverse species (Husmann et al., 2014; Li et al., 2014; Matranga et al., 2000; Pinsino et al., 2007; Sánchez Navarro et al., 2009; Stewart et

al., 2017; Sveen et al., 2019; Wenger et al., 2014), including the annelid *Lumbriculus variegatus* (Tellez-Garcia et al., 2021), and some, such as *hsp60*, which was commonly upregulated in our own treatment comparisons, function in regulating the regeneration process (Li et al., 2014; Makino et al., 2005; Patruno et al., 2001; Pei et al., 2016). As suggested by their name, heat shock proteins were initially characterized by their elicitation following heat stress, although upregulation may be triggered by a variety of stressors, and function broadly to maintain protein integrity, facilitate turnover of damaged proteins, and regulate expression of other genes involved in the CSR and innate immune response (Feder, M. E. & Hofmann, 1999; Kültz, 2020b; Richter et al., 2010; Sørensen, Kristensen, & Loeschcke, 2003). Members of this group of proteins unsurprisingly occur throughout our DEG lists, but this work is the first to our knowledge to compare their expression between injury and other stress within a single study. Given these expression patterns and known functions, they are candidates for further investigation into their possible role in conferring injury-induced improvement in stress tolerance, ideally by quantifying expression over a finer temporal scale and targeted inactivation studies.

Other DEGs of interest, particularly those shared between injured and heat-stressed worms, may be clustered via their role in a handful of broad functional pathways. Periostin (Conway et al., 2014; Ito et al., 2021) and tenascin (Chen et al., 2010; Onda et al., 1991; Sun et al., 2013), proteins involved in extracellular matrix composition and remodeling, are consistently upregulated by heat yet have been implicated in wound healing and regeneration in other animals, suggesting similarity in disruptions to tissue structure. Common downregulation of pantothenate kinase, the rate-limiting enzyme of

coenzyme A synthesis (Leonardi et al., 2005), suggests a stress-induced metabolic adjustment. Prostaglandin E synthase upregulation indicates inflammatory stimulation and may be of interest due to its demonstrated protective functions (Echeverria, Clerman, & Doré, 2005; Tanioka et al., 2000). Upregulation of prohibitin-2, a key receptor mediating mitochondrial autophagy (Wei et al., 2017), is implicated in regulating cell migration during regeneration (Nishidate et al., 2007; Rajalingam et al., 2005). These and other DEGs suggest that wound healing and regeneration pathways have roots in the CSR, which could provoke a number of intriguing hypotheses concerning the factors associated with the evolution of regeneration in annelids and other animals.

Differences in gene expression between anterior and posterior body fragments suggest a considerable degree of localized contributions to the organismal stress response. In *Caenorhabditis elegans*, a pair of anterior neurons coordinate cellular heat shock responses across the entire organism likely through neuroendocrine signaling, although cells respond autonomously in the absence of this signaling (Prahlad, Cornelius, & Morimoto, 2008). Differences in the transcriptional response or even survival under heat stress between fragments in *P. leidyi* may result from a disruption to organismal coordination of the stress response by neural structures in a similar manner, although far less is known about neuroendocrine physiology in the annelids generally. Further development of functional molecular techniques in naids and other annelids will allow further investigation of neural contributions to cellular and organismal stress responses. In syllids, the transcriptomic profiles between early anterior and posterior regeneration are also quite distinct, with the latter more closely resembling gene expression patterns of normal growth conditions than they resemble the former (Ribeiro et al., 2019).

Developmental events that follow injury vary with the location of injury in *P. leidyi* (Zattara & Bely, 2013), and posterior regeneration resembles growth in many ways in *P. leidyi,* as it also does paratomic fission (Zattara & Bely, 2011). Our findings contribute to the knowledge of expression patterns that may underly the differences between early anterior and posterior regeneration in this species. These differences appear to be largely in the expression of specific genes rather than in the biological processes those genes are associated with, per our gene ontology results. Comparisons with potential future work in other naids, including those with less regenerative capacity such as *Paranais* and *Chaetogaster* spp. (Bely & Sikes, 2010), would provide insights into candidate targets of selection contributing to regeneration gain, loss, and other variation in the taxon.

Combinations of stressors often elicit transcriptomic and physiological responses of a magnitude not predictable from those elicited separately (Crain, Kroeker, & Halpern, 2008; DeBiasse & Kelly, 2016; Gunderson et al., 2015; Holmstrup et al., 2010; Sokolova, 2021; Todgham & Stillman, 2013; Vasquez et al., 2015; Vinebrooke et al., 2004). We found that injury and heat stress act synergistically on gene expression in *P. leidyi*, with many more up- and downregulated genes than the sum of those detected in separate comparisons in either fragment of the worm body. However, the most significantly enriched GO terms were not much different than those of either stressor separately, suggesting that the synergistic effect is in the degree rather than the nature of the response. Studies of multiple-stressor interactions are often focused on stressors associated with either climate change (e.g., temperature, hypoxia, pH) or anthropogenic pollution (e.g., heavy metals, endocrine disruptors), whilst interactions between injury and any of these factors receive far less research attention. It is worth considering these

potential interactions for ecological and mechanistic reasons. Climate change may alter biotic factors such as habitat occupant density, community composition, and species interactions, along with abiotic factors such as storm frequency or intensity and hydroperiods, that affect the frequency, severity, or context of injury, including the likelihood that both injury and other forms of stress are experienced simultaneously. At the mechanistic level, induction of shared molecular pathways could lead to cross-tolerance to other stressors by injury or vice versa, as suggested by the present study and occurs between various other stressors (Collins et al., 2021; Gotcha, Terblanche, & Nyamukondiwa, 2018; Gunderson et al., 2015; Sinclair, B. J. et al., 2013; Todgham & Stillman, 2013). Predictions of animal responses to environmental dynamics that fail to incorporate injury effects may miss an important factor in the complexity of such responses.

Tables

Table 3.1. *P. leidyi* IsoSeq transcriptome features and BUSCO (Manni et al., 2021) statistics against the metazoan gene set.

No. transcripts	111961
Complete BUSCOs	847
Complete and single-copy BUSCOs	279
Complete and duplicated BUSCOs	568
Fragmented BUSCOs	4
Missing BUSCOs	103
Total BUSCO groups searched	954

Table 3.2. Number of upregulated (UR) and downregulated (DR) genes (FDR < 0.05) for selected pairwise comparisons.

	A23 x CA23	CA35 x CA23	A35 x CA35	A35 x A23	A35 x CA23	P23 x CP23	CP35 x CP23	P35 x P23	P35 x CP35	P35 x CP23	CA23 x CP23
UR	97	195	85	200	625	157	186	87	80	634	65
DR	130	208	191	263	1025	48	176	104	16	662	97
Total	227	403	276	463	1650	205	362	191	96	1296	162

Table 3.3. Number of shared upregulated (UR) and downregulated (DR) genes (FDR < 0.05) for a selected subset of overlaps between pairwise comparisons in Table 3.2.

	A23 x CA23 / CA35 x CA23	P23 x CP23 / CP35 x CP23	A35 x CA35 / P35 x CP35	A23 x CA23 / P23 x CP23	A35 x A23 / P35 x P23	CA35 x CA23 / CP35 x CP23	A35 x CA23 / P35 x CP23
UR	11	19	9	29	60	89	141
DR	7	9	10	8	59	71	214
Total	18	28	19	37	119	160	355

Table 3.4. DEGs (FDR < 0.05) shared between injury-alone and heat-alone comparisons for anterior-less worms (A23 x CA23 / CA35 x CA23).

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Direction
92163	ASH2L_HUMAN	Set1/Ash2 histone methyltransferase complex subunit ASH2	3.85E-174	+
97304	MA2B1_MOUSE	Lysosomal alpha-mannosidase	5.77E-20	+
105690				+
57865				+
70447	AGAL_MOUSE	Alpha-galactosidase A	1.46E-29	+

79043	GRP75_PONAB	Stress-70 protein, mitochondrial	0	+
111384	CH10_ORYLA	10 kDa heat shock protein, mitochondrial	4.96E-43	+
68413				+
74053	ANM5_MOUSE	Protein arginine N-methyltransferase 5	0	+
39644	PPBT_BOVIN	Alkaline phosphatase, tissue-nonspecific isozyme	1.05E-150	+
104156	TEBP_RABIT	Prostaglandin E synthase 3	9.91E-26	+
98623				-
107944	COPT1_PONAB	High affinity copper uptake protein 1	2.58E-22	-
106668				-
107656	SYWC_BOVIN	Tryptophan--tRNA ligase, cytoplasmic	1.38E-149	-
71501	PANK1_ORYSJ	Pantothenate kinase 1	5.59E-18	-
110598	HPGDS_CHICK	Hematopoietic prostaglandin D synthase	5.97E-47	-
51758	CHS_MELAT	Chitin synthase	5.08E-140	-

Table 3.5. DEGs (FDR < 0.05) shared between injury-alone and heat-alone comparisons for posterior-less worms (P23 x CP23 / CP35 x CP23).

Transcript ID	**UniProtKB / Pfam ID**	**Protein**	**E-value**	**Direction**
111384	CH10_ORYLA	10 kDa heat shock protein, mitochondrial	4.96E-43	+
83206	CH60_CHICK	60 kDa heat shock protein, mitochondrial	0	+
79043	GRP75_PONAB	Stress-70 protein, mitochondrial	0	+
22946	SYAC_MOUSE	Alanine--tRNA ligase, cytoplasmic	0	+
81398	DAG1_BOVIN	Dystroglycan 1	9.97E-08	+
88942	CKS1_MOUSE	Cyclin-dependent kinases regulatory subunit 1	1.42E-29	+
101878	DDX47_HUMAN	Probable ATP-dependent RNA helicase DDX47	0	+
109067	PHB2_MOUSE	Prohibitin-2	5.66E-156	+
77071	GLU2B_HUMAN	Glucosidase 2 subunit beta	1.28E-111	+

103977	IPYR_DROME	Inorganic pyrophosphatase	1.21E-32	+
12883	MTMR2_BOVIN	Myotubularin-related protein 2	0	+
32108	S2611_HUMAN	Sodium-independent sulfate anion transporter	1.29E-150	+
93210	DCOR_RAT	Ornithine decarboxylase	4.85E-146	+
5552				+
80232	SYEP_DROME	Bifunctional glutamate/proline--tRNA ligase	0	+
1461	SHOC2_XENLA	Leucine-rich repeat protein SHOC-2	3.29E-176	+
99280	CALM_ELEEL	Calmodulin	2.31E-82	+
98282	SRCH_RABIT	Sarcoplasmic reticulum histidine-rich calcium-binding protein	1.26E-19	+
100967	FNTA_BOVIN	Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha	2.34E-123	+
85010	HGNAT_MOUSE	Heparan-alpha-glucosaminide N-acetyltransferase	1.16E-117	-
106791	KAD1_CHICK	Adenylate kinase isoenzyme 1	3.61E-80	-
111705				-
109733	RLT1_RHIO9	Mucoricin	2.85E-06	-
96403	FCN2_HUMAN	Ficolin-2	1.17E-58	-
110358	SM20_SCHMA	20 kDa calcium-binding protein	9.31E-19	-
111157	LYPL1_HUMAN	Lysophospholipase-like protein 1	4.68E-75	-
98223	CP4F_SHEEP	Prostaglandin E2 omega-hydroxylase CYP4F21	4.18E-138	-
105387	COFI_OGAPD	Cofilin	2.45E-36	-

Table 3.6. DEGs (FDR < 0.05) shared between injury-alone comparisons for both body fragment groups (A23 x CA23 / P23 x CP23).

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Direction
96233	PUR6_CHICK	Multifunctional protein ADE2	0	+
97432	PUR6_CHICK	Multifunctional protein ADE2	0	+
88942	CKS1_MOUSE	Cyclin-dependent kinases regulatory subunit 1	1.42E-29	+
108369	GRN_DICDI	Granulin	7.03E-06	+
88157	ADH_SULAC	NAD-dependent alcohol dehydrogenase	3.72E-16	+

106647	EPDR1_HUMAN	Mammalian ependymin-related protein 1	5.47E-05	+
79013	MIOX_DANRE	Inositol oxygenase	1.16E-113	+
110851	IF5A_DROME	Eukaryotic translation initiation factor 5A	1.85E-59	+
22453	AAKG2_HUMAN	5'-AMP-activated protein kinase subunit gamma-2	8.59E-76	+
110968				+
104447	HMG2_DROME	High mobility group protein DSP1	1.03E-65	+
35544	RIR1_MOUSE	Ribonucleoside-diphosphate reductase large subunit	0	+
102670				+
78465	PF07679.19	Immunoglobulin I-set domain	8.10E-06	+
111063	H2AX_HUMAN	Histone H2AX	1.11E-77	+
111461	H2AV_XENTR	Histone H2A.V	4.18E-64	+
26720	NLK_HUMAN	Serine/threonine-protein kinase NLK	2.01E-159	+
108398	RET1_RAT	Retinol-binding protein 1	9.67E-05	+
15612	FKBP4_SPOFR	46 kDa FK506-binding nuclear protein	7.49E-28	+
93326	HDHD5_HUMAN	Haloacid dehalogenase-like hydrolase domain-containing 5	2.68E-94	+
107200	AN32A_DROPS	Acidic leucine-rich nuclear phosphoprotein 32 family member A	5.03E-47	+
79043	GRP75_PONAB	Stress-70 protein, mitochondrial	0	+
96623	ALRF2_MOUSE	Aly/REF export factor 2	8.71E-47	+
106189	PSB7_MOUSE	Proteasome subunit beta type-7	6.72E-129	+
94138	TCPE_MACFA	T-complex protein 1 subunit epsilon	0	+
111384	CH10_ORYLA	10 kDa heat shock protein, mitochondrial	4.96E-43	+
66139	DD19A_BOVIN	ATP-dependent RNA helicase DDX19A	0	+
109067	PHB2_MOUSE	Prohibitin-2	5.66E-156	+
92574	SERA_HUMAN	D-3-phosphoglycerate dehydrogenase	2.62E-145	+
98411	CAH15_MOUSE	Carbonic anhydrase 15	1.57E-48	-
91287	PUNA_GEOSE	Purine nucleoside phosphorylase 1	1.97E-17	-
80562	CEL_BOVIN	Bile salt-activated lipase	3.44E-65	-
110575	COPT1_PONAB	High affinity copper uptake protein 1	2.71E-19	-
94480				-
82046	S22A3_MOUSE	Solute carrier family 22 member 3	7.25E-58	-

81549	AQP9_HUMAN	Aquaporin-9	1.94E-52	-
96403	FCN2_HUMAN	Ficolin-2	1.17E-58	-

Table 3.7. DEGs (FDR < 0.05; max 50 shown) shared between heat-alone comparisons for both body fragment groups (CA35 x CA23 / CP35 x CP23).

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Direction
109908	CRYAB_MACFA	Alpha-crystallin B chain	1.34E-15	+
111384	CH10_ORYLA	10 kDa heat shock protein, mitochondrial	4.96E-43	+
99284	POSTN_MOUSE	Periostin	1.81E-17	+
84988	TENX_HUMAN	Tenascin-X	5.16E-29	+
77307	HSP7C_SAGOE	Heat shock cognate 71 kDa protein	0	+
108040	U2AF4_RAT	Splicing factor U2AF 26 kDa subunit	1.51E-122	+
108614	CB076_DANRE	UPF0538 protein C2orf76 homolog	6.33E-30	+
99029	CDC37_DROVI	Hsp90 co-chaperone Cdc37	3.34E-91	+
81037	STIP1_BOVIN	Stress-induced-phosphoprotein 1	2.22E-144	+
105630	GHITM_HUMAN	Growth hormone-inducible transmembrane protein	3.40E-108	+
92507				+
96808	UBE2A_MOUSE	Ubiquitin-conjugating enzyme E2 A	1.06E-82	+
93864	AHSA1_HUMAN	Activator of 90 kDa heat shock protein ATPase homolog 1	2.96E-106	+
98668	F10A1_CHICK	Hsc70-interacting protein	4.14E-52	+
103578	KTR3_YEAST	Probable mannosyltransferase KTR3	4.27E-25	+
20983	MARH6_MOUSE	E3 ubiquitin-protein ligase MARCHF6	0	+
89924				+
108657	CREB_HYDVD	Cyclic AMP-responsive element-binding protein	6.30E-11	+
71909	AHSA1_HUMAN	Activator of 90 kDa heat shock protein ATPase homolog 1	2.68E-103	+
99989	FKBP4_HUMAN	Peptidyl-prolyl cis-trans isomerase FKBP4	1.04E-116	+
110191				+

103787	SRSF7_HUMAN	Serine/arginine-rich splicing factor 7	1.66E-19	+
104156	TEBP_RABIT	Prostaglandin E synthase 3	9.91E-26	+
77997	SUV3_DANRE	ATP-dependent RNA helicase SUPV3L1, mitochondrial	0	+
82094	GDIA_MACFA	Rab GDP dissociation inhibitor alpha	0	+
86448	SMCE1_MOUSE	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	3.11E-13	-
110342				-
109078	SET_HUMAN	Protein SET	2.40E-46	-
84031	PRUN1_MOUSE	Exopolyphosphatase PRUNE1	2.20E-24	-
105147	HMG2_DROME	High mobility group protein DSP1	3.20E-59	-
85106	HSP83_DROMI	Heat shock protein 83	7.41E-45	-
110541	ATPO_PIG	ATP synthase subunit O, mitochondrial	1.82E-70	-
111993				-
110722				-
110084	PDLI7_HUMAN	PDZ and LIM domain protein 7	3.36E-11	-
106037	PF17064.8	Sleepless protein	3.60E-07	-
106668				-
99527	BASI_CHICK	Basigin	1.31E-17	-
100763	FIBA_APOPA	Fibrinogen-like protein A	2.02E-26	-
110596				-
110324				-
101142	PANK1_ORYSJ	Pantothenate kinase 1	2.50E-11	-
111639				-
111542				-
109885	SM16_SCHMA	16 kDa calcium-binding protein	1.01E-20	-
109879				-
69592				-
49152	ESN_DROME	Protein espinas	1.17E-05	-
75628	YJX4_SCHPO	CRAL-TRIO domain-containing protein C23B6.04c	5.82E-12	-
109244	QCR1_MOUSE	Cytochrome b-c1 complex subunit 1, mitochondrial	1.09E-99	-

Figures

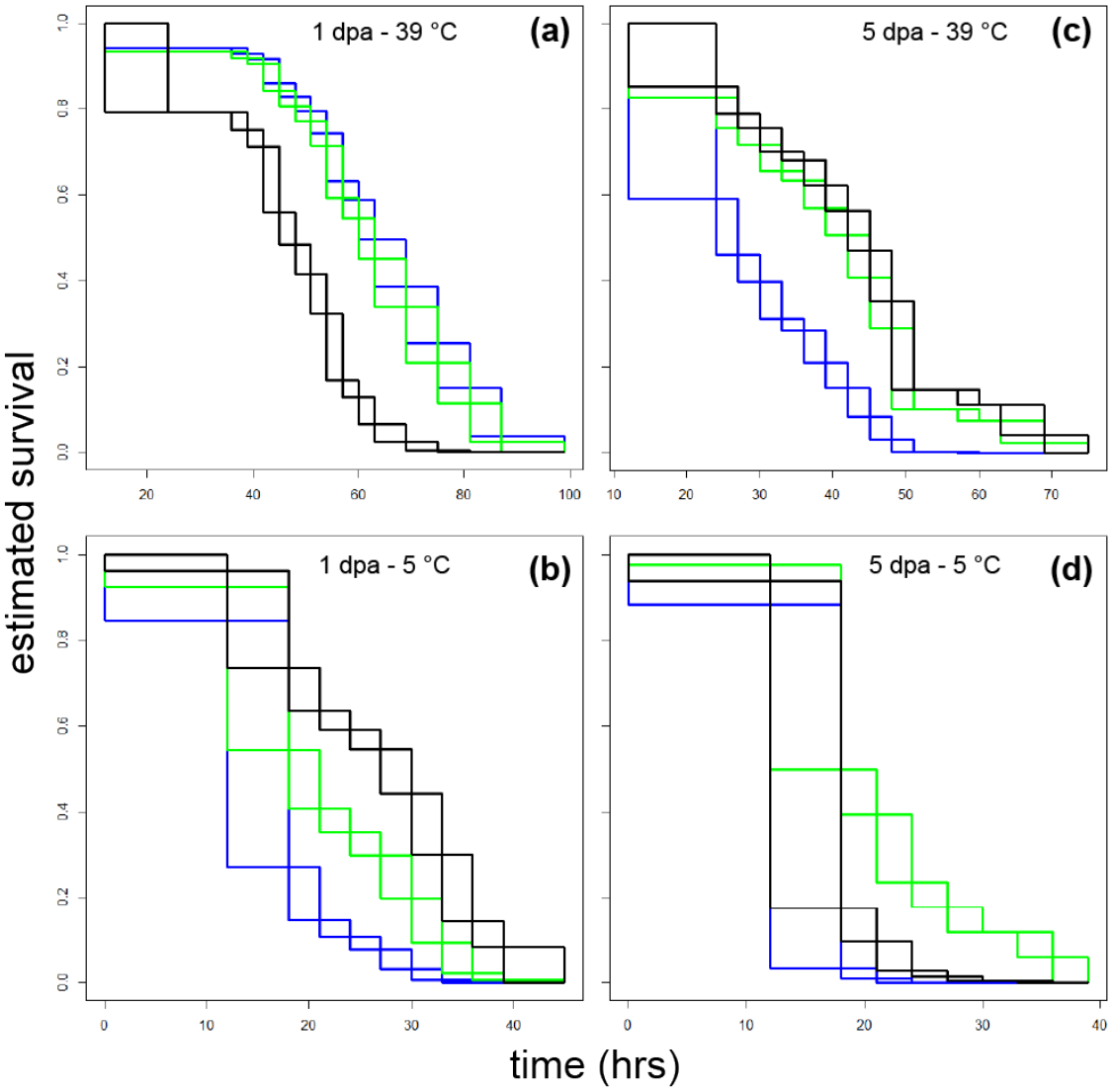

Figure 3.1. Estimated survival of worms under continuous exposure to thermal stress (39° C: a,c; 5° C: b,d) at 1 dpa (a,b) or 5 dpa (c,d). Color indicates injury condition (black = uninjured, blue = anterior amputation, green = posterior amputation). Each curve represents n = 15 worms. Survival data are interval-censored between timepoints when survival was scored in each trial, represented by the points when upper and lower bounds of each estimate curve intersect. Significant differences between both injury conditions and control were detected in (a) (both $P < 0.05$) and between anteriorly-regenerated

worms and control in (c) ($P = 0.01222$) via Kaplan-Meier survival analysis with Cox proportional hazards models.

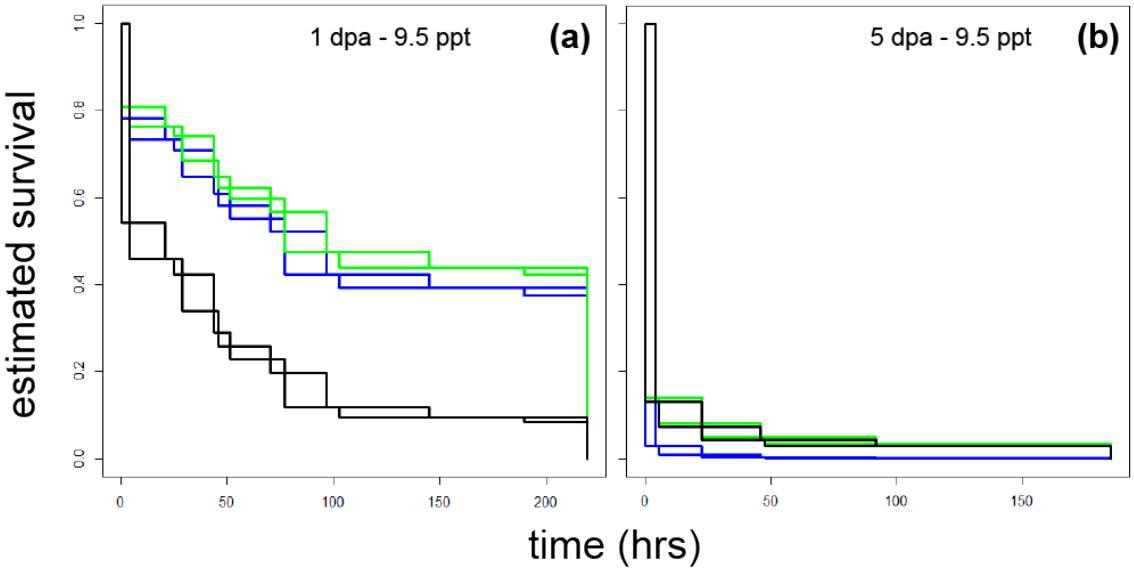

Figure 3.2. Estimated survival of worms under continuous exposure to salinity stress (9.5 ppt) at 1 dpa (a) or 5 dpa (b). Color indicates injury condition (black = uninjured, blue = anterior amputation, green = posterior amputation). Each curve represents $n = 15$ worms. Survival data are interval-censored between timepoints when survival was scored in each trial, represented by the points when upper and lower bounds of each estimate curve intersect. Significant differences between both injury conditions and controls were detected in (a) (both $P < 0.05$) via Kaplan-Meier survival analysis with Cox proportional hazards models.

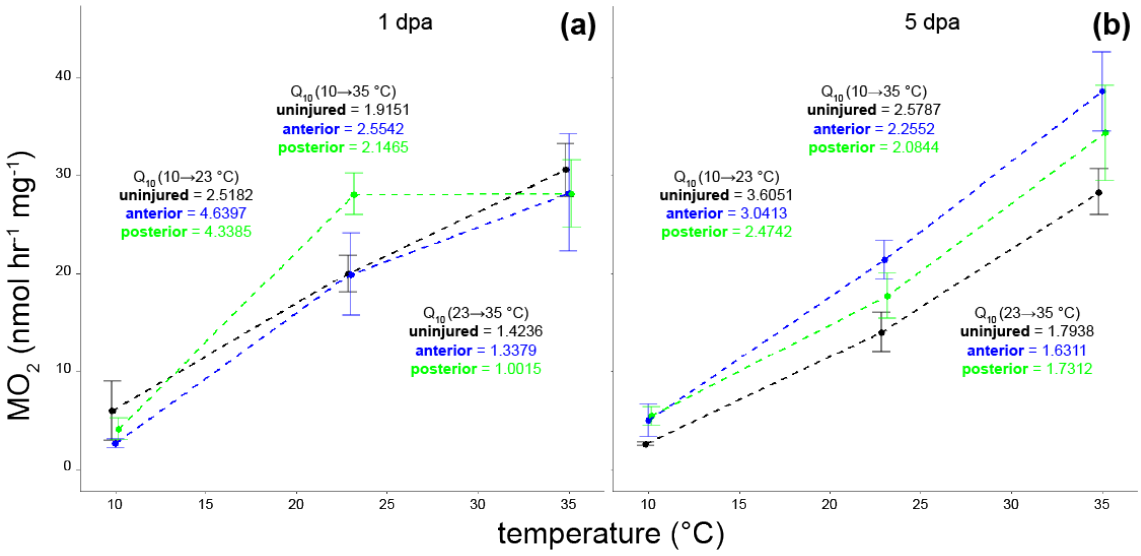

Figure 3.3. Plots of temperature versus mass-specific MO_2 for 1 dpa (a) and 5 dpa (b) worms under each injury condition (black = uninjured, blue = anterior amputation, green = posterior amputation). Bars indicate the standard error. Q_{10} of each injury condition at each time point between experimental temperatures is shown above each respective temperature interval. Each point represents n = 18 worms pooled from three separate 6-worm trials. Points at the same temperature are slightly offset for visibility.

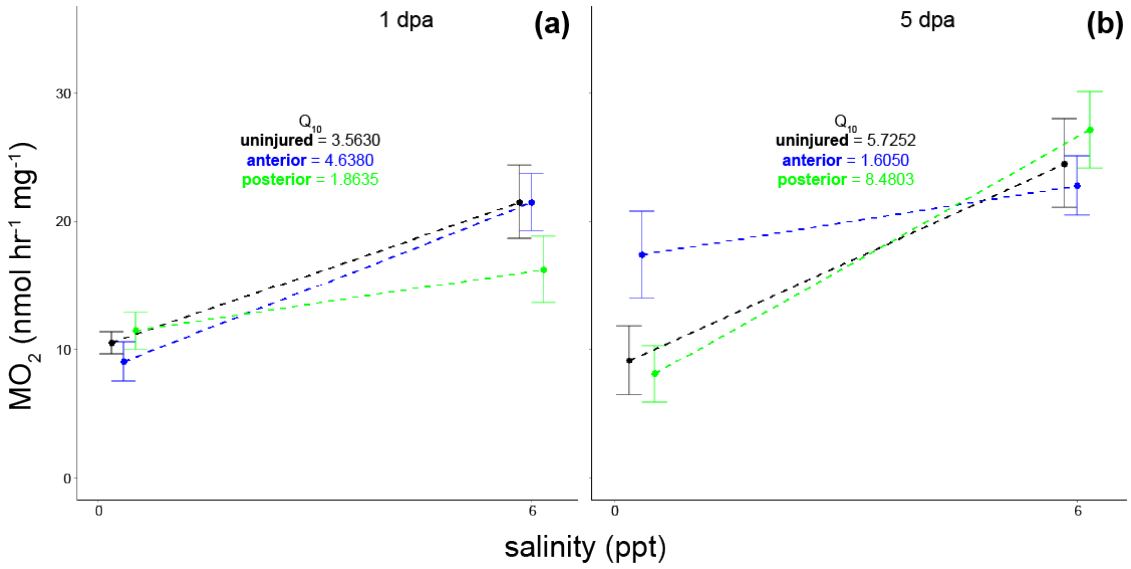

Figure 3.4. Plots of salinity versus mass-specific MO_2 for 1 dpa (a) and 5 dpa (b) worms under each injury condition (black = uninjured, blue = anterior amputation, green = posterior amputation). Bars indicate the standard error. Each point represents $n = 6$ worms from one trial (at 0.35 ppt) or $n = 18$ worms pooled from three separate 6-worm trials (at 6 ppt). Points at the same salinity level are slightly offset for visibility.

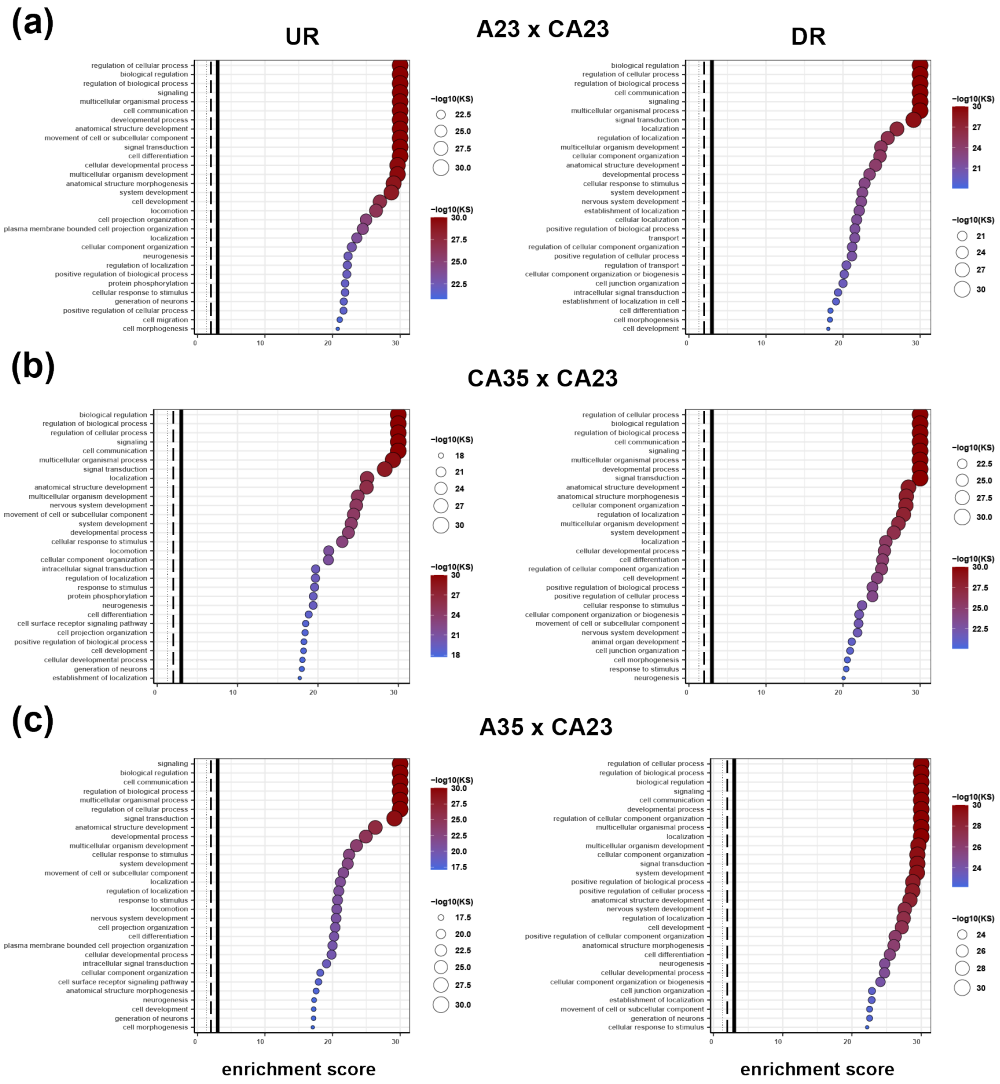

Figure 3.5. Results of gene ontology enrichment analysis for DEGs between select pairwise comparisons of the anterior-less groups: A23 x CA23 (a), CA35 x CA23 (b), A35 x CA23 (c). The top 30 most significantly enriched biological process (BP) GO terms are ranked in descending order by Kolmogorov-Smirnov *P*-value. Plots of enriched terms in upregulated DEGs (logFC > 0) are shown on the left and in downregulated DEGs (logFC < 0) on the right. Dot color and size indicate the negative log-adjusted enrichment score. To simplify plotting, enrichment scores less than 1e-30 were set to 1e-30.

Chapter 4: Investment in regeneration versus asexual reproduction is resource-dependent in a freshwater annelid

Abstract

The post-embryonic developmental processes of regeneration and asexual agametic reproduction are widespread and often co-occur in animals. These traits are thus of great ecological significance, but their physiological dynamics within species are not well understood. In naid annelids, regeneration and asexual reproduction via fission are evolutionarily related and mechanistically similar yet distinct, making these animals useful systems in which to study resource allocation strategies between the two processes. We tested how asexual reproductive investment varies as a function of somatic investment demands by repeatedly amputating individuals of the naid *Pristina leidyi*, allowing regeneration to proceed, and measuring reproductive output over time. We replicated these treatments under high and low food levels to determine to what extent the investment dynamic between regeneration and fission is affected by the resource pool. We found that reproductive output was affected by injury and regeneration frequency in a resource-dependent manner, such that only worms with less food availability exhibited reproductive deficits attributable to an injury and regeneration frequency × feeding level interaction. When reproductive output was decreased, this occurred not through a reduction in offspring quantity but a reduction in offspring quality. Unlike fission speed, regeneration speed in offspring was unaffected by any experimental variables. Our findings suggest that: 1) the resource pool is a key factor mediating the resource investment pattern between regeneration and fission in this species; 2) sacrificing per-

offspring investment rather than fecundity may be an optimal strategy if resources are limiting; 3) regeneration and fission have evolved distinct resource allocation pathways. Our results prompt further questions about the adaptive significance of these dynamics in *P. leidyi* and whether similar patterns hold true in other regenerating, asexually reproducing lineages.

Introduction

A common life history trade-off in animals is that between somatic and reproductive investment. Somatic investment may take the form of increased body mass (such as growth or storage) or elevated levels of repair, metabolism, or immune function, while reproductive investment may manifest as increased offspring number, quality, or postnatal survival. Numerous and diverse species experience decreased investment in one as investment in the other increases, as demonstrated in annelids (Aira et al., 2007), insects (e.g.: Bascuñán-García, Lara, & Córdoba-Aguilar, 2010; Kelly, 2011; Stahlschmidt et al., 2013), and vertebrates (e.g.: Dial & Fitzpatrick, 1981; Gélin et al., 2016; Larue et al., 2021; van Rooij et al., 1995). This trade-off is not universal, however, and varies substantially between species, taxa, and various intrinsic and extrinsic conditions (Glazier, 1999; Heino & Kaitala, 1999). Ecologists often hypothesize that intraorganismal competition over finite resources underlies this trade-off and others (Worley, Houle, & Barret, 2003). Animals therefore experience pressure to evolve optimal strategies for efficient allocation between processes (Perrin & Sibly, 1993), which may exhibit plastic variation in response to the resource pool itself (i.e., availability of food). The size of the resource pool has been implicated in some of the variation in patterns of somatic versus reproductive investment (Ng'oma, Perinchery, &

King, 2017; Zera & Harshman, 2001). Modeling work has explored which allocation strategies should be optimal under different resource conditions (de Jong, G. & van Noordwijk, 1992; Fischer, Dieckmann, & Taborsky, 2011; Glazier, 1999; Roff & Fairbairn, 2007; Yoshida, 2006), but current understanding of these strategies is limited by the availability of experimental data in real organisms. Such work is important to pursue, because determining how a species invests proportionally in various processes under certain conditions can generate useful insights about that species' physiological capabilities, life history strategy, and possibly evolution.

There is a notable knowledge gap pertaining to resource allocation strategies involving post-embryonic developmental processes, namely regeneration and asexual agametic reproduction (hereafter, asexual reproduction) (e.g., budding, fission, fragmentation). Both traits often co-occur in soft-bodied taxa like cnidarians, planarians, and annelids (Brockes & Kumar, 2008; Giangrande & Licciano, 2014; Kostyuchenko & Kozin, 2020; Zattara & Bely, 2016). Given the prevalence of injury in wild populations of these groups (Lindsay, 2010), allocation strategies involving regeneration and reproduction are ecologically relevant and likely have been shaped by natural selection. Both processes require investment of resources, leading to short- and possibly long-term physiological and higher-order consequences. The costs of both traumatic loss of a body part and the subsequent regeneration of that part, in which the structure and function is (at least partly) restored over time, can be wide-ranging and significant (Archie, 2013; Bely, 2010; Bernardo & Agosta, 2005; Lawrence, John M., 2010; Maginnis, 2006b; Starostová et al., 2017). For example, a considerable amount of research has investigated the effects of injury and regeneration on sexual reproduction. These effects include lower

reproductive rates or smaller litter sizes (Bernardo & Agosta, 2005; Sepulveda et al., 2008; Zajac, 1985), reduced follicle mass (French et al., 2007), lower reproductive effort (Chapple, McCoull, & Swain, 2002), skipped reproductive periods (Maiorana, 1977), extended brooding time (Zajac, 1985), or lower-quality eggs or offspring (Dial & Fitzpatrick, 1981). In some species, however, injury and regeneration have led to increases in reproductive investment (Altincicek et al., 2008; Beatty et al., 2021; Fox & McCoy, 2000). Regeneration impacts on asexual reproduction, by contrast, are not well described. Asexual reproduction by fission or fragmentation is expected to be costly as well since it involves the direct loss of somatic tissue, and the resources contained within, to create offspring. Physiological assessments of the cost of asexual reproduction, however, remain scarce, including in relation to regeneration.

Species capable of both regeneration and fission are particularly useful study systems for understanding tradeoffs between repair and asexual reproduction. This is because regeneration and fission appear to be closely related both mechanistically and evolutionarily in a number of animal groups (Brockes & Kumar, 2008; Giangrande & Licciano, 2014; Martinez, V. G. et al., 2005). In annelids, for example, fission itself most likely evolved through co-option of pathways used for regeneration (Bely & Wray, 2001; Zattara & Bely, 2016) and the developmental processes of fission closely resemble those of regeneration (Bely & Nyberg, 2010). Due to these overlaps, the physiological relationship between these processes is not necessarily zero-sum. Injury might in fact accelerate reproduction through the allocation of resources towards pathways shared by both regeneration and asexual reproduction, as hypothesized for some corals (Henry et al., 2003), or facilitate asexual reproduction by breaking individuals into pieces that then

regenerate into new individuals (Bely et al., 2014; Carter et al., 2015; Martinez-Acosta & Zoran, 2015; Mladenov, 1996; O'Dea, 2006; Padua et al., 2016; Wulff, 1991). How individuals apportion finite resources between the two may be contingent on factors including but not limited to resource abundance, frequency or severity of damage, and other life history traits. Close examination of the investment strategy employed between these two processes, which despite having a great deal in common have yet diverged in several key aspects, can provide useful insights on their adaptive value and how they may have evolved within particular ecological contexts.

Small freshwater annelids known as naids are excellent candidates for investigating resource allocation strategies of post-embryonic development. The naids, an informal grouping of the subfamilies Pristininae and Naidinae, are known for their generally strong regenerative ability. Most species that have been studied can repeatedly regenerate large portions of the body rapidly (usually within several days), repeatedly, and with high fidelity (Bely & Sikes, 2010; Berrill, 1952). Reproduction typically occurs asexually via paratomic fission, in which new head and tail features are intercalated in the body of an original "parent" worm before splitting into two "daughter" worms, although sexual reproduction can be induced by certain environmental cues (Kaliszewicz et al., 2005; Zattara & Bely, 2011, 2013). Naids reproduce asexually every few days, although the exact rate and even number of simultaneous zones of fission vary due to feeding, age, physiological condition, and environmental variables (Özpolat & Bely, 2015). Paratomy produces large, clonal offspring sequentially, permitting relatively straightforward assessments of investment in individual offspring. Regeneration and asexual reproduction are ecologically relevant traits in naids, as these animals inhabit often-turbulent habitats,

such as small, sandy or rocky streams (Brinkhurst, 1986) in which injury is likely from sediment mobility and predatory interactions (Kaliszewicz, 2003; Smith, D. P., Kennedy, & Dickson, 1991) and where asexual reproduction probably allows for rapid (re-)colonization of suitable, sufficiently defaunated or marginal patches (Hughes, 1987; Meirmans et al., 2012). The naid ecological niche lends added relevance to studies of resource allocation, as they may experience considerable physiological challenges from both erratic food availability and frequent physical disturbance.

In this study, we investigated how induced investment in regeneration affects investment in asexual reproduction in *Pristina leidyi,* a broadly-distributed naid species that is an emerging model for studying post-embryonic development (Bely & Wray, 2001; Özpolat & Bely, 2015; Özpolat et al., 2016; Smith, D. P. et al., 1991; Zattara & Bely, 2011, 2013). Like other naids, *P. leidyi* reproduces by fission, generating fully-formed clonal offspring derived directly from the original worm's somatic tissue, presenting the possibility of direct resource competition between repair (regeneration) and reproduction (fission). We hypothesized that fission would exhibit a trade-off with regeneration, with tissue amputation leading to reduced reproductive investment and the latter decreasing as a function of increasing regenerative demand (imposed by altering the number of amputations to individuals). To determine if investment in the soma (regeneration) versus reproduction is constrained by the resource pool, we manipulated food availability across the experiment, which is known to affect proliferating cell count but not pattern in this species (Zattara & Bely, 2013). We therefore hypothesized that total but not proportional investment in reproduction would be affected by food level over the long term.

Methods

Animal culture and material

Established cultures of *P. leidyi* (see Bely & Wray, 2001) were cultured at room temperature (23 ºC) in glass bowls (12 cm diameter) filled with ~150 ml of artificial spring water (1% artificial seawater) (ASpW). Strips of brown paper towels were provided as substrate. Cultures were fed once weekly with 10 mg powdered Spirulina. Half-volume water changes were administered weekly.

To generate experimental animals, 109 healthy-looking worms of similar size were pulled from a culture that had been established three weeks prior, to ensure that cultures were undergoing near exponential growth (Mohondro, Rennolds, and Bely, unpublished data), and moved to individual wells of 24-well plates filled with 1.5 mL ASpW. Each individual worm was fed once weekly with 0.15 mg Spirulina. Individuals were monitored daily for fission (zooid release). The first posterior zooid released by each worm was moved to a new 24-well plate, and these 109 worms were used as experimental animals (F_0) as described below. All F_0 worms were produced within four days of each other (Fig. 4.1A). Individual *P. leidyi* are estimated to have a lifespan of approximately one year (Bely, unpublished data) and F_0 worms born within days of each other were thus considered of approximately equivalent age.

Experiment 1: Effect of injury and feeding on survival and reproduction

To test the effects of anterior amputation injury (and regeneration) frequency on reproductive output and how these are modulated by feeding, we conducted an experiment with a 2×4 factor design, including two food levels and four injury levels.

F_0 worms were randomly assigned to one of two feeding treatments: low food (LF), which received 0.15 mg Spirulina on the first day of each week (n = 55); and high food (HF), which received 0.15 mg Spirulina on the first and fourth days of each week (n = 54). Full water changes were administered for all worms on the first and fourth day of each week.

Worms in each feeding group were randomly assigned to one of four injury treatments: injured once (1X), twice (2X), or three times (3X) during the experiment, and uninjured control (0X) worms (Fig. 4.1A), such that n was divided approximately evenly between these four (n = 13-14 per injury treatment per feeding treatment). However, due to unanticipated mortality throughout the experiment, these assignments were adjusted as needed to maintain relative balance. *P. leidyi* typically forms fission zones (FZ) at approximately two thirds the length of the body, within segments 14-16. Because it possesses the original head and represents the larger fission product, the anterior zooid is considered the "parent" and the posterior zooid, once released, is considered the "offspring", by convention. Worms can continue fissioning even after amputation of the anterior part of the body. For this experiment, all amputations were inflicted at a consistent position at the anterior end of F_0 worms. At approximately two weeks following the "birth" (release) of the last F_0 worm, all worms were anesthetized in 0.05 mM nicotine. Worms designated for injury were amputated at the junction of segments 6 and 7 using a scalpel (Fig. 4.1B). The excised anterior segment was discarded and the remainder of the worm was retained for the experiment. Control worms were anesthetized but uninjured. All worms were then transferred to clean 24-well plates in fresh ASpW. All worms including controls were not fed for one week, while injured

animals regenerated. Designated feeding schedules resumed after this week. Exactly three weeks following the first injury, worms in the 2X and 3X groups were amputated in the same fashion as previously, and all worms were again anesthetized regardless of injury treatment and not fed for one week following this point. 3X worms were amputated one final time another three weeks afterwards. The experiment was concluded at 100 days since the birth of the first F_0 worm.

Each worm was scored daily for survival and fission (release of a posterior zooid, designated F_1) across the duration of the experiment. The first F_1 produced by each F_0 worm, and the first F_1 produced following each round of amputation (regardless of whether the individual was in an injury treatment or not), was removed, imaged, and assigned to experimental treatments as described below (Fig. 4.1C). All other offspring were discarded upon discovery. Throughout this paper, the following shorthand is used to refer to time intervals during which offspring production was recorded: from F_0 birth until the day of first injury (t_0), from one week after first injury until the day of second injury (t_1), from one week after second injury until the day of third injury (t_2), and from one week after third injury until the conclusion of the experiment (t_3).

Experiment 1: Offspring quality assessments

During Experiment 1, the first F_1 worm produced by each F_0 worm at the beginning of the experiment and following each regeneration period, or at each equivalent time point for uninjured worms, was collected. On the day of discovery, these worms were anesthetized and imaged with a Zeiss Axioplan 2 microscope under a 2.5x objective and AxioVision 4.8 image processing software. Body dimensions were measured with ImageJ using the Fiji package and volume was approximated by using the

formula for a cylinder ($\pi W^2 L$) (Fig. 4.2). Whole worm length (L) was measured (in mm) by tracing from the base of the proboscis to the tip of the pygidium along the anteroposterior axis. Whole worm width (W) was averaged across three roughly equally spaced positions along the body. *P. leidyi* often develop multiple FZs along the anteroposterior body axis simultaneously, and fission that completes more anterior to other, less-developed FZs can result in one or more FZs being present along the body of the zooid. Even early stage FZs are easily identified by differences in opacity, the lack of discernible internal organs, and slight to moderate transverse invagination. We calculated total body volume, total volume contained within all FZs if present, and subtracted the latter from the former to calculate net body volume. We did not make a distinction in our calculations whether total FZ volume was derived from just one or multiple FZs. FZ volume was calculated for each zone in a similar manner to that of the whole body, but length extended only along the area where new tissue was visibly developing, and only one width measurement was taken, in approximately the center of each zone.

Following imaging, worms were randomly assigned to one of two assay groups: a "reproductive potential" assay and a "regeneration potential" assay (Fig. 4.1C).

For the "reproductive potential" assay, F_1 worms were placed individually in fresh 24-well plates filled with ASpW and maintained on the same feeding regimen as their respective F_0 worm. F_1 worms were checked daily for the appearance of a first FZ and the completion of the first fission (release of a "F_2" offspring). Time (# days) from "birth" to formation of a first FZ, from formation of the first FZ to zooid release (fission speed), and from birth to zooid release were recorded (Fig. 4.1C). After fission, the F_1 worm and all of its offspring were discarded.

For the "regeneration potential" assay, within an hour following imaging, F_1 worms were amputated in the manner described previously and transferred individually to fresh 24-well plates filled with spring water. Worms were checked daily for signs of full regeneration, assessed by the emergence of visible chaetae in the anterior-most four segments and of the proboscis. Time from injury to full regeneration (# days) (regeneration speed) was recorded (Fig. 4.1C). At the completion of regeneration, worms were discarded. Worms were not fed during this experiment. Production of any offspring during this experiment, which was common within 1-2 d following amputation if a fission zone was present at birth (common in F_1 of worms from HF F_0), was noted and offspring were immediately discarded.

Experiment 2: Direct effect of feeding on regeneration speed

We assessed how feeding level directly affects regeneration speed of individual worms (rather than that of their offspring) through an additional experiment. We pulled 24 worms from a culture (newer than previous but maintained similarly) and randomly assigned 12 each to LF and HF levels, as described above. Worms were maintained on food for two weeks and then amputated in the same manner as described above. Worms were scored daily for regeneration completion, as described above. Three LF worms and one HF worm died following amputation and were excluded from analysis.

Statistical analysis

All statistical analysis was performed in the R computing environment (R Development Core Team, 2019). The following analyses were performed on F_0 worms. Differences in F_0 mortality over the course of Experiment 1 between feeding and injury

treatments were tested using a negative binomial regression after rejecting the null hypothesis of a deviance goodness-of-fit test ($P < 0.001$). Multiple ANOVAs were run to calculate differences in fecundity (# of offspring produced) between feeding and injury treatments for different periods of time: total, t_3, t_2, t_1, and t_0 output. Feeding × injury treatment interactions were removed from the models if not significant at $\alpha = 0.05$. Post-hoc comparisons were done with Tukey's adjustment. ANOVA assumptions were checked graphically and via Bartlett's test of homogeneity of variances.

The following analyses were performed on F_1 worms. Body volume was analyzed via Type III SS ANCOVA after log-transformation of total body volume and the volume of any FZs present (plus-1 to avoid the generation of infinity outputs resulting from transformation), in order to correct for heteroscedasticity, using the "car" package (Firth et al., 2009). FZ volume was treated as a covariate. Inclusion of a F_1 worm's respective F_0 worm in the models raised the Akaike information criterion (AIC) value and was thus excluded from these models. A logistic regression was used to test the probability of F_1 being born with a FZ already present using a model containing feeding treatment, F_0 injury treatment, their interaction, and time period. Time from birth to FZ detection in the reproductive potential assay, time from FZ detection to fission completion in the reproductive potential assay (fission speed), and time from injury to regeneration completion in the regeneration potential assay (regeneration speed), were each analyzed via separate Kruskal-Wallis rank sum tests for differences between feeding, F_0 injury treatment, and time period, plus their two- and three-way interactions. Post-hoc comparisons were done with Dunn's (1964) test with the Benjamini-Hochberg (1995) correction.

The effect of food level on regeneration speed in Experiment 2 was tested using a Welch's t-test.

Results

Injury increases mortality risk

Injury significantly increased mortality ($P < 0.05$), even for worms only injured once (Fig. 4.3A). Mortality was calculated as the proportion of worms that died following their most recent injury, regardless of whether they were assigned to be injured more times or how long following the injury that they died. Across the whole experiment, only 1 out of 13 uninjured control HF worms (at age 40 d), and 0 out of 12 uninjured control LF worms died. The mortality rate following one injury was 28% and 53% in HF and LF, respectively. Mortality following the second and third injuries was 59% and 33%, respectively, for HF and was 42% and 44%, respectively, for LF. There was no significant effect of feeding ($P \sim 0.82$) or the feeding × injury interaction ($P \sim 0.72$), the latter of which was removed from the final regression model. With only a few exceptions, most worms died within two weeks following the most recent injury, although a few worms lasted a considerable time (50 or more days) following a single injury before dying in LF (Fig. A3.1). Some worms that died produced a functional zooid on the day that they were discovered to be dead, but there was no clear relationship between the likelihood of this and either feeding or injury number (Fig. A3.2).

Feeding but not injury has a strong effect on fecundity

Feeding had a robust effect on fecundity as measured at all intervals, including total fecundity over the course of the experiment (Fig. 4.3B), prior to the first injury (t_0)

(Fig. 4.3C), and over each post-injury period (Fig. 4.3D-F) (all $P < 0.001$). High food more than tripled average total reproductive output versus low food (21.78 ± 2.99 SD vs. 6.59 ± 1.54 SD) among all worms that lived to the conclusion of the experiment (Fig. 4.3B).

Injury frequency had a minimal and inconsistent effect on fecundity overall and was most apparent in HF worms. Total fecundity was not significantly affected by injury ($P = 0.191$), including its interaction with feeding ($P = 0.23$). There was a statistically insignificant decrease in fecundity in HF worms of less than one offspring with each successive injury. A similar decrease is not evident in LF worms. It was possible that any injury-induced effects on reproductive rate were temporary, and so we performed two-way ANOVAs on total offspring produced during each post-injury period. Post-injury changes in reproductive rate were more substantial than what we observed from the total fecundity data but still small and inconsistent. Injury significantly affected output following the first (t_1) ($P = 0.0355$) (Fig. 4.3D) and second (t_2) ($P = 0.0043$) (Fig. 4.3E) injuries, but the interactions with feeding were not significant. However, in the post-third injury period (t_3), the injury and feeding interaction was significant ($P = 0.0162$) (Fig. 4.3F). Reductions in periodic fecundity following recent injury are evident at t_2 and t_3, the latter of which was restricted to HF worms.

Feeding and injury affect offspring body size and fission speed but not regeneration speed

To assess the effects of injury history and feeding on offspring quality, we measured body size of all F_1 worms (at time of discovery) and assessed regeneration

speed and fission speed in subsets of F_1 produced immediately following the regeneration periods (Fig. 4.1C).

F_0 injury history, feeding, and aging affected F_1 body size (Fig. 4.4). The interaction between injury frequency and feeding was significant ($P < 0.01$), indicating a multiplicative negative effect of injury on total (Fig. 4.4A-D) and net (Fig. A3.4) log-transformed body size in LF F_1. HF F_1 were consistently larger across all timepoints ($P < 0.001$) and had larger FZs if any were present (Fig. 4.4E-H). We observed that these FZs tended to also be further developed than those in LF F_1. The largest F_1 body sizes for each feeding group were recorded at the beginning of the experiment. In LF F_1, variance tended to decrease with increasing F_0 age. The main effect of timepoint, equating to F_0 age, was significant for both total and net log-transformed body size (both $P < 0.001$), with F_1 size at t_1 and t_2 being significantly smaller than that at t_0 (Tukey's-adjusted post-hoc comparisons, both $P < 0.01$). Our plots show that this effect is likely driven by decreasing size in LF F_1.

Most F_1 worms had only one FZ if any were present, but occasionally worms possessed two at the time of collection. HF F_1 worms were more likely to possess FZs overall (103/156, 66%) versus LF F_1 (31/117, 26.5%), resulting in greater differences between average total and net body volumes. However, there were no statistical differences between total and net body volume, and so we only show total body volume (Fig. 4.4A-D) and FZ volume (Fig. 4.4E-H) here; net body volume can be found in Fig. A3.4.

Fission speed of F_1 worms was affected by F_0 feeding, injury history, and time period of birth (parental age) (Fig. 4.5). Time from first FZ appearance to zooid release

(fission speed) took just over 5 days on average and ranged from as few as 2 to as many as 19 days. The three-way interaction was significant ($P < 0.001$), indicating an increased time from the appearance of the FZ to successful fission for F_1 from older, more frequently injured LF F_0 worms. HF F_1, regardless of age or injury history, did not vary in fission speed. Time from birth to first FZ appearance took 3-4 days on average, which includes many, mostly HF F_1 that had at least one FZ when discovered, but some took as long as 23 days (Fig. A3.3). The three-way interaction term was also significant ($P < 0.001$) for the time from birth to the first FZ appearance, indicating a similar pattern as fission speed.

In contrast, regeneration speed of F_1 worms was variable but unrelated to F_0 feeding ($P = 0.319$) or injury history ($P = 0.447$) at any time point ($P = 0.34$), with no significant interactions (Fig. 4.6B-D). Regeneration of F_1 worms was completed in 5 to 17 days, but most worms regenerated by 10 days, with a median across all treatments of 6 days. In Experiment 2, regeneration speed was also not affected by food level ($P = 0.610$) and regeneration speed mostly ranged between 4 to 5 days, taking up to 7 days (Fig. 4.6A).

Discussion

In this study, we investigated the investment pattern between regeneration and paratomic fission and whether this pattern is constrained by the amount of available resources. While self-repair, a form of somatic investment, and reproduction are typically considered to be distinct and generally competitive processes in sexual organisms, the mechanistic and evolutionary relatedness between regeneration and asexual reproduction in naids like *P. leidyi* provides an opportunity to improve our understanding of the

interaction between these processes. The most prominent pattern to emerge from our data is that food availability—essentially, the size of the resource pool—not only drives total reproductive investment strongly but also modulates the impact that successive injury and regeneration events, essentially a form of induced somatic investment, have on reproductive investment. When the external resource pool is large (i.e., when worms are fed frequently), there is generally no negative effect of up to three sequential amputations of anterior segments and subsequent regeneration (involving forming new segments and morphallactic remodeling of adjacent segments). Only when the resource pool is small (i.e., when worms are fed less frequently) does injury and regeneration frequency have a significant impact on reproductive investment (albeit in a limited way, as discussed below). This pattern suggests that the resource demands of regeneration may constrain reproductive investment when food is scarce, but when food is abundant, which often stimulates asexual reproduction in diverse groups (e.g.: Gibson & Paffenhöfer, 2002; Kaliszewicz & Lipińska, 2013; Purcell et al., 2019; Tökölyi et al., 2016), such a trade-off may not occur. Thus, more food allows worms to compensate for a conflict between regeneration and fission.

Although food restriction decreased overall reproductive investment and facilitated further reductions due to injury, these injury-induced reproductive costs manifested in specific ways. Successive injuries did not affect overall fecundity, but per-offspring investment declined as measured by body size and fission speed. Thus, whenever injury reduced reproductive investment, it was through reductions in offspring quality, not quantity. We found no evidence that low food availability induces a diversion of resources from reproduction (fission) to somatic maintenance, with the caveat that we

did not measure F_0 regeneration speed or other measures of physiological condition directly. It is notable, however, that more frequently injured LF worms often exhibited more apparent signs of stress, including morphological abnormalities such as body kinks, reduced coelom volume, and darker gut pigmentation (see Fig. A3.5 for comparison). Gut pigmentation is a result of waste products and other components in chloragogenous cells that line the gut and function in energy storage, metabolism, and detoxification in other annelids (Cholewa et al., 2006; Hoeger & Kunz, 1993; Molnár et al., 2012), and so may act as a marker of elevated physiological stress. These visible markers of stress are commonly observed in older worms as well, suggesting that injury and food restriction have a similar effect on a worm's physiological condition as aging. Offspring of worms exhibiting these characteristics did not display these signs, nor did newly formed posterior tissue, although the original somatic tissue that was "passed on" to offspring via fission often retained these qualities. Frequently injured HF worms exhibited fewer of these signs, suggesting that food compensates for negative effects of injury on maintenance as well. The possibility that body size reduction, along with these other phenotypic changes, is (at least partly) an adaptive response to resource restriction—to reduce metabolic expenditure, for example (McCue, 2010)—should not be discounted. We hypothesize that reductions in offspring quality may be partly a consequence of "maternal" epigenetic transfer of physiological condition, which has been described to both beneficial and detrimental effect in gametic reproducers, including snails (Thorson et al., 2017), insects (Lockwood, Julick, & Montooth, 2017; Triggs & Knell, 2012), and zebrafish (Bautista & Burggren, 2019).

The resource allocation pattern suggested here indicates a general life history strategy in *P. leidyi* that prioritizes the production of new physiological individuals (individual animals, or ramets) over the preservation of old individuals. Even under food limitation, worms were more likely to die following injury than to exhibit any lasting reduction in reproductive rate, and injury-associated reductions in reproductive investment manifested only as reduced offspring quality, not quantity. As a small, relatively short-lived, clonally-reproducing species, minimal investment in any given ramet is likely to be optimal. Instead, prioritizing reproduction whenever resources are available in sufficient amount to produce new individuals likely provides the greatest fitness advantage, especially since clonal fission creates virtually no difference between a younger and older animal besides the freshness of the tissue. This strategy parallels that of some short-lived lizards that invest preferentially into (sexual) reproduction over regeneration after tail loss (Dial & Fitzpatrick, 1981; Fox & McCoy, 2000), as their short life span minimizes the fitness value that regeneration might provide. Thus, even very distantly related animals with entirely different anatomical and physiological traits can develop similar allocation strategies involving regeneration on the basis of comparable life history traits. With regards to asexual reproduction in particular, maximizing offspring number facilitates rapid habitat colonization, a tactic shared by other species adapted to frequently disturbed environments (Glazier, 1999; Pianka, 1976; Willis et al., 1996). As fission is clonal, prioritizing reproduction over somatic investment (e.g., growing larger) increases the mass of the genetic individual (genet) without imposing greater constraints on the ramet, such as changes in surface-to-volume ratio, allowing for maximum exploitation of resource patches and creating theoretically infinite reproductive

value, and a practically immortal genet, so long as resources allow (Hughes & Cancino, 1985). It is therefore unsurprising that food availability has such a strong effect on reproductive investment in *P. leidyi*. Multiple species of naid are known to even increase fission under predation pressure, maximizing the number of worms with a chance to survive, and increase the length of the body at the time of fission, possibly as an adaptive tactic to enhance the chances of surviving sublethal predation (Kaliszewicz, 2015).

Resource restrictions imposed by low food availability may be responsible for reductions in per-offspring investment following injury and regeneration, but what explains the gradual reductions in offspring quality over time and with increasing number of F_0 injuries? This pattern in the data may be attributable to changes in energy storage. Parent somatic tissue that is transferred directly into fission offspring includes chloragogenous cells, as described previously, and other potential sites of stored biochemical energy, which may act as buffers against nutritive, injury, or other sources of stress (Zera & Harshman, 2001). Depletion of these stores to fuel regeneration and fission under low food may deprive offspring of energy which would otherwise be contributed by maternal transfer. It is unknown to what extent energy stores normally contribute to regeneration or fission in most annelids, including how stores may be divided between physiological processes (Dales, 1969), but work in another annelid, a fireworm polychaete, indicates that regeneration can require high stored energy expenditure (Yáñez-Rivera & Méndez, 2014). Energy stores are also depleted by regeneration in some stellate echinoderms (Dobson et al., 1991; Lawrence, J. M. & Larrain, 1994). The tight relationship between fission and food availability in *P. leidyi* suggests that *P. leidyi* may operate primarily as an income breeder that turns to stores to help fuel anabolic

processes when injury imposes an unexpected energetic demand. Future work is needed to investigate the metabolic physiology of *P. leidyi* under food restriction and regeneration to clarify this phenomenon.

Ultimately, we found that regeneration is, unlike fission, largely independent of the resource pool, suggesting that fission and regeneration may requisition resources differently. Although regeneration speed varied in our study and is known to be influenced by a range of factors (Zattara & Bely, 2011), including where injury occurs and with respect to the timing of fission (Zattara & Bely, 2013), we found no clear relationship with F_0 age, feeding, or injury history. In Experiment 2, regeneration speed also did not differ between food level, supporting the notion that this invariance was not simply due to an absence of maternal effects but is particular to the process of regeneration. This finding may suggest an adaptive resource allocation strategy: fission is unnecessary for survival of the ramet, but a worm cannot feed without a mouth, one of the last structures to form during anterior regeneration (Zattara & Bely, 2011). It would be advantageous to regenerate anterior segments at a rate independent of externally available energy to maximize chances of survival before starvation. Rapid regeneration of parts necessary for feeding and digestion at a rate independent of nutritional status occurs in brittlestars (Dobson et al., 1991; Fielman et al., 1991), which suggests convergence upon an optimal regeneration investment strategy in quite different taxa. It is intriguing that such different allocation tactics exist between asexual reproduction and regeneration, as it hints towards either different metabolic pathways that have developed to fuel these similar developmental processes or different regulatory mechanisms controlling these pathways, either of which may have been subjected to past selection. However, to

understand whether regeneration's seeming resource insensitivity is a general feature of regeneration in *P. leidyi*, it will need to be investigated for posterior regeneration.

Although naids and other animals with high regenerative ability often survive the destruction of substantial body portions, we found that injury imposes some cost on self-preservation in *P. leidyi*. No worms died immediately after amputation, but injured worms were more likely to die at some point during the experiment than uninjured worms. The period following injury includes the time spent regenerating lost tissue, so death may occur from resource exhaustion by regeneration, likely compounded by the inability to feed. Repeating this experiment under similar food availability but amputating posterior segments and manipulating food during the regeneration period could address whether the inability to feed during the regeneration period contributes to mortality. Since uninjured worms were also food-restricted during the time that injured worms were regenerating, this fasting period cannot be solely responsible for mortality. However, several worms died after food had been reintroduced, raising the question of whether worms are unable to eat enough following regeneration to compensate for resource losses, or if the process of death is initiated irreversibly at some prior point for reasons unrelated to resource restrictions. As regeneration is only advantageous if an animal survives the initial loss of tissue (Goss, 1969; Reichman, 1984), the possibility that injury can induce death in some worms and not others of the same age and genotype without being due to differences in resource availability raises interesting questions about what other factors, such as epigenetic differences (Verhoeven & Preite, 2014), might differentiate clones from one another.

Our findings contribute to the broader understanding of the complex, highly variable, and often unpredictable patterns of reproductive versus somatic investment throughout animals. Although asexual reproduction offers a number of advantages with respect to resource availability, as described above, the actual response to food limitation is inconsistent between asexual animals. We found that *P. leidyi* continues to invest in reproduction, albeit with a reduced rate of fission and at possibly detrimental effect to the original worm, when food is low, a strategy shared by asexual sea anemones (Bedgood et al., 2020). However, unlike *P. leidyi*, which does not increase individual size to significant extent when food is plentiful, the asexual sea cucumber *Holothuria atra* invests in growth over reproduction when food is high (Dolmatov, 2014). Asexual hydra invest both in maintenance and reproduction when food and environment are unstable, indicating that these two investment sinks are not necessarily in conflict even under resource limitation (Schaible et al., 2011). *P. leidyi* both shares features with and differs from investment patterns in sexually reproducing animals as well. The polychaete *Polydora* continues to reproduce while regenerating (Zajac, 1985), as *P. leidyi* does, while relative investment in regeneration versus reproduction in lizards appears to vary with life history (Bernardo & Agosta, 2005), and at least one species increases per-offspring investment while regenerating (Beatty et al., 2021). Disentangling the factors driving these investment patterns remains a relevant challenge for biologists.

Figures

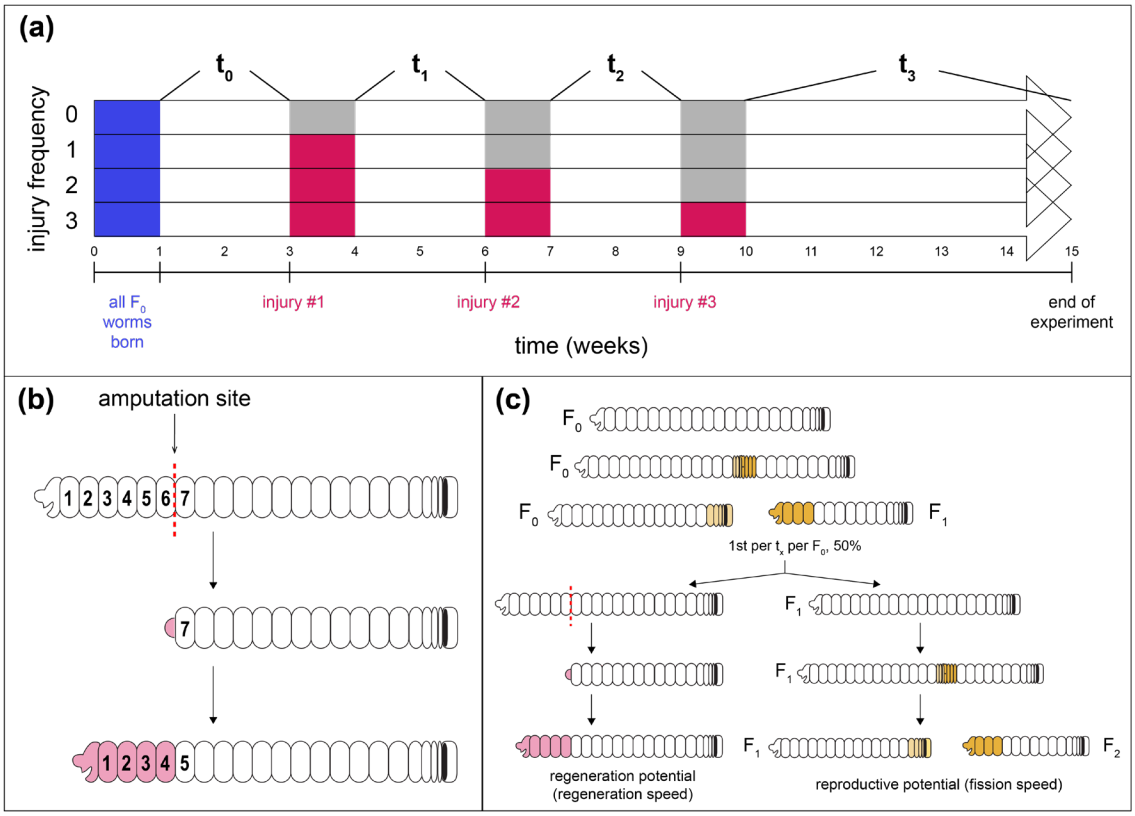

Figure 4.1. Experimental design. All treatments shown were replicated in LF and HF groups. (a) Timeline for the primary reproductive output experiment, from F_0 birth to the experiment's conclusion, for each injury frequency treatment group. Amputation injuries were performed at the same time in all worms designated to be injured and recovered for one week, without food, indicated by the magenta periods. Corresponding gray periods indicate that worms in that injury group were not injured but otherwise subject to the same conditions. (b) Schematic of amputation. Worms varied in absolute segment length. (c) Design of F_1 reproductive potential and regeneration potential assays.

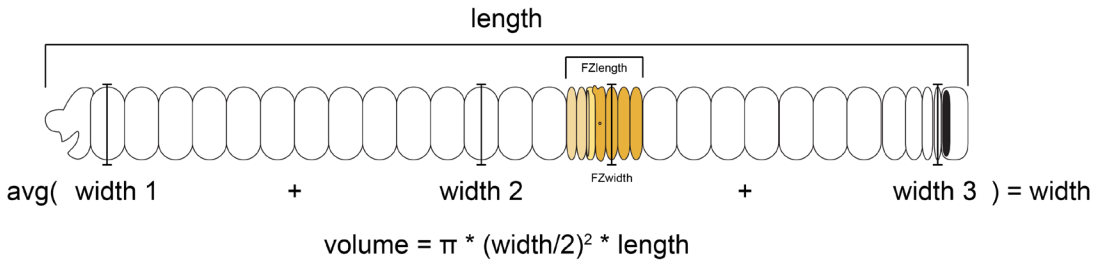

Figure 4.2. Calculation of body volume in uninjured or fully regenerated *P. leidyi* individuals. Body volume was approximated using the formula for a cylinder (see Methods).

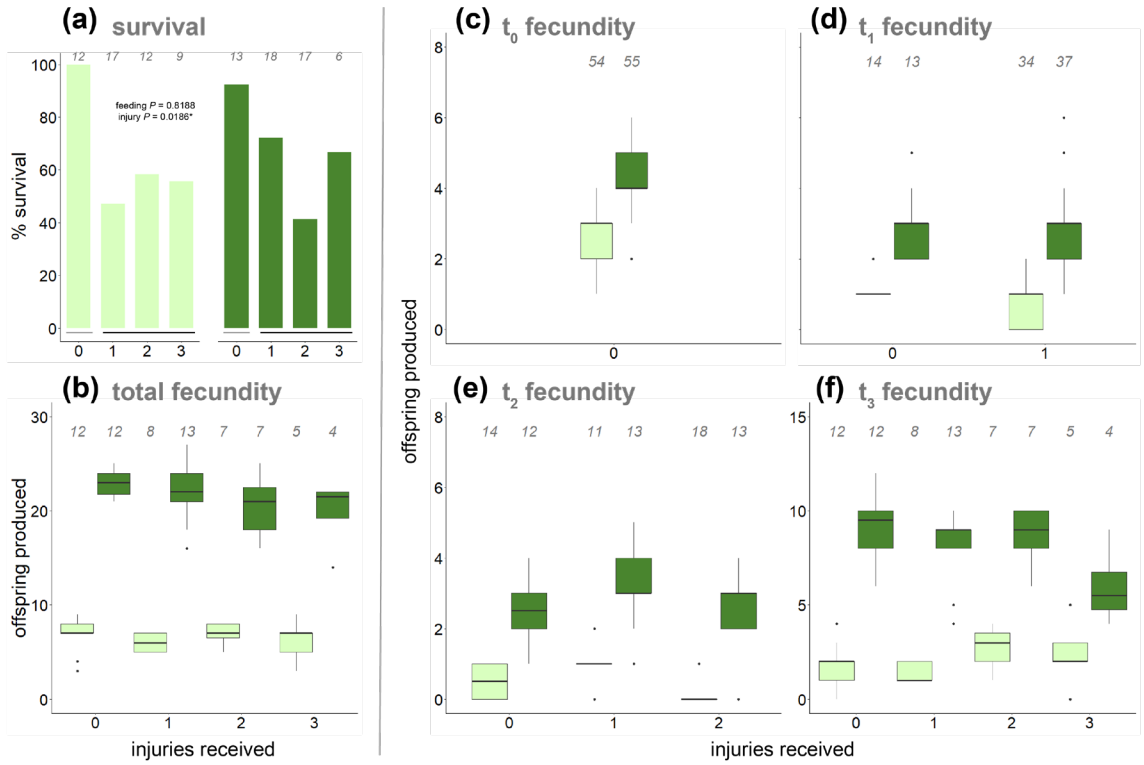

Figure 4.3. F_0 survival and fecundity as affected by number of injuries received and feeding. Color indicates feeding treatment (light = low, dark = high). Sample size is indicated over each bar. (a) Percent survival as a function of injuries received. Each column is calculated as total number of individuals that survived following that number of injuries out of the total that had experienced that number of injuries to that point (columns therefore do not strictly correspond to assigned treatments). (b-f) Fecundity, measured as number of posterior zooids produced, across the entire experiment (b) or during t_0 (c), t_1 (d), t_2 (e), or t_3 (f). Period length is 2 weeks for t_0, t_1, and t_2 and is 5 weeks for t_3 (see Fig. 4.1). Data presented include only those worms which survived to the end of each respective time period.

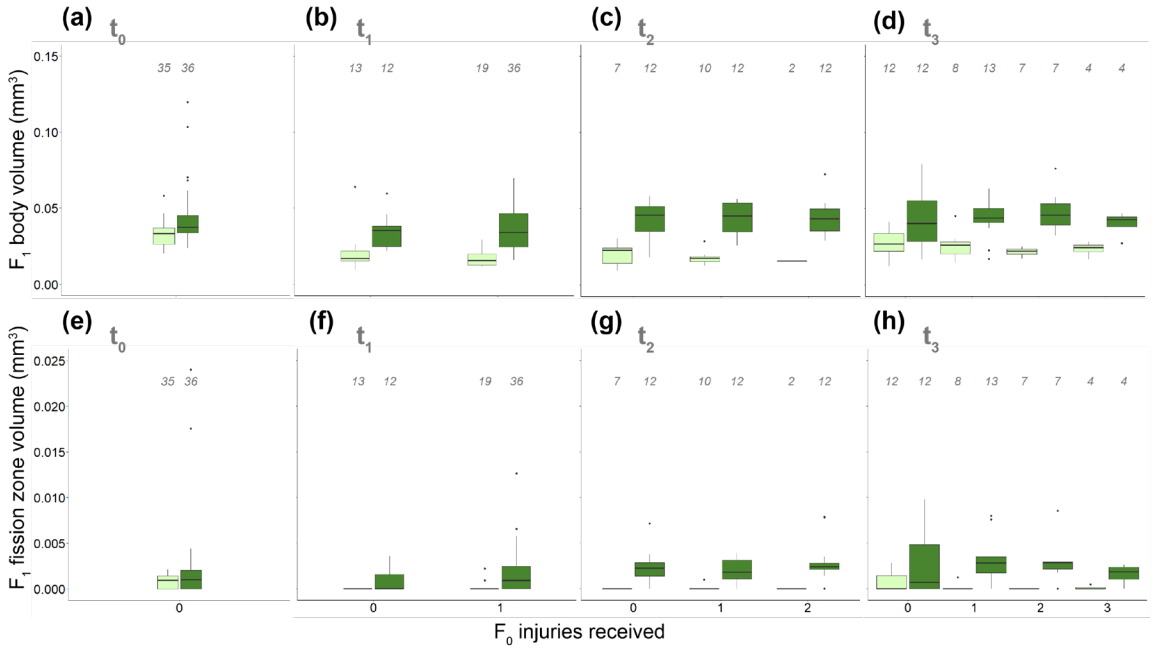

Figure 4.4. Total body volume (a-d) and FZ volume (e-h) of F_1 produced by each F_0 following the start of the experiment (t_0) (a, e) and the first (t_1) (b, f), second (t_2) (c, g), and third (t_3) (d, h) F_0 regeneration periods. See Fig. 4.2 for volume calculation methods. Color indicates F_0 feeding treatment (light = low, dark = high). Sample size is indicated over each bar. Volumes for log-transformed for analyses (see Methods).

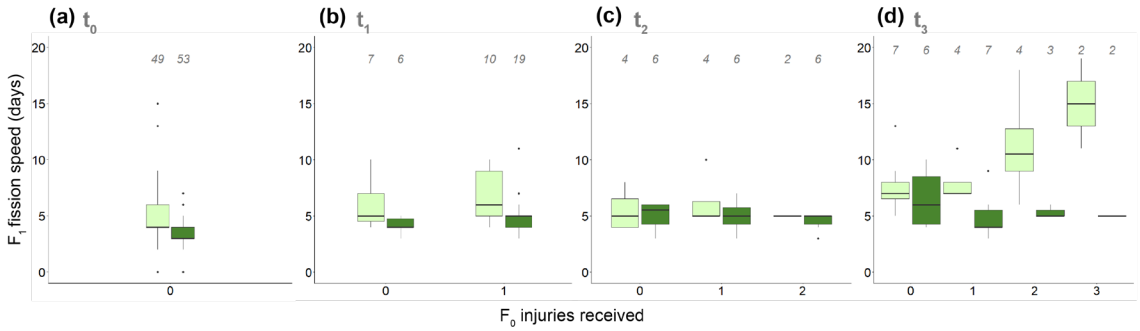

Figure 4.5. Fission speed as a function of parental feeding and injury history in F_1 produced following the start of the experiment (t_0) (a) and the first (t_1) (b), second (t_2) (c), and third (t_3) (d) F_0 regeneration periods. Color indicates feeding treatment (light = low, dark = high). Sample size is indicated over each bar. (a) Regeneration speed of worms from Experiment 2. (b-d) Regeneration speed of F_1 produced following the first (t_1) (b), second (t_2) (c), and third (t_3) (d) F_0 regeneration periods.

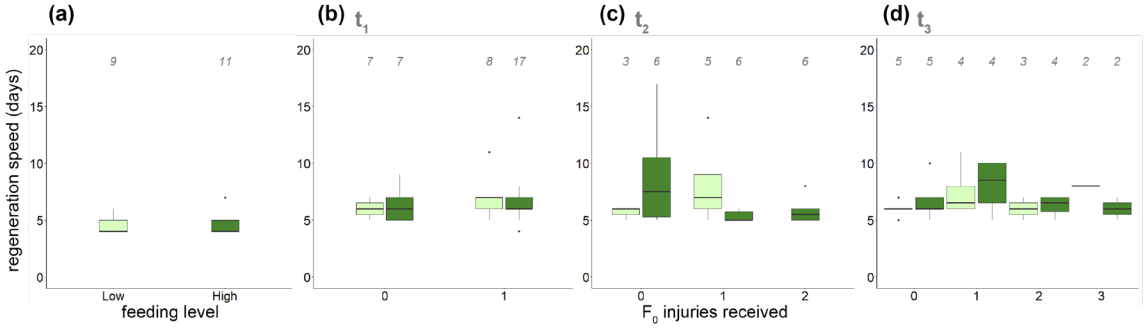

Figure 4.6. Regeneration speed as a function of own (a) or parental feeding and injury history (b-d). Color indicates feeding treatment (light = low, dark = high). Sample size is indicated over each bar. (a) Regeneration speed of worms from Experiment 2. (b-d) Regeneration speed of F_1 produced following the first (t_1) (b), second (t_2) (c), and third (t_3) (d) F_0 regeneration periods.

Appendix 1: Silhouette attributions for Fig. 2.3

Sponge – Image by Mali'o Kodis, photograph by Derek Keats. License

Cnidarian – Image by Qiang Ou. License

Annelid – Image by Noah Schlottman, photograph by Casey Dunn. License

Bryozoan – Image by Noah Schlottman, photograph by Hans de Blauwe. License

Platyhelminth – Image modified from Andreas Neudecker. License

Arthropod – Image by Almandine, vectorized by T. Michael Keesey. License

Chordate – Image by Matt Reinbold, modified by T. Michael Keesey. License

Appendix 2: Chapter 3 supplementary tables and figures

Table A2.1. Selected DEGs (FDR < 0.001): A23 vs. CA23.

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Protein coordinates	logFC	logCPM	F	P value	FDR
98623					-3.13556	5.209282	162.3944	1.83E-10	6.28E-06
98411	CAH15_MOUSE	Carbonic anhydrase 15	1.57E-48	219-1187[+]	-2.39307	4.529742	147.0926	4.09E-10	7.02E-06
104706				164-487[+]	-4.68026	5.598169	130.0723	1.17E-09	1.34E-05
96233	PUR6_CHICK	Multifunctional protein ADE2	0	157-1425[+]	3.49399	6.421227	121.0594	2.01E-09	1.73E-05
91287	PUNA_GEOSE	Purine nucleoside phosphorylase 1	1.97E-17	2-457[+]	-2.83877	5.401997	103.6322	6.57E-09	4.52E-05
111433	CAT8_MOUSE	Cathepsin 8	5.18E-11	83-439[+]	-1.65828	7.125899	97.53961	1.05E-08	6.01E-05
91191	FBP1_STRPU	Fibropellin-1	8.25E-37	36-1361[+]	-8.21738	2.801388	125.2985	2.18E-08	0.000107
97432	PUR6_CHICK	Multifunctional protein ADE2	0	132-1400[+]	4.19349	6.087589	82.98669	3.86E-08	0.000164
88942	CKS1_MOUSE	Cyclin-dependent kinases regulatory subunit 1	1.42E-29		1.390159	5.273971	80.97685	4.3E-08	0.000164
65316	PDE9A_HUMAN	High affinity cGMP-specific 3',5'-cyclic phosphodiesterase 9A	2.03E-163	256-1431[+]	1.592953	4.368625	78.40992	5.46E-08	0.000167
102787				252-1001[+]	3.772886	2.930086	78.03995	5.66E-08	0.000167
108369	GRN_DICDI	Granulin	7.03E-06		1.475071	10.38169	77.70205	5.84E-08	0.000167
102110	VPP4_HUMAN	V-type proton ATPase 116 kDa subunit a isoform 4	9.56E-108	1-1392[+]	-1.76917	6.456291	74.18268	8.23E-08	0.000217
70960	OSTA_LEUER	Organic solute transporter subunit alpha	1.63E-13	3-1184[+]	-1.83469	3.912291	70.68732	1.17E-07	0.000234
75833	IIGP5_HUMAN	Interferon-inducible GTPase 5	4.42E-53	127-1329[+]	-3.14794	3.09102	70.49707	1.19E-07	0.000234
109959	GRN_DICDI	Granulin	2.90E-05		1.567083	9.363465	70.37634	1.21E-07	0.000234
73659	VPP1_RAT	V-type proton ATPase 116 kDa subunit a1	0	1-2499[+]	-2.14321	5.262253	70.34297	1.21E-07	0.000234
88157	ADH_SULAC	NAD-dependent alcohol dehydrogenase	3.72E-16	231-1547[+]	1.410683	5.137089	70.25655	1.22E-07	0.000234
106647	EPDR1_HUMAN	Mammalian ependymin-related protein 1	5.47E-05	99-803[+]	1.91607	4.670539	68.93944	1.4E-07	0.000254
105510	DUT_RAT	Deoxyuridine 5'-triphosphate nucleotidohydrolase	1.57E-72	1-606[+]	1.665216	4.365238	67.32697	1.67E-07	0.000286
8722	GVIN1_MOUSE	Interferon-induced very large GTPase 1	0	50-5014[+]	-2.5136	4.119865	65.60689	2.01E-07	0.000328
99366	STAR5_BOVIN	StAR-related lipid transfer protein 5	2.68E-49	183-842[+]	1.447534	6.249263	56.13154	6.04E-07	0.000937
109771	GRN_DICDI	Granulin	8.24E-06		1.5513	10.27211	55.57083	6.57E-07	0.000937
79013	MIOX_DANRE	Inositol oxygenase	1.16E-113	142-996[+]	1.714183	7.339379	54.95092	6.99E-07	0.000937

111225					1.954403	10.12021	55.02424	7.25E-07	0.000937
110698	PF04103.18	CD20-like family	2.70E-07	100-858[+]	-2.78856	4.671946	54.7067	7.3E-07	0.000937
109797	GRN_DICDI	Granulin	2.26E-05		1.615888	9.313346	54.15159	7.82E-07	0.000937
79730	SGMA_DICDI	Sphingomyelin phosphodiesterase A	1.01E-69	114-2189[+]	-1.83826	4.416679	54.03349	7.85E-07	0.000937
110851	IF5A_DROME	Eukaryotic translation initiation factor 5A	1.85E-59	45-548[+]	0.62119	9.270789	53.83296	8.06E-07	0.000937
22453	AAKG2_MOUSE	5'-AMP-activated protein kinase subunit gamma-2	4.26E-70	109-3705[+]	2.072012	4.981187	53.5303	8.37E-07	0.000937
110968					1.851908	4.514405	53.45625	8.45E-07	0.000937
77962	NQO1_CAVPO	NAD(P)H dehydrogenase [quinone] 1	2.31E-14	3-1367[+]	-2.86985	5.284109	53.28075	9.01E-07	0.000967

Table A2.2. Selected DEGs (FDR < 0.001): CA35 vs. CA23.

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Protein coordinates	logFC	logCPM	F	P value	FDR
109908	CRYAB_MACFA	Alpha-crystallin B chain	1.34E-15	129-629[+]	3.314269	6.691767	174.7168	1E-10	2.74E-06
111384	CH10_ORYLA	10 kDa heat shock protein	4.96E-43	116-421[+]	2.27355	8.513635	165.1441	1.59E-10	2.74E-06
99284	POSTN_MOUSE	Periostin	1.81E-17	1-903[+]	1.876368	5.974099	141.4008	5.62E-10	6.44E-06
84988	TENX_HUMAN	Tenascin-X	5.16E-29	98-2230[+]	4.477983	6.805893	124.6449	1.67E-09	1.15E-05
77307	HSP7C_SAGOE	Heat shock cognate 71 kDa protein	0	201-2153[+]	3.516845	5.483423	122.2006	1.84E-09	1.15E-05
108040	U2AF1_BOVIN	Splicing factor U2AF 35 kDa subunit	9.49E-11	64-732[+]	1.116211	6.861008	120.618	2E-09	1.15E-05
108614	CB076_DANRE	UPF0538 protein C2orf76 homolog	6.33E-30	3-431[+]	1.551339	4.875399	114.4255	3.03E-09	1.49E-05
86448	SMCE1_MOUSE	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	3.11E-13	228-1004[+]	-1.87353	6.247321	98.34513	9.85E-09	4.23E-05
110342					-2.40205	5.491794	96.57077	1.13E-08	4.32E-05
99029	CDC37_DROVI	Hsp90 co-chaperone Cdc37	3.34E-91	140-1222[+]	1.613666	7.855046	93.13988	1.49E-08	5.13E-05
81037	STIP1_HUMAN	Stress-induced-phosphoprotein 1	1.41E-136	143-1111[+]	1.910945	6.142955	90.15807	1.91E-08	5.98E-05
105630	GHITM_HUMAN	Growth hormone-inducible transmembrane protein	3.40E-108	123-1163[+]	1.46372	6.324875	85.80455	2.78E-08	7.78E-05
92507				174-1259[+]	1.40887	5.319792	85.16439	2.95E-08	7.78E-05
109078	SET_HUMAN	SET	2.40E-46	1-483[+]	-1.39141	6.8527	80.14001	4.64E-08	0.000114
96808	UBE2A_MOUSE	Ubiquitin-conjugating enzyme E2 A	1.06E-82	152-613[+]	1.011058	5.774296	76.19659	6.75E-08	0.000155
93864	AHSA1_HUMAN	Activator of 90 kDa heat shock protein ATPase homolog 1	2.96E-106	101-1174[+]	2.935567	3.976932	75.13687	7.49E-08	0.000161

48125	PF13383.9	Methyltransferase domain	3.60E-09	182-736[+]	-9.03923	4.269381	76.32621	1.83E-07	0.000369
84031	PRUN1_MOUSE	Exopolyphosphatase PRUNE1	2.20E-24	1139-1768[+]	-1.67292	4.339537	62.90863	2.71E-07	0.000517
98668	F10A1_CHICK	Hsc70-interacting protein	4.14E-52	529-897[+]	1.21532	6.527399	60.42764	3.6E-07	0.000635
103578	KTR3_YEAST	Probable mannosyltransferase KTR3	4.27E-25	283-1347[+]	1.273476	5.427458	60.20753	3.7E-07	0.000635
20983	MARH6_MOUSE	E3 ubiquitin-protein ligase MARCHF6	0	167-3673[+]	0.763059	6.503036	58.20545	4.69E-07	0.000736
105147	HMG2_DROME	High mobility group protein DSP1	3.20E-59	119-739[+]	-1.308	7.580118	58.16186	4.71E-07	0.000736
89924					1.650298	4.355143	56.68541	5.64E-07	0.000842

Table A2.3. Selected DEGs (FDR < 0.001): A35 vs. CA35.

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Protein coordinates	logFC	logCPM	F	P value	FDR
104706				164-487[+]	-5.10594	5.598169	125.846	1.52E-09	5.23E-05
35544	RIR1_MOUSE	Ribonucleoside-diphosphate reductase large subunit	0	91-2499[+]	1.66601	4.79965	107.609	4.9E-09	8.42E-05
98623					-2.33851	5.209282	93.07389	1.5E-08	0.00013
98411	CAH15_MOUSE	Carbonic anhydrase 15	1.57E-48	219-1187[+]	-1.87425	4.529742	92.13523	1.62E-08	0.00013
107740				85-900[+]	-3.25857	6.036317	90.77952	1.89E-08	0.00013
92647	CATL2_HUMAN	Cathepsin L2	5.76E-74	121-1374[+]	-3.53544	4.251303	86.57239	2.6E-08	0.00014
102110	VPP4_HUMAN	V-type proton ATPase 116 kDa subunit a isoform 4	9.56E-108	1-1392[+]	-1.9174	6.456291	85.56251	2.84E-08	0.00014
22453	AAKG2_MOUSE	5'-AMP-activated protein kinase subunit gamma-2	4.26E-70	109-3705[+]	2.578414	4.981187	82.07861	3.89E-08	0.000167
111433	CAT8_MOUSE	Cathepsin 8	5.18E-11	83-439[+]	-1.45026	7.125899	75.14959	7.48E-08	0.000262
105249				107-1120[+]	-5.35692	2.997025	74.96622	7.61E-08	0.000262
101016	VA0D1_MOUSE	V-type proton ATPase subunit d 1	0	165-1211[+]	-1.78877	6.02138	72.02181	1.02E-07	0.000316
110166	DYLT5_MOUSE	Dynein light chain Tctex-type 5	5.03E-11	446-1012[+]	-2.11241	4.88915	71.25593	1.1E-07	0.000316
91287	PUNA_GEOSE	Purine nucleoside phosphorylase 1	1.97E-17	2-457[+]	-2.31125	5.401997	69.64589	1.3E-07	0.000345
109733	RLT1_RHIO9	Mucoricin	2.85E-06	82-876[+]	-1.94821	5.6816	68.3799	1.49E-07	0.000366
33214	S17B3_XENTR	Transcription factor Sox-17-beta.3	1.80E-26	383-3178[+]	-6.59461	1.927501	75.47229	1.82E-07	0.000405
73659	VPP1_RAT	V-type proton ATPase 116 kDa subunit a1	0	1-2499[+]	-2.10986	5.262253	66.16949	1.89E-07	0.000405
111787				3-317[+]	-1.5458	7.3398	64.17189	2.35E-07	0.000475

107646	CRYL1_PONAB	Lambda-crystallin homolog	1.48E-67	81-1052[+]	-3.18658	4.264846	63.1788	2.63E-07	0.000502
91191	FBP1_STRPU	Fibropellin-1	8.25E-37	36-1361[+]	-6.80864	2.801388	82.07929	3.03E-07	0.000534
106794	HPGDS_CHICK	Hematopoietic prostaglandin D synthase	3.64E-43	39-674[+]	-2.18042	5.984745	61.24083	3.31E-07	0.000534
105197	APRR2_ARATH	Two-component response regulator-like APRR2	0.000139	287-745[+]	-1.82309	5.465303	60.36423	3.63E-07	0.000534
87030				259-1146[+]	-5.22013	4.534726	60.5688	3.74E-07	0.000534
86237	CP2F3_CAPHI	Cytochrome P450 2F3	6.22E-68	71-1654[+]	-6.57703	2.06433	67.69667	3.75E-07	0.000534
110698				100-858[+]	-3.10683	4.671946	60.05287	3.81E-07	0.000534
96233	PUR6_CHICK	Multifunctional protein ADE2	0	157-1425[+]	2.356456	6.421227	59.96159	3.89E-07	0.000534
46538				360-872[+]	3.758685	3.21735	58.67219	4.43E-07	0.000586
110851	IF5A_DROME	Eukaryotic translation initiation factor 5A	1.85E-59	45-548[+]	0.639883	9.270789	57.05703	5.39E-07	0.000685
109959	GRN_DICDI	Granulin	2.90E-05		1.396759	9.363465	56.39421	5.84E-07	0.000717
32913	ANKAR_MOUSE	Ankyrin and armadillo repeat-containing protein	3.37E-34	239-3637[+]	-6.11223	2.597477	62.83714	6.1E-07	0.000722
70960	OSTA_LEUER	Organic solute transporter subunit alpha	1.63E-13	3-1184[+]	-1.59438	3.912291	53.81446	8.07E-07	0.000867
109797	GRN_DICDI	Granulin	2.26E-05		1.609146	9.313346	53.70235	8.28E-07	0.000867
80562	CEL_BOVIN	Bile salt-activated lipase	3.44E-65	222-2210[+]	-3.0049	3.530507	53.23576	8.7E-07	0.000867
48125				182-736[+]	8.155488	4.269381	59.9025	8.86E-07	0.000867
84165				134-826[+]	-5.22758	4.490783	53.47325	8.9E-07	0.000867
110686	VA0E1_RAT	V-type proton ATPase subunit e 1	1.29E-20		-1.39156	6.476873	53.02653	8.93E-07	0.000867
10234	LGR5_BOVIN	Leucine-rich repeat-containing G-protein coupled receptor 5	7.88E-17	1033-4074[+]	-6.88101	2.444933	58.98583	9.16E-07	0.000867
111461	H2AV_XENTR	Histone H2A.V	4.18E-64	55-441[+]	1.183739	7.734511	52.68502	9.34E-07	0.000867
8722	GVIN1_MOUSE	Interferon-induced very large GTPase 1	0	50-5014[+]	-2.19318	4.119865	52.03099	1.02E-06	0.000919
109725				140-826[+]	-2.56785	4.17308	51.26166	1.13E-06	0.000991

Table A2.4. Selected DEGs (FDR < 0.001): A35 vs. A23.

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Protein coordinates	logFC	logCPM	F	P value	FDR
77307	HSP7C_SAGOE	Heat shock cognate 71 kDa protein	0	201-2153[+]	4.017426	5.483423	147.8521	4.01E-10	1.38E-05
109908	CRYAB_MACFA	Alpha-crystallin B chain	1.34E-15	129-629[+]	2.767965	6.691767	128.1878	1.23E-09	2.12E-05

100280	MLF2_HUMAN	Myeloid leukemia factor 2	1.02E-38	138-899[+]	2.883075	4.432257	101.8239	7.53E-09	6.66E-05
108040	U2AF4_RAT	Splicing factor U2AF 26 kDa subunit	1.51E-122	64-732[+]	1.021279	6.861008	101.4409	7.76E-09	6.66E-05
110342					-2.37797	5.491794	93.4582	1.46E-08	0.0001
108614	CB076_DANRE	UPF0538 protein C2orf76 homolog	6.33E-30	3-431[+]	1.368476	4.875399	91.02239	1.78E-08	0.000102
99029	CDC37_DROVI	Hsp90 co-chaperone Cdc37	3.34E-91	140-1222[+]	1.573794	7.855046	88.61795	2.18E-08	0.000107
81037	STIP1_HUMAN	Stress-induced-phosphoprotein 1	1.41E-136	143-1111[+]	1.810509	6.142955	81.56137	4.07E-08	0.000163
111384	CH10_ORYLA	10 kDa heat shock protein, mitochondrial	4.96E-43	116-421[+]	1.553039	8.513635	81.04501	4.27E-08	0.000163
99284	POSTN_MOUSE	Periostin	1.81E-17	1-903[+]	1.367487	5.974099	77.01001	6.24E-08	0.000214
108481				1-798[+]	-1.64577	6.2247	72.67391	9.56E-08	0.000299
102690	SRCA_CHICK	Sarcalumenin	5.84E-144	3-1508[+]	-1.40179	4.806425	70.56086	1.19E-07	0.000315
98338	FACR1_DROME	Putative fatty acyl-CoA reductase CG5065	3.02E-95	72-1322[+]	-3.24524	5.618295	71.01324	1.19E-07	0.000315
84988	TENX_HUMAN	Tenascin-X	5.16E-29	98-2230[+]	3.24073	6.805893	68.07196	1.62E-07	0.000398
108060	Y2624_MYCTU	Universal stress protein Rv2624c	1.07E-08	140-541[+]	2.032426	5.312626	66.28725	1.86E-07	0.000427
105630	GHITM_HUMAN	Growth hormone-inducible transmembrane protein	3.40E-108	123-1163[+]	1.25711	6.324875	63.94843	2.41E-07	0.000486
111193	RLT1_RHIO9	Mucoricin	2.03E-05	1-381[+]	-0.98275	8.993963	63.94387	2.41E-07	0.000486
105262					-2.01744	4.116542	62.86182	2.72E-07	0.000486
20983	MARH6_MOUSE	E3 ubiquitin-protein ligase MARCHF6	0	167-3673[+]	0.792511	6.503036	62.76487	2.75E-07	0.000486
100763	FIBA_APOPA	Fibrinogen-like protein A	2.02E-26	3-1265[+]	-2.22648	3.973629	62.44863	2.85E-07	0.000486
109733	RLT1_RHIO9	Mucoricin	2.85E-06	82-876[+]	-1.84738	5.6816	61.69435	3.11E-07	0.000486
109625				3-809[+]	-1.74106	6.566724	61.69197	3.11E-07	0.000486
111639					-1.10403	6.752587	57.5401	5.08E-07	0.00073
70519	PLD3B_MACFA	PRELI domain containing protein 3B	1.38E-64	90-674[+]	1.432401	5.191892	57.26595	5.25E-07	0.00073
96224					1.697362	4.9415	57.175	5.31E-07	0.00073
98172	WIPF1_MOUSE	WAS/WASL-interacting protein family member 1	0.000505	306-1298[+]	1.561528	6.015115	55.68124	6.38E-07	0.000843
110596					-1.03506	8.150899	55.15391	6.82E-07	0.00085
33214	S17B3_XENTR	Transcription factor Sox-17-beta.3	1.80E-26	383-3178[+]	-5.89091	1.927501	61.54563	6.97E-07	0.00085
79013	MIOX_DANRE	Inositol oxygenase	1.16E-113	142-996[+]	-1.71088	7.339379	54.74167	7.18E-07	0.00085
110324					-2.82414	3.589845	53.85541	8.03E-07	0.000902
4354	TBC25_HUMAN	TBC1 domain family member 25	7.55E-108	3-2330[+]	1.496825	4.076436	53.55587	8.35E-07	0.000902
107020	BRK1B_XENLA	Probable protein BRICK1-B	3.70E-30		-1.65965	6.136484	53.50619	8.4E-07	0.000902

Table A2.5. Selected DEGs (FDR < 0.001, max 50): A35 vs. CA23.

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Protein coordinates	logFC	logCPM	F	P value	FDR
111384	CH10_ORYLA	10 kDa heat shock protein, mitochondrial	4.96E-43	116-421[+]	2.390464	8.513635	181.0038	7.51E-11	1.55E-06
98623					-3.3047	5.209282	176.2162	9.36E-11	1.55E-06
104706				164-487[+]	-6.0047	5.598169	170.0697	1.35E-10	1.55E-06
109908	CRYAB_MACFA	Alpha-crystallin B chain	1.34E-15	129-629[+]	3.10327	6.691767	155.6504	2.58E-10	2.06E-06
106668					-1.58693	8.238701	152.817	3E-10	2.06E-06
98411	CAH15_MOUSE	Carbonic anhydrase 15	1.57E-48	219-1187[+]	-2.35782	4.529742	142.7344	5.21E-10	2.99E-06
108614	CB076_DANRE	UPF0538 protein C2orf76 homolog	6.33E-30	3-431[+]	1.710804	4.875399	138.1736	6.77E-10	3.32E-06
110598	HPGDS_CHICK	Hematopoietic prostaglandin D synthase	5.97E-47	65-739[+]	-2.26208	4.627148	130.4669	1.07E-09	4.33E-06
105197	APRR2_ARATH	Two-component response regulator-like APRR2	0.000139	287-745[+]	-2.72131	5.465303	129.54	1.14E-09	4.33E-06
100763	FIBA_APOPA	Fibrinogen-like protein A	2.02E-26	3-1265[+]	-3.22164	3.973629	126.0655	1.41E-09	4.84E-06
108040	U2AF4_RAT	Splicing factor U2AF 26 kDa subunit	1.51E-122	64-732[+]	1.125859	6.861008	122.6001	1.76E-09	5.49E-06
111433	CAT8_MOUSE	Cathepsin 8	5.18E-11	83-439[+]	-1.80958	7.125899	114.8422	2.95E-09	8.19E-06
91287	PUNA_GEOSE	Purine nucleoside phosphorylase 1	1.97E-17	2-457[+]	-3.01576	5.401997	114.1128	3.1E-09	8.19E-06
111787				3-317[+]	-2.06869	7.3398	111.6382	3.68E-09	8.84E-06
97304	MA2B1_MOUSE	Lysosomal alpha-mannosidase	5.77E-20	2-481[+]	1.252745	6.902165	110.4183	4.01E-09	8.84E-06
77307	HSP7C_SAGOE	Heat shock cognate 71 kDa protein	0	201-2153[+]	3.322287	5.483423	110.3173	4.12E-09	8.84E-06
110342					-2.53174	5.491794	105.0568	5.91E-09	1.19E-05
102110	VPP4_HUMAN	V-type proton ATPase 116 kDa subunit a isoform 4	9.56E-108	1-1392[+]	-2.10265	6.456291	101.8114	7.54E-09	1.44E-05
107740				85-900[+]	-3.45183	6.036317	100.574	8.62E-09	1.56E-05
22453	AAKG2_MOUSE	5'-AMP-activated protein kinase subunit gamma-2	4.26E-70	109-3705[+]	2.865427	4.981187	97.98776	1.01E-08	1.67E-05
46538				360-872[+]	5.458466	3.21735	97.40878	1.06E-08	1.67E-05
73659	VPP1_RAT	V-type proton ATPase 116 kDa subunit a1	0	1-2499[+]	-2.58604	5.262253	97.31525	1.07E-08	1.67E-05
97910	T23O_ANOGA	Tryptophan 2,3-dioxygenase	2.20E-148	3-1319[+]	-2.08832	6.614242	96.7525	1.12E-08	1.67E-05
88942	CKS1_MOUSE	Cyclin-dependent kinases regulatory subunit 1	1.42E-29		1.516922	5.273971	95.92392	1.19E-08	1.71E-05
110698	PF04103.18	CD20-like family	2.70E-07	100-858[+]	-3.862	4.671946	90.00811	1.97E-08	2.62E-05
111639					-1.38626	6.752587	89.73018	1.99E-08	2.62E-05
105249	PF07801.14	Protein of unknown function (DUF1647)	2.60E-15	107-1120[+]	-5.83592	2.997025	88.85598	2.14E-08	2.72E-05

110686	VA0E1_RAT	V-type proton ATPase subunit e 1	1.29E-20		-1.81463	6.476873	88.42853	2.22E-08	2.72E-05
91191	FBP1_STRPU	Fibropellin-1	8.25E-37	36-1361[+]	-8.21738	2.801388	122.8469	2.47E-08	2.93E-05
99284	POSTN_MOUSE	Periostin	1.81E-17	1-903[+]	1.454337	5.974099	86.4271	2.64E-08	2.99E-05
81037	STIP1_HUMAN	Stress-induced-phosphoprotein 1	1.41E-136	143-1111[+]	1.863027	6.142955	85.8301	2.78E-08	2.99E-05
101016	VA0D1_MOUSE	V-type proton ATPase subunit d 1	0	165-1211[+]	-1.9609	6.02138	85.80336	2.78E-08	2.99E-05
87030				259-1146[+]	-6.26439	4.534726	86.07145	2.91E-08	2.99E-05
98338	FACR1_DROME	Putative fatty acyl-CoA reductase CG5065	3.02E-95	72-1322[+]	-3.59895	5.618295	85.43692	3.05E-08	2.99E-05
107944	COPT1_PONAB	High affinity copper uptake protein 1	2.58E-22	54-587[+]	-1.88225	5.829838	84.752	3.06E-08	2.99E-05
102533	GELS1_LUMTE	Gelsolin-like protein 1	6.55E-136	97-1221[+]	-2.33155	6.168723	84.47587	3.13E-08	2.99E-05
84585				192-1685[+]	-1.26196	5.401941	83.18803	3.51E-08	3.18E-05
109725				140-826[+]	-3.29367	4.17308	83.17939	3.52E-08	3.18E-05
92647	CATL2_HUMAN	Cathepsin L2	5.76E-74	121-1374[+]	-3.42745	4.251303	81.56344	4.07E-08	3.59E-05
107646	CRYL1_PONAB	Lambda-crystallin homolog	1.48E-67	81-1052[+]	-3.63628	4.264846	80.65908	4.43E-08	3.74E-05
92163	ASH2L_HUMAN	Set1/Ash2 histone methyltransferase complex subunit ASH2	3.85E-174	141-1841[+]	1.509098	4.428873	80.56423	4.47E-08	3.74E-05
110166	DYLT5_MOUSE	Dynein light chain Tctex-type 5	5.03E-11	446-1012[+]	-2.24911	4.88915	80.21822	4.61E-08	3.77E-05
111694				76-435[+]	-4.32095	5.494206	79.02894	5.49E-08	4.38E-05
109733	RLT1_RHIO9	Mucoricin	2.85E-06	82-876[+]	-2.08769	5.6816	77.97066	5.7E-08	4.38E-05
102019	KMO_DANRE	Kynurenine 3-monooxygenase	4.41E-176	101-1531[+]	-1.87691	5.534159	77.68384	5.85E-08	4.38E-05
98668	F10A1_CHICK	Hsc70-interacting protein	4.14E-52	529-897[+]	1.38292	6.527399	77.66891	5.86E-08	4.38E-05
103035				137-895[+]	-5.78803	2.821099	77.00844	6.24E-08	4.56E-05
31224	PAR14_HUMAN	Protein mono-ADP-ribosyltransferase PARP14	5.29E-101	22-3069[+]	-2.35118	3.597091	74.42569	8.03E-08	5.73E-05
106794	HPGDS_CHICK	Hematopoietic prostaglandin D synthase	3.64E-43	39-674[+]	-2.41853	5.984745	74.34577	8.17E-08	5.73E-05
105630	GHITM_HUMAN	Growth hormone-inducible transmembrane protein	3.40E-108	123-1163[+]	1.353593	6.324875	73.64685	8.68E-08	5.96E-05

Table A2.6. Selected DEGs (FDR < 0.001): P23 vs. CP23.

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Protein coordinates	logFC	logCPM	F	P value	FDR
111464	PF17064.8	Sleepless protein	1.00E-06	94-495[+]	11.41238	5.432951	379.9085	1.49E-11	2.59E-07

110872				1-489[+]	11.42187	5.395081	379.06	1.51E-11	2.59E-07
75151	DHE3_HUMAN	Glutamate dehydrogenase 1, mitochondrial	0	151-1521[+]	2.986453	7.794221	201.3073	3.11E-11	3.56E-07
111384	CH10_ORYLA	10 kDa heat shock protein, mitochondrial	4.96E-43	116-421[+]	2.058218	8.513635	137.0297	7.24E-10	5.85E-06
78425	MOT12_HUMAN	Monocarboxylate transporter 12	1.50E-07	1-339[+]	2.279155	4.80619	134.2967	8.51E-10	5.85E-06
96233	PUR6_CHICK	Multifunctional protein ADE2	0	157-1425[+]	3.712185	6.421227	130.5207	1.11E-09	6.13E-06
65815	GLSK_RAT	Glutaminase kidney isoform, mitochondrial	0	256-2238[+]	8.868308	3.875601	194.7835	1.25E-09	6.13E-06
83206	CH60_CHICK	60 kDa heat shock protein, mitochondrial	0	138-1877[+]	2.662853	7.137562	116.7814	2.58E-09	1.11E-05
108837	CHIA_MOUSE	Acidic mammalian chitinase	1.31E-53	1-864[+]	5.4376	4.965106	114.8411	3.18E-09	1.22E-05
19471	MOT12_XENTR	Monocarboxylate transporter 12	5.31E-46	453-2324[+]	2.546285	4.884283	108.0846	4.74E-09	1.63E-05
98055	TSAL_GEOSL	L-threonine ammonia-lyase	1.20E-52	59-1408[+]	8.988341	3.287691	139.6821	1.09E-08	3.4E-05
98411	CAH15_MOUSE	Carbonic anhydrase 15	1.57E-48	219-1187[+]	-1.87347	4.529742	94.87829	1.3E-08	3.71E-05
101662	SFXN1_MOUSE	Sideroflexin-1	3.78E-68	3-566[+]	1.821735	6.091986	91.71967	1.68E-08	4.44E-05
99348				127-543[+]	7.748744	5.029388	86.15183	2.92E-08	7.16E-05
110736	CNFN_XENTR	Cornifelin homolog	1.79E-12	217-651[+]	3.860537	4.081214	82.59673	3.71E-08	8.49E-05
85010	HGNAT_MOUSE	Heparan-alpha-glucosaminide N-acetyltransferase	1.16E-117	156-2096[+]	-1.44514	5.674129	80.8038	4.37E-08	9.38E-05
110333	CAPSL_MOUSE	Calcyphosin-like protein	2.85E-60	222-854[+]	1.426146	5.462118	76.68823	6.44E-08	0.00013
103759	GCSH_DROME	Glycine cleavage system H protein, mitochondrial	1.90E-45	98-607[+]	1.343571	6.117554	71.97304	1.03E-07	0.000188
94763	TSAL_GEOSL	L-threonine ammonia-lyase	2.36E-52	164-1513[+]	6.367384	3.482888	71.96221	1.04E-07	0.000188
105717				159-1175[+]	3.787605	4.246836	70.51955	1.21E-07	0.000208
91701				103-954[+]	7.082076	2.591428	91.95599	1.51E-07	0.000248
110871				183-620[+]	5.361959	4.138225	68.1194	1.6E-07	0.00025
105744	FIBA_APOPA	Fibrinogen-like protein A	0.001	2-1264[+]	-2.67943	4.137367	66.34689	1.85E-07	0.000277
97432	PUR6_CHICK	Multifunctional protein ADE2	0	132-1400[+]	3.681636	6.087589	66.03441	2.04E-07	0.000292
101323				206-1474[+]	4.094049	5.521143	65.63088	2.13E-07	0.000293
35544	RIR1_MOUSE	Ribonucleoside-diphosphate reductase large subunit	0	91-2499[+]	1.270267	4.79965	63.41512	2.56E-07	0.000338
79043	GRP75_PONAB	Stress-70 protein, mitochondrial	0	79-2124[+]	1.965587	5.369608	62.22121	2.93E-07	0.000361
97174	ASGL1_HUMAN	Isoaspartyl peptidase/L-asparaginase	6.19E-86	355-1371[+]	8.090432	3.737137	82.73233	2.94E-07	0.000361
73818					7.550435	2.609089	67.57224	3.79E-07	0.000449
55949	TECTA_MOUSE	Alpha-tectorin	4.01E-33	106-2607[+]	-2.30322	5.150658	55.18488	6.83E-07	0.000782
108398	RET1_RAT	Retinol-binding protein 1	9.67E-05	122-577[+]	1.262302	6.329141	54.19737	7.69E-07	0.000852

48789	SC5A8_MOUSE	Sodium-coupled monocarboxylate transporter 1	1.80E-84	288-2048[+]	6.15038	4.575293	54.08343	8.23E-07	0.000884

Table A2.7. Selected DEGs (FDR < 0.001): CP35 vs. CP23.

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Protein coordinates	logFC	logCPM	F	P value	FDR
111384	CH10_ORYLA	10 kDa heat shock protein, mitochondrial	4.96E-43	116-421[+]	2.542695	8.513635	202.0457	3.02E-11	1.04E-06
81037	STIP1_HUMAN	Stress-induced-phosphoprotein 1	1.41E-136	143-1111[+]	2.511713	6.142955	147.8263	3.93E-10	6.75E-06
109908	CRYAB_MACFA	Alpha-crystallin B chain	1.34E-15	129-629[+]	2.842926	6.691767	133.6341	8.85E-10	1.01E-05
108614	CB076_DANRE	UPF0538 protein C2orf76 homolog	6.33E-30	3-431[+]	1.59619	4.875399	120.6725	1.99E-09	1.71E-05
99284	POSTN_MOUSE	Periostin	1.81E-17	1-903[+]	1.693887	5.974099	117.1709	2.52E-09	1.73E-05
108040	U2AF4_RAT	Splicing factor U2AF 26 kDa subunit	1.51E-122	64-732[+]	1.080995	6.861008	113.0869	3.33E-09	1.9E-05
86448	SMCE1_MOUSE	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	3.11E-13	228-1004[+]	-1.94086	6.247321	104.7184	6.06E-09	2.97E-05
84988	TENX_HUMAN	Tenascin-X	5.16E-29	98-2230[+]	3.952697	6.805893	102.3411	7.78E-09	3.34E-05
99029	CDC37_DROVI	Hsp90 co-chaperone Cdc37	3.34E-91	140-1222[+]	1.660808	7.855046	98.20825	9.96E-09	3.8E-05
101878	DDX47_HUMAN	Probable ATP-dependent RNA helicase DDX47	0	94-1425[+]	1.199492	4.957791	95.17739	1.27E-08	4.35E-05
103787	SRSF7_HUMAN	Serine/arginine-rich splicing factor 7	1.66E-19	89-862[+]	1.489683	5.757852	88.38026	2.23E-08	6.95E-05
110342					-2.19513	5.491794	84.14404	3.23E-08	9.23E-05
83206	CH60_CHICK	60 kDa heat shock protein, mitochondrial	0	138-1877[+]	2.184468	7.137562	81.10891	4.25E-08	0.000104
105630	GHITM_HUMAN	Growth hormone-inducible transmembrane protein	3.40E-108	123-1163[+]	1.409665	6.324875	79.8691	4.76E-08	0.000104
20983	MARH6_MOUSE	E3 ubiquitin-protein ligase MARCHF6	0	167-3673[+]	0.895944	6.503036	79.81057	4.79E-08	0.000104
109078	SET_HUMAN	Protein SET	2.40E-46	1-483[+]	-1.39047	6.8527	79.64733	4.86E-08	0.000104
77307	HSP7C_SAGOE	Heat shock cognate 71 kDa protein	0	201-2153[+]	2.71944	5.483423	77.49529	6.05E-08	0.000122
98668	F10A1_CHICK	Hsc70-interacting protein	4.14E-52	529-897[+]	1.336435	6.527399	72.47677	9.76E-08	0.000186
110722					-3.05007	7.394428	71.95855	1.09E-07	0.000197
111639					-1.16444	6.752587	64.50752	2.27E-07	0.000389
110596					-1.09966	8.150899	62.06127	2.98E-07	0.000488
93864	AHSA1_HUMAN	Activator of 90 kDa heat shock protein ATPase homolog 1	2.96E-106	101-1174[+]	2.576564	3.976932	59.26629	4.13E-07	0.000645
103029				1-849[+]	1.696059	8.297292	58.15529	4.72E-07	0.000682

99989	FKBP4_HUMAN	Peptidyl-prolyl cis-trans isomerase FKBP4	1.04E-116	132-1595[+]	2.264978	6.557439	58.20937	4.77E-07	0.000682

Table A2.8. Selected DEGs (FDR < 0.001): P35 vs. P23.

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Protein coordinates	logFC	logCPM	F	P value	FDR
108614	CB076_DANRE	UPF0538 protein C2orf76 homolog	6.33E-30	3-431[+]	1.621396	4.875399	126.5038	1.37E-09	2.44E-05
109908	CRYAB_MACFA	Alpha-crystallin B chain	1.34E-15	129-629[+]	2.73551	6.691767	125.9326	1.42E-09	2.44E-05
77307	HSP7C_SAGOE	Heat shock cognate 71 kDa protein	0	201-2153[+]	3.216757	5.483423	108.099	4.82E-09	5.52E-05
110342					-2.37823	5.491794	96.23014	1.16E-08	8.97E-05
99284	POSTN_MOUSE	Periostin	1.81E-17	1-903[+]	1.517142	5.974099	94.68573	1.32E-08	8.97E-05
99029	CDC37_DROVI	Hsp90 co-chaperone Cdc37	3.34E-91	140-1222[+]	1.607016	7.855046	92.56229	1.57E-08	8.97E-05
81037	STIP1_HUMAN	Stress-induced-phosphoprotein 1	1.41E-136	143-1111[+]	1.893449	6.142955	90.00852	1.94E-08	9.51E-05
111639					-1.33402	6.752587	83.65943	3.37E-08	0.000132
108040	U2AF4_RAT	Splicing factor U2AF 26 kDa subunit	1.51E-122	64-732[+]	0.923519	6.861008	83.35667	3.46E-08	0.000132
105630	GHITM_HUMAN	Growth hormone-inducible transmembrane protein	3.40E-108	123-1163[+]	1.40584	6.324875	80.17852	4.63E-08	0.000159
110596					-1.24436	8.150899	79.03222	5.15E-08	0.000161
86448	SMCE1_MOUSE	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	3.11E-13	228-1004[+]	-1.5876	6.247321	72.4896	9.74E-08	0.000279
109078	SET_HUMAN	Protein SET	2.40E-46	1-483[+]	-1.30319	6.8527	70.89898	1.15E-07	0.000303
100280	MLF2_HUMAN	Myeloid leukemia factor 2	1.02E-38	138-899[+]	2.277786	4.432257	69.0865	1.38E-07	0.000339
20983	MARH6_MOUSE	E3 ubiquitin-protein ligase MARCHF6	0	167-3673[+]	0.821531	6.503036	67.58881	1.62E-07	0.000371

Table A2.9. Selected DEGs (FDR < 0.001): P35 vs. CP35.

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Protein coordinates	logFC	logCPM	F	P value	FDR
111464	PF17064.8	Sleepless protein	1.00E-06	94-495[+]	11.34667	5.432951	388.3452	1.28E-11	3.32E-07
110872				1-489[+]	11.05053	5.395081	365.4093	1.93E-11	3.32E-07

75151				151-1521[+]	2.848323	7.794221	185.563	6.11E-11	7E-07
65815	GLSK_RAT	Glutaminase kidney isoform, mitochondrial	0	256-2238[+]	9.402309	3.875601	230.4827	4.1E-10	3.52E-06
108837	CHIA_MOUSE	Acidic mammalian chitinase	1.31E-53	1-864[+]	5.592593	4.965106	116.9142	2.77E-09	1.66E-05
78425	MOT12_HUMAN	Monocarboxylate transporter 12	1.50E-07	1-339[+]	2.095513	4.80619	115.0716	2.9E-09	1.66E-05
110871				183-620[+]	6.947139	4.138225	99.82968	9.33E-09	4.03E-05
107185	CDD_MOUSE	Cytidine deaminase	3.19E-46	123-533[+]	1.616957	4.652353	98.96031	9.39E-09	4.03E-05
101662	SFXN1_MOUSE	Sideroflexin-1	3.78E-68	3-566[+]	1.824047	6.091986	92.58425	1.56E-08	5.97E-05
105717				159-1175[+]	4.449639	4.246836	85.37524	2.94E-08	0.000101
98055	TSAL_GEOSL	L-threonine ammonia-lyase	1.20E-52	59-1408[+]	7.84665	3.287691	109.5698	5.09E-08	0.000158
99348				127-543[+]	7.949248	5.029388	79.0615	5.52E-08	0.000158
97174	ASGL1_HUMAN	Isoaspartyl peptidase/L-asparaginase	6.19E-86	355-1371[+]	8.851938	3.737137	106.0255	6.39E-08	0.000164
91701				103-954[+]	7.345529	2.591428	104.483	6.85E-08	0.000164
19471	MOT12_XENTR	Monocarboxylate transporter 12	5.31E-46	453-2324[+]	2.071564	4.884283	75.581	7.17E-08	0.000164
103759	GCSH_DROME	Glycine cleavage system H protein, mitochondrial	1.90E-45	98-607[+]	1.273255	6.117554	65.53787	2.02E-07	0.000434
92523	ALAT2_XENTR	Alanine aminotransferase 2	0	136-1695[+]	3.761059	5.047545	65.07918	2.25E-07	0.000455
76615	COCA1_MOUSE	Collagen alpha-1(XII) chain	1.28E-22	51-1688[+]	4.080907	4.950472	61.87903	3.23E-07	0.000617
106668					0.976139	8.238701	59.8857	3.84E-07	0.000694
98411	CAH15_MOUSE	Carbonic anhydrase 15	1.57E-48	219-1187[+]	-1.44681	4.529742	57.76908	4.94E-07	0.000849
94763	TSAL_GEOSL	L-threonine ammonia-lyase	2.36E-52	164-1513[+]	5.992093	3.482888	56.67446	5.7E-07	0.000933

Table A2.10. Selected DEGs (FDR < 0.001, max 50): P35 vs. CP23.

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Protein coordinates	logFC	logCPM	F	P value	FDR
111384	CH10_ORYLA	10 kDa heat shock protein, mitochondrial	4.96E-43	116-421[+]	2.765223	8.513635	234.915	8.54E-12	2.78E-07
111464	PF17064.8	Sleepless protein	1.00E-06	94-495[+]	11.34667	5.432951	375.1865	1.62E-11	2.78E-07
110872				1-489[+]	11.05053	5.395081	352.4341	2.47E-11	2.82E-07
75151	DHE3_HUMAN	Glutamate dehydrogenase 1, mitochondrial	0	151-1521[+]	2.873574	7.794221	188.1282	5.46E-11	4.68E-07
81037	STIP1_HUMAN	Stress-induced-phosphoprotein 1	1.41E-136	143-1111[+]	2.760998	6.142955	175.8944	9.5E-11	6.53E-07

111639					-1.84449	6.752587	155.7052	2.58E-10	1.48E-06
83206	CH60_CHICK	60 kDa heat shock protein, mitochondrial	0	138-1877[+]	3.036281	7.137562	147.6354	3.97E-10	1.82E-06
110342					-2.99423	5.491794	146.4257	4.24E-10	1.82E-06
65815	GLSK_RAT	Glutaminase kidney isoform, mitochondrial	0	256-2238[+]	9.402309	3.875601	221.4316	5.35E-10	2.04E-06
109908	CRYAB_MACFA	Alpha-crystallin B chain	1.34E-15	129-629[+]	2.813381	6.691767	131.2453	1.02E-09	3.51E-06
85010	HGNAT_MOUSE	Heparan-alpha-glucosaminide N-acetyltransferase	1.16E-117	156-2096[+]	-1.84557	5.674129	128.4444	1.21E-09	3.75E-06
108614	CB076_DANRE	UPF0538 protein C2orf76 homolog	6.33E-30	3-431[+]	1.637456	4.875399	127.2344	1.31E-09	3.75E-06
109733	RLT1_RHIO9	Mucoricin	2.85E-06	82-876[+]	-2.64122	5.6816	123.349	1.68E-09	4.43E-06
77307	HSP7C_SAGOE	Heat shock cognate 71 kDa protein	0	201-2153[+]	3.375343	5.483423	114.9464	2.98E-09	7.32E-06
101878	DDX47_HUMAN	Probable ATP-dependent RNA helicase DDX47	0	94-1425[+]	1.301876	4.957791	112.3725	3.5E-09	8.01E-06
99029	CDC37_DROVI	Hsp90 co-chaperone Cdc37	3.34E-91	140-1222[+]	1.770842	7.855046	111.0026	3.85E-09	8.26E-06
78425	MOT12_HUMAN	Monocarboxylate transporter 12	1.50E-07	1-339[+]	1.993242	4.80619	104.2868	6.26E-09	1.26E-05
101662	SFXN1_MOUSE	Sideroflexin-1	3.78E-68	3-566[+]	1.92207	6.091986	101.6599	7.63E-09	1.41E-05
98411	CAH15_MOUSE	Carbonic anhydrase 15	1.57E-48	219-1187[+]	-1.92848	4.529742	100.6569	8.23E-09	1.41E-05
19471	MOT14_MOUSE	Monocarboxylate transporter 14	9.20E-28	453-2324[+]	2.443171	4.884283	100.1933	8.53E-09	1.41E-05
108837	CHIA_MOUSE	Acidic mammalian chitinase	1.31E-53	1-864[+]	5.039694	4.965106	100.9761	8.63E-09	1.41E-05
96403	FCN2_HUMAN	Ficolin-2	1.17E-58	121-999[+]	-4.14305	3.51571	98.30356	9.88E-09	1.54E-05
105630	GHITM_HUMAN	Growth hormone-inducible transmembrane protein	3.40E-108	123-1163[+]	1.540744	6.324875	95.01228	1.28E-08	1.92E-05
105744	FIBA_APOPA	Fibrinogen-like protein A	0.001	2-1264[+]	-3.2844	4.137367	93.92119	1.4E-08	2.01E-05
103759	GCSH_DROME	Glycine cleavage system H protein, mitochondrial	1.90E-45	98-607[+]	1.529375	6.117554	92.67653	1.55E-08	2.13E-05
110596					-1.33093	8.150899	90.00443	1.94E-08	2.49E-05
108040	U2AF1_BOVIN	Splicing factor U2AF 35 kDa subunit	9.49E-113	64-732[+]	0.961449	6.861008	89.91055	1.95E-08	2.49E-05
110871				183-620[+]	6.191528	4.138225	87.13494	2.62E-08	3.21E-05
111705				55-423[+]	-1.86433	6.969525	81.11622	4.24E-08	5.03E-05
110333	CAPSL_MOUSE	Calcyphosin-like protein	2.85E-60	222-854[+]	1.444778	5.462118	78.74093	5.29E-08	6.06E-05
98055	TSAL_GEOSL	L-threonine ammonia-lyas	1.20E-52	59-1408[+]	7.84665	3.287691	104.2505	6.95E-08	7.55E-05
110598	HPGDS_CHICK	Hematopoietic prostaglandin D synthase	5.97E-47	65-739[+]	-1.6368	4.627148	75.78369	7.03E-08	7.55E-05
109811				1-456[+]	2.111746	4.975141	75.27661	7.39E-08	7.69E-05
110736	CNFN_XENTR	Cornifelin homolog	1.79E-12	217-651[+]	3.656831	4.081214	74.93767	7.64E-08	7.71E-05
97174	ASGL1_HUMAN	Isoaspartyl peptidase/L-asparaginase	6.19E-86	355-1371[+]	8.851938	3.737137	101.5208	8.37E-08	8.21E-05

91701				103-954[+]	7.345529	2.591428	99.46209	9.31E-08	8.69E-05
111027	HEMTN_HIRME	Neurohemerythrin	2.84E-48	121-483[+]	-1.97599	9.452163	73.10526	9.36E-08	8.69E-05
100763	FIBA_APOPA	Fibrinogen-like protein A	2.02E-26	3-1265[+]	-2.2653	3.973629	72.44551	9.79E-08	8.85E-05
103787	SRSF7_HUMAN	Serine/arginine-rich splicing factor 7	1.66E-19	89-862[+]	1.340151	5.757852	72.10078	1.01E-07	8.85E-05
111193	RLT1_RHIO9	Mucoricin	2.03E-05	1-381[+]	-1.0399	8.993963	71.4748	1.08E-07	8.85E-05
102867				3-743[+]	-3.70894	4.422183	71.64116	1.09E-07	8.85E-05
86448	SMCE1_MOUSE	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	3.11E-13	228-1004[+]	-1.57266	6.247321	71.03724	1.13E-07	8.85E-05
111238	SIAE_MOUSE	Sialate O-acetylesterase	9.93E-26	3-563[+]	-2.50104	5.87043	71.18651	1.13E-07	8.85E-05
98668	F10A1_CHICK	Hsc70-interacting protein	4.14E-52	529-897[+]	1.321561	6.527399	71.0003	1.13E-07	8.85E-05
99989	FKBP4_HUMAN	Peptidyl-prolyl cis-trans isomerase FKBP4	1.04E-116	132-1595[+]	2.519116	6.557439	70.87245	1.17E-07	8.94E-05
99348				127-543[+]	6.983743	5.029388	70.45567	1.28E-07	9.57E-05
108398	RET1_RAT	Retinol-binding protein 1	9.67E-05	122-577[+]	1.433201	6.329141	69.48337	1.33E-07	9.69E-05
88942	CKS1_MOUSE	Cyclin-dependent kinases regulatory subunit 1	1.42E-29		1.28089	5.273971	68.17036	1.52E-07	0.000109
4822	SPPL3_MOUSE	Signal peptide peptidase-like 3	2.25E-120	178-1452[+]	1.471924	5.539888	67.81241	1.58E-07	0.000111
101323				206-1474[+]	4.166293	5.521143	67.83187	1.68E-07	0.000116

Table A2.11. Selected DEGs (FDR < 0.001): CA23 vs. CP23.

Transcript ID	UniProtKB / Pfam ID	Protein	E-value	Protein coordinates	logFC	logCPM	F	P value	FDR
110872				1-489[+]	9.250092577	5.395081	227.5491	4.62E-10	1.11E-05
111464	PF17064.8	Sleepless protein	1.00E-06	94-495[+]	9.066498742	5.432951	216.35591	6.46E-10	1.11E-05
65815	GLSK_RAT	Glutaminase kidney isoform, mitochondrial	0	256-2238[+]	8.500284729	3.8756008	176.65621	2.37E-09	2.72E-05
110778				2-394[+]	-3.66767379	4.0408414	96.382836	1.15E-08	9.88E-05
110736	CNFN_XENTR	Cornifelin homolog	1.79E-12	217-651[+]	4.126679425	4.0812143	93.016474	1.51E-08	0.000104
29004	PHP3_CAEEL	Homeobox protein php-3	1.35E-20	125-1786[+]	2.809894579	3.9573868	89.470147	2.03E-08	0.000105
111744				78-410[+]	-2.76062134	4.6909984	88.807122	2.15E-08	0.000105
91701				103-954[+]	7.998835259	2.5914279	118.07895	3.18E-08	0.000128
101323				206-1474[+]	4.713344201	5.5211429	84.255602	3.45E-08	0.000128

109520				70-759[+]	-1.3863853	6.7216249	82.545751	3.72E-08	0.000128
109025	PI16_MOUSE	Peptidase inhibitor 16	9.83E-33	120-938[+]	-5.216424	6.0503044	79.64825	5.23E-08	0.000163
98055	TSAL_GEOSL	L-threonine ammonia-lyase	1.20E-52	59-1408[+]	7.915674901	3.287691	105.96541	6.28E-08	0.00018
111490				58-567[+]	-5.56069267	5.1367829	72.87501	1E-07	0.000265
110035				42-812[+]	-6.04559056	4.4412115	72.02652	1.09E-07	0.000267
106397				78-554[+]	-9.08368312	2.9636069	166.15167	1.4E-07	0.000306
75151	DHE3_HUMAN	Glutamate dehydrogenase 1	0	151-1521[+]	1.668907666	7.7942205	68.807849	1.42E-07	0.000306
106621	TX53A_ETHRU	U-scoloptoxin(05)-Er3a	0.000427	74-511[+]	-1.85976779	6.1074206	68.128027	1.53E-07	0.000309
97174	ASGL1_HUMAN	Isoaspartyl peptidase/L-asparaginase	6.19E-86	355-1371[+]	8.396546072	3.7371368	89.956078	1.76E-07	0.000337
70009				2560-2889[-]	-1.10967967	5.2055569	63.171398	2.63E-07	0.000463
108890				158-541[+]	-1.82338368	5.5281834	62.955038	2.7E-07	0.000463
110095				63-842[+]	-8.34037953	2.497638	137.91264	3.4E-07	0.000546
108837	CHIA_MOUSE	Acidic mammalian chitinase	1.31E-53	1-864[+]	3.821258351	4.9651062	61.106331	3.5E-07	0.000546
99348				127-543[+]	6.461864622	5.0293876	59.956316	4.04E-07	0.000583
45987	PF14295.9	PAN domain	0.011	167-2392[+]	-8.53470778	2.718586	96.946352	4.07E-07	0.000583
103851				2-1303[+]	-9.8621235	4.0764944	97.876272	4.25E-07	0.000584
110658				49-438[+]	-6.13448867	4.8424905	59.036359	4.5E-07	0.000594
103128				116-475[+]	-1.62602195	5.037624	56.242583	5.95E-07	0.000731
86767				2075-2404[-]	-1.01462859	5.3818191	56.232557	5.96E-07	0.000731
111023				79-585[+]	-6.5263034	4.6159054	54.159956	8.15E-07	0.000966
18061	HUNB_DROOR	Protein hunchback	4.45E-23	74-3595[+]	7.086475018	2.2033096	59.453352	8.71E-07	0.000967
94763	TSAL_GEOSL	L-threonine ammonia-lyase	2.36E-52	164-1513[+]	5.42110532	3.4828877	53.283988	8.73E-07	0.000967

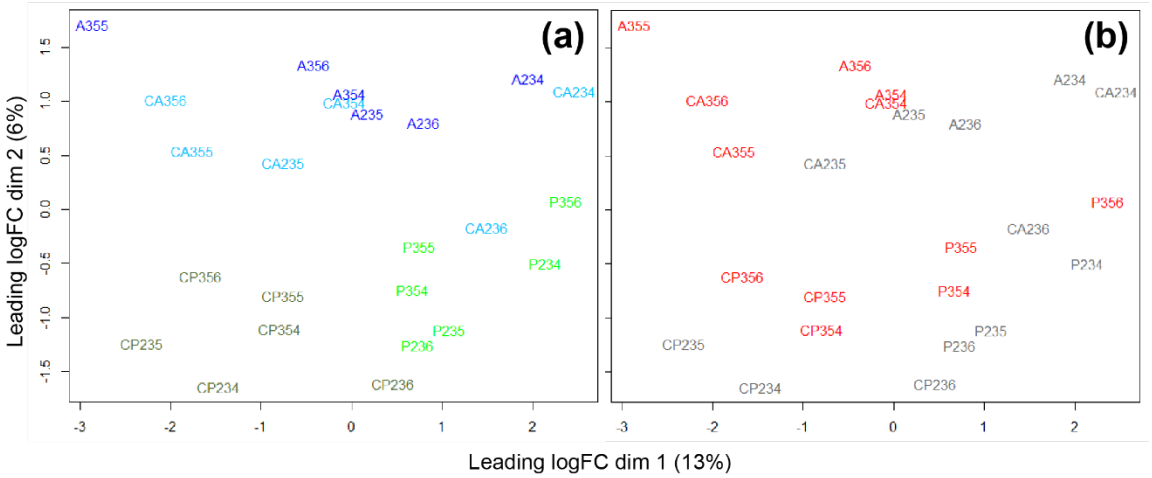

Figure A2.1. Multidimensional scaling plot of Euclidean distances between transcript expression profiles of experimental samples. Color coding corresponds to injury treatment (light blue = CA, blue = A, olive = CP, green = P; a) or temperature treatment (gray = 23 °C, red = 35 °C; b).

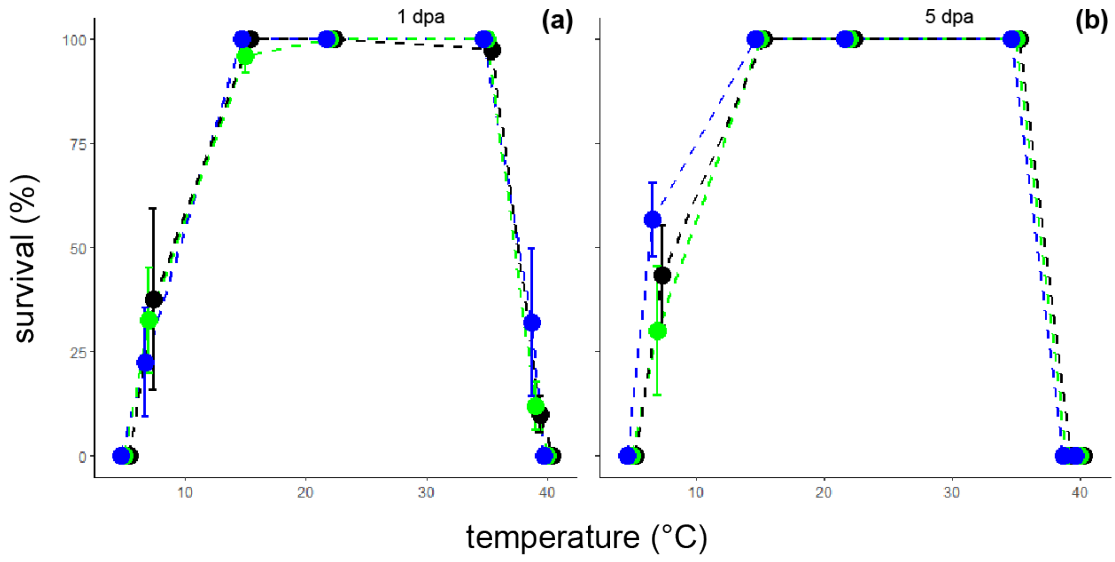

Figure A2.2. Survival percentage of worms following 48-hour exposure to temperature at 1 dpa (a) and 5 dpa (b). Color indicates injury condition (black = uninjured, blue = anterior amputation, green = posterior amputation). n = 5 for each data point. Bars indicate standard error.

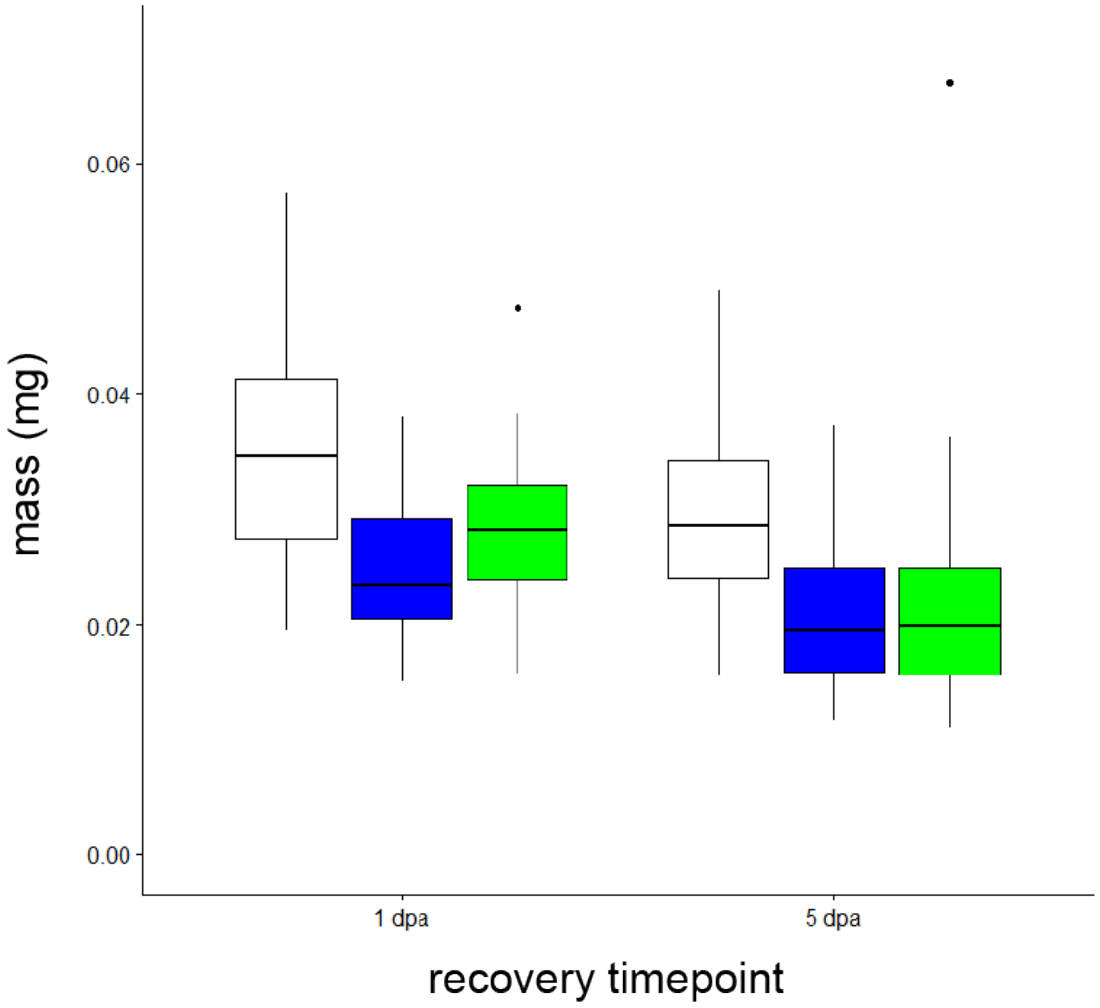

Figure A2.3. Worm mass by recovery timepoint and injury condition (white = uninjured, blue = anteriorly injured, green = posteriorly injured). Worms are pooled across all three respirometry temperature treatments. *n* = 54 for each box.

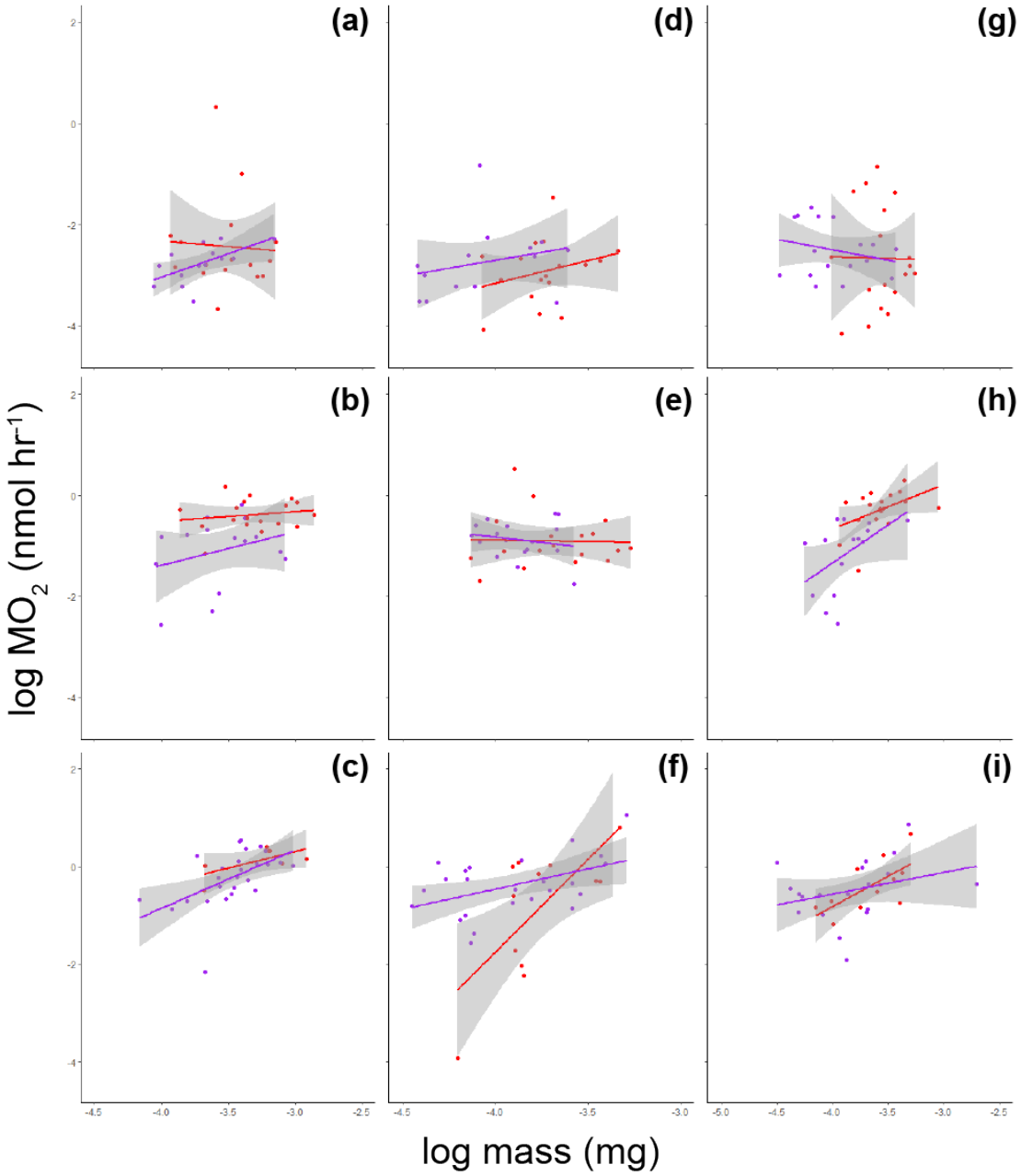

Figure A2.4. Log of oxygen consumption rate as a function of estimated worm mass between 1 dpa (red) and 5 dpa (purple) worms without injury (a-c), anteriorly injured (d-f), and posteriorly injured (g-i) at 10 (a,d,g), 23 (b,e,h), and 35 °C (c,f,i). Fitted lines are constructed using a linear method. Shading represents 95% confidence intervals.

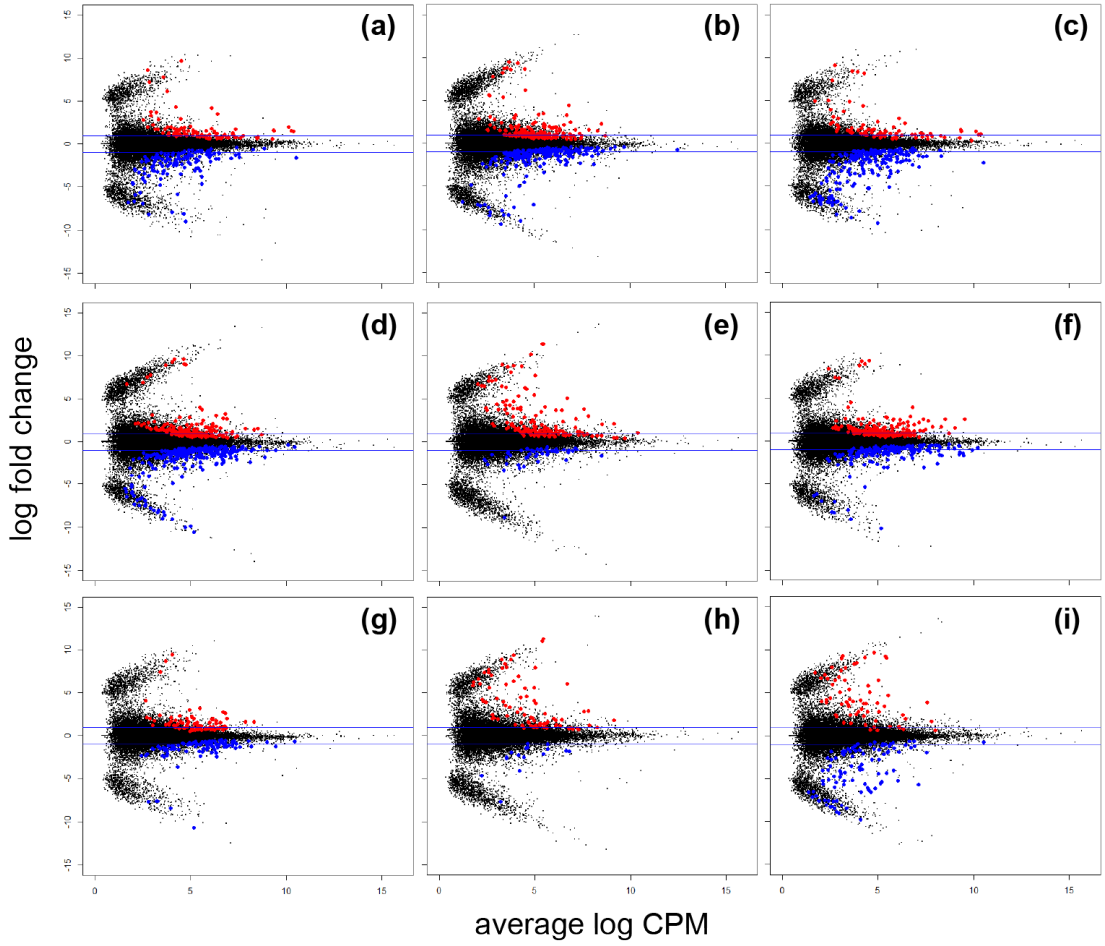

Figure A2.5. Mean-difference plots showing all filtered transcripts (dots) including those differentially expressed (red = upregulated, blue = downregulated; FDR < 0.05) for selected pairwise comparisons: A23 x CA23 (a), CA35 x CA23 (b), A35 x CA35 (c), A35 x A23 (d), P23 x CP23 (e), CP35 x CP23 (f), P35 x P23 (g), P35 x CP35 (h), CA23 x CP23 (i). Horizontal blue lines mark ±1 fold change.

Appendix 3: Chapter 4 supplementary figures

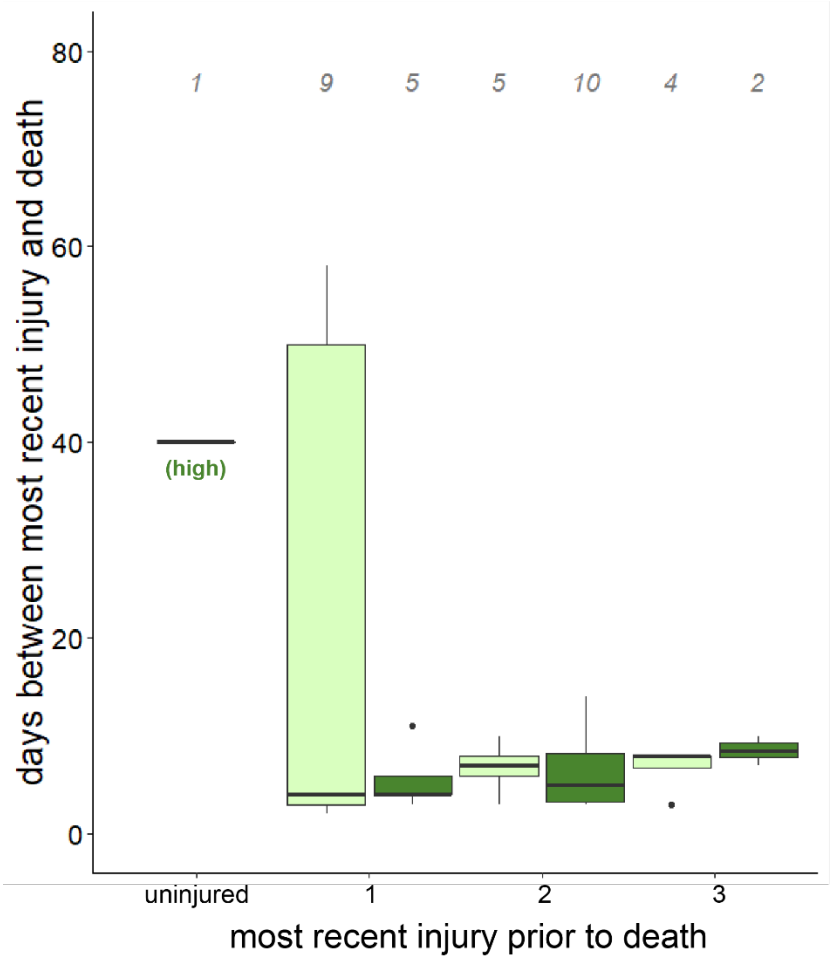

Figure A3.1. Time (in days) between most recent injury experienced and death in F_0 worms. Color indicates feeding treatment (light green = low, dark green = high). Sample size is indicated over each bar. Data include only those worms that died before the end of the experiment.

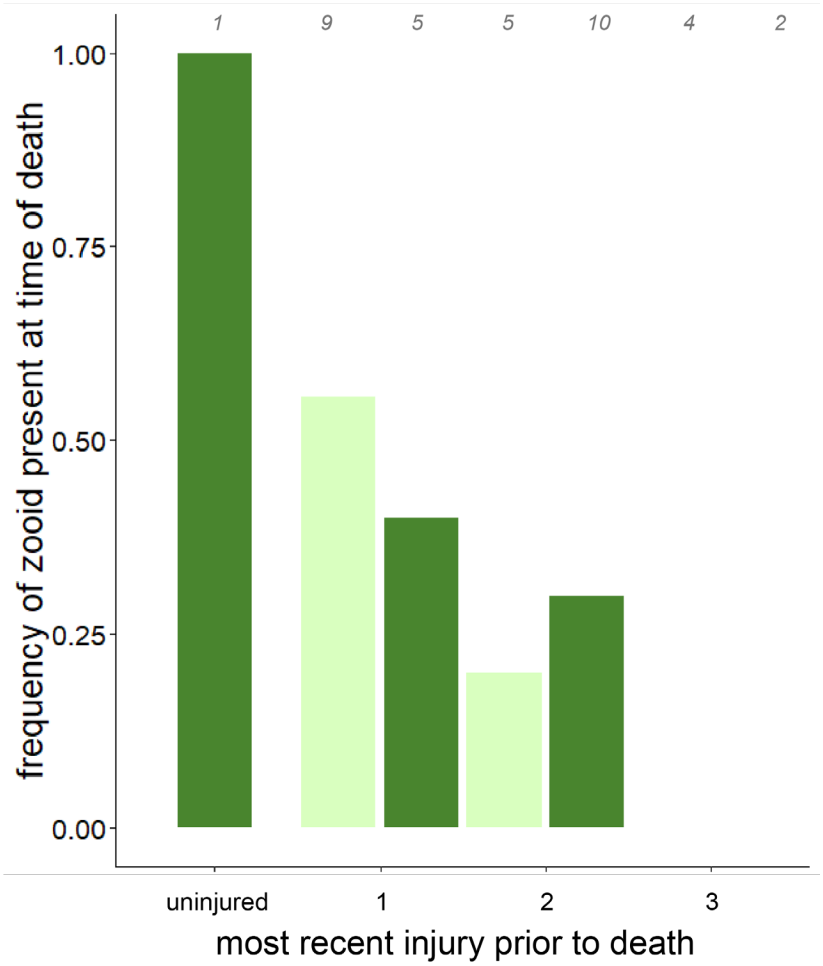

Figure A3.2. Frequency of worms that produced a living zooid at the time death was discovered in F_0 worms by the most recent injury that worm had experienced prior to death. Color indicates feeding treatment (light green = low, dark green = high). Sample size is indicated over each bar. Data include only those worms that died before the end of the experiment.

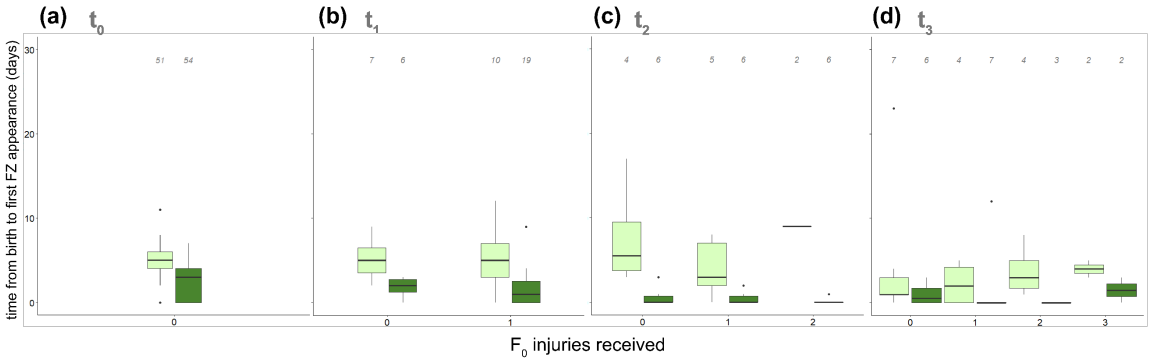

Figure A3.3. Time from birth to initial detection of the first fission zone in F_1 worms produced following the start of the experiment (t_0) (a) and the first (t_1) (b), second (t_2) (c), and third (t_3) (d) F_0 regeneration periods. Color indicates parental feeding treatment (light green = low, dark green = high). Sample size is indicated over each bar.

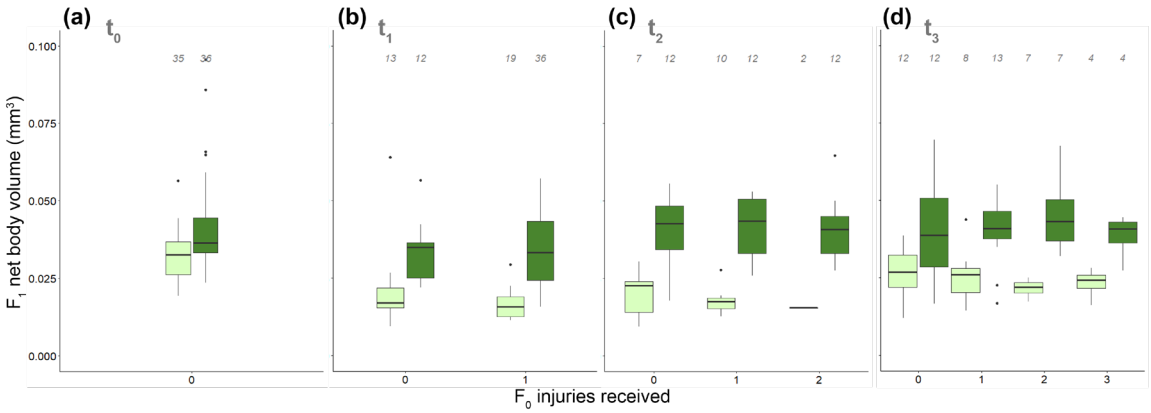

Figure A3.4. Net body volume of F_1 worms produced following the start of the experiment (t_0) (a) and the first (t_1) (b), second (t_2) (c), and third (t_3) (d) parental regeneration periods. Color indicates parental feeding treatment (light green = low, dark green = high). Sample size is indicated over each bar.

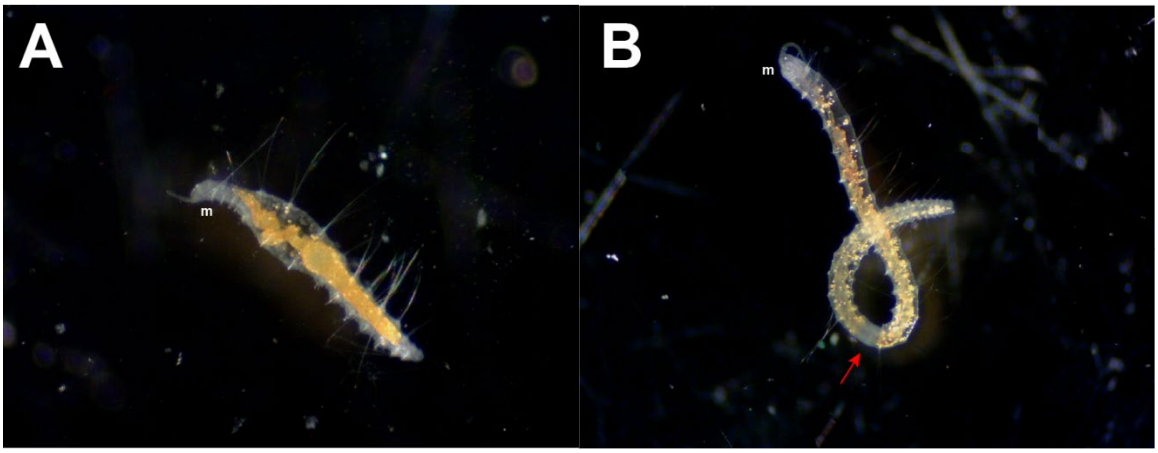

Figure A3.5. *Pristina leidyi* exhibiting signs of stress (a) versus healthy and actively fissioning (b). Note darker pigmentation of the alimentary canal, posterior truncation, and coelomic swelling in (a). m = mouth, red arrow indicates fission zone. Images not to scale.

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