ABSTRACT

Title of Document: BREAKING THE A-P AXIS: EVOLUTION OF DIVERSE ASEXUAL REPRODUCTION STRATEGIES IN *CONVOLUTRILOBA* ACOELS

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The defining characteristic of the Bilateria is the presence of a distinct head end and tail end, which defines the anterior-posterior (A-P) axis, a feature that is established during embryogenesis and generally remains unaltered during the lifetime of an organism. While a few bilaterians have evolved asexual reproduction strategies that allow them to subdivide the A-P axis, acoels in the genus *Convolutriloba* have an unparalleled ability to alter the A-P axis during modes of transverse fission, longitudinal fission, and reversed polarity budding. *Convolutriloba* acoels thus offer an exceptional opportunity to investigate the mechanisms that allow for the radical modification of an already established A-P body axis and to explore the evolution and development of diverse asexual reproduction strategies among related species. In this study, I reconstruct the evolutionary history of asexual reproduction in the *Convolutriloba* and compare the diverse modes of asexual reproduction at the level of body-wall musculature, nervous system development, and cell proliferation while

also exploring the regenerative potentials of tissues across species with different modes of asexual reproduction. In addition, I further explore the unusual process of A-P axis reversal that occurs during reversed polarity budding in *C. retrogemma* through studies of body patterning and regeneration. The results of these analyses suggest that a rich developmental toolkit of regenerative abilities, including the ability to utilize both epimorphosis and morphallaxis, to regenerate all parts of its body even from a small fragment, and to produce bifurcated A-P axes were present in the ancestor of the *Convolutriloba* allowing for the evolution of A-P axis modifications unlike any other bilaterian group. This toolkit along with the evolution of a seemingly unpatterned zone of tissue within the body of *C. retrogemma* capable of generating new anterior axes appear to have allowed this species to evolve the ability to form reversed A-P axes during budding.

BREAKING THE A-P AXIS: EVOLUTION OF DIVERSE ASEXUAL REPRODUCTION STRATEGIES IN *CONVOLUTRILOBA* ACOELS

By

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Chapter 1: Introduction

Evolution of asexual reproduction

In the vast majority of bilaterians, the anterior-posterior (A-P) axis is made during embryogenesis and then never altered for the rest of the life of the organism. However, through regeneration a fragment of the original A-P axis can re-establish a complete A-P axis, and through asexual (agametic) reproduction completely novel A-P axes can be formed. While animals utilize a diversity of asexual reproduction mechanisms that in some way alter the original A-P axis, essentially all modes of asexual reproduction fall into one of two categories, fission and budding, which differ in fundamental ways (Vorontsova and Liosner 1960; Hughes 1989). Fission occurs through the division of an animal's body into two or more parts, and fission products therefore directly inherit some fraction of the original individual's body and generally retain the primary body axis orientation of the original adult. During budding offspring develop as multicellular outgrowths from the body of an adult organism with most features, including the primary body axis, formed anew.

Asexual reproduction has evolved independently multiple times within the Metazoa. Fission tends to be common among more motile metazoans, such as annelids (Bely and Wray 2001), flatworms (Reuter and Kreshchenko 2004), and echinoderms (Uthicke 2001), while budding is most common among sessile and often colonial animals, such as cnidarian polyps (Sanyal 1966; Fischer and Hofmann 2004), bryozoans (Davenport 1891), tunicates (Berrill 1951), and pterobranch hemichordates (Dilly 1985). While animals have evolved diverse budding and fission modes, related

organisms tend to undergo similar modes of asexual reproduction (Hughes 1989). Annelids, for example, include hundreds of species that can undergo asexual reproduction (apparently stemming from many independent origins), but in nearly all cases this involves some form of transverse fission (Lasserre 1975; Schroeder and Hermans 1975). The lack of diversity in asexual reproduction strategies among related animals suggests that the developmental processes that have evolved to allow for asexual reproduction are usually stable.

Most animals that have the ability to reproduce asexually also have extensive regenerative capacities, allowing them to replace lost body regions when injured either through increased cell proliferation (epimorphosis) or by remodeling existing tissues (morphallaxis) (Morgan 1901). While most metazoan species capable of asexual reproduction also have regenerative abilities, all species that can regenerate do not have the ability to asexually reproduce. Furthermore, in many animals regeneration and fission appear to be highly similar developmental processes (Bely and Wray 2001; Martinez et al. 2004; Burton and Finnerty 2009). Together, these data suggest that asexual reproduction may, in many groups, have evolved from regeneration. An obvious difference between regeneration and asexual reproduction is the stimulus initiating each event. The regenerative process is set off by some external stimulus that leads to bodily injury and the removal of some portion of an animal's body, while asexual reproduction is self-initiated.

The mechanisms that allow for regenerative processes have been studied for over two centuries in a variety of metazoans, including *Hydra* (Trembley 1744), planarians (Morgan 1901), and amphibians (Spallanzani 1768). However, asexual

reproduction via fission and budding has received less attention though some recent efforts have been made to understand the molecular mechanisms allowing for asexual reproduction in *Hydra* (Galliot and Schmid 2002; Bode 2003), planarians (Sanchez Alvarado 2006), and annelids (Bely and Wray 2001). While recent studies comparing the molecular mechanisms between regeneration and asexual reproduction suggest that asexual reproduction is not accomplished by simply redeploying embryonic developmental pathways (Burton and Finnerty 2009), the mechanisms by which diverse asexual reproduction modes evolve remain largely unknown.

While closely related asexual species tend to undergo very similar modes of asexual reproduction, an unusual exception occurs within the acoel genus *Convolutriloba*. The four species of worms within this group are morphologically very similar (Fig. 1.1), yet they utilize three quite different modes of asexual reproduction, including transverse fission, longitudinal fission, and reversed polarity budding. *Convolutriloba* acoels thus offer a rare opportunity to investigate the developmental basis of diversification in asexual reproduction strategies among closely related species.

The Acoela

Acoels are small (~2-10 mm), free-living bilaterian worms that commonly inhabit marine and brackish environments worldwide. Acoels are generally oval or cylindrical in shape, are flattened dorso-ventrally, have a mouth (but no anus), and, instead of a gut cavity, possess a syncytium that digests food items in large vacuoles. The cerebral ganglion is quite simple unlike the more complex bilobed brains found

in most bilateral species, and the acoel nervous system is a loose net of nerve fibers distributed throughout the body.

Over the last decade, the phylogenetic position of acoels has been a controversial matter. While they were traditionally placed within the phylum Platyhelminthes (Hyman 1951), accumulating evidence in the form of 18S rDNA sequences (Ruiz-Trillo et al. 1999), mitochondrial gene sequences (Ruiz-Trillo et al. 2004), the Hox gene complement (Cook et al. 2004), and microRNAs (Sempere et al. 2006) indicates that the acoels may in fact be the most basal lineage of bilaterians. They were originally removed from the Platyhelminthes and placed into a newly erected phylum, Acoelomorpha, along with the nermatodermatid worms (Telford et al. 2003; Baguna and Riutort 2004). The Acoelomorpha has since been dismissed as paraphyletic (Wallberg et al. 2007), resulting in the elevation of the Acoela to an independent phylum basal to all other bilaterian lineages. Given this unique phylogenetic position, acoels hold critical information for inferring early steps in the origin of the Bilateria, such as the evolution of the bilaterian A-P axis. Recent studies of acoel development argue for a simple planula-like Urbilaterian (Hejnol and Martindale 2008b) and suggest the independent evolution of the mouth and anus (Hejnol and Martindale 2008a). Despite these studies and the potential insights that the study of acoel development may have on understanding early bilaterian evolution, acoels remain relatively poorly studied.

Despite early views that acoels had limited powers of regeneration (Hyman 1951), diverse acoel species have been shown to have exceptional regenerative abilities (Hanson 1967; Drobysheva 1986; Hori et al. 1999). This is likely due, in

part, to the presence of totipotent stem cells, called neoblasts, which are distributed throughout the bodies of acoels (Gschwentner et al. 2001) as well as their ability to repattern existing tissues. Neoblasts are thought to be the only mitotically active cells within the body of acoels and are responsible for the renewal of all cell types during development, growth, and regeneration (Gschwentner et al. 2001). While neoblasts seem to provide acoels with extensive regenerative abilities, most acoels do not reproduce asexually. Examples of variant forms of fission and budding have been described in a few acoel species, but asexual reproduction is not widespread and has seemingly evolved independently in these isolated cases (Reuter and Kreshchenko 2004).

Study system: Convolutriloba *acoels*

The acoel genus *Convolutriloba* includes four species of morphologically similar worms that have evolved the ability to asexually reproduce via radical axial modifications. Members of the genus are relatively large (~4-8 mm) acoelomate worms that possess a distinct trilobed tail along the posterior margin, two anterior eyespots, and symbiotic chlorophyte algae distributed throughout their body. *Convolutriloba* acoels are endemic to the reefs of the Indo-Pacific region, though specimens have recently been collected along Caribbean reefs near the Bahamas (A. Dupont, personal communication). Although only four species have been identified in the genus, *Convolutriloba* display three distinct modes of asexual reproduction (Fig. 1.2), including nearly unique examples of longitudinal fission and reversed-polarity budding. *Convolutriloba hastifera* reproduces by simple transverse fission followed by regrowth of anterior and posterior structures (Gaerber et al. 2007).

Convolutriloba longifissura reproduces by two sequential and orthogonal fission events: the first transverse, dividing the worm into anterior and posterior fragments; and the second longitudinal dividing the posterior fragment further into left and right halves (Åkesson et al. 2001). The remaining two species, *C. retrogemma* and *C. macropyga*, reproduce by a remarkable process of reversed polarity budding, in which a parent produces posterior buds with an A-P polarity reversed relative to that of the parent. (Hendelberg and Åkesson 1991; Shannon and Achatz 2007). Such diverse modes of asexual reproduction within a single genus are exceedingly rare among metazoans (Hughes 1989). In addition to providing an opportunity to study the diversification of asexual reproduction strategies, *Convolutriloba* acoels also allow for the study of the mechanisms by which bilaterians can modify their primary body axis during postembryonic development.

Present study

The general objective of this study is to identify some of the developmental mechanisms that have allowed for the diversification of asexual reproduction among *Convolutriloba* species. While studies of different modes of asexual reproduction have been conducted in a variety of metazoan species, the mechanisms that allow for the diversification of asexual reproduction among related species are not well understood. When multiple modes of asexual reproduction occur within a group, do more complex modes of asexual reproduction tend to evolve from simpler modes? Do similar changes in body morphology occur to enact these diverse modes of asexual reproduction? Given that asexual reproduction likely evolved from underlying regenerative abilities, do different regenerative capacities allow for the

evolution of diverse modes of asexual reproduction? Different asexual reproduction strategies often lead to varied modifications in the normally fixed primary body axis of adult animals. As body axes are altered and novel axes form during different forms of asexual reproduction, are adult tissues respecified through the upregulation of the same axial patterning genes involved in axis determination during embryogenesis? *Convolutriloba* acoels with diverse modes of asexual reproduction and similar morphologies provide an unusual opportunity to provide answers to these questions.

In chapter 1, I reconstruct the evolutionary relationships among the four *Convolutriloba* species using DNA sequence data and obtain comparative data on general morphogenesis, body-wall musculature, and the nervous system throughout the course of asexual reproduction in the four species of *Convolutriloba*. The results of this study reveal that reversed-polarity budding is the ancestral form of asexual reproduction in the *Convolutriloba* genus, suggesting that the simplest mode of asexual reproduction, transverse fission, is, in fact, derived. While a clear difference between fission and budding exists in the formation of the nervous system, surprisingly homologous processes occur in body-wall muscle disorganization during the different modes of asexual reproduction. The potential driving forces behind the diversification of asexual reproduction in the genus are discussed.

In chapter 2, I assess the regenerative potential of three *Convolutriloba* species, representing the different modes of asexual reproduction in the genus, to determine if any unusual regenerative abilities may have allowed for the evolution of theses diverse modes of asexual reproduction or conversely, if the diversification of

asexual reproduction has been accompanied by diversification of regenerative abilities. These regeneration experiments along with observations of cell proliferation during regeneration reveal that posterior regeneration occurs largely through a morphallactic process while anterior and lateral regeneration occur by epimorphosis in the three species. In addition, these experiments also reveal a temporal and spatial gradient of axial respecification during fission and budding and the surprising potential of posterior tissue to regenerate bifurcated anterior axes. Such a variety of regenerative abilities may have provided a developmental toolkit that allowed for the diversification of asexual reproduction within the genus.

In chapter 3, I characterize the expression of conserved axial patterning markers and the patterns of cell proliferation during reversed polarity budding in *C. retrogemma* to determine how axial respecification occurs in the region of polarity reversal. I also conduct regeneration experiments on tissues from different body regions of budding *C. retrogemma* to determine the temporal and spatial nature of axial respecification. The results of these experiments reveal the presence of a zone of tissue at the site of A-P axis reversal where adult tissues are modified. Axial patterning genes are upregulated as tissue outgrowth occurs, allowing for the generation of novel, reversed axes. The convergent mechanisms that allow for budding in *Hydra* and *C. retrogemma* are also discussed.

Significance

This study provides the first analysis of the evolutionary mechanisms and morphological modifications that allow for the diversification of asexual reproduction within closely related metazoan taxa. In addition, it also provides the first

examination of the modifications that occur during primary body axis reversal and the axial respecifications that occur during lateral subdivision of the A-P axis in asexual reproduction. While these specific axial modifications occur only in this small genus of acoels, they have important implications in the developmental potential of basal animals and bilaterians in general. Despite obvious variations in different modes of fission and budding, this study suggests that such diverse modes of asexual reproduction often utilize convergent mechanisms to allow for the formation of new tissues and final separation of the asexual progeny from the adult. In addition, apparently simple modes of asexual reproduction are not necessarily the basal condition, reinforcing the potential simplifying effects of evolution. The radical axial modifications that occur during asexual reproduction in *Convolutriloba* acoels suggest the developmental capacity for both basal and bilateral animals to alter the established A-P axis long after embryogenesis. The results presented here suggest that radical axial modifications occurring as part of asexual reproduction can evolve as a result of different regenerative abilities. Finally, these results suggest the potential for bilateral animals to selectively depolarize a portion of tissue within the body, forming a seemingly unpolarized zone of tissue within the body of an otherwise patterned adult.

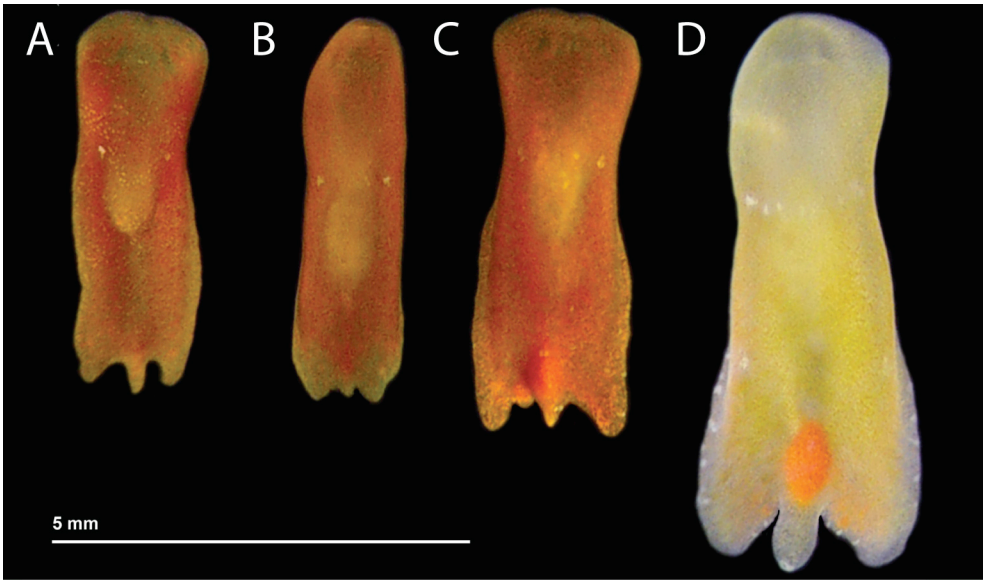

Figure 1.1. Comparative live photomicrographs of the four *Convolutriloba* species. (A) *C. hastifera* (B*) C. longifissura* (C) *C. retrogemma* (D) *C. macropyga*. Specimens were viewed on a Wild M3Z stereomicroscope and photographed with a Sony SDC-P71 digital still camera.

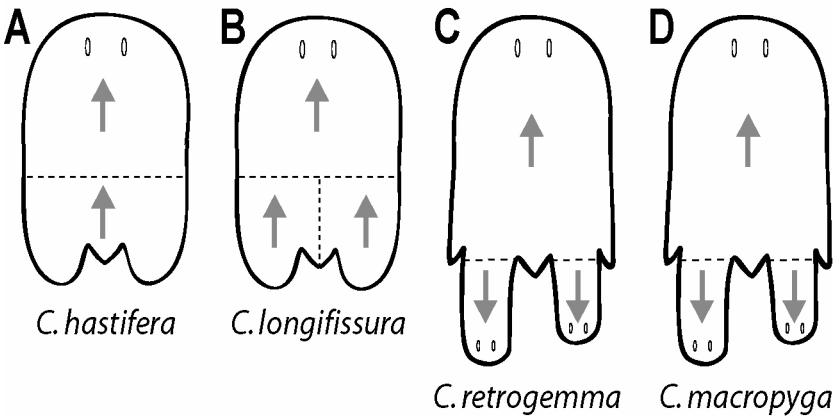

Figure 1.2. Modes of asexual reproduction among the four species of *Convolutriloba*. (A) *C. hastifera* reproduces by a single transverse fission. (B) *C. longifissura* reproduces by transverse fission followed by longitudinal fission of the posterior fragment. (C, D) *C. retrogemma* and *C. macropyga* produce buds with reversed A-P axes relative to the parental axis. Buds form from left and right budding sites and often develop asynchronously. Arrows indicate the direction of the A-P axis with arrowheads pointing anterior. Dashed lines represent the planes of separation that occur during reproduction.

Chapter 2: Radical modification of the A-P axis and the evolution of asexual reproduction in *Convolutriloba* acoels

Abstract

Acoel worms in the genus *Convolutriloba* are remarkable in that closely related, morphologically very similar species reproduce asexually by dramatically different processes. Transverse fission, longitudinal fission, and reversed-polarity budding all occur within this genus, indicating an unparalleled ability to alter the A-P axis. *Convolutriloba* thus offers an exceptional opportunity to investigate the development and evolution of asexual reproduction. Molecular phylogenetic analysis indicates that reversed-polarity budding is ancestral and fission is derived for the genus. A clear difference between budding and fission is indicated by the development of the nervous system, which forms *de novo* during budding, but regenerates largely by extensions of remaining components of the nervous system during both types of fission. Despite this and other differences between fission and budding, localized muscle disorganization coupled with behaviorally-mediated tearing are characteristic of both transverse fission and reversed-polarity budding (though not longitudinal fission), suggesting that a homologous tissue-separation mechanism underlies these two outwardly quite different asexual reproductive modes. I suggest that the ability to split the posterior axis field into two adjacent fields, manifested during both reversed-polarity budding and longitudinal fission, may have been a driving force behind the diversification of asexual reproductive mode in this group.

Introduction

Asexual agametic reproduction, such as budding and fission, has evolved numerous times among animals and can take a bewildering variety of forms (Brusca and Brusca 2003). Asexual reproduction ultimately produces multiple, discrete individuals, but intermediate stages of the process can involve remarkable modifications of the original individual's body. When new individuals develop while still physically attached to the parent (e.g., during budding and some forms of fission), the typically single, fixed primary body axis of an individual can be broken into multiple, tandemly arrayed axes (e.g., in annelids and platyhelminths undergoing paratomic fission and cnidarians undergoing strobilation), produce lateral branches (e.g., in cnidarians, hemichordates and annelids undergoing budding), be split longitudinally to give rise to adjacent parallel axes (e.g., in acoels and cestodes undergoing longitudinal fission), and even undergo a localized axis reversal to produce axes with opposite orientations (e.g., in acoels undergoing reversed-polarity budding) (Fig. 2.1). While tandemly arrayed and branched body axes are common intermediate stages among asexually reproducing animal species (Hughes 1989), longitudinal axis division and body axis reversal are extremely rare. Investigating these unusual reproductive modes can provide important insights into how novel modes of asexual reproduction evolve.

Although diverse forms of asexual reproduction occur among animals, closely related asexual species tend to exhibit similar reproductive modes (Hughes 1989). An unusual exception to this pattern occurs within the Acoela, a group of small marine worms thought to represent the most basal bilaterian lineage (Ruiz-Trillo et al. 1999;

Wallberg et al. 2007). Within the acoel genus *Convolutriloba*, dramatically different asexual reproductive modes occur among closely related, morphologically similar species. Although only four species are described for the genus, they display three distinct modes of asexual reproduction (Fig. 2.2), including nearly unique examples of longitudinal fission and reversed-polarity budding. *Convolutriloba hastifera* reproduces by simple transverse fission followed by regeneration (Gaerber et al. 2007). *Convolutriloba longifissura* reproduces by two sequential and orthogonal fission events: the first transverse, dividing the worm into anterior and posterior fragments, and the second longitudinal, dividing the posterior fragment further into left and right halves (Åkesson et al. 2001). The remaining two species, *C. retrogemma* and *C. macropyga*, reproduce by a remarkable process of reversed-polarity budding, in which a parent produces posterior buds with an anterior-posterior (A-P) polarity reversed relative to that of the parent (Hendelberg and Åkesson 1988; Shannon and Achatz 2007). While asexual reproduction occurs in all species of *Convolutriloba*, most acoels (including the closest relatives of *Convolutriloba*) do not typically undergo asexual reproduction (Åkesson et al. 2001), though many do have extensive regenerative abilities (Hanson 1967).

Convolutriloba offers a rare opportunity to investigate the developmental basis of diversification of asexual reproduction among closely related species. Thus far, the process of asexual reproduction has been described in the four species primarily based on external morphology, and some descriptions are very cursory. The internal musculature and nervous system have been described in non-reproducing adults of some species (Gschwentner et al. 2003; Gaerber et al. 2007; Shannon and

Achatz 2007), but the dynamic changes in musculature and the nervous system during asexual reproduction have only begun to be investigated in one of these (Gschwentner et al. 2003; Gaerber et al. 2007). Thus, it is currently unclear whether there are any underlying similarities between different types of reproduction, such as fission and budding, or transverse and longitudinal fission. Furthermore, the evolutionary relationships among the four species are not known, yet this information is critical to determining which reproductive mode is ancestral and which modes are derived within the genus. In order to investigate the diversification of asexual reproduction in *Convolutriloba*, I reconstructed the evolutionary relationships among species in the genus using DNA sequence data and obtained comparative data on general morphogenesis, musculature, and the nervous system throughout the course of asexual reproduction in the four species of *Convolutriloba*.

Materials and methods

Animal collections and culture

Convolutriloba specimens were collected between 2004 and 2007 from marine aquaria at several retail aquatic stores in Maryland, South Carolina, and Georgia (USA). These acoels are thought to have been introduced to commercial reef tanks along with material for the aquarium trade collected from the Indo-Pacific, the only region from which *Convolutriloba* have been described (Winsor 1990; Ishikawa and Yamasu 1992; Hirose and Hirose 2007). In total, I obtained eight different *Convolutriloba* lines, which based on asexual reproductive mode, morphology, and themolecular phylogenetic analysis represent all four species described for this genus. Acoels were cultured as isogenic lines (originally established from a single

individual) in five gallon aquaria at 24°C with a 12h:12h light:dark cycle in artificial seawater (34 ppt). To produce cultures with the fastest rates of asexual reproduction, I maintained them with full spectrum 10,000K artificial illumination (critical for growth of their symbiotic algae), high water quality (pH=8.5; 0 ppm ammonia and nitrites; <20 ppm nitrates), and active protein skimming. *Artemia* nauplii were provided weekly as food.

DNA amplification and sequencing

DNA was extracted from each acoel line using the DNeasy Tissue Extraction Kit (Qiagen, Valencia, CA, USA) and regions of three genes, the nuclear ribosomal genes 18S and 28S and the mitochondrial protein-coding gene cytochrome oxidase I (COI), were amplified by PCR. A 1071 bp 5' fragment and an 814 bp 3' fragment of 18S were amplified using primers 1F, 5R, 5F, and 9R (Carranza et al. 1997). A 1243 bp fragment of 28S was amplified using F63sq and R1411sq (Medina et al. 2001). A 1249 bp region of COI was amplified by PCR in two partly overlapping fragments. The first half was amplified using LCO1490 and HCO2198 (Folmer et al. 1994) and the second half was amplified using either COI N+ (5'-CCAGTTTTTGCAGGAGGRATYACYAT-3') or COI P+ (5'-TGAGARAATYTWACMTTATTTGTITG-3') as a forward primer and either COI O- (5'-TAATCAGTGTANCGTCGNGGTATICC-3') or COI Q- (5'-ATACCAAANCTACTCATTTCATGYCA-3') as a reverse primer. Reactions (25 µl including 0.5 units Platinum *Taq*, Invitrogen, Carlsbad, CA, USA) were amplified using the following cycling conditions: 94°C for 5 min; 35 cycles of 94°C for 30 sec,

50°C for 30 sec, 72°C for 2 min; and 72°C for 5 min. PCR products were purified using the QIAquick PCR Purification Kit or QIAquick Gel Extraction Kit (Qiagen) and sequenced on an automated sequencer. A few PCR products yielded double sequence because both acoel and symbiotic algal genes were amplified by the primers. In such cases, PCR fragments were cloned into pGEM-T (Promega, Madison, WI, USA) and resequenced to obtain unambiguous acoel sequence. Sequences were deposited in GenBank under the following accession numbers: 18S (5' fragment) -- EU710898-EU710905; 18S (3' fragment) -- EU710906-EU710913; 28S -- EU710914-EU710920; COI -- EU710921-EU710928.

Phylogenetic analyses

The final data matrix consisted of 1480 bp of 18S (in two non-adjoining fragments), 1243 bp of 28S, and 1210 bp of COI obtained from the eight *Convolutriloba* lines along with GenBank sequences for the acoels *Childia groenlandica* and *Convoluta convoluta*, the closest outgroups for which data were available for the gene markers (accession numbers for *C. groelandica*: 18S–AY078367; 28S–AY157603; COI–AJ405972; accession numbers for *C. convoluta*: 18S–AJ012524; 28S–AY218097; COI–AJ405969, AJ405970). Neither of these outgroups is known to reproduce asexually. *Convoluta convoluta* can regenerate (Drobysheva 1986), but no published information about *C. groenlandica* regeneration could be found. Gene sequences were aligned using ClustalX (v. 1.83) (Thompson et al. 1997) using default parameters and minor corrections for obvious misalignments were made manually in MacClade (v. 4.05, Sinauer Associates, Sunderland, MA,

USA). DNA sequence divergences (uncorrected pairwise distances) were calculated using PAUP* (v. 4.0b10. Sinauer Associates, Sunderland, MA, USA). Phylogenetic analyses were performed with PAUP* using maximum parsimony (MP) (branch-and-bound search) and maximum likelihood (ML) (heuristic search with 10 random sequence additions). MP clade support was evaluated by bootstrapping (Felsenstein 1985) with 1000 replicates. For the ML analysis I used a GTR + I + γ model of sequence evolution, chosen using ModelTest (v. 3.7) (Posada and Crandall 1998), with parameter values estimated by PAUP*.

Stages of asexual reproduction

For each of the four species of *Convolutriloba*, I determined the typical sequence and relative timing of events for that species' mode of asexual reproduction. For each species, 96 individuals that had just completed a round of asexual reproduction were placed singly in wells of 24-well cell-culture trays containing ~2ml artificial seawater and maintained at 24^{o}C with a 12h:12h light:dark cycle. Daily observations were made of each individual throughout the course of a complete round of asexual reproduction.

Phalloidin staining

The musculature of worms at different stages of asexual reproduction was investigated by phalloidin staining of F-actin fibers. Worms were relaxed in 3.4% $MgCl_2$ for 10 min., fixed in 3.7% formaldehyde in 0.75x phosphate buffered saline (PBS) for 30 min., washed at least three times in PBS, washed in PBTx (PBS + 0.1%

Triton X), incubated with Alexa Fluor-488 phalloidin (Invitrogen, Carlsbad, CA, USA) at 1:100 in PBTx overnight at 4°C, and then washed three times in PBS. Samples were mounted in Fluoromount-G (Southern Biotech, Birmingham, AL, USA) and imaged using a Zeiss LSM-510 confocal microscope. Images were processed and montages of multiple neighboring views were made with Adobe Photoshop (v. 7.0).

Serotonin immunostaining

Serotonin immunostaining was used to reveal the serotonergic components of the nervous system of worms at different stages of asexual reproduction. Worms were relaxed, fixed, and washed as for phalloidin stains. Samples were then blocked in 10% normal goat serum (NGS) in PBTx for 1 hour at room temperature and incubated in rabbit anti-serotonin polyclonal antibody (Sigma, St. Louis, MO, USA) at 1:100 in 10% NGS/0.9X PBTx overnight at 4°C. Samples were rinsed repeatedly in PBTx over 1 hour, incubated in Alexa Fluor-488 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) at 1:200 in 10% NGS/0.9X PBTx overnight at 4°C, rinsed three times in PBTx and rinsed once in PBS. Samples were mounted in Fluoromount-G and images were captured with a Zeiss Axioplan 2 epifluorescence microscope equipped with a Zeiss AxioCam HRc camera. Images were processed as for phalloidin staining.

Results

Phylogenetic analyses

The combined nucleotide data set (3933 bp) included 349 parsimony informative sites (18S: 80; 28S: 30; COI: 239), of which 209 sites (18S: 9; 28S: 8; COI: 192) were informative within *Convolutriloba*. The MP and ML analyses of the combined, three-gene data set produced single best trees with identical topologies (Fig. 2.3). COI alone yielded a single best tree with this same topology for both MP (well-supported by bootstrapping) and ML, but 18S alone and 28S alone yielded poorly resolved trees (data not shown). Where multiple isolates per species were obtained, they formed strongly supported clades. Relationships among *Convolutriloba* species were well resolved: *C. hastifera* is sister to *C. longifissura,* this clade is sister to *C. retrogemma*, and *C. macropyga* is the most basal lineage within the genus. Uncorrected pairwise distances for COI, the most variable marker, ranged from 7.2-14.3% among *Convolutriloba* species.

General morphology

Convolutriloba are small (4-8 mm in length), ciliated, dorsoventrally flattened marine worms with a pair of anterior eyes and a trilobed tail. They feed on prey using a ventral mouth that leads to a digestive syncytium and also possess photosynthetic endosymbionts (chlorophyte algae) distributed beneath the body wall and throughout the parenchymal tissue (Hirose and Hirose 2007). Although body organization is temporarily modified in different ways during the three modes of asexual reproduction (Fig. 2.4), non-reproducing individuals of the three species exhibit very

similar morphologies. Tri-layered sheets of muscles lie below the dorsal and ventral body wall surfaces. Ventral musculature has been described in two of the four species (Gschwentner et al. 2003; Shannon and Achatz 2007). Here I focused on dorsal musculature and found that its organization is similar across the four species (Fig. 2.5, A, D, F, and I). It consists of highly organized circular, diagonal, and longitudinal muscle fibers (from external to internal), a common pattern among many acoels (Ladurner and Rieger 2000; Hooge 2001). Serotonin staining reveals much of the central nervous system in *Convolutriloba* (Fig. 2.6A) (Gaerber et al. 2007). I found that in non-reproducing worms, the pattern of labeled serotonergic cell bodies and nerve fibers is similar across the four species. Serotonin staining reveals the dorsal, bilobed cerebral ganglion (just below the eyes) and the six longitudinal nerve cords, two of which run ventro-laterally, extending most of the length of the animal, and four of which are dorsal and medial, extending about half the length of the body. Weak serotonin staining sometimes also reveals the diffuse sub-muscular nerve net.

Asexual reproduction

Below, I describe the process of asexual reproduction in each of the four species of *Convolutriloba*, including the main stages of asexual reproduction and relative timing of events (Fig. 2.4), changes in the musculature (Fig. 2.5), and changes in the nervous system (Fig. 2.6).

Transverse fission in *C. hastifera*

That *C. hastifera* reproduces by simple transverse fission was recently reported (Gaerber et al. 2007), but the description is very cursory, being limited to one short paragraph and part of a figure, and no other published data are available on reproduction in this species. I have confirmed that *C. hastifera* reproduces by an unequal transverse fission occurring approximately three-quarters of the way down the length of the body, producing a larger anterior fragment and a smaller posterior fragment (Fig. 2.4A), and have further characterized this process. I found that when an individual prepares to fission, its posterior end flattens and adheres firmly to the substrate. Small constrictions form on each side of the animal along the future plane of fission. Eventually, one of these constrictions becomes more pronounced and a fissure forms on that side. Muscle fibers along the fission plane become disorganized ~200 μm ahead of the actual tissue separation (Fig. 2.5B), which progresses across the animal over 6-8 hours, separating it into anterior and posterior fragments. During fission the anterior end pulls forward, against the stationary tail, suggesting that behaviorally-mediated pulling may help to effect fission.

The anterior fragment inherits the mouth, the digestive syncytium, and the entire serotonergic nervous system except for the posterior ends of the two lateral nerve cords. As soon as fission occurs, this fragment swims away actively and its behavior becomes indistinguishable from that of the original animal. Tail lobes are regenerated within a day (Fig. 2.4A). The posterior ends of the lateral nerve cords extend as the animal lengthens and takes on the normal body proportions.

The posterior fragment inherits no portion of the digestive system and only short posterior tracts of the lateral nerve cords. It remains immobile and firmly attached to the substrate until it has fully regenerated. Within 6-8 hours after fission, some of the animal's symbiotic algae (or remnants thereof) form a dark spot in the center of the animal, as has been described for *C. longifissura* and *C. retrogemma* (Åkesson and Hendelberg 1989; Åkesson et al. 2001). These algal cells are presumably shuttled into the developing digestive syncytium and broken down as a temporary food source since the worm cannot feed on captured prey until the mouth forms (after the nervous system is generated). An anterior regeneration blastema, initially unpigmented, becomes visible shortly after the algal digestion mass is formed. Within this blastema, poorly organized, transverse muscles initially form (Fig. 2.5C). Subsequently transverse muscles become more organized and longitudinal and diagonal muscles are added, leading to the characteristic organized pattern of adults. Nervous system regeneration is extensive and appears to progress primarily via extensions of the remaining nervous system. Immediately after fission, the lateral nerve cord fragments extend anteriorly into the blastema and bend medially (Fig. 2.6B). They nearly meet at the midline, and here paired clusters of serotonergic cells appear, eventually forming the bilobed ganglion (Fig. 2.6C). The four medial nerve cords form last, extending posteriorly from the ganglion, coincident with eye formation. Once the nervous system has fully regenerated, the posterior fragment becomes motile.

Transverse and longitudinal fission in *C. longifissura*

C. longifissura reproduces by two sequential, orthogonal fissions (Fig. 2.4B) (Åkesson et al. 2001). The first fission is transverse and unequal, occurring at a location and by a process essentially identical to that of *C. hastifera*. The subsequent regeneration of the anterior fragment also progresses just as it does in *C. hastifera*. The development of the posterior fragment in *C. longifissura* is markedly different, however. This fragment forms a left/right pair of algal-digestion masses, revealing the establishment of two adjacent A-P axes in this fragment. A longitudinal fission that begins anteriorly and progresses posteriorly subsequently divides the fragment into left and right halves, one of which inherits the medial lobe of the tail (Bartolomaeus and Balzer 1997; Åkesson et al. 2001; note that Bartolomaeus and Balzer 1997 misinterpreted aspects of this division, as discussed in Åkesson et al. 2001). Although Gschwentner et al. (2003) described the reorganization of the ventral musculature during development of the posterior fragments, muscle dynamics during the actual fission process have not previously been described. I found that during longitudinal fission, the muscle fibers physically break along the plane of fission ~300 μm ahead of the actual tissue separation (Fig. 2.5E). Unlike transverse fission, muscle fibers do not become disorganized ahead of the fissure, and animals remain completely stationary throughout the process, showing no hint of behavioral pulling. Only after the two halves separate do the worms become motile and form a mouth.

Anterior regeneration is initiated prior to the completion of longitudinal fission, such that the posterior fragment can possess two anterior blastemas while longitudinal fission is ongoing. As in *C. hastifera*, nervous system regeneration

occurs largely by extensions of pre-existing nervous system components (Fig. 2.6) (Gaerber et al. 2007). As longitudinal fission is progressing from anterior to posterior, the two lateral nerve cord fragments extend anteriorly and medially (Fig. 2.6D). They never meet (as they do in *C. hastifera*) because of the longitudinal fissure dividing the posterior fragment. In each half of the posterior fragment, a new ganglion forms anteriorly and a short anterior piece of the new lateral nerve cord forms on the new lateral side (Fig. 2.6E). The new lateral nerve cord then extends posteriorly, as longitudinal fission progresses. Once the ganglion forms, eyes develop and the four medial nerve cords extend posteriorly from the ganglion (as in *C. hastifera*). Based on the configuration of the developing nervous system and the position of eyes within the anterior blastema, it is evident that the anterior portion of the A-P axis of each posterior fragment is initially deflected towards the fission plane (the medial plane of the original animal). The A-P axis straightens as development proceeds, presumably through differential growth establishing bilateral symmetry. Each posterior fragment becomes motile once anterior neural regeneration is complete. Only once the left and right fission products are physically separated does the trilobed tail form, possibly through morphallaxis as no obvious blastema is formed.

Reversed-polarity budding in *C. retrogemma* and *C. macropyga*

Convolutriloba retrogemma and *C. macropyga* reproduce asexually by a highly similar process of reversed-polarity budding (Fig. 2.4, C and D) in which buds are produced with an A-P polarity opposite that of the parent. Both Hendelberg and Åkesson (1988) and Shannon and Achatz (2007) describe budding as being initiated

from a pair of posterior budding sites, one on either side of the medial lobe of the tail. However, Hendelberg and Åkesson (1988) indicate that in *C. retrogemma* only one bud at a time develops per site, while Shannon and Achatz (2007) indicate that in *C. macropyga* multiple buds may be present at each budding site (such that more than two buds may be developing at once on an individual). I have cultured both species under identical conditions and find that the typical mode of budding for both is from a pair of posterior budding sites each producing one bud at a time, as initially described for *C. retrogemma*. I found that supernumerary buds form only in larger, older *C. macropyga* individuals that appear to have developed morphological abnormalities.

The first signs of budding are thickened tissue between the medial lobe and a lateral lobe of the tail and migration of symbiotic algae to this same region (Hendelberg and Åkesson 1991; Shannon and Achatz 2007). I found that these bud-initiation sites are characterized by disorganized muscle fibers, similar to those observed along the transverse fission plane in *C. hastifera* and *C. longifissura*. This region of disorganized muscle persists throughout the budding process as a narrow band of ~200 μm in the longitudinal plane and ~800 μm in the transverse plane at the boundary between the parent and developing bud (Fig. 2.5, G and J). As the bud extends, the new muscles that form in the bud take on the characteristic organized muscle pattern as soon as they are formed (Fig. 2.5, H and K). Some muscle fibers, primarily longitudinal fibers, extend across the zone of disorganized muscle and directly connect parent and bud (Fig. 2.5, G and J). When the bud has reached approximately half the length at which it will detach, an algal digestion mass forms at its center (Åkesson and Hendelberg 1989), indicating the beginning of digestive

syncytium development. Once the nervous system is formed, the mouth forms, allowing late stage buds to feed on prey even while attached to the parent (Hendelberg and Åkesson 1988; Shannon and Achatz 2007).

The nervous system of the bud develops completely *de novo* (Fig. 2.6, F and H) and no serotonin labeling is detected in the developing bud until it is approximately half the length at which it will detach. At this point, two clusters of serotonin-positive cells appear near the anterior margin of the bud, indicating the beginning of ganglion formation (Fig. 2.6, F and H). Once the ganglion is fully formed, eyes develop and the nerve cords extend from the ganglion (Fig. 2.6, G and I). Although in a few *C. macropyga* individuals with mid-stage buds the lateral nerve cords of parent and bud appeared to make contact (data not shown), in almost all individuals it appears that the nerve cords of the bud and parent remain completely separate throughout the budding process (Fig. 2.6, F and H). Around the time the ganglion forms, the bud becomes behaviorally independent of the parent and attempts to move in an opposite direction. Late-stage buds with complete nervous systems eventually tear away from the parent across the region of disorganized muscle fibers. Tearing lasts only a few seconds and results in visible wounds on both individuals. The parent and bud wound-heal, and the bud generates a trilobed tail (Fig. 2.4, C and D).

The speed of asexual reproduction in all four *Convolutriloba* species is highly variable and depends on lighting, temperature, water quality, and feeding regime (Shannon and Achatz 2007; J.M.S., pers. obs.). The minimum duration for a complete round of asexual reproduction (i.e., the entire sequence represented in Fig. 2.4 for

each species) that I recorded was 3 days for *C. hastifera* and *C. longifissura* and 5 days for *C. retrogemma* and *C. macropyga*. However, the large anterior fission product of *C. hastifera* and *C. longifissura* can initiate a new fission event within 24 hours after a prior fission, and the large parent of *C. retrogemma* and *C. macropyga* can produce a visible new bud in as little as 36 hours after the detachment of a previous bud.

Discussion

Dramatically different forms of asexual reproduction have evolved among animals, but closely related species tend to undergo similar modes of reproduction (Hughes 1989). Annelids, for example, include hundreds of species that can undergo asexual reproduction (apparently stemming from many independent origins), but in nearly every case this involves some form of transverse fission (Lasserre 1975; Schroeder and Hermans 1975). The acoel genus *Convolutriloba* is highly unusual in displaying radically different modes of asexual reproduction, including transverse fission, longitudinal fission, and reversed-polarity budding. This genus thus offers an excellent opportunity to identify features that may promote diversification of asexual reproduction mode and to identify possible underlying homologies between these different modes. Toward this end, I have generated the first molecular phylogeny of *Convolutriloba* to provide an evolutionary framework for interpreting developmental differences within this group and have characterized the sequence of morphogenetic events, body musculature changes, and nervous system changes that occur during asexual reproduction in each of the four species of the genus.

Evolutionary relationships among diverse asexual reproductive modes

Convolutriloba species are anatomically so similar that mode of asexual reproduction has been a key character used to distinguish among them (Hendelberg and Åkesson 1991; Åkesson et al. 2001; Gaerber et al. 2007). Thus, independent validation of species delineations is needed. Where multiple isolates of a species were obtained for the phylogenetic analysis (three of four species), isolates grouped according to species with strong bootstrap support. In addition, sequence divergence among the four putative species ranged from 7.2-14.2% at COI, comparable to divergence at this locus among closely related species in a variety of animal phyla (e.g., Hebert et al 2003). Because *Convolutriloba* acoels have been collected almost exclusively from reef tanks maintained for the aquarium trade and their geographic origins are virtually unknown, it is difficult to provide a definitive test of species delineations. However, these data provide the first molecular support for the current delineation of *Convolutriloba* species.

The molecular analyses provide a well-resolved phylogeny for *Convolutriloba*. The two species that undergo reversed-polarity budding, *C. macropyga* and *C. retrogemma*, are the two most basal lineages within the genus, with the remaining two species forming a clade. Parsimony mapping of reproductive mode on this topology suggests that reversed-polarity budding is ancestral for the genus and was subsequently lost in the ancestor of *C. hastifera* and *C. longifissura*, which undergo transverse fission and sequential transverse/longitudinal fission, respectively.

Reversal of the primary body axis

Asexual reproduction involving body axis reversal is extremely rare. One of these rare cases involves the anthozoan cnidarian *Nematostella vectensis*, which typically reproduces asexually by simple transverse fission but occasionally (and perhaps accidentally) develops a second oral pole, complete with a second mouth and whorl of tentacles, at the original aboral pole prior to fissioning mid-body (Reitzel et al. 2007). Among bilaterians, *C. retrogemma* and *C. macropyga* are the only known examples of species reproducing by a reversed-polarity process.

The potential of several bilaterian groups to undergo polarity reversal is nevertheless apparent from aberrant regeneration and embryogenesis. Dicephalic adult planaria have been experimentally induced by altering the Wnt/β-catenin pathway (Gurley et al. 2008; Iglesias et al. 2008; Peterson and Reddien 2008) and by treating regenerating adults with gap junction communication blockers (Nogi and Levin 2005), chick embryo extracts (Rodriguez and Flickinger 1971), and nucleic acid synthesis inhibitors (Kohl and Flickinger 1966). Dicephalic individuals have also been experimentally induced in annelids when amputations result in very short fragments or when conditions do not allow for post-amputation autotomy (Hyman 1916; Kawamoto et al. 2005). Embryonic mutations can also produce reversed A-P axes, as in *Drosophila* embryos with mutations in the *bicaudal* and *dicephalic* genes (Bull 1966; Lohs-Schardin 1982; Sutter et al. 1989). Even if experimentally induced dicephalic individuals typically do not have long-term viability, they demonstrate the broader potential for body-axis reversal among bilaterians.

The finding that *C. macropyga* and *C. retrogemma* are the two most basal lineages within *Convolutriloba* suggests that reversed-polarity budding is ancestral for the genus and evolved just once within this clade, and thus just once among bilaterians. I found all aspects of budding that I investigated to be essentially identical between the two species, supporting the homology of reversed-polarity budding between *C. macropyga* and *C. retrogemma*. In both species, buds are produced from left and right budding zones along the posterior margin of the animal. When a new bud is initiated, the muscles in this zone are disorganized and, although the bud's new musculature appears organized as soon it forms, the zone of disorganized musculature persists as a narrow band at the boundary between parent and bud throughout the budding process. The ganglion and nerve cords appear *de novo* in mid-late stage buds, and the nerve cords of the parent and bud are not connected (except in a few rare cases in *C. macropyga*). The bud eventually tears free at the region of disorganized muscle.

The disorganization of muscle fibers at the base of the bud may be critical to bud detachment by providing a zone of weakened tissue, analogous to perforations in a sheet of paper guiding a tear. That musculature disorganization occurs at the parent/bud boundary and thus in the region of axis reversal suggests that absent or atypical A-P patterning cues could be responsible for muscle disorganization. Characterizing the zone of axis reversal and elucidating its molecular basis will be critical to understanding the process of reversed-polarity budding and how it evolved.

Transverse division of the primary body axis

Simple transverse fission (or architomy) is one of the most common forms of asexual reproduction among animals. It can be effected via a range of processes, including localized tissue degradation (Mire and Venable 1999), differential cell adhesion (Kawamura and Sugino 1999), and behaviorally-mediated pulling or twisting (Crozier 1917). Because of the simplicity and prevalence of simple transverse fission, in many groups it is thought to be the first type of asexual reproduction to have evolved, subsequently being modified to produce more complicated modes of fission, such as paratomy in which tissue regeneration occurs prior to physical separation. Surprisingly, the molecular phylogeny suggests that in *Convolutriloba* simple transverse fission is a derived asexual mode rather than being ancestral.

Convolutriloba hastifera and *C. longifissura*, the two species in which transverse fission occurs, form a clade and undergo transverse fission in essentially the same way, suggesting that transverse fission is homologous between these two species. The only apparent differences in the process between the two species occur during regeneration of the posterior fragment and are clearly due to the fact that this fragment undergoes a subsequent longitudinal division only in *C. longifissura*.

Despite obvious differences between transverse fission and reversed-polarity budding, both involve tissue separation in the transverse plane and share two key similarities in the process of tissue separation. First, prior to transverse fission, the typically highly organized muscle fibers become locally disorganized along the future plane of fission, similar to the region of disorganized musculature that develops at the

parent/bud boundary during reversed-polarity budding. Second, in both processes behaviorally mediated pulling appears to be key to effecting actual tissue separation. During transverse fission, the posterior end of the animal flattens and adheres strongly to the substrate while the anterior end attempts to move forward and eventually tears free along the region of disorganized musculature, while during reversed-polarity budding the parent and bud pull away from each other, causing the tissue tearing. The actual time it takes for the two pieces to tear apart is much greater during transverse fission than during reversed polarity budding (hours versus seconds), presumably because during fission the posterior portion is stationary rather than moving in a counter-direction and the cross-sectional width of tissue that must be torn is greater.

Longitudinal division of the primary body axis

Although transverse fission is relatively common among animals, longitudinal fission is extremely rare, especially among bilaterians. Some cnidarians reproduce by longitudinal fission (Mire and Venable 1999; Raikova et al. 2007), but among bilaterians I am only aware of a cestode (Specht and Voge 1965) and *C. longifissura* undergoing longitudinal fission. The rarity of asexual division in the longitudinal axis, especially in light of the prevalence of transverse division, may derive from several simple attributes of most bilaterally symmetric animals. Most bilaterians are longer than they are wide, and thus a longitudinal division will result in a larger wound surface area than a transverse division, making the former potentially riskier and energetically more costly to repair. Additionally, accidental breaks of the animal

in the longitudinal plane are much less likely to occur than ones in a transverse plane, making regeneration following longitudinal division less likely to evolve or be selectively maintained. Most bilaterians also possess important medial structures, such as the mouth, gut, or brain, which could make longitudinal division more difficult. Finally, bilateral symmetry is often critical for efficient locomotion in motile bilaterians, and thus the loss of this symmetry after a longitudinal division could severely impair locomotory function.

It is noteworthy that in the two bilaterian examples of longitudinal fission of which I am aware, the stages that undergo longitudinal fission do not possess some of these typical bilaterian features. In *C. longifissura*, longitudinal division occurs only in the posterior fragment produced by transverse fission. This posterior fragment is wider than it is long, so that the longitudinal division results in a smaller wound surface area than would a second transverse fission. It also remains immobile until fully regenerated, eliminating any potential locomotory costs of the asymmetry. This fragment lacks a brain, mouth, and digestive syncytium, which I speculate could hinder a longitudinal division. In support of this hypothesis, when a *C. longifissura* individual is cut transversely slightly anterior to the normal fission plane (making the posterior fragment inherit some of the digestive syncytium), the posterior fragment occasionally attempts to divide longitudinally. When this occurs, the longitudinal fission proceeds across the entire fragment *except* the most anterior region, where the digestive syncytium is present (J.M.S, unpublished data). The cestode *Mycencestoides vogae* can undergo longitudinal division of the scolex, which similarly lacks a gut, has a short A-P length relative to its width, and does not

undergo active locomotion (Specht and Voge 1965; Schmidt and Todd 1978). Thus, the unusual morphological characteristics of longitudinally dividing bilaterians lend support to the hypothesized costs of dividing in this plane of fission.

The process of longitudinal fission in *C. longifissura* is surprisingly different from that of transverse fission. Behaviorally mediated tissue tearing, seen during transverse fission and reversed-polarity budding, is not possible for the longitudinal division of the posterior fragment because this fragment remains completely immobile until regeneration is complete, as do any decapitated individuals in this genus (J.M.S., unpublished data). Instead, muscle fibers along the longitudinal fission plane break in an anterior to posterior direction, slightly ahead of actual tissue separation, causing a progressive unfastening of the internal musculature along the fission plane. Longitudinal fission is a slow process (18-24 hours to completion) and occurs without any obvious signs of strain on the tissue, suggesting that apoptosis or changes in cell adhesion, rather than behaviorally mediated tissue tearing, may effect tissue separation after muscle fibers have been broken.

It is probably not coincidence that two extremely rare modes of asexual reproduction in bilaterians, longitudinal fission and reversed-polarity budding, occur within the same genus. Although outwardly quite different, longitudinal fission and reversed-polarity budding in *Convolutriloba* share a highly unusual feature: a left/right splitting of the posterior-most part of the A-P axis. In *C. longifissura* this is manifested by longitudinal fission that splits the posterior part of the A-P axis into two adjacent A-P axes. In *C. retrogemma* and *C. macropyga*, this is manifested by the left/right pair of budding zones that are exactly medio-lateral along the posterior

margin of the animal and from which the (reversed) axes of the bud are generated. If one were to superimpose *C. longifissura* and *C. retrogemma/macropyga*, the two adjacent posterior axes generated from the posterior fragment of *C. longifissura* would be coincident with the two axes of the posterior buds. I speculate that the evolution of longitudinal fission, so rare among bilaterians, was facilitated in this genus because the *Convolutriloba* ancestor already possessed the ability to split the posterior part of the axis into two, for the purpose of producing left/right posterior budding sites.

Are fission and budding distinct processes?

Essentially all modes of agametic asexual reproduction fall into one of two categories, fission and budding, which differ in fundamental ways (Hughes 1989). Fission products directly inherit some fraction of the original individual's body and therefore normally retain the primary body axis orientation of the original adult. During budding, offspring develop as multicellular outgrowths of the parent and most features of the offspring, including the primary body axis, are formed anew. In *Convolutriloba*, nervous system development differs as expected between fission and budding. During fission, whether transverse or longitudinal, new portions of the nervous system form primarily as extensions of the remaining portions of the nervous system. During budding, in contrast, the new nervous system forms entirely *de novo*, without any apparent contact with the parent's nervous system. In addition to these morphogenetic differences, budding and fission are correlated with different lifestyles. Budding is most common among sessile, often colonial metazoans, such as cnidarian polyps, bryozoans, tunicates, and pterobranch hemichordates, while fission

tends to be common among more motile, non-colonial metazoans, such as annelids, flatworms, and echinoderms. This correlation between mode of reproduction and motility may be due to the fact that bud outgrowths could impair locomotion in highly motile animals. The two *Convolutriloba* species that reproduce by reversed-polarity budding represent unusual examples of budding occurring in motile metazoans. That these species' buds are produced directly posteriorly, rather than laterally, may minimize the impact of budding on the parent's locomotion.

Despite important differences between fission and budding, the data indicate a close relationship between the two processes in *Convolutriloba*. Asexual reproduction abilities among *Convolutriloba* acoels apparently derive from a single, recent origin of asexual reproduction, since all four *Convolutriloba* species reproduce asexually (as well as sexually) but most of their close relatives as well as most acoels are strictly sexual. This suggests that asexual reproduction, whether by fission or budding, is probably homologous at some level among all four *Convolutriloba* species. Homology of fission and budding is supported by underlying similarities between the two processes, including disorganization of musculature and behaviorally mediated tearing (seen in budding and transverse fission), digestion of symbiotic algae in early-stage fission products and buds that do not yet possess a mouth (seen during budding, transverse fission, and longitudinal fission), and some indication of a dual, posterior axis field (evident during budding and longitudinal fission). Thus, although fission and budding are generally considered to be distinct modes of asexual reproduction, I demonstrate that evolutionary switching between budding and fission can occur and

that the mechanism for this may at least in part be through recruitment of homologous underlying processes that can give rise to divergent modes of reproduction.

Conclusions

Convolutriloba species are closely related but have undergone a remarkable evolutionary diversification of asexual reproductive mode, presumably in a short amount of evolutionary time. Acoels (like platyhelminths) possess totipotent stem cells called neoblasts distributed throughout their bodies and these cells are critical for regeneration and presumably for asexual reproduction as well (Gschwentner et al. 2001). Although neoblasts undoubtedly help to explain the extensive regeneration abilities of acoels, their presence cannot in and of itself explain the diversification of asexual reproductive mode since all acoels presumably possess neoblasts yet most acoels do not reproduce asexually. I hypothesize that a key innovation that permitted the diversification of asexual reproductive mode in *Convolutriloba* was the splitting of the posterior axis field into two fields, manifested as dual posterior budding zones during reversed-polarity budding and as longitudinal splitting of the posterior portion of the A-P axis during longitudinal fission. The evolution of a novel modification of the primary body axis may be a driving factor in the diversification of asexual reproduction in other animal groups as well. Understanding the molecular mechanisms underlying the malleability of the primary body axis in *Convolutriloba* and other diverse asexual clades is a critical next step for understanding the developmental basis of asexual diversification.

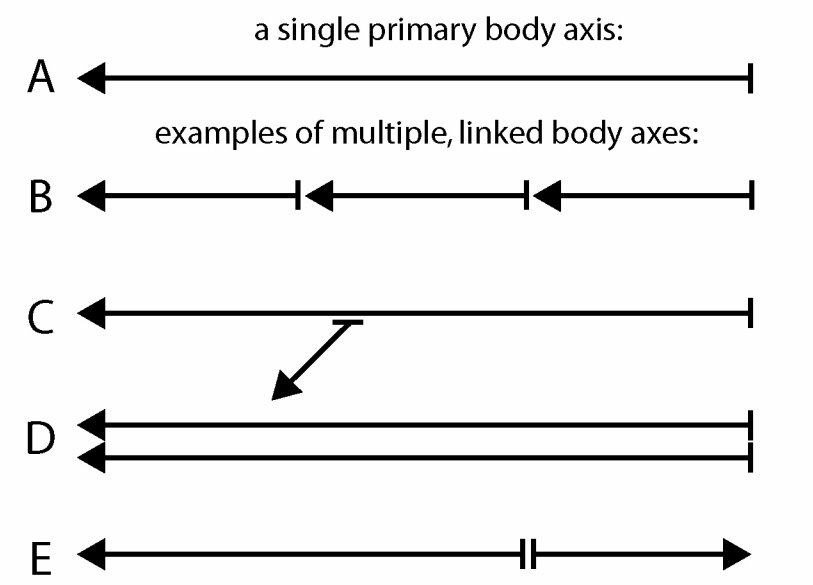

Figure 2.1. The primary body axis can be dramatically altered during asexual reproduction. (A) Typically animals have a single primary body axis, but during asexual reproduction this axis can (B) become subdivided into tandemly arrayed axes, (C) become branched, (D) become subdivided longitudinally to form two adjacent axes, or (E) give rise to axes with opposite orientations.

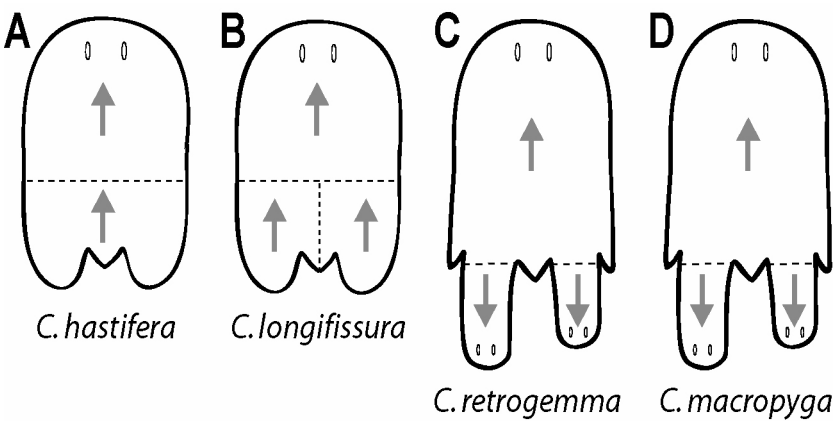

Figure 2.2. Modes of asexual reproduction among the four species of *Convolutriloba*. (A) *C. hastifera* reproduces by a single transverse fission. (B) *C. longifissura* reproduces by transverse fission followed by longitudinal fission of the posterior fragment. (C, D) *C. retrogemma* and *C. macropyga* produce buds with reversed A-P axes relative to the parent axis. Buds form from left and right budding sites and often develop asynchronously. Arrows indicate the direction of the A-P axis with arrowheads pointing anterior. Dashed lines represent the planes of separation that occur during reproduction.

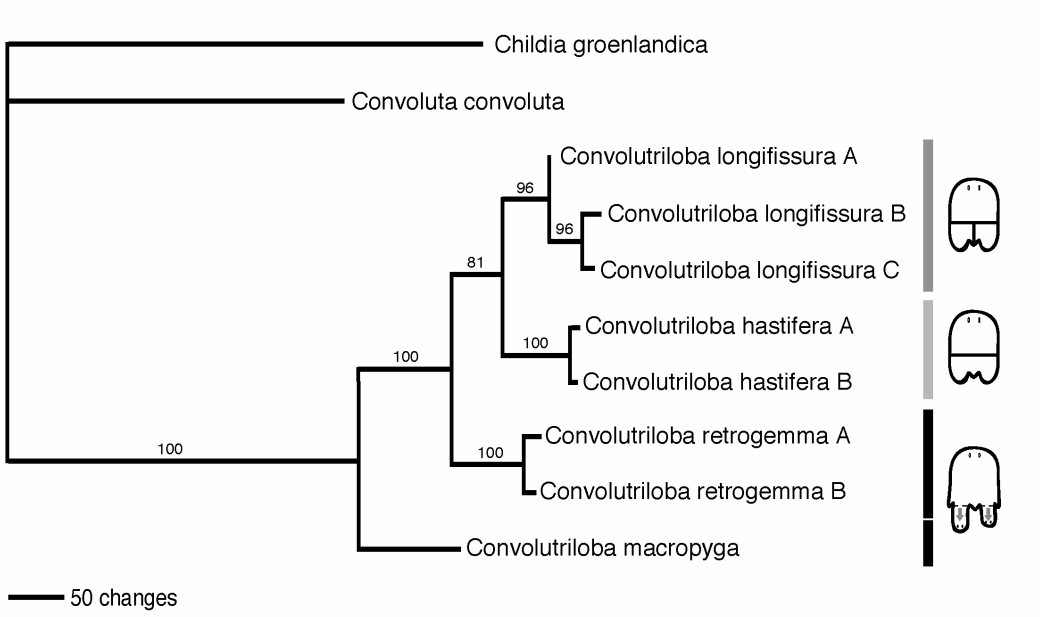

Figure 2.3. Maximum parsimony tree of *Convolutriloba* species based on the combined data set (28S, 18S, and COI). Bootstrap support values (1000 replicates) are indicated above branches. MP consistency index = 0.90, retention index = 0.74, and rescaled consistency index = 0.67. Maximum likelihood analysis produced a single best tree with an identical topology. Mapping asexual reproduction mode onto this tree suggests that reversed polarity budding is the ancestral mode of asexual reproduction in the genus.

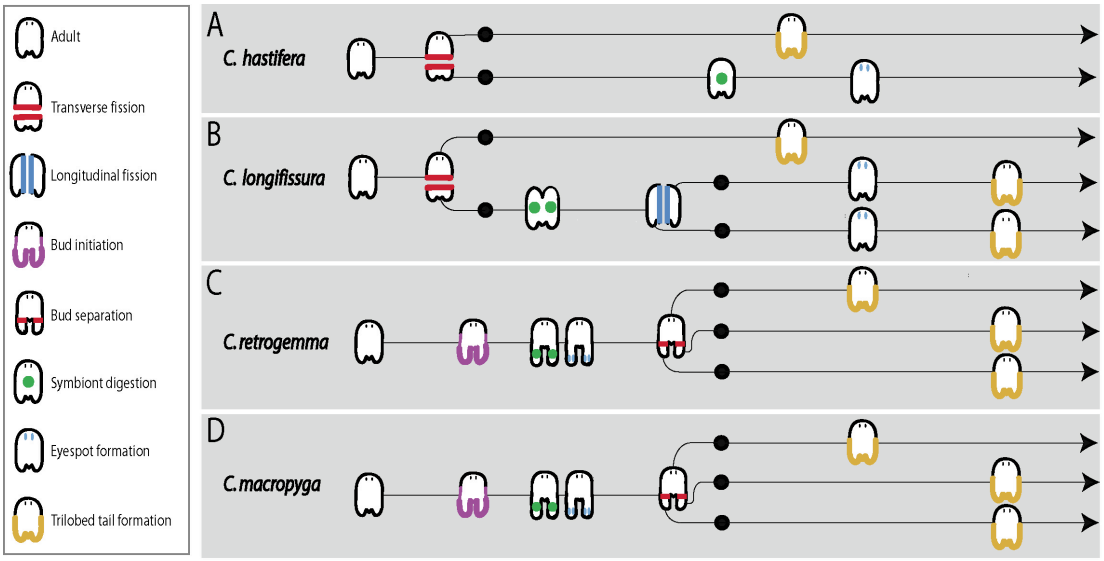

Figure 2.4. Sequence and relative timing of morphological changes during asexual reproduction among *Convolutriloba* species. Major events (left) are indicated for each of the four species (A-D) during the course of one complete round of asexual reproduction. Note that many of these events have been previously described (e.g., Hendelberg and Åkesson 1988; Hendelberg and Åkesson 1991; Åkesson et al. 2001; Shannon and Achatz 2007; see Results for details).

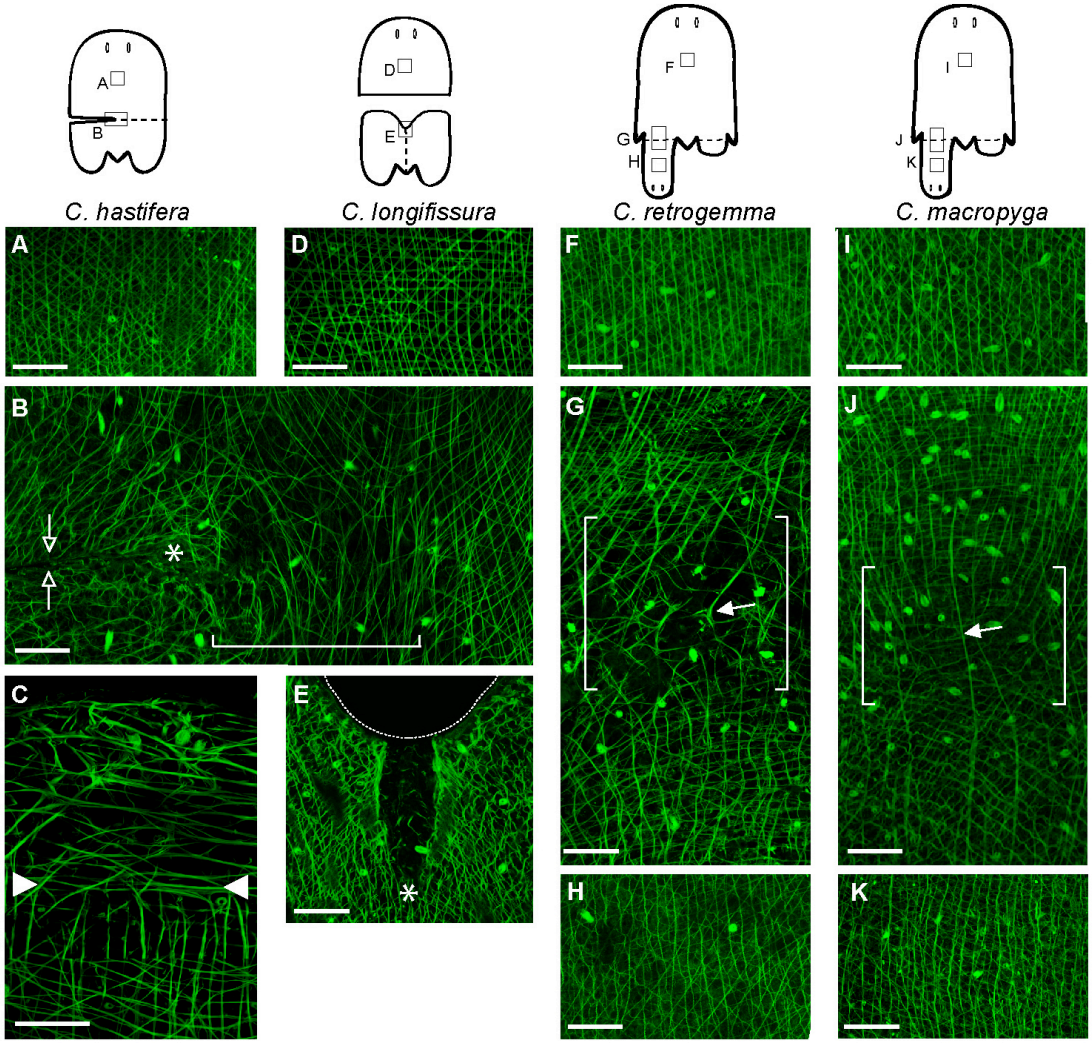

Figure 2.5. Confocal projections of phalloidin-stained muscle fibers during asexual reproduction. (A, D, F, I) Dorsal adult body-wall muscle patterns are similar in the four *Convolutriloba* species. (B) Muscle fibers become disorganized along the transverse fission plane just prior to tissue separation in *C. hastifera* (not shown) and *C. longifissura* (shown here). Bracket indicates region of disorganized muscle fibers. Open arrows point to the fissure (progressing from left to right) and indicate the plane of transverse fission. Asterisk indicates the leading edge of the fissure. (C) During

regeneration following transverse fission (shown here in *C. hastifera*), circular muscle fibers form first within the blastema (shown here), while formation of diagonal and longitudinal fibers occurs secondarily (not shown). Arrowheads mark the original plane of transverse fission. (E) During longitudinal fission in *C. longifissura* muscle fibers break along the fission plane just prior to tissue separation. Asterisk indicates the leading edge of muscle separation (progressing from top to bottom). Dotted line indicates contour of the tissue. (G, J) During reversed polarity budding in both *C. retrogemma* and *C. macropyga*, a region of disorganized muscle fibers (indicated by a bracket) develops at the base of the developing bud. Some fibers cross this zone (arrows). (I, J) In the buds of *C. retrogemma* and *C. macropyga*, muscle fibers have the typical pattern of adults. Ovoid structures on A-K are spiral muscle mantles surrounding defensive secretory structures (sagittocysts). All images are dorsal views. Scale bars are 100 μm.

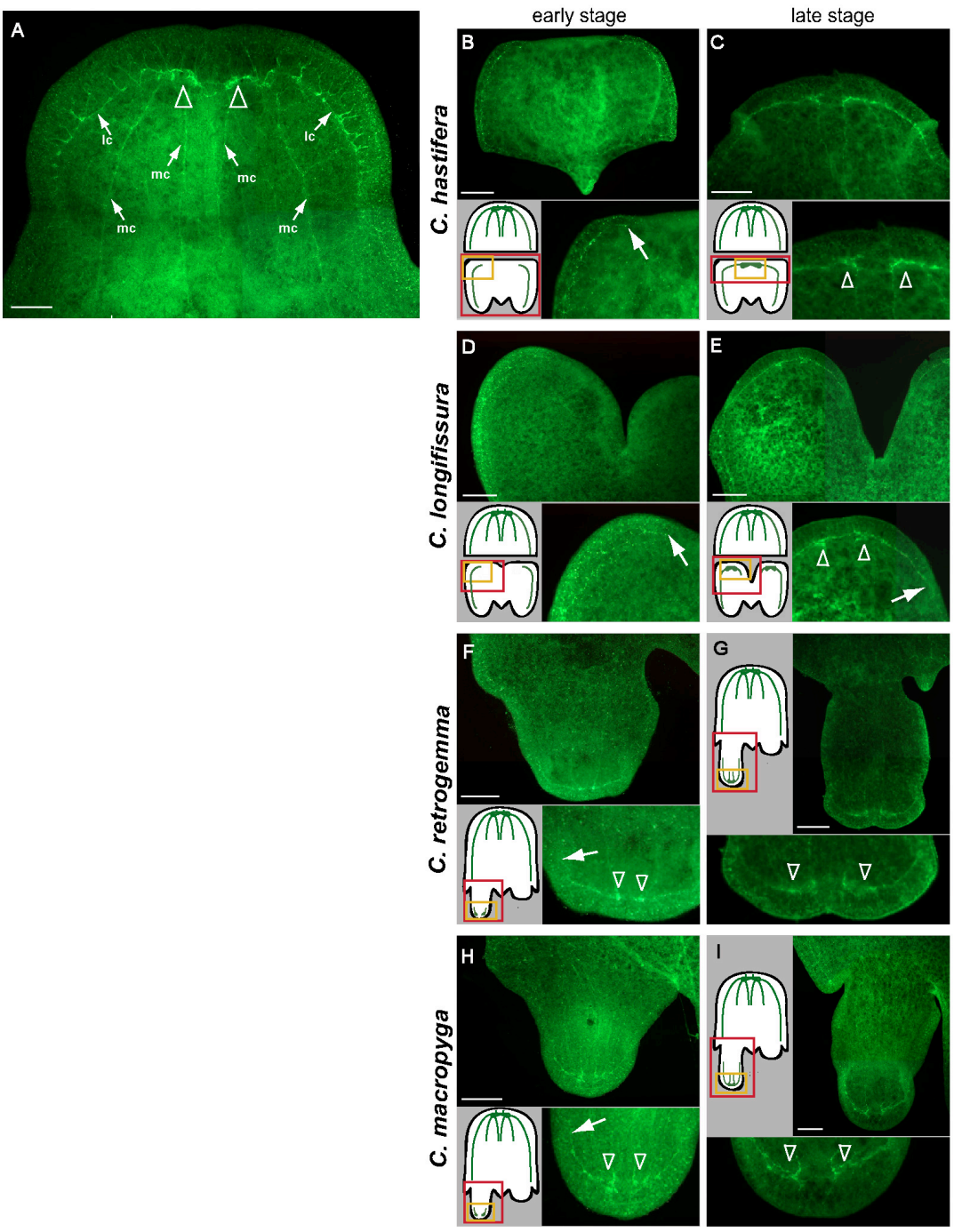

Figure 2.6. Serotonin immunostaining of the central nervous system of *Convolutriloba*. (A) The central nervous system of *Convolutriloba* (*C. retrogemma* shown here) consists of a bilobed ganglion (open arrowheads), two lateral nerve cords (lc), and four medial nerve cords (mc). (B-I) Early and late stages of nervous system

formation during asexual reproduction in the four *Convolutriloba* species. Larger, lower magnification views (red) and smaller, higher magnification views (yellow) are shown for each. Arrows indicate the ends of lateral nerve cords. Open arrowheads indicate the lobes of the ganglion. After transverse fission in *C. hastifera*, the lateral nerve cords of the posterior fragment initially extend antero-medially (B) and later the ganglion forms where they meet (C). During longitudinal fission in *C. longifissura*, the lateral nerve cords of the posterior fragment initially extend antero-medially (D), but the longitudinal fissure prevents them from meeting. The two lobes of the ganglion and the lateral nerve on the new lateral side then form (E). During reversed polarity budding in *C. retrogemma* and *C. macropyga,* the ganglion forms *de novo* followed by the formation of the nerve cords (F- I). Scale bars are 250μm.

Chapter 3: Asexual acoels possess a diverse regeneration toolkit

Abstract

Acoels in the genus *Convolutriloba* have evolved dramatically different modes of asexual reproduction, including examples of transverse fission, longitudinal fission, and reversed polarity budding. In this study, I conducted parallel regeneration trials on three of the four *Convolutriloba*, representing all three asexual modes, to elucidate the regenerative capacities that may have allowed for the evolution of such different asexual reproduction strategies. I found that all three *Convolutriloba* species have remarkable and largely similar regenerative abilities. They can regenerate anteriorly, posteriorly, and laterally, and can even regenerate from a small fraction of the original adult. They also appear to use both epimorphic and morphallactic regeneration, as they replace lost anterior and lateral body regions through an epimorphic process (producing a discrete blastema) and regenerate posterior tissues largely through a morphallactic process of tissue remodeling (with little if any increased cell proliferation). Transverse amputations during stages of fission suggest that the respecification of both anterior-posterior and left-right axes during asexual reproduction occurs progressively, rather than simultaneously, as tissues physically separate. Surprisingly, I also found that following certain amputations, all three species will regenerate anteriorly multifurcated axes (multiple heads), suggesting that the ability to produce adjacent axes through regeneration predated the origin of longitudinal fission (which similarly produces adjacent axes). Collectively, the data indicate that diversification of *Convolutriloba* asexual

reproduction was not accompanied by diversification of regenerative abilities. Instead, I suggest that the *Convolutriloba* ancestor already possessed a rich regeneration toolkit that may have allowed for extreme diversification of asexual reproduction in the group.

Introduction

A close relationship between asexual reproduction and regeneration has long been noted (Morgan 1901). Groups that have evolved asexual reproduction (e.g., by budding or fission) tend to also have high regeneration abilities, although regeneration is found in many animals that do not reproduce asexually (Morgan 1901; Sanchez Alvarado 2000). Many animal groups with extensive regenerative abilities have evolved multiple examples of asexual reproduction, including cnidarians (Holstein et al. 2003), platyhelminths (Berrill 1952; Egger et al. 2007), and annelids (Berrill 1952; Giese and Pearse 1975), all of which have evolved diverse modes of fission, budding, and fragmentation in various clades. Comparative developmental studies of regeneration and asexual reproduction have also shown that morphogenesis during regeneration and asexual reproduction is very similar, (Berrill 1952; Giese and Pearse 1975; Bely and Wray 2001; Burton and Finnerty 2009). Together, these patterns suggest that asexual reproduction strategies may often evolve from an underlying ability to regenerate lost body regions.

One of the most remarkable examples of asexual reproduction diversification among bilaterians occurs within the acoels, a group of small marine worms thought to represent the most basal bilaterian lineage (Ruiz-Trillo et al. 1999). While the group has a few representatives that reproduce asexually, most acoels do not reproduce asexually (Reuter and Kreshchenko 2004). However, the genus *Covolutriloba* is a genus of four relatively derived, symbiotic acoels that has evolved three different modes of asexual reproduction among its four species (Fig. 3.1). *Convolutriloba hastifera* reproduces by a transverse fission event (Gaerber et al. 2007); *C.*

longifissura reproduces by a transverse fission followed by a longitudinal fission in the posterior fragment (Bartolomaeus and Balzer 1997; Åkesson et al. 2001); and *C. retrogemma* and *C. macropyga* reproduce by an unusual process of reversed polarity budding (Hendelberg and Åkesson 1988; Shannon and Achatz 2007). The previous phylogenetic studies reveal that reversed polarity budding is the ancestral mode of asexual reproduction in *Convolutriloba*, suggesting that fission is the derived mode of asexual reproduction in the genus (Sikes and Bely 2008).

The axial modifications that occur as part of these asexual reproduction strategies in *Convolutriloba* are highly unusual for animals. *Convolutriloba retrogemma* and *C. macropyga* represent the only examples of primary body axis reversal among bilaterians, and *C. longifissura* represents one of only two known examples of longitudinal fission among bilaterians (Sikes and Bely 2008). Despite the diverse axial modifications that occur during these different asexual reproduction modes, several aspects of asexual reproduction are surprisingly similar across species (Sikes and Bely 2008).

While *Convolutriloba* evolved such diverse asexual reproduction strategies, their closest relatives do not reproduce asexually but do have regenerative abilities (Åkesson et al. 2001), consistent with *Convolutriloba* evolving these asexual reproduction abilities at least in part from a pre-existing ability to regenerate. Since few related animal groups display a diversity of asexual reproduction strategies, I know of no studies that have attempted to understand diversification of asexual reproduction modes through the study of comparative regeneration processes. Acoels

in this genus offer a unique opportunity to study the process by which regenerative abilities can shape the diversification of asexual reproduction modes.

Acoels, like planarians, have stem cells, called neoblasts, distributed throughout the body, and these cells are the only mitotically active cells in the adult (Gschwentner et al. 2001). In stark contrast to the extensive knowledge we now have about planarian regeneration, which has been studied at the morphological, cytological, developmental, and functional levels (Reddien and Sanchez Alvarado 2004; Sanchez Alvarado 2006), virtually nothing is known about regeneration in acoels, despite the expectation that they too have extensive regeneration abilities. No studies of body-level patterning or the dynamics of cell proliferation during regeneration of different body regions have been conducted, nor has the regenerative potential of *Convolutriloba* specifically been assessed.

I conducted comparative regeneration experiments on three of the four *Convolutriloba* species, representing all three modes of asexual reproduction in the genus, to determine whether extensive regenerative potential might underlie asexual reproduction diversity. I performed a range of amputations on these species to assess their regenerative capacity, assayed cell proliferation during regeneration to determine how acoels replace lost tissues, and investigated the expression of a conserved anterior patterning gene following amputation. Finally, I conducted tissue excisions in the three species to assess whether the extreme axial modifications that occur during asexual reproduction can be elicited through regeneration.

Materials and methods

Animal collection & culture

Convolutriloba hastifera, C. longifissura, and *C. retrogemma* were originally collected from marine aquaria housing Indo-Pacific corals at retail aquatic stores in Maryland (USA). An isogenic line of each species (originally established from a single individual) was cultured in artificial seawater (ASW) as previously described (Sikes and Bely 2008). Adult worms were maintained under full spectrum 10,000K artificial illumination (critical for growth of their symbiotic algae) and fed *Artemia* nauplii weekly as a food source. Amputated individuals and tissue fragments (see below) were illuminated but not fed.

Cell proliferation

In all three species, S-phase cells were labeled with BrdU to reveal cell proliferation dynamics during regeneration. Two different types of experiments were performed: 1) In cut-pulse-chase experiments, worms were amputated transversely at the mid-body (n=72/species) or longitudinally along the midline (n=72/species) and immediately incubated in BrdU for 2h. Worms were then maintained in ASW for 0h, 8h, or 24h (n=24/species/treatment) before fixation. 2) In pulse-cut-chase experiments, worms were incubated in BrdU for 12h (n=24/species) and then amputated transversely at the mid-body. Worms were then maintained in ASW for 48h before fixation.

For both of the above schemes, worms were incubated in 0.1 mg/ml BrdU for 2h at 25^{o}C. Worms were relaxed in 3.4% $MgCl_2$, fixed in 3.7% formaldehyde in

0.75X phosphate-buffered saline (PBS) for 30 min, washed at least three times in PBS, washed in PBTx (PBS + 0.01% Triton-X), incubated in 75% HCl at 37^{o}C for 30 min, and washed 5x in PBTx. Samples were then blocked in 10% normal goat serum (NGS) in PBTx for 1 h at room temperature and incubated in mouse anti-BrdU monoclonal antibody (Sigma, St. Louis, MO, USA) at 1:100 in 10% NGS/0.9X PBTx overnight at 4^{o}C. Samples were rinsed repeatedly in PBTx over 1h, incubated in a FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA) at 1:200 in 10% NGS/0.9X PBTx for 5h at room temperature, rinsed three times in PBTx, and finally in PBS. Samples were mounted in 70% glycerol.

Nuclear staining

To stain nuclei of all cells during regeneration in the three species, worms were relaxed in 3.4% $MgCl_2$, fixed in 3.7% formaldehyde in 0.75X PBS for 1 h at room temperature, washed at least three times in PBS, and washed in PBTx. Samples were then incubated in 0.01 mg/ml Hoescht 33258 stain (Thermo Fisher Scientific, Rockford, IL, USA) in PBTx overnight at 4^{o}C. Samples were washed three times in PBTx and finally in PBS. Samples were mounted in 70% glycerol.

Regeneration experiments

In all three species, tissue was either amputated or excised from adults. To avoid any potential anesthesia-related regeneration artifacts, no anesthesia was used for these or any other cuts performed in this study. Amputated worms and excised tissues were maintained singly in wells of 24-well cell-culture trays containing ~2ml

ASW and maintained at 24°C with a 12h:12h light:dark cycle. Daily observations were made to assess the progress of regeneration.

Three different regeneration experiments were performed: 1) In mid-body tissue excision experiments, ~0.5mm x 0.5mm fragments of tissue were excised from the adult body, between the lateral edge of the animal and the digestive syncytium (but including neither of these). The progress of regeneration was recorded daily for five days. 2) In transverse amputation experiments, adult worms, posterior fission products, and worms undergoing transverse fission were amputated transversely at the mid-body. *Convolutriloba longifissura* posterior fission products were amputated at different stages of longitudinal fission with some amputations made ~0.5 mm below the leading edge of the longitudinal fissure and others made ~0.5 mm above it. Animals were observed daily and the timing of regenerative events, such as digestion of symbiotic algae and the formation of eyespots and trilobed tails were recorded. In addition to these regeneration experiments, adult worms (n=12/species) were wounded with a partial transverse cut approximately at the plane of transverse fission (about ¾ of the way down the length of the body) and then transversely amputated at the mid-body (i.e., slightly anterior to the partial cut). Regeneration of the posterior fragments was observed after four days. 3) In body-margin tissue excision experiments, ~0.5mm x 1mm fragments of tissue were excised from the following body regions: a) mid-body between the syncytium and lateral edge, b) along the posterior margin between the lateral and central lobes, c) along the posterior margin at the central lobe, d) along the posterior margin to include the lateral margin, e)

along the lateral edge at the mid-body, and f) along the anterior edge between the eyespots. Fragments were scored after four days.

CrOtx1 *gene isolation and whole mount in situ hybridization*

I isolated a homolog of the conserved anterior patterning gene orthodenticle (*otx*) from *C. retrogemma* by first amplifying a small fragment of the homeobox from genomic DNA through PCR using the degenerate primers OTX A+ (5'-MGTAARCARMGTMGIGARMGIAC-3') and OTX B- (5'-TGAACTCTKGAYTCTGGIARATT-3'). Reactions (25μL including 0.5 U Platinum *Taq*, Invitrogen, Carlsbad, CA, USA) were performed using 30 cycles at 60^{o}C to 45^{o}C (temperature decreased 0.5^{o}C/cycle) annealing with 3 min extension, followed by 20 cycles at 50^{o}C annealing with 3 min extension. To obtain additional sequence of *CrOtx1* (thus named because a second homolog of *otx* was also identified, see Chapter 3), I performed 2 rounds of 3'RACE PCR using nested primers. Total RNA (isolated from ~50 mg adult worms using RNAwiz (Ambion, Austin, TX, USA)) was reverse transcribed and amplified using published reverse transcription and anchor primers (Frohman 1990). Gene specific primers for the first and second RACE PCR respectively were OTX D+ (5'-TTTGGAGGCTCTGTTTGGG-3') and OTX E+ (5'-ATCCGGACATCTTCATGAGG-3'). PCR products were cloned into pGEM-T (Promega, Madison, WI, USA) and sequenced. I used NCBI BLAST searches to identify related sequences and used nucleotide sequences of the homeobox regions to

generate neighbor-joining trees using PAUP* (v. 4.0b10, Sinauer Associates, Sunderland, MA, USA).

To characterize expression patterns of *CrOtx1* during regeneration, sense and antisense digoxigenin-labeled in situ riboprobes (1.4 kb in length) were generated from RACE fragments using the T7 promoter (Ambion). I used an in situ hybridization protocol based on that of Hejnol and Martindale (2008) for acoel embryos and juveniles. Worms were relaxed 10 minutes in 3.4% $MgCl_2$ prior to fixation and hybridization was carried out using 2.5 ng/μl probe at 60^{o}C. Specimens were mounted in 70% glycerol.

Nervous system immunostaining

The serotonergic components of the nervous system of relevant regeneration stages were revealed by serotonin immunostaining as previously described (Sikes and Bely 2008).

Image acquisition and processing

Images of live specimens (using DIC optics) and stained specimens were captured with a Zeiss Axioplan 2 epifluorescence microscope equipped with a Zeiss AxioCam HRc camera (Carl Zeiss Microimaging, Thornwood, NY, USA). Image processing and the generation of montages of multiple images were made with Adobe Photoshop (v. 10.0).

Results

Regenerative capabilities

All three *Convolutriloba* species could fully regenerate after transverse or longitudinal amputations as well as from small excised tissue fragments. *Convolutriloba hastifera* and *C. longifissura* complete the regenerative process about one day faster than *C. retrogemma*. Following transverse amputation, the anterior fragment regenerated a trilobed tail within 2-3 days (Fig. 3.2H), while the posterior fragment regenerated a head complete with eyespots within 4-5 days (Fig. 3.2P). Lateral regeneration after longitudinal amputation was complete after 4-5 days (Fig. 3.2T). Tissue excisions regenerated a complete head and tail within 4-5 days.

Cell proliferation

In all three species, BrdU-labeling experiments revealed similar patterns of cell proliferation during anterior and lateral regeneration, but different patterns during posterior regeneration. Following transverse cuts, posterior regeneration in all three species proceeded with little or no increase in cell proliferation associated with the wound site. After cut-pulse-chase experiments, there was labeling throughout the fragment, typical of normal growth (Gschwentner et al. 2001), but only a slight concentration of labeled cells at the posterior wound site (Fig. 3.2A-E). The small accumulations of BrdU-positive cells after the 8-hour chase may represent cell division associated with the wound healing process and not represent proliferation associated with regeneration. After pulse-cut-chase experiments, there was no concentration of labeled cells at the posterior wound site at all, indicating that

neoblasts (labeled during the initial pulse) do not accumulate or differentially proliferate at posterior wound sites (Fig. 3.2F). In addition, cell density remains low along the posterior wound site (Fig. 3.2G)

In contrast, anterior in all three species proceeded with significant increases in cell proliferation at the wound site. The cut-pulse-chase experiments revealed increasing levels of cell proliferation at the anterior wound site as the chase period increases (Fig. 3.I-M). After pulse-cut-chase experiments, there was a considerable concentration of labeled cells making up most of the regenerative blastema (Fig. 3.2N). The cut-pulse-chase experiments during lateral regeneration revealed increases in cell proliferation at the wound site similar to those observed during anterior regeneration (Fig. 3.2Q-S), and increases in cell density as the blastema appeared (Fig. 3.2O).

During anterior and lateral regeneration an initial region of clear tissue appears at the wound site that later develops into a regenerative blastema. This tissue has a high concentration of algal symbionts and initially lacks the characteristic red rhabdoid gland cells. After four to five days eyespots are visible and the regenerated tissue gains the characteristic yellow-green color of adult *Convolutriloba* individuals with normal algal symbiont concentrations. Small rhaboid gland cells appear and increase in size shortly after regeneration is complete. During posterior regeneration following transverse amputation, no obvious blastema appears yet the trilobed tail reappears at the wound site usually within three to four days, yielding an individual half its length prior to amputation. No alterations occur in the body-wide distribution of algal symbionts or rhabdoid gland cells.

Regeneration & patterning of midbody excised tissues

Mid-body tissue excision experiments (Fig. 3.3A) resulted in the regeneration of a new individual in all three species (Fig. 3.3B-D). While the excised square of tissue maintained its cuboidal shape for the first day after excision, by day two fragments took on a more circular shape (Fig. 3.3E-J). Between one to three days after excision, these tissue fragments digested some of their symbiotic algae, producing a dark spot somewhere within the body (Fig. 3.3F-H) similar to algal digestion that occurs during asexual reproduction (Åkesson and Hendelberg 1989; Hendelberg and Åkesson 1991). Within four days after excision, the first indication of eyespots became apparent and the algal digestion spot disappeared (Fig. 3.3I). By day five, the fragment had regenerated a complete trilobed tail and had completed eyespot development (Fig. 3.3J). *CrOtx1* was not expressed within the adult body before tissue excision nor in tissue fragments immediately following excision (Fig. 3.3K). However, one day after tissue excision marginal cells along one half of the perimeter of the animal expressed *CrOtx1* (Fig. 3.3L), presumably marking the future anterior end. This broad band of expression became more restricted at the anterior tip from two to four days (Fig. 3.3M-O). At five days, *CrOtx1* was no longer expressed within the body of the newly regenerated worm (Fig. 3.3P). While few serotonergic cells were visible within the first three days of regeneration after tissue excision, the entire serotonergic nervous system appeared four days after tissue excision, coincident with the formation of eyespots (Fig. 3.3R-W).

Regeneration during fission

Transverse amputation during longitudinal fission in *C. longifissura* had different outcomes depending on the fission stage. Nonreproductive adults of all three species regenerated a single head (Fig. 3.4, A-C & D). Similarly, anterior regeneration of *C. hastifera* posterior fission products led to the formation of a single head (Fig. 3.4B). However, anterior regeneration of *C. longifissura* posterior fission products resulted in two different outcomes based on the stage of longitudinal fission. A single anterior axis developed if amputations were made below the active longitudinal fission plane (Fig. 3.4, C & E), but two parallel anterior axes regenerated when amputations were made at or above the longitudinal fission plane (Fig. 3.4, C & F). Following regeneration, these parallel axes continue longitudinal fission eventually separating into two individuals. In addition, when a *C. longifissura* individual in the process of transverse fission was amputated at the mid-body (just anterior to the fission plane), two heads regenerated, a larger at the wound site produced by the transverse amputation and a smaller, slightly lateral one at the site where transverse fission had initiated (Fig. 3.4C). The width of this smaller head appeared to roughly match the extent of the transverse fissure. However, worms that were partially cut to artificially simulate a partial transverse fission event and then transversely amputated regenerated only a single head and no lateral heads (data not shown).

Regeneration of tissues excised from body margins

Body margin tissue excision experiments revealed unusual axial regeneration potentials in all three species. While small rectangular tissue fragments excised fully from within the body, that is, with four amputation planes, consistently regenerated a single axis in *C. hastifera* and *C. longifissura* (Fig. 3.5, A & G), such excisions occasionally regenerated reverse anterior axes in *C. retrogemma* (Fig. 3.5A). Additionally, tissue fragments excised along the posterior margin of the body, created as a result of three amputation planes, often regenerated reverse anterior axes in *C. retrogemma* when cuts were located between the lateral and central lobes (Fig. 3.5, B & H). While the remaining *C. retrogemma* individuals and all *C. hastifera* and *C. longifissura* individuals regenerated head(s) on only one end, many of these regenerates formed two or more heads along the anterior side (Fig. 3.5, B & I-J). Excisions around the posterior central lobe had the highest frequency of multifurcated axis regeneration in two of the three species (Fig. 3.5C). Other tissue excisions made along either the lateral or anterior margins, also created by three amputation planes, consistently regenerated a single axis in all species (Fig. 3.5E-F). Finally, tissue excisions made with only two amputation planes, retaining the original posterior and lateral edges of the animal, regenerated a single head and tail in most cases (Fig. 3.5D). Serotonin immunostaining revealed that regenerates with bifurcated anterior axes have complete central nervous systems with a bilobed ganglion and all associated nerve cords (Fig. 3.5K-L).

Discussion

Acoels in the genus *Convolutriloba* are unusual among metazoans in that closely related species have evolved very different modes of asexual reproduction. Among bilaterians, there is a close tie between regeneration and asexual reproduction, and it has been suggested that asexual reproduction evolves from an organism's pre-existing ability to regenerate (Morgan 1901; Reuter and Kreshchenko 2004). To elucidate the relationship between asexual reproduction and regeneration in *Convolutriloba*, I performed parallel regeneration trials to reveal the regenerative potential of three *Convolutriloba* acoels, each with a different mode of asexual reproduction.

Epimorphosis and morphallaxis

Animals that regenerate generally do so by utilizing one or both of two major regenerative processes: morphallaxis and epimorphosis (Morgan 1901). Morphallaxis refers to regeneration without cell division and involves the remodeling of tissues near a wound site into new structures, while epimorphosis refers to the regeneration of lost body regions through active cell proliferation and the formation of an undifferentiated mass of cells called the blastema. In *C. hastifera, C. longifissura*, and *C. retrogemma* I found increased levels of cell proliferation at the wound site during anterior and lateral regeneration but no or very limited increases in cell proliferation during posterior regeneration. Furthermore, a visible blastema largely devoid of pigmented cells forms during anterior and lateral regeneration but not in posterior regeneration, and the regeneration of the trilobed tail occurs more quickly than either anterior or lateral regeneration. These data suggest that anterior

and lateral regeneration occur through epimorphosis while posterior regeneration occurs through morphallaxis. Interestingly, these patterns of cell division are similar to those observed during the regrowth of tissues in the products of transverse fission in both *C. hastifera* and *C. longifissura* (data not shown).

While morphallactic and epimorphic processes have been described from both protostome and deuterostome bilaterians (Agata et al. 2007), cnidarians are thought to regenerate missing structures exclusively by the process of morphallaxis (Holstein, Hobmayer, and Technau 2003). Thesedata suggest that acoels, thought to represent the most basal bilaterian lineage (Ruiz-Trillo et al. 1999), have the ability to regenerate by both morphallaxis and epimorphosis, like other bilaterians. This pushes back the origin of epimorphic regeneration to the base of the Bilateria.

Pulse-chase BrdU experiments suggest two possible scenarios by which stem cells, or neoblasts, may give rise to the anterior and lateral regenerative blastemas in *Convolutriloba* acoels. Neoblasts in the adult body may undergo cell division with subsequent migration of one daughter cell to the wound site where blastema formation occurs. This mechanism of stem cell division followed by directed migration into the blastema has been described during regeneration in planarians (Salo and Baguna 1989), which have a neoblast system similar to acoels (Gschwentner et al. 2001). Another possible mechanism for the formation of the anterior blastema is increased and repeated division of neoblasts at the wound site and within the blastema itself. Such increases in the extent of cell division at the blastema are typical of vertebrate limb regeneration following dedifferentiation of cells at the wound site (Nye et al. 2003). BrdU experiments without a chase show no increase in

cell proliferation at the wound site or in the blastema suggesting little to no variation in the rate of cell division during the regenerative process. Thus, I propose that epimorphic regeneration in *Convolutriloba* occurs through increased levels of cell proliferation at the wound site and throughout the regenerative blastema until regeneration is complete.

Remarkable regenerative abilities in Convolutriloba *acoels*

Convolutriloba longifissura, C. hastifera, and *C. retrogemma* can regenerate all parts of their bodies and can even regenerate from small fragments of the original individual. The regeneration from these small fragments is exceptional as an entire animal with typical morphology and a normal central nervous system is re-established from tissue fragments approximately $1/20^{th}$ the size of the original adult. These fragments undergo extensive respecification as they must generate new anterior, posterior, and lateral edges as well as respecify the body's midline. Regeneration is quite rapid: anterior and lateral regeneration are completed within 4-5 days, posterior regeneration is complete within 2-3 days, and whole-body regeneration from a small excised fragment is accomplished within 4-5 days. The timing of anterior, posterior, and lateral regeneration are roughly comparable to anterior, posterior, and lateral development following fission (Sikes and Bely 2008). While not a requirement for extensive regenerative abilities, the presence of an evenly distributed pool of neoblasts throughout out the body likely plays an important role in providing such extensive regeneration potentials. In addition, the presence of symbiotic algae within the bodies of *Convolutriloba* acoels provide regenerating fragments with a food

source, as algae may provide animals with energy either through photosynthesis or and by being digested directly. This ability to "feed" even when the individual is devoid of most structures probably contributes to the high survival rate and rapid regeneration from even the smallest of tissue fragments in *Convolutriloba*. A similar suggestion has been made from comparisons of symbiotic and nonsymbiotic *Hydra* species (Bossert and Slobodkin 1983).

Interestingly, amputations made *during* the process of fission in *C. longifissura* indicate that the respecification of a single A-P axis into two axes during fission does not occur at once but instead occurs gradually, as fission progresses. A transverse amputation made just above the transverse fission plane after an individual has initiated the process of transverse fission results in the regeneration of a smaller lateral head in addition to the regeneration of the "normal" head of the primary A-P axis. The asymmetry in size between the lateral and main heads suggests that the lateral head is the product of a subdivided posterior axis that is half the width of the worm's body with a modified left-right axis, while the larger head is the product of regeneration along the normal A-P and left-right axes. Therefore the primary body axis of animals in the middle of transverse fission is subdivided into a second posterior A-P axis posterior to the transverse fission plane on one side, yet this same animal maintains a single intact A-P axis the entire length of the body on the other side. If a wound similar to a mid-stage transverse fissure is made on adult *C. longifissura* and the animal is then amputated above this wound, only a single head regenerates suggesting that these animals do not become competent to make twin A-P axis until transverse fission is initiated. In addition, transverse amputations made on

the posterior fission product (after transverse fission is completed) reveal the temporal nature of axial respecification during longitudinal fission. If amputations were made below the leading edge of the longitudinal fissure, regeneration of a single head occurred, but transverse fissions made at or below the edge of the longitudinal fissure resulted in the regeneration of two parallel A-P axes. Further studies exploring midline patterning and the genetic control of axial respecification will be important in understanding how longitudinal fission occurs.

Given that excised fragments of tissue from the mid-body region of adult *Convolutriloba* typically regenerated complete, normal animals, I expected excised tissues from any body region to regenerate a normal animal. However, this was not the case. In *C. retrogemma*, tissues excised along the posterior margin between the lateral and central lobe where buds are normally produced often regenerated heads on both ends, suggesting that the reversed polarity cues present in actively budding worms persist even when this region is removed from the context of the adult body. These biaxial regenerates remain intact until head regeneration is complete when the two heads eventually pull apart and subsequently regenerate a trilobed tail on both fragments, similar to the normal process of bud detachment (Åkesson and Hendelberg 1989; Sikes and Bely 2008).

Furthermore, in all three species, fragments excised from the posterior margin of an animal, especially from the center of the posterior margin (i.e., including the central posterior lobe), often regenerated with an anteriorly bifurcated or multifurcated axis, forming an animal with multiple heads and a common trilobed tail. When tissues were excised along the anterior or lateral margin, or when

excisions made along the posterior margin included a lateral edge, a single head and tail regenerated in all species. The increase in frequency of multiple heads as the excised posterior fragment was made more centrally suggests that the lateral edges of the body could provide important polarity cues that, when present in the fragment, inhibit the formation of multiple axis. These animals with anteriorly bifurcated axes resemble in form the transient, mid-stages of longitudinal fission in *C. longifissura*. However, in contrast to these fission stages as well as the reverse axis regenerates observed in *C. retrogemma*, regenerates with anteriorly multifurcated axes in all species generally do not pull apart but instead persist as multi-headed animals with a common trilobed tail. I therefore suggest that the ability to generate forked anterior axes predated the evolution of longitudinal fission in the genus. *Convolutriloba longifissura* must have then evolved some mechanism for tissue separation, such as the breakdown of body-wall muscles (Sikes and Bely 08), to allow for the successful evolution of longitudinal fission.

Conclusions

Understanding the relationship between asexual reproduction and regenerative capacity is important for understanding how asexual reproduction evolves and diversifies in animals. *Convolutriloba* acoels have evolved extraordinary diversity in asexual reproductive mode, and I find that they similarly have extensive and diverse regeneration abilities. Instead of regeneration ability diversifying in concert with asexual reproduction diversification, however, I find that most of the regeneration capabilities occurring among *Convolutriloba* species were present prior to the diversification. These data indicate that the common ancestor of *C. hastifera*, *C.*

longifissura, and *C. retrogemma* was already capable of both morphallaxis and epimorphosis, could regenerate all parts of its body even from a small fragment, and had the ability to form bifurcated anterior axes. The presence of symbiotic algae and widely distributed somatic stem cells in *Convolutriloba* were probably important in allowing for extensive and rapid tissue replacement, and the evolution of tissue-separation mechanisms was certainly an important component of reproductive diversification. In addition, I suggest that the rich regeneration toolkit of *Convolutriloba* was an important feature that allowed for the evolution and diversification of asexual reproduction strategies in this remarkable group of bilaterians.

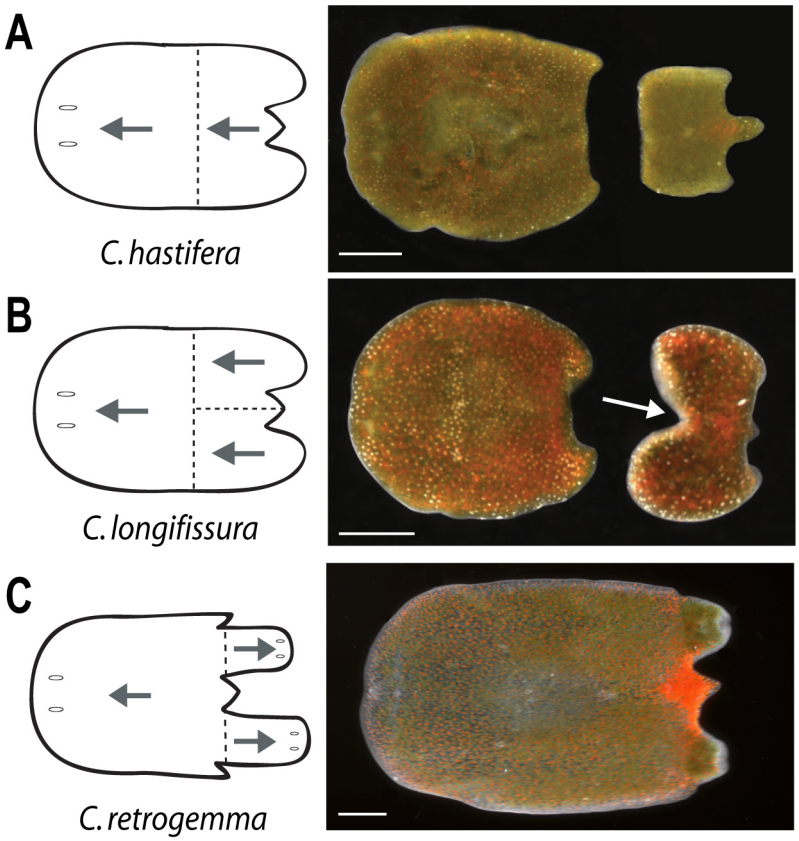

Figure. 3.1. Asexual reproduction strategies in *Convolutriloba* acoels. (A) *C. hastifera* reproduces by simple transverse fission (B) *C. longifissura* reproduces by a transverse fission followed by a subsequent longitudinal fission in the posterior fragment (indicated by white arrow). (C) *C. retrogemma* reproduces by reversed polarity budding in which two buds are produced along the posterior of an adult with and A-P axis polarity completely reversed relative to the parent. Gray arrows indicate direction of A-P polarity with all arrowheads in the anterior orientation. Scale bars = 500μm

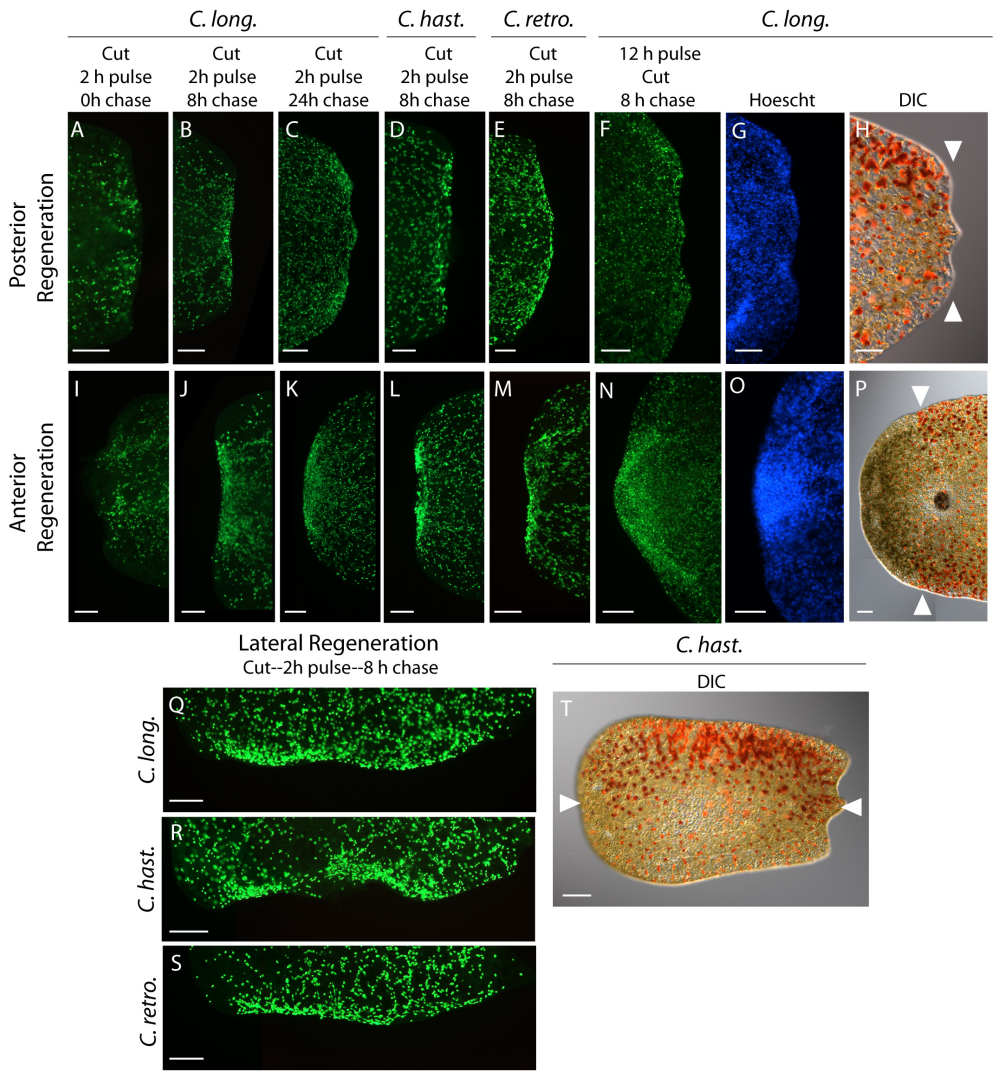

Figure 3.2. Cell proliferation during regeneration following mid-body transverse and longitudinal amputations in three *Convolutriloba* species. (A-C) BrdU labeling at the posterior wound site in *C. longifissura* anterior fragments after a 2 hr BrdU pulse followed by a(n) (A) 0 hr chase, (B) 8 hr chase;, and (C) 24 hr chase before processing. (D-E) BrdU labeling at the posterior wound site in (D) *C. hastifera* and

(E) *C. retrogemma* anterior fragments following 2 hr BrdU pulse and 8 hr chase before processing. (F) BrdU labeling of the posterior wound site in *C. longifissura* after an initial 12 hr BrdU pulse before amputation and followed by an 8 hr delay before processing after amputation. (G) Hoescht staining of the regenerated posterior end of *C. longifissura* following transverse amputation after 8 hours after amputation. (H) DIC image of the regenerated posterior end following transverse amputation in *C. longifissura* after 4 days. Arrowheads indicate the original amputation plane. (I-K) BrdU labeling at the anterior wound site in *C. longifissura* posterior fragments after a 2 hr BrdU pulse followed by a(n) (I) 0 hr chase, (J) 8 hr chase, and (K) 24 hr chase before processing. (L-M) BrdU labeling at the anterior wound site in (L) *C. hastifera* and (M) *C. retrogemma* posterior fragments after a 2 hr BrdU pulse followed by an 8 hr chase before processing. (N) BrdU labeling of the anterior wound site in C. *longifissura* after an initial 12 hr BrdU pulse before amputation and an 8 hr delay before processing after amputation. (O) Hoescht nuclear staining of the regenerating anterior end of *C. longifissura* 8 hrs after transverse amputation (P) DIC image of the regenerated anterior end following transverse fission in *C. longifissura* after 4 days. Arrowheads indicate the original amputation plane. (Q-S) BrdU labeling of the lateral wound site produced by a longitudinal amputation after a 2 hr BrdU pulse followed by an 8 hr chase before processing in (Q) *C. longifissura*, (R) *C. hastifera*, and (S) *C. retrogemma*. (T) DIC image of the regenerated lateral side following longitudinal amputation in *C. hastifera* after 4 days. Scale bars = 150 μm.

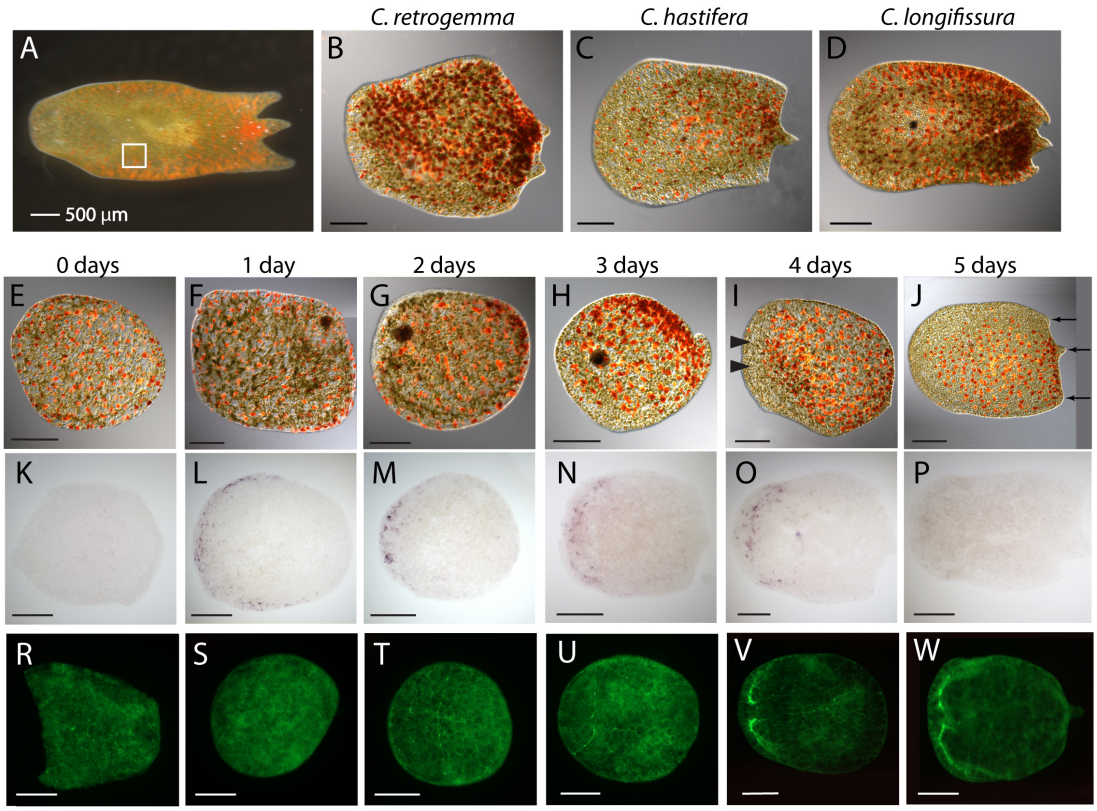

Figure 3.3. Regeneration of an excised fragment of tissue (~0.25 mm^2) from the adult body of *Convolutriloba* acoels. (A) Adult *C. hastifera* illustrating the region of excised tissue. (B-D) *Convolutriloba retrogemma, C. hastifera,* and *C. longifissura* all regenerate completely five days after tissue excision. (E-J) Tissues excised from *C. retrogemma* during regeneration over five days following excision. Dark spots within fragments through three days are digested symbiotic algae. Arrowheads indicate the regeneration of eyespots. Small arrows indicate the regeneration of the trilobed tail (K-P) *CrOtx1* expression is not present immediately following excision, but is upregulated at the anterior end of regenerating tissue fragments between day one and four. *CrOtx1* expression is absent five days after excision when regeneration

is complete. (R-W) Serotonin immunostaining reveals that a normal central nervous system regenerates four to five days after tissue excision in *C. retrogemma*. Scale bars = 125 μm.

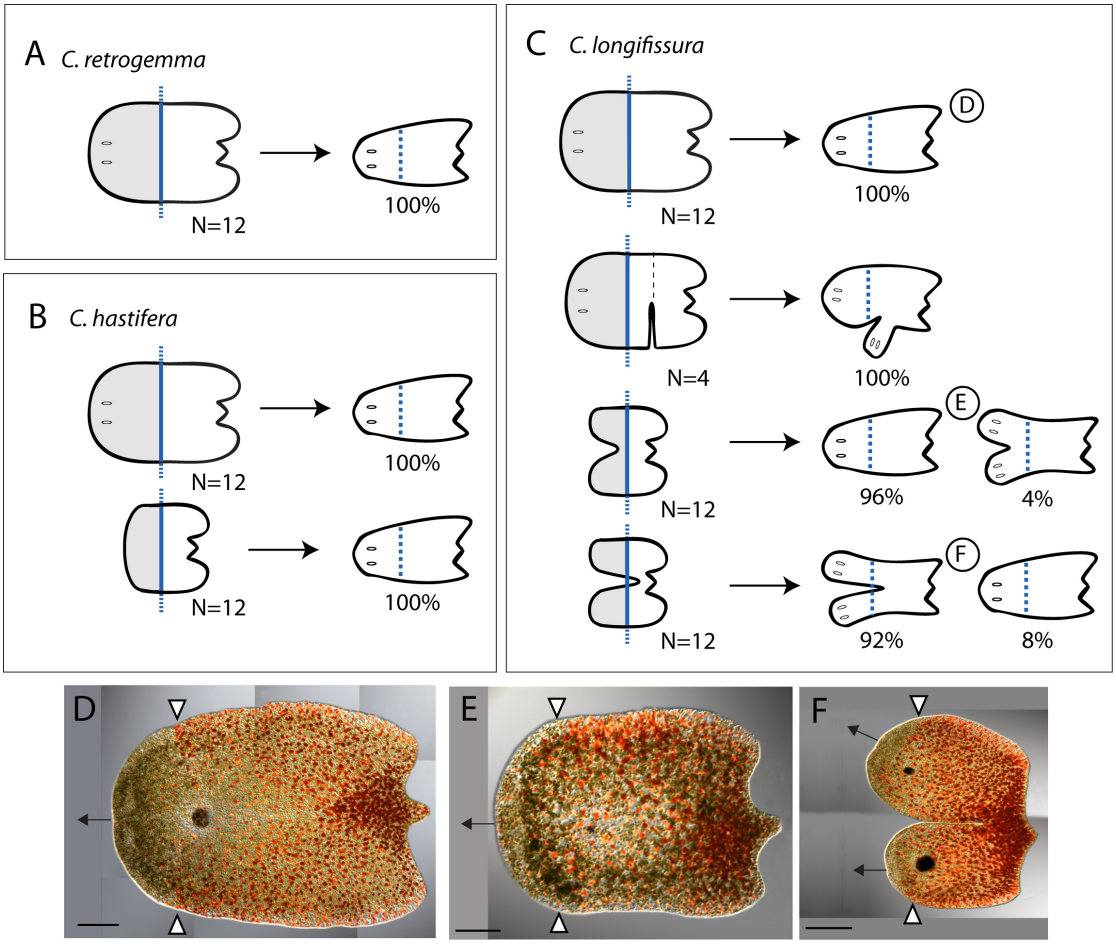

Figure 3.4. Regeneration of the posterior fragment formed after transverse amputation in three *Convolutriloba* species. (A) *C. retrogemma* always regenerates a single head following transverse amputation. (B) Both *C. hastifera* adults and posterior fission products regenerate a single head after transverse amputation. (C) Adult nonreproductive *C. longifissura* regenerate a single head, but if amputated after the initiation of transverse fission, they generate an additional lateral axis. Transverse amputation of early posterior fission products results in the formation of a single head, but amputation of late stage posterior fragments regenerate two heads adjacent to each other. (D) Regenerated *C. longifissura* following transverse amputation of a

nonreproductive adult. (E-F) *C. longifissura* posterior fission products regenerate a (F) single head if amputated below the leading edge of longitudinal fission, but regenerate (G) two adjacent heads if amputated above the leading edge of longitudinal fission. Arrowheads indicate the original amputation plane and black arrows indicate the anterior midline in all DIC panels. Scale bars = 200 μm

		N					
A	*C. hastifera*	35	100%	-	-	-	-
	C. longifissura	44	100%	-	-	-	-
	C. retrogemma	45	85%	11%	-	4%	-
B	*C. hastifera*	24	67%	33%	-	-	-
	C. longifissura	48	94%	6%	-	-	-
	C. retrogemma	60	30%	15%	3%	47%	5%
C	*C. hastifera*	33	88%	12%	-	-	-
	C. longifissura	23	48%	48%	4%	-	-
	C. retrogemma	19	16%	84%	-	-	-
D	*C. hastifera*	24	100%	-	-	-	-
	C. longifissura	24	100%	-	-	-	-
	C. retrogemma	24	96%	4%	-	-	-
E	*C. hastifera*	5	100%	-	-	-	-
	C. longifissura	5	100%	-	-	-	-
	C. retrogemma	5	100%	-	-	-	-
F	*C. hastifera*	19	100%	-	-	-	-
	C. longifissura	20	100%	-	-	-	-
	C. retrogemma	15	100%	-	-	-	-

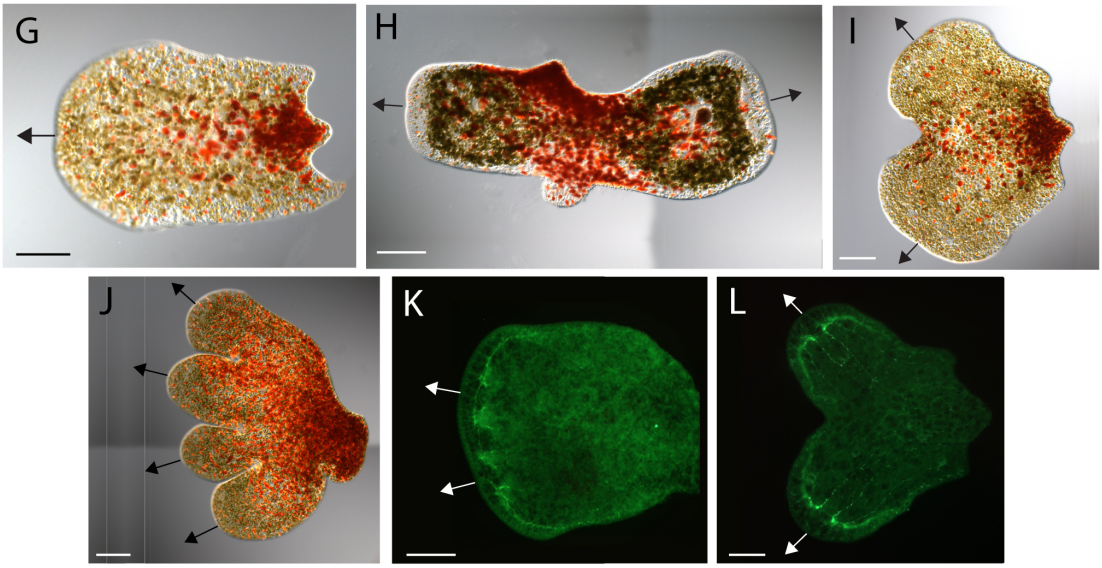

Figure 3.5. Regeneration of tissues excised from the adult body in three *Convolutriloba* species. (A) Tissue fragments excised from completely within the adult body almost always regenerate a single head and tail axis in the three *Convolutriloba* species. (B) Tissues excised from within the parental body along the posterior margin between the lateral and central lobes often regenerate a biaxial individual in *C. retrogemma* and sometimes regenerate bifurcated or multifurcated anterior axes in all three species. (C) Excised tissues along the posterior margin at the central lobe often regenerate bifrucated and multifurcated axes in all species. (D) Excisions made along the posterior margin that include the lateral edge of the adult usually regenerate a single head and single tail in the three species. (E-F) Tissues excised along the lateral edge or the anterior margin always regenerate a single head and a single tail in all three species. (G) DIC image of a regenerated tissue fragment excised from within the adult body of *C. retrogemma*. (H) DIC image of a biaxial *C. retrogemma* individual resulting after regeneration from an excised tissue fragment located along the posterior margin between the lateral and central lobes. (I) DIC image of a regenerate with a bifurcated anterior axis resulting from a tissue fragment excised from the posterior margin between the lateral and central lobes of an adult *C. retrogemma*. (J) DIC image of a rare multi-furcated regenerated tissue fragment that was excised along the posterior margin at the central lobe of *C. longifissura*. (K) Serotonin immunostaining of the central nervous system at an early stage of regeneration in a *C. retrogemm*a tissue fragment excised along the posterior margin between the lateral and central lobes reveals two adjacent bilobed ganglia. (L) Serotonin immunostaining of an individiual with a bifurcated anterior axis reveals

that each regenerated head has an intact central nervous system complete with bilobed ganglia and extending nerve cords. Arrows indicate the anterior midline in all panels. Scale bars = 150μm.

Chapter 4: Making heads from tails: Reversal of the primary body axis during asexual reproduction in *Convolutriloba retrogemma* (Acoela)

Abstract

Convolutriloba acoels are the only bilaterians known to naturally reverse the anterior-posterior (A-P) axis through asexual reproduction. In the two *Convolutriloba* species capable of reversed-polarity budding, two posterior bud sites produce buds with an A-P axis orientation completely reversed relative to the parent. I investigated this budding process in one of these species, *C. retrogemma*, with the goal of better understanding how this mode of asexual reproduction evolved. Cell proliferation assays indicate that increased cell division occurs throughout the developing bud and does not suggest a clear growth zone. Homologs of Hox and *otx*, widely conserved axial patterning genes, are initially upregulated within a hemicircular region of parental tissue during bud initiation, and their expression becomes regionalized along the A-P axis only as bud elongation occurs. This suggests a model for bud patterning that is remarkably similar to that of *Hydra* budding, even though the two are clearly not homologous. Shortly after a bud is initiated, a zone of muscle disorganization develops between the parent and bud, and this zone correlates with the polarity transition zone. Tissue excision experiments indicate that fragments containing this zone have the potential to give rise to a new body axis with reversed polarity. Surprisingly, tissue excised completely from within this zone has no regenerative potential and appears to lack polarity of its own. I hypothesize that the presence of this apparently unpolarized zone that has the

potential to generate a new A-P axis was critical for the evolution of reversed polarity budding in *C. retrogemma.*

Introduction

Bilaterians display an amazing diversity of body shapes, yet the vast majority share a fundamental feature: a distinct head end and tail end, which define the anterior-posterior (A-P) body axis. It was the evolution of a body plan with this distinct A-P axis that is thought to have allowed for the evolution of the dramatic diversity of body forms observed in the Bilateria today (Akam 1995). Over the past twenty years, it has become clear that despite variations in body form, most bilaterians use conserved developmental mechanisms, such as the Hox genes, to specify regions of the A-P axis (Gellon and McGinnis 1998).

A small fraction of bilaterians has evolved the ability to modify this single primary body axis through asexual reproductive modes such as fission or budding. In nearly all cases, asexual species do not alter the orientation of the axis, but only subdivide it into multiple tandemly arrayed axes through fission or form lateral buds off a central stalk (Hughes 1989). Only two bilaterian species, two acoel worms in the genus *Convolutriloba,* have evolved, through a single evolutionary event, the ability to naturally reverse the primary axis during asexual reproduction.

Acoels are small marine worms thought to represent the most basal bilaterian lineage (Ruiz-Trillo et al. 1999). Like typical bilaterians, these worms have a distinct anterior and posterior end, a centralized nervous system, and anteriorly located sensory structures. Although the molecular basis of acoel A-P axis development has not been investigated, the cleavage program of acoels is organized in a bilateral fashion such that macromere division occurs along a plane that will become the future A-P axis (Henry et al. 2000). These data suggest that the specification of the primary

body axis occurs very early in development. While most acoels reproduce only sexually and thus maintain a single, fixed A-P axis throughout life, acoels in the genus *Convolutriloba* have evolved diverse asexual reproductive modes, including the only occurrences of reversed-polarity budding known among the Bilateria (Hendelberg and Åkesson 1991; Shannon and Achatz 2007). These *Convolutriloba* species, *C. retrogemma* and *C. macropyga,* have two posterior budding sites from which buds develop with an A-P axis completely reversed relative to the parent (Fig. 4.1A). Both species go through similar developmental stages during budding, including the formation of a transitional zone of body wall muscle disorganization, the digestion of symbiotic algae, and *de novo* development of nervous structures (Sikes and Bely 2008). However, little is known about the amazing asexual process that allow these small worms to reverse the otherwise fixed A-P axis of bilaterians.

While *Convolutriloba* acoels are the only bilaterians known to naturally reverse their A-P axis during postembryonic development, bipolarity has been experimentally induced in several bilaterians. Dicephalic adult planaria have been induced by altering the Wnt/β-catenin pathway (Gurley et al. 2008; Iglesias et al. 2008; Peterson and Reddien 2008) and by treating regenerating adults with gap junction blockers (Nogi and Levin 2005) and nucleic acid synthesis inhibitors (Kohl and Flickinger 1966). Dicephalic annelids have also been experimentally induced when amputations result in very short fragments or when post-amputation autotomy is inhibited in amputated individuals (Hyman 1916; Kawamoto et al. 2005). Such experimental manipulations that elicit polarity reversal suggest an underlying ability

for some bilaterian groups to reverse the primary body axis, yet among bilaterians only *Convolutriloba* has evolved the ability to do so as part of normal development.

I investigated the developmental basis of reversed-polarity budding in *C. retrogemma,* one of the two species in the genus reproducing by reversed-polarity budding. To determine how buds with reversed polarity develop, I assayed patterns of cell proliferation, changes in body wall musculature, and the expression of conserved axial patterning genes during budding. To assess the axial potential of different body regions and to determine the temporal and spatial nature of axis reversal during budding, I also performed cutting experiments designed around a previously identified zone of disorganized body-wall musculature that seems to form a boundary between parent and bud at the site of polarity reversal (Sikes and Bely 08).

Materials and methods

Animal collection & culture

Convolutriloba retrogemma individuals were originally collected from a marine aquarium housing Indo-Pacific corals at a retail aquatic store in Maryland (USA). An isogenic line of this species (originally established from a single individual) was cultured as previously described (Sikes and Bely 2008). Individuals at various budding stages were pulled from these cultures for experiments.

Phalloidin staining

The musculature of worms at different stages of budding was investigated by phalloidin staining of F-actin fibers. Worms were relaxed, fixed, and washed as

previously described (Sikes and Bely 2008). Samples were incubated with Alexa Fluor-488 phalloidin (Invitrogen) at 1:100 in PBTx overnight at 4°C, and then washed three times in PBS. Samples were mounted in Fluoromount-G (Southern Biotech, Birmingham, AL, USA).

Cellular proliferation assays

To label S-phase cells during budding, worms at all stages of budding were incubated in 0.1 mg/ml bromodeoxyuridine (BrdU) for either 2 h or 8 h at 25°C. Worms were relaxed with 3.4% $MgCl_2$ for 10 min, fixed in 3.7% formaldehyde in 0.75X phosphate-buffered saline (PBS) for 30 min, washed at least three times in PBS, washed in PBTx (PBS+0.01% Triton-X), incubated in 75% HCl at 37°C for 30 min, and washed 5x in PBTx. Samples were then blocked in 10% normal goat serum (NGS) in PBTx for 1 h at room temperature and incubated in mouse anti-BrdU monoclonal antibody (Sigma, St. Louis, MO, USA) at 1:100 in 10% NGS/0.9X PBTx overnight at 4°C. Samples were rinsed repeatedly in PBTx over 1 h, incubated in a FITC-conjugated goat anti-mouse antibody (Sigma) at 1:200 in 10% NGS/0.9X PBTx overnight at 4°C, rinsed three times in PBTx, and washed multiple times in PBTx and finally in PBS. Samples were mounted in 70% glycerol.

Nervous system immunostaining

The serotonergic components of the nervous system were revealed by serotonin immunostaining. Worms were relaxed, fixed, and washed as for BrdU stains. Samples were then blocked in 10% normal goat serum (NGS) in PBTx for 1 h

at room temperature and incubated in rabbit anti-serotonin polyclonal antibody (Sigma, St. Louis, MO, USA) at 1:100 in 10% NGS/0.9X PBTx overnight at 4°C. Samples were rinsed repeatedly in PBTx over 1 h, incubated in Alexa Fluor-488 goat anti-rabbit IgG (Invitrogen) at 1:200 in 10% NGS/0.9X PBTx overnight at 4°C, rinsed three times in PBTx and once in PBS. Samples were mounted in Fluoromount-G.

Gene isolation & sequencing

To isolate Hox and *otx*-class genes, small fragments (including part of the homeobox) were amplified from genomic DNA through PCR using degenerate primers. A single round of PCR was used to amplify central Hox and *otx*, while two rounds, using nested primers, were used to amplify anterior and posterior Hox genes. All primer sequences are listed in Table 1. Primers used include HOX A+ and HOX C- for the first anterior Hox PCR, HOX A+ and HOX B- for the second anterior Hox PCR, HOX BS+ and HOX BT- for the central Hox PCR, HOX G+ and HOX I- for the first posterior Hox PCR, HOX H+ and HOX I- for the second posterior Hox PCR, and OTX A+ and OTX B- for the *otx* PCR. For the first PCR, 30 cycles were performed at 60°C to 45°C (temperature decreased 0.5°C/cycle) annealing with 3 min extension, followed by 20 cycles at 50°C annealing with 3 min extension. For the second PCR, 35 cycles were performed at 50°C annealing with 3 min extension.

To obtain additional sequences, I performed 3' RACE for all genes isolated by degenerate PCR (*CrAhox, CrCHox, CrPHox1, CrPHox2, CrOtx1, and CrOtx2*), as well as 5'RACE for all Hox genes. For both 3' and 5'RACE, total RNA (isolated from ~50 mg of actively budding worms, using RNAwiz (Ambion, Austin, TX,

USA)) was reverse transcribed and amplified using published protocols and anchor primers (Frohman 1990). For 3' RACE, gene specific primers for the first and second PCR, respectively were HOX K+ and HOX L+ for *CrAhox*, HOX U+ and HOX V+ for *CrChox*, HOX N+ and HOX P+ for *CrPhox1*, HOX O+ and HOX Q+ for *CrPhox2*, and OTX D+ and OTX E+ for both *CrOtx1* and *CrOtx2*. Multiple rounds of 5' RACE were performed for *CrAhox*, *CrPhox1*, and *CrPhox2* since only short fragments were obtained in initial reactions. The 5' RACE gene specific primers for the first and second PCR, respectively, were HOX AW- and HOX AV- for *CrAhox* round 1, HOX BL- and BK- for *CrAhox* round 2, HOX BS2- and HOX BR- for *CrAhox* round 3, HOX AS- and HOX AR- for *CrChox*, HOX AT- and HOX AU- for round 1 in both *CrPhox1* and *CrPhox2*, and HOX BN- and HOX BM- for round 2 in both *CrPhox1* and *CrPhox2*. New primers were designed from both the 3' and 5' RACE products to amplify each of the Hox genes in one piece. These primers were HOX CD+ and HOX BD- for *CrAhox*, HOX CG+ and HOX AN- for *CrChox*, HOX BU+ and HOX CT- for *CrPhox1*, and HOX BU+ and HOX BX- for *CrPhox2*. PCR products were cloned into pGEM-T or pGEM-T Easy vectors (Promega, Madison, WI, USA) and sequenced.

Phylogenetic analyses

Phylogenetic analyses were performed to determine gene orthology for the Hox and *otx* genes. I assembled the nucleotides of the homeobox from *C. retrogemma* Hox genes along with the homeoboxes of all GenBank published acoel Hox and ParaHox and assembled the homeobox nucleotides of *CrOtx1* and *CrOtx2*

with the homeoboxes of *otx* homologs from a variety of metazoan taxa. The homeobox nucleotides from *Drosophila goosecoid* and *paired,* two non-*otx* family homeobox genes, were also downloaded as outgroups. Nucleotide alignments were made using Clustal X (v. 1.83) (Thompson et al, 1997). Phylogenetic analyses were performed with PAUP* (v. 4.0b10. Sinauer Associates, Sunderland, MA, USA) using neighbor joining (NJ) and maximum parsimony (MP) (branch and bound search) for both datasets. Clade support was evaluated by bootstrapping (Felsenstein 1985) with 1000 replicates. Analyses of the Hox genes were carried out with the 180 bp of the homeobox from the following sequences: *Symsagittifera roscoffensis, SrHox1*—AY117547, *SrHox4/5*—AY117548, *SrHoxpost*—AY117549, *SrCdx*—AY117550; *Paratomella rubra, PrHox1*—AY282605, *PrPostA*—AY282606, *PrPostB*—AY282607, *PrCdx*--AY282608. Analyses of the *otx* family genes were carried out with the 180 bp of the homeobox from the following sequences: *Hydra vulgaris, CnOtx*—AF114441; *Podocoryne carnea, Pc-Otx*—AF160992; *Nematostella vectensis, Nv-Otx*—AY465181; *Pristina leidyi, Pl-Otx1*—AF336056, *Pl-Otx2*—AF336057; *Mus musculus, otx1*—NM_011023, *otx2*—NM_144841; *Drosophila melanogaster, otd*—X58983, *goosecoid*—U52968, *paired*—AH001027.

Whole mount in situ hybridization

To characterize expression patterns of Hox and *otx* homologs, sense and antisense digoxigenin-labeled in situ riboprobes were generated from RACE or combined RACE + degenerate fragments using the T7 promoter (Ambion). Probe lengths were: *CrAhox*, 1.3kb, *CrChox*, 861 bp, *CrPhox1*, 1.4 kb, *CrPhox2*, 1.6 kb,

CrOtx1, 1.4 kb, and *CrOtx2*, 1.3 kb. I used an in situ hybridization protocol based on that of Hejnol and Martindale (2008) for acoel embryos and juveniles, with worms relaxed 10 minutes in 3.4% $MgCl_2$ prior to fixation and hybridization carried out using 2.5 ng/μl probe at 60°C (for *CrOtx1, CrOtx2, and CrPhox1*) or 52°C (for *CrAhox, CrChox, and CrPhox2*). Specimens were mounted in 70% glycerol.

Regeneration experiments

I used cutting experiments to assess the polarity of tissues within the body of actively budding *C. retrogemma*. Amputation and tissue excisions were performed on unanaesthetized worms from actively budding cultures. Amputated worms and excised tissues were maintained singly in wells of 24-well cell-culture trays containing ~2ml artificial seawater and maintained at 24°C with a 12h:12h light:dark cycle. Daily observations were made to assess the progress and polarity of regeneration. Amputated regions were not provided with food but were illuminated (to allow algal symbionts to photosynthesize).

To assess the stability of polarity reversal between the parent and bud, I excised ~1mm^2 regions of tissue that spanned the zone of muscle disorganization (between parent and bud) and included tissues from both the parent and bud from 96 actively budding adult worms with mid- to late-stage buds. Ninety-six control excisions on comparable worms (and in some cases the same worm) were made fully within the parent's body while another 96 were performed within the body of the bud.

To assess the regenerative potential of tissue within the zone of muscle disorganization, I excised tissue wholly from within this zone. To do this, I excised

~1mm x 0.5mm fragments of tissue that spanned the zone of disorganized musculature from 24 late-stage budding adults and then cut this piece in half lengthwise, producing two longitudinal strips of tissue. One of these was immediately stained with phalloidin to allow visualization of the location of the disorganized muscle region. Based on this pattern a ~0.01mm x 0.25mm region of tissue from the other half was amputated according to morphological landmarks to ensure all tissue retained was located wholly within the region of muscle disorganization. Twenty-four similar sized regions of tissue with the normal pattern of muscle organization were excised from the parental body as controls.

Imaging

BrdU, in situ hybridization, serotonin, and regeneration samples were imaged with a Zeiss Axioplan 2 epifluorescence microscope equipped with a Zeiss AxioCam HRc camera (Carl Zeiss MicroImaging, Thornwood, NY, USA). Images of phalloidin samples were imaged using a Leica SP5 X confocal microscope (Leica Microsystems, Bannockburn, IL, USA). Image processing and (when necessary) the assembly of photo-montages of multiple neighboring views were made with Adobe Photoshop (v. 10.0).

Results

Musculature

In non-budding adults, muscle fibers are well organized into the normal interlaced pattern along the entire posterior margin of the animal (Fig. 4.1B). Shortly after the first morphological signs of bud initiation, a C-shaped band of muscle

disorganization develops at the base of the developing bud (Fig. 4.1C), forming a distinct zone between the organized musculature of the bud and that of the parent. This zone persists throughout bud elongation (Fig. 4.1D), and when the bud physically separates from the parent, tissue separation occurs within this zone. Following bud detachment, the parent's wound heals and no muscle disorganization is apparent until another round of budding commences.

Cell proliferation

BrdU labeling reveals that cell proliferation increases when buds are initiated and remains high throughout the bud elongation process as compared to background levels of cell division in the adult body (Fig. 4.1E-G). Slightly higher levels of proliferation occur along a subterminal crescent of cells along the anterior lateral edges of the bud, yet there is no obvious growth zone at the base of the bud. Extended pulses of BrdU (8 hours) at early stages of budding label a majority (though not all) of the cells that make up the bud (data not shown).

Cloning and phylogenetic analyses

Through degenerate and RACE PCR, I recovered from *C. retrogemma* two homologs of *orthodenticle* (*CrOtx1* and *CrOtx2)* and four Hox homologs, one anterior-class (*CrAhox*), one central-class (*CrChox*), and two posterior-class (*CrPhox1* and *CrPhox2*) genes. Additionally, short fragments of one additional anterior-class gene and one additional central-class Hox gene were isolated but I was unable to obtain enough sequence to use these in phylogenetic analyses or to permit

expression studies. Phylogenetic analyses using the homeobox domains of the isolated Hox genes result in a neighbor-joining tree and a maximum parsimony tree with similar topologies. *CrAhox* groups with anterior-class genes, *CrChox* groups with central-class genes, and *CrPhox1* and *CrPhox2* group with the posterior-class genes (Fig. 4.2A), all with strong support. Phylogenetic analyses place both *otx* homologs within an *otx* clade with strong bootstrap support (Fig 4.2B).

Gene expression

In situ hybridizations indicate that all six genes are expressed dynamically throughout the budding process in specific fields of the developing bud and/or associated with the developing nervous system (Fig. 4.3). *CrOtx1, CrOtx2, CrAhox, CrPhox2*, and *CrPhox2* are expressed at the bud site beginning at the earliest sign of bud formation, evident as a slight thickening of tissue at the posterior margin of the animal (Fig. 4.3, A-C & E-F). Although somewhat variable, early expression occurs in a roughly hemi-circular patch of adult tissue (with the diameter of the hemicircle falling along the parent's posterior margin). Although these genes are initially all expressed in the same patch of tissue, as the bud elongates gene expression is compartmentalized along the newly forming A-P axis of the developing bud (Fig. 4.3, A-C & E-F). *CrOtx1* is expressed along the anterior tip during early and mid-stage budding, but shows more diffuse expression across the bud at later stages just prior to separating from the parent (Fig. 4.3A). *CrPhox1* is expressed in an arc of cells at the base of the bud in early budding stages but is expressed along the middle of the bud's developing A-P axis in mid and late-stage buds (Fig. 4.3E). *CrPhox2* is expressed in

a similar arc of tissue at the base of the bud in early stages and maintains the similar pattern of mid-posterior expression throughout the remainder of the budding process (Fig. 4.3F). *CrOtx2, CrAhox*, and *CrChox* show expression associated with the developing central nervous system during bud elongation (Fig. 4.3B-D).

Regeneration experiments

I excised tissue fragments that spanned across the zone of muscle disorganization, including tissues from both the parent's and bud's body, to assess the stability of polarity reversal between the parent and bud. Most of these amputations (90%) regenerated biaxially, producing a fully developed head on either end of the excised tissue. Control tissue fragments of similar size and shape but excised wholly within the body of either the parent or the bud almost always regenerated a normal worm, with a single head and tail on opposite ends (97% - tissue from parent; 96% - tissue from bud) (Fig. 4.4A). Serotonin immunostaining of the central nervous system reveals that the tissues excised from body tissues regenerates a single bilobed ganglion and lateral nerve cords at the anterior end (Fig. 4.4B), while the excised tissue spanning the zone of muscle disorganization regenerate a complete central nervous system on both ends (Fig. 4.4C). The tissue fragments excised from the parental body express the anterior patterning marker *CrOtx1* at the anterior end during regeneration (Fig. 4.4D), but tissues excised across the disorganized muscle express *CrOtx1* at both regenerating ends (Fig. 4.4E).

Given that amputations spanning the polarity transition zone gave rise to biaxial regenerates, I initiated additional experiments to determine the developmental

potential of the tissues within this zone when it is wholly removed from the context of adult or bud tissue. I excised a small piece of tissue located within the region of disorganized muscle as well as a control fragment of tissue, of similar size and shape, from the parental body (Fig. 4.5A). While almost all of the small tissue fragments from the parental body regenerated a normal head and tail after 3 days (Fig. 4.5B), 75% of the fragments excised from within the zone of disorganized muscle failed to regenerate after 10 days (Fig. 4.5C). These non-regenerating fragments exhibited a small number of disorganized serotonergic cells and no clear pattern of nerves (Fig. 4.5D). Normal patterns of serotonin were observed for those fragments undergoing complete regeneration (data not shown).

Discussion

Reversed-polarity budding in C. retrogemma

Two acoel worms in the genus *Convolutriloba* are the only bilaterians that have evolved, through a single evolutionary event, the ability to naturally reverse the primary axis during asexual reproduction (Hendelberg and Åkesson 1988; Shannon and Achatz 2007). To help understand this remarkable process, I investigated cell proliferation, musculature changes, and axial patterning gene expression during reversed polarity budding in *C. retrogemma* and also performed cutting experiments to clarify the nature of the boundary between parent and bud.

Cell proliferation occurs in the bud throughout the budding process. Increased cell division was obvious at the site of bud initiation and within the extending bud, as compared to levels in the parent's body. Even with extended BrdU pulses, however,

not every cell in the bud was labeled, suggesting that some parental cells may become incorporated into the bud along with cell proliferation. C*onvolutriloba retrogemma* buds thus appear to form largely through the rapid multiplication of somatic stem cells in the bud itself though there may also be a contribution by parental cells that get directly incorporated into the bud.

I previously described the presence of a zone of muscle disorganization between the parental tissue and the tissue of the developing bud in *C. retrogemma* (Sikes and Bely 2008). The regeneration data suggest that this region of muscle disorganization is coincident with the site of polarity reversal. I have thus named this region the polarity transition zone (PTZ). In this study, I found that this zone of disorganized muscle fibers is a transient feature and not permanent in adult animals. The region develops at bud initiation and persists until bud detachment, but disappears as the parent heals the wound that formed at bud separation. This suggests that the PTZ is recreated for each round of budding and is initiated at a sub-terminal region within the parent's body at the initiation of subsequent budding events. I have also found that when actively budding adults are starved, they cease to bud and regain the normal pattern of muscle fibers along the entire posterior margin until they return to a nutritional state that allows for budding when the zone of muscle disorganization reappears (pers. obs.). Thus, it appears that this zone of muscle disorganization forms as a temporary boundary between parent and bud rather than being a permanent feature of the posterior end of *C. retrogemma* and is a critical feature allowing for reversed polarity budding.

I found that conserved axial patterning genes, namely Hox and *otx* homologs, are dynamically expressed within the developing bud of *C. retrogemma*. Expression of Hox and *otx* homologs was undetectable by in situ hybridization in the parental body prior to bud initiation. At bud initiation, a thickening of tissue at the future bud site occurred before any visible bud outgrowth, yet at this stage already most of the axial patterning genes I investigated (*CrOtx1, CrOtx2, CrAhox, CrPhox1*, and *CrPhox2*) were expressed within the same patch of parental tissues. Subsequent to bud initiation, during early and mid-stage budding, *CrOtx1* was upregulated at the anterior tip of the elongating bud, while *CrPhox1* and *CrPhox2* expression were restricted to body regions near the bud's posterior as predicted by their conserved role in axial specification. *CrOtx2*, *CrAhox*, and *CrChox* showed expression patterns associated with the development of nervous structures, including the bilobed ganglion at early stages and along the lateral nerve cords at mid-stage budding, which is consistent with the conserved role of Hox genes and *otx* in nervous system patterning (Keynes and Krumlauf 1994; Kourakis et al. 1997; Leuzinger et al. 1998). At later budding stages just before bud detachment, *CrOtx1* expression was more diffuse and not localized to any body region. Both *CrPhox1* and *CrPhox2* were expressed at body regions between the posterior and middle of the late-stage bud. *CrChox* and *CrOtx2* maintained expression patterns along the lateral nerve cords through late-stage budding, while *CrAhox* was not expressed in late stages. The expression of A-P patterning genes apparently well within the body of the parent and prior to the formation of the zone of disorganized musculature suggests the intriguing possibility that parental tissue may be converted to a bud fate when the bud is first initiated.

Although the primary goal of elucidating the expression patterns of these axial patterning genes was to inform us about the process of reversed polarity budding, these data represent the first expression data for Hox genes in an acoel and, as such, provide important information on the evolution of bilaterian Hox gene expression. As previous studies have found (Cook et al. 2004), acoels have a clear homolog of the central class of Hox genes, supporting the notion that the bilaterian ancestor possessed all 3 Hox gene classes. In addition, I recovered some additional *C. retrogemma* Hox genes indicative of independent duplications within each of these classes that appear to be restricted within this acoel species and not part of the Hox duplication event that occurred in other bilaterians. The 3 classes of Hox genes are expressed roughly in three regions along the bud's A-P axis, with *CrAhox* in the anterior, *CrChox* associated with nervous components in the center of the bud, and *CrPhox1* and *CrPhox2* in the more posterior regions of the bud. These data indicate that Hox gene patterning along the A-P axis was clearly present at the base of the bilaterian clade and therefore existed prior to the common ancestor of the Lophotrochozoa, Ecdysozoa, and Deuterostomia.

I used cutting experiments to assess the axial polarity of tissues within the body of *C. retrogemma*. Tissue fragments excised across the zone of disorganized musculature, that include normally patterned tissues of both the parent and the bud on either end, regenerate heads in both directions, suggesting that even when removed from the body, tissues maintain their original A-P orientation and regenerate based on the axial polarity they had within the animal. Following bud detachment, the adult and bud each have a wound that includes part of the PTZ since the bud tears away

from the adult at this zone of muscle disorganization. Upon detachment, wound healing quickly occurs and the region of muscle disorganization disappears, allowing for the development of a normal posterior end in both the parent and bud. However, when tissues are excised from the parental body immediately following bud detachment, the edge located at the site of the PTZ always regenerates heads (data not shown), suggesting that edges within a PTZ, when excised from the parental body, default to the development of a single anterior axis. This implies that the body of intact worms somehow signals the conclusion of a budding event, allowing for wound healing and subsequent formation of a normal posterior margin.

While tissues of the parent and bud each have the potential to regenerate a new axis with opposing polarity, I find that the PTZ's potential to generate axes is not inherent to the tissue but dependent on attached patterned tissue of either the parent or bud. When fragments of tissue located wholly within the region of disorganized muscle are excised from the body, they rarely regenerate and are incapable of forming a new worm. These tissue fragments that fail to regenerate begin digestion of symbiotic algae but do not increase in size or develop central nervous system structures. By contrast, excisions of similar size from any other part of the worm's body fully regenerate a complete animal within 2-3 days. Thus, it appears that the potential to give rise to anteriorly-oriented axes is not inherent to the PTZ itself. I suggest that the PTZ is a region of tissue that is not truly part of either the parent or the bud and that lacks polarity of its own, suggesting that cues from adjacent tissues may give the PTZ its axis-generating potential.

Convergence in budding mechanisms

Although budding occurs in a variety of metazoan species, the process has been studied in very few, with *Hydra* as the only animal receiving significant attention. Although certainly not homologous, budding in *Hydra* and *C. retrogemma* share a number of features. Classical experimentation on *Hydra* along with more recent molecular studies have suggested mechanisms by which *Hydra* buds are initiated, elongated, and regionalized (Webster and Hamilton 1972). Comparing the findings for *C. retrogemma* budding to a recent model *Hydra* budding indicates at least three striking similarities. First, in both *Hydra* and *C. retrogemma* a circular or semicircular patch of adult tissue appears to be converted to bud fate prior to bud elongation. A bud in *Hydra* is initiated as a circular field located along the stalk of an adult animal, with positional values of the future bud established as concentric rings emanating from the center of the circular field (Berking 2003). Regional differentiation follows in these concentric rings, with the innermost field fated to become the head and serving as the focus for bud outgrowth. Similarly, in *C. retrogemma*, axial patterning gene expression suggests that bud initiation occurs when a patch of parental tissue is converted to bud fate along the posterior margin of an adult. The shape of this patch is a semicircle, or possibly a circle if dorsal and ventral sides are interpreted as the two halves of the circle. Although I find no evidence for concentric rings of expression in *C. retrogemma*, the spatial pattern of the expression does suggest a central focus point, as in *Hydra*.. In both *Hydra* and *C. retrogemma*, buds appear to form through a two-stage process, with establishment of positional values (e.g., axial gene expression) occurring first followed by bud

outgrowth and regional differentiation. Secondly, positional cues that occur at bud initiation appear to be set up at the same time from head to tail in both *Hydra* and *C. retrogemma*. Working models suggest that both animals coincidentally pattern all future body regions, as indicated in *C. retrogemma* by the finding that anterior, central, and posterior axial markers are expressed at approximately the same time. Finally, the differentiation of the most posterior structures, the basal disc in *Hydra* and the trilobed tail in *C. retrogemma* occurs after bud detachment in both animals (Sersig and Lesh-Laurie 1981). These striking similarities in these two animals that have independently gained the ability to reproduce by budding suggests that the evolution of budding has involved significant convergence, despite important differences in body plan organization, lifestyle, and mode of budding. Further studies of the budding process in other metazoans will enable us to determine if these similar processes occur in all species that utilize budding as a means of asexual reproduction.

Conclusions

Convolutriloba acoels are the only bilaterians known to naturally develop a completely reversed A-P axis after its establishment in embryogenesis. These data suggest that this reversal of the budding axis in *C. retrogemma* does not occur in a single step and is not as simple as taking a parental A-P axis and reversing it. An important intermediate step, the ability to generate potentially unpolarized tissues that simultaneously express anterior and posterior patterning genes and thus have axis-generating potential within the adult body, seems essential in allowing these worms to accomplish this remarkable axis reversal. Given that this polarity transition zone seems to give rise to novel, reversed axes, the reversed A-P axis of the bud may not

be the product of a developmentally reversed parental axis, but may be derived from this unpolarized region of tissue that has the axial patterning potential to allow for the outgrowth of an entirely new A-P axis. Further studies into the genetic and developmental mechanisms by which *C. retrogemma* develops this potentially unpolarized zone within its body is a critical next step to understanding this novel and unique process.

Table 4.1. PCR primer sequences used to amplify Hox and *otx* genes from *C. retrogemma*

Primer	Sequence (5′-3′)
HOX A+	GGTMGTACTAAYTTYACIAAYAA
HOX B-	CCYTCYTTTARTARYTTYTTYTTGYTTCAT
HOX C-	CCYTCYTTTARTARYTTYTTYTG
HOX G+	WSTMGTAARAARMGIMGICCITA
HOX H+	AARMGTMGTCCTTAYCCIAARAA
HOX I-	RTGYTGYTTYTTRTTYTTCAT
HOX K+	CAACAGATACTTGACACGCGC
HOX L+	GCTAGGAGAATCGAAATAGC
HOX N+	CACCAAGAACCAAACACTGG
HOX O+	CCTACATAACAAGAGAGCG
HOX P+	CGAAGACTGGAAATCGCACGG
HOX Q+	AACTTGACAGATCGTCAGG
HOX U+	CGAGATTGCCAACCTGCTGGC
HOX V+	GCTGGCACTAACCGAGCGAC
HOX AN-	TTACAAACCATAATTACTAGG
HOX AR-	CGATCCTCCTGCGTCTGGTGAGGTACCTG
HOX AS-	GTGCCAGCAGGTTGGCAATCTCGATCCTC
HOX AT-	CGGGTGATGTGGGGGTGCGGGAGGTAC
HOX AU-	AGGTGCATAGCCGGGTGACCACCGGGC
HOX AV-	CTTGGGTTTCGTTCAGAGTCAACGAAGTTGC
HOX AW-	ACCACTGCACGGTTCTCAATTAACGCCAG
HOX BD-	CATGAACGCGGCGAGGAAAATACG
HOX BK-	CCACGGCACTGGCGTGGTACTGGC
HOX BL-	CCCCTGGACCCAAATGACATCCCGC
HOX BM-	GACTGTGTGAGACGGTTCCTCCGCC
HOX BN-	GTTTGGTTCTTGGTGTATGGCCGTCG
HOX BR-	CCTTACGTCCTGTACTCCTTGAGAAC
HOX BS+	GARYTVGARAARGARTT
HOX BS2-	GGCCCTGTTACTGAGCCTGGACTCA
HOX BT-	CKNCKRWTYTGRAACCA
HOX BU+	GGAACAGGTCACGCGGCGAGCGTC
HOX BX-	GAACAACATTTAAGCCACTGCACCT
HOX CD+	CCGACGGCCGCAGTCAATATCAACG
HOX CG+	CCCACCACGCAACTTCGGCGGGCTCG
HOX CT-	TTGGTACACGTTTCATGGCGGAAAA
OTX A+	MGTAARCARMGTMGIGARMGIAC
OTX B-	TGAACTCTKGAYTCTGGIARATT
OTX D+	TTTGGAGGCTCTGTTTGGG
OTX E+	ATCCGGACATCTTCATGAGG

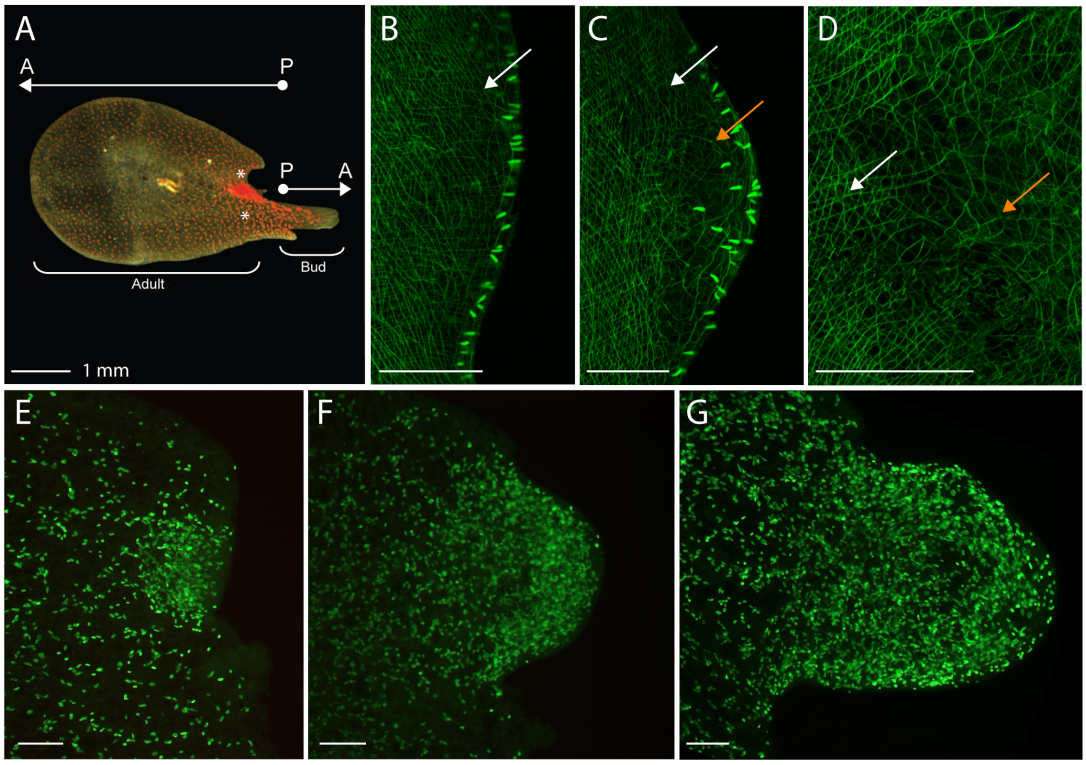

Figure 4.1. Reversed polarity budding in *C. retrogemma*. (A) Buds are produced with an A-P orientation completely reversed relative to the parent and are initiated (often asynchronously) from two bilaterally symmetrical budding positions at the posterior of the animal (asterisks). (B) Phalloidin staining of body wall musculature reveals normal pattern of musculature (white arrow) along the posterior margin in non-budding *C. retrogemma*. (C) A region of disorganized muscle (orange arrow) appears along the posterior margin of the adult upon bud initiation while the normal pattern of musculature persists in the adult body (white arrow). (D) The zone of muscle disorganization (orange arrow) forms a boundary between the organized musculature of the parent (white arrow) and bud and persists throughout bud elongation. (E) BrdU labeling of S-phase cells reveal a region of increased cell proliferation along the posterior margin of an adult as bud initiation begins. (F) Cells

forming a crescent shape in mid-stage buds show an increased rate of division compared to the background levels of cell proliferation within the adult body. (G) Late-stage buds maintain a crescent-like shape of slightly higher levels of cell proliferation when compared to the body of the parent. Scale bars = 100 μm unless otherwise noted.

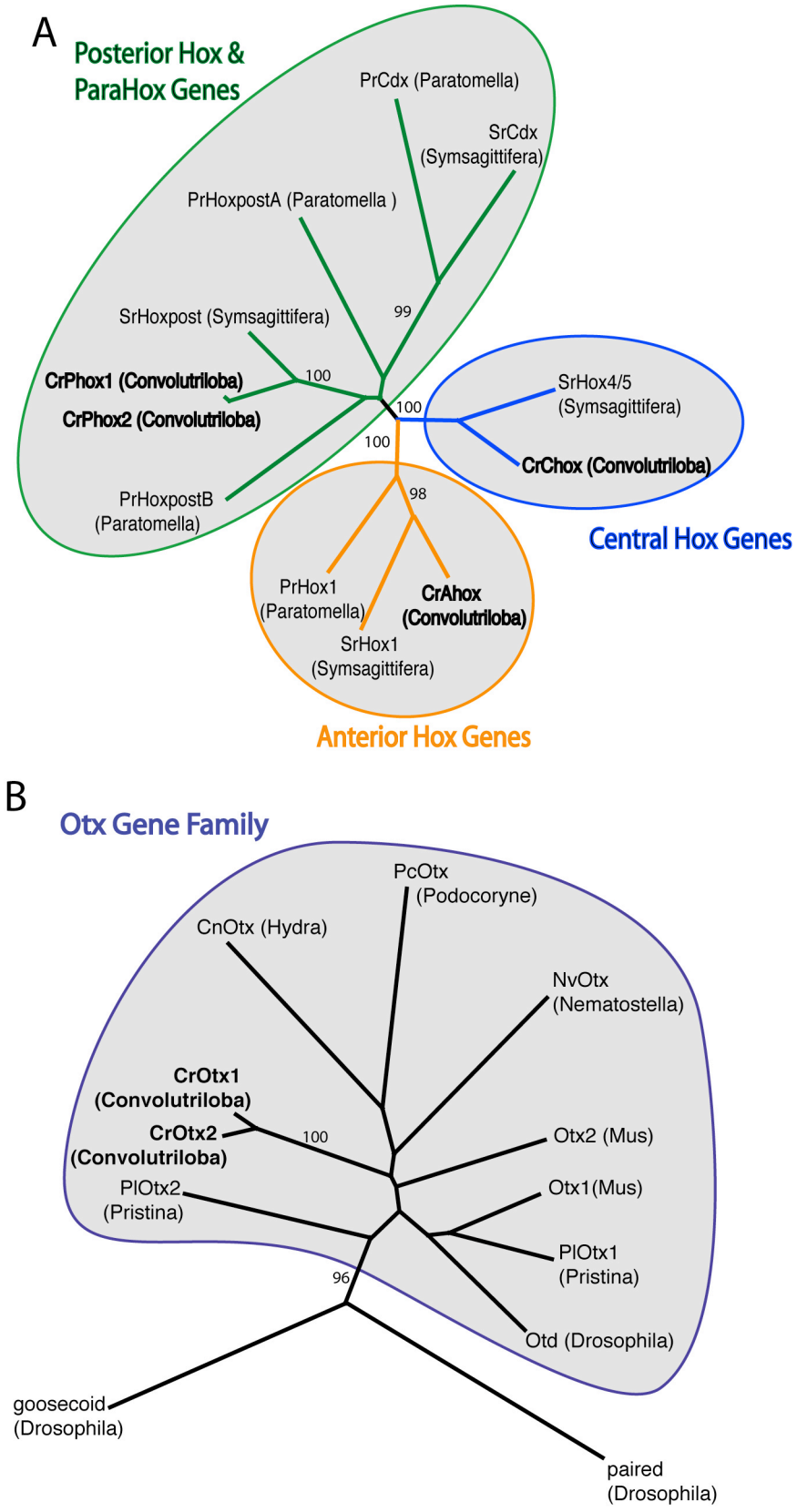
A
Posterior Hox &
ParaHox Genes
PrCdx (Paratomella)
SrCdx
(Symsagittifera)
PrHoxpostA (Paratomella)
SrHoxpost (Symsagittifera)
99
CrPhox1 (Convolutriloba)
100
CrPhox2 (Convolutriloba)
100
SrHox4/5
(Symsagittifera)
100
CrChox (Convolutriloba)
PrHoxpostB
(Paratomella)
98
Central Hox Genes
PrHox1
(Paratomella)
CrAhox
(Convolutriloba)
SrHox1
(Symsagittifera)
Anterior Hox Genes
B
Otx Gene Family
PcOtx
(Podocoryne)
CnOtx (Hydra)
NvOtx
(Nematostella)
CrOtx1
(Convolutriloba)
CrOtx2
(Convolutriloba)
100
Otx2 (Mus)
PlOtx2
(Pristina)
Otx1(Mus)
PlOtx1
(Pristina)
96
Otd (Drosophila)
goosecoid
(Drosophila)
paired
(Drosophila)

Figure 4.2. Phylogenetic analysis of *C. retrogemma* Hox and *otx* genes. (A) Neighbor-joining consensus tree depicting the relationship of the homeobox of Hox and ParaHox genes indentified in acoels. (B) Neighbor-joining consensus tree of the homeobox region of metazoan *orthodenticle* genes. Numbers on branches are bootstrap support values (1000 replicates). *Convolutriloba* sequences from this study are indicated in bold.

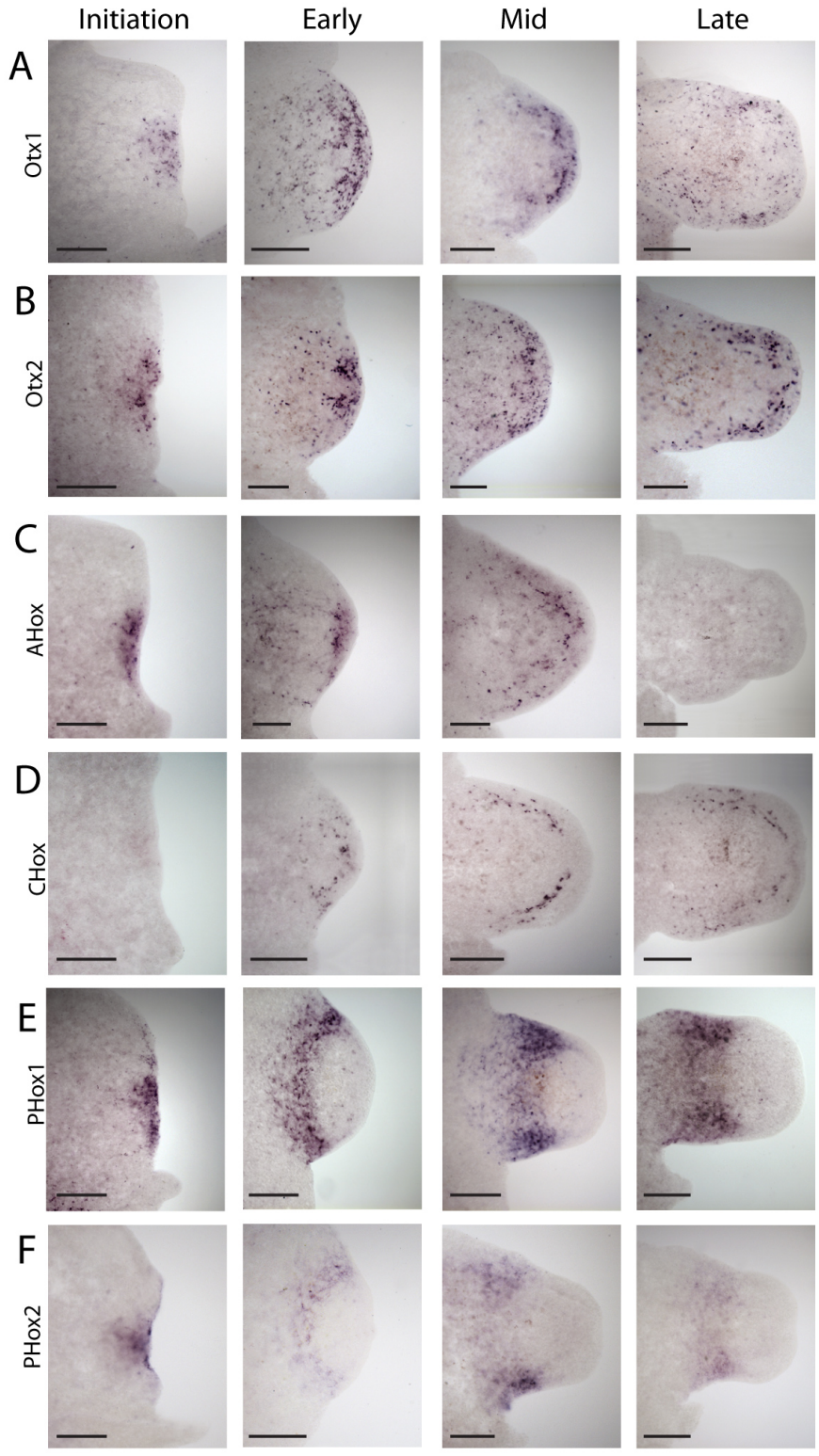
Initiation
Early
Mid
Late
A
Otx1
B
Otx2
C
AHox
D
CHox
E
PHox1
F
PHox2

Figure 4.3. Expression patterns of conserved axial body-patterning genes during stages of reversed-polarity budding in *C. retrogemma*. (A) *CrOtx1* is expressed within the parental tissue as buds are initiated, expressed at the anterior tip of early and mid-stage buds, and expressed in cells scattered throughout the entire bud in later stages. (B) *CrOtx2* is expressed at the posterior margin of the parent during bud initiation and is expressed in patterns associated with ganglion development in early stages and lateral nerve development in later stages. (C) *CrAhox* is expressed at the posterior margin of the parent during bud initiation and is expressed in association with ganglion development at early- to mid-stages but is not expressed in late-stage buds. (D) *CrChox* is not expressed before bud outgrowth occurs, but shows expression patterns associated with the developing lateral nerve cords in all budding stages. (E-F) Both *CrPhox1* and *CrPhox2* are expressed at the posterior margin of the parent as buds are initiated, expressed in a hemicircular posterior domain in early-stage buds, and expressed in a stripe of tissue just posterior to the midbody in mid- and late-stage buds. Scale bars = 150 μm.

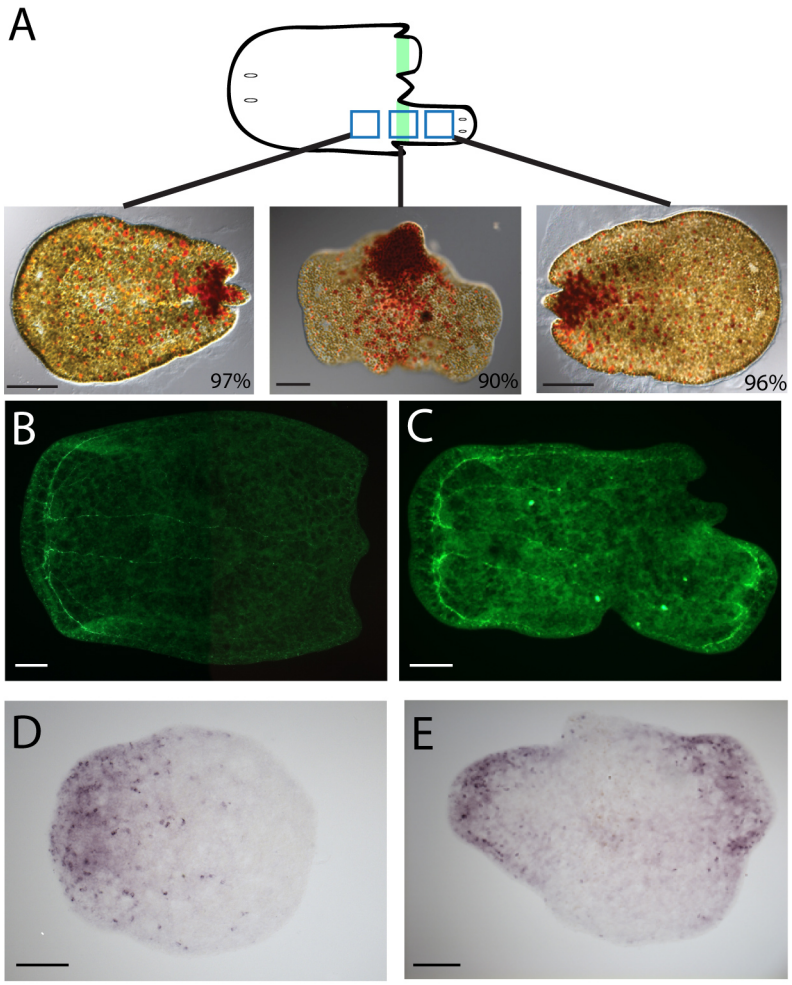

Figure 4.4. Regenerative potential of tissues across the polarity transition zone in *C. retrogemma*. (A) Excised regions of tissue (~1 mm^2) located fully within either the parent or the bud regenerate a single head and single tail, but excised regions that span the polarity transition zone (green shaded area) regenerate heads in both directions. (B) Serotonin immunostaining of a regenerated tissue fragment from the adult body reveals a single bilobed ganglion with associated nerve cords, shown five days after excision. (C) Serotonin immunostaining of a regenerated tissue fragment that spanned the polarity transition zone reveals bilobed ganglia and nerve cords on both ends, shown five days after excision. (D) In situ hybridization of *CrOtx1* reveals

expression localized at only one end of a regenerating tissue fragment excised completely from the parental body, shown three days after excision. (E) Anterior patterning, as revealed by *CrOtx1* expression, occurs at both ends of regenerating fragments when excisions spanned the zone of muscle disorganization, shown three days after excision. Scale bars = 150 μm.

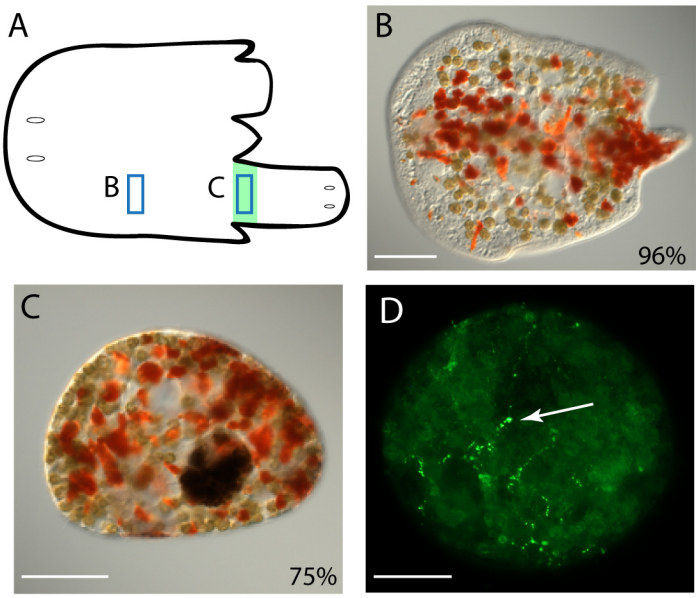

Figure 4.5. Regenerative potential of tissues located completely within the polarity transition zone of *C. retrogemma*. (A) Small regions of tissue (~0.01mm x 0.25 mm) were excised from within the parental body and completely within the zone of disorganized muscle at the base of mid to late stage buds. (B) Tissue excised from the parental body regenerates a complete head and tail within three days. (C) Tissue excised from completely within the zone of muscle disorganization fails to regenerate after 10 days, but does digest symbiotic algae (dark spot). (D) Tissue fragments that fail to regenerate have a disorganized pattern of serotonergic cells (white arrow) as revealed by serotonin immunostaining. Scale bars = 100µm.

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Curriculum Vitae

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EDUCATION

August 2009 — **University of Maryland,** College Park, MD USA
Ph.D. in Behavior, Ecology, Evolution, and Systematics

May 1998 — **Furman University**, Greenville, SC USA
Bachelor of Science, *magna cum laude,* in Biology

CERTIFICATIONS & COURSEWORK

June 2005 — Comparative Invertebrate Embryology
Friday Harbor Labs, University of Washington USA

June 2001 — Advanced Placement Teaching Certification
Environmental Science and Biology

May 1998 — South Carolina Secondary School Certification (Grades 7-12)
Biology, General Science, Gifted and Talented

PROFESSIONAL EXPERIENCE

2003-2009 — **University of Maryland,** College Park, MD USA
Advisor: Dr. Alexandra Bely
Dissertation project: Breaking the A-P axis: Evolution of diverse asexual reproduction strategies in *Convolutriloba* acoels

2003-2009 — University of Maryland, College Park, MD USA
Teaching/Research Assistant

- Instructed lab and discussion sections in undergraduate-level environmental science, ecology, and invertebrate zoology courses
- Conducted research on the evolution of regeneration on oligochaete annelids

2006-2007 **Duke University Talent Identification Program (TIP)**, Durham, NC
Instructor
- Taught gifted and talented high and middle school students in marine biology at the Duke Marine Lab summer program
- Teaching Assistant (Summer 2003)—physiological ecology

1998-2003 **Eastside High School**, Taylors, SC
Science Teacher
- Taught courses in biology, marine science, physical science, AP biology, and AP environmental science

1995-1997 **Furman University,** Greenville, SC
Laboratory/Teaching Assistant
- Assisted in laboratory preparation and instruction, Introductory Biology and Chemistry, Chordate Morphology and Development

HONORS/AWARDS

2008	Integrating Evolution, Development, and Genomics Biannual Conference Student Abstract Award
2008	Jacob Goldhaber Travel Grant, University of Maryland Graduate School
2007	University of Maryland Best Student Presentation of Research Award (BEES)
2003-2005	University of Maryland Darwin Fellowship
2005	University of Maryland Graduate Teaching Award given by the Department of Biology
2004-2005	University of Maryland Distinguished Teaching Assistant
2004	University of Maryland Janie Pritchard Award for Graduate Teaching Assistants
2002-2003	Eastside High School Teacher of the Year
1999-2001	Who's Who Among America's Teachers
1998	Who's Who Among American Colleges and Universities
1998	Phi Beta Kappa, Gamma Chapter of South Carolina
1997	Kappa Delta Pi, Honors Society in Education

PUBLICATIONS

Sikes, J.M. and A.E. Bely. 2008. Radical modification of the A-P axis and the evolution of asexual reproduction in *Convolutriloba* acoels. *Evolution and Development* 10 (5): 619-631.

Bely A.E. and J.M. Sikes. 2009. Repeated evolutionary losses of head regeneration in a group of asexual annelids. *(in prep).*

Sikes J.M. and A.E. Bely. 2009. Making heads from tails: Reversal of the primary body axis during asexual reproduction in *Convolutriloba retrogemma* (Acoela). *(in prep).*

Sikes, J.M. and A.E. Bely. 2009. Asexual acoels possess a diverse regeneration toolkit. *(in prep).*

RESEARCH PRESENTATIONS

Fall 2008	University of Maryland Bioscience Research Day, College Park, MD USA, Poster Presentation: "Making heads from tails: Reversal of the primary body axis in the basal bilaterian *Convolutriloba retrogemma*."
Summer 2008	Integrating Evolution, Development, and Genomics Biannual Conference, Berkeley, CA USA, Poster Presentation: "Radical modification of the A-P axis and the evolution of asexual reproduction in *Convolutriloba* acoels."
Winter 2007	Society for Integrative and Comparative Biology Annual Conference Phoenix, AZ USA, Oral Presentation: "Reversed polarity budding in a basal bilaterian (Acoel: Acoelomorpha)"
Summer 2006	Integrating Evolution, Development, and Genomics Biannual Conference, Berkeley, CA USA, Poster Presentation: "Development and evolution of diverse asexual reproductive strategies in *Convolutriloba* acoels."
Winter 2005	Society for Integrative and Comparative Biology Annual Conference, San Diego, CA USA, Poster Presentation: "Regeneration and asexual reproduction in acoels of the genus *Convolutriloba*"

SECONDARY EDUCATION EXPERIENCE (1998-2003):

GRANTS RECEIVED

2002-2003 **Toyota Tapestry Grant Award**

- $10,000 Grant award funding "Monitoring & Managing the Smooth Coneflower, *Echinacea laevigata"*
- Cooperative project with the SC Department of Natural Resources to develop a management plan for the endangered smooth coneflower in upstate SC USA

2001-2003 **Village Green Technology Innovation Grant Award**

- $50,000 Challenge grant award funding "The Peace Project"
- Interdisciplinary project providing technology to develop intercultural understanding, ecological awareness, and global awareness

2000-2001 **South Carolina EIA Grant Award**

- $1,000 grant award funding "Creating Coastal Conditions in the Classroom"
- Marine science project providing hands-on marine habitats within an inland classroom for data collection and observation