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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Quantitative approaches to reproductive and locomotion behaviors in
planarians and mouth opening in *Hydra***

A thesis submitted in partial satisfaction of the
requirements for the degree
Master of Science

in

Biology

by

Jason Alexander Carter

Committee in charge:

Professor Eva-Maria S. Collins, Chair
Professor James Posakony
Professor Gürol Süel

2015

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Chair

University of California, San Diego

2015

DEDICATION

*To my parents, Jeff and Patty, and my sister, Kaitie
for their love and unwavering support*

EPIGRAPH

He attacked everything in life with a mix of extraordinary genius and naive incompetence, and it was often difficult to tell which was which.

—Douglas Adams

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ACKNOWLEDGEMENTS

Two and a half years ago, Dr. Collins gave a wide-eyed freshman, armed with little more than a vague notion of what research actually was, the opportunity to join her lab. That I have come so far, in such short a time, is a testament to Eva-Maria's mentorship. Without her dedication, passion for science and education, constant pushing and, most importantly, patience to put up with my endless typos, I would not be where I am today. Through my experiences here, I have acquired not only a wealth of knowledge, of which only a small fraction is directly related to worms, but a love of science and the desire to pursue a career in research.

I would also like to thank Olivier Cochet-Escartin, who was always happy to lend a helping hand, poke holes in my theories, and talk about exponentials. Furthermore, I owe thanks to Phuong Truong, who helped me image more worms than I care to count, to My Dang, who helped take care of more worms than I could possibly count, and to Angel Leu, who ran far to many *in situs* on my behalf. I would also like to thank the members of the Collins lab who made the past few years so enjoyable.

Finally, I would like to thank all of the wonderful co-authors I have had the privilege of working with over the past few years. Without their hard-work, none of this would have been possible:

Chapter 2 has been submitted for publication of the material. Carter, Jason; Lind, Christine; Truong, Mai P.; and Collins, Eva-Maria S. "To Each Their Own. Reproductive Strategies and Success of Three Common Planarian Species: *Schmidtea mediterranea*, *Dugesia japonica* and *Dugesia tigrina*". The thesis author was the primary investigator and author of this paper.

Chapter 3 has been submitted for publication of the material. Carter, Jason; Sinha, Joydeb; Cochet-Escartin, Olivier; and Collins, Eva-Maria S. "A *slo-1* homolog is required for the normal ethanol response in the planarian species *Schmidtea mediterranea*." The thesis author was the primary investigator and author of this paper.

Chapter 4 has been submitted for publication of the material. Carter, Jason*; Hyland, Callen*; Steele, Robert E.; and Collins, Eva-Maria S. "A Physical Model for

Mouth Opening in *Hydra*". *These authors contributed equally to this work. The thesis author was one of the primary investigators and the primary author of this paper.

ABSTRACT OF THE THESIS

Quantitative approaches to reproductive and locomotion behaviors in planarians and mouth opening in *Hydra*

by

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Master of Science in Biology

University of California, San Diego, 2015

Professor Eva-Maria S. Collins, Chair

Planarians and *Hydra* are famous for their incredible regenerative abilities and are considered classical organisms for studies in stem cell biology and regeneration. Besides these areas, both organisms have also emerged as popular models for evolution, aging and toxicology. In this thesis, I use a variety of quantitative methods to address three behavioral questions in these organisms; (1) how is planarian asexual reproductive behavior regulated, (2) what is the molecular mechanism controlling ethanol-induced behavior in planarians, and (3) what are the physical processes governing *Hydra* mouth opening.

To answer the first question, we utilize the results of a long-term, large-scale experiment tracking tens-of-thousands of individual reproductive events to gain un-

precedented insight into the life history strategies of three common planarian species: *Schmidtea mediterranea*, *Dugesia japonica*, and *Dugesia tigrina*. We find that, despite sharing a common anatomy and reproducing exclusively through binary fission, each species employs a distinct reproductive strategy on the population level. With regards to the second question, we develop quantitative methods to compare the behavioral responses of planarians exposed to exogenous ethanol and identify two genes that are necessary for a drunken behavioral response to acute ethanol exposure in planarians. Finally, we develop the first dynamical understanding of mouth opening in *Hydra*, a process which has been extensively studied physiologically and that requires tearing a new hole in the animals epithelium every time it wants to eat.

Together, these studies demonstrate the power of quantitative methods to provide novel insights into diverse questions ranging from the population to the organismal tissue and molecular levels.

Chapter 1

Introduction

Planarians are a type of flatworm of the phylum *Platyhelminthes* long renowned for their incredible regenerative abilities [65, 101, 105]. Mediated by a large pluripotent stem cell population (comprising nearly 30% of all cells), planarians can accomplish incredible feats of regeneration following amputation, including the *de novo* production of a complete digestive system, central nervous system and a complex brain composed of approximately 10,000 neurons [2, 75, 77]. These regenerative abilities enable small tissue fragments taken from any portion of an adult planarian to regenerate into complete worms [19, 65, 75, 77]. Due to these stem cells and regenerative ability, planarians are one of the few complex organisms to reproduce asexually *via* binary fission, wherein the animal tears itself into two pieces, each of which regenerates into a complete animal within approximately one week [4, 47, 48, 81].

Like planarians, *Hydra* has gained historical attention for its regenerative ability and potential immortality [60, 89]. *Hydra* are a freshwater cnidarian polyp with a radially symmetric body column [9]. At the head of the body column is a ring of tentacles and a dome-shaped hypostome, at the center of which is a mouth. The body structure of *Hydra* itself is morphologically simple, composed of two epithelial tissue layers (ectoderm and endoderm) separated by a thin layer of extracellular matrix, which is known as the mesoglea [13, 50, 55]. Despite lacking a central nervous system, *Hydra* do possess various nerve cells scattered throughout the epithelial tissue layers, which can stimulate coordinate contraction of two antagonistic sets of muscle processes, which are located in the ectoderm and endoderm, respectively [12, 43]. These muscles comprise the animal's hydrostatic skeleton and give rise to its structure and behavior [49].

In this thesis, we employ techniques from quantitative biology, including automated image analysis and tracking, mathematical modeling and statistics, to answer three questions relating to various aspects of planarian and *Hydra* behavior ranging from the population level to molecular level. (1) How is asexual reproduction regulated in populations of complex, multicellular organisms and does it bear similarities to the regulation of asexual reproduction in unicellular organisms? In order to answer this question, we generate entire life histories for planarians, a complex multicellular organism, on a level previously only seen for unicellular organisms. (2) What is the molecular mechanism of neuronal control of planarian locomotion behavior in re-

sponse to acute exposure to exogenous ethanol? In addressing this question, we find that planarians are uniquely suited for pharmacological studies examining the molecular mechanism by which drugs interact with the central nervous system in a way that is directly applicable to humans. (3) What are the forces governing mouth opening behavior in *Hydra*, which requires a new hole to be torn in the epithelial tissue of the *Hydra* each time it wishes to open its mouth? Although *Hydra* mouth opening has been well documented physiologically, our study represents the first biomechanical understanding of the actual opening process.

In the first chapter of this thesis, using data collected from over 23,000 individual reproductive events spanning six years, we present the first statistical, comparative analysis of reproductive strategies in the planarian species *Schmidtea mediterranea*, *Dugesia japonica*, and *Dugesia tigrina*. We find that, despite the anatomical similarity of all three species and the fact that each reproduces *via* transverse binary fission, each species employs a distinct strategy for optimizing reproductive success. Furthermore, we found that models developed in bacterial systems to describe cell cycle length could be directly applied to describe the time between reproductive events in planarian asexual reproduction.

In the second chapter, the molecular mechanism responsible for the planarian ethanol response was examined. Combining molecular and genetic techniques to knockdown specific proteins with quantitative analysis of behavioral phenotypes, two planarian homologs of the *slo1* gene, encoding big potassium ion channel isoforms, were identified as necessary for ethanol sensitivity. Furthermore, we found that both planarian homologs displayed a high degree of conservation with the human *slo1* gene, suggesting that planarians could represent a powerful invertebrate animal system for pharmacological studies examining the molecular targets and effects of ethanol and other drugs on the nervous system.

In the third chapter, we take advantage of transgenic *Hydra* expressing fluorescent proteins in the epithelial tissue and confocal microscopy to perform the first dynamical analysis of the mouth opening process. Mouth opening in *Hydra* is unique insofar as the mouth is not a permanent opening and must be formed by ripping through two sealed layers of epithelial tissue with each opening. Previous studies have examined only the morphology and physiology of the *Hydra* mouth, but

little was known about how the mouth opening process actually works. We find that a simple phenomenological model is capable of reliably describing the mouth opening curve, as well as predicting several biologically relevant parameters of the *Hydra* mouth. Further examination of the biomechanics involved with mouth opening suggests that mouth opening is an active process driven by the musculature, which we predict must exert forces on the order of a few nN to achieve opening. Overall, this study illustrates that the simplicity of the *Hydra* body structure, combined with the accessibility of *in vivo* experiments, make *Hydra* a powerful model organism for quantitative biomechanical studies on the cellular and tissue levels.

In sum, these three studies demonstrate the powerful insight a quantitative approach can provide to a wide variety of biological problems ranging from the population level to the molecular level. Furthermore, the studies presented in this thesis demonstrate that these two classical regenerative organisms allow us to study fundamental problems in biology beyond regeneration.

Chapter 2

To Each His Own

Reproductive Strategies and Success of Three Common
Planarian Species: *Schmidtea mediterranea*, *Dugesia*
japonica, and *Dugesia tigrina*

2.1 Abstract

Planarians are the most complex animals with the ability to regenerate complete organisms from small tissue pieces. This ability allows them to reproduce by splitting themselves into a head and a tail piece, making them a rare example of asexual reproduction via transverse fission in multi-cellular organisms. Due to the stochastic nature of long reproductive cycles, which range from days to months, few and primarily qualitative studies have been conducted to understand the reproductive behaviors of asexual planarians. We have executed the largest long-term study on planarian asexual reproduction to date, tracking more than 23,000 reproductive events of three common planarian species found in Europe, North America, and Asia, respectively: *Schmidtea mediterranea*, *Dugesia tigrina*, and *Dugesia japonica*. This unique data collection allowed us to perform a detailed statistical analysis of their reproductive strategies. Since the three species share a similar anatomy and mode of reproduction by transverse division, we were surprised to find that each species had acquired its own distinct strategy for optimizing its reproductive success. We statistically examined each strategy, associated trade-offs, and the potential regulatory mechanisms on the population level. Interestingly, models for cell cycle length regulation in unicellular organisms could be directly applied to describe reproductive cycle lengths of planarians, despite the difference in underlying biological mechanisms. Finally, we examined the ecological implications of each strategy through intra- and inter-species competition experiments and found that *D. japonica* outcompeted the other two species due to its relatively equal distribution of resources on head and tail pieces, its cannibalistic behaviors and ability to thrive in crowded environments. These results show that this species would pose a serious threat to endogenous planarian populations if accidentally introduced in their habitats.

2.2 Introduction

The reproductive strategy of a particular species plays an important role in determining its evolutionary fitness for a given habitat. Understanding how different reproductive strategies for closely-related species have evolved to optimize their reproductive success in response to different environmental challenges represents a fundamental problem of evolutionary biology and life history theory [87, 88]. Because an individual can only ingest a certain amount of energy, it needs to optimize how that energy is allocated to growth and reproduction. Ultimately, it will face trade-offs between allocated resources to offspring and parent, number of offspring, frequency of reproduction, and adult and offspring mortality [84, 87]. Thus, every time an animal reproduces it should accomplish this in a way that maximizes the number of offspring that will survive and continue to reproduce [41]. While many studies exist on life history strategies in sexual animals, *e.g.* Refs. [82, 83, 85], little is known in the context of multicellular asexually reproducing organisms.

Here we compare the reproductive strategies of three closely related freshwater planarian species found in Southern Europe [4, 6, 24], North America [6, 23], and East Asia [46, 66], respectively: *Schmidtea mediterranea* (S), *Dugesia tigrina* (G), and *Dugesia japonica* (J). All three species are used as model organisms in the fields of regenerative medicine and stem cell biology [1, 65]. Each species also reproduces asexually by splitting into a head and a tail piece and subsequently regenerating the missing body structures within roughly a week, a feat made possible by their large pluripotent stem cell population [28, 65, 105].

Four previous studies on the reproductive dynamics of G-planarians exist which are relevant to our work [3, 23, 78, 81] and whose key results can be summarized as follows: Isolated G-worms grew, as expected, exponentially and faster than grouped G-worms (0.079/day for isolated *vs.* 0.029/day for grouped cannibalistic and 0.075/day for grouped non-cannibalistic worms). The average length of the reproductive cycle was determined to be approximately 11 days with significant variability among individuals, the reproduction rate was found to be independent of density but dependent on the absolute number of animals. The primary reason for mortality during the growth phase was cannibalism, with smaller tails having a higher

probability of being ingested than larger ones, and the probability of cannibalism was found to increase with increasing worm density. Finally, regarding size, worms of various sizes were found to reproduce, generally producing a larger head piece and a smaller tail piece at division (70% *vs.* 30% [81]). In addition, heads hardly grew between consecutive divisions whereas tails grew substantially.

However, one discrepancy exists between the findings in Refs. [3, 78] compared to Refs. [23, 81]: While the former showed that heads had shorter reproductive cycles than tails, the opposite was reported in [23, 81]. Possible explanations may be a difference in strain as well as in observation time (25-30 days in [23, 81] versus 102 days in [3, 78]). In addition, a large variability in the reproductive behaviors among worm families was observed and each family tree only comprised 4-10 animals at the end of the experiment [23].

This discrepancy and the stochastic nature of planarian reproduction emphasize the importance of large-scale longitudinal studies to gain reliable insight into the reproductive strategies of these species. Using such a large-scale, long-term experiment [97] tracking thousands of reproductive events, we have previously studied, both experimentally and theoretically, the life history strategy of S-planarians in great detail [28, 70, 98]. Statistical analysis of the reproductive behavior of individual worms in isolation revealed that S-planarians exhibit a randomized reproductive strategy with respect to the number of offspring produced per reproductive event. The distribution of offspring was well described by a maximum relative entropy principle for certain types of worms within the population [70]. Furthermore, we showed that S-planarians appear to regulate their reproductive cycles partially through their size at birth and partially through a stochastic term whose nature remains unknown [28, 98].

However, it remained to be determined whether the observed reproductive behaviors were particular to that planarian species or whether they constituted a general “planarian strategy” as a consequence of their anatomy and reproduction by transverse division. To address this question, we studied two other species, J- and G-worms, which originate from North America and East Asia, respectively, allowing us to compare three species from three continents. Despite these geographic differences, all three species share a similar morphology, food and habitat preferences (slow running and still water), and reproduce by transverse division. It is therefore not ob-

vious whether all three species would share the same strategy or whether they had evolved different solutions to the same problem based on the ecological differences of their natural habitats. Moreover, G- and J-planarians have been reported as invasive species [4, 16, 24, 46, 66]. In fact, *D. tigrina* can now frequently be found in Europe, often co-habiting with other planarian species [37] and *D. japonica* can be found across Japan and China [66].

Since our last study on S-worm reproduction dynamics [98], we have more than doubled our S-worm data and are now exceeding 15,000 individuals. Additionally, we have tracked more than a combined 8,500 asexual reproductive events from the two other planarian species. Although detailed statistical analysis of asexual reproduction has been carried out on single-cell systems, the results presented in this paper represent an original large-scale, long term comparative analysis of asexual reproductive strategies in higher organisms. To our surprise we found that each species has acquired a unique reproductive strategy. While S-planarians exhibited infrequent reproductive events with large clutch sizes, G-planarians primarily underwent rapid consecutive divisions producing only one small and one large offspring. Finally, J-worms divided roughly symmetrically, thus optimizing resource allocation and survival of both offspring. Interestingly, the regulation of reproductive cycles could largely be explained with models originally developed for cell cycle control [45]. Finally, a pairwise competition of members of the three species in confined environments demonstrated that the most successful life strategy in direct competition was based on the ability to distribute resources equally among offspring, eat one’s competitor, and sustain crowded environments. This finding has important implications for species preservation regulations as it demonstrates how accidental human introduction of foreign species into new habitats can potentially wipe out endogenous populations.

2.3 Materials and Methods

2.3.1 Planarian Maintenance

Three species of freshwater planarians: *Schmidtea mediterranea* (S), *Dugesia japonica* (J), and *Dugesia tigrina* (G) were used in all experiments. G-planarians in

principle can produce either sexually or asexually. However, given a complete lack of eggs found throughout our experiments, we assume that our strains reproduced only asexually. S- and J- worms were clonal strains. S- and J-species were established lab colonies, originally from Europe and China, respectively. G-worms were purchased from Ward’s Science and caught in the wild in Wisconsin, U.S.A. Worms were kept in the dark at 20° C in an incubator (Panasonic), except for during feeding (once per week), cleaning (twice per week) and counting (once per week). To assure that food was not a limiting resource, worms were fed in excess with organic liver purchased from a local butcher.

2.3.2 Isolated Worm Experiments

Five S-worms, two J-worms and two G-worms were randomly selected from the lab planarian stock. These “founder” worms were placed individually in petri dishes (100mm diameter x 20mm height, Fisher Scientific) containing 25mL of planarian water [17]. Upon asexual reproduction, the reproductive event was recorded in the SAPling database as described in detail in Ref. [97]. Worm reproductive waiting times (RWTs) and the type of reproductive event (fission vs fragmentation) were recorded as previously described in Ref. [70]. In all, 15,030 S-, 6,229 J- and 2,492 G-worm reproductive events were recorded over the course of 70 months.

2.3.3 Image Acquisition and Area Analysis

Worms were imaged using a Leica stereo microscope (Leica Microsystems, Wetzlar, Germany) with a Basler A601f CCD camera (Basler AG, Ahrensburg, Germany) and Basler Pylon Viewer (Basler AG, Ahrensburg, Germany). Cannibalism assays were performed by placing a petri dish under this camera, cutting a worm with a razor blade and imaging at 0.2 frames per second until either all pieces had been consumed or one hour had elapsed. In the isolated worm experiment, all offspring of a subset of randomly selected worms (2,143 S-, 2,128 J-, and 340 G-pieces) were imaged within 3 days of asexual reproduction. In these experiments, worm pieces were separated post-imaging into individual petri dishes and all pieces returned to the incubator. Because planarians are approximately two-dimensional, their size was determined by recording

their areas [98], as determined by a custom MATLAB (MathWorks, Natwick, MA) script. Area of the worm at division was approximated by summing the areas of all offspring.

2.3.4 Interacting Population Experiments

Planarian populations were maintained in Tupperware boxes containing 100 or 200 mL of planarian water. As in Ref. [28], planarians were counted by pipetting individual worms into a petri dish while simultaneously recording the number of worms with a palm click counter. For each experiment, a minimum of two boxes with identical conditions were used to account for variability in the randomly chosen initial worm population. Food was provided in excess once per week as to not be a limiting resource. In competition experiments, two different species, specifically J- vs. S-, G- vs S-, and J- vs G-planarians, were kept together under the same conditions described in this section.

2.3.5 Growth Rates and Competition Analysis

For each trial, population counts were binned into approximately 14 day time periods and averaged. Actual time periods ranged from 13-15 days depending on when the worms were counted. Only populations exceeding 20 planarians were included as outlined in Ref. [28]. N_0 was fixed at 20 worms for all fits. When scaling was necessary, the data was scaled by volume because we found that the critical carrying capacity scaled by volume (Supplemental Figure 1) rather than by area, as previously suggested in Ref. [8]. The mean population for each trial was calculated by averaging the populations of the boxes in that set. As some trials ran for a longer time period than others, the later mean populations were calculated by averaging over the continuing trials. The planarian populations that exhibited growth were fitted with the logistic growth equation [28].

2.4 Results and Discussion

To gain a better understanding of the reproductive strategies employed by each of the three planarian species (S (Figure 2.1(a)), J (Figure 2.1(b)) and G (Figure 2.1(c)), we first examined asexual reproduction rates and types exhibited by individual worms kept in isolation. Isolation was considered optimal for growth because it allows for the elimination of direct competition for food and space as factors influencing reproduction. In addition, we eliminate possible growth inhibition of individual worms stimulated by worm-worm interactions, as suggested in Ref. [8].

Tracking the reproductive behaviors of thousands of individual worms growing in isolation was enabled by our SAPling database system, which is described in detail in Ref. [97]. In recording the reproductive events of 15,030 S-, 6,229 J- and 2,492 G-worm, we obtained unprecedented statistical insight into the asexual reproductive strategies for each of the three planarian species.

2.4.1 Fissions, Fragmentations and Reproductive Waiting Times

All three planarian species reproduce asexually by spontaneously splitting themselves perpendicular to the anterior-posterior body axis to create two daughter worms, one (H)ead- and one (T)ail-piece, from a single worm (Figure 2.1(a-d)). The H-piece contains the eyes, brain, and usually the pharynx (eating tube), whereas the T-piece has to regenerate these structures *de novo*. We refer to a single splitting event (*i.e.* two daughter pieces produced at the same time from a single worm) as a **division**. At each reproductive event a worm produces multiple (≥ 2) offspring, each of which regenerate into an adult worm within about a week. The time between the birth of an offspring and the next reproductive event of that offspring is denoted as the **Reproductive Waiting Time (RWT)** (Figure 2.1(d)). Following the nomenclature established in our previous work, we use the term **Teilung** to refer to a reproductive event in general [70, 98].

For a detailed analysis of reproductive strategies it is necessary to further differentiate types of Teilung based on the number of offspring produced per event. As in our previous works, we distinguish **fissions (fi)**, in which only one H- and one T-piece are produced, from **fragmentations (fra)**, in which at least three offspring

are produced, i.e. a H-, T-, and a (M)iddle (trunk)piece [70, 98]. In essence, a fra-event is nothing else than several consecutive divisions that occurred so rapidly one after another that the worm did not have enough time to fully regenerate between them, thus leading to the existence of M-pieces without well-defined tail morphology. In our previous work, we determined the minimum time for regeneration to be 6 days [28, 70, 98]. Therefore, if a worms divides again within 5 days since its last division, we consider the entire event as a fra-Teilung and the total RWT of that worm is the sum of the times between divisions (Figure 2.1(d)).

We have previously shown that the RWTs of S-planarians differ significantly depending on whether the worm was itself born as a H- or a T-piece [28, 70, 98]. Because all three species share the same anatomical differences between H- and T-offspring, we expected to find a similar trend for the other two species. Indeed, for all species, T-worms were found to possess significantly longer (roughly double) RWTs as compared to their respective H-counterparts (Figure 2.1(e)). A comparison of the probability distribution function (PDF) for the RWTs of H- and T-worms for each species revealed that T-worms generally have a higher probability of reaching long RWTs and a wider spread of RWTs compared to their H-worm counterparts (Figure 2.1(f-h)). S-species T-worms exhibited the longest recorded RWTs, which were, on average, 2-3 times longer than those observed for T-worms of the other two species. The longest recorded S-species T-worm RWT was 300 days, whereas the maximum RWTs for J- and G-species T-worms were 148 and 67 days, respectively (Figure 2.1(f-h)). Similarly, the spread of H-worm RWTs for S-worms was significantly larger than those of H-worms from either of the other two species, with the longest recorded RWTs for S-species H-worms being 4-10 times longer than the longest observed RWTs in J- and G-species H-worms, respectively (Figure 2.1(f-h)).

Interestingly, all T-worm PDFs seemed to display a plateau in the frequency of short RWTs while no similiar trend was present for H-worms. Based on our previous work dealing with memory effects in S-species T-worms, we hypothesized that the origin of this plateau for the other species was similarly due to the difference in RWTs for T-worms that were produced from H-worms (T_H) *versus* T-worms that were produced from T-worms (T_T)[28]. Indeed, our data demonstrates that for all three species, T_T -worms have a statistically significantly longer average RWT when

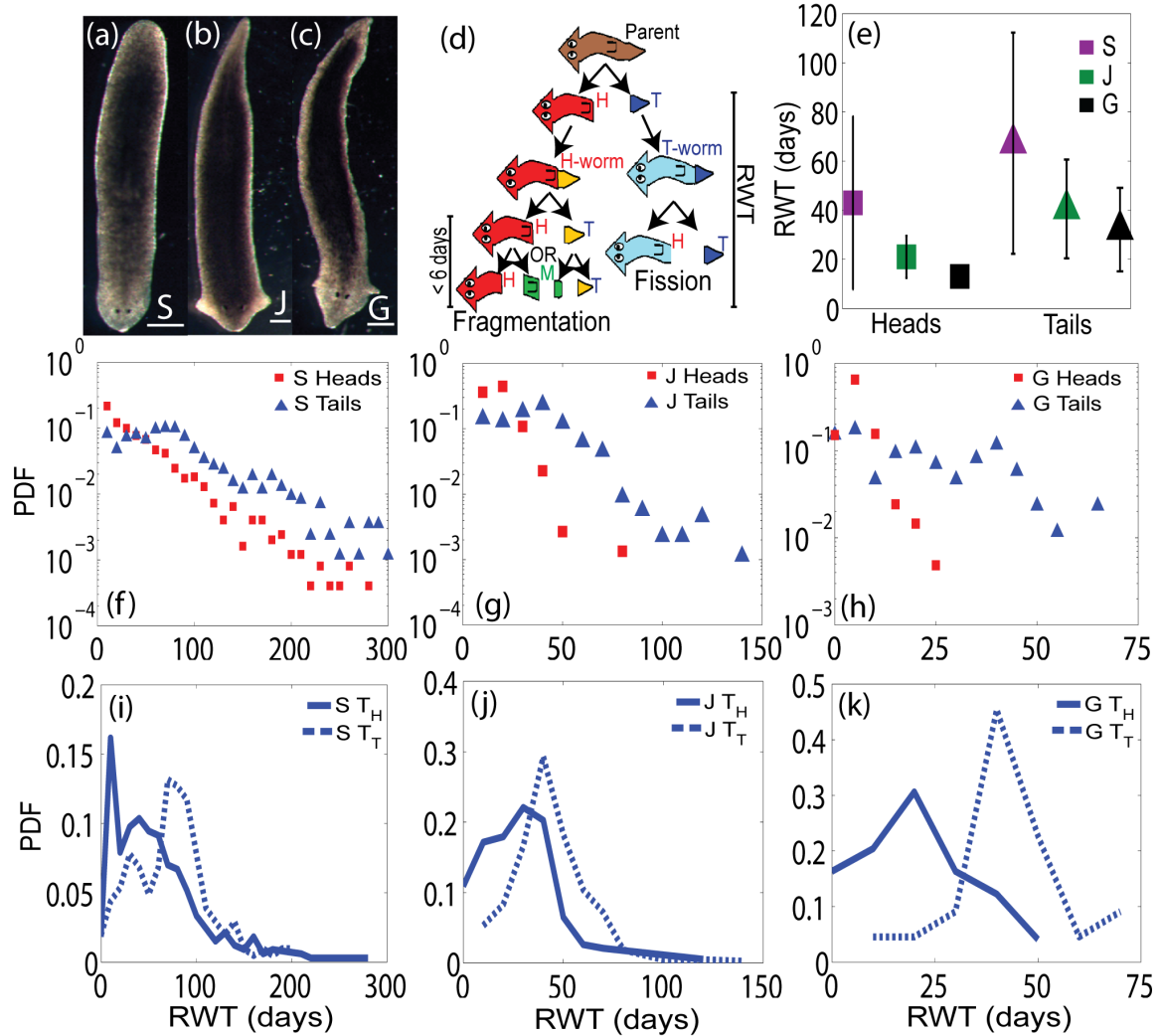


Figure 2.1: Reproductive behaviors and -waiting times. Representative images of (a) *S. mediterranea* (S), (b) *D. japonica* (J), and (c) *D. tigrina* (G). Scale bar corresponds to 1 mm. (d) Schematic illustrating reproductive behaviors and naming convention used. (e) Average RWTs of all reproductive events for H- and T-worms for each of the three species. Error bars represent STD . (f) Probability distribution function (PDF) of RWTs for all reproductive events for H- and T-worms for S-, (g) J- and (h) G-planarians. (i) Probability distribution function (PDF) of RWTs for all reproductive events for T-worms divided between those with H-parents (T_H) and T-parents (T_T) for S-, (j) J- and (k) G-planarians. The differences in the distributions for of T_H and T_T worms of all three species were found to be statistically significant ($p < 0.0001$) using a Student's T-test. In all, 15,030 S-, 6,229 J- and 2,492 G-worms were examined

compared to T_H worms of the same species (Figure 2.1(i-k)). Thus, a parent-memory effect exists in all three species, whose biological origin remains to be determined [28]. Of note, this does not seem to be an aging effect since the H- and T-identity of grandparents do not seem to matter.

In summary, the RWTs of all three planarian species are strongly influenced by whether a worm was born as a H- or T-piece. T-pieces of all three species have, on average, substantially longer RWTs than H-pieces of the same species, in agreement with previous reports on G-worm reproduction [78]. Furthermore, for all three species, T-pieces whose parent was a T-worm show significantly longer RWTs than T-pieces of the same species whose parent was an H-worm. Further inter-species comparison of RWTs shows that S- planarians exhibit the longest and widest-spread RWTs, while G-worms have the shortest RWTs of the three species for both H- and T-worms.

2.4.2 Isolated Worms: Worm and Teilung Types

RWTs strongly correlate with the type of worm (H- *vs.* T-worm) undergoing the Teilung for each of the three species, with T-pieces generally exhibiting longer RWTs (Figure 2.1(f-h)). In addition, we have previously shown that, in S-worms, the length of a worm's RWT correlates with the type of Teilung the worm ultimately undergoes, *e.g.* the RWT *preceding* a fission is usually shorter than the RWT *preceding* a fragmentation [70, 98]. Taken together, this implies that the RWT of a given worm depends on: (1) the Teilung type that a worm ultimately undergoes (fi *vs.* fra), and (2) the type of worm (H *vs.* T) that an individual was born as. In this section, we elaborate on the distinctions between worm type and Teilung type, which will become important when we examine reproductive strategies in the following section.

Teilung types Reproductive events can be split into fi- and fra-Teilungs, leading to either two (1 H- and 1 T-piece) or multiple offspring (1 H-piece, 1 T-piece and 1 or more M-pieces) per reproductive event (Figure 2.1(d)). We have previously shown that fragmentations are an important part of the reproductive strategy of S-worms [70, 98]. Both H- and T-worms have the potential to undergo a fragmentation, though S-species H-worms fragment more often than T-worms do. A S-worm may produce anywhere between one and seven M-pieces in a single Teilung [98]. Therefore,

we here further distinguish fra-Teilungs in which two or more M-pieces are produced from those fra Teilungs in which only one middle piece is produced. We refer to the former, in which 4 or more offspring are produced in a single reproductive event, as a **Multiple Fragmentation (multi-fra)** Teilung. We refer to the latter, in which 3 offspring are produced simply as a fra Teilung. Because multi-fra producing more than two M-pieces are rare in S-planarians [98] and the other two species do not exhibit multi-fra events at all (Figure 2.2(a)), we do not further differentiate multi-fra based on the number of pieces produced in the event.

Worm types In addition to separating Teilungs based on the number of daughter pieces produced (fi *vs.* fra *vs.* multi-fra), we further distinguish Teilungs based on the type of worm undergoing Teilung. Worm type is determined by the H- or T-identity of the worm undergoing a Teilung (Figure 2.1(d)). Strictly speaking, not all worms fall into a H- or T-worm identity as some are M-worms produced in fra-Teilungs (Figure 2.1(d)). However, as fra-events are rare in J- and G- worms (Figure 2.2(a)), we simplify our classification of worm types throughout this paper by treating M-pieces as T-pieces unless otherwise specified. Accordingly, we distinguish fissions that occurred from H-worms (**H-fi**) and those that occurred from T-worms (or M-worms) (**T-fi**), and H-fragmentation and T-fragmentation (**H-fra** and **T-fra**, respectively). We do not differentiate between worm types for multi-fra events given the rarity of multi-fra events in J- and G-worms. That is, we refer to any reproductive event in which 4 or more offspring are produced simply as multi-fra, regardless of the worm type undergoing that reproductive event.

2.4.3 Isolated Worms: Basic Reproductive Strategies

An inter-species comparison of the frequency with which each species employs the aforementioned Teilung types demonstrate that the three species utilize distinct reproductive strategies. Furthermore, an examination of the relationships between Teilung type, RWTs, area at Teilung (A_T) and the fraction of the worms area invested in the H-daughter piece at Teilung provides insight into possible trade-offs associated with each reproductive strategy.

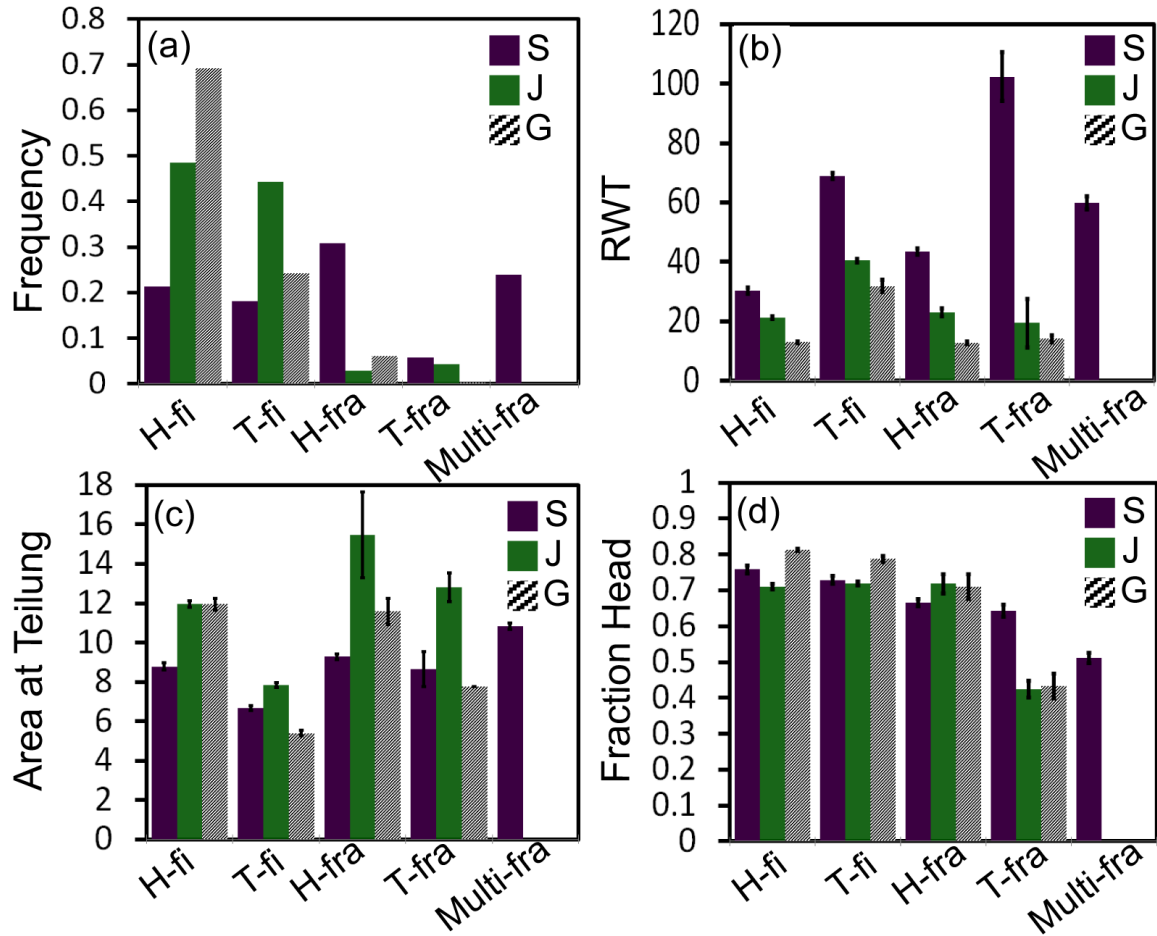


Figure 2.2: Reproductive modes differ among the three planarian species. (a) Frequency of Teilung types ($N_S=15,030, N_J=6,229, N_G=2,492$). (b) RWT depends on Teilung type ($N_S=15,030, N_J=6,229, N_G=2,492$). (c) Area at Teilung ($N_S=898, N_J=616, N_G=288$). (d) Percentage of the worm undergoing Teilung's area allocated to the H-piece immediately following Teilung (H-daughter area divided by total area of the worm at Teilung; $N_S=898, N_J=616, N_G=288$). Error bars indicate SEM.

S-planarians S-planarians rely heavily on H-fra ($\sim 35\%$) and multi-fra ($\sim 25\%$) with H-fi and T-fi making up the remaining $\sim 40\%$ (Figure 2.2(a)). This result is interesting as it shows that fragmentations are a much more important aspect of S-planarian reproduction ($\sim 60\%$) than we previously reported ($\sim 30\%$) [70]. In fact, we had speculated in Ref. [70] that we may see an increase in fragmentations in S-planarians over time, because our optimization model suggested multi-fra producing 2-3 tails/middles per Teilung to be optimal for the reproductive success of S-planarians under the same conditions. The reliance of S-planarians on H-fra as the predominate mode of reproduction maximizes the number of offspring produced per Teilung, but comes with the associated cost of longer RWTs and substantially less investment in the survival of the H-offspring (Figure 2.2(b,d)). That is, although these Teilungs are higher yield, they happen less frequently. In the wild, this could increase the risk of being eaten by a predator before reproduction has occurred. As we have shown previously [98], even S-worms with a small area at Teilung have a non-zero probability of fragmenting, leading to small offspring and causing all pieces, including H-pieces, to exhibit a slower consecutive RWT, which decreases the chance that any offspring will survive to continue reproducing even further. In summary, the S-planarian reproductive strategy emphasizes high-yield reproductive events at the cost of Teilung frequency and the amount of resources invested into individual offsprings (Figure 2.3(a,d)). This is conceptually similar to organisms which increase their clutch area at the expense of parental care [36]. In the wild, one would then expect that this increase in number of offspring produced per event would come at the cost of decreased survival rates for all offspring.

G-planarians Like S-worms, G-worms optimize the number of offspring produced, although in an entirely different manner. G-planarians showed the lowest percentage of H-fra and T-fra ($< 5\%$ combined) and never underwent multi-fra reproductive events. G-worms employ H-fi as the predominate reproductive method (Figure 2.2(a)), which enables the maintenance of large H-worms through successive Teilungs and allowing for the shortest average H-worm RWTs to be approximately 10 days (in excellent agreement with previous studies [3, 23]) for any of the three species (Figure 2.2(b,c)). Correspondingly, G-worms' area at Teilung is maintained

by a large investment in the H-piece ($\sim 80\%$) at the expense of T-offspring investment (Figure 2.2(d)), in agreement with previous studies [3, 23, 81]. Of note, those G-species T-worms which undergo fra- events have RWTs comparable to G-species H-worms undergoing fission and significantly shorter than the RWTs of the other two species. These T-worms are also significantly larger than T-worms undergoing fission. We speculate that G-worms are able to produce small T-pieces without compromising population growth in the wild, because only a fraction of the worms are cannibalistic [3, 78] and in their natural habitat T-pieces may be able to hide underneath rocks or plants and thus be protected from larger H-pieces [3, 78] (see also Section 2.4.8). In summary, G-worms predominantly undergo H-fi events, whereby they invest heavily in the area of the H-offspring, allowing the H-offspring to quickly regrow and drop off successive new T-worms in a repetitive high-yield cycle (Figure 2.3(c,d)).

J-planarians Finally, J-worms rarely fragmented and instead reproduced predominantly through equal numbers of H- and T-fi (Figure 2.2(a)). Accordingly, J-planarians invest the least, $\sim 70\%$ on average, in the H-offspring during fission when compared to the other two species. This means that J-species T-worms tend to be more similar in size and RWTs when compared to J-species H-worms. Because J-planarians are cannibalistic and were observed to aggressively pursue food-size worm pieces of their own kind (Supplemental Movie 1), this reproductive strategy of producing predominantly large offspring may help ensure offspring survival. This effect is further discussed in Section 2.4.8. Overall, J-planarians seem to adopt an intermediate reproductive strategy. Namely, they avoid high-yield, high-risk fragmentations and asymmetric fissions, in favor of reproducing through proportionate amounts of H- and T-fi Teilungs, in which H- and T-offspring receive similar investment of parental resources leading to intermediate RWTs. Of the three species, J-worms invest the most in their T-pieces, suggesting an attempt to optimize the number of pieces that survive at the cost producing less offspring (Figure 2.3(b,d)).

In conclusion, despite sharing the same anatomical mechanism of dividing in two pieces to reproduce, each planarian species has evolved a unique strategy to do so. The RWTs of the three species follow the trend $RWT_S \gg RWT_J > RWT_G$ (Figure 2.2(b)), with S-planarians sacrificing the number of reproductive events in

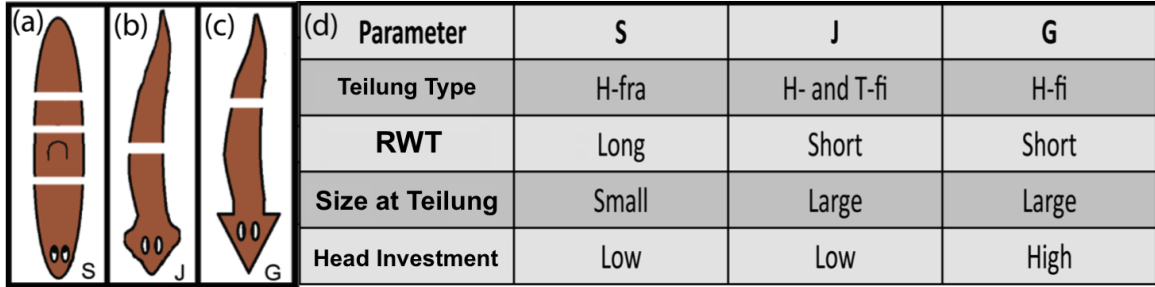


Figure 2.3: Each species of planarian exhibits a distinctive asexual reproductive strategy. Schematic showing predominate reproductive strategy for (a) S-, (b) J- and (c) G-worms. S-worms rely on high-yield but infrequent fragmentations, J-worms rely equally on H- and T-fi events and G-worms rely predominately on high-frequency H-fi. (d) Table summarizing reproductive strategies and consequent trade-offs.

favor of a greater number of offspring (N) in each event ($N_S \gg N_J = N_G$, Figure 2.2(a)), although these offspring are generally much smaller ($A_S \ll A_J = A_G$, Figure 2.2(c)). Finally, we found that S-planarians and J-planarians invest the least and greatest amount of energy into ensuring the survival of the H-offspring ($E(H)$; $E(H)_G > E(H)_S > E(H)_J$, Figure 2.2(d)). Thus, we conclude each species evolved a unique reproductive strategy optimized for its natural habitat and each strategy can be summarized as follows: (1) S-planarians primarily undergo high-yield H-fra and multi-fra reproductive events (Figure 2.3(a)), (2) J-planarians reproduce roughly equally through H- and T-fi (Figure 2.3(b)), and (3) G-planarians rely predominately upon H-fi (Figure 2.3(c)).

2.4.4 Isolated Worms: Relating RWTs and Size at Teilung

Next, we asked whether we could relate the observed reproductive strategies to the average worm size at Teilung. To this end, we imaged all daughter pieces following a Teilung to estimate the area of 898 S-, 616 J- and 288 G-worms at the time they underwent Teilung. Combining this data with the long-term tracking of individual worms, we were able to correlate the **area at Teilung** of an individual with the *preceding* RWT of that individual (Figure 2.4). A linear correlation (slope= m) between preceding RWTs and area at Teilung would imply that worms grow continuously until undergoing a reproductive event. Thus, the longer a worm waits to

reproduce, the larger it should be when it ultimately does reproduce. We found a weak linear correlation between preceding RWTs and area at Teilung for S- and J-species H- and T-worms and G-species T-worms (in all cases, $m \leq 0.05$). Thus, while worms which waited longer to divide were on average large at Teilung, this effect was not strong and may also depend upon the size at birth (Figure 2.4(a-c)).

Surprisingly, however, we observed a strong negative correlation ($m = -0.56$) for G-species H-worms (Figure 2.4(c)), implying that G-worms decrease in area at Teilung with increasing preceding RWT. Although counter-intuitive, this result can largely be accounted for by the short RWTs of G-worms (mostly ≤ 20 days). Given that worms are fed once a week and regeneration requires 7 days, such short RWTs indicate that little actual growth of an individual G-planarian H-worm occurred before it underwent another Teilung. Therefore, since growth between successive generations is negligible, worms of different sizes reproduced roughly with the same RWTs, in agreement with previous findings [3]. Only H-worms that were much smaller took longer to divide than larger pieces, leading to the observed distribution (Figure 2.3(c)). This is consistent with the model of G-reproductive strategies proposed earlier, *i.e.* rapid reproduction via dropping of small T-pieces through successive fast H-fi (Figure 2.3(c)).

Given the drastic area differences between the three species, a direct comparison of absolute areas at Teilung (S: $\sim 6 \text{ mm}^2$, J: $\sim 12 \text{ mm}^2$, G: $\sim 12 \text{ mm}^2$) is unlikely to yield any meaningful results (Figure 2.2(c)). The distribution of areas at Teilung, however, does reflect how individuals in each population allocate their resources during reproduction and thus reflects each reproductive strategy. For S-planarians, the PDF of area at Teilung show that, on average, H-worms divide larger than T-worms, consistent with our previous results [98]. Notably, there is a large distribution of the S-worm areas (H-worms: $\sim 5 \text{ mm}^2$ to $\sim 20 \text{ mm}^2$; T-worms: $\sim 2 \text{ mm}^2$ through $\sim 12 \text{ mm}^2$), likely due to differences in area of worms undergoing fission and fragmentation events (Figure 2.2(c,d)).

For J-worms, T- and H- worms exhibit two distinct peaks at $\sim 5 \text{ mm}^2$ and $\sim 13 \text{ mm}^2$, respectively. Similar to the S-species, the J-species T-worm peak occurs at a significantly smaller area than the H-worm peak. In both cases, the distributions are narrower than those for S-worms (Figure 2.4(e)), due to J-worms primarily fissioning (Figure 2.2(a)). The tail of the distribution toward larger areas likely corresponds to

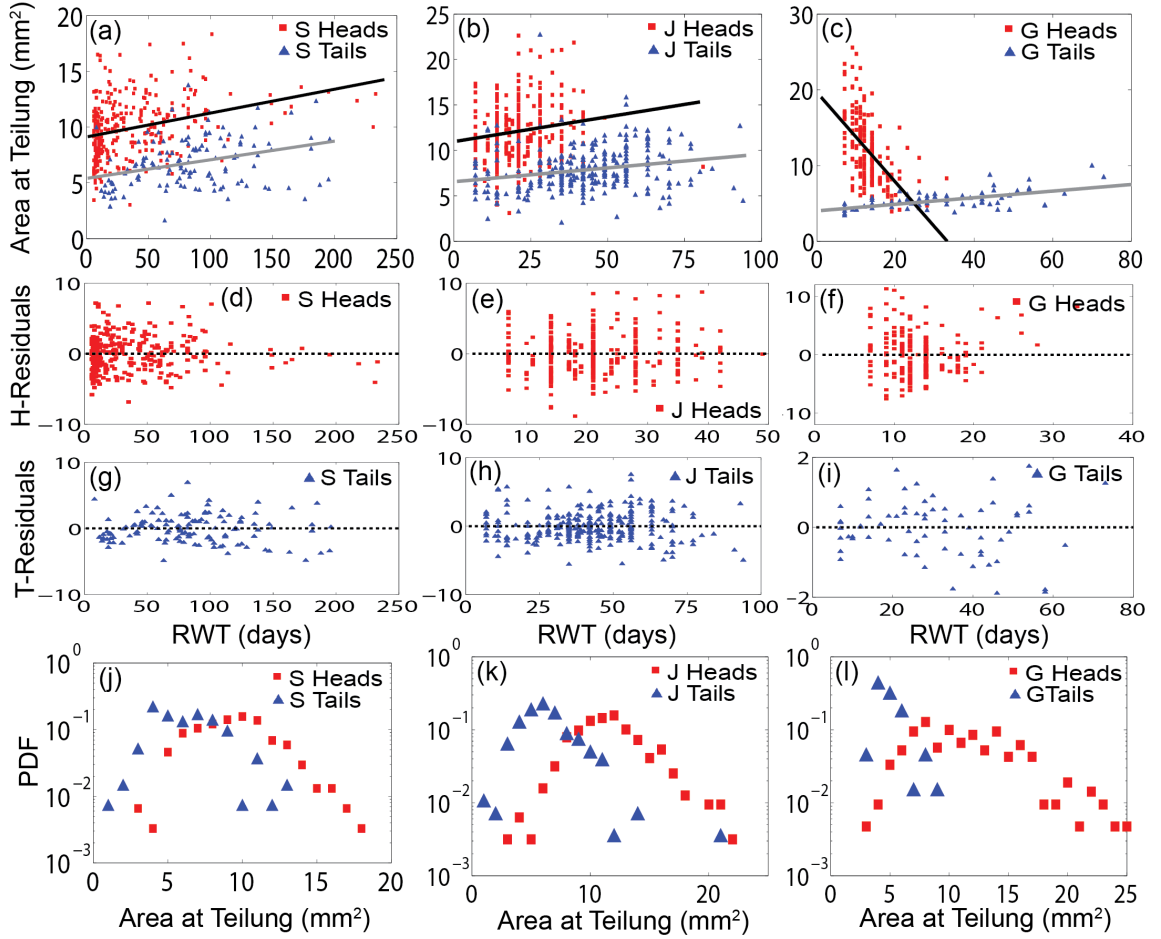


Figure 2.4: RWTs and area at Teilung. Area at Teilung *vs.* RWT with linear best fit lines for (a) S_H ($y = 0.02x + 9.1$; $R^2=0.10$), S_T ($y = 0.2x + 5.4$; $R^2=0.11$), (b) J_H ($y = 0.05x + 11$; $R^2=0.03$), J_T ($y = 0.03x + 6.6$; $R^2=0.05$), and (c) G_H ($y = -.59x + 20$; $R^2=.30$), G_T ($y = 0.04x + 4.0$; $R^2=0.36$). Non-skewed residuals indicate linear fits are appropriate for (d) S-species H-worms, (e) J-species H-worms, (f) G-species H-worms, (g) S-species T-worms, (h) J-species T-worms, and (i) G-species T-worms. Area at division PDFs for (j) S_H (N=610) and S_T (N=288), (k) J_H (N=317) and J_T (N= 299), and (l) G_H (N= 211) and G_T (N= 70). For all species, H-worms are plotted as red squares and T-worms are shown as blue triangles. H-worm best-fit lines are shown in black while T-worm best-fit lines are shown in gray.

the minority of worms undergoing fragmentation (Figure 2.4(e)).

Finally, G-worms show a narrow distribution of areas at Teilung for T-worms (centered around 5mm^2) and a larger spread of Teilung-areas for H-worms (ranging from $\sim 5\text{mm}^2$ to $\sim 25\text{mm}^2$). Interestingly, G-species T-worms show the smallest area range, with all areas less than 10mm^2 (Figure 2.4(f)). These areas at Teilung reflect our earlier observation that G-species H-worms divide rapidly without time for substantial growth (Figure 2.3(c,d)). Thus, after several division cycles, the absolute area at Teilung is smaller. It is not immediately obvious why G-worm tails do not grow to larger areas before dividing. Potentially, the frequency of Teilung is regulated differently from that of S- or J-worms; see Section 2.4.6.

2.4.5 Isolated Worms: Relating RWTs, Size at Birth, and Death Statistics

As mentioned above, producing small offspring is likely to decrease that offspring's chance of survival. To quantitatively study the **relationship between area at birth and survival rates**, we imaged 2,143 S-pieces, 2,128 J-pieces and 340 G-pieces at birth (immediately following Teilung). Combined with the RWTs of these worms, we can ask whether trade-offs exist between the reproduction frequency, size and number of offspring, and the worms' survival rates.

For H-worms of all three species and J-species T-worms, the relationship between area at birth and following RWT is best fit by a decreasing exponential, indicating that the larger a worm is at birth, the faster it will divide, in agreement with the observed positive exponential growth rates in S-worms [98]. Interestingly, in S- and G-planarians, little correlation is found between T-worm area at birth and following RWT (Figure 2.5(a-c)).

As expected from Figure 2.2(d) showing that H-worms are larger than T-worms at Teilung, the PDFs of area at birth show for all species that the birth-area of H-worms are generally larger than those of T-worms (Figure 2.5(j-l)). J-worms show the most similarity between H- and T-worm area at birth (H: 1mm^2 to 20mm^2 ; T: 1mm^2 to 15mm^2). In contrast, both S- and G-worms show a much narrower range of T-worm areas at birth (S: $\sim 1\text{mm}^2$ to $\sim 5\text{mm}^2$; G: $\sim 1\text{mm}^2$ to $\sim 5\text{mm}^2$),

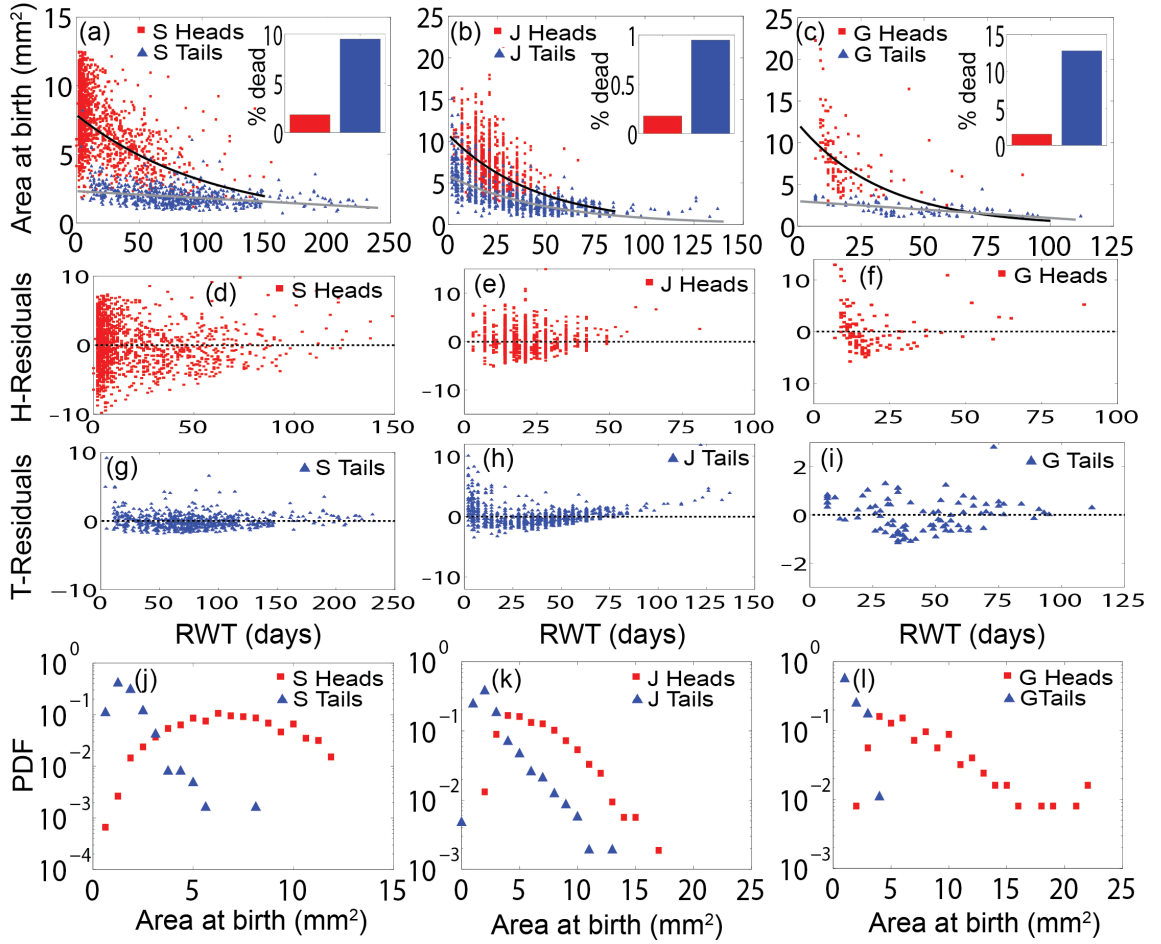


Figure 2.5: Tradeoffs between RWTs, area and survival. RWT *vs.* area at birth with exponential best fit lines for H-worms of all species and J-species T-worms. Linear best fit lines for S- and G-species T-worms. (a) S_H ($y=12.3e^{-0.009t}$; $R^2=0.32$), S_T ($y=-0.008x+3.7$; $R^2=0.15$), (b) J_H ($y=10.8e^{-0.023t}$; $R^2=0.28$), J_T ($y=5.8e^{-0.02t}$; $R^2=0.57$), and (c) G_H ($y=12.3e^{-.03t}$; $R^2=0.22$), G_T ($y=-.02x+3.0$; $R^2=0.35$). Insets show percent death for H- and T-worms of each species. (d-i) Residuals corresponding to best fit lines. Residuals are slightly skewed for exponential fit for J-species T-worms, but a linear fit (not shown) was worse. (j) PDF of area at birth for S_H ($N=1,514$) and S_T ($N=629$), (k) J_H ($N=1,066$) and J_T ($N=1,062$), and (l) G_H ($N=248$) and G_T ($N=92$). For all species, H-worms are plotted as red squares and T-worms are shown as blue triangles. H-worm best fit lines are shown in black while T-worm best fit lines are shown in gray for all species.

while H-worms have a larger span (S: $\sim 1 \text{ mm}^2$ to $\sim 12 \text{ mm}^2$; G: $\sim 3 \text{ mm}^2$ to $\sim 20 \text{ mm}^2$) (Figure 2.5(j-l)). A similar difference in size for T-pieces and H-pieces has previously been reported for G-worms [81].

The differences in size at birth, comparing T- and H-worms for each species, is reflected in their respective death rates. Specifically, all T-worms have a higher death rate (S: $\sim 10\%$; J: $\sim 1\%$; G: $\sim 12\%$) than H-worms of the same species (S: $\sim 2\%$; J: $\sim 0.1\%$; G: $\sim 2\%$). Inter-species comparison shows that both H- and T- worms of S- and G- worms have similar death rate, whereas J- species H- and T-worms have death rates approximately an order of magnitude smaller (Figure 2.5(a-c) insets). This is most likely a consequence of the equal allocation of resources between J-species H- and T-worms, whereas this allocation is much more variable in the other species.

2.4.6 Isolated Worms: Size at Teilung and determinants of RWTs

Size at birth may at least partially determine a worm’s RWT, *i.e.* the larger a worm is when it is born, the shorter it takes for it to divide. In fact, we have previously shown for S-planarians that the size at birth and a size-independent random element contribute equally to determine RWTs [98]. Given the substantially different reproductive strategies of the three species, we wondered whether birth size was a similarly important factor for the other two species or whether their RWTs were determined by other mechanisms.

This question of size and growth control mechanisms has been extensively studied in the context of single cells. Historically, there have been two models for size control, a “sizer” and a “timer” model [14, 42, 45, 92]. According to the “sizer” model, cells divide upon reaching some size-threshold (either in terms of absolute size or as a function of the size at birth). Alternately, in the “timer” model, an internal clock controls when a cell divides and consequently its size at divisions [69, 91, 100]. Recently a third mechanism, an “adder” model, in which cells add a constant volume (area in our case) regardless of initial size, has been shown to accurately describe certain bacteria strains [45, 92].

To determine whether our planarian species growth dynamics could be de-

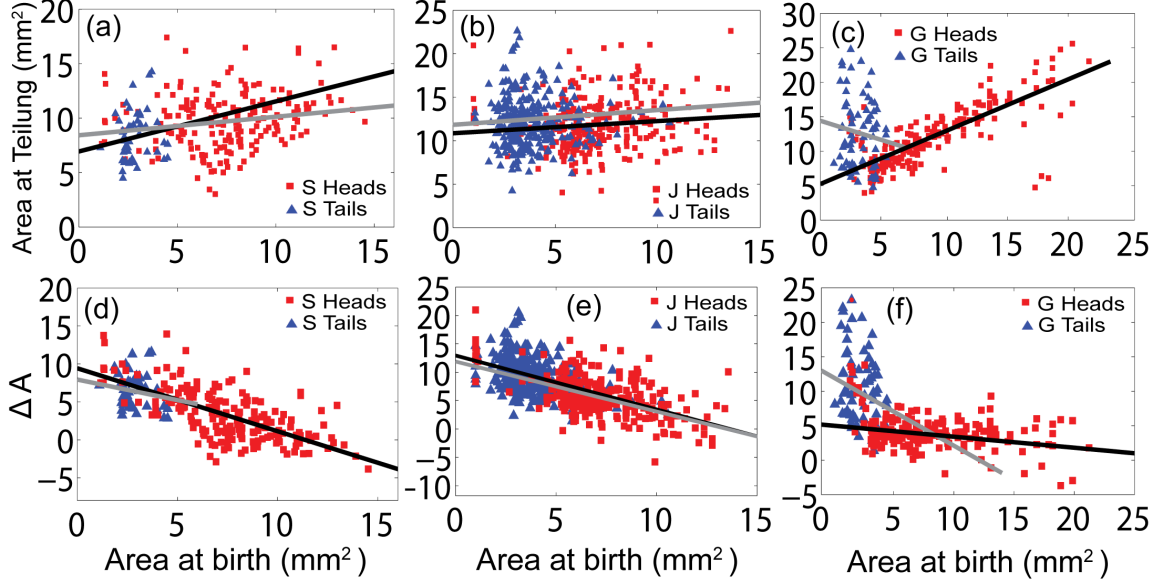


Figure 2.6: Relationship between area at birth, area at Teilung, and area added. Area at birth *vs.* area at Teilung with linear best-fit lines for (a) S_H ($y=0.46*x+7.4$), S_T ($y=0.17*x+8.6$), (b) J_H ($y=0.14*x+11$), J_T ($y=0.17*x+12$), and (c) G_H ($y=0.74*x+6$), G_T ($y=-0.55*x+14$). Relationship between area at birth (A_B) and amount of growth ($\Delta = A_D - A_T$) for (d) S_H ($y=-0.63*x+8.6$), S_T ($y=-0.54*x+7.4$), (e) J_H ($y=-0.95*x+12$), J_T ($y=-0.88*x+11$), and (f) G_H ($y=-0.06*x+5$), G_T ($y=-0.99*x+12$). In all panels, H-worm best linear fits are indicated with black and T-worm best fit lines are indicated with gray. S_H (N=206), S_T (N=50), J_H (N=281), J_T (N=286), G_H (N=208), and G_T (N=71).

scribed by either of these three models, we studied the relationship between area at birth (A_B) and area at Teilung (A_T). S- and J-species H- and T-worms similarly demonstrate weak linear correlations between these two areas (Figure 2.6(a-b)). The lack of a strong trend suggests that other factors besides area at birth significantly influence when, and at what size, a worm undergoes Teilung. G-worms, however, behave differently with T-worms showing effectively no trend and H-worms showing a strong linear correlation (Figure 2.6(c)). Thus, S- and J-worms regulate the timing of their Teilungs in a similar manner while G-worms employ another strategy.

We next examined the relationship between A_B of a particular worm and the area gained by that worm before undergoing Teilung ($\Delta A = A_T - A_B$) (Figure 2.6(d-e)). Based on the assumption that all three planarian species exhibit exponential growth (as we have shown experimentally for S-planarians [98]), for each of the pro-

posed models, we would expect: (1) if RWTs were regulated by a sizer, there would be a negative linear slope because larger worms need to add less mass to reach the size threshold, (2) if the timer model was correct, we would observe a positive linear slope as larger worms grew faster, and (3) if an adder mechanism was at play, the slope would be equal to zero as growth is independent of birth size [45].

As expected from the positive correlation of A_B and A_T , we observed a negative correlation between ΔA and A_B for S- and J-worms, with little difference between H- and T-worms. As the slopes of the best fit lines for these species are strongly negative, it appears as though both employ a sizer-like regulation strategy for Teilung frequency. The stronger negative trends for J-worms compared to S-worms (H: -0.95 *vs.* -0.63; T: -0.88 *vs.* -0.54) suggests that J-worm regulation is nearly entirely dependent on birth size (Figure 2.6(d-e)), whereas for S-worms it is roughly half, in agreement with our previous study [98]. The finding that J-worms, which divide the most symmetric of all three species, are size-controlled is interesting as sizer-like control of reproduction is also the determinant mechanism in fission yeast, which undergo roughly symmetric binary fission [26, 45].

In contrast, G-planarian H- worm growth seems to be largely independent of birth size ($m=-0.06$), suggesting that G-species H-worm reproduction follows an adder mechanism in which a constant area is added regardless of the size at birth. Interestingly, the average added area for G-species H-worms ($\sim 5 \text{ mm}^2$) directly corresponds to the average T-worm size at birth ($\leq 5 \text{ mm}^2$) (Figure 2.6(f) and Figure 2.5(l)), providing strong support for a model of G-reproduction in which H-worms successively grow and drop-off small T-pieces. G-species T-worms on the other hand seem to exhibit a near-perfect sizer regulation with $m=-0.99$ (Figure 2.6(f)). The relationship between A_B and ΔA of G-species H- and T-worms resembles that observed in the mother and daughter pieces, respectively, of budding yeast [32, 45].

In conclusion, despite the vast differences in the underlying biology between planarians and single-cell bacterial systems, both cases of asexual reproduction *via* binary division can be described by the same mechanism.

2.4.7 Isolated Worms: Species population growth rates under idealized conditions

Having examined the trade-offs of each reproductive strategy for individual worms and the mechanisms by which each reproductive strategy may arise, we were then curious how each reproductive strategy fared on the population level and, to this end, determined the growth rate of the population of isolated worms for each species. When worms are kept in isolation, there are no limiting factors (*e.g.* competition for food or space) which prevent individuals from growing or undergoing Teilung as quickly as possible, thus allowing us to directly measure the optimal population growth rate of each species based on their respective strategies under idealized conditions.

As two offspring are produced in each division, we expect population growth to be exponential. Since it becomes experimentally difficult to track individuals of an exponentially increasing population at higher generations, we recorded individual worms only through the first 10 generations per worm family ($2^{10}=1024$ worms per family). As worms were removed from the experiment after reaching higher generations, total population growth artificially begins to slow as soon as the first worms in the population exceed generation 10. Therefore, population growth rates were extracted from the period of exponential growth before worms are removed. Given the large spread of RWTs (Figure 2.2(b)), the time to reach the 10th generation varies substantially between species. Therefore, S- and J- population growth were measured for 200 days while G-growth was measured for only 100 days (Figure 2.7).

The combined population of isolated worms (all H- and T-worms alive at time t) grew exponentially with a rate of 0.017 (1/day), 0.026 (1/day) and 0.031 (1/day) for S-, J-, and G-worms, respectively (Table 1, Figure 2.7(d)). Interestingly, G-worms appear to exhibit the fastest overall population growth when kept in isolation, despite having fewer offspring per Teilung compared to S-planarians and investing significantly less in T-pieces compared to J-planarians. The rapid population growth of G-worms is likely due to short RWTs of both G-species H- and T-worms compared to the other two species (Figure 2.2(b)). Notably, this means that G-worms maximize the total number of worms present in the population in each point in time,

Table 2.1: Table summarizing growth rates for S-, J- and G-worm populations with individual worms cultured in isolation. Growth rates are given for the total population (H-worms and T-worms combined), the population of H-worms alone and the population of T-worms alone. It is important to keep in mind that the population of H-worms grows only when a T-worm divides. Thus, a higher division rate in H-worms corresponds directly with a higher growth rate in the T-worm population of that species. Given that H-worms have shorter RWTs for all three species, this effect explains why T-worm populations of each species grow faster than the corresponding H-worm population, though the effect is most drastic in G-worms. STE is reported for each growth condition.

	Total Population	Heads	Tails
S	0.017 ± 0.0013	0.016 ± 0.0001	0.018 ± 0.0002
J	0.026 ± 0.0003	0.025 ± 0.0002	0.027 ± 0.0003
G	0.031 ± 0.0009	0.026 ± 0.0010	0.034 ± 0.0008

which we directly assay. However, it does not necessarily mean that that G-worms also maximize the total biomass of the population at any point in time as their reproductive strategy leads to the presence of many small worms in the population (Supplemental Figure 2 and [23]). Of note, these results agree qualitatively but not quantitatively with previous studies of G-worm reproduction. Our measured growth rate is substantially slower ($0.031/\text{day}$ *vs* $0.079/\text{day}$) [3, 23]. This difference may be due to difference in temperature (20°C *vs.* $23\text{-}25^\circ\text{C}$, which influences growth dynamics [28, 47], a decreased reproductive rate of cannibalistic G-worms compared to non-cannibalistic ones [3] and the large variability in clones that was previously observed [23] as all our data was based on only two founder worms for this species (see Methods).

Surprisingly, S-planarians show a substantially slower growth rate than either J- or G-planarians despite on average producing significantly more offspring per Teilung. This may be partially due to a higher death rate of S-planarians at early generations in this experiment. As deaths also grow exponentially, the effect of early deaths on absolute worm numbers would be significant. For example, a death at generation 3 would mean that $2^7=128$ worms were missing by generation 10. Since we fixed our starting population size in the fits (see Methods), this effectively lowers the growth rate.

As before, we also studied H- and T- subpopulations separately for each species

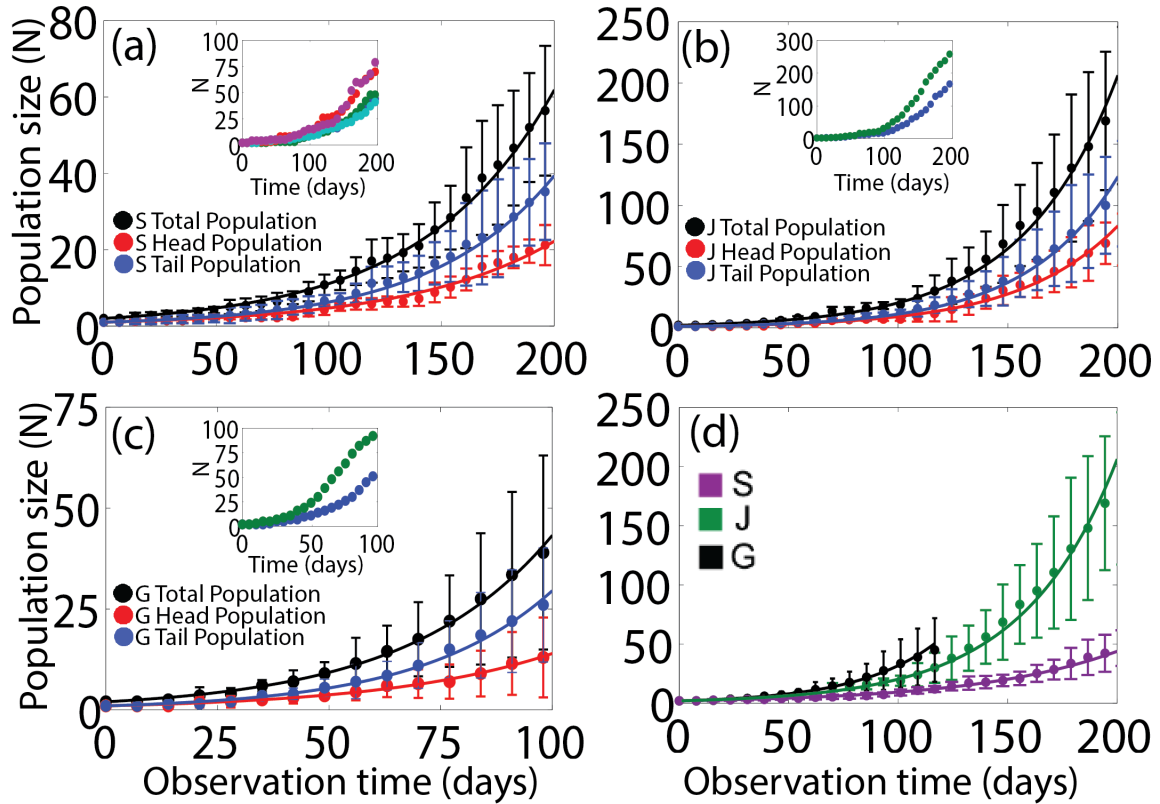


Figure 2.7: Population growth rates for planarians in isolation. (a) S-worms (5 families), (b) J-worms (2 families), and (c) G-worms (2 families). H-worm population size is plotted in red, T-worm population size is plotted in blue and total population (H- and T-worms) is shown in black. Average of all families are plotted with error bars representing the STD. Insets show variability between single families for each species. (d) Total population growth rates for worms kept in isolation for S- (purple), J- (green) and G-worms (black).

and found that the H-population growth rates for S-, J- and G- worms were equal to 0.016 (1/day), 0.025 (1/day) and 0.026 (1/day), respectively. For T-worms of each species, the population growth rates were found to be 0.018 (1/day), 0.027 (1/day) and 0.034 (1/day) for S-, J- and G-worms, respectively (Figure 2.7(a-c)). For all species, the growth of the population of H-worms was slower than the growth of the T-worm population (Figure 2.7(a-c)). Although initially counterintuitive considering that H-worms grow significantly faster and undergo Teilung more often than T-worms, it is important to remember that new T-pieces come only from H-worm Teilungs and vice versa. Therefore, the faster H-worms divide, the faster the T-population grows. The difference between H- and T-worms was most drastic for G-worms (0.026 *vs.* 0.034), while, as expected from Figure 2.2 (a-d), insignificant for J-planarians (0.026 *vs.* 0.027) (Figure 6(a-c)). Given the model of G-planarian reproduction in which H-worms quickly grow and drop off small tails in rapid succession (Figure 2.3(c)), it is unsurprising to see such a difference in the growth of H- and T-populations in G-worm populations.

2.4.8 Interacting Worms: Species growth rates and competition

Now that we have developed an understanding of how the three species perform in isolation and which reproductive strategies they employ to optimize their reproductive success, we wanted to investigate how these strategies would pay off during (1) intra- and (2) inter-species competition for resources.

Intra-species competition Similar to our previous work on S-planarians [28], we grew worms of the same species together in confinement and determined their growth rate and carrying capacities (Figure 2.8 (a)). The population size (N) of each species was counted on a weekly basis (Figure 2.8). Data was fitted with the logistic growth equation [28]:

$$N(t) = \frac{N_c N_0 e^{\gamma t}}{N_c + N_0 (e^{\gamma t} - 1)} \quad (2.1)$$

Where $N(t)$ is the population size at time t , N_C the carrying capacity, N_0

the initial population, and γ the growth rate. We found growth rates were equal to 0.025 (1/day), 0.023 (1/day) and 0.017 (1/day) for S-, J- and G-worms, respectively (Table 1). Of note, at early stages, S-worms had the fastest population growth, possibly owing to their high fragmentation frequency. Since S-worms are not cannibalistic, producing many small offspring is advantageous. In addition, starting with 20 founders in the box instead of 5 founders as in our isolation experiments, increases the chance of having more “fast growers” which would have a large influence on population growth given the observed variations in reproductive behaviors between individuals (Figure 2.8(a) Inset). Correspondingly, we found that S-worms grow more slowly in isolation than during competition (0.017 (1/day) *vs.* 0.025 (1/day)), J-worms grow at similar rates in both isolation and during competition (0.026 (1/day) *vs.* 0.023 (1/day)), while G-worms grow much faster in isolation than during competition (0.031 (1/day) *vs.* 0.017 (1/day)). This difference can potentially be explained by the fact that G-worms are cannibalistic (Supplemental Movie 2) and that their small tails cannot escape in our experimental setup of confinement in a box without hiding places, in agreement with previous laboratory studies of that species [3, 78]. Finally, in the case of cannibalistic J-worms (Supplemental Movie 1), the production of relatively large T-pieces appears to minimize the adverse effects of cannibalism. J-worms, in contrast to G-worms, can be found in either running water or still water. The ability of J-worms to be successful in our assay and in still water habitats requires the production of much larger T-pieces as Ts remain in proximity with cannibalistic H-pieces. Based on this data, we were curious to see whether the fast reproduction rate of S-planarians would give a competitive edge sufficient to outcompete the other two species or if the cannibalistic tendencies of J- and G-worms would matter most. In addition, since the carrying capacity was found to be twice as high for J-planarians than for the other two species, we expected G-worms to perform the poorest in competition because of their slower growth rate and low carrying capacity.

Inter-species competition To determine how the worms’ different reproductive strategies would perform in competition, we directly co-cultured $N_0 = 20$ worms for each of two species (J- *vs.* G-, S- *vs.* J- and S- *vs.* G-planarians). This set-up allowed members of both species to compete with one another for food, space and

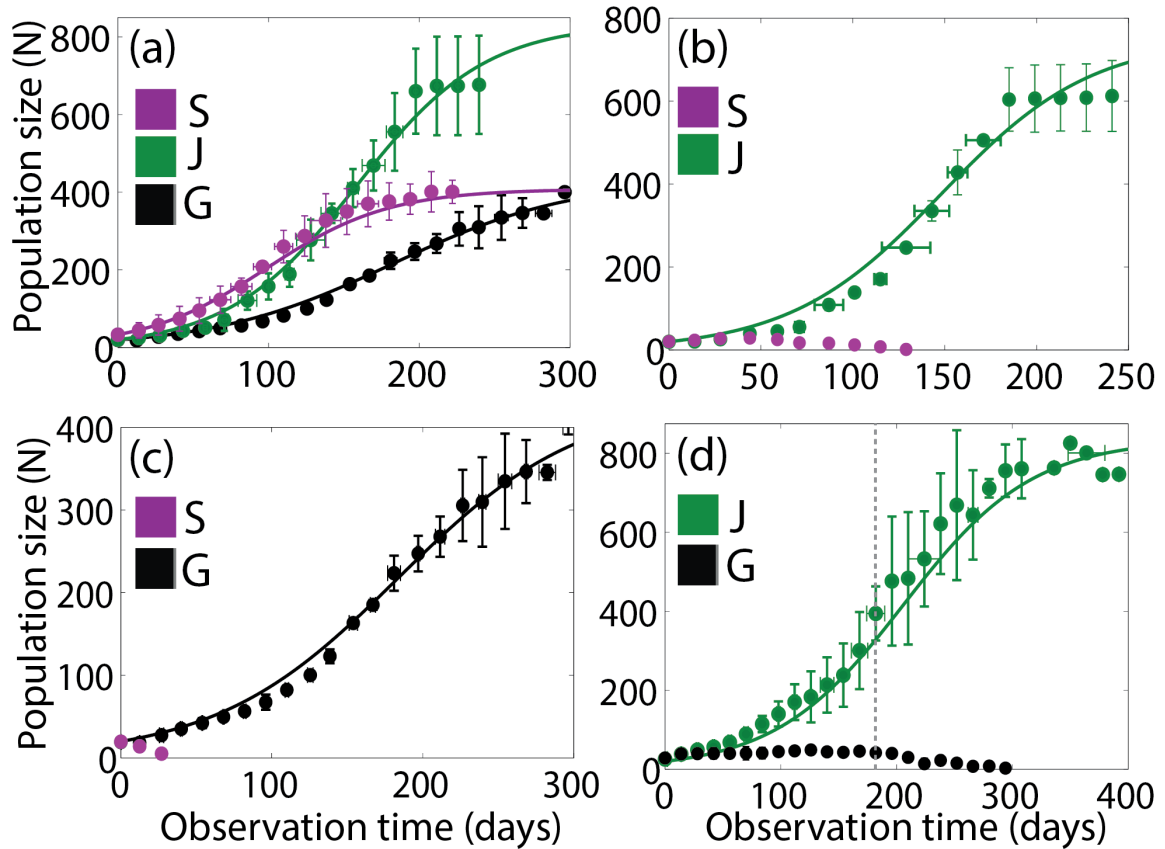


Figure 2.8: Population growth rates for competition experiments in which two species of planarians were kept in direct contact. (a) Composite of population growth rates for worms kept in direct contact with other members of their own species for S- (purple), J- (green) and G-worms (black). Error bars represent the *STD* of two independent experiments. (b) Competition results between S- and J-worms. S-worms die out after approximately 125 days and J-worms continue to grow until they reach their carrying capacity (2 boxes). (c) When S-worms are competed against G-worms, S-worms die out extremely quickly (≤ 50 days) (3 boxes). (d) When J- and G-worms were kept together, G-worms began to die out when the total population of the box exceeded the carrying capacity for G-worms around day 180 (indicated by the dashed gray line). After approximately 300 days, all G-worms are extinct. In all figures, error bars denote *STD*.

Table 2.2: Table summarizing fit parameters for S-, J- and G-worm populations in competition. J- and G-worm growth rates were obtained from J- vs. S- and G- vs. S-worm competition experiments in which S-worms died out quickly enough not to effect overall population growth. In J- vs. G-worm competition experiments, J-worms eventually wiped out all G-worms. Therefore, the carrying capacity was determined only by J-worms.

Parameter	S	J	J (<i>vs.</i> G)	G
R^2	0.86	0.96	0.95	0.98
N_C	407 ± 9	835 ± 53	856 ± 53	432 ± 21
γ (1/day)	0.025 ± 0.0007	0.023 ± 0.0008	0.017 ± 0.0007	0.017 ± 0.0006
N_0	33	20	26	19.3

other resources. When S- and J-worms were kept together, S-worms failed to grow much beyond the initial population and ultimately died out before reaching 150 days (Figure 2.8 (b)). The presence of S-worms seemed to have little effect on the growth rate of J-worms before reaching their carrying capacity at approximately 800 worms per container (Figure 2.8 (b) and Table 1). Notably, for the first ~ 40 days each species show similar population sizes, followed by a steady decrease in the number of S-worms until the entire S-worm population died out. Although S-worms by themselves exhibited a faster growth rather than J-worms, their reproductive strategy of producing many small pieces was disadvantageous in competition with J-planarians, which were found to rapidly eat small worm fragments. Interestingly, J-worms do not appear to eat full S-worms (Figure 2.9 (a-e) and Supplemental Movie 3).

Similarly, S *vs.* G competition experiments showed that the growth rate of the G-population was negligibly affected by the presence of S-worms. Here, S-worms died out even faster, within 50 days of being placed with G-worms (Figure 2.8 (c)). This can be largely attributed to the size difference between S- and G-worms. S-population extinction is ultimately achieved through rapid cannibalism of small S-worms pieces by G-worms (Figure 2.9(f-j) and Supplemental Movie 4). Again, cannibalism of full S-worms by G-worms was not observed.

Finally, in J- *vs.* G- competition experiments, J-worms ultimately reached their carrying capacity while G-worms died out over the course of approximately 300 days. The growth rate of J-worms was reduced by the prolonged presence of G-worms (growth rate of 0.017 (1/day) *vs.* 0.023 (1/day)) (Table 2). We propose that the prolonged presence of G-worms may owe itself to the similarity in area between J-

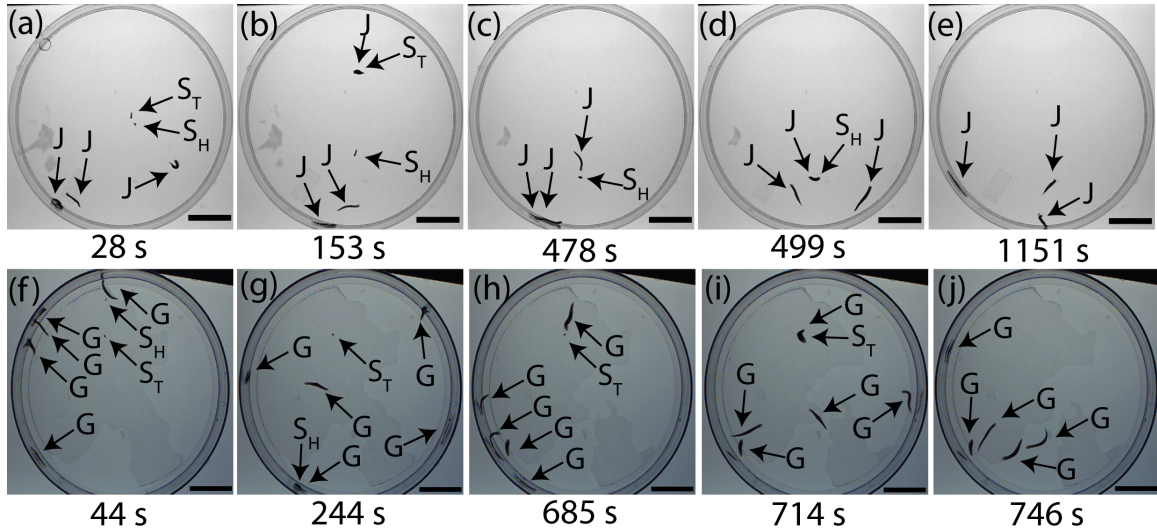


Figure 2.9: J- and G-planarians are cannibalistic of S-planarians. Sequence showing cannibalism of cut S-worms by (a-e) J-worms and (f-j) G-worms. Scale bars correspond to 10 mm. Image sequences are taken from Supplemental Movies 3 and 4.

and G-species H-pieces, making it more difficult to eat each other. Interestingly, the decline of G-worms beginning around 200 days corresponded with the total population (J+G worms) reaching 400 worms- the carrying capacity of G-worms and only half the carrying capacity of J-worms. Therefore, we hypothesize that the ability of J-planarians to survive under crowded conditions leads to their ultimate reproductive success over G-worms.

2.5 Conclusions and Outlook

Through a detailed examination of over 23,000 asexual reproductive events spanning a nearly six year period, we have shown that S-, J- and G-planarians each exhibit distinct reproductive strategies, despite all reproducing asexually through binary division. Each reproductive strategy had intrinsic trade-offs between clutch size (number of offspring produced per Teilung), time between reproductive events (RWTs), parental investment, and corresponding offspring survival rates. We also tested the relative success of each species' reproductive strategy, both as an isolated population and in direct competition with another species. Surprisingly, we found

that any advantage gained from a successful reproductive strategy was outweighed by cannibalistic effects and the ability to thrive at higher population densities. As planarian species are frequently introduced into novel habitats, whether intentionally or accidentally, these results suggest potential serious ecological implications with invasive species. Finally, we found that various models for cell size regulation of single-celled organisms can be directly applied to describe the regulation of size at division and RWTs for asexual multicellular organisms. Specifically, we reported that the RWTs of S- and J- planarians seem to be regulated by size control, whereas the RWTs of G-planarians appeared to be regulated by adding a constant area between reproductive events regardless of their size at birth. It will be an interesting avenue of future research to relate these findings on the organismal and population level to the underlying physio-chemical machinery which regulates planarian division. Little is known about how planarians regulate where they divide along the anterior-posterior body axis, what initiates division, and how many divisions a worm undergoes per Teilung. Melantonin has been implicated as a possible regulator of division initiation [63, 98, 111], but how this regulation works remains unknown since treatment with melatonin directly does not initiate spontaneous division. The next steps for a better understanding of planarian asexual reproduction therefore requires molecular studies of the regulatory mechanisms to enable a reconstruction of our population data from the underlying molecular and mechanical determinants.

Chapter 2 has been submitted for publication of the material. Carter, Jason; Lind, Christine; Truong, Mai P.; and Collins, Eva-Maria S. “To Each Their Own. Reproductive Strategies and Success of Three Common Planarian Species: *Schmidtea mediterranea*, *Dugesia japonica* and *Dugesia tigrina*”. The thesis author was the primary investigator and author of this paper.

2.6 Supplementary

Movie S1: J-worms are cannibalistic and quickly eat small fragments of other J-worms. The movies shows three large J-worms completely consuming one small T-piece within 10 minutes of cut. In addition, they are beginning to consume a much larger H-piece. Movie was filmed at 1 frame per second and is played back at 40 frames per second.

Movie S2: G-worms are cannibalistic and quickly eat small fragments of other G-worms. The movies shows eight medium-sized G-worms completely consuming two small G-worm fragments. Movie was filmed at 1 frame per second and is played back at 40 frames per second.

Movie S3: J-worms quickly eat small fragments of S-worms. The movie shows three large J-worms completely consuming both halves of a cut S-worm. Movie was filmed at 1 frame per second and is played back at 40 frames per second.

Movie S4: G-worms quickly eat small fragments of S-worms. The movie shows five large G-worms consuming both halves of a cut S-worm. Movie was filmed at 1 frame per second and is played back at 40 frames per second.

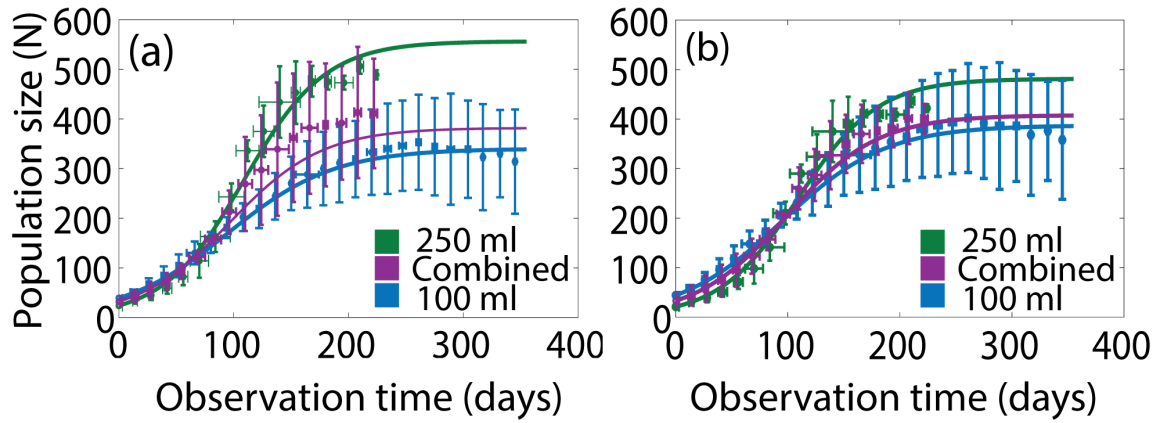


Figure 2.10: Supplemental Figure 1: Critical carrying capacity scales by volume and not by area. Interacting populations of S-worms were kept in either 100 or 200 mL of planarian water and the number of worms present in the population was counted weekly. The population size was then binned into 14 day periods and scaled according to either the **(a)** area of the container or **(b)** volume of the container. The population growth for each condition was fitted with the logistic growth equation as described in Section 2.5. Better fits are clearly obtained when scaling by volume instead of by area. Therefore, the critical carrying capacity for planarians scales by volume rather than by area as previously suggested in Ref. [3].

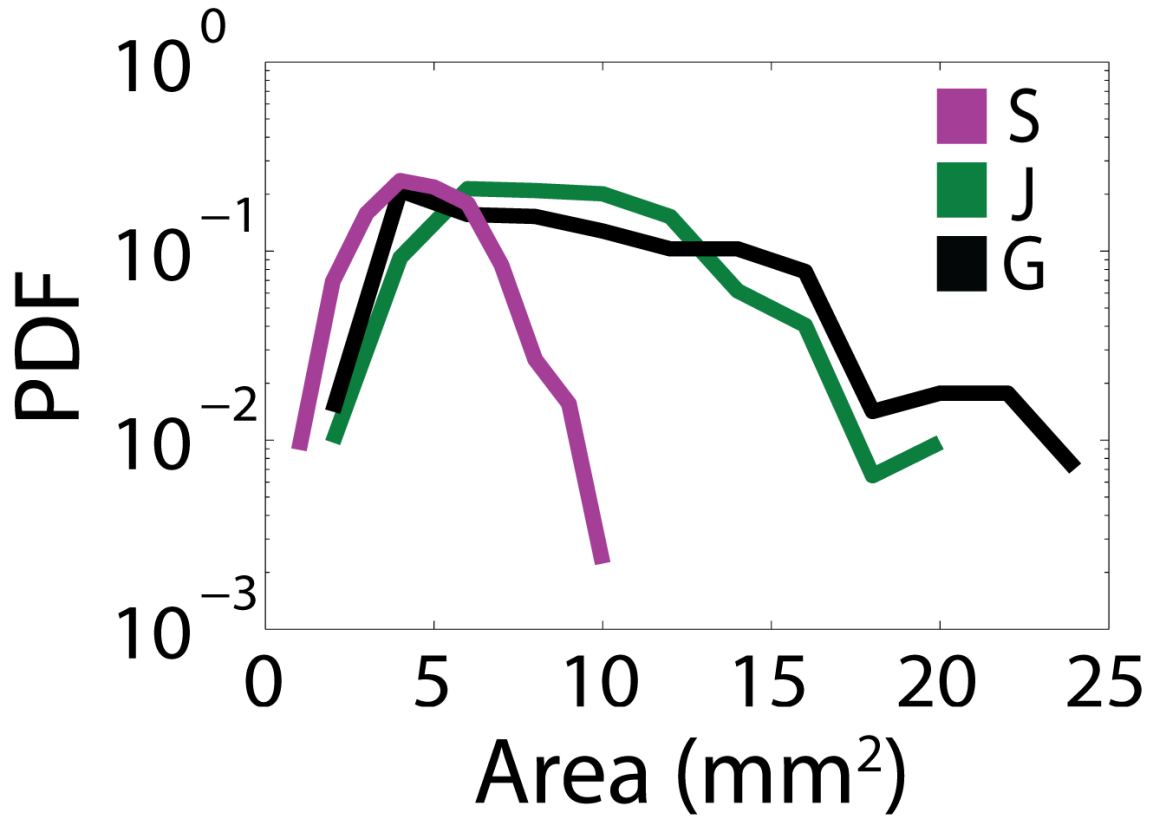


Figure 2.11: Supplemental Figure 2: Distribution of worm areas in a population at critical carrying capacity. (a) S-, J- and G-worms were cultured in containers with direct contact to other members of the same species. Once the carrying capacity was reached, 50 worms of each species were randomly chosen and imaged as described. The area of each worm was determined as described in Section 2.3. The probability distribution function (PDF) for the areas of individual worms shows a relatively narrow distribution of small sizes for S-worms (purple), and wider distributions with larger areas for J-worms (green) and G-worms (black).

Chapter 3

A *slo-1* Homolog is Required for the Normal Ethanol
Response in the Planarian Species *Schmidtea mediterranea*

3.1 Abstract

The incredible regenerative abilities of planarians, combined with the relative complexity of the planarian central nervous system, make them a potentially unique model organism for studying the interactions between various drugs and the brain. Furthermore, given the widespread use of alcohol today, the elucidation of the molecular mechanisms by which ethanol effects the brain is of the utmost importance. In this study, we show that planarians possess two homologs (*Smed-slo1-A* and *Smed-slo1-B*) of the *slo1* gene, which has previously been shown to encode for ethanol-sensitive BK ion channels that are highly conserved between invertebrates and vertebrates, including humans. Through qualitative and quantitative analysis of locomotion phenotypes and RNAi mediated knockdowns, we show that *Smed-slo1-A* and *Smed-slo1-B* are necessary for the normal ethanol response in planarians and that worms lacking both *slo1* homologs acquire a resistance to acute ethanol exposure at a concentration of 1%. The ability of two highly-conserved genes to jointly regulate the ethanol response of planarians indicates that planarians may be uniquely suited for studying the molecular interactions of ethanol with the central nervous system.

3.2 Introduction

Despite the widespread use of alcohol, little is currently understood about the molecular mechanisms involved in the interactions between ethanol and the human brain. Given the array of various side effects associated with alcohol consumption including changes long-term dependency and withdrawal symptoms, there is a great interest in determining how intoxication affects the brain and central nervous system. Developing a molecular understanding of these mechanisms is crucial to unraveling how acute alcohol exposure gives rise to its associated behaviors [51]. However, the simplicity of the structure of ethanol and consequently wide array of potential targets for ethanol interaction, has made the elucidation of the molecular targets of ethanol difficult [44].

In recent years, planarians have emerged as an intriguing model organism for studying the effects of ethanol and other toxins [38, 57, 73, 95]. Planarians are

particularly promising model organisms for neurotoxicology studies given that they are one of the simplest organisms possessing a brain with substantial parallels with that of humans [11, 95]. For example, the planarian central nervous system utilizes many of the same neurotransmitters found in humans, including GABA, which has been shown to be involved in the behavioral effects of acute ethanol exposure in humans [30, 31]. Furthermore, the planarian nervous system has been shown to behaviorally respond to a variety of drugs in ways that parallel mammals, such as sensitization, withdrawal and environmental place conditioning [67, 72, 95]. Thus, planarians have the potential to become a powerful animal model for pharmacological studies with a direct relevance to humans [11, 38].

However, recent studies investigating the interactions of ethanol with planarians have been limited to effects of ethanol on planarian behavior and regeneration [57, 90, 95]. In order to further develop planarians as a model system for understanding the effects of ethanol on the central nervous system, we need to understand the molecular mechanisms by which ethanol interacts with the planarian brain. One such potential mechanism of ethanol interaction in planarians are big potassium (BK) ion channels encoded by the *slowpoke* gene family that have previously been shown to be sensitive to ethanol and highly conserved between invertebrates and vertebrates [20, 99].

BK channels are composed of a pore-forming tetramer of α subunits and a cytoplasmic C-terminal β domain, which is responsible for the calcium and voltage-gated regulation of the channel [99, 112]. Physiologically, BK channels have been implicated in a variety of diverse functions, including neurotransmitter release, modulating neuronal excitability, and stimulating smooth muscle contraction in organisms ranging from *C. elegans* to humans [71, 106, 112]. Small alcohols, including ethanol, are believed to bind to a distinct pocket in the cytoplasmic β domain of the BK ion channel leading to an increase in the probability of channel opening and thereby altering the functionality of the neuron [27, 99]. Furthermore, studies in *Caenorhabditis elegans* have shown that BK channels encoded by the *slo1* gene regulate the behavioral response to acute ethanol exposure, both in locomotion phenotypes and egg-laying frequencies [15, 22, 107].

As such, we explored whether the behavioral response to acute ethanol ex-

posure the planarian *Schmidtea mediterranea* could be regulated by a similar molecular mechanism. We found that the planarian genome possesses two *slo1* homologs, *Smed-slo1-A* and *Smed-slo1-B*, both of which are expressed in wild-type worms and highly conserved with respect to *slo1* sequences in other invertebrates and mammals, particularly in the regions thought to be involved with ethanol sensitivity [10]. Furthermore, through RNAi mediated knock down studies, we find that either of the two sequences is sufficient for the behavioral response to ethanol exposure and that the knock down of both sequences leads to behavioral ethanol resistance.

To our knowledge, these results provide the first mechanistic understanding of the interactions between ethanol and the planarian central nervous system. Furthermore, our results indicate that, given the high degree of conservation in the mechanism of ethanol sensitivity, planarians may represent a powerful animal system for future mechanistic pharmacological studies [11, 38].

3.3 Materials and Methods

3.3.1 Planarian Culture

Small asexual *S. mediterranea* were used for all experiments. Planarians were maintained at 20°C in planarian water [65], fed weekly with organic liver paste, and cleaned twice a week [93]. Animals were kept in the dark except for feeding, cleaning, and experiments. Planarians were starved for at least 3 days before any behavioral experiments and at least a week before *in situ* hybridization to minimize non-specific background staining [94].

3.3.2 Slo-1 Cloning, Riboprobes and RNA Interference

Planarian *slo1* homologs were found using NCBI and BLAST query to search the planarian genome database [76] against the published *C.elegans slo1* sequence [29]. Based on these genomic screens, two potential *slo1* homologs were identified in *S. mediterranea*, which we termed *Smed-slo1-A* (mk4.000108.08.01) and *Smed-slo1-B* (mk4.003838.00.01), respectively. Both candidate sequences were cross-checked against the known *slo1* sequences of several other species, including humans. These

slo1 sequences were obtained from previously published studies archived in the NCBI database and subjected to phylogenetic analysis [25].

The software Primer3 [79] was used to design oligonucleotide primer pairs for both candidate planarian *slo1* sequences. Each sequence was obtained using PCR on *S. mediterranea* cDNA and subsequently cloned into the pPR-T4P vector. Vectors containing either the *Smed-slo1-A* or the *Smed-slo1-B* sequences were transformed into DH5 α E.coli for amplification. Plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA, USA; 27104) and used to synthesize digoxigenin-labeled riboprobes for *in situ* hybridization.

For RNA interference (RNAi) experiments, organic liver paste was mixed with HT115 (DE3) bacterial cells (final concentration $\sim 20\%$ by volume) expressing the dsRNA of interest and mixed with 1 μ l of food coloring immediately prior to feeding. Worms were fed for approximately 2 hours until food coloring was visible in the gut [94]. Four independent planarian populations were maintained for RNAi experiments, each of which were fed a dsRNA to knock down the following proteins: *Unc22*, *Smed-slo1-A*, *Smed-slo1-B*, or both *Smed-slo1-A* and *Smed-slo1-B* (*Smed-slo1-A/B*). *Unc22* is a *C. elegans* gene not found in planarians and serves as a control for RNAi experiments [74, 94]. All worms in RNAi studies were fed twice per week and cleaned three times per week for a total of 4 weeks (8 feedings).

3.3.3 Whole-mount *In Situ* Hybridization

Whole-mount *in situ* hybridization studies were performed as previously described [68, 94]. In brief, 7-day starved worms were treated with 2% HCl in phosphate buffered saline (PBS), followed by fixation with 4% paraformaldehyde (PFA) in PBS. Tissue permeability was increased through treatment with a 1:1,000 Proteinase K: 0.1% Triton X in PBS solution (Invitrogen 25530-049) prior to overnight bleaching in 6% H_2O_2 in 100% MeOH at room temperature in direct light. *In situ* hybridization used the digoxigenin probes described in the previous section and development in a high salt alkaline phosphatase buffer (100mM Tris, pH 9.5; 100mM NaCl; 50mM MgCl₂; 0.1% tween-20; 3.5 μ l/ml 5-bromo-4-chloro-3-indoyl phosphate (Roche 11383221001) and 4.5 μ l/ml nitro blue tetrazolium (Roche 11383213001) with

10% polyvinyl alcohol. Non-specific background was removed following development by multiple washes with 100% ethanol over the course of 30-60 minutes. Samples were transferred to a 1:1 1xPBSTx:glycerol solution for storage and imaging using a Leica stereo microscope (Leica Microsystems, Wetzlar, Germany) with a Basler A601f CCD camera (Basler AG, Ahrensburg, Germany) and Basler Pylon Viewer software (Basler AG, Ahrensburg, Germany).

3.3.4 Regeneration Assay

Four *Unc22* RNAi worms, five *Smed-slo1-A* RNAi worms, five *Smed-slo1-B* RNAi worms and five *Smed-slo1-A/B* worms were decapitated with a razor blade posterior to the eyes 4 days after the 8th RNAi feeding. Following decapitation, posterior pieces were immediately imaged (on Day 0) and individuals separated into single wells of a 12-well plate. These tails were subsequently maintained as described in Section 3.3.1 without feeding and imaged daily, using the Lieca Stereo microscope mentioned above, through Day 8 [94]. The number of eyes present on each day of regeneration were manually counted and recorded.

3.3.5 Behavioral Assay

Petri dishes containing 30 mL of planarian water were placed on a cold LED light panel underneath a Basler A601f CCD camera mounted to a custom built 2-D tracking system controlled *via* a GeckoDrive G540 4-Axis Step Motor System (GeckoDrive, Inc., CA, USA). Imaging and automated tracking of the individual worm using a custom written LabVIEW script (National Instruments, Tx, USA) was manually started simultaneously with the addition of one worm into the petri dish. Following a short rest period, this process was repeated twice with petri dishes containing 30 mL of 0.5% and 1% ethanol in planarian water. In each case, worms were tracked for 5 minutes while automatically recording the position of the worms center of mass, the corresponding elapsed time, and raw images in 1-second intervals.

Position and time data from the tracking of individual worms was used to determine the instantaneous speed of the center of mass for each worm using a custom MATLAB (MathWorks, Natwick, MA) script. To account for variation in speed based

worm size, we normalized the speed of each worm by the length of the worm while gliding. Normalized instantaneous speeds were binned into 20 second intervals and the average of all worms calculated at each time point. Area analysis was performed on the images recorded from the behavioral assay using standard image analysis techniques with ImageJ 1.48 (NIH, Bethesda, MD) and MATLAB.

3.4 Results and Discussion

3.4.1 Molecular characterization and phylogeny of 2 *slo1* homologs in planarians

The *slo1* gene has been extensively studied in *C. elegans*, particularly in the context of the behavioral ethanol response. Specifically, *C. elegans* lacking the *slo1* gene, through either naturally occurring mutations [22] or specific genetic knock-outs [107], were found to have reduced sensitivity to exogenous ethanol as measured by locomotion phenotypes and egg-laying frequencies [15]. BK channels, however, are not the only target of ethanol in *C. elegans*, as several other pathways effected by the presence of ethanol have been identified [40, 61]. Nonetheless, these studies demonstrate the importance of the *slo1* gene in modulating the effect of ethanol on *C. elegans* behavior.

To test whether the planarian ethanol response was also dependent on a homologous *slo1* gene, we performed a BLAST search against the planarian genome [76] with the known *C. elegans slo1* sequence from the NCBI database [29]. Using this method, we identified two potential planarian *slo1* homologs (Supplementary Figure 1), which we named *Smed-slo1-A* and *Smed-slo1-B*.

In order to better understand whether the ability of the *slo1* gene to regulate the *C. elegans* behavioral ethanol response is conserved in planarians, we also examined whether our planarian sequences contained a recently identified alcohol-sensing site. This 14 amino acid alcohol binding pocket was previously identified in the cytoplasmic domain of the mouse *slo1* BK ion channel and hypothesized to be responsible for conferring the ethanol sensitivity of the channel [10]. To determine the alcohol-sensitivity of both *Smed-slo1-A* and *Smed-slo1-B*, we aligned both planarian

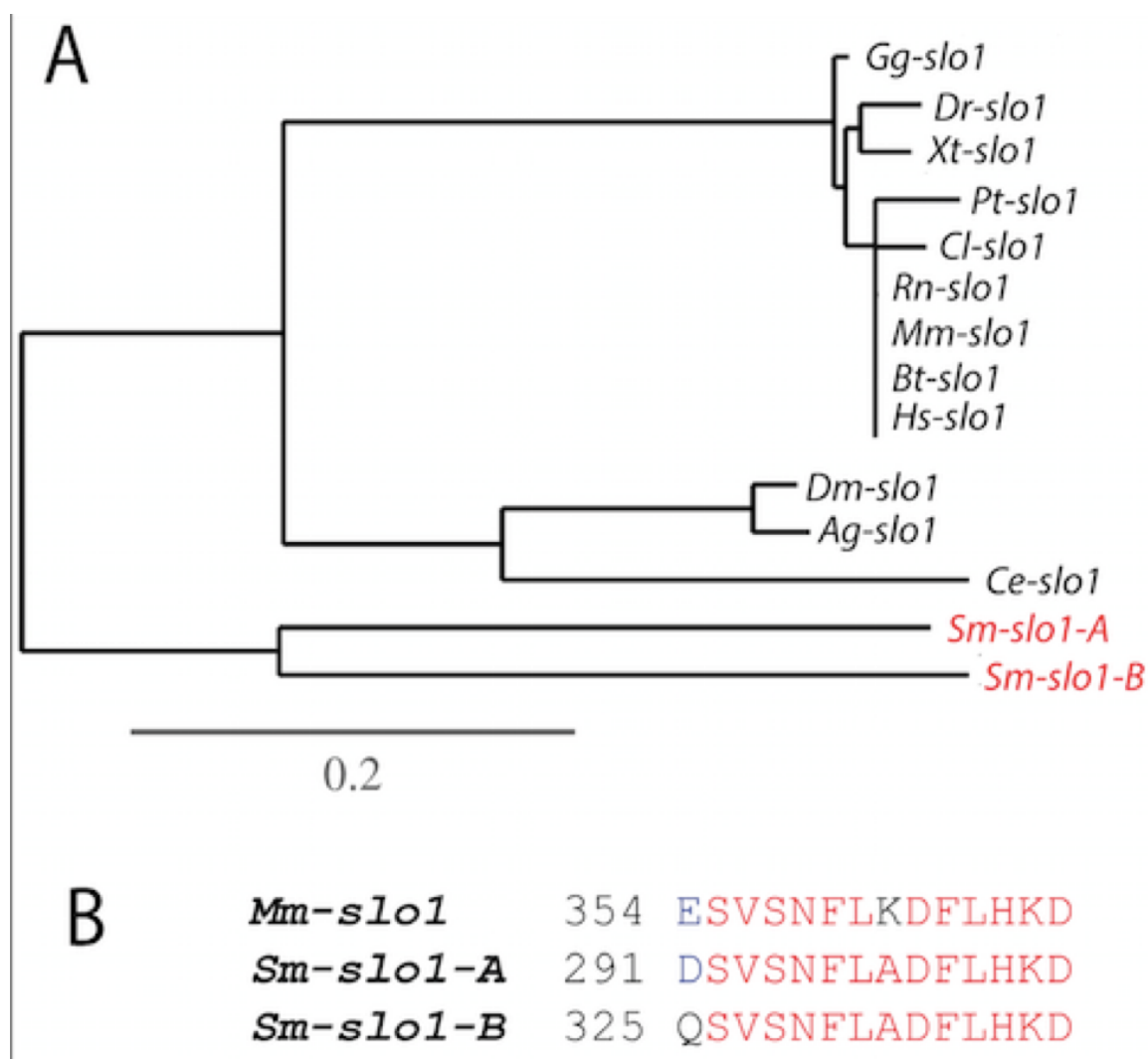


Figure 3.1: Phylogeny of *slo1* homologs. (A) Planarians have 2 *slo1* homologs, which we termed *Smed-slo1-A* and *Smed-slo1-B* (red). Both *slo1-A* and *Smed-slo1-B* appear to be closely related to one another, as well as *Drosophila melanogaster* and *Caenorhabditis elegans*. Phylogeny consists of the nematode *Caenorhabditis elegans* (Ce), the arthropods *Drosophila melanogaster* (Dm) and *Anopheles gambiae* (Ag), the vertebrates *Xenopus (Silurana) tropicalis* (Xt) and *Danio rerio* (Dr), and the mammals *Gallus gallus* (Gg), *Pan troglodytes* (Pt), *Canis lupus familiaris* (Cl), *Mus musculus* (Mm), *Rattus norvegicus* (Rn), *Bos taurus* (Bt), and *Homo sapiens* (Hs). Scale bar represents amino acid substitutions per site. (B) Alignment of the ethanol-sensitive region of *slo1* in *Mus musculus* with *Smed-slo1-A* and *Smed-slo1-B*. Both planarian sequences show a high-level of conservation with the mouse sequence in this region, indicating that the ethanol-sensitivity of the mouse binding pocket is likely conserved in both planarian sequences. Identical amino acids in at least two of the sequences are shown in red and conserved substitutions in at least two of the sequences are shown in blue. Non-conserved mutations are shown in black.

sequences with the mouse ethanol-sensitive sequence (Figure 4.1 B) and found that 12 of the 14 amino acids in this hypothesized alcohol binding pocket in *slo1* were conserved between all three sequences. Conserved residues included S357 and N358 in the mouse sequence, which were speculated to be of particular importance in the formation of the ethanol binding pocket [10].

Furthermore, *Smed-slo1-A* and *Smed-slo1-B* differed from each other by only one amino acid, a conserved mutation from glutamic acid in mice to aspartic acid in *Smed-slo1-A* and an unconserved mutation to glutamine in *Smed-slo1-B*. Surprisingly, we found that K361 in the mouse sequence, which was thought to undergo hydrogen bonding with ethanol within the pocket, was mutated to an alanine in both planarian sequences (Figure 4.1 B). As alanine lacks a bulky functional group and is thereby likely to preserve the overall shape of the binding pocket, we hypothesize that both sequences may still have a functional ethanol-sensing domain. However, without the hydrogen binding provided by the lysine residue in mice [10], we hypothesize that *Smed-slo1-A* and *Smed-slo1-B* may bind ethanol with a lower affinity.

Bukia *et al.* [10] also suggested seven other regions in the mouse *slo1* sequence which could potentially interact with ethanol and which were spatially close to the identified alcohol binding pocket. Of these seven sequence regions, five were highly conserved in both planarian sequences (Supplementary Figure 2). As the two unconserved regions were spatially farther away from the predicted alcohol binding pocket, the local structure and functionality of the binding pocket is likely still conserved [10]. Taken together, this data suggests that *Smed-slo1-A* and *Smed-slo1-B* are functional, ethanol-sensitive *slo1* sequences. Consequently, we examined the expression profile and functional roles of both planarian homologs throughout this paper.

3.4.2 Qualitative characterization of *Smed-slo1-A* and *Smed-slo1-B* knockdowns

To determine the function of *Smed-slo1-A* and *Smed-slo1-B*, we knocked down each sequence individually (*Smed-slo1-A(RNAi)* and *Smed-slo1-B(RNAi)*) and both sequences together (*Smed-slo1-A/B(RNAi)*) to test for functional redundancies. The *C. elegans Unc22* gene, which is absent in planarians [74], was used as a control for

all RNAi experiments. Using these *slo1* knockdown animals, we began by testing whether either of our planarian homologs were necessary for phenotypically normal locomotion.

Normal planarian locomotion in water consists of cilia-driven gliding in which the length of the worm remains relatively constant (Figure 4.3 A,F and Supplementary Movie 1). We found that that (*Smed-slo1-A(RNAi)*, *Smed-slo1-B(RNAi)*) and *Smed-slo1-A/B(RNAi)* worms all exhibited qualitatively normal gliding locomotion in water (Supplementary Movies 2-4). This indicates that neither of the two planarian *slo1* genes is required for normal locomotion.

When placed in 1% ethanol, planarians exhibit a “drunken” phenotype, which consists of musculature-driven oscillations in the length of the worm. More specifically, worms displaying the drunken phenotype undergo characteristic elongations (~ 1.2 times the length of the worm during gliding) and contractions (~ 0.8 times the length of the worm during gliding) with a period of approximately 4 seconds (Figure 4.3 B,F and Supplementary Movie 5). This drunken phenotype strongly resembles dynamin knockout and mianserin (a serotonin antagonist) induced locomotion phenotypes [21, 94]. A concentration of 1% was chosen for these studies as previous studies have shown that ethanol is not lethal at this concentration, but does result in a significant locomotion impairment without complete loss of coordinated behavior [18, 57, 95]. If either of our two potential planarian *slo1* sequences are responsible for the ethanol response of the worm, we would expect that worms without the *slo1* protein do not display the drunken phenotype in response to acute ethanol exposure.

Qualitatively, *Smed-slo1-A(RNAi)* and *Smed-slo1-B(RNAi)* worms still display the drunken phenotype when placed in 1% ethanol (Figure 4.3 C-D and Supplementary Movies 6-7). This drunken phenotype, however, was less pronounced in single *slo1* knockdown worms than in *Unc22(RNAi)* worms, as measured by the frequency and amplitude of elongation-contraction cycles (Figure 4.3 F). Furthermore, the loss of the gliding phenotype was not due to destruction of the cilia as a result of acute ethanol exposure (Supplemental Figure 3 A-C), as previously reported with one hour treatments in 3% ethanol [90]. Overall, these results indicate that a single *slo1* knockdown is not sufficient to abolish the worm’s ethanol response and, therefore, that expression of either of the two sequences is sufficient for the behavioral ethanol

response.

In contrast, *Smed-slo1-A/B(RNAi)* worms did not exhibit the drunken phenotype in 1% ethanol (Supplemental Movie 8), instead displaying a seemingly normal gliding phenotype without substantial oscillations in length (Figure 4.3 E-F). That *Smed-slo1-A/B(RNAi)* worms lose the drunken phenotype in 1% ethanol suggests that the expression of at least one of the two *slo1* sequences is necessary for ethanol sensitivity in planarians and that knockdown of one *slo1* sequence can be compensated for by the expression of the other sequence.

3.4.3 Slo-1 knockdowns show quantitatively different locomotion phenotypes in ethanol

Qualitatively, it is difficult to characterize planarian locomotion phenotypes at ethanol concentrations below 1% because worms intermittently display both the gliding and drunken phenotypes at lower concentrations (Supplementary Movie 9). By taking advantage of the differences in locomotion speed between gliding and the drunken phenotype, we were able to quantitatively characterize locomotion in intermediate ethanol concentrations, despite the lack of distinctive qualitative phenotypes. The ability to analyze the locomotion phenotypes at lower concentrations of ethanol provides further insight into the sensitivity of the planarian *slo1* genes. Employing a custom-built center of mass tracking system to calculate the planarian speeds [93], we were able to characterize and compare locomotion of worms in water, 1% ethanol, and 0.5% ethanol, the lowest concentration found to be capable of inducing a mixture of the two locomotion phenotypes (Figure 4.10).

As expected, *Unc-22(RNAi)* control worms displayed three distinctive speeds corresponding to the three conditions (water, 0.5% ethanol, and 1% ethanol). In water, the gliding phenotype resulted in a relatively constant average speed (~ 0.4 body lengths per second) throughout the entirety of the trial (Figure 4.10 A). Exposure to 0.5% and 1% ethanol resulted in marked decreases in average speed, with worms in 1% ethanol displaying the slowest speed (~ 0.2 body lengths per second) and worms in 0.5% ethanol displaying an intermediate speed (~ 0.3 body lengths per second).

Smed-slo1-A(RNAi) and *Smed-slo1-B(RNAi)* worms showed a slower average

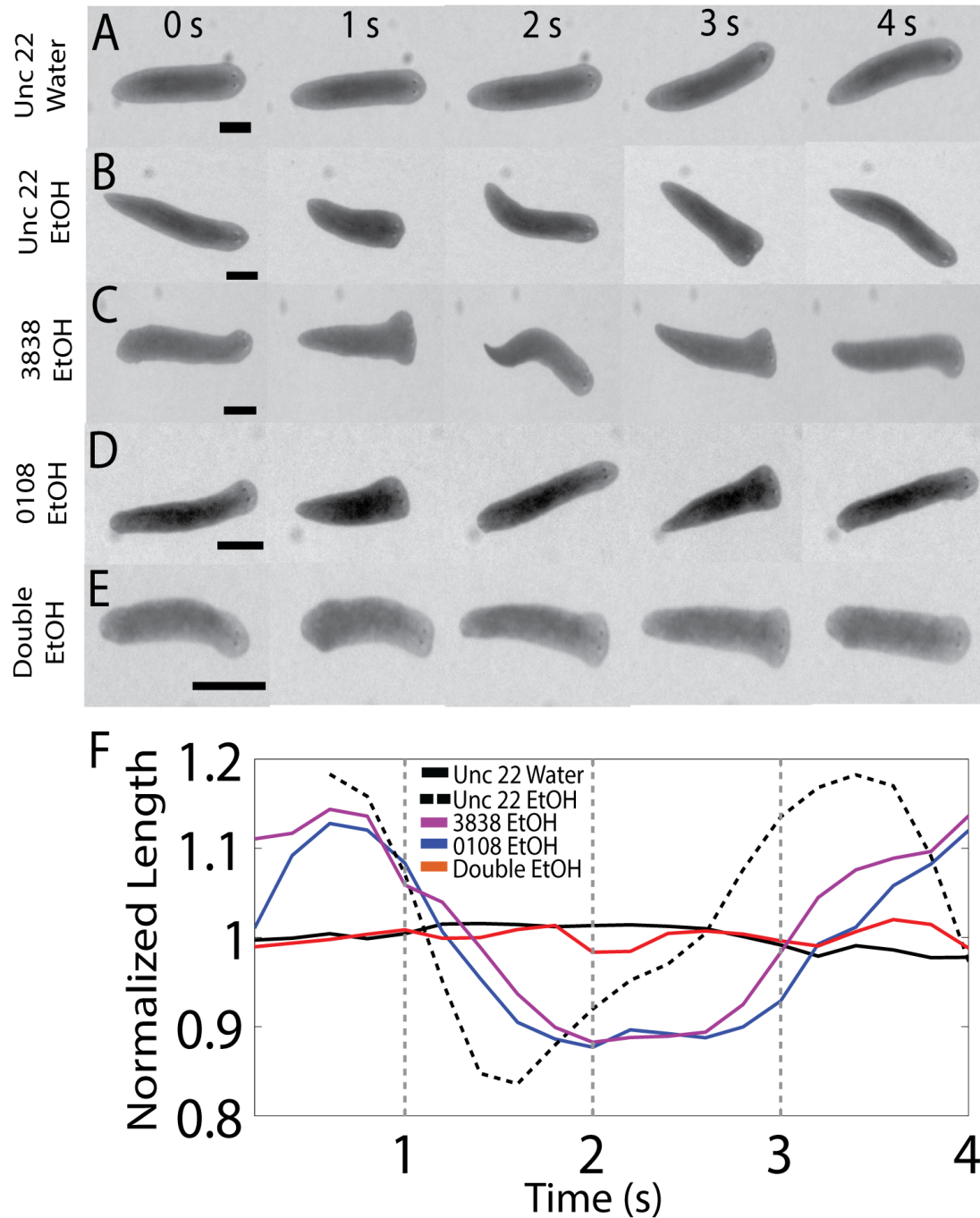


Figure 3.2: Planarian *Slo-1* is involved in the planarian ethanol response. Representative images of planarian locomotion for (a) Unc-22 RNAi control worms in water, (b) Unc-22 RNAi control worms in 1% ethanol, (c) *Smed-slo1-A* RNAi control worms in 1% ethanol, (d) *Smed-slo1-B* RNAi control worms in 1% ethanol, and (e) *Smed-slo1-A/B* RNAi control worms in 1% ethanol. (f) Normalized length along anterior/posterior axis sampled at 5 frames per second for the 4 second interval shown in representative images. Scale bars: 1mm)

speed (approximately 0.3 body lengths/sec) than the *Unc-22* control worms (approximately 0.4 body lengths/sec) in water (Figure 4.10 A-C). However, this is likely due to differences in individual worms within the respective populations (Figure 4.10 D). *Smed-slo1-A(RNAi)* and *Smed-slo1-B(RNAi)* worms also showed little reduction in speed in 0.5% ethanol when compared to their average speeds in water, indicating that the knockdown of one of the two *slo1* sequences may be sufficient to reduce sensitivity to low concentrations of ethanol. *Smed-slo1-A(RNAi)* and *Smed-slo1-B(RNAi)* worms, however, did show reductions in speed in 1% ethanol comparable to the reductions in speed of control worms in 1% ethanol. Thus, single *slo1* knockdowns may provide some resistance to low levels of ethanol, but are clearly insufficient to reduce sensitivity to higher concentrations of ethanol.

Surprisingly, *Smed-slo1-A/B(RNAi)* worms showed comparable speeds in water, 0.5% ethanol and 1% ethanol (Figure 4.10 D). This result suggests that the knockdown of both planarian *slo1* sequences leads to the acquisition of ethanol resistance at concentrations up to 1%, indicating that either of the two sequences is sufficient to provide behavioral ethanol sensitivity in planarians. Therefore, either *Smed-slo1-A* or *Smed-slo1-B* is necessary for the qualitatively and quantitatively normal ethanol locomotion response in planarians.

As exposure to 1% ethanol has previously been shown to slow development of normal behavior in head-regenerating planarians, we were curious as to whether the *slo1* gene is involved with normal head regeneration in planarians [57]. We found that the *slo1* gene is not necessary for normal head regeneration following amputation anterior to the pharynx, as all RNAi treated worms regenerated normally over the course of one week (Supplemental Figure 4).

3.5 Conclusions

We have shown that the freshwater planarian species *S. mediterranea* possesses two homologs (*Smed-slo1-A* and *Smed-slo1-B*) of the *slo1* gene, which belong to a family of genes encoding ethanol-sensitive BK ion channels that are widely conserved in mammals, including humans. *Smed-slo1-A* and *Smed-slo1-B* both contain a highly conserved sequence found to create an ethanol-binding pocket in the mouse *slo1* pro-

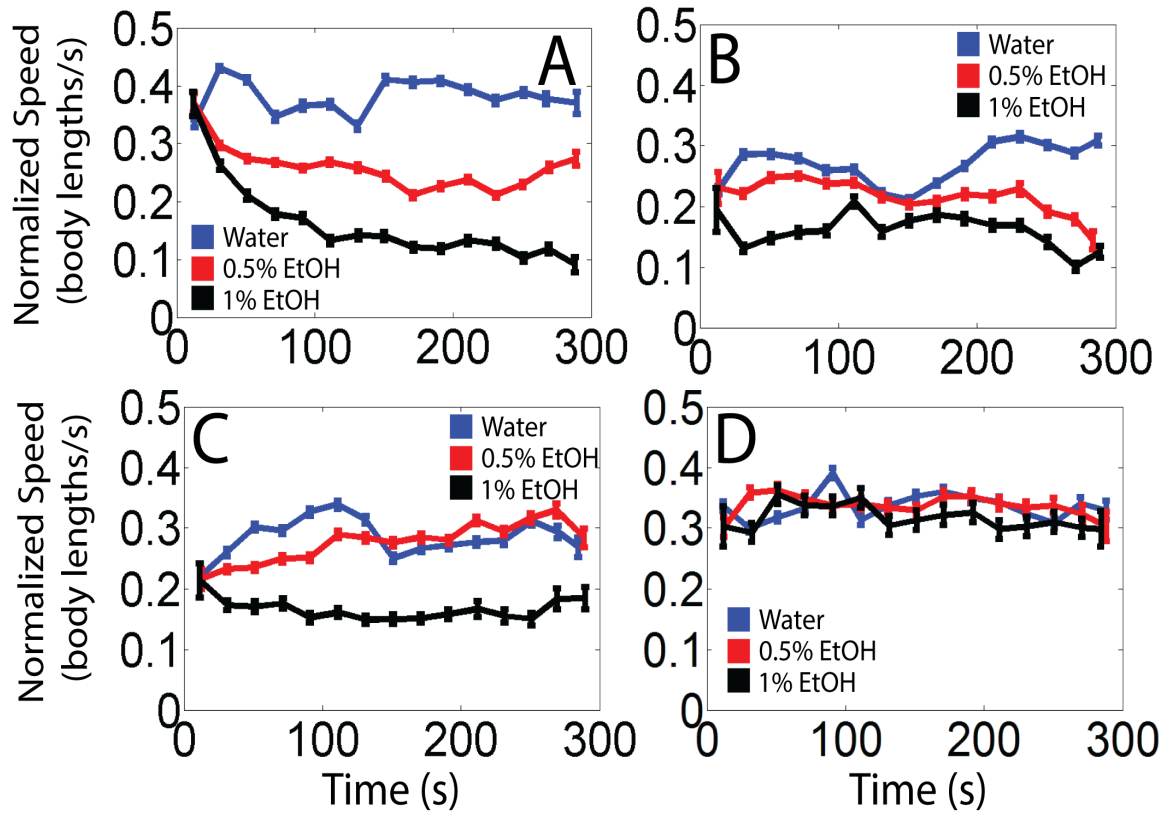


Figure 3.3: Planarian *Slo-1* knock-downs show ethanol resistance. Planarian velocities obtained through center-of-mass tracking normalized by planarian length at maximum extension in water, 0.5% ethanol and 1% ethanol for (a) *Unc-22* RNAi worms (N=9), (b) *Smed-slo1-A* RNAi worms (N=15), (c) *Smed-slo1-B* RNAi worms (N=9), and (d) *Smed-slo1-A/B* RNAi worms (N=8).

tein. Simultaneous knockdown of both *Smed-slo1-A* and *Smed-slo1-B*, however, did lead to a qualitative and quantitative resistance to ethanol. Thus, our studies indicate that planarian *slo1* homologs are necessary for the behavioral ethanol response. Given the high degree of conservation between planarian and mammalian *slo1* sequences and functionality, as well as the complexity of the planarian nervous system, planarians represent a potentially intriguing new model system for studying the effects of alcohol on the central nervous system on the molecular level.

Chapter 3 has been submitted for publication of the material. Carter, Jason; Sinha, Joydeb; Cochet-Escartin, Olivier; and Collins, Eva-Maria S. “A *slo-1* homolog is required for the normal ethanol response in the planarian species *Schmidtea mediterranea*.” The thesis author was the primary investigator and author of this paper.

3.6 Supplementary

Supplementary Movie 1: *Unc22(RNAi)* worms in water. Worms were fed *Unc22* RNAi liver for a total of 8 feedings over the course of 4 weeks. As *Unc22* is a *C. elegans* gene not found in planarians, *Unc22(RNAi)* worms served as a control for all experiments. Control worms in water exhibit the gliding phenotype. Movie is recorded at 1 fps and played at 10 fps. Scale bar is 200 μm .

Supplementary Movie 2: *Smed-slo1-A(RNAi)* worms in water. *Smed-slo1-A(RNAi)* single knockdown worms in water show the normal gliding phenotype in water, indicating that *Smed-slo1-A* is not necessary for normal locomotion in water. Movie is recorded at 1 fps and played at 10 fps. Scale bar is 200 μm .

Supplementary Movie 3: *Smed-slo1-B(RNAi)* worms in water *Smed-slo1-B(RNAi)* single knockdown worms in water show the normal gliding phenotype in water, indicating that *Smed-slo1-B* is not necessary for normal locomotion in water. Movie is recorded at 1 fps and played at 10 fps. Scale bar is 200 μm .

Supplementary Movie 4: *Smed-slo1-A/B(RNAi)* worms in water *Smed-slo1-A/B(RNAi)* single knockdown worms in water show the normal gliding phenotype in water, indicating that neither *Smed-slo1-A* nor *Smed-slo1-B* is not necessary for normal locomotion in water. Movie is recorded at 1 fps and played at 10 fps. Scale bar is 200 μm .

Supplementary Movie 5: *Unc22(RNAi)* worms in 1% ethanol Control worms in 1% ethanol display a distinctive drunken phenotype consisting of oscillations in length throughout locomotion. Movie is recorded at 1 fps and played at 10 fps. Scale bar is 200 μm .

Supplementary Movie 6: *Smed-slo1-A(RNAi)* RNAi worms in 1% ethanol *Smed-slo1-A(RNAi)* single knockdown worms in 1% ethanol still display the drunken phenotype, although the frequency and amplitude of the length oscillations are somewhat reduced compared to control worms. This indicates that *Smed-slo1-A* is sufficient for the drunken behavioral response in 1% ethanol. Movie is recorded at 1 fps and played at 10 fps. Scale bar is 200 μm .

Supplementary Movie 7: *Smed-slo1-B(RNAi)* worms in 1% ethanol *Smed-slo1-B(RNAi)* single knockdown worms in 1% ethanol still display the drunken phe-

notype, although the frequency and amplitude of the length oscillations are somewhat reduced compared to control worms. This indicates that *Smed-slo1-B* is sufficient for the drunken behavioral response in 1% ethanol. Movie is recorded at 1 fps and played at 10 fps. Scale bar is 200 μm .

Supplementary Movie 8: *Smed-slo1-A/B(RNAi)* worms in 1% ethanol *Smed-slo1-A/B(RNAi)* single knockdown worms in 1% ethanol do not display the drunken phenotype. This indicates that expression of either *Smed-slo1-A* or *Smed-slo1-B* is necessary for the drunken behavioral response in 1% ethanol. Movie is recorded at 1 fps and played at 10 fps. Scale bar is 200 μm .

Supplementary Movie 9: *Unc22(RNAi)* worms in 0.5% ethanol Control worms in 0.5% ethanol intermittently display the drunken and gliding phenotypes. Movie is recorded at 1 fps and played at 10 fps. Scale bar is 200 μm .

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Sm-slo1-A 1 -----MSNLSISSGCKFKQQLER-WYIFLAISACQL
Sm-slo1-B 1 -----MESNQLCKENR-WYIFLISASIEF
Ce-slo1 1 MGRIYSPSQSKGFNQPYGYPMNCNLSRWRVEMTEEDRKQLEBRVWCFLLSSLTTEFC

Sm-slo1-A 33 CGILLGCFYRVTONLSWERNPNVNSKLN-----DSCSRPDFI
Sm-slo1-B 25 FGISSVFYRFIVETLAYRRERDEWKNRLLNKENPLTETITVTETENCDQKILE
Ce-slo1 58 ASVILVIMIRVYTHCCQRREREFVEFEP-----APEAVQINNGSKHA

Sm-slo1-A 74 LTRFDD-----DVRHVEISNCF-----DLKDNSGELISGQNLTG
Sm-slo1-B 82 PTFEKRGSDDLRFNSNPMTDSPVTSYTKTNSHNLWILEAKDWAGEIISGQNGTC
Ce-slo1 102 ESETDP-----FLKQEEKHLGWM-----EAKDWAGEIISGQSLTG

Sm-slo1-A 110 TWSLWCI SIGSIATYVLSVVDGLT-EVCVKWNRSLTCLDLAFNITLWYFF
Sm-slo1-B 138 AVLISVGSALSGSLIYVFETSLQGVTFGLCKWSESYTCCDLAFNITLWYFF
Ce-slo1 139 EFLVLAVFVLSIGSLIYFYDAS-FQNFQVETCTEPWQSPSQCDLGFNIFFLWYF

Sm-slo1-A 165 LRFLASNDKLCFELYSFVDLTTFPSFVLYVGRVIGLRFVVRMLTSLDVV
Sm-slo1-B 194 GLRMIAADRFEFWELYSLVVLTVPFAETSLNRMWGLRFALRLISFTDV
Ce-slo1 194 RLRRTAASDRVWFLLEMYSNLDFTTPPSVAIYLRNMGREFRALRLMTVPDV

Sm-slo1-A 221 GFLNVKSSSSIRLCHLETFVSLVTASGFLHVESSGCPFI-IDNGQNIFLDF
Sm-slo1-B 250 LVELNLIKSSSKIRLCHLVLTSLVLTSAEHLHELGLDPE-QTNKONKTVFD
Ce-slo1 250 LQMLNLIKSSSIRLTQLVLTIEVAVCLTGAGLVHLENGSEFFKEFINPHLITVAD

Sm-slo1-A 276 YVILVITISVGYGDIICPFTGKLMMEFICGLALFANSIPEMFELGSRNRYR
Sm-slo1-B 305 YTFMIAATMTVGYGDIICFWQGLMMIEFLLGLALFANSPEHIEIIGNRRY
Ce-slo1 306 VYFVIVTMTVGYGDIICFTLCGLFMIFFILGLAFASVPEIADIGNRRY

Sm-slo1-A 332 NPFRKQKDKCHITLCGHITYDSVSNFLADFLHKREVDVVKIVINNFPPELELKN
Sm-slo1-B 361 GGHKREIGRQHILCGHITFSVSNFLADFLHKRDVDVQVILVDIICENILQ
Ce-slo1 362 GGHKREIGRQHIVCGHITFSVSNFLADFLHEDRDDVDVEVFLHVVVDIELE

Sm-slo1-A 387 VLRKYFHVEFVQSGNSNDLSRIQLONADACLANKYSPPDPAEAANI
Sm-slo1-B 417 SLLKKNINQVQFQGSVMNNDIARVQVEVADACLICPKYSMPDAEDAAN
Ce-slo1 418 FLKRHFHTKVPEFTGVMDQLDSRVKGGDADACLILANKYSTNPDAEDAAN

Sm-slo1-A 440 LRVSIKNFCPRIKVQLQYHNKAYLNIIPSWDWKRGDAVCLAEKLGFIQAQ
Sm-slo1-B 469 IMRVISIKNYCPTIRVIVQLMQYHNKAYLNIIPSWDWRGDDVCLAEKLGFIQAQ
Ce-slo1 470 IMRVISIKNYSSTIRVIVQLMQYHNKAYLNIIPSWDWKRGDDVCLAEKLGFIQAQ

Sm-slo1-A 496 CLAPGFSLLANLFTVRSFKDANLPNWLNTYLEGAGMEMYSEYSPAFHGCTFAE
Sm-slo1-B 525 SCLAPGFSTLLANLFTVRSFKYHHLPEWVNCYVEGASLEMYTEYLSPSFHCITFT
Ce-slo1 526 SCLAPGFSTMMANLFTVRSFKTSITPDWNLNTYLEGAGMEMYTEYLSPSFVGVTEP

Sm-slo1-A 552 AVAMSFYKLLTAETKS--TVNEENSTAINPKNDIKEPGMQGFIAQSADFS
Sm-slo1-B 581 EAVQCCTHNLILAVERSNNRNLSSTAINPKRPFETETQCFPIASADS
Ce-slo1 582 EAVDLENSLGLLLAVERD--EENECNAINPKRPFETETQCFPIAQSADS

Sm-slo1-A 606 KRAFFECRCHLNHR-YKYKQCSAAHKQFO-NTLRLR-----KFETTKSRH
Sm-slo1-B 637 AKRAFVCMACHMS---CKSVKFCNQSSQHQ-DILYNIVS-----CSWMMFNIN
Ce-slo1 636 VKRAFECCKCHDDIKDVSLLKKCKCKNLALRRNTKSTAAARATDVLQCFQPC

Sm-slo1-A 653 SK-----ESKGDSESKVKLRN-----RNIEPNERKRPHNIRNO
Sm-slo1-B 684 KKS-----LIERSSSKPFTIEN-----KKEFGQSARKRSQAL
Ce-slo1 692 NPAGPMHGLGQVQVLRINQSSTSSTHINTSLRFAYEIKLMPSSSGFRNSMSI

Sm-slo1-A 689 KSFRLRENEQIER----RFDTGMFHWCPERPIELVNLSDQLKMCVFSNRIY
Sm-slo1-B 720 NDTIRHHRFNECQQR----RSDSTGMFWCSENEFESALITNKDDLSTVFSNHI
Ce-slo1 748 PPDGRGVPSKDFEQQFQDMKQYDSTGMFWCPSNIEDCLLR-HQAAMTVLNGHY

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Figure 3.4: SupplementalFigure 1: *Smed-slo1-A* and *Smed-slo1-B* are *slo1* homologs. Two potential planarian *slo1* homologs were identified by BLASTing the *C. elegans slo1* sequence against the genome of *S. mediterranea*. The identified potential homologs (*Smed-slo1-A* and *Smed-slo1-B*) were then aligned with the *C. elegans slo1* sequence (*Ce-slo1*) to confirm the planarian sequences were actually *slo1* homologs. Amino acids identical in at least two sequences are shown highlighted in black while conserved residues are highlighted in grey. Non-conserved amino acids are shown with no background color. A high degree of conservation with the *C. elegans* sequence indicates that both *Smed-slo1-A* and *Smed-slo1-B* are likely *slo1* homologs.

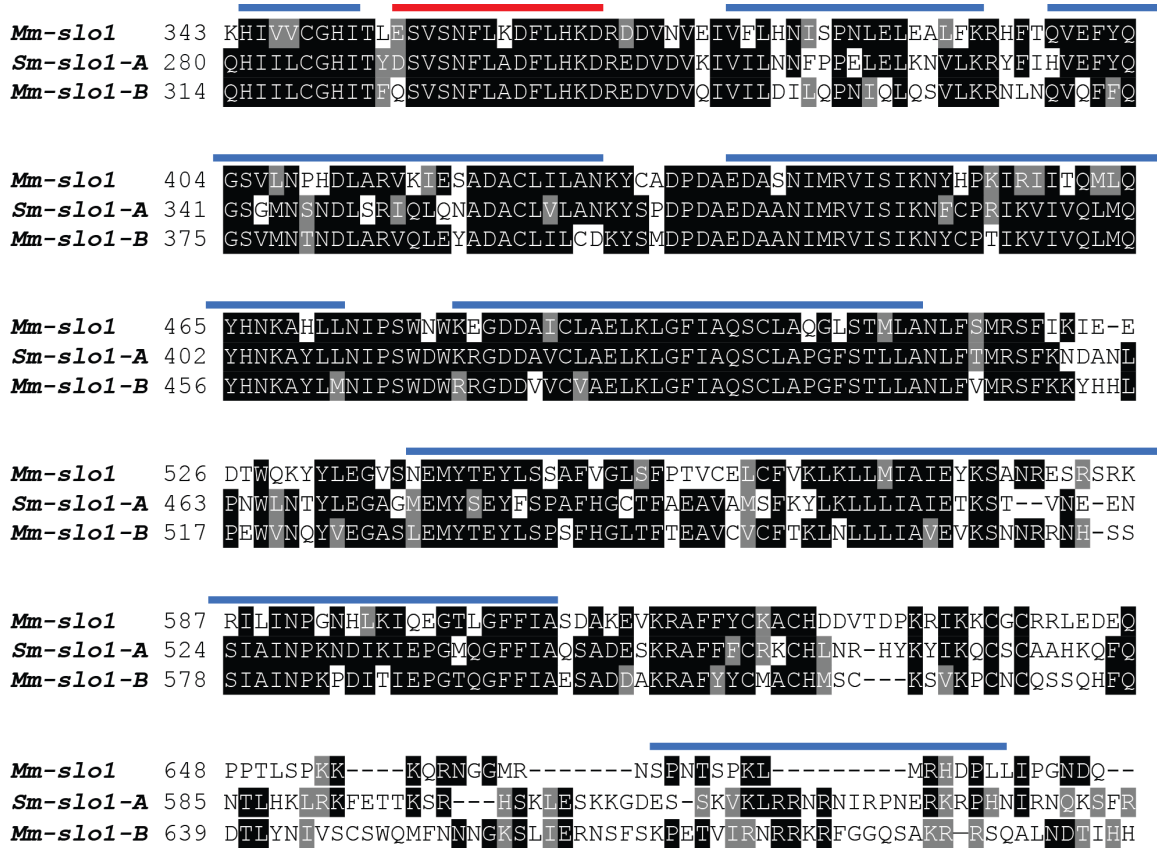


Figure 3.5: Supplemental Figure 2: Conservation of ethanol-sensitive regions in *Smed-slo1-A* and *Smed-slo1-B*. In Bukia *et al.* [10], 1 potential ethanol binding pocket was identified with the potential to provide the ethanol sensitivity of the mouse *slo1* BK channel. A further 7 sequences surrounding the pocket with the potential to effect the binding pocket were also identified. In order to determine whether *Smed-slo1-A* and *Smed-slo1-B* contained a conserved alcohol-sensitive region, the mouse *slo1* sequence (*Smed-slo1*) was aligned with *Smed-slo1-A* and *Smed-slo1-B*. The hypothesized ethanol binding pocket is indicated in red, while the other 7 surrounding sequences are indicated in blue. Identical amino acids in at least two of the sequences are highlighted in black while the conserved amino acids are highlighted in grey. Non-conserved mutations are shown with no background color. A high degree of conservation in *Smed-slo1-A* and *Smed-slo1-B* at the hypothesized ethanol binding pocket, as well as 5 of the 7 potential interacting sequences, indicates that the ethanol-sensitive functionality of the *slo1* protein is likely conserved in both planarian homolgs.

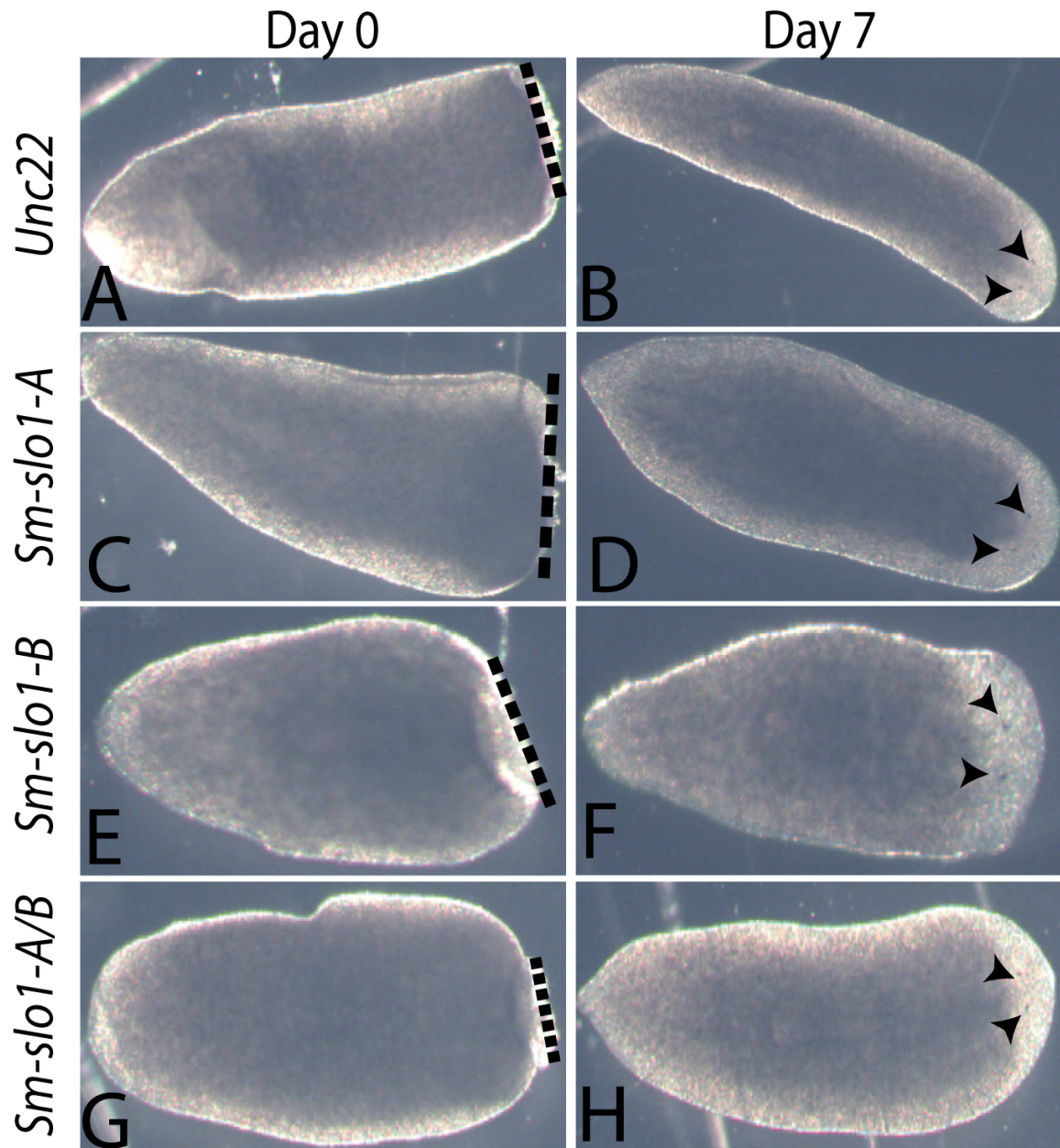


Figure 3.6: Supplemental Figure 3: *Smed-slo1-A* and *Smed-slo1-B* are not required for normal head regeneration following amputation. (A),(B) *Unc22*, (C),(D) *Smed-slo1-A*, (E),(F) *Smed-slo1-B*, and (G),(H) *Smed-slo1-A/B* RNAi fed worms were decapitated (amputation plane shown as dashed black line) immediately posterior to the eyes after 8 feedings on Day 0. All worms showed normal head regeneration, with 2 eyes appearing by Day 7. Eyes are indicated by black arrowheads. Scale bar is 1 mm.

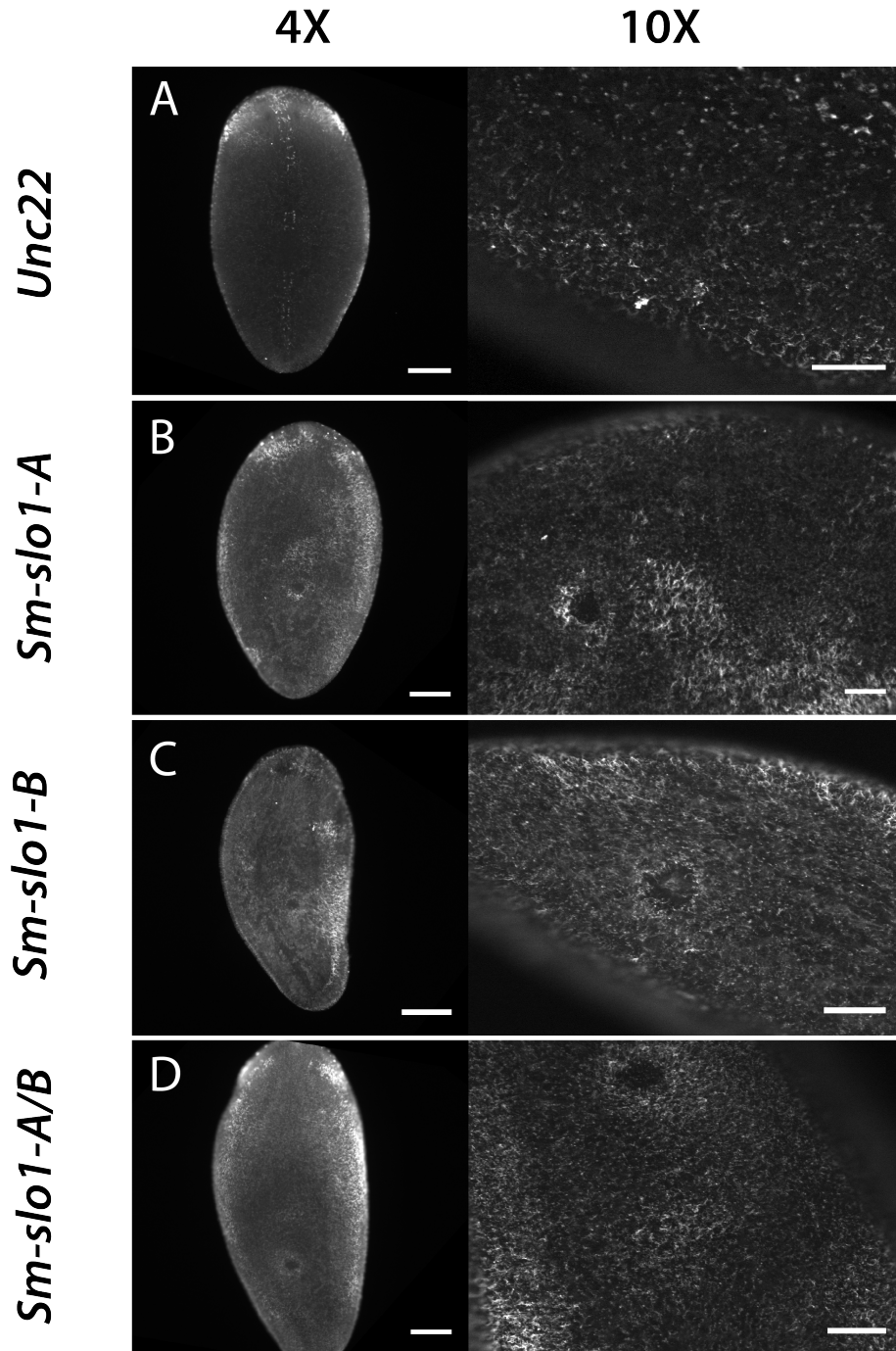


Figure 3.7: Supplemental Figure 4: RNAi treatment does not effect cilia structure. Worms were fed (A) *Unc-22*, (B) *Smed-slo1-A*, (C) *Smed-slo1-B*, and (D) *Smed-slo1-A/B* RNAi liver 8 times over the course of 4 weeks, stained with an anti-tubulin antibody and imaged at 4x and 10x. Cilia, which are required for the normal gliding phenotype, are intact in all worms following RNAi feedings. These results indicate that RNAi does not interfere with the ability of worms to undergo the normal gliding phenotype.

Chapter 4

A Physical Model for Mouth Opening in *Hydra*

4.1 Abstract

Hydra, a simple freshwater animal famous for its regenerative capabilities, must tear a hole through its epithelial tissue each time it open its mouth. This mouth opening process occurs spontaneously, as a means of regulating internal osmotic pressure and as a feeding response, which can be induced with reduced glutathione. We take advantage of the availability of transgenic *Hydra* lines and perform the first dynamical analysis of *Hydra* mouth opening, which we hypothesize is an active process driven by the contraction of radial muscles located in the ectodermal epithelial tissue layer. We find that a simple phenomenological model reproducibly fits the experimentally derived opening data and predicts several biologically important parameters of the opening process. Further modeling suggests that the maximum mouth opening area is determined by the initial tension generated by the ectodermal radial muscles, but that the amount of tension which can be built up within the hypostome is determined by the circular muscles of the endoderm. Finally, we estimate that the forces required to achieve opening are on the order of a few nN and that the rate of opening is directly related to the elastic modulus of the epithelial tissues, which measure to be on the order of a few kPa.

4.2 Introduction

Hydra, a freshwater cnidarian polyp, has a morphologically simple body plan. Its radially symmetric, tubular body column is composed of two epithelial layers, the endoderm and ectoderm, which are separated by a layer of extracellular matrix called the mesoglea [9, 96]. The apical end of the polyp consists of a ring of tentacles and the dome-shaped hypostome, which contains the axial organizer and the mouth. The mouth of *Hydra* is a remarkable structure. Unlike in most animals, it is not a permanent opening. Although the position of the mouth is defined by morphologically distinct groups of cells in both endoderm and ectoderm [13, 96, 108, 109], when closed, the mouth is a continuous epithelial sheet, sealed with septate junctions [5, 13, 86]. During mouth opening, a breach in the epithelia at the tip of the hypostome rapidly expands, sometimes wider than the body column, allowing a *Hydra* polyp to engulf

prey larger than itself [13]. Mouth opening occurs during ingestion of prey, egestion of indigestible material, and as a means of osmotic regulation [7, 13]. The feeding response of *Hydra* can be evoked chemically using reduced glutathione and is regarded as a classical model system for environmental chemical biology [33, 39, 54].

Although the structure of the *Hydra* mouth has been studied with light and electron microscopy [13], the biomechanics and cellular dynamics of mouth opening have remained unexplored. One study suggested that dramatic cellular rearrangements would be necessary to accommodate expansion of the mouth [13]. However, others have demonstrated that an immunocytochemically distinct group of endodermal cells remains at the rim of the expanding opening [96]. The contraction of radially oriented ectodermal epitheliomuscular processes is thought to generate the force for hypostome rupture and expansion, while contraction of the antagonistic epitheliomuscular processes in the endoderm would drive closing [43, 110].

In the present study, we have used live imaging of hypostomes from transgenic animals expressing fluorescent proteins in endodermal and ectodermal epithelial cells [35] to investigate the kinetics and biomechanics of *Hydra* mouth opening. By tracking individual cells during spontaneous openings we show that cells maintain existing contacts and do not undergo rearrangement. Cell shape analysis further confirms that mouth expansion occurs exclusively through viscoelastic deformation of cells. Based on the kinetics of mouth expansion, we derive a phenomenological model for mouth opening with four fit parameters, two of which can be identified with initial and maximum opening areas while the other two are indicative of the opening kinetics. We show that the opening expands from a single rupture point through a combination of passive viscoelastic recoil and active contraction of epitheliomuscular processes. The maximum opening size is determined by the initial tension generated by the radial muscles in the ectoderm, but the amount of tension that can be built up is controlled by the antagonistic endodermal circular muscles which oppose opening. We find that the forces required for mouth opening are on the order of a few nN. This model provides a biomechanical framework for understanding the remarkable plasticity of the *Hydra* mouth. More importantly, our study illustrates that *Hydra* is a powerful system for quantitative biomechanical studies of cell and tissue behaviors *in vivo* due to the ease of experimental accessibility and the simplicity of the *Hydra*

body structure [62].

4.3 Materials and Methods

4.3.1 Animal care and transgenic lines

Mass cultures of transgenic *Hydra vulgaris* lines (GFP-ectoderm/DsRed2-endoderm [35] and Wnt3 promoter::GFP [64]) were maintained in *Hydra* culture medium (1.0 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Spectrum Chemical, Gardena, CA), 0.1 mM $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$, 0.03 mM KNO_3 , 0.5 mM NaHCO_3 , 0.08 mM MgSO_4 (Fisher Scientific, Fairlawn, NJ)) using standard procedures [53]. Animals were fed three times per week on freshly hatched *Artemia nauplii* (Brine Shrimp Direct, Ogden, UT) and starved for 1-3 days before use in experiments.

4.3.2 Image acquisition

Mouth opening was visualized in transgenic animals using fluorescence confocal microscopy. Ectoderm and endoderm mouth openings were visualized separately through the use of transgenic *Hydra* expressing GFP and DsRed2 in each tissue layer [35], respectively. For imaging studies, starved animals were decapitated immediately below the tentacles with a scalpel, allowed to heal for at least two hours and manually positioned in a drop of *Hydra* medium between two glass coverslips separated by a single layer of double-sided tape. All imaging was performed on an Olympus IX81 inverted microscope equipped with an Olympus 10x/0.40 UPlanSApo objective, an Olympus DSU spinning disk unit (Olympus Optical, Tokyo, Japan), and a Prior ProScan motorized XY stage (Prior Scientific Inc., Rockland, MA). Digital images were captured with a Hamamatsu Orca-ER CCD camera (Hamamatsu Photonics, Shizuoko, Japan) using SlideBook software (Intelligent Imaging Innovations Inc., Denver, CO) running on a Dell Precision 690 PC. High-speed single-channel image sequences of mouth opening were acquired using the camera's streaming mode, achieving a frame rate between 150 and 450 ms. Generally, spontaneous mouth opening events were recorded, however a feeding response was induced in a subset of experiments by flowing *Hydra* medium containing 0.1 mM reduced L-glutathione

(Sigma-Aldrich, St. Louis, MO) into the imaging chamber [13, 54]. Two-channel movies were recorded using the same approach and equipment, but alternating between GFP and DsRed2 channels throughout the course of a single mouth opening resulted in a slower frame rate of 5.5 s in these experiments.

4.3.3 Data analysis

The mouth opening area for both single and two channel movies was calculated from binarized images created using a custom adaptive thresholding algorithm in MATLAB (MathWorks, Natick MA). In all, 19 ectoderm openings from 14 individual *Hydra* and 15 endoderm openings from 8 individual *Hydra* were recorded using the single-channel method and analyzed using this method. Additionally, 9 openings from 6 individual *Hydra* were taken from two-channel movies and analyzed. The area of mouth opening in each frame was calculated and normalized to the maximum opening size of that opening sequence. As it is difficult to specify the precise moment at which opening begins, areas were aligned at the point of 50% of maximum opening size using a custom MATLAB script. Aligned area values were then binned into 7-second intervals and plotted as a function of time.

In the transgenic animals, the fluorescent protein is distributed throughout the cytoplasm. However, because ectodermal cells contain large vacuoles, the fluorescent signal is localized to the perimeter of the cell and the nucleus, as GFP is small enough to pass through nuclear pores, allowing us to identify the border of the cell and the nucleus. Thus, in these transgenic *Hydra*, nuclei and cell borders in the ectoderm are easily identifiable and thereby can be tracked without additional labeling. 80 individual ectoderm nuclei taken from 3 individual *Hydra* were selected and manually tracked in every frame throughout mouth opening. Position and time data were recorded in MATLAB. Ectoderm nuclei speeds were calculated and plotted against their instantaneous distance from the center of the mouth opening. As cell vacuoles are readily visible in endoderm movies while cell nuclei are not, 60 vacuoles from 3 *Hydra* were instead tracked and used to calculate cell speeds for the endoderm. In both ectoderm and endoderm movies, the global movement of the mouth was quantified in the same manner described above for cell nuclei and subtracted from the

movement of individual cells. Changes in ectoderm cell shape during mouth opening were quantified by manually tracing ectodermal cell borders and automatically calculating the aspect ratio using standard image analysis methods in ImageJ (NIH).

4.3.4 Tissue separation

Methods for separating endodermal and ectodermal tissues were adapted from published protocols [50, 55]. Body columns were prepared from adult polyps by cutting immediately below the tentacles and above the budding zone with a scalpel. The tissue separation solution consisted of *Hydra* culture medium that was adjusted to pH 2.5 with 2N HCl immediately before use and kept on ice. Body columns were placed in a plastic tube containing separation solution for 2 min on ice before being transferred to a room temperature hypertonic medium (3.6 mM KCl, 1.2 mM MgSO₄, 12.5 mM TES (Fisher Scientific, Fairlawn, NJ), 6.0 mM CaCl₂ (Spectrum Laboratories, Inc., Rancho Dominguez, CA), 6.0 mM Na-citrate (LabChem Inc., Zelienople PA), 6.0 mM Na-pyruvate (Alfa Aesar, Ward Hill MA), pH 6.9). Within 1-2 minutes of this transfer, the ectoderm contracts into a ring and the endoderm into a rod as previously described [50]. The two tissue layers were gently separated from each other using fine forceps, then cut into 150-400 μ m pieces with a scalpel. Pieces prepared in this manner typically rounded up into spheres within 1-2 hours. Of note, this is the first time that transgenic *Hydra vulgaris* have been used for this kind of tissue separation.

4.3.5 Elastic modulus measurement

The elastic modulus (E) of ectodermal and endodermal tissue was determined by parallel plate compression as previously described for zebrafish embryonic tissues [34, 59, 80]. In brief, a single tissue sphere (containing only endoderm or ectoderm) was placed between two parallel plates and a fixed small deformation was applied. The upper compression plate was connected to a Cahn model D-200 digital recording electrobalance (Cerritos, CA) through a nickel-chromium wire to allow measurement of weight (force) changes using MicroScan software (Thermo Electron Corporation, Waltham MA). In successive trials, spheres were compressed to var-

ious degrees. Tissues were compressed for 10 seconds, decompressed and allowed to reach equilibrium before any subsequent compressions. Plates were coated with either polydimethylsiloxane (PDMS) (Ellsworth) or polyhydroxyethylmethacrylate (polyHEMA) (Sigma) to prevent tissue adhesion. Tissue geometry (deformation) was imaged using a Leica S8 APO dissecting microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Basler A601f CCD camera (Basler AG, Ahrensburg, Germany) and a custom MATLAB image acquisition routine. Geometry of tissue pieces was analyzed with ImageJ software. While endoderm spheres were reliably solid spheres, ectoderm spheres sometimes contained tissue folds. Therefore, post-compression, spheres were checked for pockets of empty space which would affect the elastic properties by imaging a z-stack on a confocal microscope. The Young's modulus E was calculated as previously described in Ref. [59] using a Hertzian model:

$$E = \frac{3 \cdot A \cdot \Delta F_{El}}{4 \cdot R^3}$$

Where A is the original radius of the tissue sphere before compression, F_{El} is the elastic force, and R is the radius of the contact between the tissue sphere and the compression plate.

4.4 Results and Discussion

Hydra is radially symmetric and consists of an outer ectoderm and an inner endoderm, separated by the mesoglea. The ectodermal layer is significantly thinner than the endodermal layer, whose cells are more columnar. The *Hydra* head is composed of a dome-shaped hypostome surrounded by a ring of tentacles (Fig 4.1 A). In the center of the hypostome is the mouth, which is sealed with septate cell-cell junctions when closed (Fig 4.1 A-B and Ref. [13]). During mouth opening, muscle processes in the ectoderm pull radially on the hypostome leading to a symmetric circular mouth opening (Fig 4.1 B-E).

It has previously been shown that the *Hydra* mouth opens for feeding, regurgitation of undigested material and for regulation of osmotic pressure [7, 13]. In this study, we found that the mouth also opens without external cues or the

need of osmotic regulation, as even freshly cut heads exhibit mouth opening (Supplemental Movies 1-4). We will therefore refer to this opening as spontaneous mouth opening. While the physiological basis of spontaneous mouth opening remains unclear, a quantitative comparison of the opening dynamics of spontaneous and reduced glutathione-induced opening (Supplemental Fig. 1 A-C, Supplemental Movie 5) shows that they have similar dynamics. Thus studying spontaneous mouth opening provides insight into mouth opening in general and has the experimental advantage of not requiring chemical or mechanical perturbation of the system. Additionally, the imaging set-up did not have any observable effect on mouth opening dynamics (Supplemental Fig. 1 D-F).

4.4.1 Mouth opening kinetics

To understand the opening of the *Hydra* mouth, we first examined the kinetics of spontaneous mouth opening (Supplementary Movies 1-3). Both ectodermal and endodermal tissue layers were individually imaged throughout the mouth opening process and the area of mouth opening determined. Although the *Hydra* head and particularly the hypostome region are three-dimensional, little motion takes place along the axis perpendicular to mouth opening (Supplemental Movie 4), allowing us to treat mouth opening as a two-dimensional process in the following analysis.

Because the beginning of opening is difficult to define, opening dynamics were aligned in time by the point at which the area of mouth opening achieved 50% of the maximum opening area (Fig 4.2 A). The maximum opening area was found to vary between preparations (Fig 4.2 A), and even the same preparation could open multiple times within the imaging period to different degrees (Supplemental Fig. 2 A-B). However, normalization of the area by the maximum area obtained in a given opening sequence caused all curves to collapse, indicating that the rate of opening was the same between all openings (Fig 4.2 B). Both ectodermal and endodermal tissue opened at a nearly identical rate (Fig 4.2 C), which was expected given that they are attached to one another through the mesoglea. The entire opening was complete within approximately 60 seconds, with the fastest opening rate occurring during the middle third of the opening.

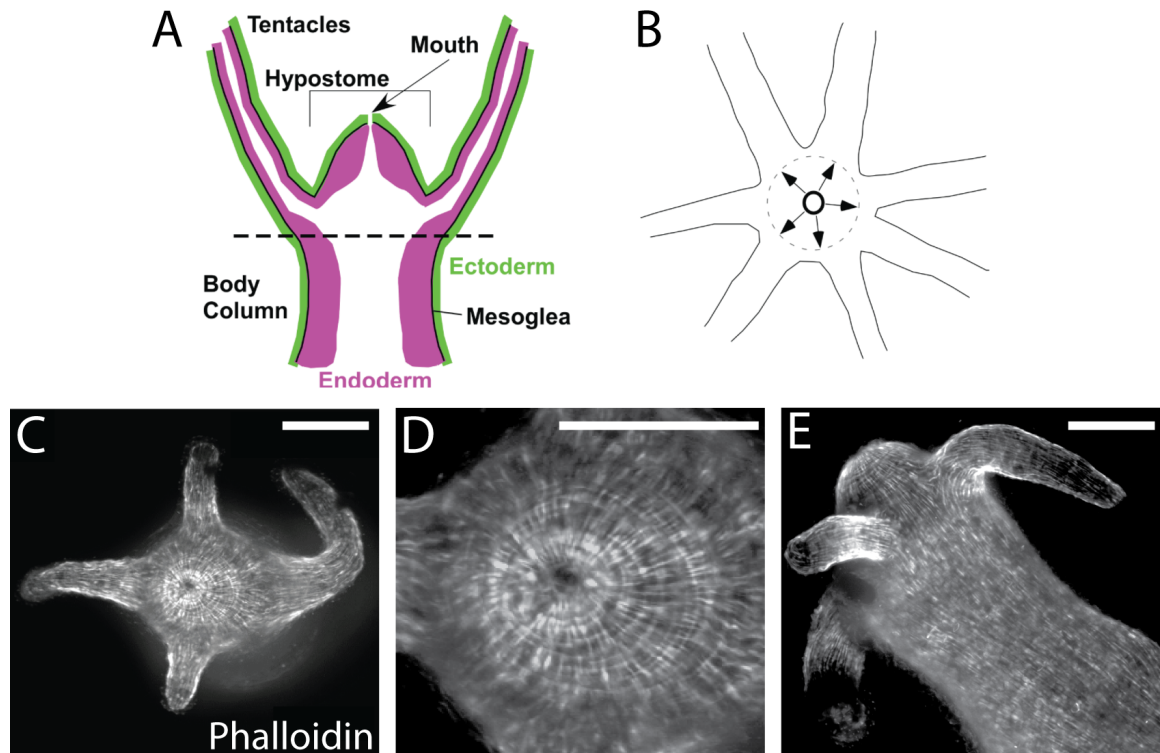


Figure 4.1: The *Hydra* mouth (A) Schematic showing the apical region of *Hydra* in cross-section, including ectodermal (green) and endodermal (magenta) epithelia, and mesoglea (black). The dashed line indicates the position of head amputation prior to imaging. (B) View of the hypostome from above. Arrows indicate direction of mouth expansion during opening. The dashed line indicates a potential maximum opening position of the mouth. (C) *Hydra* stained with rhodamine phalloidin to visualize radial and concentric muscle processes in ectoderm and endoderm, respectively. (D) Higher magnification of hypostome in (C). The radial ectodermal and circular endodermal processes are clearly visible. (E) Side-view of phalloidin-stained muscle-processes in the *Hydra* hypostome. Scale bars are 200 μm .

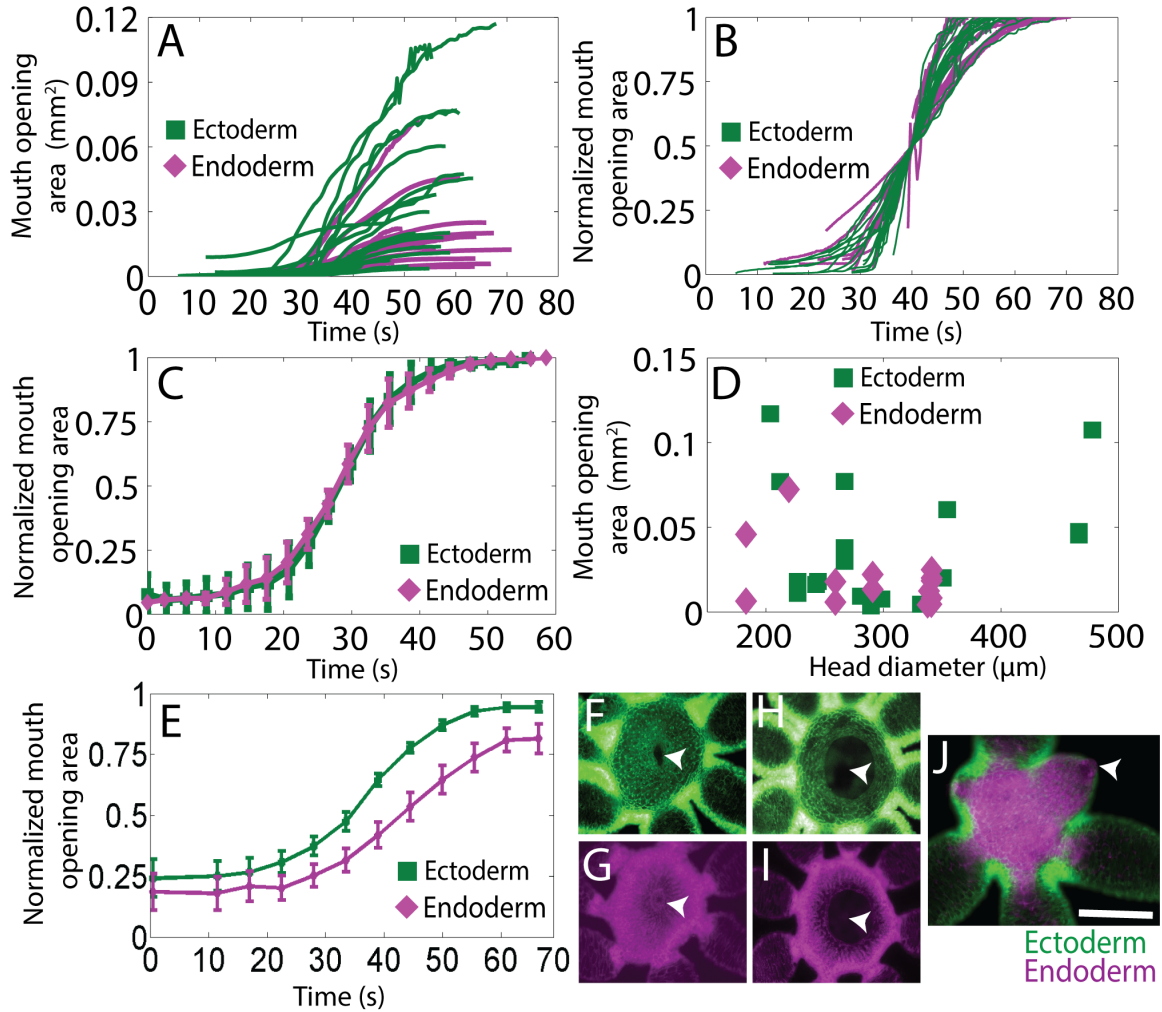


Figure 4.2: *Hydra* mouth opening dynamics. (A) Raw opening data for the ectoderm and endoderm epithelial tissue layers, aligned at the point in time at which the mouth opening area reached 50% of its maximum value. (B) Raw opening data normalized by the maximum opening area show that the rate of opening is conserved between ectoderm and endoderm. (C) Normalized opening data for both ectoderm and endoderm recorded separately from different preparations binned into 7-second intervals and averaged. (D) No correlation exists between head size and maximum mouth opening area. (E) Mouth areas for both ectoderm and endoderm from two-channel movies were normalized by the maximum opening area of the ectoderm. Ectoderm mouth opening appears to begin before endoderm opening and the ectoderm ultimately opens more widely than endoderm. (F-G) Representative images from a two-channel movie showing ectoderm opening (F) has already begun to open while the endoderm remains closed (G). (H-I) Representative images from a two-channel movie showing both ectoderm (H) and endoderm (I) at the point of maximum opening. (J) Representative side-view image from a two-channel movie with ectoderm and endoderm merged at the point of maximum opening. Arrowheads indicate the center of the mouth opening. Scale bar is 200 μm. In all panels, ectoderm is shown in green and endoderm in magenta. Error bars denote SEM.

Given the variability in the maximum opening area, we were curious whether the size of the animal determined the extent of mouth opening, as one may assume that larger animals have more or stronger muscle processes capable of exerting a larger opening force. To test this, we examined the relationship between the maximum opening area and the size of the head for both ectoderm and endoderm movies. However, no correlation was found between head size and mouth opening size (Fig. 4.2 D). In addition, heads from buds, *i.e.* asexual offspring which have not yet separated from the mother polyp, which are significantly smaller than heads from adults, were found to exhibit comparable kinetics to adults (Supplemental Figure 1 G-I, Supplemental Movie 6). An alternative explanation for different opening sizes is differences in initial tensions of the radial muscles; this option is explored in more detail in the final section.

Due to the fact that the radial muscles are located in the ectoderm, and that the mesoglea, connecting both epithelial layers, is easily stretched, it is conceptually possible that the ectodermal tissue may begin opening before the endodermal tissue. To investigate this hypothesis, we acquired two-channel movies of individual mouth opening sequences, wherein we imaged the ectodermal and endodermal tissues of the same animal in alternating order (Supplemental Movie 3). Quantitative analysis of these two channel movies clearly showed that although both tissue layers open with a nearly identical rate, the ectoderm began to open prior to the endoderm and that the endoderm opened to only about 80% of the ectoderm area at the point of maximum opening (Fig 4.2 E-I). One possible explanation for the difference between maximum ectoderm and endoderm opening size is that the endoderm begins to bulge out through the ectoderm opening at sufficiently large opening areas (Fig 4.2 J, Supplemental Movie 4).

4.4.2 Cellular rearrangement is not involved in hypostome opening

The most detailed study on *Hydra* mouth opening to date suggested that mouth opening may require cellular rearrangements [13]. This hypothesis, however, was not tested experimentally due to lack of *in vivo* imaging tools. With the ability

to do time lapse imaging on transgenic animals with fluorescent epithelial cells, we were able to test this hypothesis directly (Fig 4.3).

Our movies show that mouth opening occurs in about a minute, suggesting that cell rearrangement is highly unlikely (Fig 4.3, Supplemental Movies 1-2), as it would require much longer timescales. In addition, movies of transgenic *Hydra* in which GFP is expressed under the control of the *Hydra* Wnt3 (HyWnt3) promotor, which is expressed only in the ectodermal cells at the tip of the hypostome (Fig 4.3 A and Ref. [64]) show that at the point of maximum opening, GFP positive cells remain at the edge of the opened mouth, indicating that no rearrangement occurs (Fig 4.3 B-C).

To quantify cell trajectories and speeds during mouth opening, individual nuclei were tracked throughout the opening process (Fig 4.3 D-F). The tracks of these nuclei clearly show that the relative arrangement of cells is maintained throughout the opening process, confirming the absence of cellular rearrangement (Fig 4.3 G). The speeds of cell nuclei in the ectoderm and of cell vacuoles in the endoderm were found to decrease as a function of their distance from the center (Fig 4.3 H). The endodermal data are significantly noisier than the ectoderm data because it was difficult to track the position of the vacuoles with the same accuracy as cell nuclei. If the tissue would behave as a passive continuous prestressed elastic sheet, we would expect the cell speeds to decrease as $1/r$ as a function of their position from the hypostome center, with r the radius of the mouth opening [58]. As evident from the plots in (Fig 4.3 H), cell speeds do follow a $1/r$ dependence, in agreement with our notion that mouth opening is an active process.

To achieve mouth opening, cells either need to rearrange or deform substantially. As cellular rearrangement does not occur during mouth opening, the change in opening area should be directly reflected in a change in cell shapes, with cells being compressed perpendicularly to the mouth boundary and elongating parallel to the boundary. Indeed, cell shape changes correlated perfectly with mouth opening changes and the average aspect ratio of cells doubled from 1.5 to 3 (Fig 4.3 L-M), as quantified through manual tracking of cell morphologies (Fig 4.3 I-K). Of note, mouth opening was not perfectly circular (Supplemental Figure 3), indicating local differences in tensions, which were also evident in local differences in cell deformations

during opening (Fig 4.3 I-K). The origin of this asymmetry is not entirely clear as *Hydra* and its head are thought of as radially symmetric; one possible explanation are local differences in the strength of cell-cell junctions or in circular muscle tension (see below).

In summary, our dynamical studies conclusively confirmed that mouth opening is achieved by cell stretching, as proposed by Ref. [96], and not cell rearrangement, as proposed by [13]. The sigmoidal shape of the mouth area versus time curve (Fig. 4.2A), however, indicates that mouth opening in *Hydra* is more than a simple release of pre-existing tensions.

4.4.3 Phenomenological model

The sigmoidal shape of the normalized mouth opening area versus time curve resembles a logistic equation (Fig 4.1 C). As little is known about *Hydra* musculature outside of its general anatomy (Fig 4.4 A and Refs. [43, 49, 52]), we began with a phenomenological approach to fit the curves with a modified logistic equation which has been previously used in Ref. [103] to describe the pressure-volume relationship observed in mammalian lung inflation:

$$A(t) = a + \frac{b}{1 + e^{\left(\frac{-(t-c)}{d}\right)}} \quad (4.1)$$

where $A(t)$ is the area of the mouth as a function of time, t is the time from the initiation of the opening process, and a, b, c and d are fit parameters. Mathematically, parameter a corresponds to the lower asymptote, b defines the upper asymptote, c sets the inflection point (the point at which the sign of the second derivative changes) and d is related to the length of the range of rapid increase (Supplemental Figure 4 and Ref. [103]).

Time-shifted and normalized ectoderm and endoderm mouth opening area *vs.* time curves (Fig 4.1 C) were fitted with Eq 1. For ectoderm, we found $a=0.046$, $b=0.93$, $c=28.7$ and $d=4.19$ fit the experimental data with an R^2 value of 0.9997. Furthermore, we found that the endoderm opening rate could similarly be fitted with $a=0.02$, $b=0.99$, $c=28.23$ and $d=5.7$, giving an associated R^2 value of 0.9981 (Fig 4.4

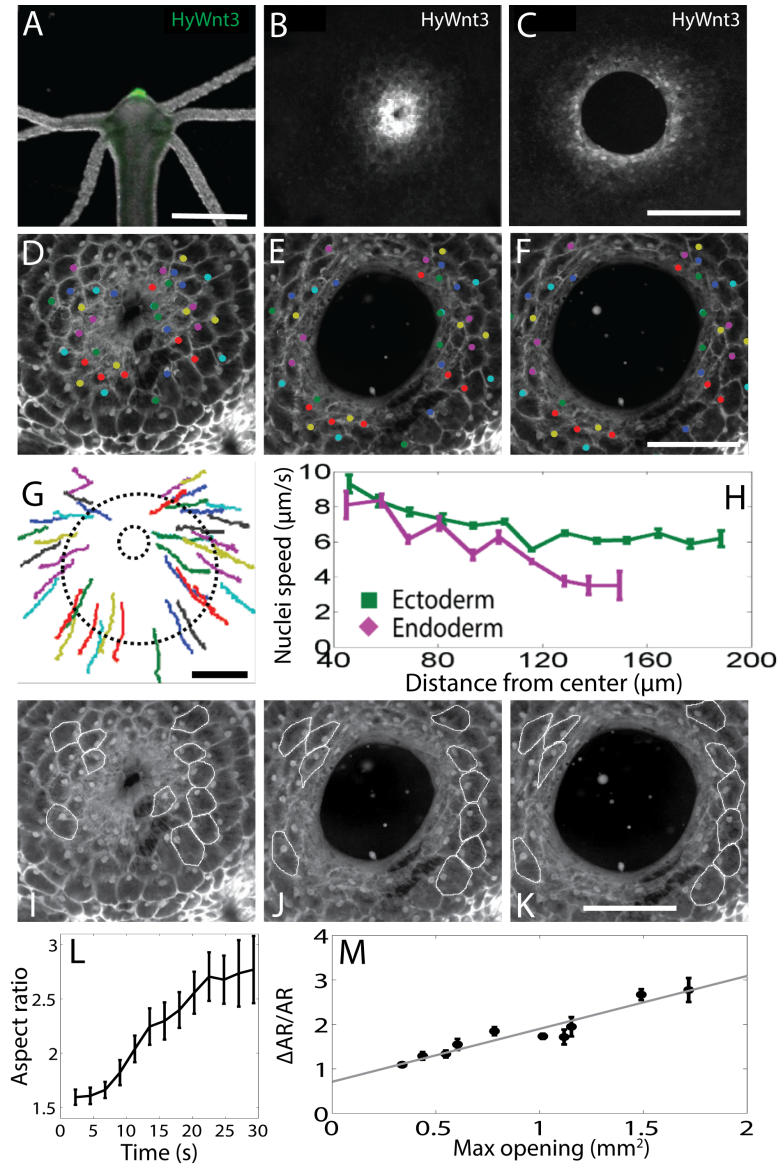


Figure 4.3: Mouth opening involves changes in cell morphology but not cell repositioning. (A) Expression of GFP driven by the Wnt3 promoter is confined to the cells at the tip of the hypostome. (B) The mouth of Wnt3-GFP *Hydra* immediately before mouth opening (0 seconds) and (C) at the point of maximum opening (approximately 40 seconds). (D-F) Representative images showing positions of labeled ectoderm nuclei at 0, 15 and 30 seconds. (G) Tracks of individual nuclei recorded every frame for approximately 40 seconds through mouth opening show no cell rearrangement. (H) Quantification of the speeds of ectoderm cell nuclei and endoderm vacuoles throughout mouth opening as a function of their distance from the center of the hypostome. (I-K) Representative images showing outlines of individual cells at 0, 15 and 30 seconds of mouth opening. (L) Change in aspect ratio of ectoderm cells through mouth opening. (M) The maximum opening area of the mouth is directly related to the change in aspect ratio of individual cells. Error bars indicate *SEM*. Scale bar is 500 μm in (A) and 200 μm in (C), (F) and (K).

B).

Parameters a and b , corresponding to the lower and upper asymptotes, respectively, can be biologically identified with the initial and the maximum mouth opening areas. Given that the *Hydra* mouth is fully sealed when closed, one would expect a to be zero. Similarly, given that we fit normalized area curves, we expect b to equal 1. Both a and b were determined to have these expected values within their 95% confidence intervals.

Parameter c , in units of time, defines the inflection point of the curve (the point at which the concavity of the curve changes). Finally, parameter d is related to the length (duration) of the region in which the majority of the opening process occurs. In our case, this region spans from approximately 20 seconds to about 35 seconds (Fig 4.4 B) and is defined by the points of intersection of a tangent to the $A - t$ curve through the inflection point with the lower and upper asymptotes (Supplemental Figure 4 and Ref. [103]). The points of intersection are then given by:

$$L_a = c - 2d \quad (4.2)$$

$$L_b = c + 2d \quad (4.3)$$

Solving these equations using our calculated fit parameters, the model predicts that the point of maximum rate of change in curvature should occur around 20 seconds into the opening process. From the area *vs.* time curve, this region should begin approximately when the ectoderm reaches an area of about 15% of its maximum opening area (Fig 4.4 B, gray line).

We have already seen that the ectoderm opens before the endoderm (Fig 4.2 E-G, Supplemental Movie 7). Therefore, one might expect that L_a occurs at the point at which the endoderm begins to rupture and both tissue layers are free to open together. To test this hypothesis, we quantified the critical threshold area (A_{cr}) of the ectoderm required to induce opening of the endoderm from our two-channel movies (Fig 4.4 B, black line). We found that A_{cr} is equal to about 15.6% of the maximum opening area, with a standard error of approximately 3%. Thus, the point at which our model predicts the region of fastest opening to begin corresponds closely to the point

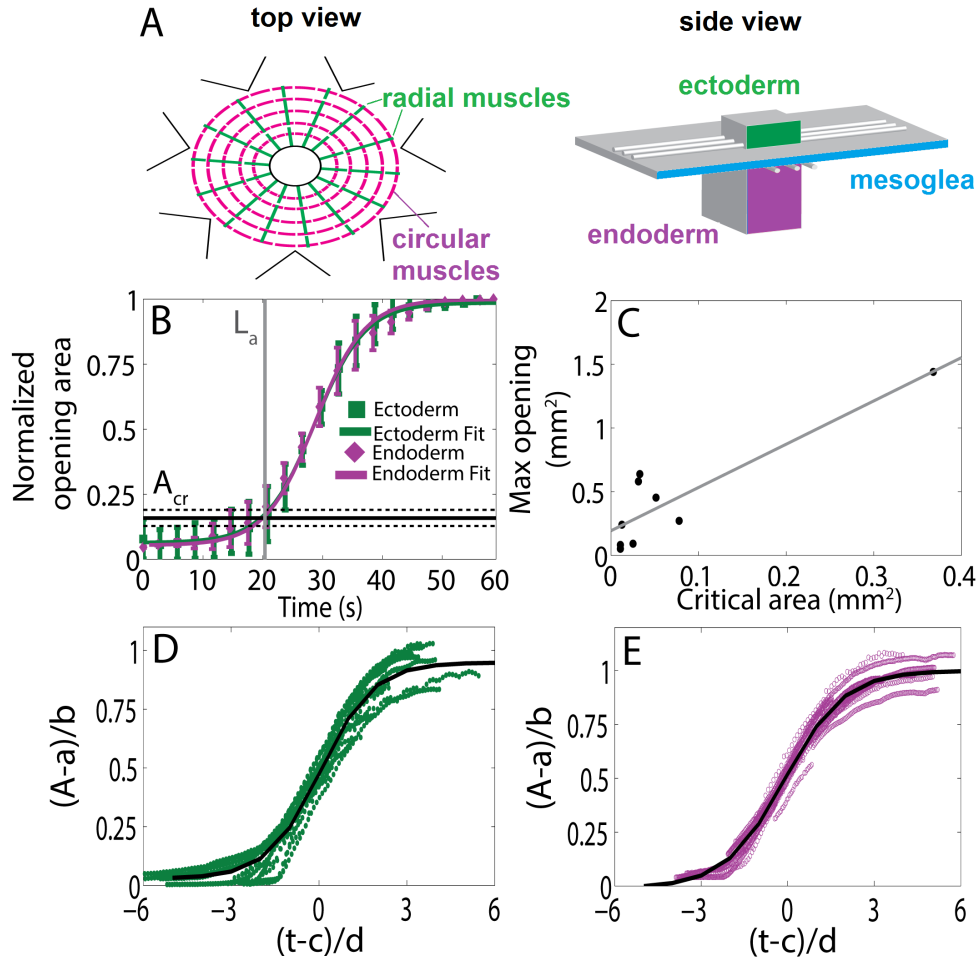


Figure 4.4: Modeling mouth opening. (A) Schematic showing orientation of the muscle processes in the hypostome prior to opening. (B) Ectoderm and endoderm opening data aligned by 50% of the maximum opening value, binned into 7-second intervals and fitted according to Eq. 1. The point at which the maximum change in curvature occurs, $L_a = c - 2d$, is shown in gray and the ectodermal critical area (A_{cr}) at which the endoderm begins opening is shown in black. The dashed black lines represent the SEM of the critical radius. (C) Relationship between the critical area and the maximum opening area. (D) Ectoderm and (E) endoderm opening data normalized by fit parameters and fit according to Eq. 1. Ectoderm $R^2=0.981$ and endoderm $R^2=0.978$.

at which the endoderm begins to open, demonstrating that this phenomenological model is able to make biologically meaningful predictions. Furthermore, we found that the threshold area was directly related to the maximum opening area (Fig 4.4 C), suggesting that the circular muscles which work to keep the endoderm closed, are involved in setting the maximum opening area.

Parameter d is related to the length of time in which the majority of opening occurs and thereby inversely proportional to the rate of opening. That is, a faster opening would lead to a steeper area *vs.* time curve and thus smaller value of d . On the other hand, the rate of opening for an elastic sheet directly reflects the mechanical properties of that sheet: the stiffer the sheet, the more force is required to obtain a certain opening, and thus the steeper the curve. In other words, we expect d to be inversely proportional to the stiffness of ectodermal and endodermal tissues, respectively. Normalizing the raw mouth opening area and corresponding time by our fit parameters allows us to collapse the individual curves for both ectoderm and endoderm (Fig 4.4 D-E). Fitting these collapsed curves, we find that the endoderm and ectoderm have d values of 1.034 and 0.900, respectively. Thus, our model predicts an approximately 1:1.15 ratio relationship between the stiffness of endoderm and ectoderm tissue, respectively.

It is possible to experimentally completely separate the two *Hydra* epithelial tissues (Supplemental Figure 5 (A-F)), allowing us to independently measure their elastic properties using parallel plate compression (Supplemental Figure 5 G-K and Ref. [59]). Of note, both ectoderm and endoderm tissue remains fully viable after separation, as demonstrated by complete *Hydra* regeneration following recombination of separated tissues (Supplemental Figure 6). From the compression experiments, we estimated the elastic moduli of ectodermal and endodermal tissue to be $4,940 \text{ Pa} \pm 563 \text{ Pa}$ (mean $\pm$ SEM) and $4,390 \pm 228 \text{ Pa}$, respectively (Supplemental Figure 5 (L-M)). This translates to a ectoderm:endoderm elastic moduli ratio of approximately 1:1.13. Considering the standard errors associated with these measurements [59, 80], this is well within the range of the predicted 1:1.15 ratio, confirming the prediction of our simple model.

In summary, we found that a simple phenomenological model is able to accurately describe the opening rate of the *Hydra* mouth and predict biologically relevant

parameters, including the ectodermal threshold radius at which the endoderm begins to open and the ratio of ectoderm and endoderm elastic moduli.

This phenomenological model, however, provides little insight about the nature of the forces acting on the mouth during opening. Thus, in the remainder of this paper, we will briefly discuss three additional components that are important for an understanding of *Hydra* mouth opening: (1) how the initial tension of the system, and thereby the maximum opening area, is controlled, (2) the biological origin of the exponential expansion of the mouth through the first 30 seconds, and (3) the order of magnitude of the forces exerted by *Hydra* muscles required to pull the mouth open.

4.4.4 What are the forces governing mouth opening?

1. Initiation of rupture and control of maximum opening size Previous studies have shown that *Hydra* lacking a nervous system are unable to open their mouths, either spontaneously or as a feeding response [12]. The necessity of the nervous system in stimulating mouth opening suggests that mouth opening is an active process whereby neuronal signals control coordinated muscles contractions. Given the orientation of the muscles in the hypostome, it is reasonable to assume that contraction of the ectodermal radial muscles provide an opening force, while contraction of the endodermal circular muscles provide a closing force for the mouth. When the mouth is fully closed, septate junctions connect cells in both epithelial sheets. Thus, for mouth opening to occur, the opening force exerted by the radial muscles must overcome the forces holding both ectoderm and endoderm closed. In the ectoderm, the sealing force is that of the septate junctions. In the endoderm, septate junctions also hold the cells together, but the circular muscles exert the primary closing force. Therefore, rupture occurs only when the opening force from the radial muscles exceeds both the sum of the forces required to break the septate junctions and the closing force exerted by the circular muscles. In cases of sequential mouth opening where the mouth does not completely close (Supplementary Figure 2), we can neglect the septate junctions.

Prior to rupture, the hypostome can be thought of as a pre-stressed elastic sheet. That is, before rupture, the tension in the hypostome increases until rupture

occurs. Thus, the initial tension in the system is set by the radial muscles and the amount of tension that can be built up in the system is controlled by the septate junctions and circular muscles. As the force can vary stochastically between individuals and even over time within the same animal, the initial tension in the system for any given opening is also set stochastically. This leads to the observed variability in maximum opening sizes both between different *Hydra* (Fig 4.2 A) and between different openings within the same *Hydra* (Supplementary Figure 2).

2. Biological origins of exponential growth through the first half of opening

Prior to rupture, no mouth opening occurs and therefore there is no net force acting on the system. At the point of rupture, mouth opening begins and the net force is equal to the difference between the forces exerted by the radial and circular muscles. As the hypostome is a viscoelastic system, the net force is directly related to the velocity of opening. Thus, an increase in the velocity of opening as observed in our experimental data in the first 30 seconds implies an increase in net force acting on mouth opening. Such an increase in net force is achieved if the difference between the radial and circular muscles increases. This could for example be realized through different tension-length dependencies of the two muscles groups. At the inflection point, if the force of the circular muscles were to become negligible, then the net force of opening would be dependent only on the force from the radial muscles. Then, as the force exerted by the radial muscles decreased through opening, so would the net force acting on opening. The passive recoil of the system as the radial force decreases would then explain the second half of the mouth opening curve.

3. Order of magnitude of forces acting during mouth opening Assuming that the force exerted by the circular muscles becomes negligible at the inflection point, then the force of the radial muscles at the inflection point is roughly equal to the elastic force required to compress the tissues to the point necessary to achieve the maximum opening, as supported by the proportionality of initial tension, as measured by the critical radius and the maximum opening area (Figure 4.4 D). Furthermore, as we know the elastic properties of viable *Hydra* epithelial tissue (Supplemental Figures 5 and 6), we can approximate the radial muscle force at the inflection point to reach

a desired maximum opening area. By definition, the elastic modulus (E), is equal to

$$E = \frac{\sigma}{\epsilon} \approx \frac{\frac{F_{El}}{A}}{\frac{\Delta l}{L}} \quad (4.4)$$

where σ represents the stress, ϵ represents the strain, F_{El} is the elastic force, A is the area, Δl is the change in radial length, and L is the original radial length. Rearranging Eq. 8, we get the relation:

$$F_{El} \approx EA \frac{\Delta l}{L} \quad (4.5)$$

Plugging in the average of the elastic moduli for ectoderm and endodermal tissue in *Hydra* (Supplemental Figure 5 L-M) and using the change in aspect ratio of single cells to estimate $\frac{\Delta l}{L}$ (Figure 4.3 L), we estimated the elastic force, and thereby the force of the radial muscles at the inflection point, to be approximately 1-2 nN. This estimate for the radial force is on the same order of magnitude as the forces required to separate two cells sealed by tight junctions [102], thus forces in the nN range are a reasonable estimate.

In summary, we found that (1) the initial tension in the system is defined by the force of the radial muscles at the beginning of opening, which equal to the maximum value between (a) F_{sj} and (b) $F_{circ}(t=0)$. Thus, we can explain differences in maximum opening sizes in terms of both stochastic (a) cellular variations in septate junction numbers or strength, and/or (b) variation in the strength of endodermal circular muscles. (2) The exponential increase in opening rate in the first 30 seconds of mouth opening can be related to an increasing net force acting on the mouth that arises from the difference in the rates at which radial and circular muscles lose their ability to exert force. (3) Finally, we estimated the forces exerted by the *Hydra* muscles on the hypostome throughout mouth opening to be on the order of several nanoNewtons.

4.5 Conclusions

To our knowledge, this work represents the first biomechanical study examining the dynamics of *Hydra* tissues *in vivo*. Taking advantage of transgenic *Hydra*

expressing fluorescent proteins in both epithelial layers, we quantified *Hydra* mouth opening kinetics and showed that opening is an active process, mediated by antagonistic radial and circular muscle groups, and characterized by a sigmoidal mouth area *vs.* time curve. Mouth opening was found to be accompanied by substantial changes in cellular morphology, but not cell rearrangement, signifying a purely elastic deformation. A simple phenomenological model was able to accurately capture the kinetics of mouth opening and correctly predicted both the threshold opening radius of the ectoderm required to initiate endoderm opening, and the ratio between ectodermal and endodermal elastic moduli, which were measured independently through tissue parallel plate compression experiments. Furthermore, by examining the forces governing *Hydra* mouth opening, we determined the biological basis for the shape of the opening curve and the maximum opening area, leading to an estimate of the forces generated by the *Hydra* muscles on the order of a few nN. Finally, this study illustrates that the structural simplicity and the availability of *in vivo* labeling techniques make *Hydra* a well-suited model for fundamental biomechanical processes on both the cellular and tissue level [56].

Chapter 4 has been submitted for publication of the material. Carter, Jason^{*}; Hyland, Callen^{*}; Steele, Robert E.; and Collins, Eva-Maria S. “A Physical Model for Mouth Opening in *Hydra*”. ^{*}These authors contributed equally to this work. The thesis author was one of the primary investigators and the primary author of this paper.

4.6 Supplementary

Supplemental Movie 1: Ectoderm mouth opening Single-channel movie showing spontaneous opening and closing of the ectodermal epithelial layer. As GFP expressed in the ectoderm is concentrated around the borders of the cell and in the nucleus, individual cells with their respective nuclei are easily identifiable. Movie is recorded at 2.2 fps and played at 10 fps. Scale bar is 200 μm .

Supplemental Movie 2: Endoderm mouth opening Single-channel movie showing spontaneous opening and closing of the endodermal epithelial layer. DsRed2, which forms a tetramer too large to pass through the nuclear pore, is not concentrated along the edge of individual cells, making individual cells and nuclei difficult to identify. Movie is recorded at 2.2 fps and played at 10 fps. Scale bar is 200 μm .

Supplemental Movie 3: Ectoderm and endoderm mouth opening Two-channel movie showing spontaneous opening and closing of both ectoderm (green) and endoderm (magenta). Ectoderm and endoderm are recorded by alternating between fluorescence channels at the fastest possible frame rate of 0.18 fps. Movie is played at 10 fps and the scale bar is 200 μm .

Supplemental Movie 4: Sideview movie of mouth opening Two-channel movie recorded from the side of the hypostome showing spontaneous opening and closing of both ectoderm (green) and endoderm (magenta). The endoderm can clearly be seen protruding through the ectoderm layer at the point of maximum opening. Movie is recorded at 0.18 fps and played at 10 fps. The scale bar is 100 μm .

Supplemental Movie 5: Feeding response Single-channel movie showing a reduced glutathione-induced opening of the ectodermal epithelial layer. Toward the end of this movie, the hypostome turns itself inside-out. Opening to this extent was observed only in a subset of reduced glutathione-induced opening. Movie is recorded at 2.2 fps and played at 10 fps. Scale bar is 200 μm .

Supplemental Movie 6: Bud mouth opening Single-channel movie showing spontaneous opening and closing of the ectodermal epithelial layer in a *Hydra* bud. Bud mouth opening dynamics are comparable to the opening dynamics of adult *Hydra* mouths, indicating that opening is independent of head size. Movie is recorded at 2.2 fps and played at 10 fps. Scale bar is 200 μm .

Supplemental Movie 7: Failed mouth opening Two-channel movie recorded from the side of the hypostome showing the spontaneous opening and closing of both ectoderm (green) and endoderm (magenta). As the ectoderm begins to open, the endoderm remains sealed and the mouth opening fails. Movie is recorded at 0.18 fps and played at 10 fps. The scale bar is 100 μm .

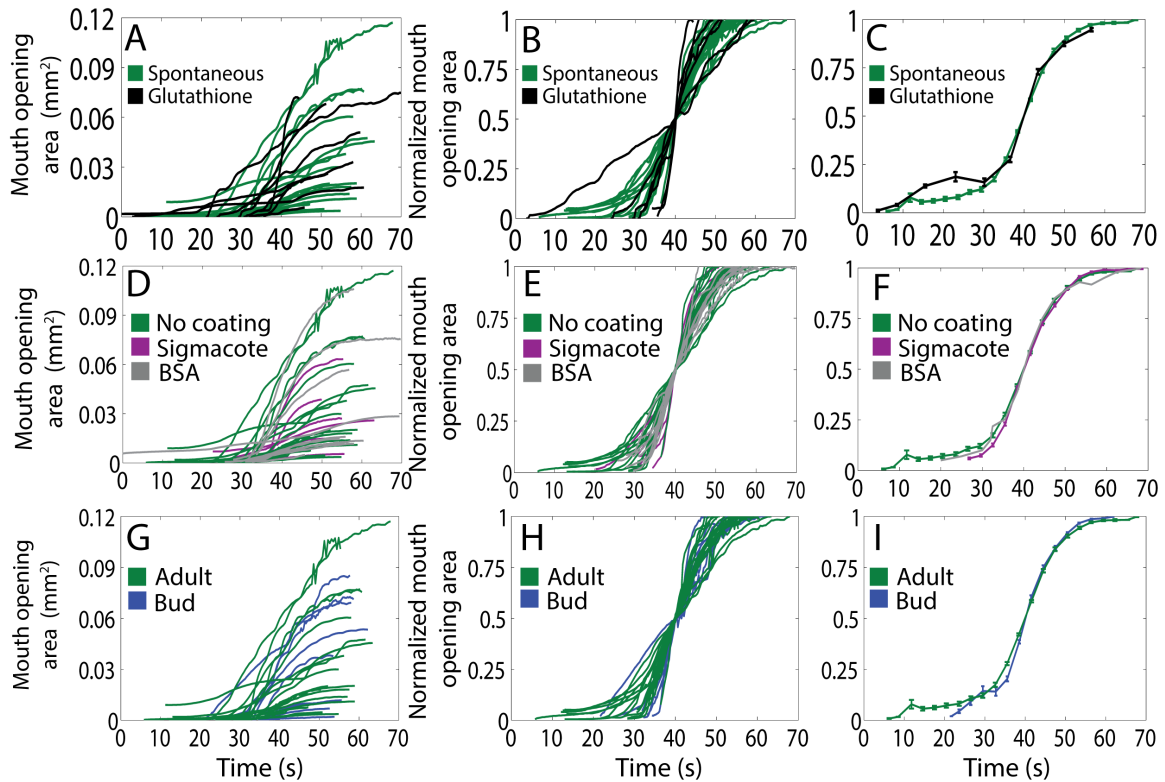


Figure 4.5: Supplemental Figure 1: Opening dynamics are conserved under different conditions. Ectoderm reduced glutathione-induced mouth openings ($N=8$) are comparable to spontaneous ectoderm mouth openings ($N=19$) in terms of (A) absolute size, and (B) and (C) opening rates. To ensure that friction between the hypostome and the cover slip during imaging did not change mouth opening dynamics, cover slips were coated with sigmacote ($N=9$) and BSA ($N=7$), respectively, and opening dynamics were compared to data using uncoated slides. No difference was observed between the data with respect to (D) absolute size or (E) and (F), opening rate. Opening dynamics are not influenced by differences of head size as indicated by the similarity in (G) absolute size and (H) and (I) opening rate between adult *Hydra* and buds ($N=10$).

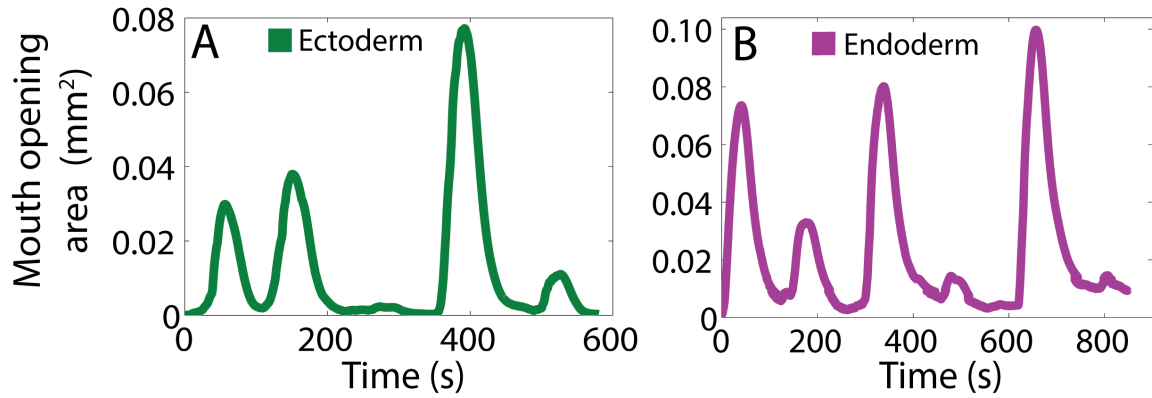


Figure 4.6: Supplemental Figure 2: Successive mouth openings in *Hydra*. Both the (A) ectoderm and (B) endoderm are capable of opening to varying sizes in succession without fully closing regardless of the previous opening size. Ectoderm and endoderm data are from two different animals.

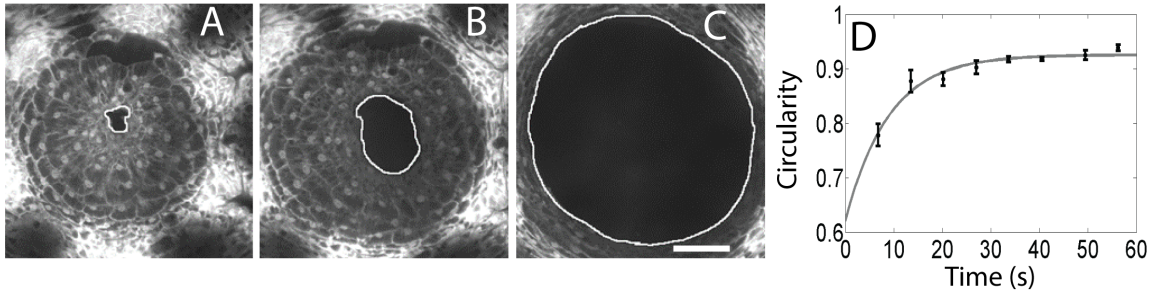


Figure 4.7: Supplemental Figure 3: Mouth circularity increases throughout opening. Representative images showing the outline of the mouth at (A) 0 seconds, (B) 30 and (C) 60 seconds of opening. (D) Circularity, defined as $4\pi \cdot \frac{\text{Area}}{\text{Perimeter}^2}$, was then calculated using ImageJ, binned and plotted as a function of time. A circularity value of 1 corresponds to a perfect circle, while lower values correspond to increasingly less circular shapes. The circularity *vs.* time curve was then fit according to $C(t) = 1 - 0.3 \cdot e^{(-0.11 \cdot t)} - 0.074$ with $R^2 = 0.96$, where $C(t)$ is circularity as a function of time and t is time since the beginning of the opening sequence. Interestingly, circularity increases through the first approximately 20 seconds of opening before plateauing around a circularity value of 0.92. This would indicate the beginning of opening is asymmetric, with some muscle process beginning to pull before others. After approximately 20 seconds, which is roughly the point at which the endoderm ruptures, circularity reaches its maximum value indicating that all muscles are pulling roughly equally at that point.

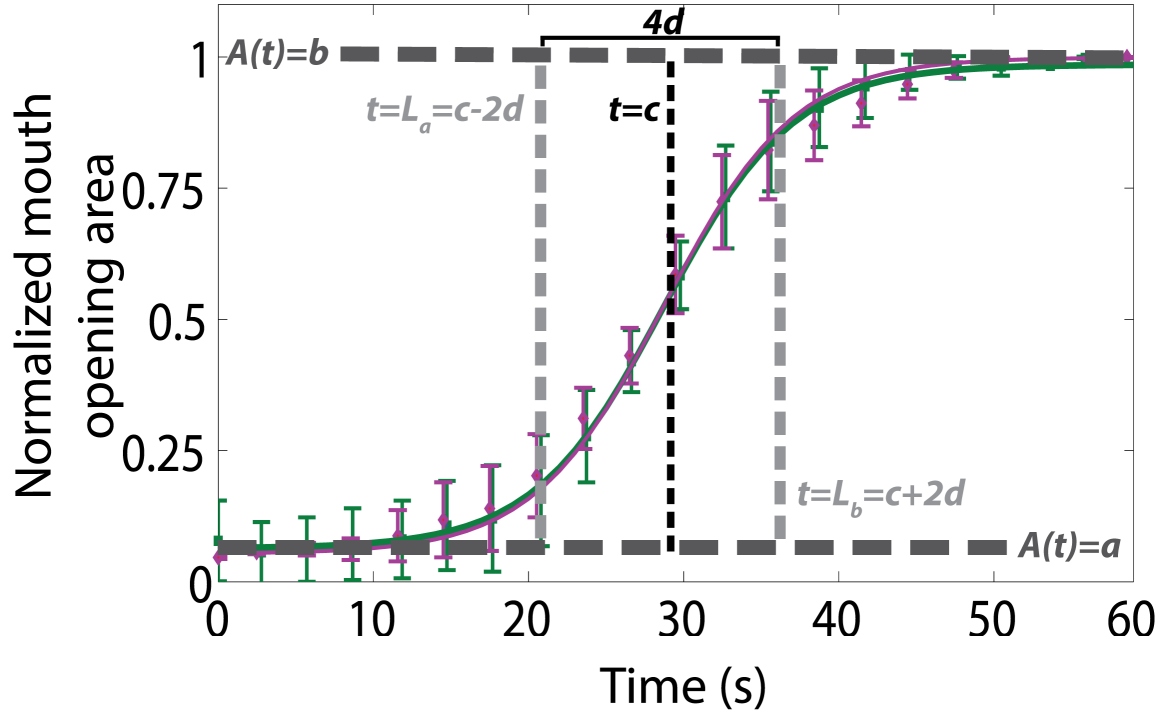


Figure 4.8: Supplemental Figure 4: Schematic showing effect of fit parameters. The normalized mouth opening curve fit according to Eq. 1. Parameters a and b set the values of the lower and upper asymptotes, respectively. Parameter c defines the time at which the inflection point occurs. Parameter d is related to the length of the region in which the majority of mouth opening occurs (between $L_a = c - 2d$ and $L_b = c + 2d$).

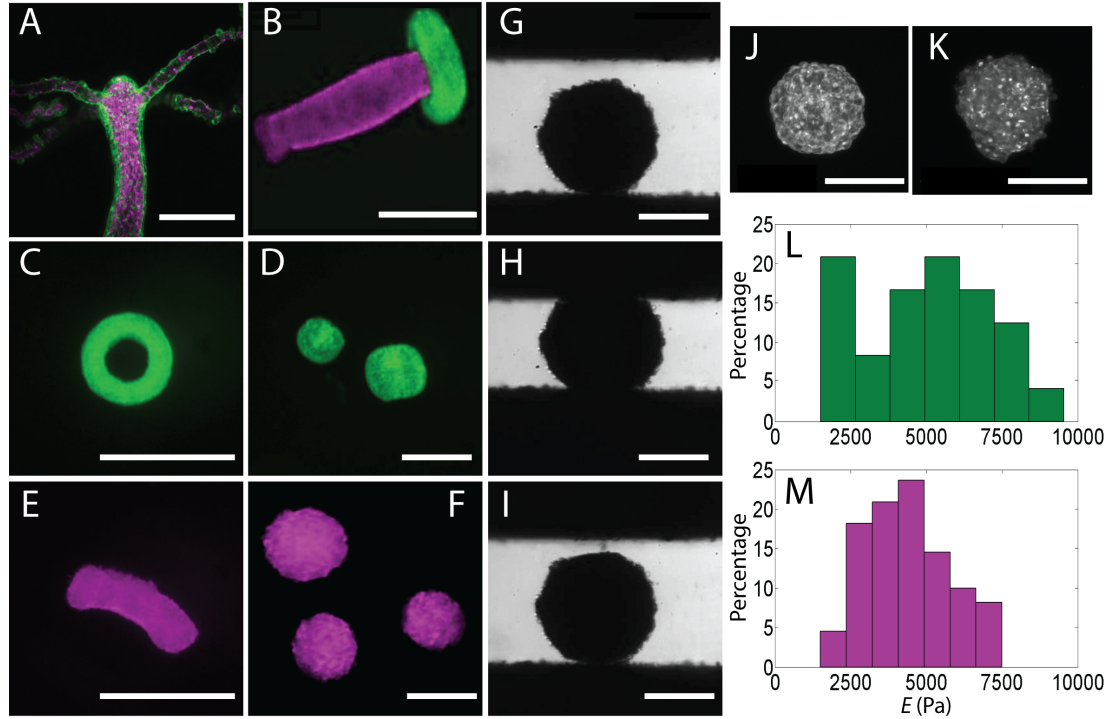


Figure 4.9: Supplemental Figure 5: Measurement of tissue elastic properties. (A) Representative image of an adult transgenic *Hydra* expressing GFP in the ectoderm and DsRed2 in the endoderm. (B) Following treatment with a low pH solution to dissolve the mesoglea, contraction of radial muscles in the ectoderm leads to the “rolling-up” of the ectoderm independent of the endoderm. (C) The ectoderm ring is then isolated from the (E) endoderm rods. Isolated tissue samples are manually cut and allowed to round up into ectoderm (D) and endoderm (F) spheres. The tissue spheres were loaded into a parallel plate compression apparatus [59] for measurements of the elastic modulus. (G-J) Representative images of compression experiments taken immediately prior to compression (G), at the point of maximum compression (H), and 11 seconds following compression (I). Because both tissues are polarized in the animal, it was possible that they did not form solid tissue spheres but may possess internal folds and hollow pockets, which would change their mechanical properties. To ensure the homogeneity of ectoderm (J) and endoderm (K) spheres prior to compression experiments, aggregates were imaged on a confocal microscope and checked for imperfections. Aggregates with obvious structural inhomogeneities were not included in experiments. (M-L) Histogram showing elastic modulus results as calculated using a Hertzian model for (L) ectoderm and (M) endoderm. The calculated elastic modulus of ectoderm tissue ranged from 2,000 Pa to nearly 10,000 Pa with a mean of 4,943 Pa and standard error of 563 Pa. Endoderm tissue was found to be, on average, slightly softer than ectoderm tissue, with a mean of $4,390 \pm 228$ Pa and a distribution between approximately 2,000 Pa and 7,500 Pa. Comparatively, human epithelial cells have been previously reported to have an elastic modulus approximately equal to 5 kPa [104]. Scale bar is 500 μm in (A) and (B), 200 μm in (C) and (E), and 100 μm in (D), (F) and (G)-(K).

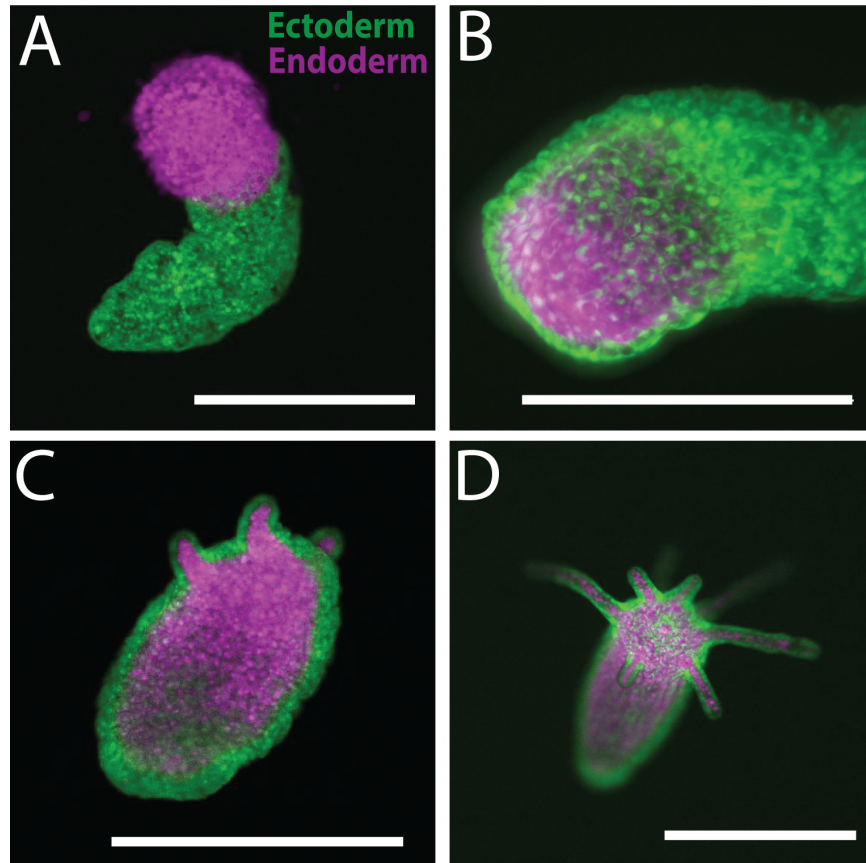


Figure 4.10: Supplemental Figure 6: Formation of single-tissue spheres does not effect cell viability. Spheres containing only ectodermal or endodermal tissue were created as done for TST measurements. One ectoderm and one endoderm sphere were then placed against each other and imaged after (A) 2 hours, (B) 18 hours, (C) 48 hours and (D) 66 hours. As the mixed spheres were able to regenerate a complete *Hydra*, the dissociation treatment used to obtain tissue samples for TST clearly does not effect the viability of the tissue. This result confirms that the elasticity values obtained from our TST measurements are reflective of healthy, adult tissue. Scale bars are 200 μm .

Chapter 5

Conclusions

In this thesis, we have used quantitative methods to provide insight into three behavioral questions in planarians and *Hydra*. We found that (1) *Schmidtea mediterranea*, *Dugesia japonica*, and *Dugesia tigrina* each display distinct reproductive strategies despite all reproducing through binary fission and that these strategies can be described according to models developed to describe asexual reproduction in unicellular organisms, (2) two *slo1* homologs are necessary for the behavioral response of planarians to acute ethanol exposure and (3) the *Hydra* mouth opening process can be described according to a simple phenomenological model.

In summary, we have demonstrated both the value of quantitative multidisciplinary approaches to fundamental questions in biology spanning several levels of complexity, from molecules to populations. Secondly, we have shown that planarians and *Hydra* are valuable model organisms for a wide range of biological questions beyond regeneration.

Chapter 6

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