REGENERATION IN THE PILIDIUM

by

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THESIS ABSTRACT

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Ability to regenerate is found in many groups of metazoans but the majority of research is focused on adults from just a few taxa, such as planarians and hydra (Agata and Inoue, 2012; Bely et al., 2014). Increasing the diversity of study organisms and life stages can reveal new and interesting aspects of regeneration mechanisms. This study focuses on regeneration of the nemertean pilidium larva. The planktotrophic pilidium of *Maculaura alaskensis* provides a unique model in which to observe several components of the regeneration process. Here I have documented a timeline for regeneration and have begun to evaluate the cells responsible for regenerative success. This study has revealed the interplay between regeneration and degeneration, a tradeoff between larval and juvenile structures, as well as the important relationship between global versus local signaling in proliferation and differentiation responses.

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CHAPTER I
INTRODUCTION

Regeneration spans a series of continua: in functionality, from physiological to reparative; in magnitude, from a single cell to an entire organism; and also in mechanism, in that the single term encompasses disparate processes. For example, the human body regenerates the entire lining of the small intestine every 5-7 days, and this physiological maintenance is driven by intestinal stem cells (Barker, 2013). In contrast, holothurians (sea cucumbers) can regenerate their gut after defensive evisceration by dedifferentiating myoepithelial cells of the mesothelium (Mashanov and Garcia-arraras, 2014). Imagine plotting regeneration on a three dimensional space using function, magnitude and mechanism as axes. Human intestinal epithelium regeneration is physiological, tissue level, and is mediated by endogenous $LRG5^+$ stem cells in the base of the intestinal crypt (Barker, 2013). Conversely, sea cucumber gut regeneration is reparative following a traumatic event, involves the regeneration of an entire organ, and is driven by dedifferentiation (Mashanov and Garcia-arraras, 2014). These two examples of gut regeneration would lie distant from one another in this three-dimensional space. Next we could add some of the many criteria that limit or enhance regeneration – for example, age (Porrello et al., 2011; Timchenko, 2009) and neuronal innervation (Pirotte et al., 2015). Quickly, the diagram becomes littered with distantly related events. Documenting the mechanisms of regeneration in a variety of systems will provide a better understanding of conserved and diversified mechanisms and potentially lead to the possibility of inducing regeneration in tissues otherwise incapable of it.

Injury for long-lived planktonic larvae is likely, and therefore tissue reorganization and regeneration is expected, but very few types of marine invertebrate larvae have been surveyed for their ability to regenerate (reviewed in Vickery et al., 2001). Studying regeneration in invertebrate larvae (which are small and sometimes semi-transparent) offers on opportunity to simultaneously track cell migration and proliferation while observing the regeneration of structures and restoration of function at the organismal level. Here I present the results of regeneration assays on the nemertean pilidium larva. The nemertean pilidium larva spends weeks to months in the plankton,

during which time the juvenile worm forms inside the larval body from a series of initially isolated rudiments. The fully grown juvenile erupts from the larval body in a catastrophic metamorphosis, and many juveniles consume their larval body. As the juvenile is formed, the larval body continues to grow aided by the putative stem cells. These putative stem cells could contribute to the maintenance and successful regeneration of the larval body, but until now the direct evidence was lacking. Here I document the capacity and timeline for regeneration after surgical removal of the larval apical organ or lappets, and identify the source population of cells utilized in regeneration of nemertean pilidium larva.

CHAPTER II
REGENERATION IN THE PILIDIUM

INTRODUCTION

Often regenerative biology is discussed in terms of regenerative medicine or the transplantation of stem cells to induce recovery in a lost or damaged structure. However, these transplantation and regeneration experiments offer unique insight into the flexibility of developmental pathways. Independent of the function (physiological or reparative), magnitude, or mechanism (stem cell mediated or not) regeneration permits the reactivation of developmental pathways. Regeneration and transplantation studies within invertebrates, including nemerteans, has shown the reactivation of developmental patterning and genes (Bierne, 1990; Loosli et al., 1996).

Nemerteans and the pilidium larva

Nemerteans, commonly referred to as ribbon worms, are characterized by soft unsegmented bodies and a long eversible proboscis housed in a special cavity — the rhynchocoel. They are predatory worms found primarily in marine environments. Recent evaluation reveals that there are approximately 1,300 described species of nemerteans (Kajihara et al., 2008; Zhang, 2013) and 113 nemertean species are found in Southern Oregon (Hiebert, 2016). This includes both described and undescribed species, as well as some only known in their larval form.

Nemerteans have been referred to as the champions of regeneration. For example, *Ramphogordius sanguineus* is capable of regenerating an entirely new individual from a small fragment of tissue only millimeters in size (Coe, 1929). While this is an astonishing display of regenerative ability, it does not describe regeneration across the phylum. Most other species where regeneration had been assessed are not capable of anterior regeneration, though posterior regeneration and proboscis regeneration are common (Gibson, 1972). Members of the phylum Nemertea are prime candidate for studying the evolution and restrictions of regeneration (Bely et al., 2014). Until the present study, nothing has been published on larval regeneration in this phylum.

The Pilidiophora, named for their unique hat-like pilidium larva, include the order Heteronemertea and the family Hubrechtidae, a total of about 450 species (Andrade et al., 2012; Andrade et al., 2014). Some of the characteristic features of this spiralian larva are the paired lappets, apical organ and a blind gut (Hiebert and Maslakova, 2015; Maslakova, 2010). Larval development in a typical pilidiophoran with planktotrophic development takes weeks months in the plankton, after which the juvenile emerges and consumes its larval body (e.g. Maslakova, 2010). It has been observed that even in a single culture, development is asynchronous, therefore rather than referring to absolute age of the larvae, it is more fitting to refer to key developmental events (such as the formation of different juvenile rudiments). The development of several species of pilidiophorans are now described from fertilization to metamorphosis, and the staging scheme proposed by Maslakova (2010) can be used to compare developmental stages across species.

Observations of regeneration in pilidium

Injury for a long-lived planktotrophic larva, like the pilidium, is likely and therefore tissue reorganization and growth in the form of regeneration can be expected. Preliminary observations by others suggest that regeneration of the larval body is possible, but varies between structures and possibly between

Figure 1. **Wild caught pilidium with injured left lappet.** (A) Wild caught pilidium from Charleston, Oregon, collected in November 2016. (B) Same larva from the plankton regenerated the injured lappet in ~2 weeks.

species (George von Dassow, Eduardo Zattara, personal communication). In November 2016 a damaged pilidium was collected from the plankton and allowed to regenerate in the lab (Figure 1). Within approximately two weeks the pilidium had regenerated its entire lappet confirming injury and subsequent regeneration can occur *in situ*.

Regeneration of two distinct structures

In this study I characterize the regeneration, or lack thereof, in two distinct larval structures – lateral lappets and apical organ. The lappets are paired feeding structures, characteristic of the pilidium larva. The lappets are spanned by the primary ciliary band (as well as inner ciliary bands). The lappets play a critical role in larval function and are used in both swimming and feeding (von Dassow et al., 2013). The primary ciliary band is composed of several rows of multi-ciliated epidermal cells, interspersed with monociliated cells with a single sensory cilium surrounded by a microvillar collar. Cilia covering most of the rest of the pilidial epidermis are shorter and less dense than those in the primary (and other) ciliary bands (von Dassow et al., 2013). Lappets are flexible and contractile structures that contain a sophisticated muscular apparatus, including a major muscle strand underlying the primary ciliary band, constrictor bands at the lappet base, radial smooth and striated fibers, a neuronal network, and a major serotonergic nerve cord running along the lappet margin (Hindinger et al., 2013; Maslakova, 2010; von Dassow et al., 2013).

The apical organ is an anterior cup-like epidermal structure that consists of columnar ciliated cells from which originates a thick tuft of long non-motile cilia; it may act as a rudder or have a sensory function (Cantell et al., 1982). Cells of the apical organ are clearly distinct from the squamous epithelial cells of the larval epidermis. A pair of serotonergic neurons are frequently associated with the apical organ (Maslakova, 2010). A thick apical muscle connects the apical organ to the esophagus which allows the larva to contract the apical organ into the episphere.

Aside from the structural and functional differences between these two larval structures, each is formed during different stages of pilidial development. The apical tuft is visible 27 hpf during gastrulation while the lappets are only beginning to form 72 hpf (Maslakova, 2010). The apical organ is derived from the apical most daughters of the first quartet micromeres while the lappets are derived from 1st, 2nd, and 3rd quartet micromeres (Henry and Martindale, 1998; von Dassow and Maslakova, in prep.). Both structures grow over the duration of the larval period, each supported by a population of putative stem cells (Bird et al., 2014).

The putative stem cells described by Bird et al. (2014) are clustered in several distinct regions, most notably in the anterior and posterior axils and around the periphery of the apical organ, and are responsible for the growth of larval body. Occasional proliferative cells are also found at the lappet end of the buccal ridges, near the esophagus-stomach sphincter, subepidermally within the primary ciliary band of the lappets, and behind the buccal ridges (Bird et al., 2014). In the case of both larval and adult organisms, regeneration activates several pathways otherwise reserved for development (e.g. Birnbaum and Alvarado, 2008). Therefore, it is likely that the putative stem cells in the pilidium could contribute to the successful regeneration of the larval body and rebuild structures formed earlier in development.

Removal of the lateral lappets maintains the source population of putative stem cells required for lappet growth. In contrast, removing the apical organ and immediately adjacent tissue simultaneously removes the apical organ and its source population of putative stem cells responsible for its growth. Thus, one might predict different potential for regeneration of these structures (Bird et al. 2014).

The characterization of regeneration in these two distinct structures provides an opportunity to study variation in regenerative processes and adaptation of developmental programming. The two primary aims of this study are to document the previously undescribed regeneration in the nemertean pilidium and to attempt to identify the cell populations that contribute to successful regeneration.

METHODS

Collecting adults and culturing larvae

Maculaura alaskensis inhabits intertidal mudflats from Washington to Northern California and is commonly found in Charleston, OR (Hiebert and Maslakova, 2015b). Like other members of the Pilidiophora, *M. alaskensis* has a pilidium larva. The larval development of this species from fertilization to metamorphosis is described in detail by Maslakova (2010), and preliminary observations (G. von Dassow, personal communication) suggest that larvae of this species are capable of regenerating. Adults were collected from the intertidal mudflats in Charleston, OR from March-September 2016 and March-June 2017. The gametes of ripe individuals are visible through the body

wall. The extracted oocytes were fertilized by sperm suspended in filtered sea water (FSW). Larvae were cultured in FSW at a concentration of ~1 larva/mL at ambient sea temperature (10-12°C) and fed *Rhodomonas lens* as described by Maslakova (2010).

Microsurgery

Larvae were selected at the cerebral-organ-disc stage (~2-3 weeks post fertilization) for microdissection. At the cerebral-organ-disc stage, the larval body has reached its maximum size (Svetlana Maslakova, personal communication) and the larva has begun to make significant investments in its developing juvenile (Maslakova, 2010). Each larva was photographed live before and after dissection. Larvae were individually cut using a glass microdissection needle (P-97 Micropipette Puller, Shutter Instrument Company, 1.0 mm OD x 0.5 mm ID glass capillary; Heat:480, Pull:500, Velocity:30, Time:100) - removing either the entire apical organ and immediately adjacent portion of the dome or the majority of a lappet.

The larvae were cultured individually (or in small groups separated by type of surgery) in a maximum concentration of ~1 larvae/2 mL at ambient sea table temperature. Larvae cultured in small volumes (24-well plates) are susceptible to infection, so smaller cultures received antibiotics (20 μg/mL streptomycin and/or ampicillin). The water was changed every 2-3 days, the larvae were fed *Rhodomonas lens* (Hiebert and Maslakova, 2015a; Maslakova, 2010).

Serotonergic neurons and muscle labeling

Regeneration of the lappet and apical organ requires both the restoration of structure and function. In order to visualize to what extent muscle and serotonergic nervous system were restored after surgery I performed fluorescent labeling and confocal microscopy by following the procedure described by Maslakova (2010).

Larvae were relaxed for 15 min (1:1 0.37M $MgCl_2$ in FSW) before fixation (4% paraformaldehyde in FSW for 30 min). Preserved larvae were rinsed in several changes of 1X Phosphate Buffered Saline (PBS, pH 7.4). Larvae were then permeabilized in three 10 min washes of PBS with 0.1% Triton X-100 (PBT).

Permeabilized larvae were incubated in 5% Normal Goat Serum (in PBT with 0.1% BSA) (Jackson Immunoresearch) for 2 hours at room temperature to block non-specific binding. Larvae were then washed in several changes of PBT/BSA followed by incubation in rabbit-anti-5HT primary antibody (diluted 1:500 in PBT/BSA, ImmunoStar Cat. # 20080) for 2h at room temperature or overnight at 4ºC. Larvae were then washed again in three 10 min changes of PBT/BSA followed by a 2h incubation at room temperature in secondary antibody Alexa Fluor 488 goat-anti-rabbit (Molecular Probes) diluted 1:600 in PBT/BSA. In the final 30 min of incubation, 1 U of Bodipy FL Phallacidin per 100 µL of PBT/BSA, and nuclear dye Hoechst 33342 (1 µM) were added to stain the f-actin and nuclei. Stained larvae were washed again in three 10 min changes of PBS and stored for imaging. Fixed and labeled larvae were imaged using an Olympus Fluoview FV-1000 laser scanning confocal microscope (optics described below).

BrdU assay and visualization

5-bromo-2'-deoxyuridine (BrdU) is a synthetic nucleotide (thymidine analogue) that is inserted into DNA during replication or repair. In these experiments BrdU is used to trace cell proliferation during growth and regeneration. Protocol for BrdU pulse/chase was adapted from Bird et al. (2014). The BrdU pulse and BrdU pulse/chase experiments all incorporate BrdU into proliferating cells immediately before treatment (Figure 2A). *M. alaskensis* pilidia were incubated in 0.05mg/ml BrdU (Sigma, St. Louis, MO, USA; B5002) in FSW for 24 hours. After 24 hours the larvae were washed in several changes of filtered sea water and divided into four treatment groups – Control Pulse, Regenerating Pulse, Control Chase, Regenerating Chase. Larvae in the Control Pulse group (controls are omitted from the diagram for clarity) were relaxed and fixed (as previously described) following removal from the BrdU. Larvae in the Regenerating Pulse group underwent microsurgery to remove a single lappet before relaxation and fixation. Together the Pulse treatments identify proliferative cells before regeneration and confirm their location immediately following microsurgery. Fixed "pulse larvae" were washed in several changes of PBS before visualization.
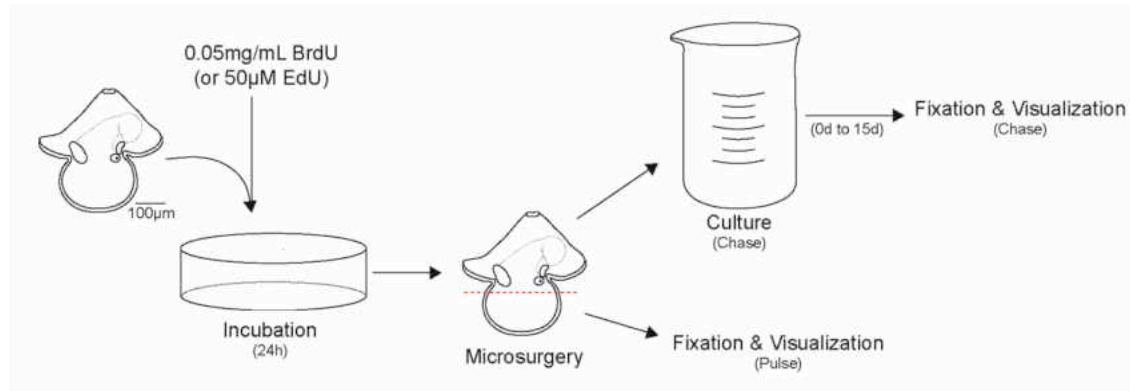
Figure 2. **BrdU Pulse-Chase Workflow**. BrdU Pulse and Pulse-Chase workflow for tracking cell proliferation in regenerating lappets. Larvae are initially incubated in 0.05 mg/mL BrdU (or 50μM EdU) for 24h to incorporate the thymine analogue into the actively proliferating cells. Larvae are then washed out of the BrdU and undergo microsurgery to remove a single lappet or the apical organ. Larvae for the pulse experiment are fixed immediately for visualization following the BrdU pulse (control) and after the microdissection (regenerating). Larvae for the pulse-chase are cultured for (0-15d) before fixation and visualization.

Larvae in the Control Chase treatment group were cultured at maximum concentration of ~1 larvae/2mL FSW (as previously described). Larvae in the Regeneration Chase treatment group were individually cut using a glass microdissection needle immediately following washes out of BrdU. The larvae were then cultured at a maximum concentration of ~1 larvae/2mL FSW as previously described. During the course of the chase, 8-12 larvae were removed for each time point and fixed for visualization from both the control and regenerating groups (24h, 48h, 4d, 6d, 12d and 15d post surgery) and washed in several changes of PBS.

Visualization followed the procedure described by Bird et al., (2014) and is the same for both the pulse and chase experiments. Following BrdU incorporation, an initial incubation in 1.0N HCl for 15-25 min denatures the DNA, later allowing the primary antibody to locate the thymidine analogue. Following incubation in the acid, the pH is neutralized with several changes of 0.1M $Na_2B_4O_7$ over 20 min. The larvae were permeabilized in %1 Triton X-100 in PBS (PBT, several changes over 30 min) in preparation for incubation with normal goat serum (NGS 5-10%, in PBT with 0.1% BSA for two hours) to block non-specific binding. The BrdU-tagged specimens were then incubated with mouse anti-BrdU monoclonal antibody (Becton Dickinson, Franklin Lakes, NJ, USA, diluted 1:100 in PBT/BSA) at 4ºC overnight, briefly washed in PBT/BSA (3x10 min), and incubated with Alexa Fluor 488 goat-anti-mouse antibody (A-21141, Invitrogen, 1:500 in PBT) for two hours at room temperature. In the final 30 min

of incubation, nuclear dye Hoechst 33342 (1 μM) was added to stain the nuclei. Larvae were then washed in several changes of PBS and stored for imaging. Fixed and labeled larvae were then photographed using an Olympus Fluoview FV-1000 laser scanning confocal microscope.

Serotonergic neurons and EdU chase

The goal of this experiment was to determine if serotonergic neurons present in the regenerated lappet differentiated from axillary putative stem cells. Unlike BrdU, EdU utilizes click chemistry to visualize proliferative cells and does not require denaturing of the DNA. The HCl-denaturation process has been shown to negatively affect additional antibody labeling.

Larvae were incubated in 10-50 μM EdU (5-ethyl-2'-deoxyuridine, Click-it EdU Kit, Invitrogen C10086) for 6h. After incubation the larvae were removed from the EdU, rinsed in FSW, and divided into two treatment groups: control and regenerating. The regenerating group underwent lappet microdissection (as previously described). Control and regenerating larvae were cultured separately as previously described. After 15 days (average time to lappet regeneration) the larvae were relaxed for 30 min in 0.34M $MgCl_2$ then fixed in 4% paraformaldehyde in filtered sea water (Electron Microscopy Science, Hatfield, PA, USA). The larvae were washed in several changes of 1X PBS.

The visualization procedure was adapted from EdU click-iT Kit (Invitrogen). The EdU visualization protocol is followed by the serotonergic neuron staining as described above. Hoechst 33342 nuclear dye (1 μM in PBT/BSA) was added in the final 30min of secondary antibody incubation. Stained larvae were washed again in three 10 min changes of PBS. Fixed and labeled larvae were imaged using an Olympus Fluoview FV-1000 laser scanning confocal microscope.

Microscopy

Live larvae were photographed on a Leica DFC 400 digital camera mounted on an Olympus BX51 microscope equipped with DIC.

Fluorescently labeled larvae were examined with an Olympus Fluoview 1000 laser scanning confocal (Olympus America, Center Valley, PA, USA) mounted on an

Olympus IX81 inverted microscope. Images were taken either with a UPlanSApo 20x (NA 0.75) or UPlanFL 40× oil (NA 1.3) lens and stacks of 0.75µm optical sections were collected.

Measurements

Measurements of several key structures in the larval body and the developing juvenile serve as metrics for regeneration progress. The three key measurements that provide insight are the ciliary band length, lappet surface area, and approximated cephalic disc volume. All measurements are extracted from images taken using a Leica DFC 400 digital camera mounted on an Olympus BX51 microscope equipped with DIC, then imported into ImageJ v. 1.51h (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) for image processing.

The ciliary band length is measured along the margin of the lappet and terminates at the connection to the anterior and posterior lobes. The lappet surface area is defined here as the area defined by the margin of the lappet and the transverse lappet muscle located at base of lappet in intact larvae. The volume of the cephalic imaginal disc is extrapolated from the maximum cross sectional area of the cephalic imaginal disc taken from the focal plane of the lappet using the equation $A^{\frac{3}{2}} \propto V,$ where A is the cross sectional area and V is the extrapolated imaginal disc volume. The measurements and metric extrapolations represent general trends and should not be interpreted as absolute.

Image processing and analysis

Stacks from confocal microscopy were imported into ImageJ v. 1.51h (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) for image processing and false-coloring was applied in Photoshop CS6.

Proliferating cells were counted from the BrdU pulse/chase experiments using Imaris 8.4.1 image analysis software (Bitplane, Oxford Instruments). Images were analyzed using the Spot detection algorithm to identify and quantify BrdU$^+$ nuclei. BrdU$^+$ nuclei average 4.80µm in diameter and range in their brightness depending on the amount of BrdU. All images were initially smoothed using a 0.5µm Gaussian filter. The Spot detection algorithm was used to initially identify peaks of Alexa Fluor 488 with a

diameter of 3.60µm-7.2µm. The search region was localized to a single lappet, anterior axil, or posterior axil using X, Y, and Z Position filters. Each image was then checked manually to add BrdU^{+} nuclei or remove misidentifications. Counts were obtained for the entire lappet (including axils), anterior axil only, and posterior axil only.

Statistics

The pairwise comparison of regeneration success rates, z-test of two proportion with Bonferroni correction, and calculation of standard error were conducted using SPSS Statistics software.

RESULTS

Regeneration success

The present study evaluates regeneration of a lappet and the apical organ. A single lappet regenerates in ~ 2 weeks with a 100% success rate (Figure 3). In contrast, the apical organ regenerates in < 50% of instances of injury but over a shorter period of time (Figure 3).

One hundred and six pilidia were scored on their apical organ regeneration success. The total population had a 41% success rate which serves as a baseline to test for significant changes in regenerative ability under different conditions. Changing cut site reveals that increasing cut depth diminishes regeneration success to < 10% (p < 0.005), while making a
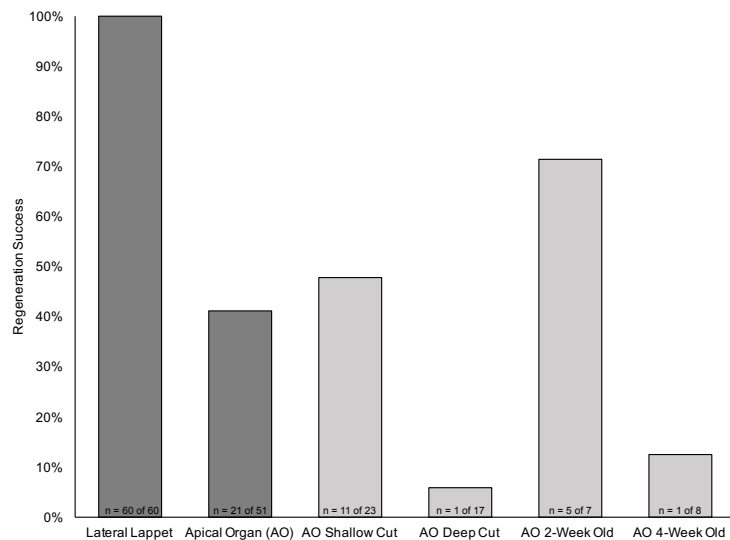
Figure 3. **Lappet and apical organ regeneration success**. Regeneration success rates for *Maculaura alaskensis* pilidium following removal of a single lappet or the apical organ and immediately adjacent epidermis. Dark gray columns show total regeneration success; light gray columns show variation in apical organ (AO) regenerative success under multiple treatments. * Increasing cut depth and increasing age significantly decrease regeneration success (p < 0.005). * 2-week old pilidia have a significantly increased rate of regeneration compared to all pilidia (p < 0.005). Significance based on z-test of two proportion with Bonferroni correction

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shallow cut does not significantly improve regenerative success over 41% (p > 0.05). Similarly, age at the time of apical organ removal plays a significant role in successful apical organ regeneration. A 2-week old pilidium is over 5x as likely to regenerate in comparison to a 4-week old pilidium (p < 0.005). The pairwise comparison (z-test of two proportion with Bonferroni correction) shows that increasing cut depth and age significantly and negatively influence regeneration success while younger pilidia are significantly more successful at regenerating.

Unsuccessful apical organ regeneration is further classified as wound healing. In the absence of an apical organ the pilidium continues to develop a juvenile and will eventually go through metamorphosis (data not shown). Until the present study apical organ regeneration was not observed in *Maculaura alaskensis*.

Lateral lappet regeneration

In order to determine the success rate and timeline for lappet regeneration I documented morphological changes in 40 pilidia following lappet removal. Additionally, I followed six cohorts of regenerating pilidia and fixed 8-12 larvae at each of the various time points for immunohistochemistry. Structural recovery of the lappet was measured as the size of the lappet and the reconnection of the primary ciliary band. The lappet achieves structural regeneration in an average of 2 weeks, when the regenerating lappet matches the size of the non-regenerating lappet.

The initial phase of lappet regeneration is the re-establishment of the primary ciliary band followed by growth of the lappet to return to its original size (Figure 4). The first phase is completed in as little as four days and the growth phase takes up the remainder of the two weeks. Before removal of the left lappet both larvae (Figure 4A and 4G) are at the cerebral-organ-disc stage. At this stage the larva is simultaneously investing in the maintenance of both the larval body and growth of the developing juvenile. Figure 4A-F depicts the key stages of regeneration following complete lappet removal. After complete lappet removal (Figure 4B) within 24h the larva has begun to heal the severed edges of the epithelium (Figure 4C). By 4 days post-microsurgery the ciliary band is reconnected and the lappet begins to grow in size (Figure 4D-4F). Lappet
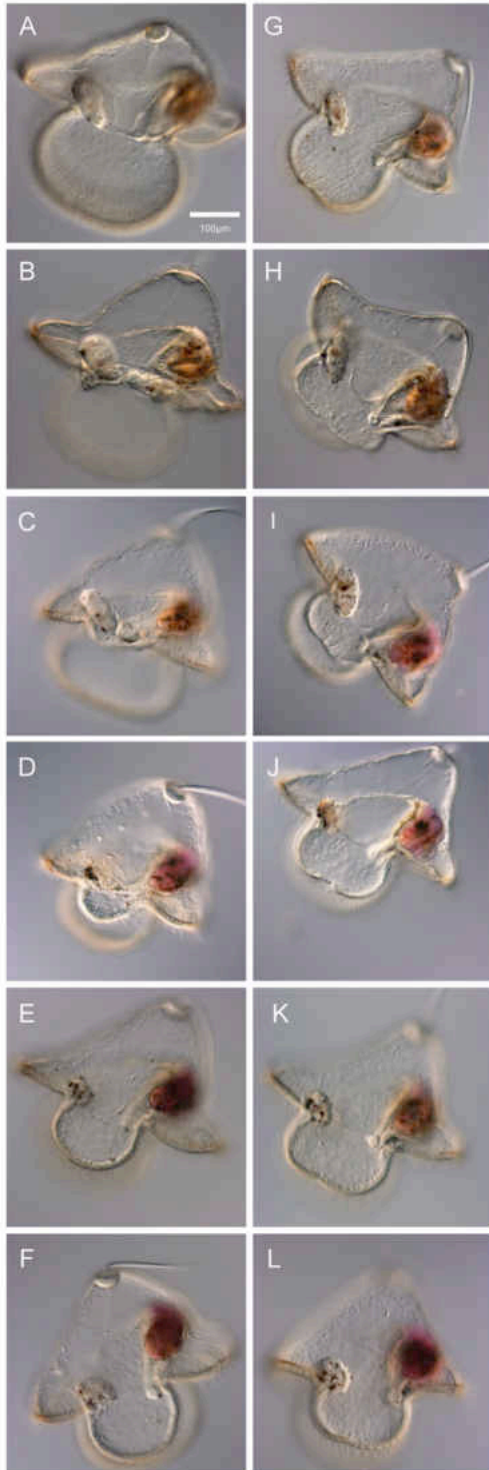
regeneration after removing approximately half of the lappet highlights the reconnection of the ciliary band (Figure 4G-4L). Here the first morphological sign of regeneration is still the reconnection of the ciliary band (Figure 4H-K) however, it occurs over a longer time period (complete connection achieved by day 8, Figure 4K). Here the severed ends of the ciliary band spread around the lappet and eventually reconnect (Figure 4I-K).

The restoration of function is determined by the reconnection of the marginal ciliary nerve (Lacalli and West, 1985), musculature, and the reappearance of the collar cells characteristic of the ciliary band (von Dassow et al., 2013). When the lappet is removed the marginal ciliary nerve is severed (Figure 5B and 5B') but restored as early as 24-48h post surgery (Figure 5C and 5C'). Co-labeling with EdU and anti-serotonin antibody shows that proliferative cell populations marked with EdU before lappet removal only contribute to a small number of serotonergic neurons in the regenerated lappet (Figure 5F). Figure 5F shows at least six serotonergic neurons (identified by the neuron cell body) but only

Figure 4. **Lappet Regeneration.** Lateral view DIC images of lappet regeneration in two individual pilidia over 10 days, (A-F) complete lappet removal (G-L) partial lappet removal. Images taken before (A and G) and immediately following the micro surgery (B and H). C and I are 24h post-surgery, D and J are 4d post surgery, E is 7 days and K is 8 days post-surgery, and F and L are 10d post-surgery. For complete lappet removal (A-F) the ciliary band is re-established early (C) and the majority of the regeneration time is spent re-growing the removed lappet (D-F). For the incomplete lappet removal (G-L) the larva spends 8 days attempting to reconnect its ciliary band from the two severed ends (I-K, day 7 not shown). In both larvae the developing cephalic imaginal disc on the side of surgery is reduced in size between days 3 and 4 of regeneration (D and J) but increases in size again by day 7 and 8 (E and K).

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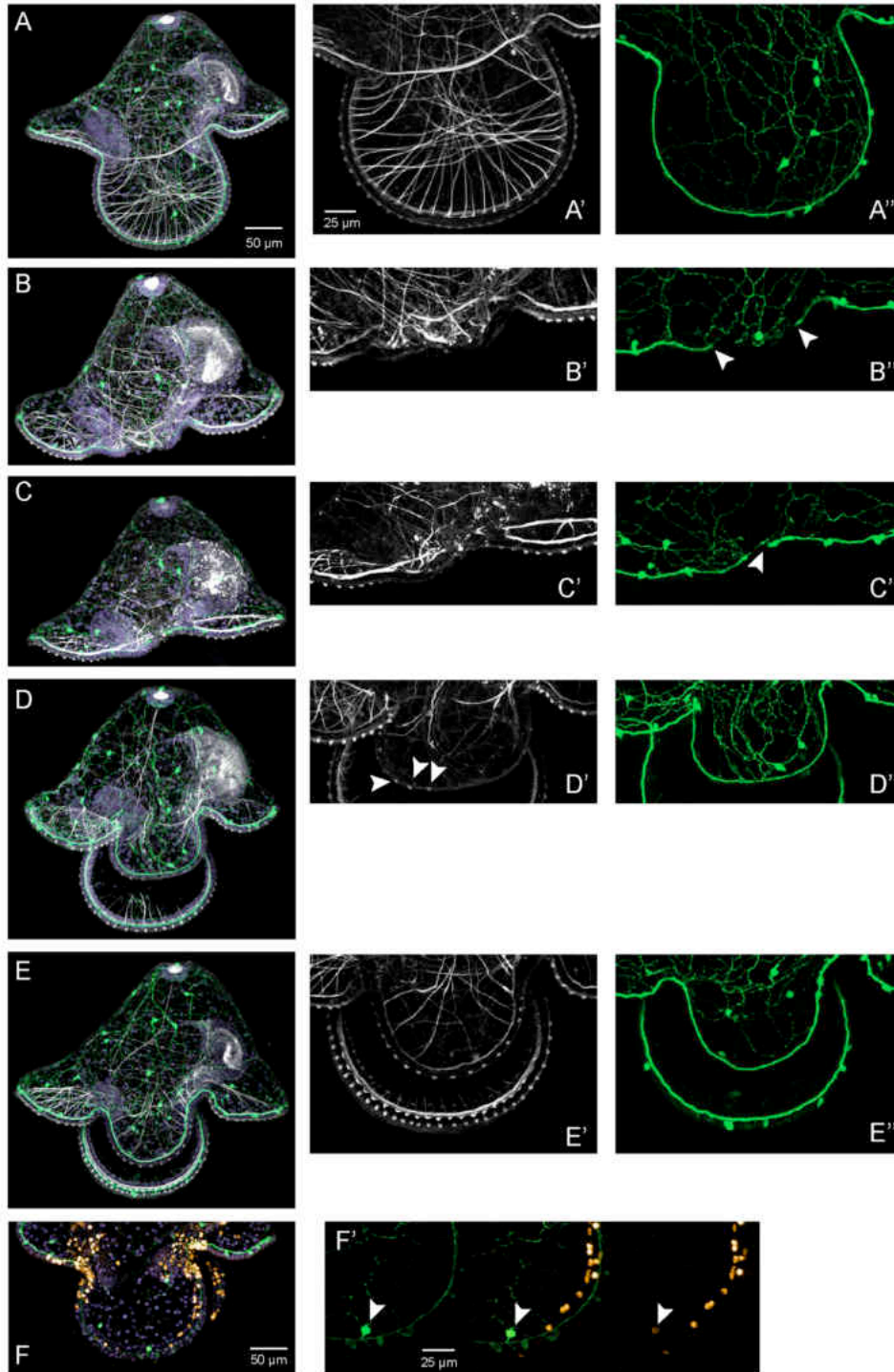
Figure 5. **Structural recovery of lappet.** Confocal images of pilidium larvae following lappet microsurgery. (A-D) Labeled with phalloidin (white), anti-serotonin antibody (green), and hoechst (purple). (A) Control larva with (A') uniformly spaced colar cells, latticed musculature extending from muscle along the margin of the lappet, and unbroken neuron (A'') connections running the length of the ciliary band. (B) Left lateral view of larva immediately following lappet microsurgery, (B') loss of microvilli collars of the ciliary band , (B'') disconnected serotonergic neurons. (C) 24h post-microsurgery, (C') two almost reconnected serotonergic neurons, (C'') no microvilli collars at the healing site. (D) 72h post-microsurgery, (D') reconnected marginal ciliary nerve, (D'') reappearance of microvilli collars of ciliary band. (E) 5d post microsurgery, (E'') reappearance of musculature in the lappet. (F) Regenerated lappet labeled with phalloidin (white), anti-serotonin (green), and hoechst (purple). (F') Separates channels for EdU (yellow), merge, and anti-serotonin antibody (green). White arrows highlight regenerated neuron derived from a proliferative cell before microsurgery that is EdU-positive.

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one of them is co-labeled with EdU. Therefore, although some serotonergic neurons are descendants of the proliferative cells initially labeled with EdU, others are not – they either migrate into the regenerating organ or descend from cells triggered to proliferate after injury. It is conceivable that the EdU signal has been diluted through successive rounds of cell division, but it would require many more divisions than there are cells to account for to eliminate detectable label. After the reconnection of the marginal ciliary nerve, the cells along the margin of the regenerating ciliary band begin to produce the microvilli characteristic of collar cells of the ciliary band (Figure 5D”). Still later, the characteristic musculature of the lappet (Figure 5A’) begins to reappear (Figure 5E’’). This data suggests that the reconnection of the marginal ciliary neuron could be prerequisite to differentiation of cells in the newly formed lappet or that axon regrowth occurs quicker that formation of a new epithelia.

Qualitative observations of regeneration are further supported by measuring the length of the ciliary band in the early stages of regeneration (Figure 6A) and the surface area of the lappet (Figure 6B) in five regenerating and five control larvae. The controls remain relatively constant for ciliary band length but show some variation in lappet surface area. The regenerating and not-regenerating measurements compare the two sides of a regenerating larva. At the start of the experiment when the lappet is initially removed, the not-regenerating lappet is not significantly different from the control in either its ciliary band length or lappet surface area. The regenerating lappet is removed which makes its ciliary band length and lappet surface area significantly different from the control and not-regenerating side. Over the 7-days the regenerating lappet increases its ciliary band length and lappet surface area with the most significant change occurring between Day 3 and 4 (Figure 6). The not-regenerating lappet decreases its size (both ciliary band length and lappet surface area).

The transition from Day 3-4 appears to be a critical point in lappet regeneration. Figure 6C shows the change in cephalic imaginal disc size which reaches its minimum at day 4 (regenerating) and day 5 (not-regenerating). Early in lappet regeneration (day 0-3) (Figure 4B-D and Figure 4H-J) the larva is reestablishing the ciliary band and is decreasing its imaginal disc size (Figure 6C). After day 3 (Figure 4D-F and Figure 4J-L) the lappet is increasing in surface area and the cephalic imaginal disc increases in size
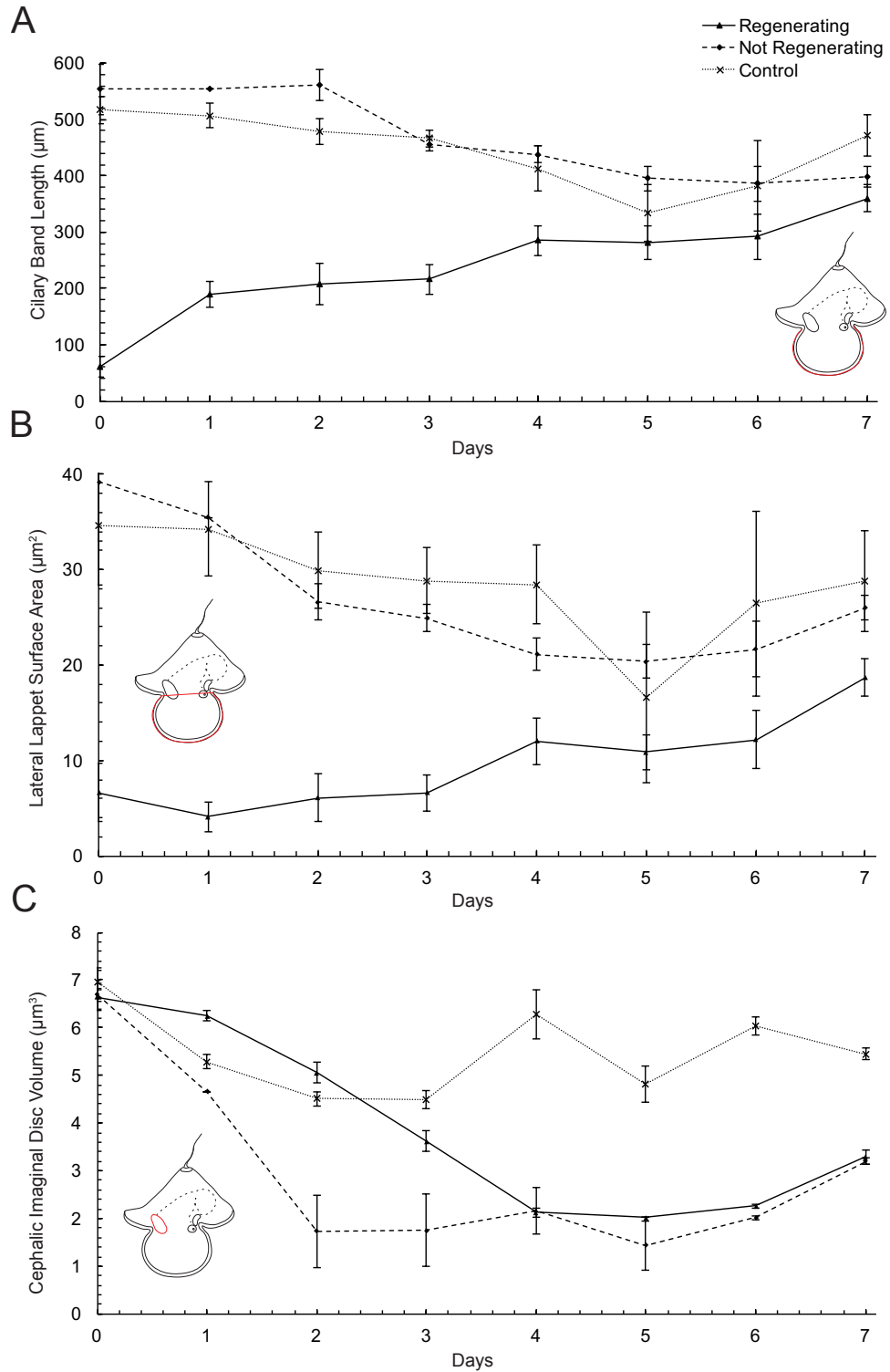
Figure 6. **Quantification of key structures in regeneration.** Measurements taken from lateral DIC images of 5 pilidia for each treatment to quantify regeneration progress in key structures taken from larvae that have not undergone microsurgery (Control), the regenerating side, and non regenerating side of larvae that have had their left lappet removed. (A) Linear measurement of the ciliary band taken from the anterior axil to the posterior axil running along the ciliary band. (B) Lappet surface area traces the ciliary band from anterior to posterior axil and crosses the larval body in line with a muscle running between the two axil regions. (C) Estimated cephalic imaginal disc area.

(Figure 6C). Figure 4D and 4J show that during lappet regeneration the cephalic imaginal discs (on both the side of the surgery and the "control side") decrease in size reestablishing the ciliary band and is decreasing its imaginal disc size (Figure 6C). After day 3 (Figure 4D-F and Figure 4J-L) the lappet is increasing in surface area and the cephalic imaginal disc increases in size (Figure 6C). Figure 4D and 4J show that during lappet regeneration the cephalic imaginal discs (on both the side of the surgery and the "control side") decrease in size and lose their original morphological structure. Cells appear to be released from the shrinking imaginal discs. It is unclear whether these are the mesenchymal cells that reside on outside of the imaginal disc facing the center of the larva or if they are epithelial imaginal disc cells that have escaped their junctions. The change of imaginal disc morphology is also characterized by the condensing of pigment from the amnion into one central region. The cephalic imaginal disc is almost lost by day 3-4 (Figure 4D and 4J) but begins to increase in size again by day 7 (Figure 4E and 4K).

Source cells for lappet regeneration

Light microscopy reveals the first observable change during regeneration of the lappet is a reestablishment of the intact ciliary band, closely followed by the gain of surface area to restore the size of the lappet. The ciliary band is extended from the cut sites and meets in the middle. This pattern of extension from the axil is expected based on the normal pilidial growth patterns described by Bird et al. (2014).

Proliferating cells originate from the anterior and posterior axils following removal of the right lateral lappet (Figure 7). The non-regenerating lappet (Figure 7B-E) serves as comparison to the regenerating lappet (Figure 7F-I). Figure 7B and 7F highlight the location of proliferative putative stem cells immediately following a 24h BrdU pulse and lappet microsurgery. After 24h (Figure 7G) proliferation increases on the regenerating lappet and there appear to be increasingly more BrdU-positive cells on the regenerating in comparison to the non-regenerating side. (Figure 7H and 7I). Progeny of cells from the initially labeled putative axil stem cell populations extend down the margin of the lappet and along the ciliary band of the anterior and posterior lobes of the non-regenerating lappet (Figure 7B-E). The regenerating lappet

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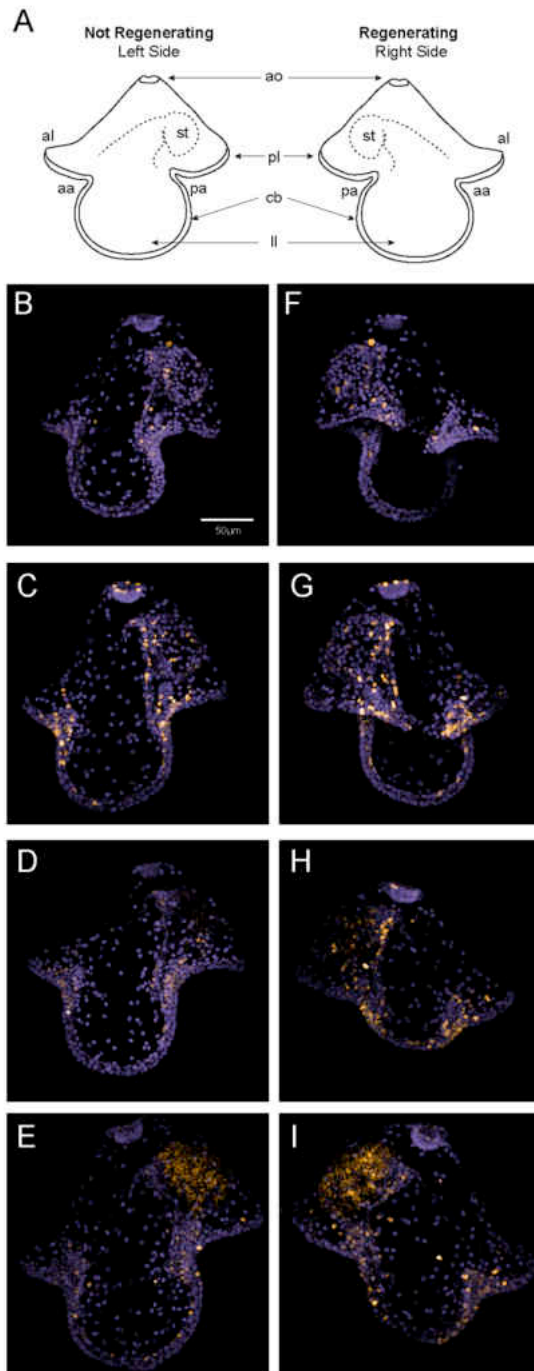
Figure 7. **Proliferation in response to lappet removal.** Comparison of proliferation as shown in yellow (BrdU) with a Hoechst background (purple). (A) Diagram compares the two views of an individual larva, from the left side (not regenerating) and right side (regenerating). This diagram shows the key larval structures – apical organ (ao), stomach (st), anterior lobe (al), posterior lobe (pl), ciliary band (cb), lateral lappets (ll) and the anterior (aa) and posterior (pa) axils. (B-E) Non-regenerating side of the pilidium at 0, 1, 2, and 4 days post-microsurgery. (F-I) Regenerating side of the pilidium at 0, 1, 2, and 4 days. In both the non regenerating and regenerating sides of the larva cells proliferate in both axil regions (yellow). Proliferation extends along the regenerating edge of the lappet from the axil regions (G-I).

follows similar proliferation patterns extending along what will become the ciliary band (Figure 7F-I).

BrdU-positive cells were counted following removal of the lappet. The number of nuclei containing BrdU over time represents both the putative stem cells originally labeled and their progeny. Proliferation from the axils is quantified by counting the number of nuclei immediately following a 24h BrdU pulse, and after a 1, 2, 4, and 6-day chase. The total proliferation represents proliferation from both the anterior and posterior axils and any additional proliferative cells in the lappet. The total rate of proliferation remains relatively constant in control larvae (Figure 8A). The regenerating and not regenerating sides of a regenerating larva show an increased rate of total proliferation over the control (Figure 8A). Unlike the control, where proliferation from the posterior axil is greater than the proliferation from the anterior axil (Figure 8B), proliferation in the regenerating larva is more evenly dispersed between the anterior and posterior axils (Figure 8B).
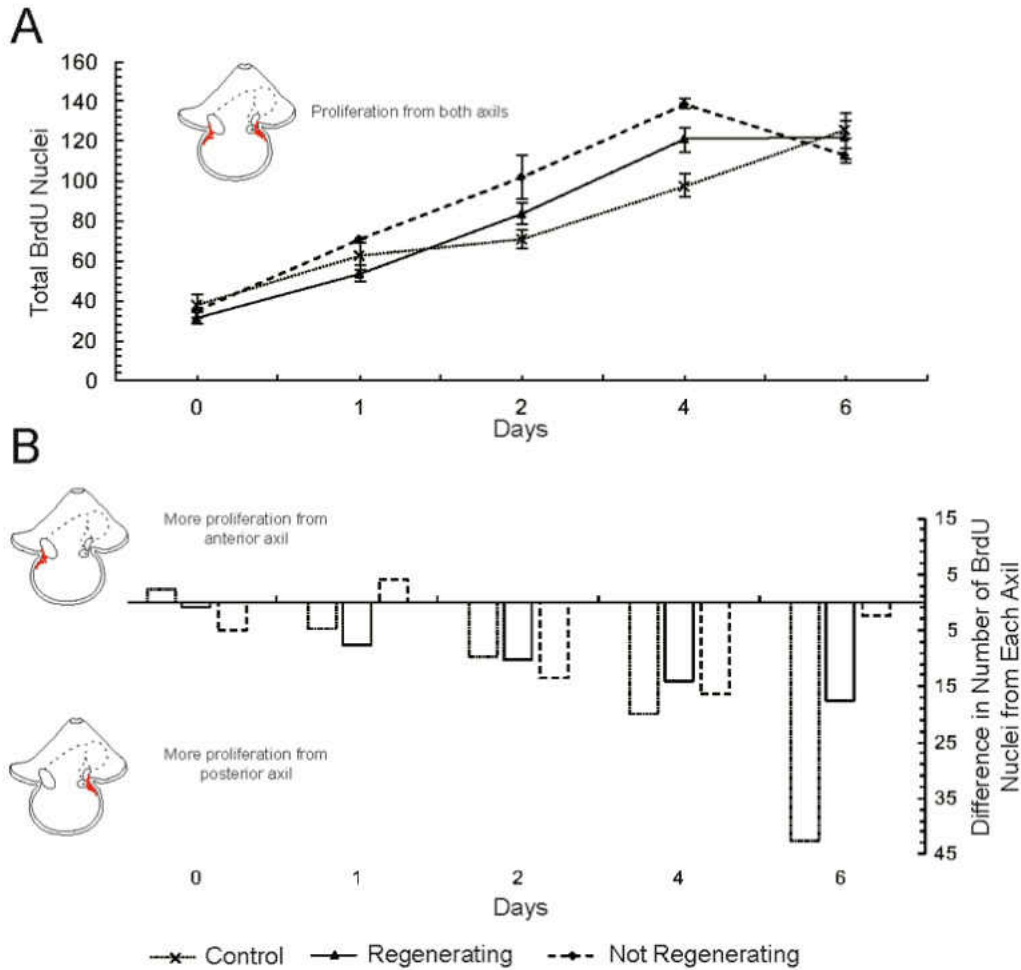
Figure 8. **Proliferation from the axil regions to the lappet in response to microsurgery.** BrdU$^+$ nuclei were counted using the Imaris Spot Detection algorithm at 0, 1, 2, 4, and 6 days post-microsurgery. (A) The total number of BrdU expressing nuclei in the axils and lappet for the control, and both the regenerating and non-regenerating lappets of larvae post-microsurgery. (B) Difference in number of BrdU nuclei between the anterior and posterior axils.

Apical organ regeneration

My results show that several factors predict the success of apical organ regeneration. Increasing the cut depth significantly decreases regeneration success (Figure 3), while making a shallow cut does not significantly improve regeneration. The more significant factor in regeneration success is the age of the pilidium at the time of microsurgery. Two-week old pilidia regenerate with 71% success, while in four-week old pilidia the regeneration success drops to 12% (Figure 3).

Apical organ regeneration occurs within 5-7 days (Figure 9) of apical organ removal though signs suggesting successful regeneration appear as early as 48-72h post-microsurgery (Figure 9B and 9C). These early signs include increased numbers of

migratory cells possessing extensive membrane and cytoskeletal protrusions and the thickening of a region of the healed dome. I observed increased number of mesenchymal cells in the larval episphere (Figure 9B and 9H) in response to apical organ removal. Successful regeneration is often accompanied by presence of a small patch of what appear to be mesenchymal cells on the interior of the healed episphere (Figure 9C).

While the muscle connecting the apical organ to the esophagus (Figure 9G) functions to pull the apical organ inward, it appears to be neither necessary nor sufficient for regeneration. Initially, the regenerated apical organ is connected to the larval body by a chain of mesenchymal cells (Figure 9F) which may be ultimately replaced by muscle or differentiate into muscle cells (Figure 9G) or not (Figure 9E). Some larvae that fail to regenerate are able to reestablish a muscle connection between the esophagus and the
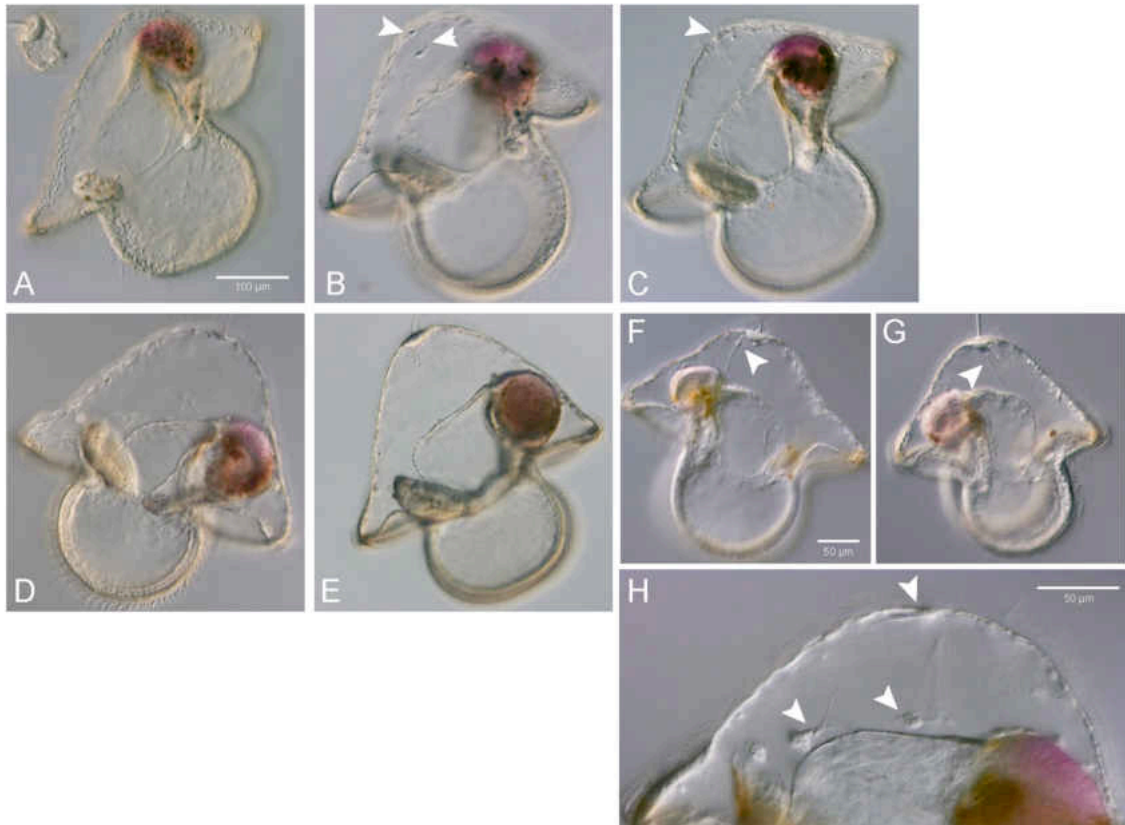
Figure 9. **Apical organ regeneration**. Lateral view DIC images of apical organ regeneration in an individual pilidium at 0d, 3d, 5d, 7d and 18d post-microsurgery (A-E). (A) Immediately following apical organ removal with apical organ superimposed to scale. (B) 3d increased numbers of migratory cells on the interior of the episphere. (C) early evidence of regenerating apical organ at 5d is a single sensory cilium and either the thickening of the epithelial cells or mesenchymal cells underneath. Regenerated early stage apical organ at 7-days (D) and 18-days (E) post-microsurgery. (F) 6d post-microsurgery the apical organ is re-established and a string of mesenchymal cells connect the new apical organ to the esophagus. (G) By 9-days post-microsurgery the mesenchymal connection is replaced by a muscular connection to the esophagus. (H) Example morphology of mesenchymal cells observed in the episphere 4-days after apical organ removal.

dome of the episphere; in this case the larva is able to contract the healed dome towards the esophagus as it would normally pull the apical organ in (data not shown). Perhaps even more surprising are the instances where the apical organ regenerates but the muscular connection is not re-established (Figure 10C'). Similar to the observation made during lappet regeneration, the imaginal discs often diminish during the course of apical organ regeneration (data not shown), but this is not the case for all individuals. Figure 9A-E shows apical organ regeneration without observed decrease in imaginal disc size, but rather growth and ultimately fusion of the imaginal discs (Figure 9E).

Figure 10. **Serotonergic nervous system and musculature in apical organ regeneration.** Confocal images of pilidium larvae stained with phalloidin (white), hoechst (purple) and anti-serotonin antibody (green). Larvae were cut at 2-weeks old and fixed after a 15 day regeneration period (A, A', A'') Control larva with a single serotonergic neuron near the apical organ. (B) Regenerated apical organ with reconnected muscle (B') and a single serotonergic neuron near the apical organ (B''). (C) Regenerated apical organ without reconnection of the muscle (C') and a single serotonergic neuron in the new apical organ (C''). (D) Apical organ regeneration with muscle reconnection (D') but without a serotonergic neuron in the new apical organ (D''). (E) No apical organ regeneration or muscle reconnection (E') but serotonergic neurons in the healed epidermis (E'').

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The apical organ of a pilidium contains at least one but often two serotonergic neurons and a web of dendrites surround the apical plate (Lacalli and West, 1985) (Figure 10A''). Figure 10 shows a gradient of regeneration success 17 days after apical organ microsurgery. Complete apical organ regeneration (Figure 10B) is characterized by the recovery of the apical tuft, muscle connecting to the esophagus (Figure 10B'), apical cup, and its associated serotonergic neurons (Figure 10B''). Intermediate regeneration success shows a deficit in muscle reconnection (Figure 10C') and a lack of associated serotonergic neurons (Figure 10D''). Complete lack of apical organ regeneration (Figure 10E) means the apical cup, tuft and associated apical muscle fail to regenerate (Figure 10E'), though serotonergic neurons may be present in the healed epidermis (Figure 10E'').

Source cells for apical organ regeneration

Putative stem cells in the periphery of the apical cup (Figure 11A) contribute to the growth of the apical organ (Bird et al., 2014). An apical cut removes all proliferative cells from the apical region. Figure 11 shows the proliferative response 15 days after removal of the apical organ and immediately adjacent epidermal tissue. When the apical organ does not regenerate (Figure 11C), all the cells in the healed episphere lack BrdU. Similarly, the regenerated apical organ lacks significant BrdU signal (Figure 11B).
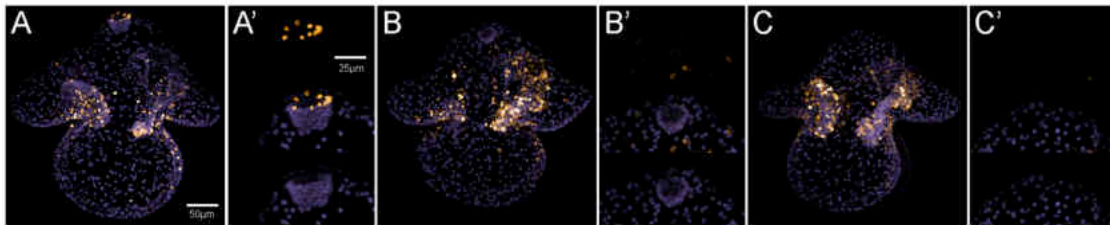
Figure 11. **Proliferation in response to apical organ removal.** Confocal images of pilidium larvae stained with hoechst (purple) and BrdU (yellow) following a 15-day chase. (A) Control larva with BrdU-labeled cells in the apical organ — a distinct population from the proliferative cells in other areas of the larval body. (A') separate and merged channels (top: hoechst, middle: merged, bottom: BrdU). (B) Apical organ regeneration (B') Some faint BrdU but most cells lack it completely. (C) No apical organ regeneration. (C') Complete lack of BrdU in any cells found in the wound site.

DISCUSSION

Comparing the regenerative capacity of two different structures provides insight into the developmental plasticity of the pilidium. The pilidium larva is capable of regenerating both the lappets and apical organ, but with varying success: the lappets

regenerate 100% of the time within 2 weeks, while the apical organ regenerates <50% of the time and to varying degrees of success but will regenerate completely in less than a week. The lappet regeneration is strongly supported by proliferative cells that contribute normally to the growth of the lappet. In contrast the apical organ is derived *de novo* from a still elusive source.

Lateral lappet regeneration

Together light microscopy and immunohistochemistry illustrate a sequence of events required for successful regeneration. Immediately following lappet microsurgery, the pilidium uses muscular larval epidermis or assembles a transient contractile ring around the cut edge and begins to heal over the cut edges at the wound site. Within 24 to 48 hours the marginal ciliary nerve, which runs the length of the circumoral ciliary band is reconnected. Once the serotonergic neurons have been reconnected, cells on the margin of the regenerating lappet produced the characteristic microvillar collars of the ciliary band. These collars are produced by collar cells on the margin of the ciliary band (von Dassow et al., 2013). The differentiation of cells in the ciliary band marks the transition priority to growth. This transition overlaps with two other important transitions – degeneration of the cephalic imaginal disc and proliferation from the anterior and posterior axils.

Regeneration is an energetically costly event. The bulk of the lappet is composed of large multi-ciliated cells and the ciliary band is composed of many densely packed cells (von Dassow et al., 2013). At the cerebral organ disc stage, the organism is investing in both its larval body and the developing imaginal discs, which will ultimately fuse to form the juvenile. Co-occurring with the differentiation of the ciliary band cells on the margin of the regenerating lappet is the disassembly of the cephalic imaginal disc and the peak of proliferation. At day 4 in regeneration the cephalic imaginal disc on the regenerating side of the larva has reached its smallest estimated volume and after this transition, both the growth rate (determined by the change in ciliary band length and lappet surface area) and proliferation of putative stem cells from the axils (quantity of BrdU$^+$ cells) slow.

Counting BrdU-positive cells in control larvae shows an increased proliferation form the posterior axil over the anterior axil during this stage of development. While this was echoed in both the regenerating and non-regenerating sides of a larva post-microsurgery the degree of difference is diminished. Overall proliferation is increased in both the non-regenerating and regenerating sides of a pilidium in comparison to the control, but the proliferation from the anterior axil is increased to more closely match the proliferation from the posterior axil. Change in relative proliferation rates during regeneration highlights a shift in developmental programming, while the similarity between regenerating and non-regenerating sides of the larva shows a lack of signal specificity. If the signal for increased proliferation were dramatically increased in the regenerating lappet over the non-regenerating lappet, then the signal could be considered specific. In the case of lappet regeneration in the pilidia, increase in proliferation is ubiquitous and therefore being received by cells on both sides of the larval body.

Several invertebrate larvae undergo dynamic growth of both their larval body and developing juvenile in response to food availability. For example, the feeding bryozoan cyphonautes larva will lose its juvenile rudiment when starved and regain it when a steady food supply is re-established (Strathmann et al., 2008). Also, *Dendraster excentricus* has been shown to differentially invest in the larval body or the juvenile rudiment depending on food availability (Strathmann et al., 1992). Under a high food regime a larva will invest in the developing juvenile but when food is scarce, the pluteus increases its ciliary band length by extending its arm length to increase food capture (Hart and Strathmann, 1994; Strathmann et al., 1992). Other planktotrophic larvae show developmental delays due to starvation, e.g. two species of polychaete larvae lose juvenile structures and metamorphic competence in response to food availability (Pawlik and Mense, 1994; Toonen et al., 2017). In these examples, the resorption of the developing juvenile or loss of metamorphic competency is explained by starvation. The pilidium also exhibits some phenotypic plasticity in response to food availability. Starved pilidia will delay the development of their imaginal discs or drive established imaginal discs to shrink (data not shown). During regeneration oft he lappet and degeneration of the imaginal discs the larva maintains the ability to feed. In the case of the apparent decrease in size and loss of original morphological structure with subsequent

re-growth of the cephalic imaginal discs, it is possible that the energetic demands of lappet regeneration require additional resources or that the signal prompting increased axillary proliferation is the nutritional deficit resulting from loss of a lappet. The lost cells of the imaginal discs could be cannibalized for resources or their removal could allows reallocation of resources they would otherwise use up. The degeneration of the imaginal discs could be attributed to the activity of the mesenchymal cells resorbing cells of the imaginal disc or cells of the imaginal disc undergoing epithelial to mesenchymal transition, leaving the imaginal discs. In the latter case, it may be possible, that some of the cells formerly comprising the disc, participate in rebuilding the new structure (i.e. the lateral lappet). Finally, it is possible that cells of the imaginal disc are undergoing programmed cell death in response to some global signal related to the injury. It is clear however, that after the imaginal discs reach minimum size at day 3 or 4 of regeneration, the cephalic imaginal discs ultimately increase in size and continue to develop.

The putative stem cells in the anterior and posterior axils of the pilidium, labeled in this study with BrdU or EdU, have been previously shown to contribute to the growth of the larval body and the imaginal discs (Bird et al., 2014). This study shows that these proliferative cells differentiate into serotonergic neurons during lappet regeneration. In addition to a few serotonergic neurons in the regenerated lateral lappet that contain the EdU – that is, descendants of proliferative cells labeled before microsurgery – there are several neurons that lack it. The lack of EdU in the nuclei of neurons in the regenerated lappet could mean the label was diluted over successive rounds of replication, or that those neurons were not derived from the putative stem cell population. Cell migration occurs during regeneration (Zattara et al., 2016) and neuron progenitors and 'young neurons' have been shown to migrate over long distances (1-2 mm) before differentiating (Lois and Alvarez-buylla, 2016). It is possible that these differentiated neurons migrated or extended from an existing cell body in another region in the larval body, or that they were derived from quiescent neural progenitors. As the larval body increases in size the number of serotonergic neurons increases (data not shown). When a lappet is removed some of the serotonergic neurons are lost but after two weeks, regenerating larvae have the same number of serotonergic neurons as non-regenerating larvae of the same age (data not shown). Regardless of the origin of the second population of serotonergic

neurons, the presence of two types (EdU$^+$ and EdU$^-$) suggests multiple mechanisms involved in lappet regeneration.

Variation in apical organ regeneration success

In contrast to the extension of an existing structure with retained putative stem cell support in lappet regeneration, removal of the apical organ and immediately adjacent epithelial tissue removes the putative stem cells that reside in and are responsible for the growth of the apical organ.

The apical organ is conserved across various groups of invertebrate larvae, including cnidarians, annelids, molluscs, flatworms and nemerteans (Marlow et al., 2014). For many phyla, the sensory cells associated with the apical organ are involved in settlement (Conzelmann et al., 2013; Hadfield et al., 2000; Rentzsch et al., 2008). Nemerteans undergo catastrophic metamorphosis consuming their larval body (including the apical organ) and it is unclear whether the apical organ plays a direct role in metamorphosis as it does in other invertebrate larvae. Preliminary observations show that a larva that has undergone microsurgery to remove the apical organ continues to develop through to metamorphosis in its absence (data not shown). In fact, apical organ removal appears to initiate metamorphosis in late stage pilidia (data not shown). Still slightly less than half of larvae that have their apical organ removed will regenerate it. As previously stated, the function of the apical organ in nemerteans remains unknown but its function must provide enough support to the larval life phase that it is worth regenerating.

The precise conditions under which the pilidium is able to regenerate its apical organ remain elusive. The overall low rate and high variability of apical organ regeneration suggests that it is not a simple extension of existing tissues, as in the lappet. The BrdU-chase experiment shows that the putative stem cells proliferating 24h prior to microsurgery may not be responsible for regeneration of the apical organ. It is possible that the signal has faded through subsequent rounds of division. Alternatively, it may be that the proliferative cells present before surgery are not involved in regeneration of the apical organ. Instead some population of differentiated (or undifferentiated but quiescent) cells become proliferative following injury and contribute to the apical organ.

Manipulating the starting conditions significantly changes the success of apical organ regeneration. Changing the cut depth from deep (close to the axil putative stem cells) to shallow (distant from axil putative stem cells) shows that proximity to a stem cell population alone does not increase the probability of regenerating. It is possible that the retained structure of the pilidial episphere (maintained by the shallow cut) is the key to regenerative success. Preliminary observations suggested that the retention of the muscle connecting the apical organ to the esophagus may contribute to the success of regeneration. For example, the maintained connection could provide a transportation route or sustain structural and organizational cues. However, further experiments demonstrated that the presence of the apical muscle is neither necessary nor sufficient for regeneration of the apical organ. It is also possible that the deep cut removes too many mesenchymal cells from a limited pool. The regenerating apical organ may compete for recruitment of the remaining mesenchymal cells with other structures, e.g. imaginal discs.

In addition to the depth of cut, pilidial age appears to be an important factor influencing regenerative outcome. By studying the association between age and regeneration it may be possibly to determine the underlying genetic or epigenetic transitions associated with loss of regenerative ability. It is well documented that regeneration occurs more readily in younger individuals across different phyla (Reviewed in Yun, 2015). In fact, mammals are able to regenerate digit tips up until puberty (Han et al., 2008) and neonatal mice can regenerate cardiomyocytes until post-embryonic day-7 (P7) stage of development (Porrello et al., 2011) while adults are not able to regenerate these structures. In the present study, a difference of two weeks altered regeneration success by almost 60%. The age of the pilidium could negatively contribute to its ability to reactivate the developmental pathways required for apical organ creation. During later stages of development, regeneration of the apical organ may no longer be a high priority. For example, if the apical organ is used as a rudder for young pilidia, then the fully developed lobes and lappets could steer the larva independent of the apical organ. As previously stated the apical organ is first observed 27 hpf and its growth is maintained by a distinct population of putative stem cells. When these putative stem cells are removed it is possible that the majority of remaining cells in the larval body do not maintain the potency to produce a new apical organ, and those that do have the ability, do not always

find their way to the right place. This study has shown that some cells in the larval body do retain that potency or are able to dedifferentiate to produce a new apical organ. For those cells the difficulty is then in arriving at the correct destination and receiving the appropriate signals to initiate regeneration.

Comparison of regeneration in two different larval structures

Regeneration across phyla is composed of two main components, first the recruitment of cells and second the re-patterning and growth of the new structure (Birnbaum and Alvarado, 2008). In pilidial lappet regeneration it is clear that the putative stem cells in the anterior and posterior axils are the source population for building the new tissue. The re-patterning takes place within the first week of regeneration – reconnection of the marginal ciliary neuron (24-48h post-microsurgery), differentiation of collar cells in the ciliary band (48-72h post-microsurgery), and the re-establishment to the musculature (after 3d). By day 4 the re-patterning has transitions into the growth and expansion of the lappet. The transition from day 3 to 4 is supported by proliferation rate and the decrease in imaginal disc size. The proliferation rate slows after day three when simultaneously the cephalic imaginal disc reaches its minimum size. The limiting factor in rate of lappet regeneration is the re-patterning and possibly energetics required for the growth of a new structure. In contrast the source population or the recruitment of cells to regenerate the apical organ is the limiting factor.

Proximity to the larval growth zones (axils) likely explains consistent regeneration of the lappets. Regeneration of the apical organ likely requires cell migration after injury, which may partially explain inconsistent regeneration. In apical organ regeneration there is an observed increase in migratory cells, though the specific origin of these mesenchymal cells is unclear. The limiting factor in apical organ regeneration appears to be the successful and reliable recruitment of these (or other) cells. In the event that the required cells are recruited (or possibly local cells are dedifferentiated) the re-patterning and growth of the apical organ can take place.

Developmental plasticity of the pilidium

The difference in regenerative abilities between these two structures hints of the limitations of developmental plasticity in the pilidium. Regeneration may be limited by the ability of cells (stem cells or local differentiated cells) to reactivate developmental programming. The putative stem cells of the axils actively contribute to the growth of the lappet, ciliary band, and the marginal ciliary neuron, which makes successful regeneration possible. Here the developmental programming is stretched only a little to extend an existing structure with a retained source of proliferative cells. In contrast, apical organ regeneration requires diversion from the developmental trajectory of the remaining cells. Apical organ regeneration shows increased developmental plasticity over lappet regeneration. The loss of apical organ source cells requires that the right conditions (whether that be molecular, temporal, or spatial cues) drive an otherwise non-contributing cell to change its developmental trajectory and produce a new apical organ.

In addition to the change of developmental trajectory required for regeneration of the larval lappet and apical organ this study highlights the dynamic growth of the developing juvenile. The juvenile worm develops from a series of imaginal discs that invaginate from the larval body. The strain of regeneration not only pauses growth of the imaginal discs but also a causes them to degenerate. When the initial stages of regeneration are complete the imaginal discs begin to increase in size and progress through development.

This study shows that pilidia are capable of modifying its developmental program to accommodate injury and strain. Regeneration of the lappet highlights the larva's ability to modify existing patterns to regrow a lost structure, while regeneration of the apical organ suggests the ability to reactivate developmental pathways for *de* novo regeneration. Furthermore, the dynamic growth and pauses in the development of the juvenile shows that this ability extends past programs specific to growth and maintenance of the larval body.

CHAPTER III
CONCLUSION

Here I have documented the previously undescribed regeneration of two different structures in the nemertean pilidium larva. Regeneration requires the recruitment of replacement cells and the re-patterning of the lost structure. The pilidium is able to regenerate several larval structures with varying degrees of success. The lappets utilize retained putative stem cells from the axils to support the regenerating structure. The re-attachment of the marginal ciliary nerve is the initial step in the re-pattering of the lappet. After the severed nerve had been reconnected, the cells of what will become the ciliary band differentiate. Still later in regeneration, the lappet regains it musculature. Lappet regeneration has a stable source population of cells and is able to quickly reconnect severed structures to initiate re-patterning.

Regeneration of a lappet is the extension of an existing structure, while regeneration of the apical organ requires independent development of a differentiated structure. Here, the source cells limit the success of apical organ regeneration. Apical organ microsurgery removes the putative stem cells responsible for apical organ growth. Successful regeneration requires migratory pluripotent cells to not only reach the appropriate destination but also receive the required signals to differentiate. Alternatively, local cells could dedifferentiate or transdifferentiate. Re-patterning for apical organ regeneration requires the ability to access and reactivate developmental programming initially observed within the earliest stages of development. Both the definitive source cells and re-patterning mechanisms of apical organ regeneration remain elusive.

Comparing the strategies employed by the pilidium for the regeneration of two distinct structures provides insight into not only the regenerative mechanism but also the overall developmental plasticity of the pilidium. Regeneration of the lappets shows flexibility of proliferative regulation. In response to injury, proliferation increases across all four sets of larval axils, not just on the regenerating side of the lappet. This pattern of ubiquitous increase in proliferation suggests a lack of specificity of signal or response, the entire larva is responding to the injury, not just the wound site. Conversely, low success observed in apical organ regeneration suggests that the ability to reactivate

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developmental patterning is limited. Are younger pilidia more successful at apical organ regeneration because they are closer to the initial differentiation of apical cells (1st quartet micromeres) than epithelial and mesenchymal cells of later stage pilidia? Is a particular cell type or signal required to initiate apical organ re-patterning? Regardless of the answer to these questions, it is clear that there are boundaries on developmental plasticity of the pilidium. In addition to the plasticity of the developmental programming in the larval body, the developing juvenile exhibits flexibility. During both lappet and apical organ regeneration the the imaginal discs decrease and subsequently increase in size. In lappet regeneration this correlates with proliferation rates and growth rates.

This work documents for the first time regeneration in the pilidium larva and offers some clues to the differences in regeneration success of various structures. Lappet regeneration utilizes physiological maintenance associated with lappet growth. The process is driven by putative stem cell proliferation and re-patterned by existing structures in response to injury. In contrast, the apical organ is unable to utilize the same pathways that maintain its cell population and growth overtime suggesting an alternative mechanism either supported by migratory stem cells or local de-differentiated cells. Documenting regeneration in the pilidia has identified the importance of developmental plasticity and has highlighted the relationship between regeneration and degeneration. Continued research into the specific cellular mechanisms and genetic modifications made during these events with further progress our understanding of limitations to regeneration.

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