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Making Hopeful Monsters - Pharmacological Disruption of the
Oral-Aboral Body Axis in the Moon Jellyfish *Aurelia coerulea* (Scyphozoa)
and its Implications for Cnidarian Body Plan Evolution

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy in Biology

by

Hayden Patrick Speck

2026

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ABSTRACT OF THE DISSERTATION

Making Hopeful Monsters - Pharmacological Disruption of the
Oral-Aboral Body Axis in the Moon Jellyfish *Aurelia coerulea* (Scyphozoa)
and its Implications for Cnidarian Body Plan Evolution

by

Hayden Patrick Speck

Doctor of Philosophy in Biology

University of California, Los Angeles, 2026

Professor David K. Jacobs, Chair

The primary body axis organizes animal development, and signals based on the secreted proteins Wnt encode positional information in cells according to their distance from signaling centers at the poles of the body column ensuring they differentiate and form appropriate structures. The Wnt gene family and the component genes of the canonical Wnt signaling pathway such as GSK-3 β are conserved across animals, and likely defined the body axis of the last common ancestor of animals. The cnidarian moon jellyfish *Aurelia coerulea* alternates between a sexually reproducing nektonic medusa and asexually reproducing, epifaunal polyp. Thus, *A. coerulea* defines its primary body axis multiple times throughout its lifecycle. Hyperactivating canonical Wnt signaling with the GSK-3 β inhibitor 1-Azakenpaullone in *A. coerulea* polyps results in the conversion of the aboral pole to a functioning oral pole with no

change at the oral pole. Under similar treatment, other cnidarian polyps maintain their aboral pole and enlarge their original oral pole. The transition from the polyp to ephyra (immature medusa) in scyphozoa is known as strobilation - where the body column is divided into segments and then each segment metamorphoses into an ephyra. Aza incubation during strobilation represses segment formation, but does not alter the rate at which the morphogenetic signal propagates along the body column. However, the progress of metamorphosis along the body column is slowed by Aza incubation and can proceed in the absence of segment formation, suggesting that segmentation and metamorphosis are separate processes. The alterations brought on by Aza incubation suggest that true jellyfish (Scyphozoa) and hydroid polyps (Hydrozoa) regulate their body columns differently. In addition, alterations in canonical Wnt signaling produce medusa shapes reminiscent of other Medusozoan classes suggesting developmental mechanisms of evolutionary change ("phenocopies").

The dissertation of Hayden Patrick Speck is approved.

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University of California, Los Angeles

2026

DEDICATION

For my family and friends found, lost and found again

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LIST OF ABBREVIATIONS

ASW	Artificial Seawater
Aza	1-Azakenpaullone, an inhibitor of GSK-3 β
cWnt	canonical Wnt signaling, or the Wnt/ β -catenin signaling pathway
DMSO	Dimethyl Sulfoxide
MMI	5-methoxy-2-methylindole
O-A axis	Oral-Aboral axis (primary body axis) of cnidarians
RGB	Red, Green and Blue colorspace
Wnt	a portmanteau of the <i>Drosophila</i> gene <i>wingless</i> , and mouse gene <i>int1</i> - pronounced “wint”

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Ancient evolutionary origin of animal ciliary and microvillar structures, National Human Genome Research Institute Training and Career Development Virtual Annual Meeting, Remote, March 2020

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National Human Genome Research Institute Training and Career Development Virtual Annual Meeting, Remote, April 2021

Pseudoscience in the Age of Misinformation: the Origins, Challenges, and Potential Solutions,
Department of Ecology and Evolutionary Biology, UCLA, May 2021

Wnt signaling and the evolution of the medusa life stage in the moon jellyfish Aurelia coerulea
(Cnidaria; Scyphozoa), Department of Ecology and Evolutionary Biology, UCLA, Feb 2023

The Effects of cWnt Signaling on Neural Development in Moon Jellyfish, *Marc Lubman*,
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Silly Questions, Smart Bruins: The Little Mermaid vs UCLA Doctoral candidate Hayden Speck,
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Chapter 1 Pharmacological induction of mirror image oral poles in polyps of the invasive moon jellyfish *Aurelia coerulea* (Scyphozoa)

ABSTRACT

The primary body axis is an ancient organizational feature of Animalian development that may have served as a mechanism to generate novel body plans in evolutionary history. A common feature of all animals is the use of a cWnt based organizer at one pole of the primary body axis. Manipulation of cWnt signaling, such as its hyperactivation by GSK-3 β inhibition, can produce radical changes in body plan, such as the induction of ectopic oral structures along the body column. In the polyps of the moon jellyfish *Aurelia coerulea*, GSK-3 β inhibition through 1-Azakenpaullone application results in the conversion of the aboral pole into a new oral zone yielding bipolar polyps. This conversion appears to be concentration dependent, with lesser concentrations of Aza only partially suppressing the aboral identity at the aboral pole, resulting in a pole with both foot and mouth structures. This response differs from other cnidarian polyps studied thus far, which tend to see a dramatic expansion of the original oral zone with paullone exposure. The lack of expansion of the original polyp oral zone in *A. coerulea* may be indicative of differences in developmental regulation between the Scyphozoa and Hydrozoa, two cnidarian classes which diverged hundreds of millions of years ago. The response of *A. coerulea* polyps to Aza incubation may be of further practical significance, as it could allow the rapid expansion of clonal polyp populations for laboratory research.

INTRODUCTION

Based on the importance of the primary body axis in animal development, we set out to establish a protocol for the use of the GSK-3 β inhibitor 1-Azakenpaullone with *Aurelia coerulea* polyps, and determine any concentration dependent outcomes. We did this by incubating our animals with a variety of concentrations of Azakenpaullone over an extended period and

recording the emergence of ectopic oral structures and changes in the body column of the polyps. We also sought to determine potential targets for further gene expression analysis studies by establishing Wnt gene expression profiles in existing transcriptomic data for the Long Beach, California *A. coerulea* strain and comparing them to previous studies in *Aurelia*. The results of this chapter form a framework for the further manipulation of the *A. coerulea* lifecycle found in Chapters 2 and 3 using Azakenpaullone.

The oral-aboral axis-a common animal feature

Differentiation of cells and structures along a body axis is one of the primary means by which complex multicellular lifeforms organize themselves in development. In animals, the most ancient body axis is the primary body axis, which in bilaterians runs from the anterior to posterior pole of the body column (Lee et al., 2006; Ryan and Baxeavanis, 2007). This axis uses biochemical signals emanating from a signaling center known as an “organizer”, located at one pole of the body column, to encode positional information in cells along the body column (Hayward et al., 2015; Kraus et al., 2007). Organizers were first detected via transplantation experiments where transplanted patches of tissue induce the formation of the structures they were normally associated with at their new location (Spemann and Mangold (née Pröscholdt), 1924). In animals, many of the primary signals emanating from the organizer rely on canonical Wnt signaling-an ancient signaling pathway found in all animals (Holzem et al., 2024). Chemical and transgenic manipulation of this pathway can suppress or enhance the appearance of anterior and posterior features of animals, creating organisms with multiple heads, or lacking heads entirely (reviewed in Petersen and Reddien, 2009). The universality of this signaling pathway in animals and the role it plays in determining body form make cWnt an intriguing mechanism by which novel body forms could have arisen throughout evolutionary history (Duffy, 2011; Loh et al., 2016).

By investigating organisms such as cnidarians (the phylum of jellyfish, anemones, hydroids and their relatives) that diverged long ago from well known bilaterian models, we can better understand cWnt's potential role in body plan diversification. Cnidarians are the sister taxon to Bilateria, sharing the endoderm, ectoderm and oral opening with Bilaterians, but lack an anal pore or mesodermally derived structures. Despite their differences, cnidarians have been shown to use the same mechanisms in patterning their oral-aboral axis that bilaterians use to organize their anterior-posterior axis, strongly supporting the homology of these systems in the cnidarian-bilaterian clade (Lebedeva et al., 2021; Lee et al., 2006). These features include the definition of one pole (posterior in Bilateria, oral in Cnidaria) of the body column by a cWnt based organizer (Holstein, 2012; Holzem et al., 2024).

Overview of cWnt signaling

The Wnt family of genes are secreted ligands that bind to a variety of receptors. In humans, the “canonical” Wnt (cWnt) signaling pathway sees a Wnt family ligand bind to the membrane bound LRP-5/6 and *frizzled* receptors (Krishnamurthy and Kurzrock, 2018). This recruits the *disheveled* protein and a protein complex composed of Axin, APC and Glycogen Synthases Kinase 3 β (GSK-3 β) (Gordon and Nusse, 2006). This complex degrades β -catenin proteins in the cytosol, but is inactivated while Wnt is bound to *frizzled*. Subsequently, β -catenin accumulates in the cytosol and is transported into the nucleus. In the nucleus, β -catenin binds to *tcf/lef* transcription factors to regulate gene expression. This form of Wnt signaling is also referred to as Wnt/ β -catenin signaling.

cWnt is also known to affect signaling pathways independent of β -catenin. These “non-canonical” pathways include the Wnt/Ca²⁺ and the Wnt/Planar Cell Polarity (PCP) pathway. In Wnt/Ca²⁺ signaling, Wnt binding to *frizzled* alters the activity of Phospholipase C (*PLC*) resulting

in an increase in cytoplasmic Ca^{2+} , in turn activating CamKII and Protein Kinase C, activating various transcription factors (De, 2011). The PCP pathway sees *wnt* ligands bind to *frizzled*, recruiting *dishevelled*, activating cytoskeletal changes and downstream cascades including Rac-1/JNK and DAAM/RhoA/ROCK (Chae and Bothwell, 2018). Wnt signaling is also known to interact with the *sonic hedgehog* and *notch* pathways, both of which are important for development (reviewed in Krishnamurthy and Kuzrock 2018).

Secreted Wnt ligands act as a morphogen establishing patterning along the anterior-posterior axis in bilaterians, as well as the homologous Oral-Aboral (O-A) axis in cnidarians. Increased cWnt signaling results in a repression of anterior features in Bilateria and an expansion of oral regions in cnidarians (Duffy et al., 2010; Kiecker and Niehrs, 2001). Additionally, both canonical and noncanonical Wnt signaling interact with tentacle formation in the cnidarian *Hydra* (Philipp et al., 2009). cWnt signaling also impacts stem cell differentiation in bilaterians and hydrozoans, and errors in Wnt signaling are involved in tumorigenesis (Polakis, 2012; Teo et al., 2006; Van Camp et al., 2014).

Wnt gene family evolution

There are twelve subfamilies of Wnt ligand, with some animal lineages such as Deuterostoma (Schubert et al., 2000) and Lophotrochozoa having taxa with most subfamilies present, while some lineages like Ctenophora have members with significantly fewer Wnt families (Cho et al., 2010; Holstein, 2012; Kusserow et al., 2005). Cnidarian genomes show a full complement of Wnt ligands in Anthozoa (Khalturin et al., 2019; Kusserow et al., 2005). An early study of cnidarian genomes has found evidence for only seven shared genes between most cnidarians and bilaterians, with significant duplications of Wnt lineages occurring in the Medusozoa subphylum (Hensel et al., 2014). However, the most recent survey seems to indicate only Wnt9 and Wnt10 are absent in *Aurelia aurita/coerulea* (Khalturin et al., 2019). All

together, these results suggest that all Wnt subfamilies were found in the Cnidarian-Bilaterian ancestor

1-Azakenpaullone

1-Azakenpaullone (Aza) is a potent inhibitor of GSK-3 β kinase activity (Kunick et al., 2004). By inhibiting degradation complex activity, Aza and related chemicals effectively mimics the effects of an increase in canonical Wnt/ β -catenin signaling in a wide variety of taxa, including Bilaterians, Poriferans, and Cnidarians (Teo et al., 2006; Windsor and Leys, 2010). GSK-3 β is also involved in other signaling cascades such as the Insulin/IGF-1 related pathway (reviewed in Duda et al., 2020; Medina and Wandosell, 2011).

GSK-3 β inhibition in cnidarians

cWnt signaling's role in polyp body formation is relatively well studied in non-scyphozoan Cnidarian model systems. Many studies have shown cWnt signaling to determine the Oral-Aboral axis during gastrulation and is involved in defining the oral zone of the animal. Hyperactivation of this pathway during gastrulation, regeneration, or sometimes merely in the adult animal, can result in the ectopic development of oral features and extension of the oral zone. Similar to many bilaterian animals, cnidarians determine their anterior-posterior axis as a result of the egg's initial animal-vegetable polar axis, with anterior structures occurring at the animal pole (reviewed in Martindale, 2005). This, along with *Hox* gene expression studies along the body column (Ryan et al., 2007), provides compelling evidence that the cnidarian O-A axis is homologous to the anterior-posterior axis of bilaterians.

NEMATOSTELLA

Gastrulation in *Nematostella* shows increased nuclear β -catenin localization at the site of gastrulation in a cWnt dependent manner, with the blastopore directly developing into the oral opening of the planula (and later polyp) (Wikramanayake et al., 2003). Knockout of oral associated gene brachyury in gastrulating *Nematostella* embryos greatly reduces expression of several Wnt ligands, with embryos failing to elongate post gastrulation (Servetnick et al., 2017) , while cWnt agonist treatment yielded “oralized” embryos (Trevino et al., 2011), and cWnt hyperactivation in late gastrulae yield bipolar planulae and polyps (Kraus et al., 2016). cWnt hyperactivation in *Nematostella* polyps during regeneration yields ectopic oral structures including mouths and stunted tentacles at the site of injury (Trevino et al., 2011). Normal polyps exposed to cWnt agonist showed no response after 8 days (Trevino et al., 2011).

HYDROZOA

Hydrozoa are medusozoan falling outside the clade containing scyphozoan and cubozoan jellyfish (Kayal et al., 2018), but some such as *Hydra* have lost the medusa phase (Daly et al., 2007). *Hydra* Wnt expression is associated with the oral “organizer” of the polyp hypostome, and Wnt expression also occurs in the hypostomes of nascent buds off the animal’s body column. *Hydra* Wnts have a similar expression pattern during regeneration, and transplanted Wnt3 treated tissue yield animals with secondary body axes (Lengfeld et al., 2009). Adult polyps subject to Alsterpaullone treatment proliferated oral structures (tentacles) progressively down the body column, showing that cWnt signaling actively maintains the body axis in *Hydra* beyond its initial role in axis formation (Reddy et al., 2019). *Hydra* has lost the planula stage, with fertilized eggs encysting and developing into polyps shortly before hatching (Martin et al., 1997), making cWnt manipulation in the embryo difficult. Beyond its role in oral region determination, Wnt ligands and associated genes are actively expressed at the tips of *Hydra* normal and ectopically induced tentacles (Philipp et al., 2009).

Hydractinia is colonial, with the feet of individual polyps/zooids connected by a stolon network. cWnt hyperactivation during embryogenesis and settlement oralizes the resulting animals and suppresses foot structures like stolons (Duffy et al., 2012). cWnt hyperactivation in pre-gastrulae results in multipolar planula which transform into multipolar polyps (Plickert et al., 2006). cWnt hyperactivation during regeneration in decapitated *Hydractinia* results in both fragments regenerating mouths at the site of injury (Duffy et al., 2010). Similar to *Hydra*, GSK-3 β hyperactivation substantially increases the oral region of the mature polyp, however *Hydractinia* shows an enlarged hypostome and suppressed stolon formation (Müller et al., 2007; Teo et al., 2006). Polyps arising from treated colonial stolon mats originate as atypically large buds that split and develop into multiple polyps, many of which are multipolar.

There is some variety in response to cWnt hyperactivation in Hydrozoans, as seen in *Dynamena pumila*. *D. pumila* has apolar gastrulation which does not respond to variation in the cWnt signaling pathway, and while the O-A axis of the planula of *D. pumila* appears to be determined by Wnt signaling, planula subject to cWnt hyperactivation do not develop multiple poles (Vetrova et al., 2022). Intriguingly, cWnt signaling may determine the form of colonies in *D. pumila*. Normally a mature *D. pumila* colony grows as a single stalk with zooids branching off of it, however cWnt hyperactivation suppresses stalk growth and generates a stolon with zooids growing directly out of it (Bagaeva et al., 2019). This is consistent with work in *Hydractinia* which identified expression of several Wnt ligands in stolons (Hensel et al., 2014) and cWnt hyperactivation induces enormous polyp anlagen from the stolon plate (Muller et al., 2007).

The Aurelia coerulea polyp

Aurelia polyps exhibit polarization along the oral-aboral axis, in a broadly similar body plan to other cnidarian polyps. The oral pole consists of the mouth (hypostome) set into a flat region at the apex of the body column (oral disk). On the margins of the oral disc is a single ring

of tentacles. The calyx is a conical region below the oral disc which contains most of the gastric cavity. The foot occupies the aboral pole, and consists of one or more tendrils which adhere to surfaces, anchoring the animal. *Aurelia* polyps can translocate along the substrate by means of their stolons, which attach to the substrate and retract. The old foot elongates and eventually breaks away as endodermal cells transfer to the stolon which becomes the new foot (Gilchrist, 1937). Polyps can asexually give rise to further polyps via budding, where a lump of tissue from the lower body column evaginates and forms a new oral structure. As the bud matures, the connection between it and the parent polyp narrows and eventually breaks (Gilchrist, 1937; Schiariti et al., 2014). See Fig 1 for a diagram of basic polyp external anatomy.

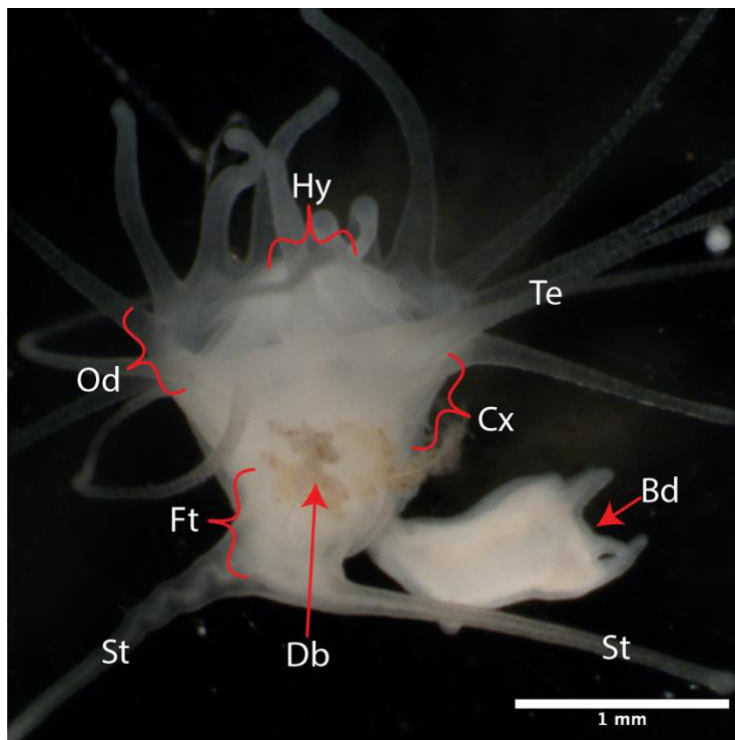


Figure 1 - Anatomical diagram of *Aurelia coerulea* polyp

A polyp on day 0 of the experiment with a pre-existing bud. Natural buds tend to have their own body column and maintain an attachment by their foot to the lower portion of the parent animal. Tentacles and stolons are both long tendrils of tissue, however stolons tend to have a visible core of dense endoderm, while tentacles are more uniformly transparent. Abbreviations - Cx - Calyx, Db - Debris, Ft - Foot, Hy - Hypostome, Od - Oral Disc, St - Stolon, Te - Tentacle.

Hypotheses

Based on the effects of GSK-3 β inhibition on other cnidarian polyps, we expect Aza incubation to have an “oralizing” impact on *A. coerulea* polyps, with higher concentrations of Aza having a more severe impact. Hydrazoan polyps see a dramatic expansion of the oral zone of the animal, with tentacles developing along the body column, so we assumed *Aurelia coerulea* polyps would show a similar response.

While we observe an oralizing effect of Aza on *Aurelia* polyps, unlike in Hydrozoan polyps, the original oral pole appears unchanged. Rather, Aza induces the conversion of the aboral pole of the polyp into a new oral pole, similar to the impact of Aza on Hydrozoan and Anthozoan larvae or regeneration in their polyps. At high concentrations, the foot converts wholesale into a mouth that remains fused to the parent animal. Lower concentrations of Aza appear to partially repress the aboral identity of the foot. The difference in responses between *Aurelia coerulea* and the differing cnidarian classes appear to show differences in body plan formation and maintenance. In *Aurelia coerulea*, the oral region of the polyp is relatively insensitive to GSK-3 β inhibition, while the foot is quite sensitive, the opposite case of Hydrozoan and Anthozoan polyps which show the persistence of the aboral pole throughout Aza exposure. Whether this difference is unique to *A. coerulea* or common to Scyphozoans remains to be seen. We also determine a number of Wnt genes as potential targets for further gene expression analysis, particularly in combination with Aza, in order to determine the role cWnt signaling plays in determining the primary body axis of *Aurelia polyps* and support previously published expression patterns.

METHODS

Concentration trial

Solutions of 0 (control), 0.125, 0.25, 0.5, 1, and 2 μ M 1-Azakenpaullone (Sigma-Aldrich A3734) (Aza) in 0.1% DMSO and seawater were prepared. 20 animals per condition were used, for a total sample size of 120. One animal was added to each well of five 24-well (4x6) plates. The six wells in each row were randomly assigned one of the six Aza concentrations, with 4 of each condition present on each plate. Animals were added to the wells and starved 48 hours prior to the start of the experiment. Mature polyps with a single oral pole and no buds were selected for the experiment, however some animals formed buds during the 48 adjustment period.

The trial was conducted for 42 days, during which animals were not fed. Animals were not fed to minimize the need for water changes. Every 1-2 days, the location of plates within the incubator was shuffled, and plates were rotated 180°. Animals were photographed once every 5-8 days. Each image was graded for the presence of new oral pole structures-*buds* were defined as oral poles (oral openings surrounded by a ring of tentacles) with a calyx that was separate from the calyx of the parent polyp, and *second mouths* were defined as oral poles sharing the same calyx of the original polyp, oriented in the direction of the original aboral pole.

Strobilation testing

Twelve bipolar polyps generated by 2 μ M Aza exposure were exposed to 5-methoxy-2-methylindole (MMI), a potent inducer of strobilation in *Aurelia* and other Scyphozoa (Fuchs et al., 2014; Helm and Dunn, 2017). A 50 mM MMI stock solution in 100% ethanol was added to seawater at a 1:1000 dilution, with each animal in their own well. After 24 hours, the MMI solution was removed and replaced with seawater. The strobilation progress of the animals was checked after 2 weeks.

Aurelia coerulea cWnt related orthologs and transcriptomics

Orthologs were identified using *Orthofinder2* (Emms and Kelly, 2019, 2018) to compare *A. coerulea* gene models from Gold et al. 2019 against a database including gene models from *Alatina alata*, *Calvadozia cruxmelitensis*, *Cassiopea xamachana*, *Homo sapiens*, *Hydra vulgaris*, and *Nematostella vectensis* retrieved from NCBI. Putative orthologs were further vetted by examining the domain structure of sequences using NCBI's Conserved Domain Database (Wang et al., 2023). The inventory of Wnt genes in Gold et al.'s *Aurelia coerulea* was compared against the inventory of Wnt genes found in *Aurelia* genomic and transcriptomic data found in the literature (Brekman et al., 2015; Khalturin et al., 2019).

Transcriptomic data from Gold et al. 2019 was used to investigate Wnt gene expression across the *Aurelia coerulea* lifecycle. The *Bioconductor* package *edgeR* (Robinson et al., 2010) was used to filter and normalize the data, with a threshold of at least one read assigned to a gene across at least two samples per lifestage. Wnt gene expression profiles were then clustered by lifestage.

RESULTS

Animals exhibited a range of responses corresponding to the concentration of Aza they were exposed to. All animals survived for the duration of the experiment. Bud production reached a peak at 0.5 (μM), with 12/20 producing at least one bud. Animals at 1 μM and 2 μM Aza produced secondary oral poles that remained fused to the main body column, rather than developing a separate calyx (19/20 for 1 μM and 19/20 for 2 μM). Of those animals that produced a bud, most produced only one bud, however 3/20 animals at 0.5 μM and 1/20 animals at 0.125 μM Aza produced two buds post Aza exposure. See Fig 2 for summary of results and Fig 3 for a timeline of new oral pole development. Most budding animals produced buds by day 12, while most animals that formed a second mouth had a distinct oral pore surrounded by tentacle buds by day 20.

Ectopic tentacles were uncommon-only 4/20 animals at 2 μM developed any tentacles at points away from any oral disc. Additionally, in 3 of 4 cases, these ectopic tentacles were closely situated to the secondary oral zone at the former aboral pole, rather than the original oral pole. The remaining case of ectopic tentacle formation seems to be a product of the fusion and gradual separation of the original and secondary oral fields rather than the independent extension of the original zone (see Fig S7). Ectopic tentacles also had the tendency to join nearby oral discs due to tissue reorganization, making the appearance of a hugely extended oral pole with tentacles along the majority of the body column, as in *Hydra*, unlikely.

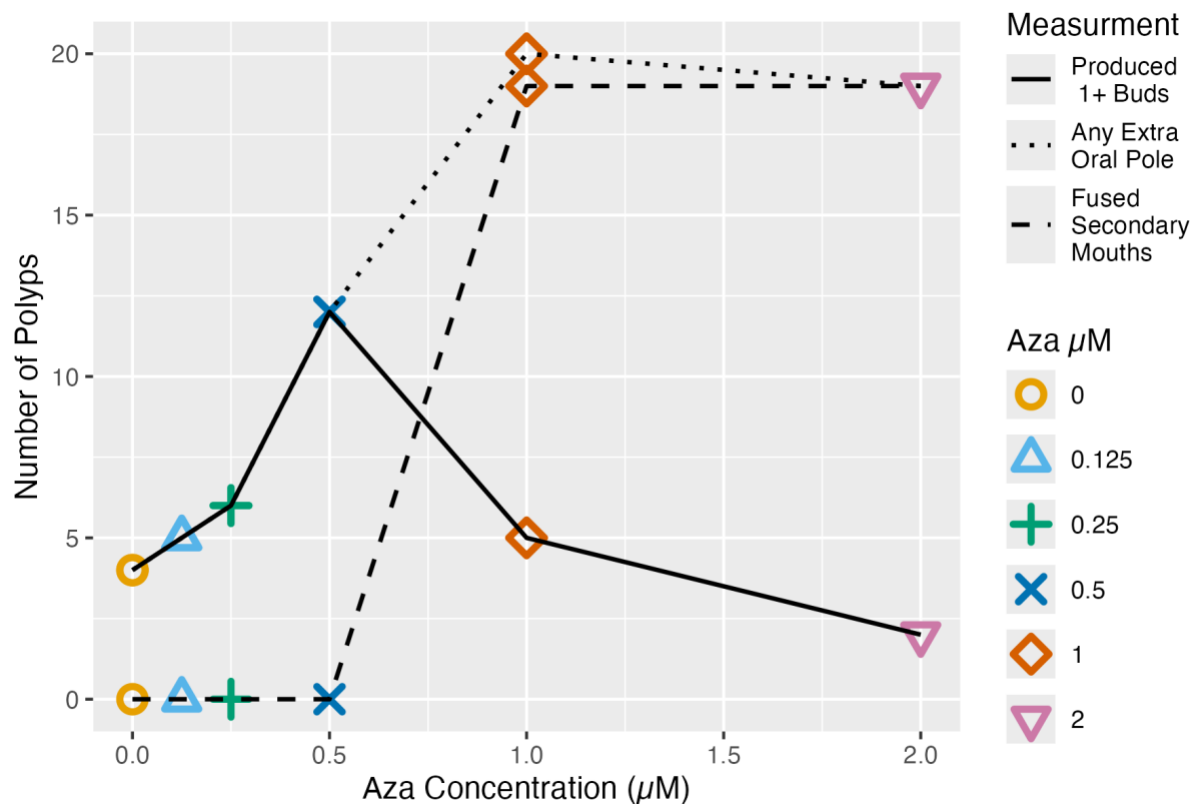


Figure 2 - Oral Pole formation in response to Aza

The proportion of polyps producing at least one additional oral pole rose with Aza concentration. Animals in Aza concentrations $> 0.5 \mu\text{M}$ tended to produce secondary oral poles that were fused to the body column of the parent polyp, as opposed to having their own distinct calyx. All conditions had $n = 20$.

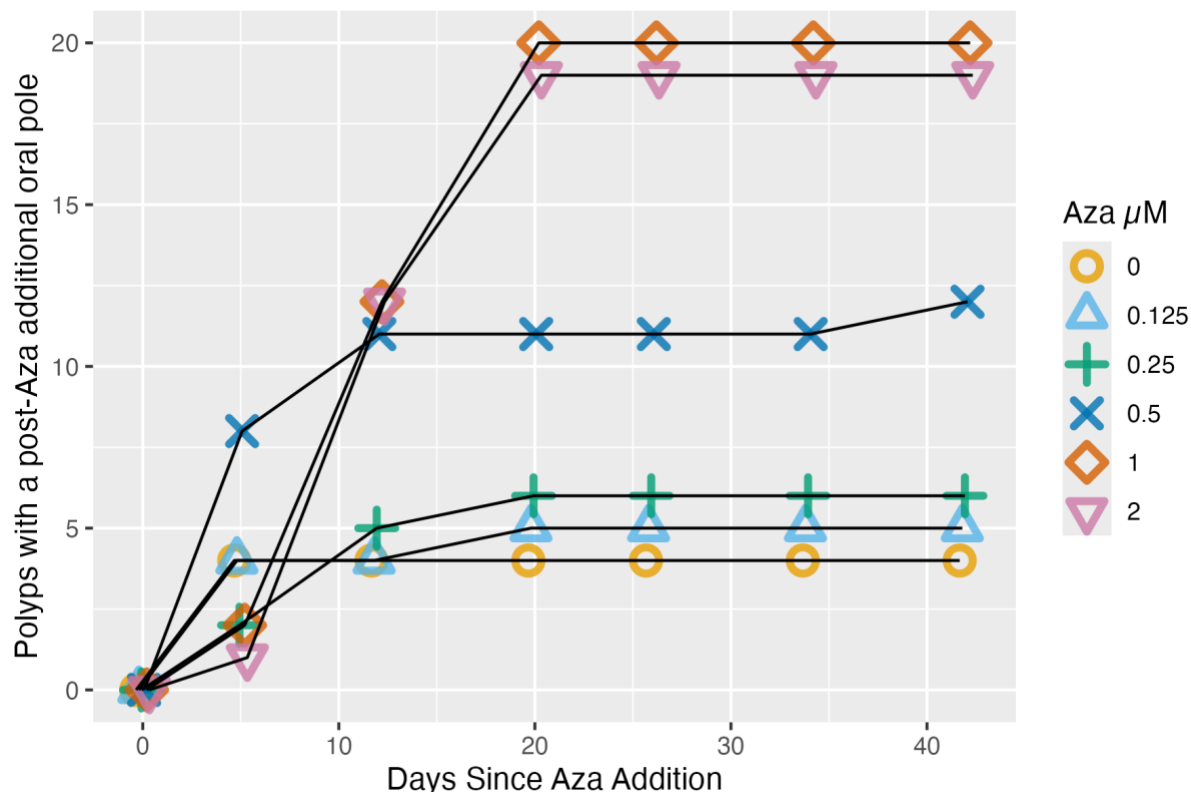


Figure 3 - Timeline of additional oral pole formation

Almost all budding animals produced a bud by day 12. By day 20, animals that produced a second mouth had developed a distinct oral pole in the place of the foot.

Statistical analysis of Aza impact

There was no significant difference in the number of animals with buds at the start of the experiment between conditions (Fisher's Exact Test p-value = 0.6778). Conditions significantly differed in the number of polyps that developed one or more buds, a second mouth, or any new oral pole (bud or second mouth) post Aza addition (Fisher p-value = 0.0214, <2.2e-16, and 8.094e-12 respectively). Pairwise Fisher Tests with Bonferroni correction shows a significant difference between 0.5 μM and 2 μM in terms of buds created post Aza (adjusted p-value = 0.033) and a difference between the high concentration conditions (1 μM and 2 μM) and all other conditions for secondary mouth formation (p-values < 1e-8). When considering the formation of any additional secondary poles, the high concentration conditions differed from 0-

0.25 μM , however 0.5 μM did not differ significantly from 2 μM . 0.5 μM did not differ significantly from the lower concentration conditions

Concentration dependent induction of secondary mouth formation

The aboral pole of 1 μM animals tended to maintain some foot characteristics, while 2 μM animals tended to see the wholesale conversion of their aboral pole into a new mouth. Half (10/20) of 1 μM animals appeared to convert half their aboral pole into one or more oral poles (see Fig 4a), while maintaining the other half as elongated foot tissue, while only 1/20 2 μM animal did the same (Fisher test p-value =0.003342). It should be noted that these projections did not adhere to surfaces of the well.

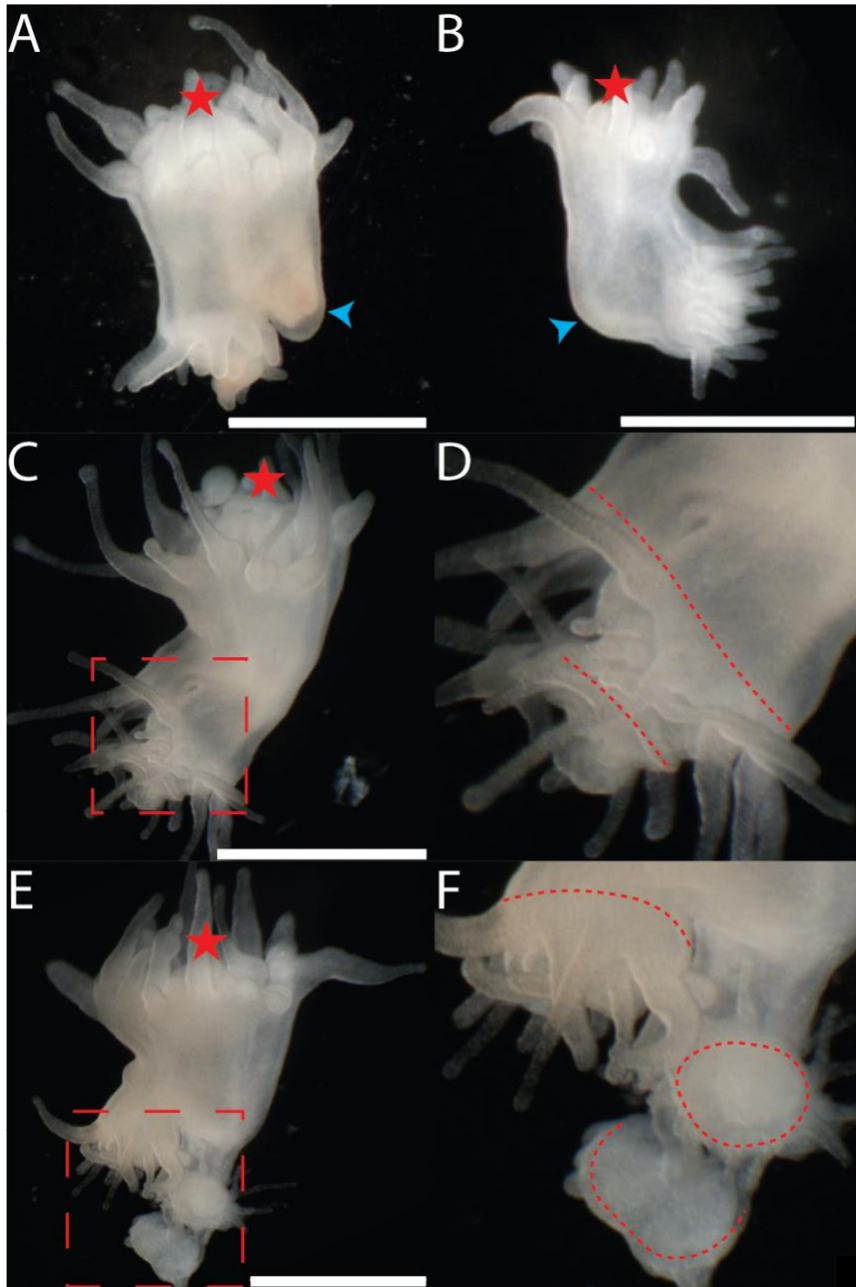


Figure 4 - Phenotypes for 1 μM Aza incubation

Animals incubated at 1 μM tend to show incomplete repression of aboral identity, resulting in disrupted secondary oral pole formation. Scale bars indicate 1 mm, red stars indicate the animal's original oral pole. **A)** Partial conversion of the foot, where half the foot converts into a new oral pole, and the other half of the foot (indicated by teal arrow) regresses to be a dense condensation of tissue. **B)** A developing foot nub indicated by teal arrow. **C)** A bipolar individual with layers of oral structures developing along the remnant of the foot. **D)** A close up of C where red dotted lines indicate oral discs. The distal oral region retreats toward the oral region formed from the base of the calyx. As the oral regions approach each other, they can fuse into a single oral zone **E)** This animal is somewhat intermediate between A and C, where half the foot has

converted to a single oral region. The other half has retained an elongated foot-like structure and shows rings of oral structures along the elongated tissue. **F)** A close up of E, with red dotted lines indicating oral discs

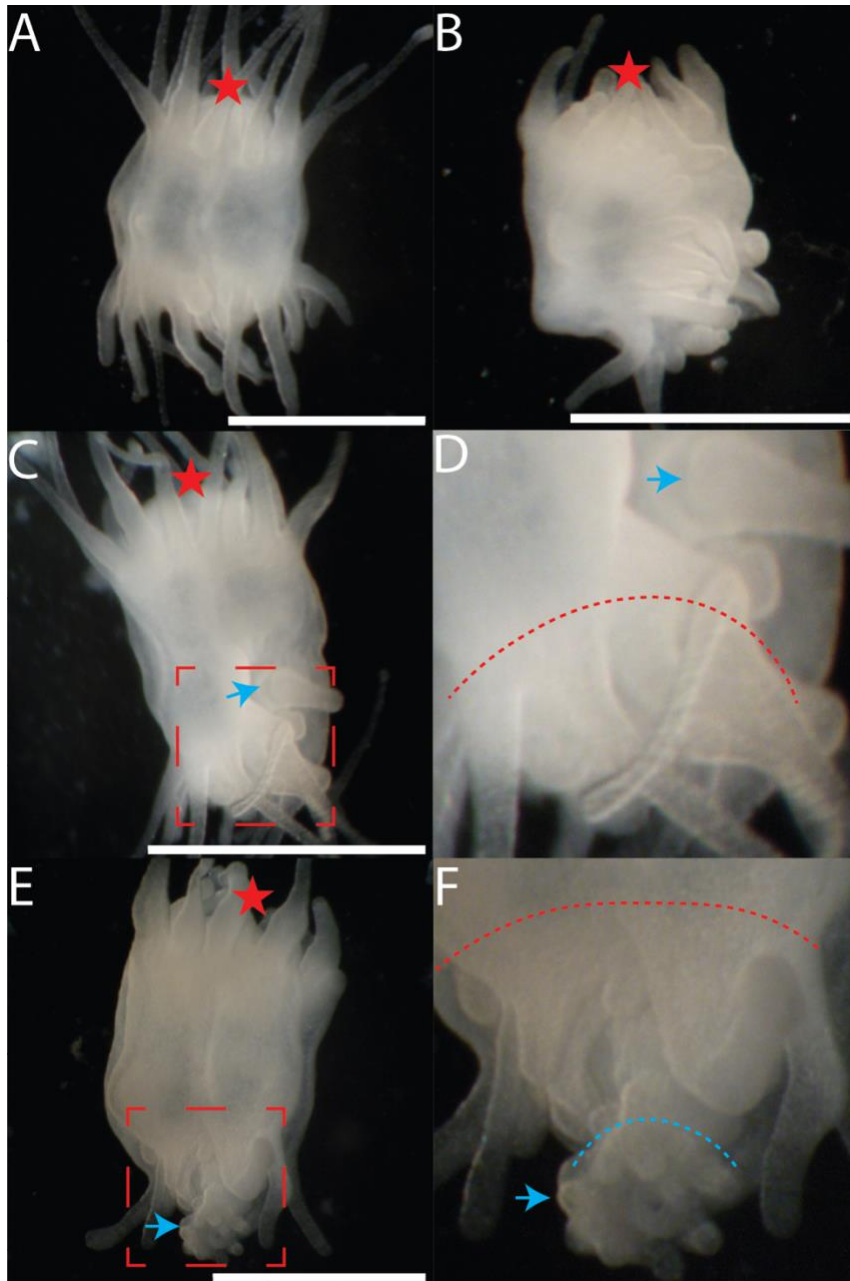


Figure 5 - Phenotypes for 2 μ M Aza

At 2 μ M Aza, foot structures are even more suppressed than at 1 μ M. Red stars indicate the original polyp oral pole. **A)** A bipolar ephyra with a full conversion of foot tissue to an oral region. **B)** An animal with a mostly repressed foot as in Fig 4b. **C)** An animal with an ectopic tentacle located somewhat at a distance from the secondary mouth. Ectopic tentacles can merge with

existing secondary oral structures and be incorporated with the tentacle ring along the oral disk. **D)** A closeup of C. **E)** An animal with several rings of tentacles. **F)** a close up of the secondary oral pole showing the ring of tentacles at the base of the calyx indicated by a dashed red line. The dashed cyan line indicates an additional ring of tentacles around an oral disc, similar to Fig 4c. The distal most mouth developed and receded toward the base of the calyx.

Secondary mouths have normal neuronal and developmental responses

Secondary mouths appeared to function as normal mouth structures and signaling centers. Secondary mouths occasionally withdrew tentacles and placed them in their mouths, a behavior consistent with normal polyp feeding. All bipolar polyps exposed to MMI strobilated from both oral poles in all animals (12/12). Such strobilation produced bipolar strobila stacks, with the oral poles of resulting ephyrae oriented toward the corresponding oral pole. Ephyra resulting from both poles exhibited twitching behavior consistent with attempted swim strokes. For further investigation of strobilation in bipolar animals see Chapters 2 and 3.

Wnt gene inventory in Aurelia

Existing genomic and transcriptomic resources for *Aurelia* show a fair degree of variation in the presence and absence of Wnt family genes. Khalturin et al. 2019 shows the most complete inventory of Wnt genes, missing only Wnt 9 & 10. Gold et al. 2019 shows the most missing Wnt genes, with Wnt 6,7, 9,10, & A lacking orthologs. In addition, many of the orthologs found in Gold et al. 2019 are only partial gene models, lacking either start or stop codons, or having large regions of “N”s.

Table 1 - Comparison of published Wnt data in the Aurelia genus

Note that Khalturin et al. 2019 and Gold et al. 2019 use gene models to call the presence or absence of genes, while Brekhman et al. 2015 uses a de novo transcriptome assembly, making it difficult to say if a gene is definitively absent. Presence is indicated by a “+” sign, and letters indicate the presence of multiple homologs. Absence is indicated by a “-“ sign. Gold et al. 2019 gene models are marked with an “*” to indicate an incomplete gene model as called by NCBI’s Conserved Domain database

Wnts	Khalturin 2019 – Roscoff - “<i>aurita</i>” / <i>coerulea</i>	Brekhman 2015 – Red Sea <i>aurita</i>	Gold 2019- Long Beach <i>coerulea</i> gene model number
1	+	+	1579.2
2	+	+	145.5
3	+	+	385.14
4	a, b	-	1722.5 (b)
5	+	a, b	915.2*
6	+	-	-
7	a, b	a	-
8	a,b	+	292.1 (b)*
9	-	single Wnt 9/10 detected	-
10	-	single Wnt 9/10 detected	-
11	a, b	+	675.38 (a); 8462.1 (b)*; 1688.5 (b)
16	+	a, b, c	3321.1*; 4362.1*
A	+	-	-
Missing families	9, 10	4, 6, A	6, 7, 9, 10, A

Gene Expression Analysis

Wnt genes in Gold et al. 2019 break into three broad groups across *A. coerulea*'s lifecycle. Wnts broadly grouped into three classifications, roughly corresponding to high planula expression followed by downregulation, low planula expression followed by increased expression starting in the polyp, and a group which saw low expression in the polyp and upregulation during strobilation (see Fig 6)

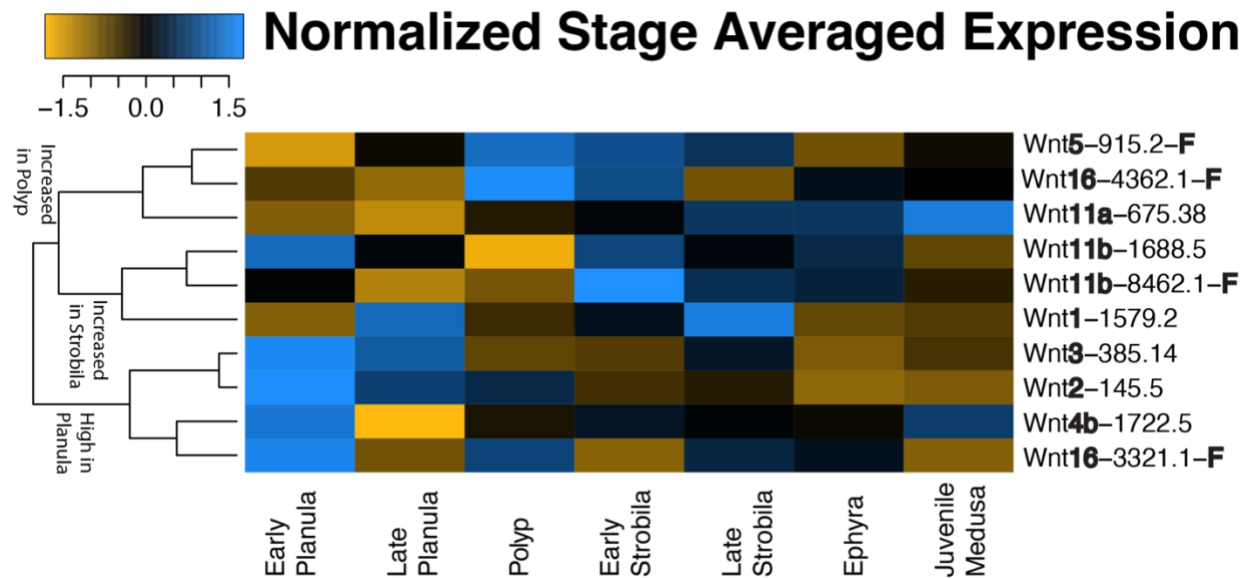


Figure 6 - Wnt gene expression across the *A. coerulea* lifecycle

Gene models followed by “F” indicate fragmentary gene models in Gold et al. 2019. Clustering groups Wnt genes into three groups highly expressed in the planula (2, 3, 4b & a 16 fragment), upregulated in the polyp relative to the planula (5, 11a, & a 16 fragment) and a group strongly upregulated in the strobila phases relative to the polyp (1, and two 11b’s)

DISCUSSION

Aza exposure promotes oral pole formation from the foot tissue of *A. coerulea* polyps in a concentration dependent manner. Animals exposed to 1 μ M or greater concentrations of Aza saw the near complete conversion of foot tissue into an oral disc, resulting in an oral disc fused to the original body column and oriented in the same direction of the foot. At 0.5 μ M, new oral structures were generated from the foot, however they tended to form their own body column and behaved similarly to normal buds. This appears to reflect a common pathway between the Aza induced formation of new buds and secondary mouths, with secondary mouths being the result of more extreme repression of the foot.

This can be extended to include the differences between 1 μ M and 2 μ M secondary mouths, with 1 μ M animals showing partial repression of foot characteristics, and 2 μ M animals showing near complete repression of aboral identity. The multiple layers of oral structures seen in 1 μ M animals could be the result of the foot's active signal patterning disrupting the Aza induced oral field into separate smaller fields.

Aza's effect on cnidarians

A. coerulea's response to Aza incubation contrasts with the response of other cnidarian's exposure to Aza. In Hydrozoan polyps and *Nematostella* embryos, GSK-3 β inhibiting paullones and Lithium induce an expansion of the original oral zone (Lee et al., 2006; Marlow et al., 2013; Plickert et al., 2006). The response of *Aurelia coerulea* polyps is more similar to that of *Hydractinia* (Muller et al., 2007) and *Nematostella* (Kraus et al., 2016) larvae or regenerating *Hydractinia* polyps-the creation of additional oral poles, rather than the expansion of the existing oral pole. The observed phenotypes do bear some similarity to the phenotypes of transgenic *Nematostella* in which degradation complex genes are knocked out (Kraus et al., 2016), however, the additional oral pole generated in *Nematostella* polyps form at the mid body, rather than converting the mature aboral pole to a new oral identity

The original oral pole appears to be unchanged by Aza exposure suggesting that GSK-3 β activity is not involved in maintaining the polyp oral pole, or that GSK-3 β is so active in the calyx tissue that Aza exposure cannot meaningfully repress its activity. The hydrozoan *Hydra* appears to define its body column with high Wnt3 expression at the oral pole and widespread expression of the cWnt antagonist *dickkopf* along the body column, the expression of which is disrupted by GSK-3 β expression (Guder et al., 2006). Further work localizing gene expression with RNA *in situ* hybridization or immunohistochemistry showing the nuclear uptake of β -catenin could further illuminate if cWnt is involved in the maintenance of the polyp oral pole.

The lack of change in *Aurelia*'s oral pole may reflect differences in the regulation of growth between *A. coerulea* and Hydrozoans, perhaps indicating differences in cell proliferation patterns. Hydrozoans and Scyphozoans differ in how they replace cells in their body. Hydrozoan polyps have populations of pluripotent stem cells known as i-cells located in the lower body column, and GSK-3 β inhibition appears to trigger differentiation into neurons and cnidocytes (Teo et al., 2006). Scyphozoa have no known stem cell populations and appear to rely on the transdifferentiation of somatic cells to replace lost cells (Gold and Jacobs, 2013).

GSK-3 β inhibition studies in other scyphozoans could help elucidate whether this difference is specific to *Aurelia coerulea* or reflective of different developmental processes between Hydrozoa and Scyphozoa. *A. coerulea*'s foot is very dynamic as it constantly generates and degenerates adhesive tendrils and stolons to facilitate the polyp's locomotion, and small fragments left behind by that locomotion can give rise to complete polyps (Gilchrist, 1937; Schiariti et al., 2014). This dynamism and regenerative ability is not found in all Scyphozoans, for instance *Cassiopea xamachana* polyps are unable to reattach once removed from surfaces (personal communication Aki Ohdera, Vivian Li). Comparisons of modes of asexual reproduction show *A. coerulea* to have a large degree of reproductive flexibility when compared to other scyphozoans, even among other *Aurelia* species (Schiariti et al., 2014). This flexibility and the dynamism of its foot may have facilitated *A. coerulea*'s invasion of new habitats around the world.

Wnt expression in Aurelia coerulea lifecycle

The expression profile of Wnt genes (Fig 6) appears broadly consistent with previous cWnt work in cnidarians, although further work on the location of gene expression is needed to enable a full comparison and explore the effect of Aza on cWnt signaling in *A. coerulea*. Wnt expression appears relatively variable between Hydrozoans and Anthozoans, with previous

studies only finding a shared expression pattern for Wnt 1, 3, and 7 in the polyp out of the twelve known *wnt* subfamilies (Hensel et al., 2014). Various Wnts, including Wnt3 are known to define the oral pole in *Hydra* polyps including the regeneration and budding of new oral poles (Lengfeld et al., 2009), suggesting that Wnt3's upregulation in *A. coerulea* planulae may be associated with the establishment of the oral-aboral axis.

For *Aurelia* specifically, the Wnt expression patterns seen in the Gold et al. 2019 data seem somewhat consistent with previous transcriptomic studies. Brekhman et al. 2015 identified Wnt 2, 3 and 16 as being highly expressed in the planula. Both Khalturin et al. 2019 and Brekhman et al. 2015 show high Wnt11a and b expression in the medusa stage, and the Gold et al. 2019 data shows increased expression of Wnt11a and the two 11b's in the strobila through medusae relative to the late planula and polyp. However, these comparisons are somewhat limited by inconsistencies in the stages sampled between studies, (e.g. Khalturin et al. 2019 excludes planula and ephyra data) and apparent differences in which Wnt subfamilies are present in the different populations (see Table 1). Based on the high expression in planulae in the Brekhman and Gold datasets, and its presence in the oral pole of various cnidarian polyps (Hensel et al., 2014), Wnt3 seems like an informative marker of oral pole development in *A. coerulea*. Meanwhile, the increased expression of Wnt11a & 11b in the transition to the medusa lifestage in the Gold, Brekhman and Khalturin datasets suggest they may be useful in studying the oral-aboral axis of medusae and developing ephyrae.

Practical application in establishing clonal polyp lines

By encouraging the formation of new oral poles, Aza exposure could be used to rapidly generate clonal *A. coerulea* polyp lines for research. As Aza affects gene expression and does not knockout any particular gene, it should have minimal impact on the genetics of the polyps

involved. Well fed polyps subjected to rounds of Aza exposure could rapidly produce a population of *A. coerulea* polyps.

Currently, *Aurelia* research is somewhat stymied by the genetic diversity of the animals used in research—researchers appear to use a variety of strains derived from disparate marine laboratories (as in Khalturin et al., 2019), or wild caught animals (as in He et al., 2015). When combined with the cryptic diversity of species in the *Aurelia* genus (Dawson, 2003; Dawson and Jacobs, 2001; Scorrano et al., 2017) and continued use of the name “*aurita*” for many non-*Aurelia aurita* strains in the literature (e.g. Khalturin et al. 2019 refers to Roscoff strain *Aurelia* as *aurita*, but phylogenetics shows the Roscoff strain grouping with Japanese and Southern Californian strains of *A. coerulea* see Gold et al., 2019), it can be difficult to apply results of a study from a different lab to one’s own animals. Removing this genetic diversity by establishing one or more laboratory strains of *A. coerulea* could improve the transfer of techniques and resources between labs and the reproducibility of studies.

Conclusion

We have established a protocol to consistently convert the aboral pole of *Aurelia coerulea* to an oral pole, either wholly or partially suppressing the aboral pole’s identity. The novel response of *Aurelia coerulea* polyps to Aza exposure shows an intriguing difference from the polyps of well studied Anthozoan and Hydrozoan model organisms. The bipolar polyps generated by *A. coerulea* when exposed to 1-Azakenpaullone appear more similar to the response of Hydrozoan and Anthozoan larvae (i.e. the induction of new oral poles) than that of Hydrozoan polyps, which show expansion of the original oral region. This difference in response may be due to underlying differences in cell proliferation along the body axis between Scyphozoans and these other cnidarian groups. Alternatively, it may be a result of *A. coerulea*’s dynamic and highly regenerative foot which uses its growth and degrowth to locomote across

surfaces and fragments of which are capable of generating new polyps. Further study of Aza exposure in other Scyphozoans and the expression of Wnt genes in *A. coerulea* and other Scyphozoa should help determine the underlying reasons for this dramatic difference. The protocol outlined here should allow further study of oral pole formation in *A. coerulea* polyps and could potentially be used to generate large numbers of clonal organisms for use in laboratory studies.

SUPPLEMENTARY FIGURES

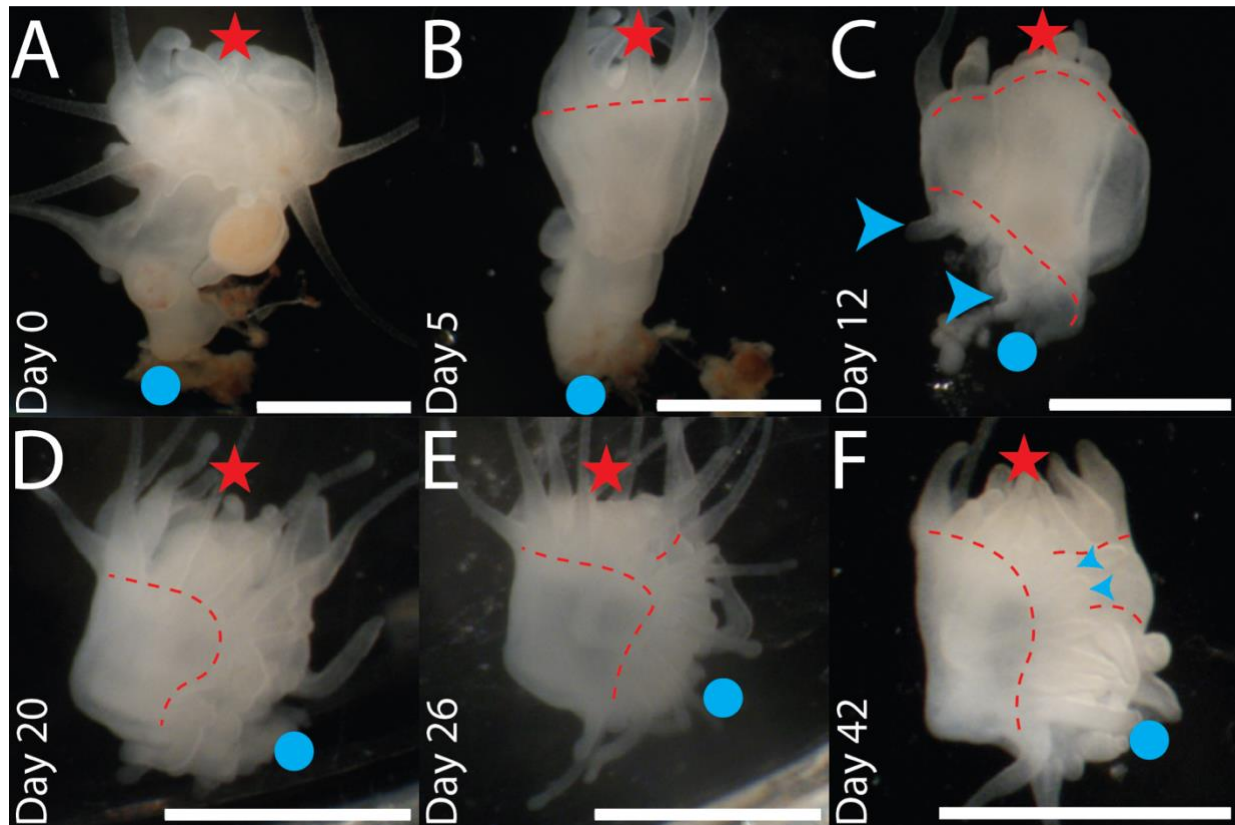


Figure 7 SUPPLEMENTARY - Potential fusion and separation of multiple oral regions in an individual

The original oral pole is indicated by a red star, and the original aboral pole is indicated by a cyan circle. Red dashed lines indicate ridges of tissue with tentacles sprouting from them, such as the oral disc. Cyan arrows in C indicate developing tentacle buds, and the cyan arrows in F show tentacles not associated with either the original or secondary oral pole. **D)** shows a large ridge of tentacled tissue that appears to connect the two oral poles. This connection appears to persist through **F)** however, the secondary and primary oral poles appear better organized, with more distinct oral discs. This may reflect a separation of oral zones that had merged due to the polyp's small size. Only one animal in the experiment exhibited this phenotype. White bar scale bar is 1 mm long.

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Chapter 2 - Early Aza exposure suppresses segmentation and triggers secondary oral pole formation in *Aurelia coerulea* strobilation

ABSTRACT

Strobilation is the process by which Scyphozoan polyps (epifaunal, asexually reproducing) generate medusae (planktonic, sexually reproducing). Strobilation consists of two mechanisms that operate contemporaneously - *segmentation*, which divides the body column into segments, and *metamorphosis*, which is the transformation of these segments into ephyra (immature medusae). These processes can be likened to the development of the primary body axis in bilaterian model systems (vertebrates and arthropods), which see a two fold process of *segmentation*, dividing the body column into units known as metamers, and then *specification*, which assigns identity to metamers. Adding the GSK3 β inhibitor 1-Azakenpaullone (Aza) during *Aurelia coerulea* (moon jellyfish) strobilation yields very different phenotypes depending on the time in when it is introduced. Simultaneously exposing animals to Aza and inducing strobilation triggers polyp oral pole formation from the foot (akin to asexual reproduction via budding), suppressing segmentation and delaying metamorphosis. However, late addition of Aza increases the body length of forming ephyrae without altering the body length of already formed ephyrae, and does not induce the formation of secondary oral poles. These results appear consistent with a multipart model of strobilation. The early phase would involve the aboral region of the animal developing a field to suppress new polyp oral pole formation and define the lowermost boundary of segmentation. Once the region of tissue which will become ephyra tissue is designated, segmentation proceeds as a morphogenetic signal traveling toward the aboral pole induces segment furrow formation. Each furrow would serve as an oral signaling center generating a cWnt gradient to define the location of the next furrow along the body column. This would have the cWnt signal operate as a morphogen in the classic French Flag patterning model for each segment. This model uses a biochemical gradient to divide a 1-

dimensional axis into multiple regions via as genes are expressed or repressed in a concentration dependent manner. This would allow *A. coerulea* polyps to persist post strobilation and prevent malformations in ephyra's primary body axis that would arise from simultaneous budding and strobilation.

INTRODUCTION

Scyphozoan ("true jellyfish") polyps generate new oral poles as part of budding (a polyp giving rise to a polyp) and strobilation-a form of asexual reproduction by which a polyp divides its body column into transverse sections. Each section develops into an ephyrae, except the aboral-most section which reforms polyp oral structures (Arai, 1997). This process adds successive segments or metameres¹, each with their own oral pole, beginning at the original polyp oral pole and proceeding toward the aboral pole. Following segment formation, the oral-most segments metamorphose into ephyrae. This subdivision into metameres followed by differentiation into ephyrae is analogous to formation and subsequent "specification" via gene expression (e.g. *Hox* genes) of insect and vertebrate metameres during axial patterning. Additionally, strobilation diverts resources and polyp tissue away from the asexual production of polyps and to the creation of ephyrae which potentially form a suite of sexually reproducing clonal medusae. The presence of budding and strobilation requires the maintenance of two separate developmental programs in the polyp.

Strobilation and metamerization

¹ We use the term "segmentation" throughout this work to refer to the process in strobilation whereby the polyp divides its body column into circular rings of tissue about the body axis, in keeping with most of the scientific literature on strobilation. This should not be taken to mean that strobila segments are homologous with the segments in vertebrates, arthropods, annelids etc.

Unlike segmentation during typical bilaterian embryogenesis, the compartments generated by strobilation separate into independent organisms of two different identities-either ephyra or polyp. Generally, only the aboral-most region of a strobila stack returns to the polyp condition, however strobila with multiple polyp segments have been reported (see Fig S24) (Haeckel, 1881; Helm, 2018). Such changes are conceptually comparable to alteration of metamere specification, which in Bilateria can result from homoeotic mutations.

Polydisc strobilation, where segmentation of the body column and assignment of a suite of these segments to an ephyra/medusa identity distinct from the polyp, is unique to Scyphozoa. Cubozoa and Staurozoa, successive outgroups to Scyphozoa, typically metamorphically convert the polyp to a medusa, although in some Cubozoa separation of a single ephyra/medusa from a polyp has been observed (Toshino et al., 2015). In contrast, the polyps of Hydrozoa (the other major Medusazoan group), form medusae by budding along the body column or a stolon, while maintaining their normal polyp anatomy (Boero et al., 1997; Daly et al., 2007). Thus segmentation and “polydisc strobilation,” the production of multiple ephyrae along the polyp body axis, appears unique to Scyphozoa within the Medusozoa.

The nature of Scyphozoan metamerism

Well before the advent of molecular biology and evolutionary developmental biology, metameric changes were considered to be potential mechanisms to generate new body plans. Bateson in his *Materials for the Studies of Variation* (1896) posited the metamere as a basic unit of discontinuous variation, which could be changed in number, size, or identity (homoeosis) during development and provide the variable fuel for evolution. When considering jellyfish, Bateson, Haeckel and others studied the variation of radial “metameric” structures of medusae such as umbrellar canals and oral lobes (Bateson, 1894; Ehrenberg, 1836; Haeckel, 1881; Romanes, 1876). Recent workers have argued that the radial compartments of the cnidarian

anemone *Nematostella* operate as metameres, suggesting the Cnidarian-Bilaterian ancestor likely possessed polarized metameres (He et al., 2023). However, here we focus on axial metamerism during strobilation, not radial metamerism.

Axial metamerism and simultaneous vs serial patterning

Bilaterian developmental studies provide potential models of axial metamerism and patterning. Primary body axis patterning in cnidarians and bilaterians share many features, including early definition by cWnt signaling and patterning by *Hox* genes (DuBuc et al., 2018). Segmenting bilaterian animals divide their body column into stable regions of gene expression which triggers compartmentalization and the formation of specific metamere types.

A primary difference between methods of segment formation is the time each segment forms relative to one another. In *Drosophila* (fruitfly) and other long-germ insects, first the axial polarity of the body column is specified by an anterior-posterior gradient of *bicoid* and a posterior-anterior gradient of *nanos*. The interaction of these gradients activates and represses the expression of different genes at different concentrations, defining a stable pattern of regional gene expression along the body axis, in a pattern reminiscent of the coloration of the French Flag (Driever and Nüsslein-Volhard, 1988; Ephrussi and Johnston, 2004; Wang and Lehmann, 1991; Wolpert, 1969). Regionally expressed genes are called “gap” genes, as their knockout results in the deletion of multiple adjacent segments (Ephrussi and St. Johnston 2004). Following gap gene expression, stripes of “pair rule” gene expression define the boundaries of every segment along the body column simultaneously. Within each segment, French Flag morphogen patterning by the morphogen *wingless* (a homolog of Wnt) define the polarity of each segment, specifying the anterior and posterior regions of the segment. This is followed by the specification of segment identity by *Hox* gene expression see (Akam, 1998; Clark et al., 2019) for further discussion.

In contrast to the simultaneous segmentation and specification of *Drosophila*, most segmented animals serially form their segments, not simultaneously. Vertebrate somitogenesis and shortgerm arthropod body column segmentation, use a “clock and wavefront” mechanism where the progressive growth of tissue at the posterior end of the animal pushes earlier formed cells along a morphogen gradient (Baker et al., 2006; Clark et al., 2019b; Cooke and Zeeman, 1976). As cells pass the determination front, they begin to form segments and are assigned segment identity by *Hox* gene expression (Aulehla et al., 2003; DuBuc et al., 2018; Imura et al., 2009; Moreno and Kintner, 2004). At regular time intervals the posterior zone of cell proliferation sends pulses of gene transcription toward the anterior pole, providing a “clock” time reference for segment formation. The distance the gradient travels during a “clock tick” directly affects the size of segments formed. Slowing the determination front’s progress increases the number and decreases the size of metameres, while speeding up the front has opposite effects (Dubrulle et al., 2001). In short-germ insects, bands of *wingless* expression in each segment define segment polarity, similar to their role in *Drosophila* (Beermann et al., 2011; Nagy and Carroll, 1994).

The mechanisms underlying strobilation are much less well understood than those of bilaterian axial patterning. However, study of genes upregulated during strobilation has yielded a number of genes associated with strobilation (see Table S8), some of which are unique to Scyphozoa. Temperature appears to be the primary trigger of strobilation induction, which is presumed to ensure the proper seasonality of ephyrae release and subsequent jellyfish blooms (Fuchs et al., 2014; Sukhoputova et al., 2019). Strobilation induction in *Aurelia coerulea* sees polyp oral expression of a presumptively *Aurelia* specific morphogen CL390 (Fig S30). After building up in the oral disc, CL390 expression rapidly expands within 1-2 days to encompass the region of polyp tissue which will undergo segmentation (Fig S30, Fuchs et al., 2014). This initial regional designation is then followed by a slower division of the body column into metameres, beginning at the oral pole of the animal and progressing aborally. We term the

portion of the body column where conscription into the segmentation field is taking place as the Segment Addition Zone (SAZ). This appears to correspond to the region of tissue which will segment in the next 24 hours (Kroiher et al., 2003, Fig S32). We refer to this procession of fate determination down the body column as a “morphogenetic” wave². This morphogenetic wave is accompanied by a dramatic increase in cellular proliferation and expression of several genes which may serve a function similar to segment polarity genes in *Drosophila* (Fujii et al., 2024, see Fig S30-31). Metamorphosis of uppermost segments into ephyrae may begin while lower metamers are still forming, particularly if the animal is large enough to produce many segments. This study compares the relative timing of specific features of segmentation and metamorphosis to determine their relationship to one another.

Spatial vs temporal models of metamere generation

We seek to determine what aspects (if any) of the simultaneous model and serial model of segmentation are applicable to the segmentation pattern of strobilation. The effects of *bicoid* and *nanos* expression which facilitate *Drosophila* axial determination bear some similarities to the generation of new oral poles resulting from hyperactivating cWnt in cnidarian larvae (Plickert et al., 2006) and exposing *Aurelia coerulea* polyps to Azakenpaullone (chapter 1). The deletion of *bicoid* suppresses anterior structures, while mislocalization of *nanos* to the anterior pole generates two posterior poles fused together in “mirror image” embryos (Driever and Nüsslein-Volhard, 1988; Struhl, 1989; Wang and Lehmann, 1991). On the other hand, the clock and wavefront model sequentially adds segments at the posterior (aboral) end of the segmenting

² We use “morphogenetic wave” here in reference to the concept of the morphogenetic field, as the wave itself is not visible. It is an invisible change that precedes morphogenesis and has been inferred by indirect means like tissue cutting and transplantation. It is a related term to morphogenesis (the actual formation of structures), but is a distinct concept.

region, just as occurs in strobilation, while *Drosophila* segmentation occurs simultaneously across the embryo. We assess the applicability of a purely spatial (French Flag) model which depends on morphogen gradients to define polarity, to a temporal (Clock and Wavefront) model which freezes successive temporal information along an axis to produce compartments, to the mode of segment formation observed in strobilation.

1-Azakenpallone and GSK3 β

1-Azakenpallone (Aza) has been shown to affect pole formation in *Aurelia* polyps and affect segmentation during strobilation. Azakenpallone is part of the paullone chemical family and is commonly used to inhibit the Glycogen Synthase Kinase 3 β (GSK3 β) enzyme and hyperactivate canonical Wnt signaling, producing phenotypes similar to those induced by exposure to the GSK3 β inhibitor lithium (Müller et al., 2007; Plickert et al., 2006). Aza incubation in *Aurelia coerulea* polyps induces oral pole formation from foot tissue (chapter 1), while Aza incubation during strobilation seems to suppress segmentation (Khalturin et al., 2019). The inhibition of GSK3 β by paullones and lithium affect patterning along the primary axis in various cnidarians, including *Nematostella*, *Hydractinia* and *Hydra* likely by its impact on canonical Wnt signaling (Marlow et al., 2013; Müller et al., 2007; Plickert et al., 2006).

cWnt signaling

Canonical Wnt signaling (cWnt) is an ancient signaling mechanism found across Metazoa which defines the anterior-posterior body axis in bilaterians and the oral-aboral axis in cnidarians (Petersen and Reddien, 2009). Secreted Wnt ligands bind to a membrane bound receptor complex composed of LRP/Frizzled (Krishnamurthy and Kurzrock, 2018). This triggers the phosphorylation and inactivation of a degradation complex incorporating the enzyme GSK3 β . The degradation complex normally destroys cytoplasmic β -catenin, and its inactivation

allows β -catenin to enter the nucleus and alter gene expression (reviewed in (McCubrey et al., 2014)). The chemical 1-Azakenpaullone (Aza) inhibits the GSK3 β degradation complex, allowing β -catenin to accumulate as if a Wnt ligand were bound to the receptor complex (Kunick et al., 2004). In hydrozoans, high cWnt activity determines the future oral pole in larvae, the polyp oral pole and patterns the oral-aboral axis in hydrozoan medusae (Müller et al., 2007; Nawrocki and Cartwright, 2013; Reddy et al., 2019; Sanders and Cartwright, 2015). In addition, cWnt appears responsible for patterning the proximal-distal axis in ephyrae, showing expression on the lateral edge of the oral surfaces of segments in early strobila, expression which is later restricted to and arm and lappet lobes as metamorphosis progresses (Fuchs et al., 2014; Khalturin et al., 2019b; Kraus et al., 2015; Wang, 2013; Zorina, 2018) See Table S8 for further discussion of gene expression patterns throughout strobilation.

Aurelia coerulea

The *Aurelia* genus or “moon jelly” is actually speciose with at least 27 often cryptic species (Dawson and Jacobs, 2001; Lawley et al., 2021), that are significantly genetically divergent (Gold et al., 2019) . Despite their similar appearance, these species differ in a number of characteristics such as temperature of strobilation induction (Schroth et al., 2002). These likely relate to seasonal/environmental developmental response, with plasticity within a species potentially determining the niche and range of the numerous species within the genus. As polydisc strobilators, the number of ephyrae produced by a single *Aurelia* polyp in one cycle of strobilation is largely dependent on the size of the polyp body column, with larger polyps yielding more segments, and therefore ephyrae (Helm, 2018; Kroiher et al., 2003).

Aurelia coerulea is a species of moon jellyfish native to the north-western Pacific (Japan, China, Korea). *A. coerulea* polyps display a number of modes of asexual reproduction, including budding of offspring polyps from the foot/calyx (Schiariti et al., 2014). Due to its hardiness and

flexible lifecycle, *A. coerulea* has invaded locations around the world, including Southern California, the Mediterranean and Australia (Dong, 2019). These invasions have a variety of negative impacts, including medusae clogging powerplant water intakes (Wang et al., 2023), nematocyst stings damaging the gills of fish in aquaculture (Baxter et al., 2011) and medusae disrupting fisheries (Bosch-Belmar et al., 2020).

We investigate the impact of Aza exposure during segmentation and metamorphosis by exposing *A. coerulea* strobila to Aza at different time points post strobilation. By comparing the relative time of formation of characteristics associated with the segmentation and metamorphosis processes, we can determine how the subprocesses of strobilation relate to each other and to polyp bud formation. If segmentation, metamorphosis and oral pole formation operate independently of one another, segmentation suppression via Aza exposure should not affect ephyra metamorphosis or secondary oral pole formation.

We find that metamorphosis can continue even as segmentation is repressed, and that the formation of secondary poles from foot tissue appears to have an antagonistic relationship with strobilation. Early Aza addition to strobilation induces the formation of massive ephyra anlagen along the body column and the formation of secondary mouths from foot tissue which can themselves strobilate and fuse with ephyrae formed from the original oral pole, yielding bipolar ephyrae. Later addition of Aza increases the body length of forming animals but does not induce the formation of mouths from the secondary pole, and does not affect the boundaries of segments formed prior to Aza addition.

Based on these results, we propose a “serial French Flag” model of segmentation along the body axis during strobilation. The first phase of this model following induction sees the animal develop a field at the foot which serves to both define the lower boundary of strobilation and prevent the formation of new polyp oral poles (i.e. budding) during strobilation. This field is likely based on cWnt signaling suppression, based on its time sensitive response to Aza

incubation. After the region of the strobila stack has been defined, segments form in response to a morphogenetic signal which passes down the body column in a wave-like manner. The interaction of this wave with an oral based signal determines the overall length of the segments formed, with each new segment forming an oral signaling center. The threshold at which the segmentation signal induces segment formation is sensitive to Aza incubation, making it likely to be facilitated by GSK3 β activity and cWnt signaling in a French Flag manner. This work can be expanded on in the future via gene expression assays like qPCR and RNA *in situ* hybridization to verify the role of cWnt in this process and determine the spatio-temporal pattern of gene expression relative to the oral poles as well as verifying the downstream genes affected by Aza addition.

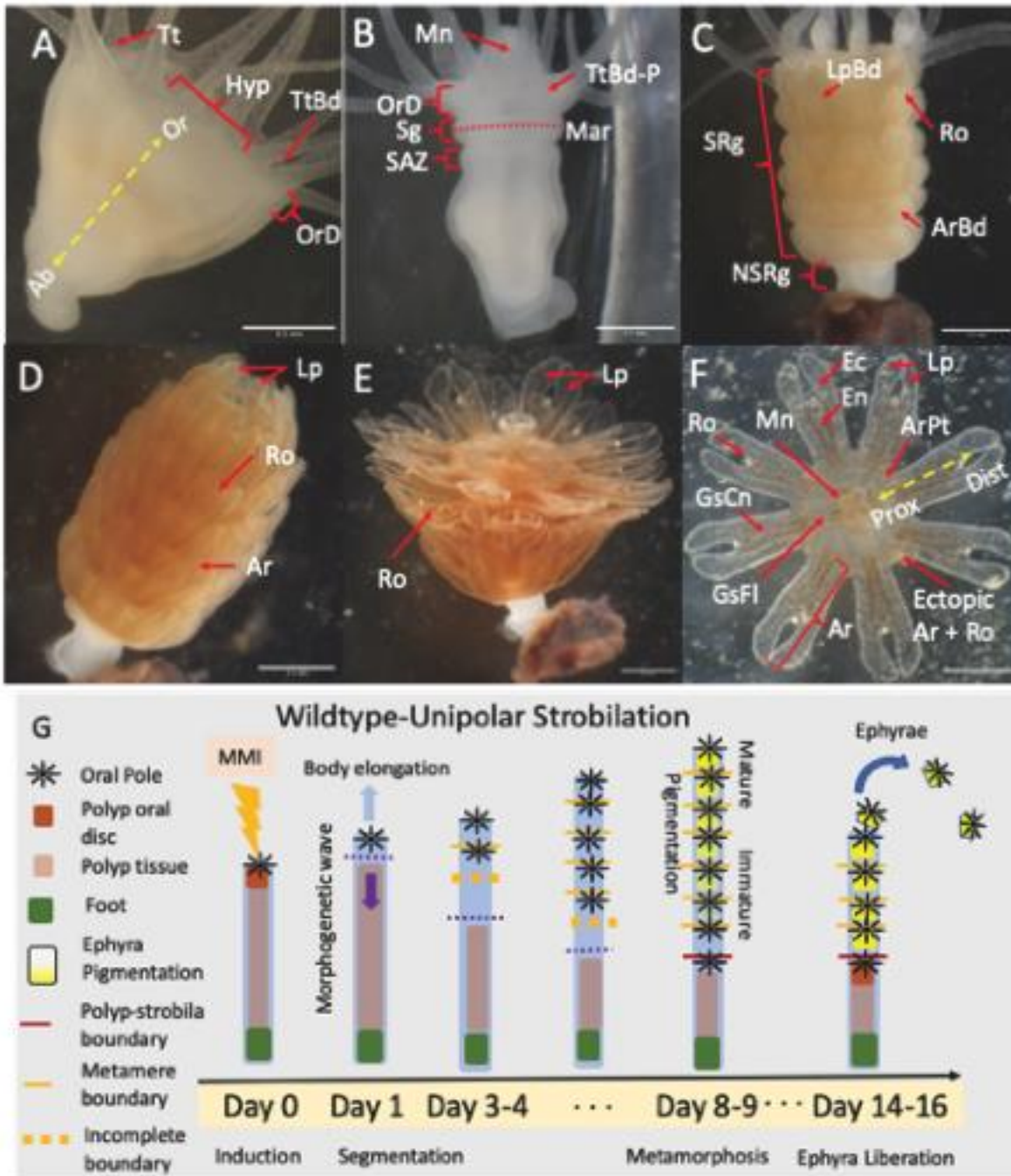


Figure 8 - Progression of wildtype strobilation

Scale bar shows 0.5mm. **A)** Pre-strobila **B)** Segmentation **C)** Early Metamorphosis **D)** Late Metamorphosis **E)** Mature Ephyrae on Strobila **F)** Liberated Ephyra. Abbreviations - Ab-Aboral pole, Ar-Arm, ArPt-Arm Pit, Bd-Bud, GsCn-Gastric Canal, GsFl-Gastric Filament, Hyp-Hypostome, Lp-Lappet, Mar-circumferential margin, Mn-Manubrium, Or-Oral expression within structure, OrD-Oral Disc, Ro-Rhopalia, SAZ-Segment Addition Zone, Sg-Segments, (n)SgRn-region of strobila that will (not) segment, Tt-Tentacle, TtBd-P-primary tentacle buds, perradial/interradial, the first 8 tentacles that form in polyp morphogenesis. **G)** Schematic of

Strobilation-shows elements not easily visible in photographs, such as the progression of the morphogenetic wave. MMI refers to the chemical strobilation inducer 5-methoxy-2-methylindole.

METHODS

Experimental methods

Aurelia coerulea polyps used in the experiment were from the Jacobs lab colony maintained at UCLA, originally established with animals from Cabrillo Marine Aquarium (San Pedro, California). Animals were grown at 15-16° C, 33-36 ppt and kept on a 12:12 light:dark schedule. Animals were fed *ad libitum* with brine shrimp 3x a week. Polyps selected were ~3 mm long with at least 8 tentacles, with their feet cleaned of algae as much as feasible without damaging the animals. Prior to experiment start, animals were added to ten 24-well plates filled with 1 mL of solution, with one polyp per plate and given 48 hours to attach. Even after this time, many animals did not attach to the plate. Animals were starved the entire time they were in the wells to limit waste build up and the need for water changes. Animals were kept in a humidified incubator set to conditions similar to their growth conditions throughout the experiment. The location of the plates within the incubator was randomized and their orientation was flipped by 180° every day to minimize higher light exposure and evaporation on the edges closest to the light sources in the incubator. To further counter plate edge effects, different days of addition were laid out in different columns across the plates according to the scheme laid out in Table 2.

Table 2 - Experimental layout of 24 well plates

1 animal was added to each well of ten 24 well plates, for a total of 240 animals. The day of Aza addition was determined by column, with each plate having a different layout to minimize plate effects and effects from location on the plate. The first set of 5 plates had Aza Day 0, 1, 3, 4, and 10 (intended to be Aza Day 2), and the second set had Aza Day 5-9 animals. Control animals were present on every plate

Plate	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
1	Control	Day 0	Day 1	Day 10*	Day 3	Day 4
2	Day 4	Control	Day 0	Day 1	Day 10*	Day 3
3	Day 3	Day 4	Control	Day 0	Day 1	Day 10*
4	Day 10*	Day 3	Day 4	Control	Day 0	Day 1
5	Day 1	Day 10*	Day 3	Day 4	Control	Day 0
6	Control	Day 5	Day 6	Day 7	Day 8	Day 9
7	Day 9	Control	Day 5	Day 6	Day 7	Day 8
8	Day 8	Day 9	Control	Day 5	Day 6	Day 7
9	Day 7	Day 8	Day 9	Control	Day 5	Day 6
10	Day 6	Day 7	Day 8	Day 9	Control	Day 5

Table 3 - Example layout of experimental plate - Plate 1

Cell represent wells of the 24 well plate and indicate the date of Aza addition to the well. Day of Aza addition corresponded to columns.

Plate 1	Col 1	Col 2	Col 3	Col 4	Col 5	Col 6
Row A	Control	Az Day 0	Az Day 1	Az Day 10	Az Day 3	Az Day 4
Row B	Control	Az Day 0	Az Day 1	Az Day 10	Az Day 3	Az Day 4
Row C	Control	Az Day 0	Az Day 1	Az Day 10	Az Day 3	Az Day 4
Row D	Control	Az Day 0	Az Day 1	Az Day 10	Az Day 3	Az Day 4

Stock solutions of 2mM 1-Azakenpaullone (Sigma-Aldrich A3734) in DMSO, and 50mM 5-methoxy-2methylindole (MMI) (Sigma-Aldrich M15451) in ethanol were created and stored at -20° C. Working solutions of 2 μ M Aza, 50 μ M MMI, and 2 μ M Aza + 50 μ M MMI were created by adding stock solutions to artificial seawater (ASW recipe in Table S5) in a ratio of 1:1000. A control solution of 0.1% DMSO in ASW was also prepared. Working and control solutions were stored at 4° C and protected from light.

Strobilation was induced by adding the MMI + Aza working solution to the Day 0 wells and the MMI solution to all other wells. On Day 1, the Day 0 and 1 animals' solution was swapped for the solution only containing Aza, and all other conditions were swapped to the control solution. Every day a new set of animals had their control solution swapped for the Aza solution. There was one exception - Day 2 animals were not swapped on the originally planned day, and instead had their solution changed at Day 10 (see Table 3). As ephyrae were liberated from strobila they were photographed and removed from their wells with a 3mL pipette to minimize their disturbance to the strobilating animals.

On their initial transfer to the wells, some animals inverted themselves - exposing their gut epithelium while having their mouth around their foot. Most animals un-inverted themselves by Day 1 or 2, but four remained inverted throughout the experiment, rendering their anatomy unobservable. Ignoring inverted animals, a total of 236 specimens were used in the experiment. There were 40 control animals divided across all plates, 19 animals for Days 1, 5, 9, and 10, and 20 animals for all other days.

Animals were photographed daily on a Leica MZ 12 dissection scope, using an OMAX 18 MP A35180U3 camera run by Touplite for Mac (ToupTek Photonics). Scale bars were added with *Fiji/ImageJ*. Figures were created using Microsoft Powerpoint and/or Adobe Illustrator.

Variables

First appearance of different characteristics was recorded from the photos taken. Characteristics were chosen to track the progress of segmentation and metamorphosis during strobilation, as well as the appearance of secondary oral poles. Unfortunately, the posture of animals sometimes obscured certain characters. As we did not wish to disturb or damage the animals with frequent handling, unobserved characters on a particular date were recorded as

“na”. Because of this missing data, in order to say a character formed on a particular day we required that the characteristic was recorded as absent the day before it was first observed.

SEGMENTATION VARIABLES ANALYZED:

1) *Date of first* and 2) *final segment formed* - dates when the first and the final sharp constriction along the body column appear.

Note that this includes segmentation from strobilation at secondary poles for animals with multiple oral poles. Many animals that formed a single primary segment had multiple segments form at a secondary pole

3) *Number of Primary Segments*-the number of ephyra forming segments that formed along the original body axis, rather than segments that formed from any secondary oral pole formed from foot tissue.

METAMORPHOSIS VARIABLES ANALYZED:

4) *Date statocyst spotted*-day when the first statolith-a mass of yellowish crystals at the distal end of the rhopalium-was first observed

5) *Date of lappets spotted*-date when lappet buds-nubs of tissue projecting from the armbud- were first observed.

6) *Date of pigmentation*-date when the strobilating region of the animal shifted from predominantly white (corresponding to polyp tissue), to a yellowish brown. This is not the time when the animal achieved its final pigmentation level, which is a strong orange-brown and more difficult to gauge(see Fig 8c vs 8e).

7) *Date* and 8) *Number of Ephyrae liberated*-the date the first ephyra broke free of the strobila stack and the number of ephyrae which detached from the strobila stack. This reflects the maturation of the ephyrae's neural and muscular systems-as the ephyrae mature, they begin to make swimming strokes by bringing their arms together. This motion eventually breaks them free from the manubrium of the ephyrae lower on the strobila stack.

ADDITIONAL VARIABLES INCLUDE:

9) *Date of Strobilation Initiation*- date when the first signs of strobilation were observed. These signs were primarily the elongation of the body column and the swelling of the base of primary tentacles on the oral disc.

10) *Presence of secondary oral pole* -required distinct tentacle buds arranged around an oral opening.

Statistical Analysis

Statistical analyses and plotting was conducted in R. Principle Component Analysis (PCA) was performed with *MissMDA* and *FactoMineR* packages (Josse and Husson 2016, Lê et al. 2008). Normality was assessed via QQ-plot and the data was found to be nonparametric for all variables, except for the *number of primary segments*. The residuals of the average *number of primary segments* was analyzed by the linear model (PrimarySegments ~ DayOfAddition) using the packages *sandwich* and *lmttest* (Zeileis et al. 2019, Hothorn et al 2015).

Response to the Aza incubation were analyzed by Kruskal-Wallis (KW) rank sum test from the package *stats* for all variables except *presence of secondary pole*. All-vs-all multiple comparisons were conducted by two-tailed Dunn's test with Bonferroni correction from the *DescTools* package. These results were used to generate multiple comparison labeling using the *cld* package where samples that share a letter in their label are not significantly different from one another. KW and Dunn's test were also used in a permutation function, which permuted the dataset's labels and recorded the results 99,999 times, to generate a permuted null distribution of 10e5 values (including the experimental result). Multiple comparison labels were generated from the results of a two-tailed comparison of the rank of the experimental values within the generated null distribution.

Potential biases in the data resulting from the distribution of early conditions to only half the plates were addressed in a number of ways. Variables were analyzed in terms of both the

date Aza was added to wells and their row, column and plate. The control animals on plates 1-5 were compared to the control of plates 6-10. Also, Aza Day 9 animals (found on plates 6-10) were compared to Aza Day 10 animals (found on plates 1-5) as these were the two latest days of addition in the experiment. Samples were also permuted within their day of addition to conduct a permutation test on the effects of plate, row and column on the variables of interest.

RESULTS

Strobilation proceeded well and control animals showed phenotypes typical of our *A. coerulea* colony. By Day 16, 33 of 40 control animals had released one or more ephyrae, releasing a total of 125 out of 189 ephyrae. All animals survived through Day 16. Animals from treatments showing changes relative to wildtype are shown in Figs 9-13. Aza Day 0 & 1 animals saw the development of secondary oral poles from the foot. Early Aza incubation resulted in the expansion of segment size and disruption of radial structures (e.g. arms, rhopalia, lappets) by the formation of ectopic structures. The impact of Aza incubation decreased the later in time the Aza was introduced to the animals. See Fig 9-13 for discussion of phenotypes generated at each time point. When considering radial structures such as statoliths and lappets, this study focuses on their time of appearance, in order to show the impact of Aza/cWnt on the primary body axis, rather than considering changes in radial symmetry.

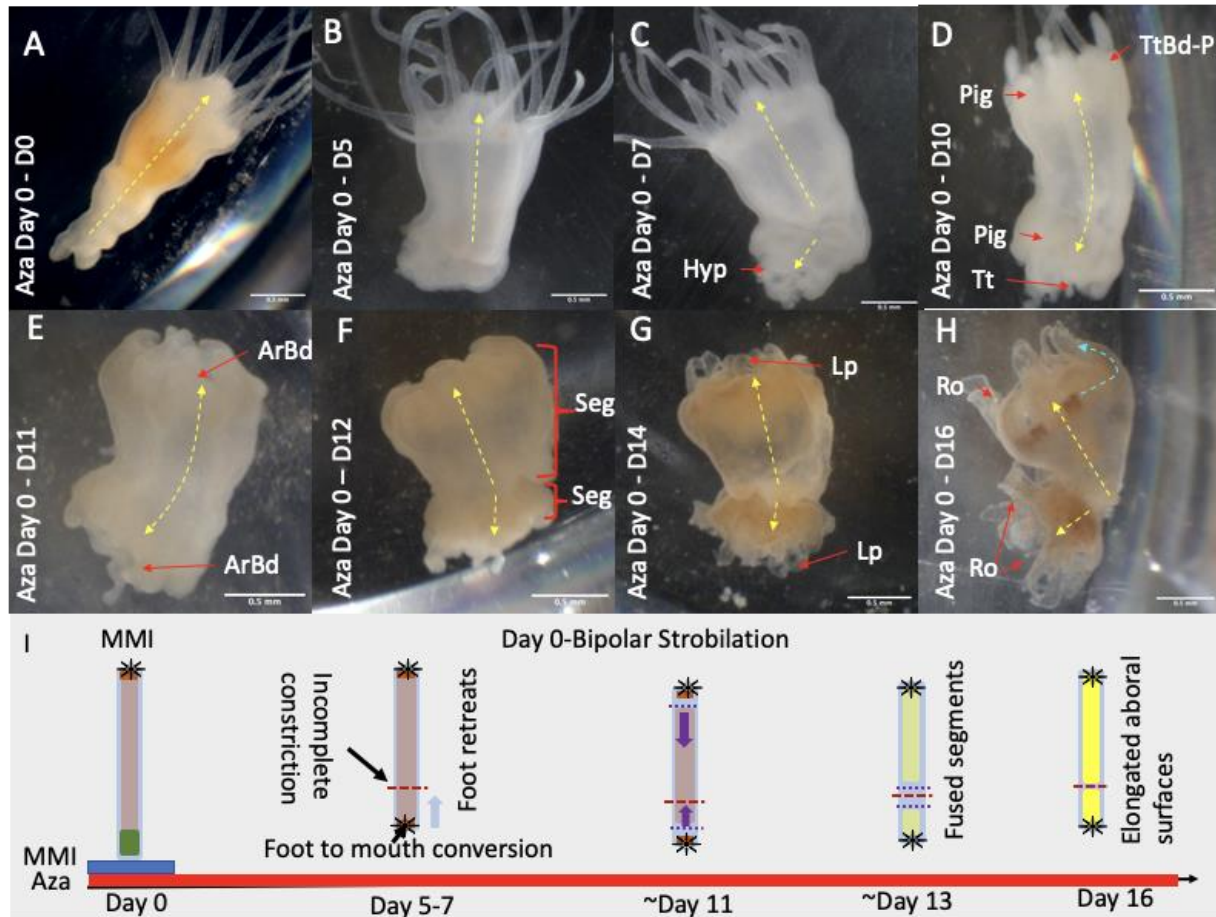


Figure 9- Aza Day 0 individual

Morphology labeled as in Fig 8. Labeling of photos are formatted as *Aza Day #* (day Aza added to animals) - *D #* (day photo taken). Aza Day 0 saw the foot convert into a new oral pole and the metamorphosis of the entire polyp to ephyra tissue (n = 20/20). 10 of 20 animals featured a clear distinction between the ephyrae of the original and secondary poles as above, while 7 of 20 lacked a complete constriction, resulting in bipolar ephyrae (see Fig S26 for an example). 3 of 20 animals failed to form distinct arms and lappets around their oral surface, instead forming numerous ectopic rhophalia and lappets along their aboral surfaces. Disruption to radial structures was common, with all animals showing either disruption to the arrangement of radial features (as in the secondary pole above) or ectopic structures.

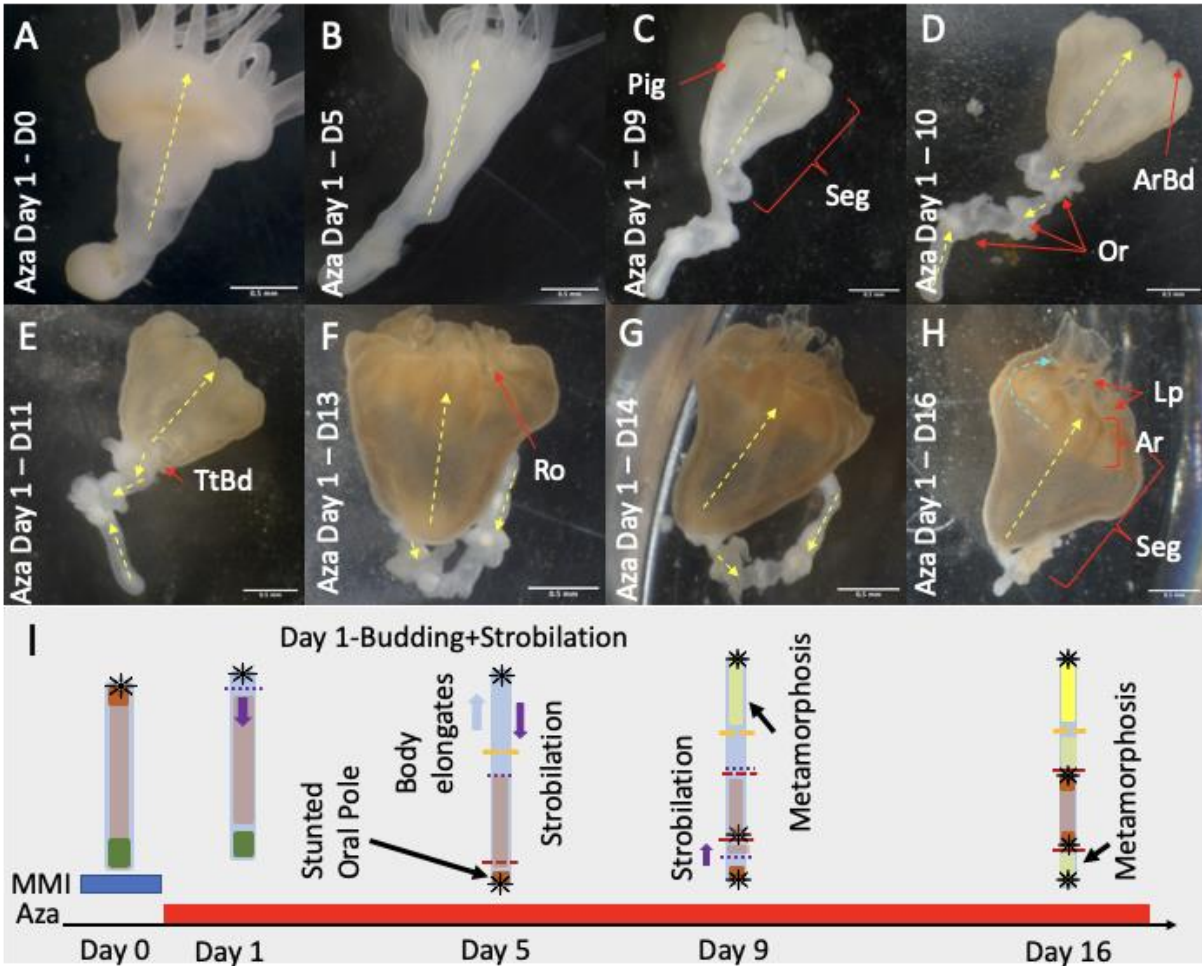


Figure 10 - Aza Day 1 individual

The foot of the animal develops rings of oral features (the small yellow arrows in C-G) which begin strobilation later than the primary oral pole. The majority of body tissue is devoted to a single large ephyra. The elongated aboral pole limits the ephyra arms' ability to lie parallel to the oral disc when relaxed, resulting in a prolate shape similar to the bells of staurozoans and cubozoans (notice the curled posture of the arms in f-h). Phenotypes included an enlarged first segment which preserved the geometry of the oral structures (as above, 8/19) and an enlarged first segment which featured ectopic rhopalial and lappet structures along the aboral surfaces of the first segment (11/19). Errors in radial symmetry were common, with all strobila showing disruption to at least one segment of the strobila stack (as seen in the formation of oral structures in D above)

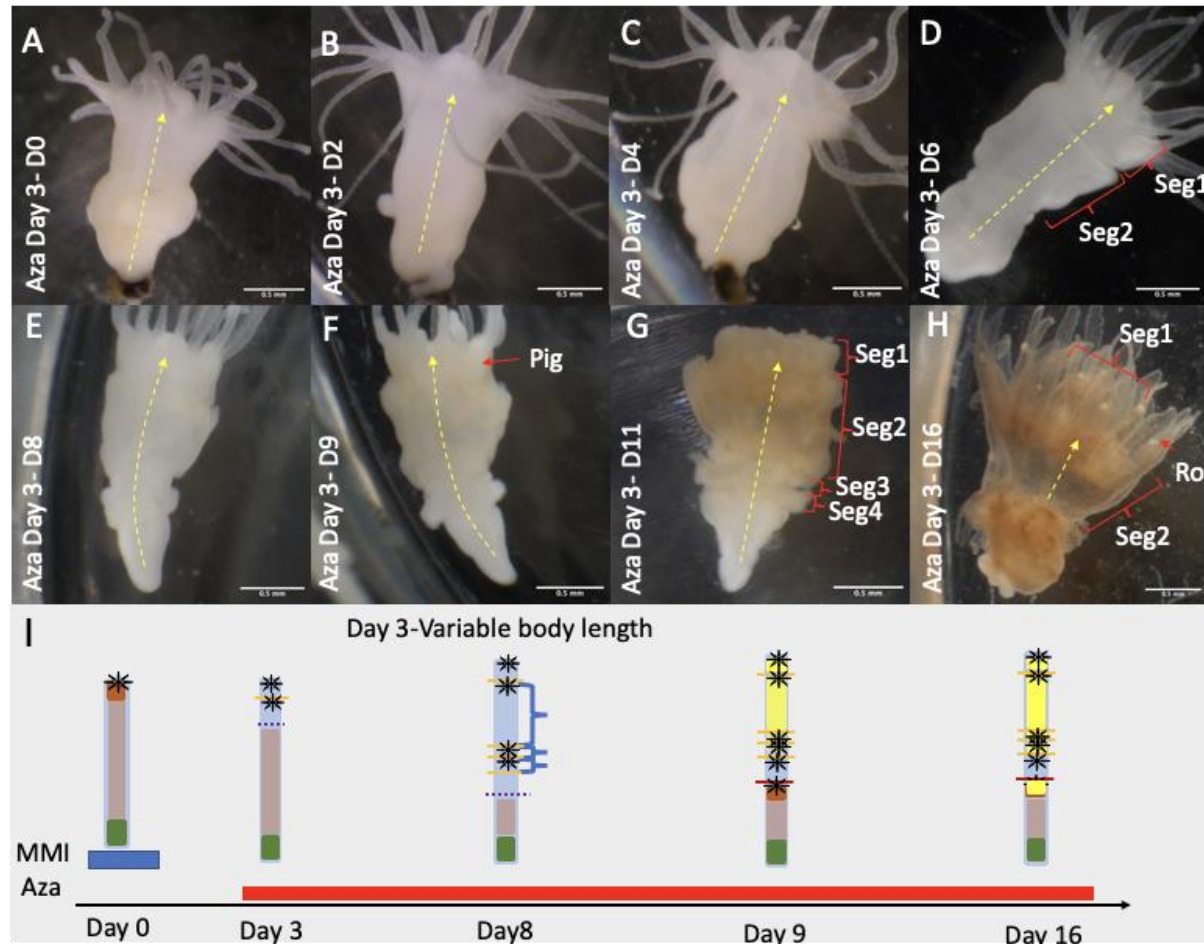


Figure 11 - Aza Day 3 individual

The second segment added shows an extremely elongated aboral pole, while the arms of segments 3-4 fail to form properly. In contrast the first segment formed resembles a wild-type ephyra. Phenotypes included enlarged segments with some disruption to radial structures (as above, $n = 10/20$), enlarged segments with no disruption to radial structures ($7/20$), fusion of multiple segments into a single ephyra ($2/20$), and minimal change in size but disruption to radial structures ($1/20$)

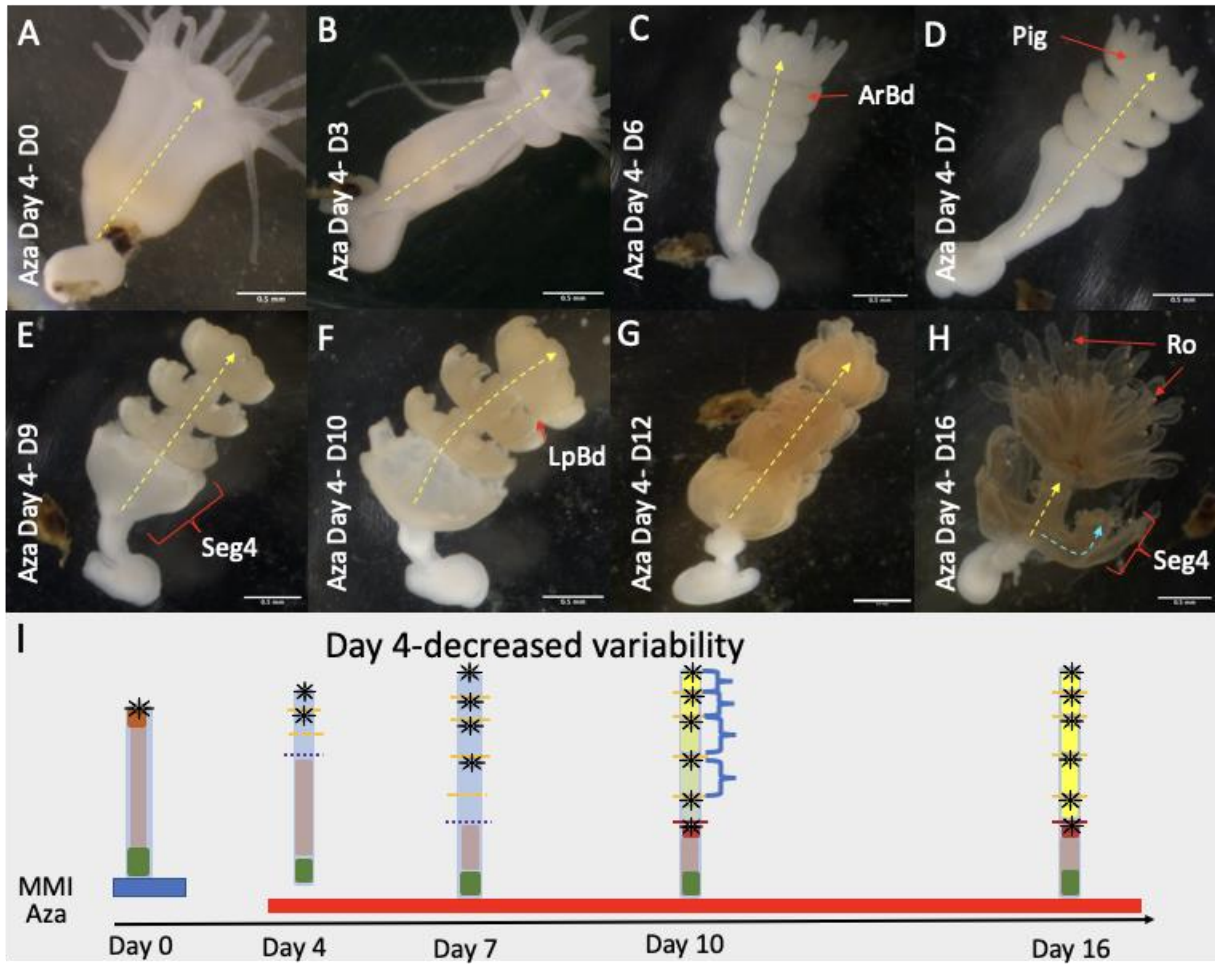


Figure 12 - Aza Day 4 individual

The development of segments formed before the day of Aza addition appear phenotypically normal, while the lowest segment has an elongated oral pole and increased size. Despite the increased size of the lowest ephyra, it appears to have a relatively normal body plan with clearly defined arms, lappets and rhopalia. Most ephyrae showed an enlarged segment size with no disruption to radial structures (as above, $n = 14/20$), some showed enlarged segments with disruption to radial structures ($5/20$) and one showed no impact on body size but did show disruption in radial features ($1/20$)

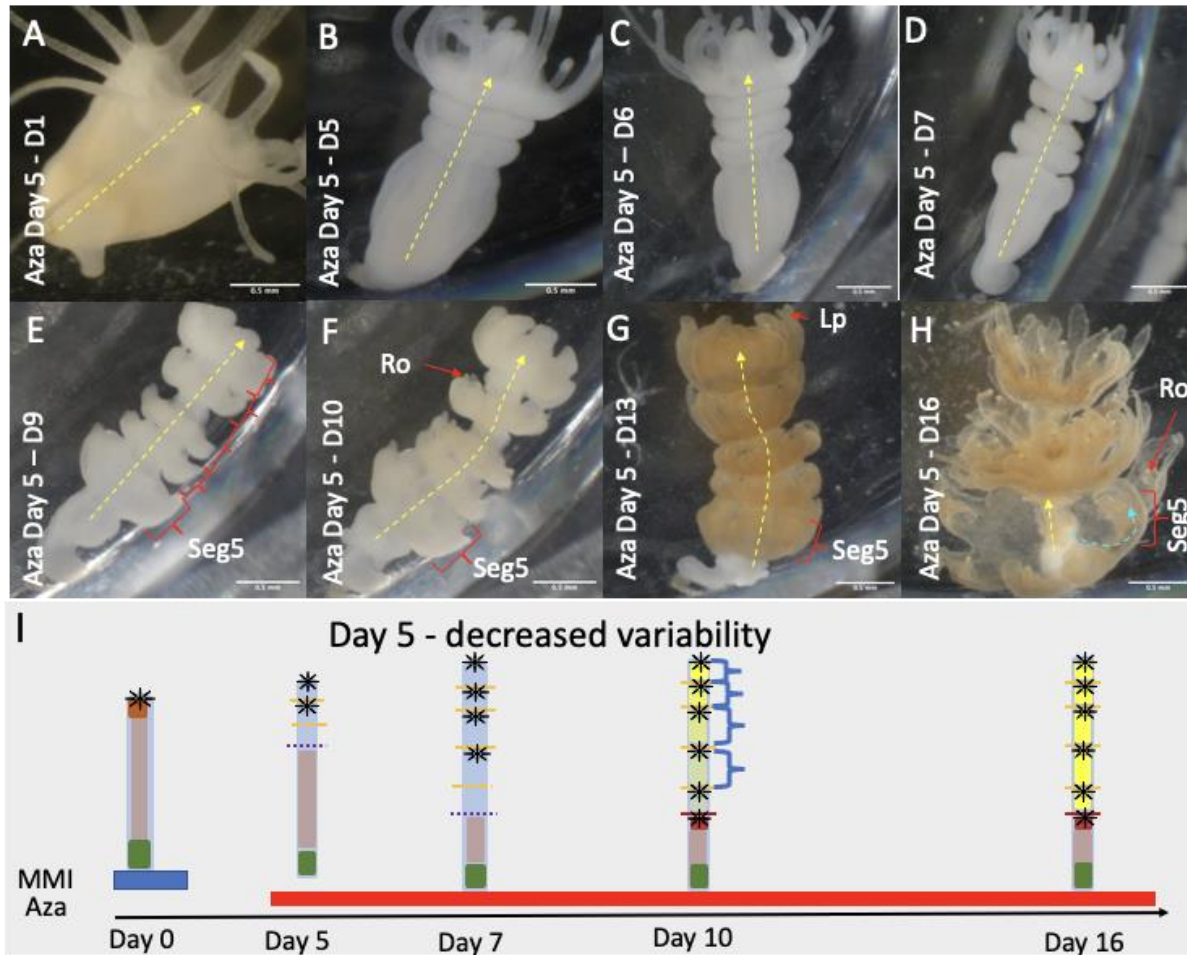


Figure 13 - Aza Day 5 individual

Shows variation in segment interval and width along the strobila column. 3 segments were already formed on day of addition (B), the third of which appears to have an enlarged manubrium. For H) the two uppermost ephyrae had liberated on Day 15 and were thus not photographed. Seg5 increased in size relative to older segments on stack, but once again has well defined ephyral features like arms, lappets and rhopalia. Most animals showed enlarged segments ($n = 13/19$), while a few showed enlarged segments and disorganized radial structures ($2/19$), and several appeared normal ($4/19$).

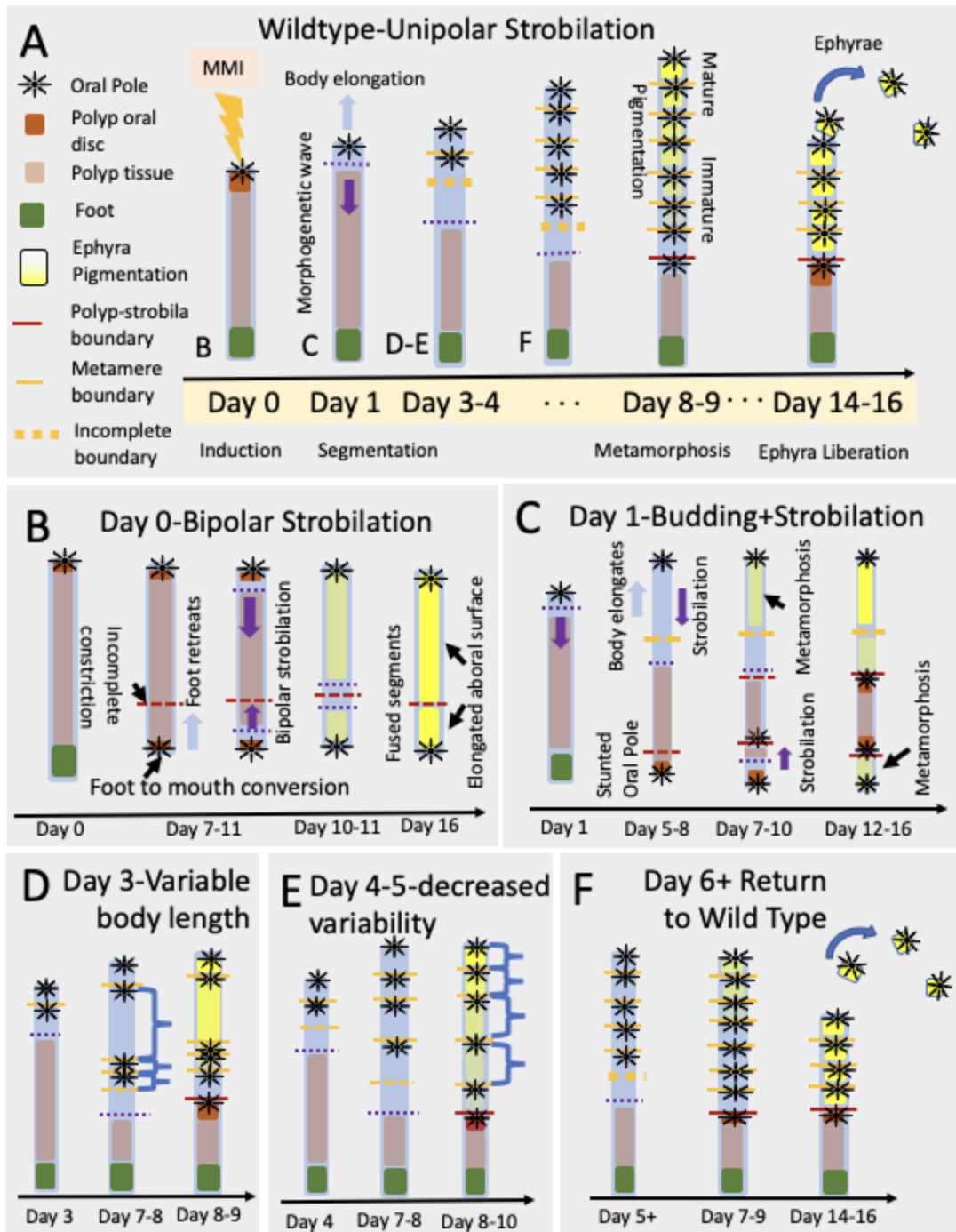


Figure 14 - Summary of strobilation disruption with 1-Azakenpallone

A- Induction of Wild Type, **B-F** addition of AZA at successively later times after induction- Strobilation induced at Day 0 with MMI for all animals. **A)** *Control/Wild type sequence of significant events in strobilation*. Strobilation induced at day 0 by a 24 hour incubation with 5-methoxy-2-methylindole (MMI). Adding Aza at different time points relative to strobilation induction results disrupts the segmentation and metamorphosis of segments produced after Aza introduction. Aza Day 6-10 animals show similar phenotype to control sequence. Normally, strobilation takes a little over two weeks from the introduction of MMI to release of ephyrae. **B-F)**

Later introduction of Aza has a successively smaller magnitude effect on segmentation and affects animals farther down the body column than earlier Aza introduction.

Plate effects were not significant for almost all variables

Tests to determine the impact of plate position and identity showed no significance in most cases (see Table S6 for the results of all Kruskal Wallis and permutation tests). Plate 1-5 controls were not significantly different from Plate 6-10 for all variables, nor were Day 9 animals significantly different from Day 10 animals ($p > 0.05$). Row and column were found to be not significant for all variables. Plate ID was found to be significant for *date of first segmentation*, *date of strobilation initiation*, *date of pigmentation*, and *number of primary segments*, but only *pigmentation* and *date of final segment formation* were found to be significant with permutation testing. Permuted Dunn's tests only showed significant pairwise differences for *date of pigmentation* after Bonferroni correction (see Table S7).

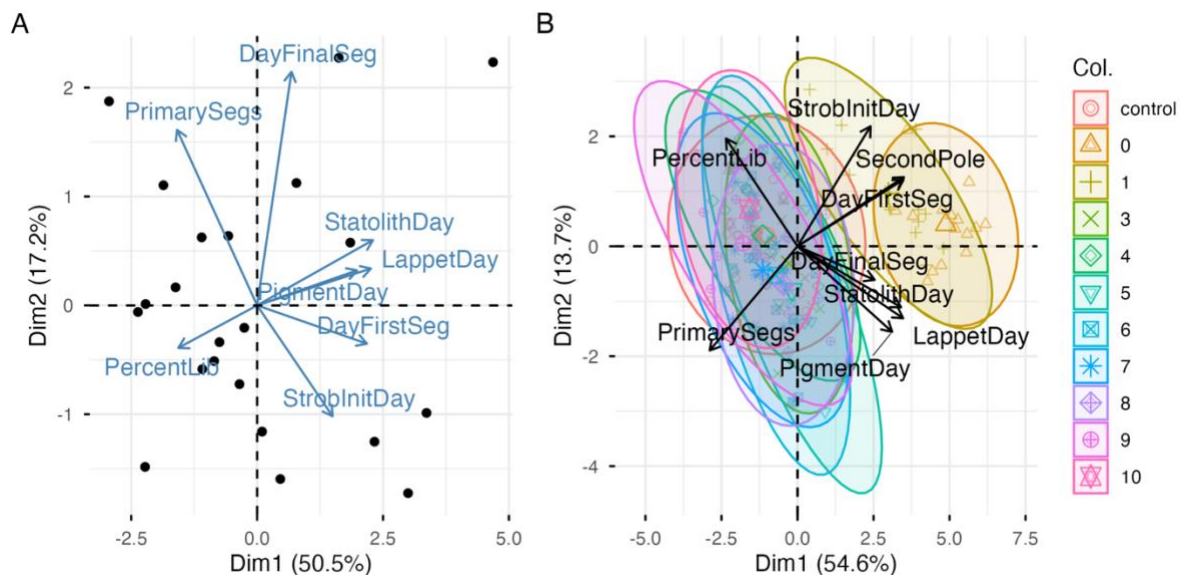


Figure 15 - PCA of strobilation variables

A) PCA for control animals with no missing data $n = 22/40$. **B)** PCA for all animals without missing data $n = 168/236$. We see early strobilation variables (*Initiation*, *First Segment*) and metamorphic variables (*Pigmentation*, *Lappet*, *Statolith*) group together. Dim 2 appears to be highly influenced by the number of segments formed, which is highly related to the *day of final*

segmentation in A), but is not in B) due to Aza treatment. *Presence of a second pole* was omitted from A as only one control animal had a second pole. PCA shows a negative relationship between beginning of segmentation variables (*initiation day, first segment day*) and *number of primary segments*, and a negative relationship between metamorphic variables (*statolith formation, lappet formation and pigmentation*) and the *percentage of liberated ephyrae*.

PCA shows covariance of segmentation and metamorphic variables

Loadings of variables on principle components showed a high covariance of metamorphic variables and initiation of segmentation variables to one another (Fig 15). Day 0 animals (yellow triangles in Fig 15b) were clearly separated from control and Day 3+ animals, while Day 1 animals heavily overlapped with Day 0 animals and somewhat overlapped with Day 3+ animals. This general pattern is also seen when considering a single variable at a time (Figs 16 & 17).

Segmentation Variables

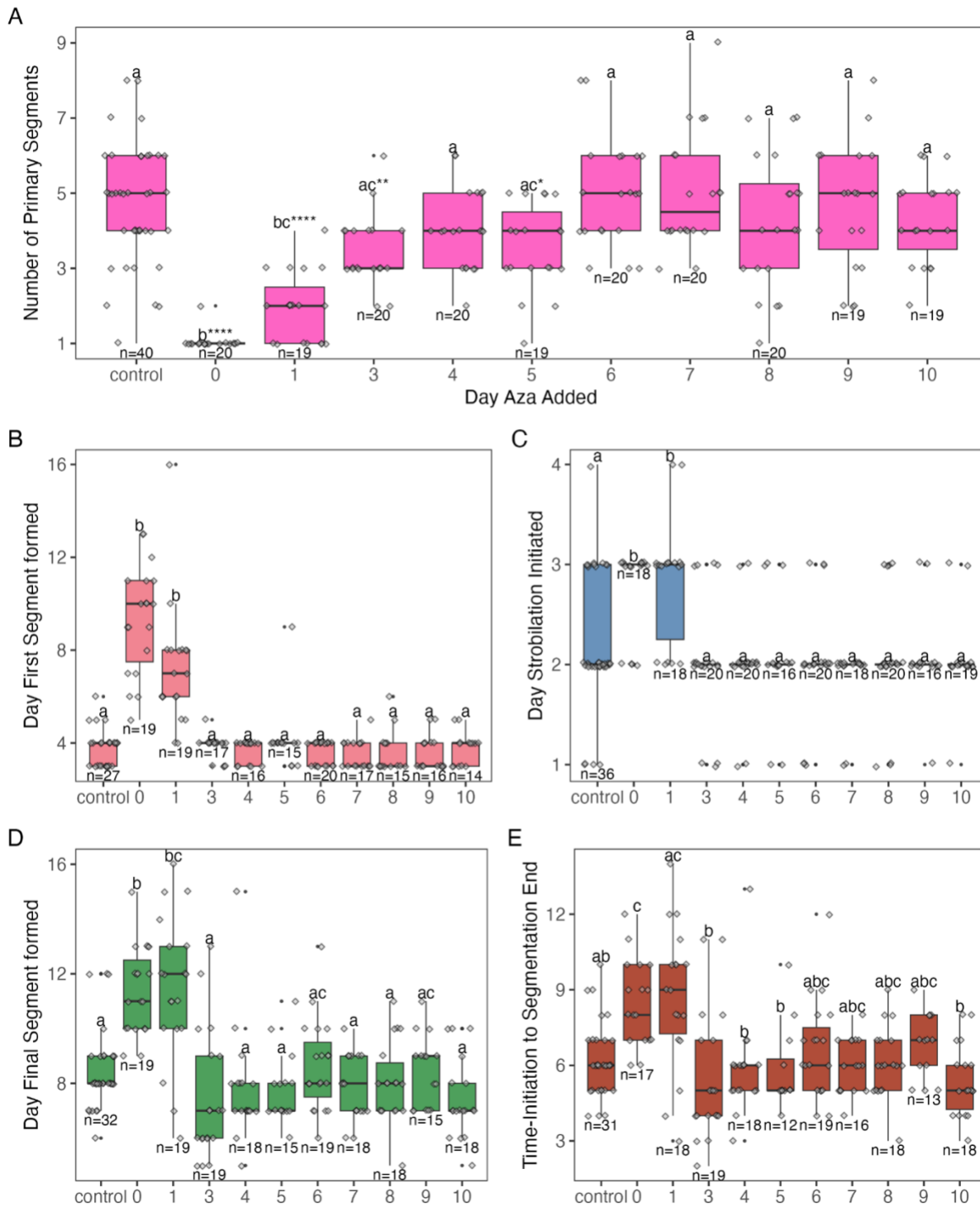


Figure 16 - Segmentation Variables (Y axis), Day of Aza addition (X axis)

Letters above bars correspond to multiple comparison grouping determined by Dunn's test with Bonferroni correction. Groups that share a letter cannot be differentiated from each other. For the comparison of *Primary Segment Number*, asterisks correspond to p-value of the linear

model considering number of *primary segments* as a function of *day of Aza addition*, treating *Aza day* as a categorical variable, with * <0.05, ** < 0.01, *** <0.001, and **** < 1e-4. *Segmentation Duration* is shown in E), corresponding to the difference in time between *Strobilation Initiation* and *day of Final segmentation*. All units correspond to days, except for part A which measures segments.

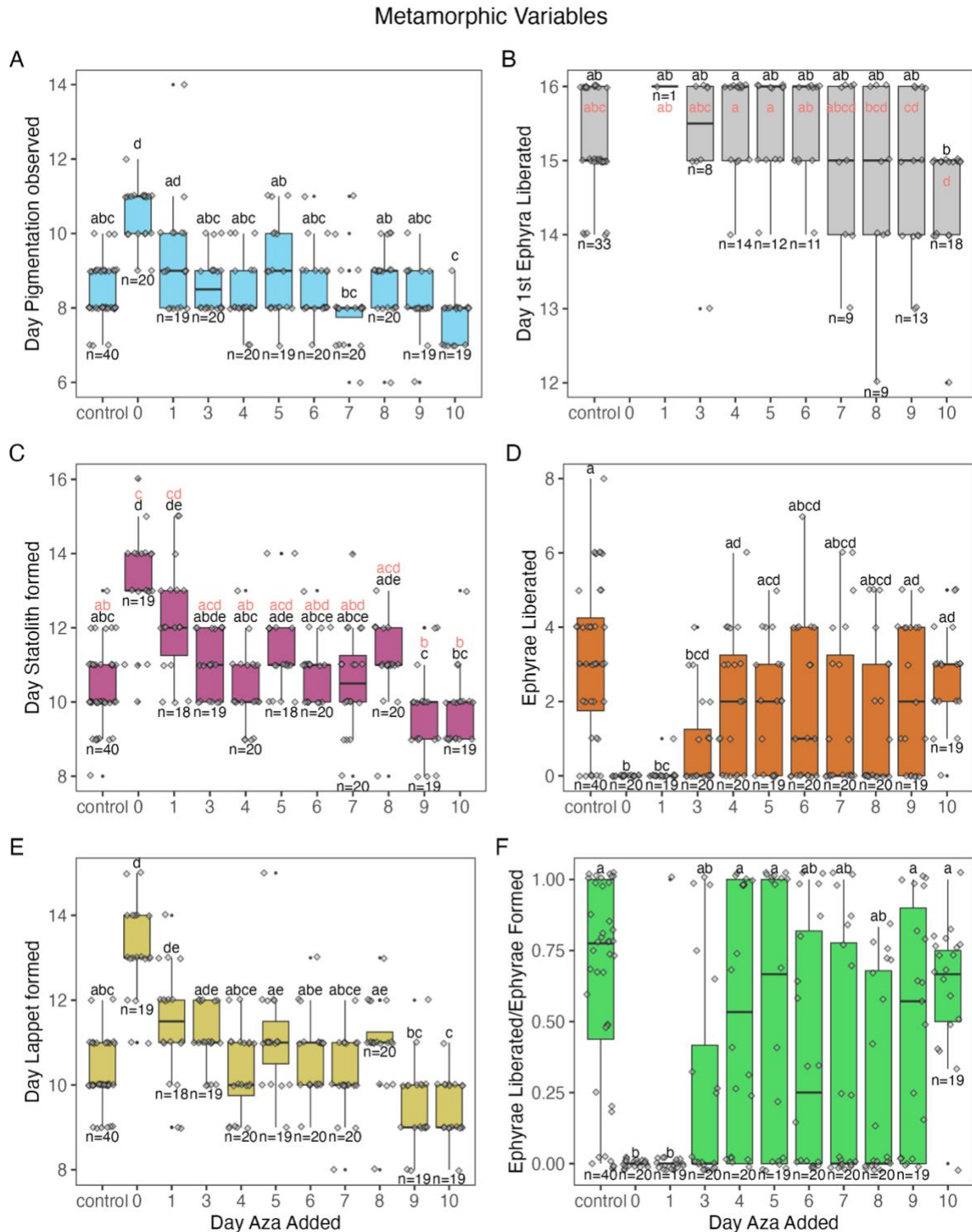


Figure 17 - Metamorphic variables (Y axis), Day of Aza addition (X axis)

Letters above bar graph correspond to multiple comparison labels, categories that share a label cannot be called significantly different by Dunn's Test as in Fig 16.

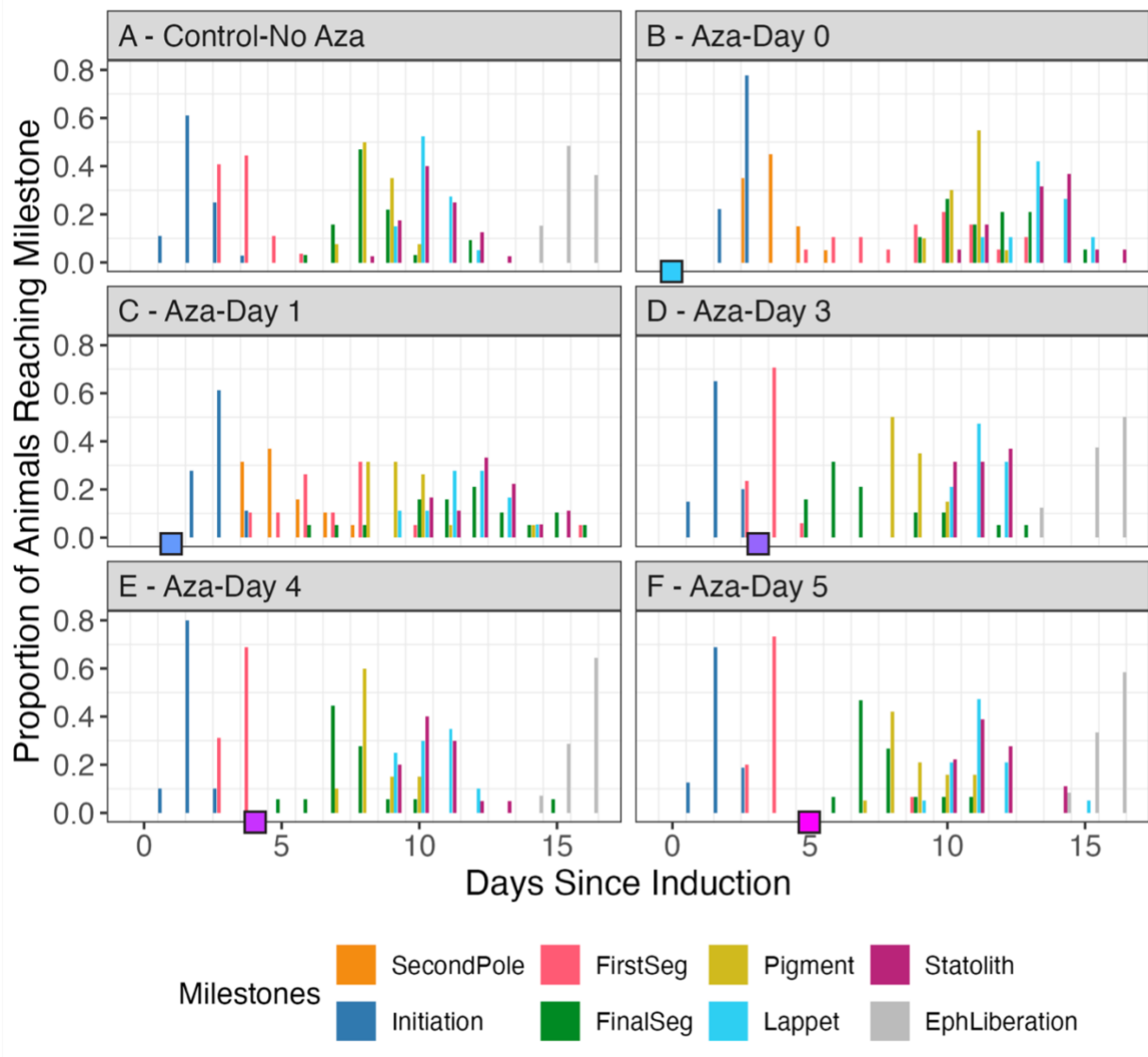


Figure 18- Histogram of time strobila reached developmental milestones

Bars correspond to X/Y where X = number of treated animals reaching the milestone at that timepoint and Y = all animals in the condition which reached that milestone. Instances where a single animal per condition reached a developmental milestone were omitted for clarity, but included in Fig S25. Fig S25 also shows Days 6-10. **A)** The control profile shows relatively distinct phases- 1-*strobilation initiation*, 2-segmentation start (*day of first segment formation*), 3 - end of segmentation (*date of final segment formation, date of pigmentation*) 4- distal development (*date of statolith formation, lappet formation*) and 5-ephyra release (*date first ephyra liberated*). **B&C)** Early Aza incubation appears to significantly delay all phases and triggers formation of an oral pole from the foot. **D-F)** Later incubation shows a return to control timing (similar to A). Colored squares on the x-axis show the day of Aza addition.

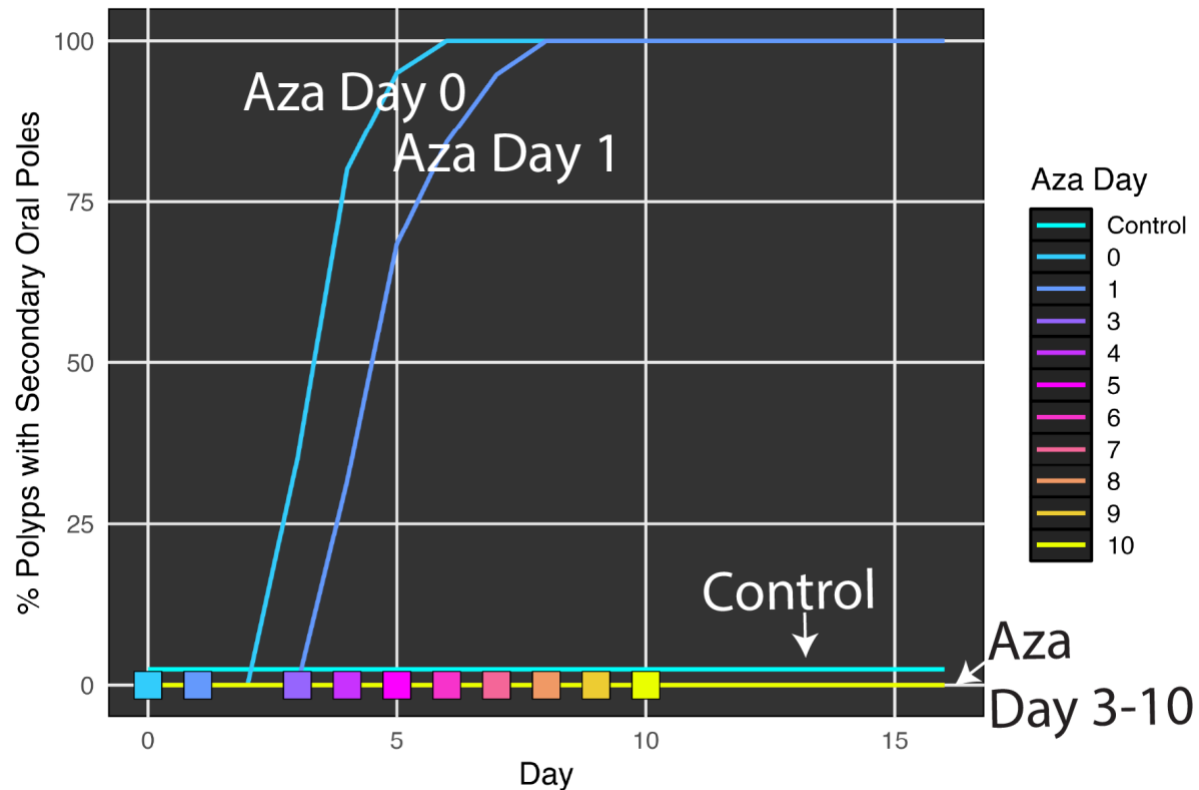


Figure 19 - Timeline of secondary oral pole formation

Day 0 and Day 1 treatments are the only ones to generate a substantial proportion of secondary poles. Colored squares along the x-axis correspond to the day of Aza addition.

Secondary pole formation is inhibited in late strobila

All Day 0 (20/20) and Day 1 (19/19) animals formed secondary oral poles (defined as forming a distinct oral structure surrounded by tentacle buds oriented in the former aboral direction) from foot tissue, while no Day 3+ animals formed secondary oral poles (see Fig 19). A single control animal (1/40) began with two mouths in its oral disc. These mouths were maintained throughout strobilation, yielding an ephyrae consistent with descriptions of “double monster” ephyrae (Vannucci 1957) and several ephyra with unusually large numbers of arms and distorted umbrellar surfaces. Day 3+ animals failed to form secondary poles even after all ephyrae had liberated. Day 0 animals tended to form an indistinct segment furrow between the primary and secondary poles (Fig 9), which only grew distinct towards the very end of

strobilation (Fig 9h). Day 0's secondary poles were oriented in the opposite direction of the original oral pole (Fig 9c-g). Day 1 animals tended to form distinct furrows between the original and secondary poles, similar to the furrows formed between a parent polyp and an offspring (Fig 10). Such poles tended to be smaller than day 0 secondary poles, and their orientation was not always predictable (see yellow arrows in Fig 10e)

Early Aza incubation delays and suppresses segmentation

Early Aza incubation strongly delays segmentation and reduces the number of segments formed while increasing the size of segments. Day 0 and 1 animals formed fewer segments than Day 3+ and control specimens (KW p-value $2.44\text{e-}17$, permuted p-value $1\text{e-}05$) (Fig 16 & 20). Linear modeling showed a significant difference in the mean residual of the number of segments vs. the control in Day 0, 1, 3, and 5 animals (see Table S4). While Day 4 animals did not form a significantly different number of segments, some animals did form enlarged segments farther down the strobila stack (Fig 16 and Fig 9-13, summarized in Fig 14). *Date of strobilation initiation*, *first segment* formation and *final segment* formation were all later in Day 0 and 1 animals than in control and Day 3+ animals (all KW p-values $< 1\text{e-}10$, all permuted p-values = $1\text{e-}5$) (Fig 16, see Table S6 for exact values).

Segmentation appeared highly sensitive to the timing of Aza addition. Ephyrae from the primary oral pole in Day 0 and 1 had extremely elongated aboral poles, resulting from the conversion of polyp tissue into ephyra tissue without segmentation. The geometry of the resulting umbrella appeared to be a direct carry over from the polyp body column, rather than a proportional rearrangement of ephyra features to create an enlarged disc (Fig 9h & 10h). Later addition of Aza saw enlarged ephyrae created further down the body column in a mostly proportional manner (Fig 11h-13h, see Fig S27 for side-by-side comparison of strobila with enlarged segments at various positions along the body column)

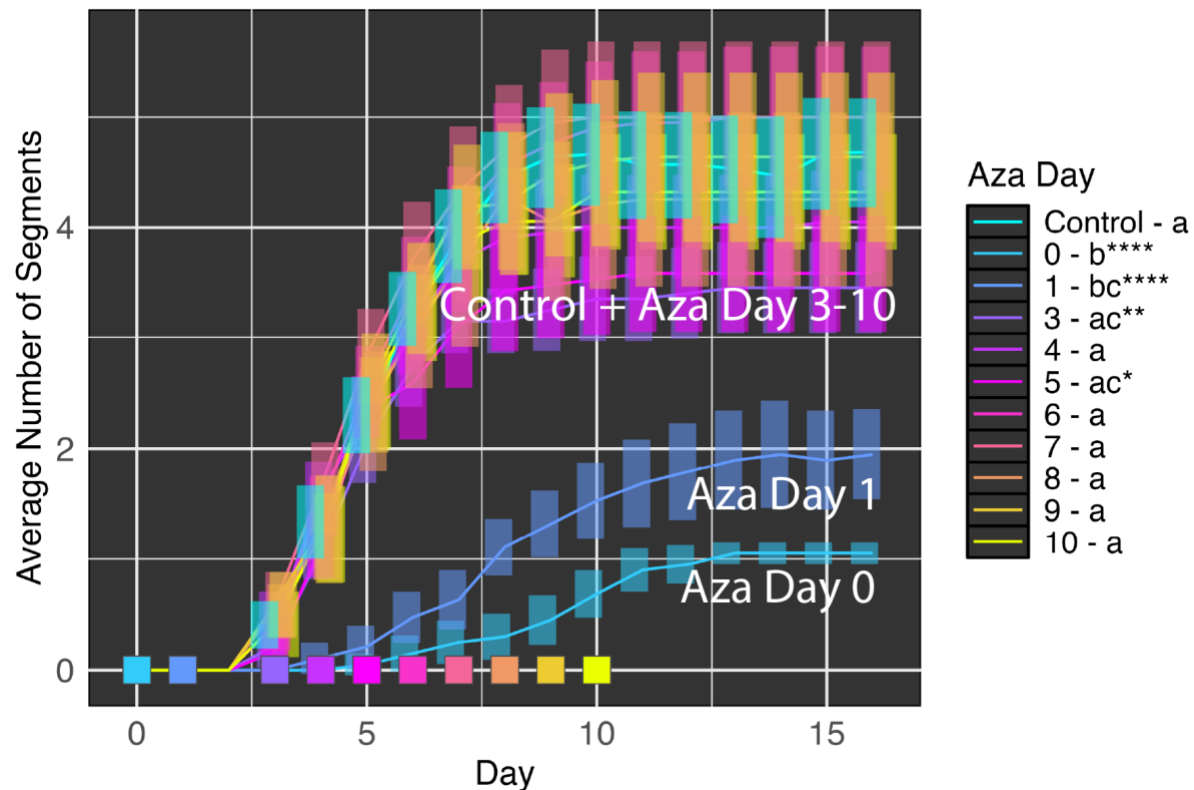


Figure 20 - Mean number of segments accumulated per polyp through time

Labeling in caption includes Day of addition followed by multiple comparison groups from Dunn's test and significance values from the linear model (Number of Primary Segments ~ AdditionDay), as shown in Table S4. Significance coding as in Fig 16a. Error bars show 95% confidence intervals and are slightly offset so as to not obscure other bars.

Aza incubation delays metamorphosis

The metamorphic variables of *statolith formation*, *lappet formation* and *pigmentation appearance* during development covaried (Fig 15). The variables showed slight differences in behavior-Aza Day 0 & 1 lappet and statolith appearance dates differed significantly from the control, while for pigmentation only Day 0 differed significantly from control (Fig 17a-c).

Occasionally animals in a Day 3+ condition were not differentiable from Day 1 animals, however there did not appear to be a straightforward relationship between the date of addition and which groups differed.

Liberated ephyrae were analyzed both by the number of individuals liberated by Day 16 and the proportion of ephyrae segments that formed on a strobila which were ultimately liberated. Day 0 animals did not liberate any ephyrae, while only a single Aza Day 1 animal released an ephyra. Of the remaining conditions only Aza Day 3 animals significantly differed in the number of ephyrae they released when compared to the control (Fig 17d & f, Fig 21).

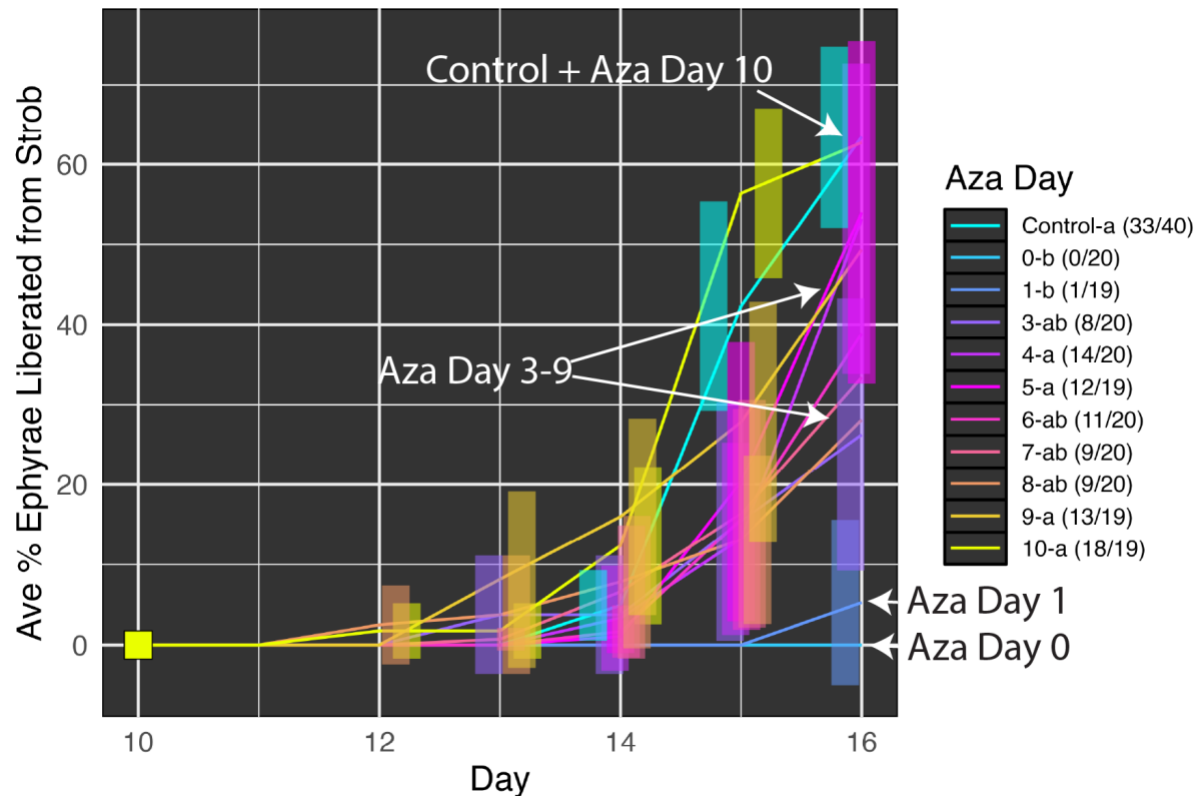


Figure 21 - Percentage of segments/ephyrae formed that become liberated ephyrae by condition

Multiple comparison labeling from KW test of percentage liberated ephyrae at day 16 follows day of Aza addition in legend and is then followed by a fraction in parentheses showing how many strobila released any number of ephyrae. Note that animals that did not liberate ephyrae were included in the average. For individual strobila release patterns see Fig S28.

DISCUSSION

Metamorphosis proceeds even if segmentation is repressed

The original oral pole of all Aza Day 0 & 1 animals formed ephyrae anlagen with an extended aboral surface. The regions of the polyp body column that would have formed additional segments were instead incorporated as an elongated umbrellar surface similar to that of cubazoan or staurozoan medusae. Even in bipolar ephyrae formation, (Fig S26) ephyrae formed with distinct arms, lappets and rhopalia. Neural and muscular development was relatively normal despite the lack of released ephyrae, as the giant ephyrae displayed contractile movements consistent with swimming strokes.

Aza incubation also disrupted the formation of radial structures during metamorphosis, with a variety of ectopic rhopalia and lappets forming along the animals. This effect appeared most severe in early Aza Days, and was less common in late Aza conditions (see captions of Figs 9-13 for further discussion). This would be congruent with the patterns of Wnt expression in strobilation shown in Khalturin et al. 2019, Wang 2013, and Zorina 2018, which showed high expression along the rim of segments and in the arms and lappets of metamorphosing segments.

Both metamorphosis and segmentation were delayed by early Aza incubation, with variables within these groups covarying somewhat (Fig 15). The strong covariance of the metamorphic variables pigmentation, lappet and statolith formation suggests they are directly related to one another. The visible gradation of pigmentation along the body column of unsegmented animals (Fig 9d), along with the gradation of pigmentation between segments along a strobila stack (seen in Fig 9c, 11d-g & 11 d-g) suggest that metamorphosis is triggered by a signal progressing from the oral pole aborally along the body column, potentially related to the morphogenetic wave triggered by strobilation induction.

Our results suggest that metamorphosis and segmentation proceed separately from each other down the body column. Tracking the formation and metamorphosis of each segment of a strobila separately may provide better resolution of this process. As speed of segmentation

varies along the body column, (see Fig 33, Kroihner et al., 2003) determining the speed of metamorphosis of individual segments may allow us to assess if metamorphosis strongly covaries with segmentation, as would occur if both processes are controlled by the morphogenetic wave. This topic is further examined in Chapter 3.

cWnt signaling potentially mediates between strobilation and polyp oral pole formation

A possible explanation for the lack of secondary pole formation in Aza Day 3+ animals is a buildup of cWnt antagonists in the foot triggered by strobilation induction. Aza Day 0 animals generated bipolar animals with fused epithelia, similar to the bipolar polyps formed from polyps exposed to high concentrations of Aza in Chapter 1. However the size of these new poles varied depending on the concentration of Aza they were exposed to. The foot of Aza Day 1 animals converted into small bud structures which were often separated from the base of the parent animal by a slender stalk of tissue (Fig 10h) similar to the secondary poles induced by exposure to intermediate concentrations of Aza in Chapter 1. This decreased response and lack of response to Aza in Aza Day 3+ animals would be consistent with cWnt inhibition building in the foot as strobilation progresses.

The rapid increase of resistance to pole formation in the foot appears to occur on a similar timeline to that of CL390 gene expression early in strobilation (Fuchs et al. 2014). During induction, CL390 expression builds in the oral disc, until it rapidly expands to define the region of the body column which will undergo segmentation (Fig S30, Table S8). This expression is maintained throughout segmentation and is absent in ephyrae. Another gene, CL1188, appears in polyp tissue and its expression retreats to the foot during strobilation. CL1188, or a gene controlled by CL1188, may be the cWnt antagonist responsible for the foot field.

An aboral gradient opposing oral segmentation signals would prevent mirror image strobila forming, as in Aza Day 3+ cases. The aboral-most segment in *A. aurita* strobila takes the longest time to form of all segments (see Fig 33b, Kroihner et al. 2003), which could be due to spillover from an anti-segmentation field in the foot. This would inhibit furrow formation in the forming segment, requiring a longer time for the signals to build to the point where a final furrow between the segment and strobila could form. Likewise, this would explain why foot tissue from strobila has no ability to induce strobilation when fed or grafted to uninduced polyps, while tissue from segments induces strobilation in uninduced polyps (Fig 33a, Fuchs et al. 2014).

Strobilation model of segmentation-define the region, divide with a series of flags

Early Aza incubation resulted in fewer, larger segments, while late hyperactivation did not affect the body axis of segments formed prior to Aza incubation. Thus our observations require both a spatial and temporal explanation. However, segments were not produced at a steady frequency between conditions (see Fig 20), making the presence of a timekeeping “clock” as in the clock and wavefront model unlikely. From a spatial perspective, the increase in size and decrease in number of segments is consistent with an increased morphogenetic gradient emanating from the oral surface of each subsequent segment (See Fig 22a). This would be conceptually similar to the role *wingless* plays in short-germ band insect segmentation, where bands of *wingless* define the posterior pole of segments which are progressively added in an anterior to posterior direction (Beermann et al., 2011; Nagy and Carroll, 1994). However, in the case of *Aurelia*, the cWnt signal would be responsible not only for segment polarity, but also for length of each individual segment. Our results indicate that segment boundaries are not modified by Aza addition post formation, which is more consistent with a serial model of segment addition than a French flag model of simultaneous segment boundary definition.

Based on these results, we propose a two step model of segmentation during strobilation. The first step sees a rapid diffusion of CL390 to define the oral region of the body column which will undergo segmentation. As part of this, an anti-segmentation field emanating from the foot forms based on cWnt suppression (Fig 22b). This foot field limits the progress of the later segmentation signals and prevents the formation of new oral poles (both those created by budding and ectopically induced by Aza exposure). The second phase sees the morphogenetic wave progress steadily down the body axis, as seen in Kroiher et al. 2003, in a cell proliferation dependent manner as seen in Fuji et al. 2024. Tissue on the oral side of this wave is committed to become a segment while tissue on the aboral side of the wave maintains a polyp identity.

The wave interacts with a gradient of cWnt activity emanating from the oral pole. Once the cWnt activity decreases to a threshold value, a furrow forms to define the aboral boundary of the segment. Once a furrow is formed, a new oral signaling center forms on the oral surface of the tissue below it, generating a new cWnt gradient, and the pattern repeats (Fig 22c). Adding Aza hyperactivates cWnt signaling globally in the animal, shifting the threshold value of cWnt activity for furrow formation toward the foot (Fig 22d). This suppresses segment formation along an expanded region of tissue, and results in ephyrae with enlarged umbrellar surfaces. Even though segment formation is suppressed in this elongated segment, it is still able to undergo metamorphosis, indicating a degree of independence between segmentation and metamorphosis. For further discussion of the elements of this model, see Supplementary Section 2.

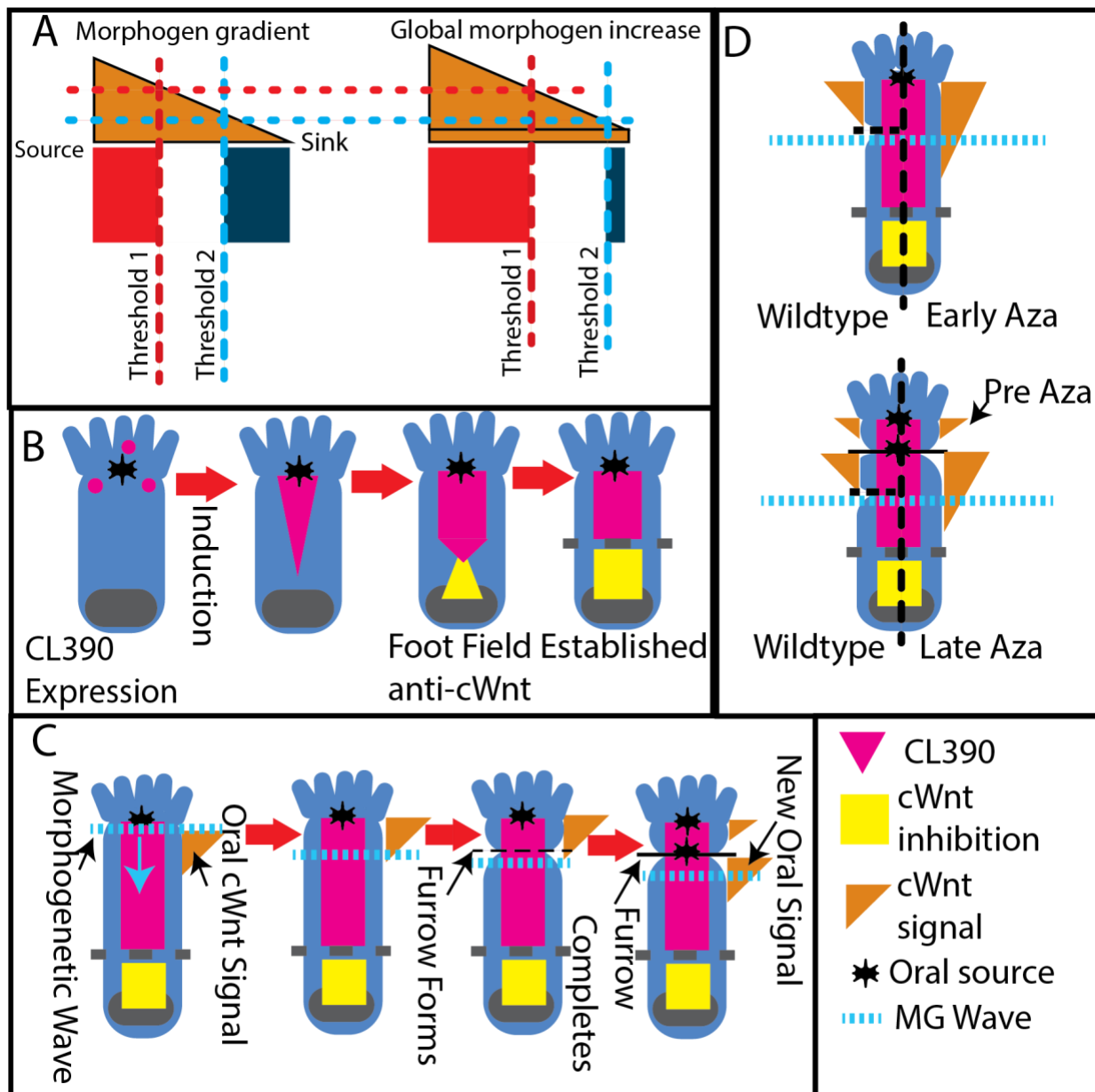


Figure 22- Two step process of segmentation in strobilation

A) French Flag model of regionalization - A morphogen activates a series of genes in a concentration dependent manner. In areas not exposed to the appropriate concentration of morphogen, the regional genes are repressed. A gradient of the morphogen along the body axis can separate the body into 3 (or more) regions -High signal (gene 1 on), Medium signal (gene 2 on) and Low signal (gene 3 on). If the concentration of the morphogen involved were governed by cWnt signaling, hyperactivating cWnt with Aza would be consistent with a global increase in morphogen concentration, altering the location where the thresholds are met along the body. Based on Sharpe, 2019; Wolpert, 1969. **B) Strobilation step 1- defining the foot and strobila stack** - This model assumes that the oral pole of the scyphopolyp is defined by high cWnt activity, as occurs in many cnidarian polyps and Hydrozoan medusae (Marlow et al., 2013; Müller et al., 2007; Nawrocki and Cartwright, 2013; Sanders and Cartwright, 2015). CL390 expression rapidly spreads from the oral pole (as in Fuchs et al., 2014; Wang, 2013). A cWnt

repressive field emanating from the foot activates. This field maintains polyp identity at the aboral pole throughout strobilation. **C) Strobilation step 2 - segment formation** A cWnt independent morphogenetic wave emanating from the mouth travels down the body column (as demonstrated in Fujii et al., 2024b; Kroihner et al., 2003). The oral pole generates a cWnt dependent morphogenetic gradient which defines the lower boundary of the segment. When the morphogenetic wave reaches the critical threshold of cWnt activity, a furrow between the upper and lower segment forms. Once the furrow has progressed sufficiently, a new oral signaling center is created and generates a new gradient of cWnt to define the next segment in the stack. **D) Aza addition suppresses segment formation and displaces furrows aborally.** Global hyperactivation of cWnt by Aza extends the oral cWnt gradient farther down the body column, meaning the morphogenetic wave must travel farther to reach the critical threshold needed to form a furrow. Aza incubation does not affect the segment boundaries which have already been passed by the morphogenetic wave.

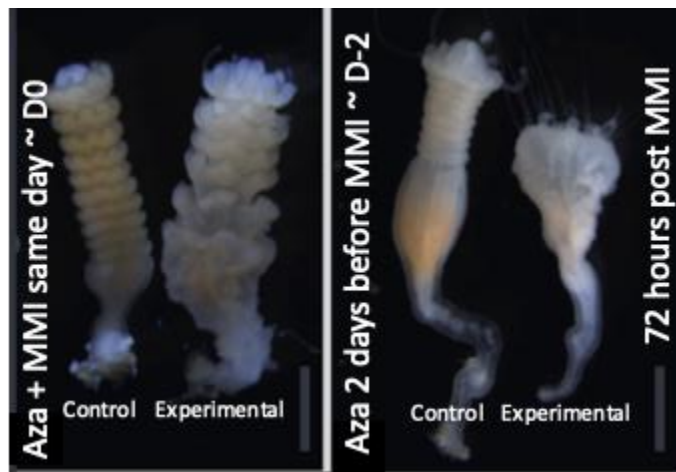


Figure 23 - Khalturin Aza strobilation incubation results

Left-result of simultaneous exposure to Aza and MMI, equivalent to our D0 condition. *Right* result of exposing the animal to Aza for 2 days and then adding MMI. Photos were taken 72 hours after MMI addition. Figure modified from Khalturin et al. 2019.

Comparison with Khalturin et al. 2019's results

Our results confirm Aza's repressive effect on segmentation as noted in Khalturin et al. 2019 (see Fig 23 for animals from Khalturin et al. 2019). Both studies demonstrate segment number is reduced more by earlier Aza incubation. However, these studies differ on the time of Aza addition needed to produce particular phenotypes, likely due genetic factors in the *Aurelia* strains/species examined. In general, our Southern California animals showed slower

strobilation and a higher sensitivity to Aza exposure. For further discussion of differences between the studies, see Supplementary Section 1.

In evolution - switching between asexual and sexual life stages

Based on the role of cWnt in defining the oral pole of cnidarian polyps, a cWnt based tradeoff between strobilation and budding would be evolutionarily advantageous to *Aurelia* polyps by preventing different reproductive pathways competing with one another for tissue. Both strobilation and budding require the parent polyp to donate a large amount of tissue to their offspring. When a body column has multiple oral signaling centers during strobilation, multiple ephyra anlagen can fuse into a single individual, resulting in ephyrae that do not swim well (see Vannucci, 1957 for an example). While both ephyrae and buds are clonal offspring, ephyrae develop into sexually reproducing medusae. Repressing budding during strobilation therefore devotes more of the animal's resources toward sexual reproduction. This could introduce evolutionary dynamics seen in animals with both clonal and sexual reproduction. In these cases stable environments select for clonal reproduction and unstable environments favor sexual reproduction (as seen in Jokela et al., 2009).

Phenocopies

The umbrellar surfaces of ephyrae generated by these experiments appear similar in geometry to those of Staurozoan and Cubozoan medusae, leading us to consider these phenotypes to be phenocopies. When wildtype *A. coerulea* ephyrae relax, they adopt a snow flake-like shape, with the plane of their arms roughly parallel to the plane of their inner disc (see individual in Fig 8f and the uppermost ephyra on the stack of 8e as examples). The giant ephyrae created in Aza Day 0 & 1 appear incapable of lying flat in this manner. Instead their arms lie orthogonal to the plane of their inner disc (see Figs 9h & 10h, also seen in Haeckel

1881 sketches see Fig S24). This curled posture appears to be the consequence of the enlarged umbrellar surface propping the arms at an upwards angle, and resembles the mature medusae of cubozoans and staurozoan as well as juvenile medusae generated by monodisc strobilation in *Morbakka virulenta* (Cubozoa) (Straehler-Pohl and Jarms, 2022, 2005; Toshino et al., 2015). The geometry of medusa umbrellas directly alters the hydrodynamics of the animal, affecting its swimming and foraging patterns. The impact of Aza on the geometry of the ephyra umbrellas is further considered in chapter 3.

Conclusion

Our results support that metamorphosis and segmentation during strobilation are separate processes with a common starting point, analogous to bilaterian metamer formation and specification. Previous observations show that strobila segments can transform into polyps rather than ephyrae when under stress (Helm, 2018) suggest that segmentation does not always commit tissue to undergo metamorphosis. Our own results demonstrate that tissue can metamorphose even when the segments that would normally form within it are repressed.

We suggest that the lower boundary of strobilation is governed by the creation of an aboral cWnt inhibitory field following induction, which simultaneously represses budding from the foot and maintains the foot's polyp identity. This field would allow polyps to persist through multiple rounds of growth and strobilation, and would also inhibit extraneous polyp oral signaling centers which would deform ephyrae. Incubation with the GSK3 β inhibitor 1-Azakenpaullone while simultaneously inducing strobilation converts foot tissue into oral tissue while the field is still weak or absent. Once converted, this new oral pole may strobilate along with the original oral pole. This indicates cWnt signaling may govern a tradeoff between a purely asexual reproductive pathway (budding) and an asexual + sexual reproductive pathway (strobilation).

The foot repressive field and its relationship to cWnt can be verified in the future by localizing cWnt related gene expression during strobilation, particularly in the foot of the animal.

The tradeoff between budding and strobilation may have a significant impact on a polyp population's ability to both sustain itself via budding and to generate mass releases of medusae ("blooms"). Given *Aurelia coerulea*'s success as an invasive species across the world, and the disruption to human activity its blooms can inflict (fishing, tourism, powerplant operations, etc. see Dong 2019, Marchessaux et al. 2023 for summaries), the question of *A. coerulea* lifecycle regulation has serious economic implications. Further work on the regulation of budding vs. strobilation may give further insight into polyp and medusa control strategies.

SUPPLEMENTARY TABLES

Table 4 SUPPLEMENTARY - Linear model comparing residuals of number of segments to condition as a categorical variable

Bolded rows pass the Bonferroni corrected p-value threshold for an alpha of 0.05.

	Normal LM				Robust LM			
	Estimate	Std. Error	t value	Pr(> t)	Std. Error	t value	Pr(> t)	
(intercept-control)	4.675	0.21120823	22.1345537	2.10E-58	0.2548349	18.3452107	1.41E-46	
Day0	-3.625	0.36582339	-9.9091531	1.93E-19	0.25994693	-13.945154	2.86E-32	
Day1	-2.7276316	0.37218602	-7.3286783	4.13E-12	0.33327868	-8.1842365	2.03E-14	
Day3	-1.225	0.36582339	-3.3486104	0.00095207	0.33450521	-3.6621253	0.00031184	
Day4	-0.625	0.36582339	-1.7084747	0.08892736	0.35067659	-1.782269	0.07605384	
Day5	-1.0960526	0.37218602	-2.9449055	0.00356971	0.37545202	-2.9192881	0.00386474	
Day6	0.325	0.36582339	0.88840683	0.37527071	0.4192185	0.77525204	0.43900475	
Day7	0.325	0.36582339	0.88840683	0.37527071	0.43859395	0.7410043	0.45946348	
Day8	-0.425	0.36582339	-1.1617628	0.24656275	0.48579158	-0.8748608	0.38258257	
Day9	-0.0434211	0.37218602	-0.1166649	0.90722965	0.47616615	-0.0911889	0.92742365	
Day10	-0.3592105	0.37218602	-0.9651371	0.33551234	0.37328245	-0.9623022	0.33693086	

Table 5 SUPPLEMENTARY - Recipe for 1 L of Artificial Seawater

Based on MBL 1 recipe from the Biological Bulletin Compendia website. Add deionized water to 1L the mark, wait until all solids dissolved before using. NaHCO₃ should be added last.

Ingredient	mM	g/L
NaCl	423	24.72
KCl	9	0.67
CaCl ₂	9.27	1.36

MgCl2	22.94	4.66
MgSO4 Hexahydrate	25.50	6.29
NaHCO3	2.14	0.18

Table 6 SUPPLEMENTARY - Summary of tests of significance for all variables

All cells show p-values. Values highlighted in red are <0.05. All variables were found to be significant by Kruskal Wallis test (KW) and permuted Kruskal Wallis (pKW) testing when grouped by *Day of Aza addition*. Comparison of Controls of plates 1-5 (A) with plates 6-10 (B) showed no significant difference. Comparison of D9 animals (on plates 1-5) vs D10 (plates 6-10) showed no significant difference. KW when grouped by Plate found several variables to be significant, but only *Pigmentation Day* was found to be significant with regard to plate identity by pKW. *Date of Final segmentation* was found to be significant by plate pKW, but not normal KW testing. For pairwise examination of plate permuted results see Table S7

	p-values							
Variables	By Aza Day KW	Aza Day pKW	Control A v B - KW	D9 v D10 - KW	By Cols KW	By Rows KW	By Plate KW	By Plate pKW
# Prim Segs	2.44E-17	1.00E-05	0.06016452	0.08548857	0.53159224	0.84687984	0.00053461	0.14297
1st Seg Day	1.73E-16	1.00E-05	0.23570337	0.05161779	0.07786628	0.53872091	4.93E-05	0.48721
Strob Init Day	9.55E-08	1.00E-05	0.27661629	0.31672377	0.42851788	0.25356068	0.01744775	0.25578
Final Seg Day	2.48E-11	1.00E-05	0.85903743	0.22011339	0.07506461	0.7862874	0.12232614	0.04979
Seg Duration	6.38E-07	1.00E-05	0.16252175	0.15461483	0.07890455	0.90281134	0.27887983	0.11697
Pig Day	1.75E-12	1.00E-05	0.71545157	0.91851228	0.22014702	0.86608417	0.00249955	1.00E-04
Lib Eph Day	0.0108414	0.00649	0.76501706	0.70171107	0.10421895	0.65934402	0.5789306	0.42734
Stato Day	5.58E-14	1.00E-05	0.35886254	0.34411877	0.75449336	0.17880046	0.16839262	0.06976
Lappet Day	1.99E-17	1.00E-05	0.66663815	0.7234838	0.25490588	0.24672086	0.56239087	0.31958
# Lib Eph	1.08E-10	1.00E-05	0.15128178	0.64036017	0.79350971	0.27384761	0.69745042	0.07887
% Lib Eph	1.43E-09	1.00E-05	0.45934108	0.10929009	0.85012675	0.33251551	0.64363602	0.23135

Table 7 SUPPLEMENTARY - Pairwise comparisons of permuted Dunn's tests by plate for date of pigmentation and final segmentation

For a false discovery rate of 0.05, an alpha of 1.11×10^{-3} derived by Bonferroni correction ($0.05/45$) was used. Red highlighted cells pass the Bonferroni corrected alpha. Plate comparisons in format X(First plate number).(Second plate number). *Date of final segmentation* was found to not have any significant comparisons post Bonferroni, while *date of pigmentation* saw several differences between plates to be significant. Most differences for pigmentation were found between plates 1 & 2 and other plates as well as plate 6 from plate 3, 5, and 8. The overall pattern of differences is unclear. Future study could measure the intensity of pigmentation under standard lighting conditions to avoid treating pigmentation as a categorical variable and hopefully avoid any human error that could cause the significant differences seen here.

Plate comparisons	Pigmentation By Plate	Date of Final Segmentation By Plate
X2.1	0.02418	0.40758
X3.1	7.00E-05	0.40338
X4.1	0.00022	0.12689
X5.1	2.00E-05	0.07008
X6.1	0.23366	0.03291
X7.1	0.00044	0.28653
X8.1	1.00E-05	0.32365
X9.1	4.00E-05	0.40514
X10.1	0.00821	0.00896
X3.2	0.03109	0.31637
X4.2	0.08441	0.08352
X5.2	0.03103	0.04408
X6.2	0.11018	0.01799
X7.2	0.08575	0.38416
X8.2	0.00078	0.23486
X9.2	0.02514	0.30473

X10.2	0.31596	0.00412
X4.3	0.30952	0.18968
X5.3	0.49173	0.11293
X6.3	0.00098	0.06353
X7.3	0.31627	0.20294
X8.3	0.09637	0.4282
X9.3	0.46171	0.48725
X10.3	0.09131	0.01966
X5.4	0.30186	0.36757
X6.4	0.00433	0.29468
X7.4	0.49502	0.03165
X8.4	0.0351	0.20569
X9.4	0.27177	0.14639
X10.4	0.19648	0.1346
X6.5	0.00094	0.43851
X7.5	0.30884	0.01277
X8.5	0.10076	0.11749
X9.5	0.46897	0.07787
X10.5	0.08665	0.23618
X7.6	0.01462	0.01283
X8.6	0.00015	0.10532
X9.6	0.00364	0.07052
X10.6	0.078	0.29721

X8.7	0.06623	0.17538
X9.7	0.31301	0.22783
X10.7	0.23393	0.00312
X9.8	0.15297	0.41929
X10.8	0.01338	0.03822
X10.9	0.11193	0.02287

SUPPLEMENTARY FIGURES

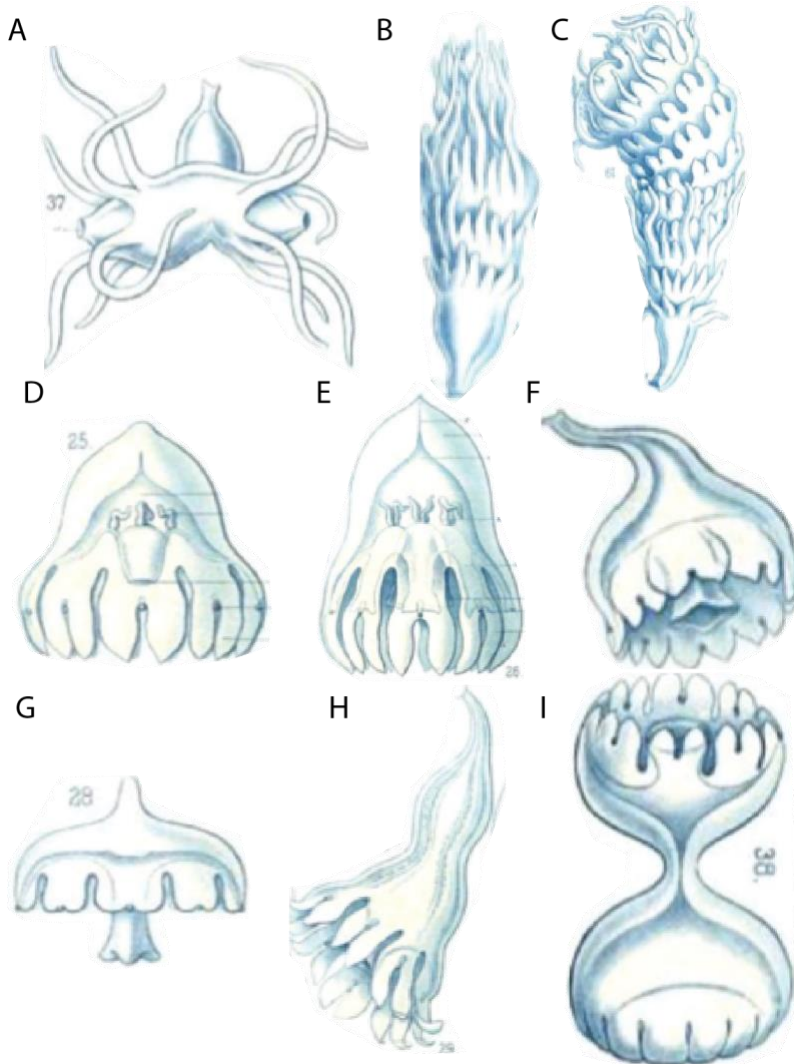
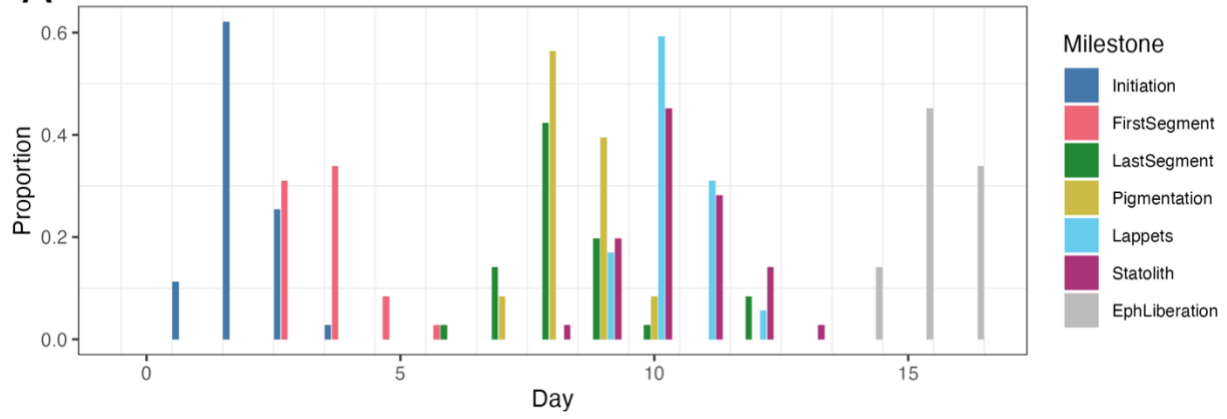


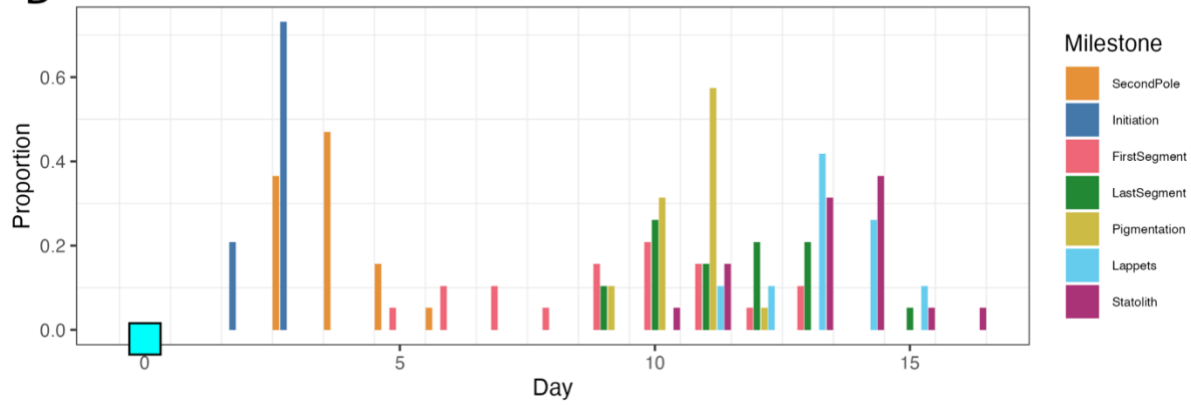
Figure 24 SUPPLEMENTARY - Ectopic polyp oral disc, modified from Metagenesis und Hypogenesis von *Aurelia aurita*

Plate section of Haeckel's sketches of wild caught strobila and ephyra malformations **A)** a bipolar polyp that has maintained its foot structure **B)** & **C)** strobilae where polyp oral structures are forming in place of ephyra structures for some segments. **D – H)** ephyra with dramatically elongated aboral poles, **I)** a bipolar polyp fused together by the ectoderm of the umbrellar surface. D-I show phenotypes remarkably similar ephyra shown in Fig 8-13 and S27

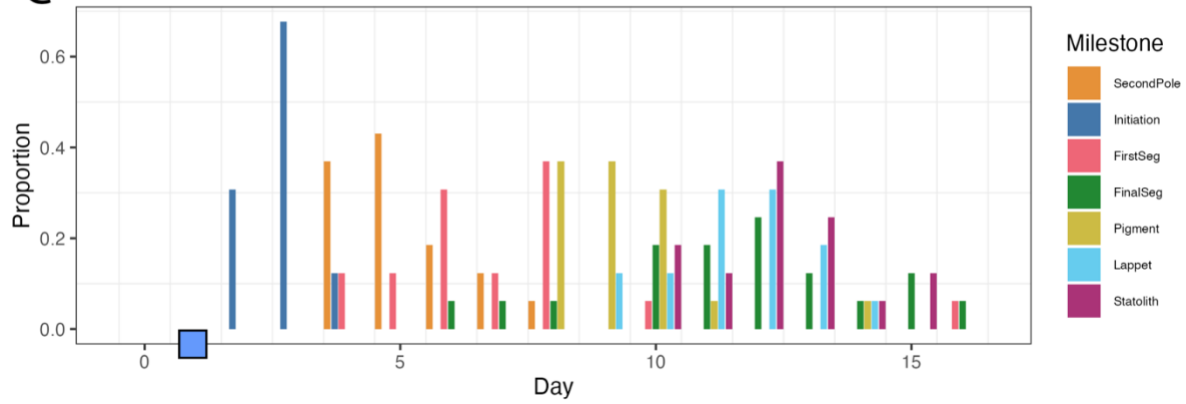
A Control Strobilation Timeline



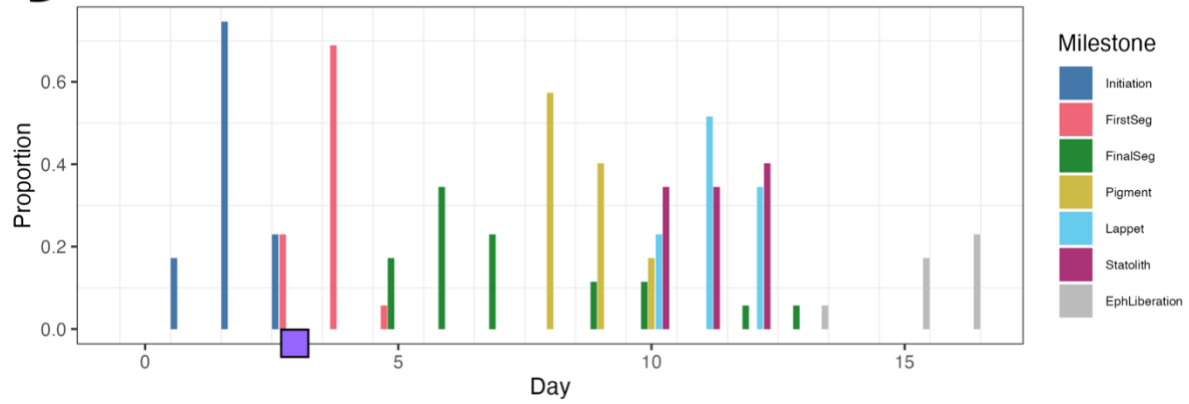
B Day 0 Strobilation Timeline



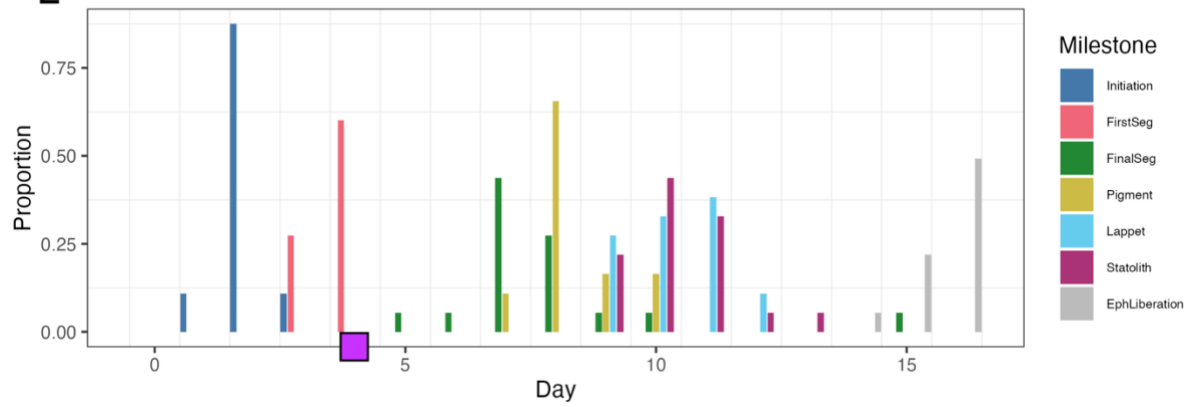
C D1 Strobilation Timeline



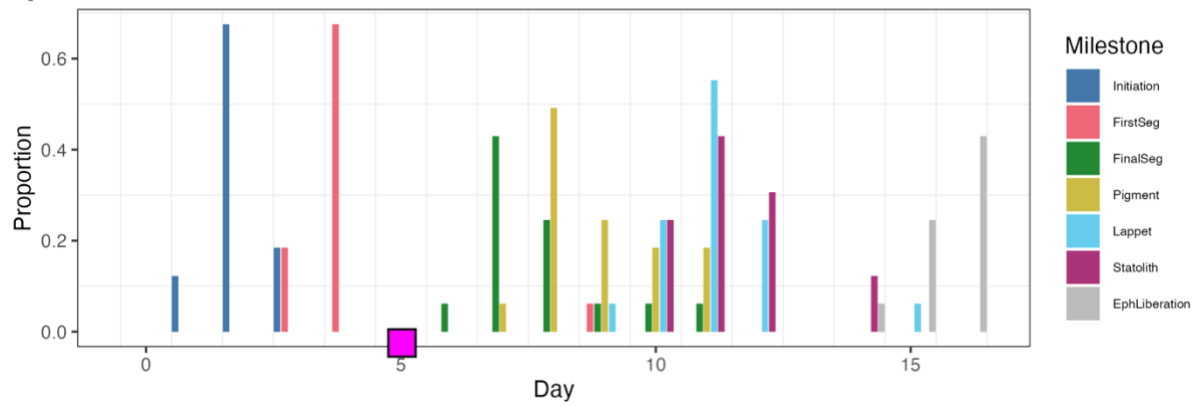
D D3 Strobilation Timeline



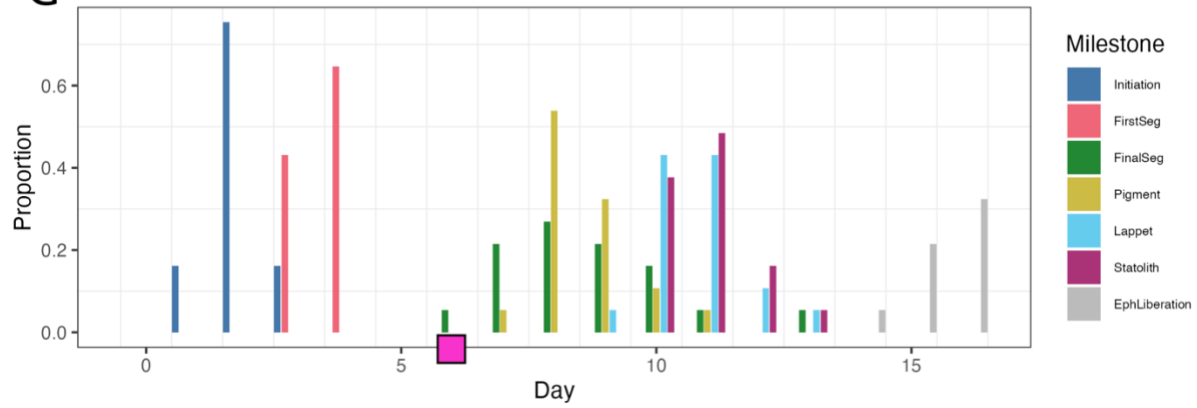
E D4 Strobilation Timeline



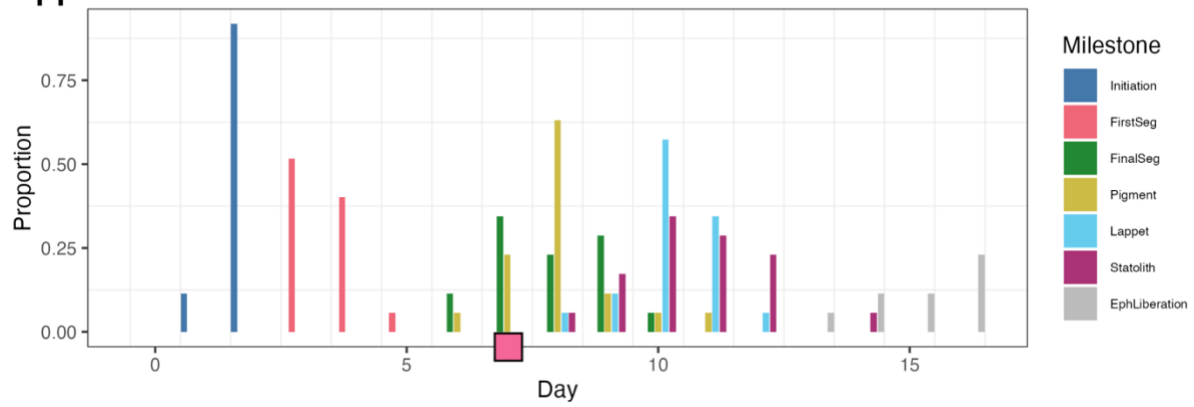
F D5 Strobilation Timeline



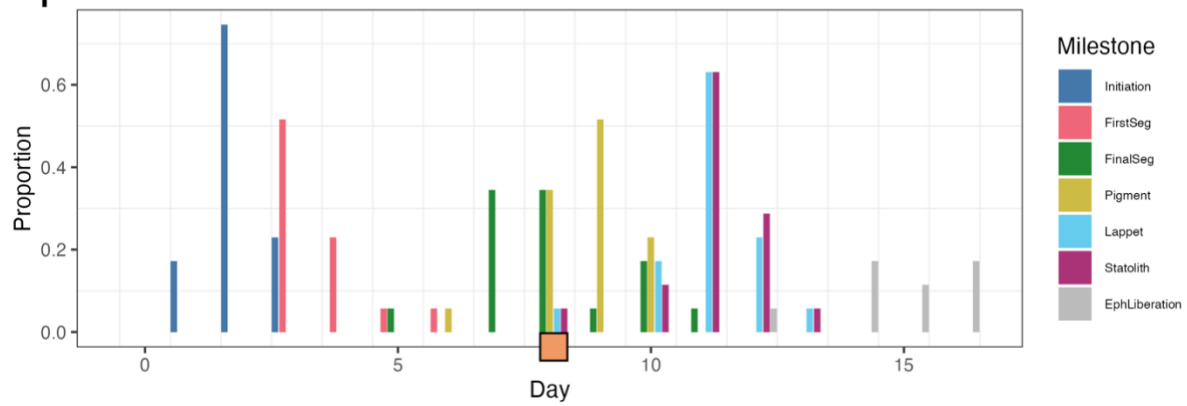
G D6 Strobilation Timeline



H D7 Strobilation Timeline



I D8 Strobilation Timeline



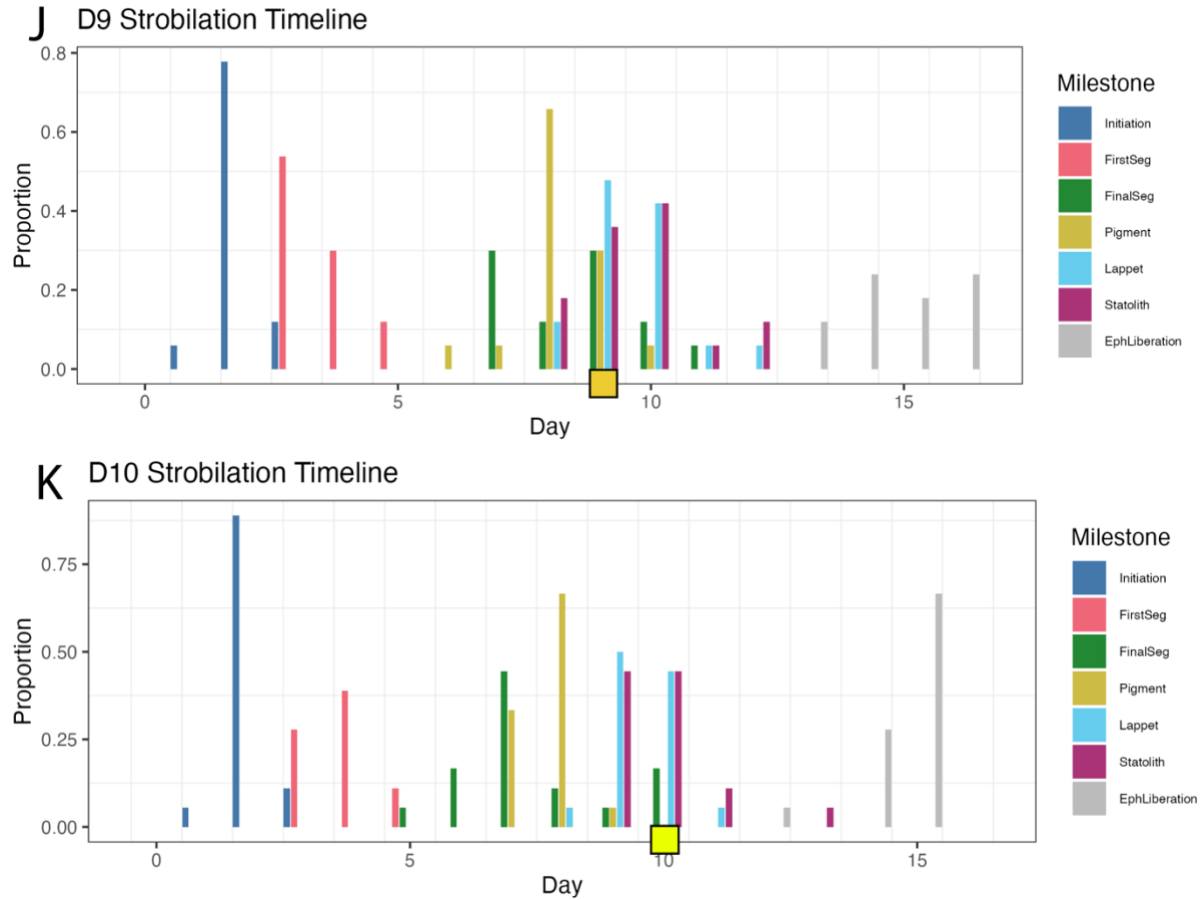


Figure 25 SUPPLEMENTARY - Histogram of developmental milestones for all conditions

Note that these histograms show (# animals reaching milestone at timepoint)/(all animals in condition). Colored squares show day of Aza addition.

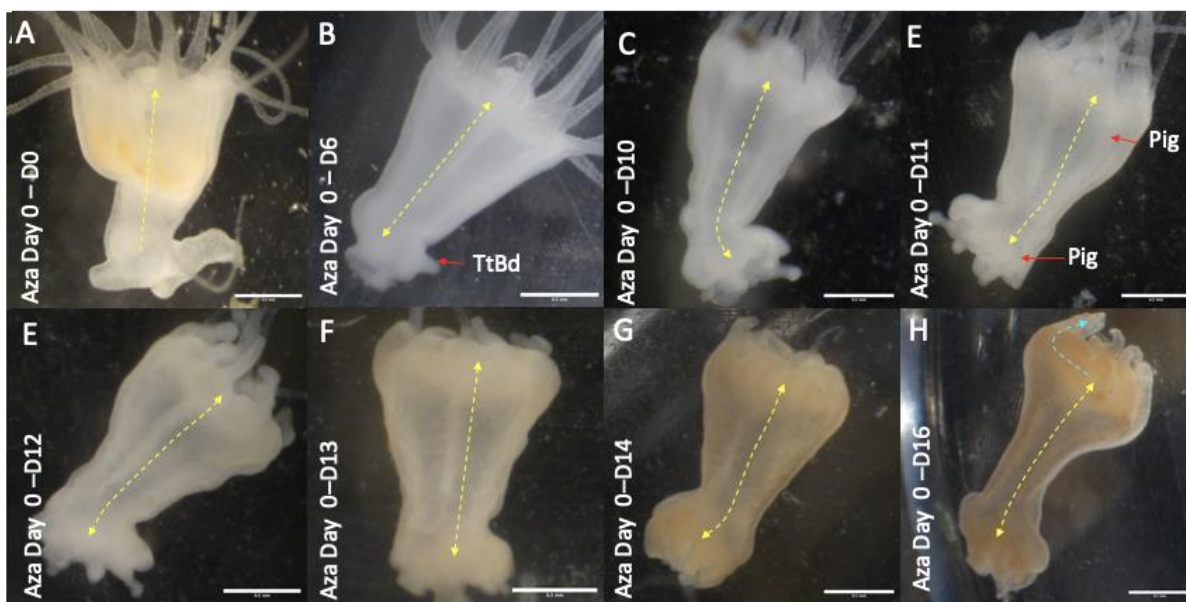


Figure 26 SUPPLEMENTARY - Bipolar ephyrae

Animal shows incomplete constriction between new and original oral pole. Animal began with a somewhat distinct furrow, but lost it during development

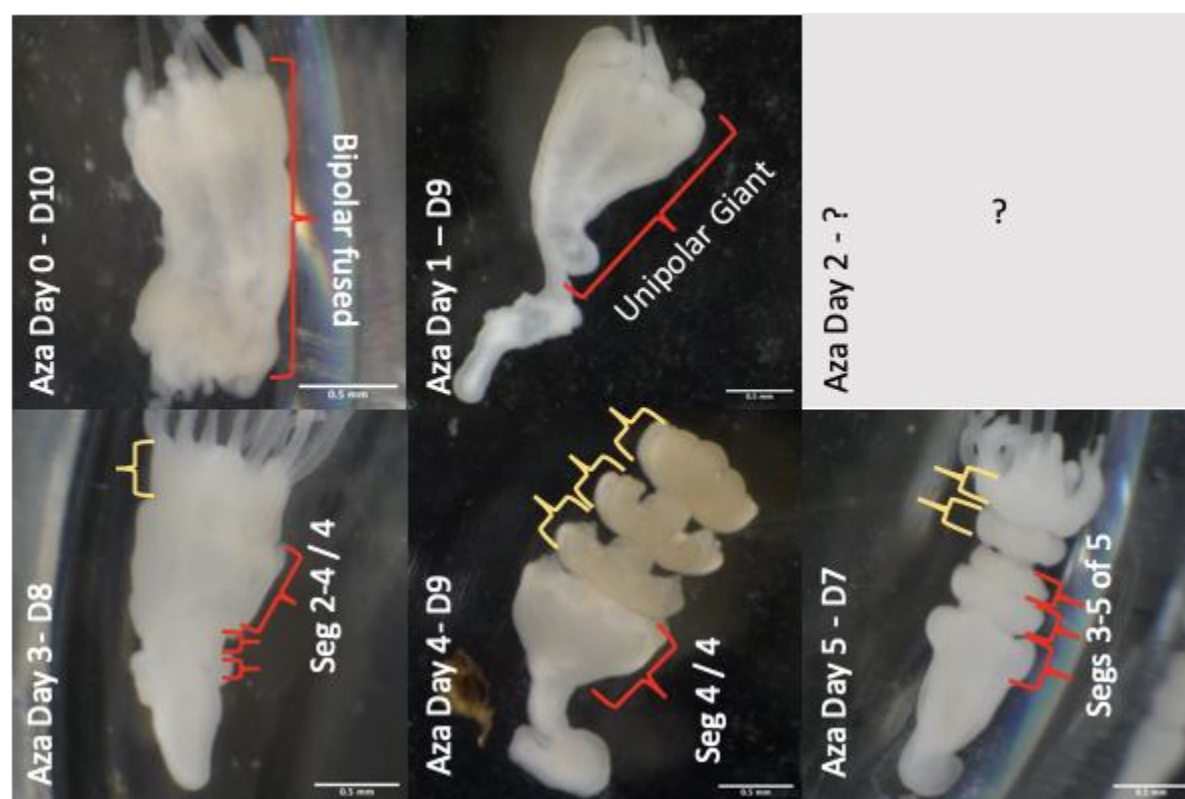


Figure 27 SUPPLEMENTARY - Side-by-side comparison of disruption to segment intervals

As strobilation progresses, disrupted segmentation is predominantly shifted aborally, consistent with pattern formation beginning at the oral pole and traveling toward the foot. Red brackets indicate affected segments, yellow segments indicate normal segmentation intervals.

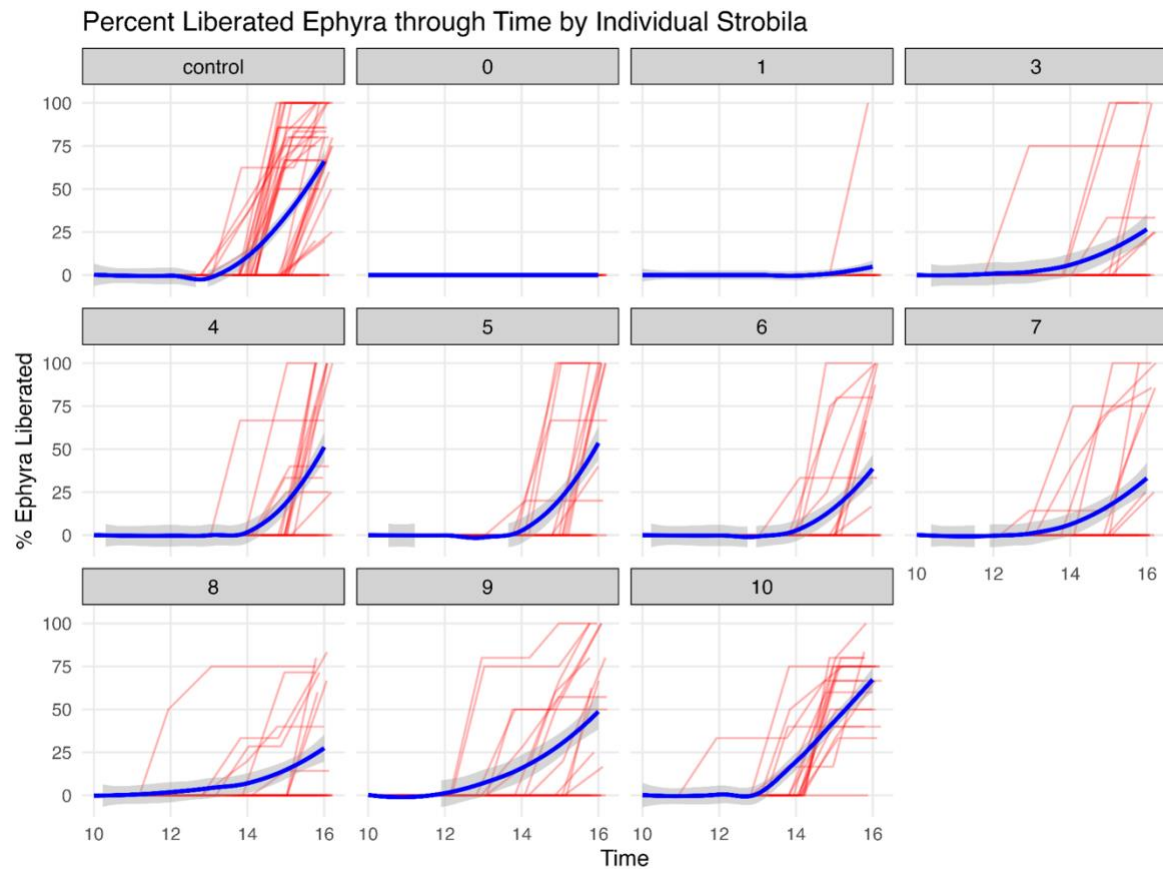


Figure 28 SUPPLEMENTARY - Percentage of ephyrae released from strobila by condition
Red lines indicate percentage of an individual strobila's ephyrae that have been released. Loess curve in blue.

SUPPLEMENTARY SECTION 1

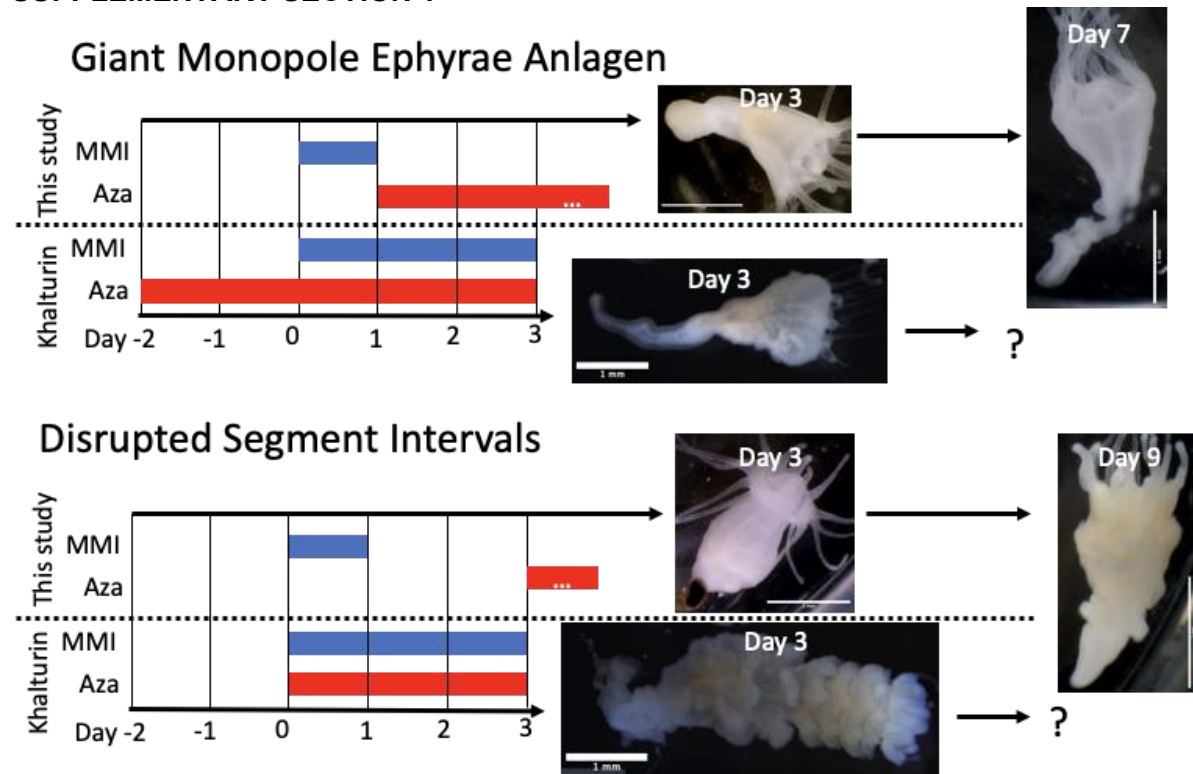


Figure 29 SUPPLEMENTARY - Comparison of experimental design with Khalturin et al. 2019

Left - A timeline of conditions used by this study and Khalturin et al. 2019 to generate the corresponding phenotypes. Blue bar corresponds to animal exposure to MMI (1 μ M in Khalturin, 50 μ M in ours), with time of MMI addition defining “Day 0” of the experiment. Red bar corresponds to incubation with 2 μ M Aza, with “...” indicating continued exposure post Day 3. *Middle* photos show animals 72 hours post MMI induction, *Right* photos show our Southern California strain D1 and D3 animals at a stage in their development comparable to the Khalturin animals. All scale bars correspond to 1 mm. Khalturin photos modified from Khalturin et al. 2019.

Discussion of Khalturin et al. 2019’s results

Khalturin et al. 2019 showed images of Aza incubated *Aurelia* polyps that were exposed to Aza different times. Their two experimental conditions were a pre-incubation in 2 μ M Aza for two days prior to 1 μ M MMI induction (Aza Day -2), and a simultaneous exposure to MMI and Aza (Aza Day 0). Animals were photographed 72 hours after MMI induction. Animals in Aza Day -2 formed a single large ephyrae anlagen, while Aza Day 0 animals formed a number of enlarged segments (see Khalturin Supplementary Info 3.5).

Our animals appear to strobilate more slowly than Khalturin et al.'s. 72 hours post induction, Khalturin et al.'s presented control animals have 10 and 8 segments, while their experimental D0 animal shows 6 segments (Fig S29 right side). In our experiment, only 11/40 of the control animals had formed a single segment at that time. The closest phenotypes we obtained to their Aza Day -2 case (forming single giant monopolar ephyrae) would be our Aza Day 1 case, while their Aza Day 0 case (disruption to strobila segment size) would be closest to our Aza Day 3+ cases. Khalturin et al.'s sustained MMI incubation could potentially explain some of the differences between our results-the continued strobilation induction signal could have counteracted Aza's segment repressive effects.

In addition to experimental conditions, environmental and genetic factors could explain differences between our animals. The Khalturin animals appear significantly larger than our Southern California animals. Size has a strong impact on the number of ephyra produced, with larger animals producing more ephyrae (Helm, 2018; Kroiher et al., 2003). Temperature prior to strobilation induction could also play a role, as exposure to cold results in increased induction-related gene (e.g. CL390, RxR) expression and can trigger strobilation (Fuchs et al., 2014d; Wang, 2013). It is also likely that there are genetic differences between the animals, as Khalturin et al. worked with three strains-*Aurelia aurita* from the Baltic and White Sea, along with the "Roscoff" strain derived from the a population of *Aurelia coerulea* that invaded France. We worked with a strain of *Aurelia coerulea* derived from the Long Beach-San Pedro population in Southern California.

It is also conceivable that the conditions of the UCLA colony have selected for a lower sensitivity to MMI and greater budding potential, increasing responsiveness to Aza. Our culture has existed for over 20 years, and during that time has been almost entirely sustained by polyp asexual reproduction, with no sexual reproduction by medusae. Strobilating animals lose a large portion of their tissue to ephyrae, which, in our colony, are ultimately used for experiments and

do not breed. This would lower the fitness of strobilation prone animals in comparison to strobilation resistant polyps, which would be able to devote more tissue to budded offspring. This could select for clonal lines that more easily bud (analogous to forming a new polyp oral pole from the foot via Aza exposure) and respond less to strobilation cues.

SUPPLEMENTARY SECTION 2

Model talk - strobilation gene expression in space and time

The following section discusses elements believed to operate in strobilation and their expression in time and space.

Induction and the CL390 wave

Induction includes retinoic acid signaling and the morphogen CL390. Retinoic Acid Receptor (RxR) is upregulated during induction, and the orally expressed Retinol Dehydrogenase 2 (*Rdh2*, an enzyme involved in retinoic acid production) increases in expression, while *Rdh1* (also oral) decreases (Fig S30, Table S8) (Fuchs et al., 2014; Wang, 2013). Incubating strobila with retinol induces strobilation, and retinol treatment of strobila results in elongated Wnt/BMP expression regions in the strobila (Wang, 2013). In addition to RA signaling, induction sees an increase in CL390 expression. This expression builds around the mouth and then rapidly expands into the future segmenting region of the strobila (Fuchs et al., 2014d). The chemical inducer used in this study, 5-methoxy-2-methylindole, is thought to mimic the indole rings of tryptophans in the CL390 peptide, and found to induce strobilation across a wide variety of Scyphozoa and Cubozoa (Fuchs et al., 2014d; Helm and Dunn, 2017; Yamamori et al., 2017)). Either CL390 or another secreted signal found in the segments of strobila is sufficient to induce segmentation when tissue from the segments is fed to uninduced polyps (Fuchs et al., 2014d).

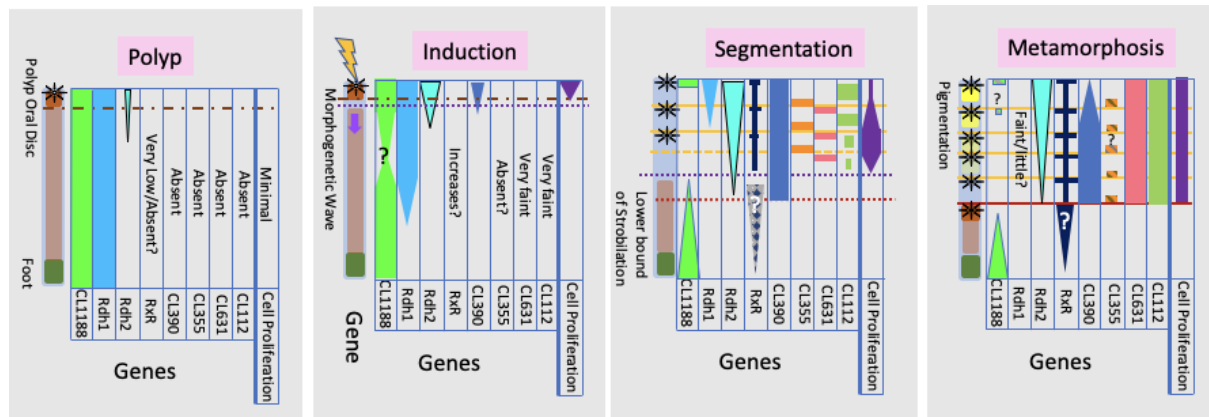


Figure 30 SUPPLEMENTARY - Representation of gene expression domains and regions of cell proliferation through strobilation

For further treatment of gene expression during medusa generation see Table S8. Width of the colored bars show strength of RNA *in situ* hybridization staining for gene expression or BrdU staining for cell proliferation. **CL1188** –appears to be expressed in polyp tissues and the manubrium of ephyrae. **Rdx1** – Fading expression throughout strobilation, seemingly associated with the oral pole of the scyphistoma. **Rdx2** – As Rdx1 expression fades, Rdx2 expands from the oral pole, potentially taking over retinoic acid signaling. **RxR** –Expression induced by cold exposure aids in strobilation induction timing. Plays role in ephyral distal-proximal patterning associated with arm buds and rhopalia through metamorphosis. **CL390** – With induction, CL390 rapidly diffuses from the oral disc to encompass the region of the body column that will generate metameres. **CL631 & CL355**– have expression patterns similar to segment polarity genes in *Drosophila* development, expressed in stripes at the oral (CL355) and aboral (CL355) ends of segments. **CL112**–expressed in the endoderm of developing segments.

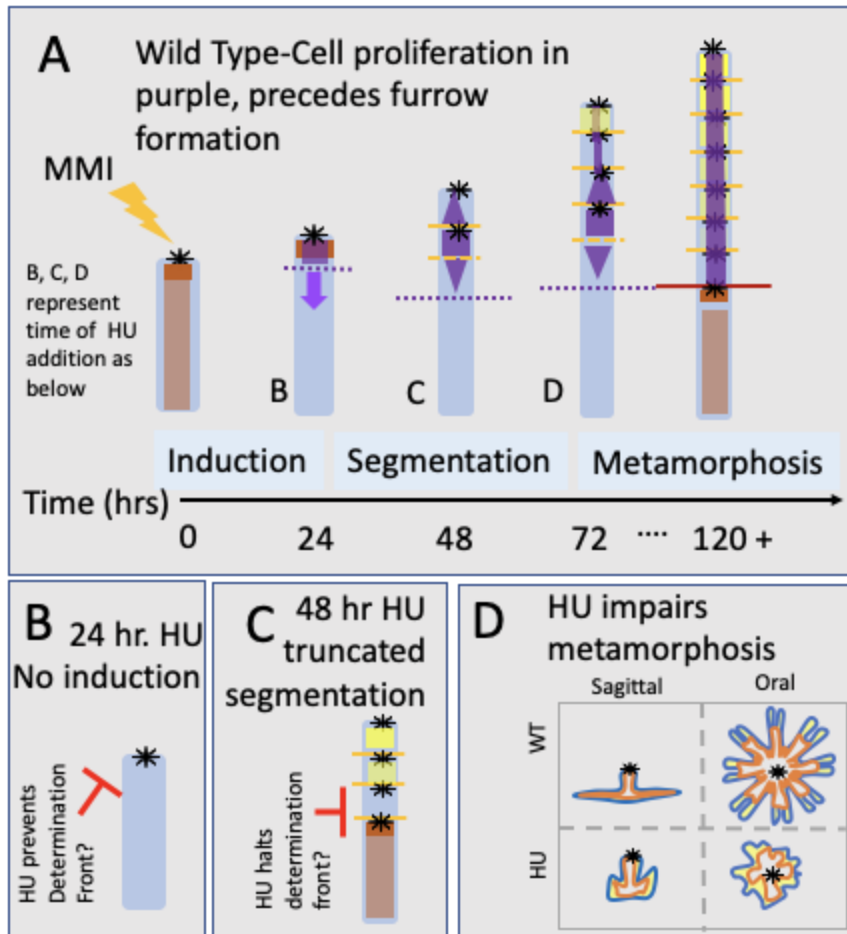


Figure 31 SUPPLEMENTARY - Cell proliferation is required for initiation/continuation of strobilation, and for metamorphosis. Summary of Fuji et al. 2024

A) Wildtype sequence, with hydroxyurea (HU) added at points B, C, and D to inhibit cell proliferation. *Wild type* cell proliferation proceeds from the oral to aboral pole. Proliferation initiates prior to segment formation, roughly corresponding to the region of determined tissue in Kroehler et al. 2000. Highest proliferation occurs in forming segments; and continues in already formed segments. **B)** 24 hr HU addition-Inhibition of cell proliferation within 0-24 hrs of induction by MMI blocks strobilation all together. **C)** 48 hr HU addition-HU application 48-72 hrs post MMI induction limits metamere formation to the Segment Addition Zone at the time of HU addition. **D)** HU application during Metamorphosis creates malformed Ephyrae with thickened mesoglea and stunted arms. Ectoderm = blue, endoderm = orange, yellow = mesoglea, gray = gastric cavity. Oral pole indicated by star. HU application to mature strobila did not affect ephyra release. Experiment in *A. coerulea* from the Seto Inland Sea, Japan.

Progressive cell proliferation

Cell proliferation proceeds along the oral-aboral axis, preceding segment formation at a distance comparable to the determined region of the Segment Addition Zone (SAZ). Blocking cell proliferation appears to halt the morphogenetic wave, halting segmentation outside the SAZ

(see Fig S31, Fujii et al., 2024). Proliferation occurs throughout metamorphosis and blocking proliferation results in malformed ephyrae with shortened distal-proximal axes. It is unclear whether the proliferation is a continuation of proliferation associated with segmentation. Sources indicated in Table S7.

The Morphogenetic Wave

An invisible field that progresses along the body column from oral to aboral pole. Kroiher et al. showed that tissue up to ~0.5 mm ahead of the most recently formed segment furrow is fated to form segments (Fig S32). This wave appears to be partially reliant on cellular proliferation. Fuji et al pharmacologically inhibited proliferation with Hydroxyurea (HU) (Fig S32). When applied up to 24 hours after strobilation induction, strobilation was completely blocked, while adding HU between 48 to 72 hours appeared to halt further segmentation outside of the current SAZ. This results in 1-3 segments being added to the strobila as the segments in the SAZ finish maturation. Cell proliferation is greatly increased in the SAZ and already formed segments relative to unsegmented regions of the segmentation zone.

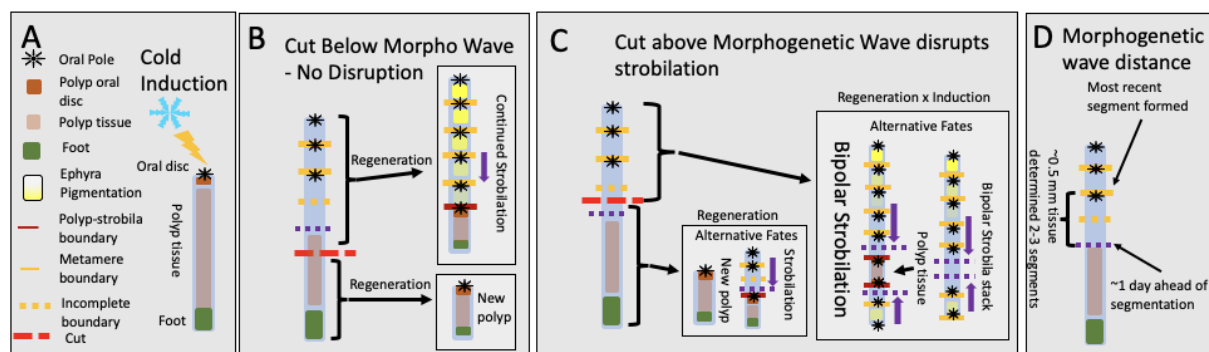


Figure 32 SUPPLEMENTARY - Cutting experiments determine distance of tissue subject to segment fate determination prior to segmentation. Summary of Kroiher et al. 2000

Cutting experiments show evidence for morphogenetic wave in strobilation. Fate determination occurs roughly ~1 day before segments form. **A)** Kroiher induced strobilation by incubating at low temperature, and sectioned strobilating animals at different points along the body column relative to the most recent segment formed. Wild type strobilation as Fig 14a. **B)** Strobila cut > 0.5 mm tended to regenerate polyp tissue at the aboral end of strobila stack, and the aboral

fragment would regenerate as a non-strobilating polyp. **C)** Strobila cut < 0.5 mm tended to strobilate from the aboral pole in an aboral-oral direction and the original strobila stack will continue strobilating in an oral-aboral direction, yielding bipolar strobila. Some cases had polyp tissue between the strobila stacks, while others had strobila stacks directly connected to each other. The aboral end of these cut strobila could either regenerate a polyp oral pole, or begin strobilating in the original oral-aboral direction. **D)** Based on these cutting results, tissue up to 0.5 mm from the most recent segment formed is determined to a strobila fate. This determination front proceeds the progress of visible segments by roughly 1 day, corresponding to 2-3 new segments/ephyrae (~0.18 mm/ephyra). Experiment in *Aurelia* sp. from the North Sea.

In our experiment, when Aza was added throughout segmentation, the segments with elongated aboral poles tended not to be the segment being formed at the time of addition, and segments that would have been forming in the SAZ at the time of addition show less severe changes relative to the last segment formed on the stack. This suggests that GSK-3 β activity/cWnt signaling most affects tissue in the segmenting region that has not yet been reached by the morphogenetic wave.

Foot based inhibition field

The insensitivity of advanced strobila feet to Aza incubation in this study suggests that cWnt is inhibited in the foot post induction. The graded response of the feet of Aza Day 0 animals (complete conversion) compared to Aza Day 1 animals (partial conversion) and Aza Day 3 (no conversion) suggests that this inhibition builds over the first several days following induction.

A short range cWnt antagonistic signal from the foot could also delay the formation of the final segment of the strobila stack due to spill over from the nonsegmenting region. The expansion of the oral based CL390 signal would be halted by the short range antagonistic foot signal. This would account for Kroihner et al.'s observation of the relative rate of formation of segments along the body column (Fig 33b), and Fuchs et al.'s observation that feeding segment tissue to polyps induces strobilation, while feeding foot tissue does not induce strobilation (Fig

33a). Future studies could examine if simultaneously feeding strobila foot tissue to polyps during the induction process represses strobilation.

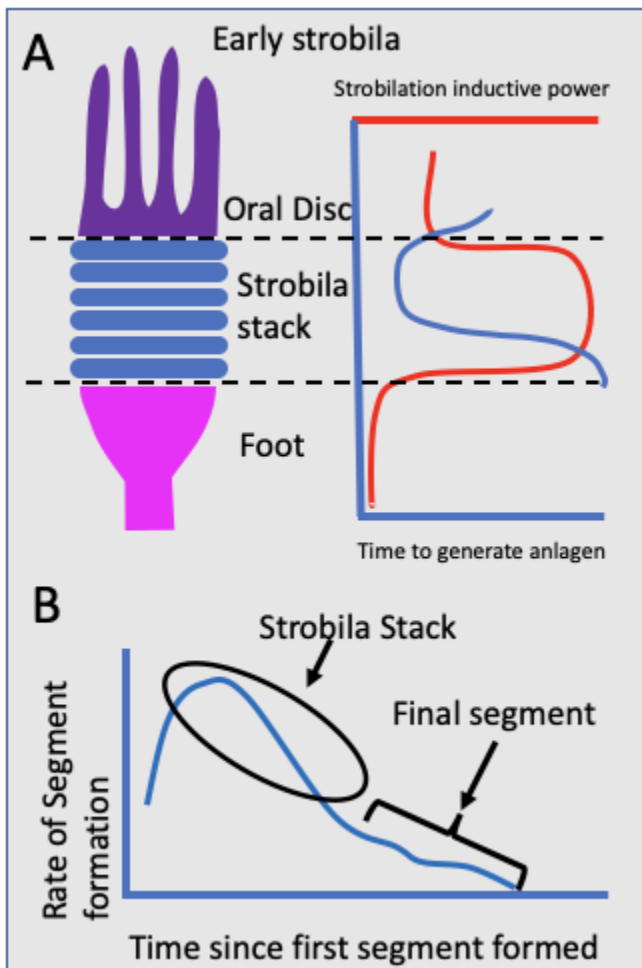


Figure 33 SUPPLEMENTARY - Differential strobilation potential along the strobila body column as determined by tissue feeding/grafting and segment formation rate

A) Fuchs et al. 2014 measured strobilation inductive potential by feeding and grafting tissue from different regions of early strobila to nonstrobilating polyps and measuring the proportion which strobilated. Results indicate a high strobilation inductive power in strobila segment tissue, a medial inductive power in tissue from the uppermost ephyrae, and very little inductive power in foot tissue. Inductive power is likely the product of a diffusive inductive signal emanating from the oral pole and an inhibitory signal emanating from the aboral pole. Red line indicates inductive potential while blue line shows speed of segment formation based on Kroehler et al 2003. **B)** A generalization of Kroehler et al.'s rate of segment formation. The rate of segment formation appears to follow the inferred inducer concentration from Fuchs et al. The second through penultimate segments form the most rapidly, while the segment at the aboral end of the stack takes the most time to form, and the segment at the oral pole taking an intermediate amount of time to form.

Table 8 SUPPLEMENTARY - Table of gene expression and cell proliferation during polyp-medusa transition

Stage specific columns (in order)-Localization, Tissue Layer, Evidence of Expression Pattern, Category of Gene/Function. General columns-Strains of *Aurelia coerulea* used, Sources/References. *Localization*-Ab-Aboral expression within structure, *Ar*-Arm, *ArPt*-ArmPit, *Bd*-Bud, *EaS*-Expressed at Stage, no localization available, *GsCn*-Gastric Canal, *GsFI*-Gastric Filament, *Hyp*-Hypostome, *Lp*-Lappet, *Mar*-circumferential margin, *Mn*-Manubrium, *Or*-Oral expression within structure, *OrD*-Oral Disc, *Ro*-Rhopalia, *SAZ*-Segment Addition Zone, *Sg*-Segments, *(n)SgRn*-region of strobila that will (not) segment, *Tt*-Tentacle, *TtBd-P*-primary tentacle buds, perradial/interradial, the first 8 tentacles that form in polyp morphogenesis. *Unv*-Universal in tissue mentioned, with heightened expression in other structures mentioned. Asterisk indicates conflict between ISH and other expression data. Cell proliferation is based on BrdU or comparable stain, not ISH. *Tissue*-Ec-Ectoderm, En-Endoderm, *-minor expression, *Quality of Evidence*- A-peer reviewed high quality ISH, B-nonpeer reviewed high quality ISH, peer reviewed low quality ISH, C-low quality nonpeer reviewed ISH, or general expression data such as RNAseq, qPCR etc, I-inferred based on behavior of gene in sequential stages but no direct evidence, S-speculation based on behavior of gene in related taxa or evolutionarily conserved role. *Category*- ARG-Anterior-Posterior Gradient, Bas-Basal replacement rate, Cold-expression induced by cold temperature, DI-Distal-Proximal axis patterning, Gap-denote large region of developing org (analogy to *Dros* gap genes), Ind-Strobilation Induction, MtM-strong striated expression pattern along body column corresponding to metamerites, POrAb-polyp Oral-Aboral polarity (analogy to *Dros* maternal genes e.g. *bicoid*, *nanos*), SeNe-suspected in sensory-neural development, SgPI-oral-aboral polarity of individual segments/ephyrae (analogy to *Dros* segment polarity genes e.g. *wingless/Wnt*). *Strain* – Ros-Roscoff, Fr; SP-San Pedro, USA; SIS-Seto Inland Sea, Jp; ? – likely Roscoff but uncertain. *Sources*-C1-chapter 1, F-Fuchs et al. 2014, Fj-Fuji et al. 2024, G5-Gold et al. 2015, G9- Gold et al. 2019, Kh-Khalturin et al. 2019, Kr-Kraus et al. 2015, N0-Nakanishi et al. 2010, N5-Nakanishi et al. 2015, W-Wang 2017, Z-Zorina 2018

Notes on certain gene expression patterns- RxR is described in the text of F and W to not be expressed in polyps, however cDNA gel electrophoresis and qPCR in Fuchs' Fig 3 and 5 show expression in the polyp stage. Presumably this expression can be ascribed to build up of RxR in response to cold exposure. Fuchs' Fig S3 shows some RxR staining in the nonsegmenting region but does not discuss it, potentially due to nonspecific staining.

	POLYP				INDUCTION				SEGMENTATION				METAMORPHOSIS				EPHYRA				SOURCE	
Gene	Loc	Tissue	Evid	Cat	Loc	Tissue	Evid	Cat	Loc	Tissue	Evid	Cat	Loc	Tissue	Evid	Cat	Loc	Tissue	Evid	Cat	Strain	Source
Rdh1	Unv, Hyp	Ec,En	A	POrAb	Unv	Ec, En	C	POrAb	NSR	Ec, En	A	POrAb	NSRn	Ec, En	C	POrAb, gap	non*	na	A	na	Ros	W,F
Rdh2	Hyp	En	A	POrAb	Hyp	En	C	POrAb	OrD, Sg	En	A	APG, gap	SgRn	En	C	POrAb, gap	Mn	En	A	SgPl	Ros	W,F
CL1188	Unv	Ec	B	?	EaS	?	C	?	Hyp, NSRn	Ec	B	gap	NSRn	Ec	B	Gap	Mn	Ec	B	SgPl	Ros	W
CL390	OrDisc	Ec	C	Cold	OrD	Ec	A	Ind, gap	OrD, SgRn	Ec	A	gap	SgRn	Ec	A	Gap	non	na	A	na	Ros	W,F
CL355	non	na	A	na	EaS	?	C	?	OrD, SgAb, SAZ	Ec	B	SgPl, MtM	SgRn, Mn	Ec	C	SgPl	Unv, Mn	Ec	B	SgPl	Ros	W
CL631	non	na	A	na	EaS	?	C	?	SgOr, SAZ	Ec	A	SgPl, MtM	SgRn	Ec	B	SgPl	Unv	Ec	A	?	Ros	W,F
CL112	non	na	A	na	EaS	?	C	?	OrD, Sg, SAZ	En	A	MtM	SgRn	En	B	SgPl	Mn	En	A	SgPl	Ros	W,F
Wnt11a	EaS	?	C	?	EaS	?	C	?	OrD, SgMarOr	Ec	A	DI, MtM	LpBdOr	Ec	A	DI, SgPl	Lp	Ec	I	DI	Ros, SP	W,Kh,C1/G9
BMP5/8	TtBd-P	En	A	DI	TtBd-P	En	C	DI	ArBdOr, TtBd-P	En	C	DI	LpBdOr	En	A	DI, SgPl	GsCn, ArPt	En	A	DI	?, Ros	Kr,Kh,W
gata	?	na	na	na	?	na	na	na	SgAb	Ec	A	SgPl, MtM	SgAb	Ec	I	SgPl	Unv, Mn	Ec, En	A	SeNe	?	Kr
otx	?	na	na	na	?	na	na	na	SgOr	Ec	A	SgPl, MtM	SgOr, Ro	Ec	I	SeNe, SgPl	Ro, SgOr	Ec	A	SeNe	?, SP	Kr,N0
Dkk-like	non	na	C	na	EaS	?	C	?	EaS	?	C	?	EaS	?	C	?	GsFl	En	B	DI?	Ros	W
Wnt4	EaS	?	C	?	EaS	?	C	?	EaS	?	C	?	LpBd	Ec	C	DI	Lp	Ec	B	DI	Ros, SP	Z,C1/G9
RxR	non*	?	A	Cold	EaS	?	C	Therm, gap	Sg, ArBd	Ec, En	A	DI	Unv, Ro	Ec, En	A	DI, SeNe	Ro,Lp,Mn	?	A	SeNe	Ros	W,F
eya	EaS	?	C	?	Eas	?	I	?	Eas	?	C	?	Ro, Man	Ec, En*	A	SeNe	Ro, Mn,	Ec,En	A	SeNe	SP	N5
so	EaS	?	C	?	Eas	?	I	?	Eas	?	C	?	Ro,	Ec	A	SeNe	Ro	Ect	A	SeNe	SP	N5
Optix	EaS	?	C	?	Eas	?	I	?	Eas	?	C	?	Ro, Unv	En	A	?	Unv	En, Ec	A	?	SP	N5
PaxA	EaS	?	C	?	Eas	?	I	?	Eas	?	C	?	ArAb	Ec	A	SeNe	ArAb	Ec	A	SeNe	SP	N5
PaxB	EaS	?	C	?	Eas	?	I	?	Eas	?	C	?	non	na	A	na	Ro	Ec	A	SeNe	SP	N5

	POLYP				INDUCTION				SEGMENTATION				METAMORPHOSIS				EPHYRA				SOURCE	
Gene	Loc	Tissue	Evid	Cat	Loc	Tissue	Evid	Cat	Loc	Tissue	Evid	Cat	Loc	Tissue	Evid	Cat	Loc	Tissue	Evid	Cat	Strain	Source
Rdh1	Unv, Hyp	Ec, En	A	POrAb	Unv	Ec, En	C	POrAb	NSR	Ec, En	A	POrAb	NSRn	Ec, En	C	POrAb, gap	non*	na	A	na	Ros	W, F
Rdh2	Hyp	En	A	POrAb	Hyp	En	C	POrAb	OrD, Sg	En	A	APG, gap	SgRn	En	C	POrAb, gap	Mn	En	A	SgPI	Ros	W, F
CL1188	Unv	Ec	B	?	EaS	?	C	?	Hyp, NSRn	Ec	B	gap	NSRn	Ec	B	Gap	Mn	Ec	B	SgPI	Ros	W
brn3	?	na	na	na	?	na	na	na	?	na	na	na	Ro, SgAb	Ec	A	SeNe	Ro, SgAb	Ec	A	SeNe	SP	N0
pit1	?	na	na	na	?	na	na	na	?	na	na	na	Ro	Ec, En	A	SeNe	Ro	Ec, En	A	SeNe	SP	N0
twist	Tt	En	S	?	?	na	na	na	?	na	na	na	?	na	na	na	GsCn, ArPt	En	A	?	?	Kr
tbx4/5	Tt	En	S	?	?	na	na	na	?	na	na	na	?	na	na	na	GsCn, ArPt	En	A	?	?	Kr
brac	Hyp	En	S	?	?	na	na	na	?	na	na	na	?	na	na	na	Mn	Ec	A	?	?	Kr
foxA	Hyp	En	S	?	?	na	na	na	?	na	na	na	?	na	na	na	Mn	Ec, En	A	?	?	Kr
otp	Hyp	Ec	S	?	?	na	na	na	?	na	na	na	?	na	na	na	Mn, Ar	Ec	A	SeNe	?	Kr
ETS	Tb	Ec	B	DI	Tb	Ec	I	DI	SgOr	Ec	B	DI	ArPt	Ec	B	DI	ArPt	Ec	B	DI	?	W
Ptc	non	na	B	na	EaS	?	C	?	SgMar, SAZ	Ec	B	M	SgRn	Ec	B	?	Ro	Ec	B	SeNe	?	W
cell prolifer	Tt, Unv	Ec, En	A	Bas, Gro	OrD	Ec, En	A	Ind	Sg, SAZ	Ec, En	A	Ind, Gro	Unv, Ro	Ec, En	A	Gro, SeNe	Ro	Ec	A	SeNe	SP, SIS	N5, G5, Fj

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Chapter 3 - Segmentation and Pigmentation during Metamorphosis propagate at different rates
along the body column of *Aurelia coerulea* (Cnidaria, Scyphozoa) strobila

ABSTRACT

Developmental processes are dynamic in both space and time, using time variable signals to encode information in cells, ensuring their proper identity and maturation. Strobilation, the process by which scyphozoan polyps transition from a sessile polyp to planktonic medusae is seemingly governed by the progression of signal along the 1-D axis of the body column. This is seen in both *segmentation* by the addition of transverse metamerous progressively farther from the mouth of the animal, and *metamorphosis* where segments at the top of the strobila stack develop ephyra features before lower segments. Despite operating in the same direction and in response to a common cue, the signaling connection between these processes is not clear. This study compares the movement of these processes along the body column by measuring the distance segments form from the mouth and quantifying the color change that accompanies metamorphosis in ephyra tissue. It finds that the rate at which the two processes propagate down the body column significantly differ under normal conditions, and these processes are affected independently by GSK-3 β inhibition via 1-Azakenpaullone (Aza) incubation. While Aza suppresses the number of segments formed, it does not appear to alter the rate at which the underlying signal moves through the body. In contrast, Aza slows the progression of pigmentation down the body to roughly that of segmentation, where normally it moves some 2-3 times faster than segmentation. These results indicate the method by which the segmentation and metamorphic signal are conveyed along the body differ, and that the two processes may be controlled by differing signals, or at the very least, that there are additional factors modulating that signal in one of the processes. These results suggest developmental mechanistic pathways

with the potential to evolve and generate the diversity of medusa bell shapes and biomechanical function found across Scyphozoans, Cubozoans and Staurozoans.

INTRODUCTION

The spatial and temporal aspects of development are key to understanding how organisms replicate patterns of cells, tissues and complex structures. Spatial and temporal developmental patterns encode positional information, regulating cell differentiation and cycling. As development is a dynamic process, it is important to consider how developmental signals propagate through tissue to create physical patterns. We examine developmental processes taking place along an axis where monitoring the physical location of each process helps clarify the relationship between processes. Biological activity progressing along a body axis through time can form morphogenetic waves or developmental morphogen fronts that progress along the body column, (see an example in Fig 34) (Di Talia and Vergassola, 2022; Hayden et al., 2022). By measuring the location of thresholds of biological activity, we visually track propagation of underlying developmental signals along the organism.

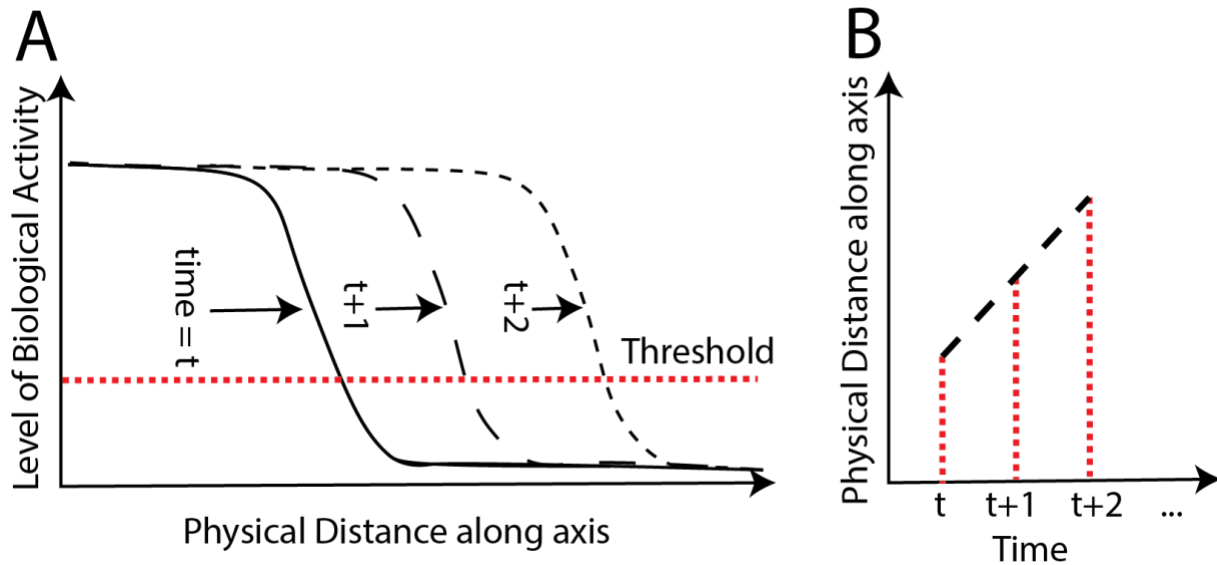


Figure 34 - Simplified propagation of a traveling front during development

Curves of some quantifiable biological activity along a body axis at different timepoints shown in **A**), including a threshold level of activity shown in red. The position at which the level of biological activity meets or exceeds this threshold is charted in **B**), with the rate of progression along the body column shown by the slope of the dashed black line.

Spatiotemporal aspects of strobilation

Strobilation appears to consist of two visually apparent processes that spread progressively from the polyp oral pole towards the aboral pole. In *segmentation*, furrows transversely divide the body column into defined regions (segments) (Spangenberg, 1965). These defined regions mature during *metamorphosis*, where polyp tissue transitions into ephyra tissue and forms the structures necessary for a nektonic lifestyle such as sensory structures for orientation and rhopalia/lappets for propulsion. In *Aurelia coerulea* these life stages strongly differ in coloration, starting as off-white in the polyp and ending as a strong orange-brown in ephyrae (see Fig 35). This color shift appears to correspond to the maturity of segments, as older segments near the mouth are more intensely orange and have more mature sensory and locomotor structures than the younger segments at the foot.

We quantitatively track this color shift to estimate the spatiotemporal dynamics of metamorphosis, which can then be directly compared to the progression of segmentation along the body axis. The relationship between these processes can be investigated by interfering with the propagation of these processes down the body column, such as by the suppression of segment formation by 1-Azakenpaullone (chapter 2, Khalturin et al., 2019). If segmentation and metamorphosis are the response to a single morphogenetic signal front traveling away from the mouth, their propagation along the body column should change in parallel with each other. However, if the propagation rate of these processes respond differently to chemical interference, it would suggest that the methods by which the “segmentation front” and “metamorphosis front” move along the body column differ. This would support the hypothesis that segmentation and metamorphosis are both triggered by strobilation induction, but have a degree of independence from one another.

Genetics of strobilation

Some of the genetic pathways controlling strobilation induction and segmentation in *Aurelia* are known. In short, induction appears to occur in response to retinoic acid signaling and the build-up of the presumptive signaling gene product CL390 near the oral pole (Fuchs et al., 2014; Wang, 2013). Once a threshold is reached, CL390 expression rapidly spreads along the body column, defining the region of the animal which will form the strobila stack. The upper and lower boundaries of segments appear to be defined by CL631 and CL355 expression, which advances down the body column at a slower rate than CL390 (Fuchs et al., 2014; Wang, 2013)). We consider the formation of a furrow between segments a response to a threshold level of signal or biological activity being reached, such that similar levels of activity are present at the formation of each segment. Metamorphosis follows segmentation, where new neural and sensory structures form following expression of transcription factors such as Brn3 and *sine*

occulis (Nakanishi et al., 2010, 2015). For a more thorough discussion of strobilation genetics, see Chapter 2 Supplementary information.

Pigmentation in strobilation

The coloration of ephyra appears to be the result of melanin production. The pigmentation of well fed polyps is the result of canthaxin, a keto-caretenoid found in the arthropod food of polyps, while the brownish color of ephyra was determined to be melanin via liquid chromatography (Branden et al., 1980). It has been argued that the melanin production directly controls ephyra morphogenesis by depleting tyrosine concentration in a segment. As melanin has a lower affinity for iodine than tyrosine, iodine that would normally react with tyrosine instead iodinate downstream molecules and produces a change in gene expression (Berking et al., 2002). However, this remains speculative as the target of iodination has not been found (Helm, 2018). Experimental manipulation of retinoic acid signaling and the bacterial community of *A. aurita* has managed to yield unpigmented strobila segments, however the segments and resulting ephyrae suffer from malformations (Jensen et al., 2025) Whether or not the transition to ephyra tissue is directly governed by pigmentation, the timing of metamorphosis and the appearance of orange-brown coloration are closely associated with one another in *Aurelia coerulea* (see chapter 2, Fig 16 and 17 for comparison of timing of pigmentation and formation of ephyra structures), allowing us to use pigmentation as a proxy for the progression of metamorphosis.

The RGB color space

In standard 8-bit encoding of the Red Green Blue (RGB) color space, as a pixel becomes more orange, the values for each channel change in a predictable manner, enabling us to track the spatio-temporal spread of the orange-brown coloration in ephyrae. For each pixel

there is a separate intensity value for the red, green and blue channels from 0-255, with 0 indicating an absence of the color. The combination of these primary color channels creates our perception of color including secondary and tertiary colors such as orange. In the RGB color space, orange (and its shade, brown) result from a high R, a mid G and a low B. Colors along the spectrum from white to black are created when the intensities of $R = G = B$, with pure white lying at (255, 255, 255). Accordingly, for whitish polyp tissue to become orange, we would expect the ratio R/B to start around 1 and increase as the orange coloration grows stronger.

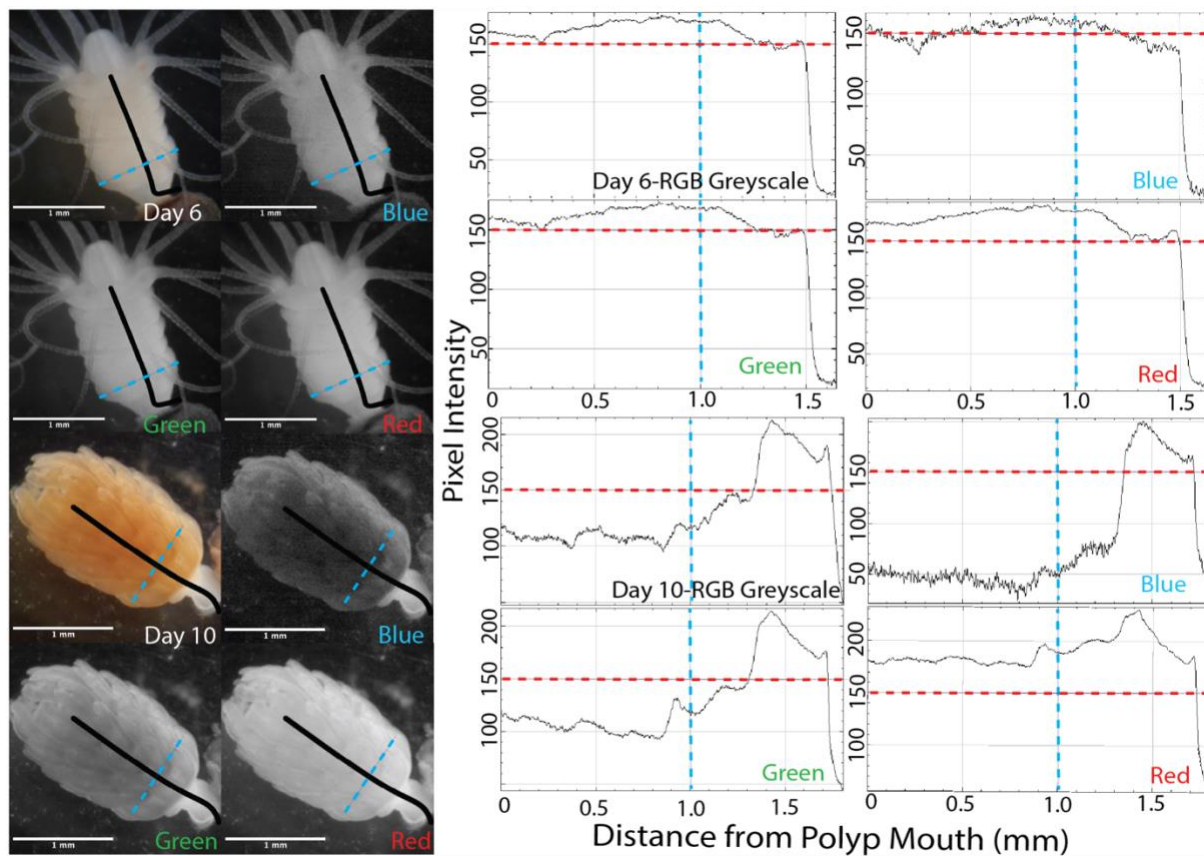


Figure 35 - Intensities of Red, Green and Blue channels in strobilation

A control individual observed at day 6 and day 10 after strobilation induction. The black line (40 pixels wide) on photos of the individual shows the transect measured with greyscale images corresponding to the Red, Green and Blue channels. A scale bar shows 1 mm, and a blue dashed line corresponds to 1 mm away from the original oral disc ("Distance from Polyp Mouth"). Graphs show average intensity of the 40 pixel moving sample along the transect from the mouth (0 mm) to the foot (~1.5 mm). The graphs show average 8-bit intensities from 0 to

255 on the y-axis. The blue dashed lines indicate the 1 mm mark, while the red dashed line marks an intensity of 150. On day 6, there is a mostly uniform off-white coloration along the body column and the R/B ratio is low ($R/B \sim 1$). By day 10, the orange pigmentation strongly increases the red to blue ratio, ($R/B \sim 3$), while the foot tissue maintains its polyp identity and whitish coloration ($R/B \sim 1$, with all three channels having high intensity). Sharp decline across all channels beyond the foot ($\sim 1.7\text{mm}$) is a result of measuring black background for illustrative purposes. Further figures illustrate the temporal dynamics of these color changes associated with the metamorphic wave across control and experimental animals.

The shift in channel intensities accompanying metamorphosis allows us to measure the spatial progression of pigmentation along the oral-aboral axis (see Fig 35), providing a proxy that quantifies the transition from polyp to ephyra tissue. As both segmentation and metamorphosis begin at the oral end of the primary body axis and progress towards the foot, we can track the progress of the two processes through time by recording the distance from the mouth where segments form and the most distant position from the mouth where = tissue has reached a particular level of pigmentation. This allows calculation and comparison of the “velocities” with which these processes travel down the body column.

Our comparison of the propagation of segmentation and pigmentation yields several findings. Under control conditions, segmentation and pigmentation move away from the mouth along the body column at different speeds (mm/day), with pigmentation having a significantly steeper slope (higher rate of progression) than segmentation. Aza incubation shortly after strobilation induction does not significantly affect the rate at which the “segmentation front” moves along the animal, but does reduce the rate of the “pigmentation front” to a rate not statistically significant from that of the segmentation front. This supports the hypothesis that the signaling pathways controlling pigmentation (and by extension metamorphosis) and those controlling segmentation are not the same or are subject to differing additional factors, despite their common induction pathway.

This is significant because changes in the progression of segmentation or metamorphosis result in large differences in the number and shape of ephyra produced in strobilation. Bell shape directly impacts the hydrodynamics of medusae, which in turn affects a medusa's suitability for a filter feeder or fast moving predator lifestyle (Costello et al., 2008). This may relate to the evolution of the diverse bell-shapes found among true jellyfish (Scyphozoa) and their closest Medusozoan relatives (the box jellies of Cubozoa and stalked jellies of Staurozoa) in the clade Acraspeda (Kayal et al., 2018).

METHODS

Data gathering

Data used was gathered from Chapter 2's photo dataset. Linear measurements were taken from individual strobila whose body axis lay roughly parallel to the floor of the well with photos at 4912 x 3684 resolution at a standard magnification, against a black background.

1-Azakenpaullone (Aza) is a strong selective inhibitor of GSK-3 β (Kunick et al., 2004) which has been demonstrated to hyperactivate canonical Wnt signaling in a variety of model organisms (Meyers et al., 2012) including cnidarians such as the anemone *Nematostella vectensis* (Röttinger et al., 2012) and the hydrozoan *Hydractinia echinata* (Plickert et al., 2006). Aza has been demonstrated to disrupt segment formation in *Aurelia* in a time dependent manner (Khalturin et al., 2019), but its impact on metamorphosis has not previously been studied in detail.

Animals were induced to strobilate using 50 μ M 5-methoxy-2-methylindole and strobilation was altered by replacing the artificial seawater of experimental animals with 2 μ M Aza at various time points. There were six conditions-a control which lacked Aza, a condition where Aza was added simultaneously with the methylindole (referred to as Aza Day 0), and

groups where Aza was added on day 1,3,4, & 10 (referred to as Aza Day 1 etc). Time is generally measured in days post induction, with induction occurring on day 0.

Linear measurements

Distance and color intensity measurements were conducted in *ImageJ2* using *Fiji* (Schindelin et al., 2012; Schroeder et al., 2021). Distance of segments from the mouth was measured with a spline starting at the upper surface of the oral disc and running along the midline of the animal. Segment diameter was measured as a line running transversely across the body column, from the base of one nascent arm to another. Segment length was defined as the length of a line between the oral (subumbrellar) surface of the armpit to the peak of the segment's aboral surface (umbrella) when drawn parallel to the oral-aboral axis. Distance of a segment from the original oral pole was defined as the distance from the upper surface of the polyp oral disc to the peak of the segment's umbrellar surface (see Fig S46 for a visual breakdown). All measures of dimension were taken in pixels and converted into mm.

A. coerulea body length can be difficult to measure in living, unrelaxed strobila, as they contract their body column when disturbed and their ephyra segments may begin to move. To account for this, measurements were only taken on animals that displayed an elongated body, lying parallel to the floor of the well. Measurements ceased once the arms of the ephyrae began to lie parallel to their oral disc, as the ephyrae began actively “swimming” while attached to the strobila stack, making body length difficult to judge.

The foot of the animal presents an additional difficulty to measures of body length. *A. coerulea* polyps locomote across surfaces by extending long tendrils of tissue which adhere to surfaces known as stolons. Once the stolon attaches the polyp transfers the bulk of its foot tissues to the stolon while gradually moving away from the old foot, which shrinks and

eventually breaks free (Gilchrist, 1937). Body length was measured along the body column from the surface of the oral disc to the nearest point of adhesion at the foot.

Statistics for linear measurements

Means of the length of the body column of the strobila, oral-aboral axis of segments and segment diameter of segments per condition were compared to the control via Dunnett test. The first segment of each strobila was compared to the first segment of the control group, and so on (denoted as segment number 1, number 2, etc. when counting from the original oral pole).

Dunnett tests (Dunnett, 1955, 1964) were conducted using the R package *DescTools* (Signorell et al., 2025) for the initial and final body length, as well as the average dimensions of segments during the segmentation process.

Image analysis of pigmentation

RGB channel intensity was measured along a transect following the midline, beginning at the mouth of the animal and ending at the terminus of the foot where it contacts the plate. In instances where there were multiple points of contact between the foot and the polyp (e.g. if a polyp had stolons in addition to its foot), the point of adhesion closest to the mouth was considered the terminus. Individual transects shorter than 1 mm were discarded, as were all day-condition combinations with fewer than 8 samples. This resulted in a dataset of a total of 1438 transects (individuals at a particular time point) and sufficient observations for 93 combinations of condition and time out of a possible 102 for 17 days post induction and 6 conditions. This consisted of average intensity readings at 1906183 locations for R and B. Transects averaged 1326 readings and 1.76 mm in length.

RGB intensity readings are affected by factors beyond the build up of pigment which were accounted for in the following manners. Shadows negatively affect a pixel's intensity in all

channels while thicker tissue is more opaque than thin tissue (see Fig S45). In this study, images were taken against a black background, so thinner tissues tended to have lower intensity values. Using a ratio of R/B helped account for factors that affected the intensity of all channels.

When gathering intensity data along the transect we did our best to avoid shadows and tissue folds which transversed the midline of the body.

The intensity of the R, G and B channels along these transects were calculated by averaging each channel across the 40 pixel width of the line. Intensity readings were scaled such that the maximum average intensity of each channel for the control condition was 100 (corresponding to the remnant polyp tissue at the foot). For days 14-16, the maximum was set at the average of the maximum values of day 10-13 were used, as the number of strobila for which all ephyrae remained attached to their parent strobila dropped below the threshold by day 14. These scaled values were then used as data for analyses and to calculate the progression of color down the animal. We divided the average red intensity by the blue intensity at each point along the transect. To control for changes in length between measurements, we assigned readings into 101 proportionate groups corresponding to 0-100% of the total length of the transect. The percentile length, time and R/B value were charted in a 3D space and visualized as surfaces in R using the packages *rgl* and *plotly* (see Fig 36, 41 and 47) (Murdoch et al., 2026; Sievert, 2019).

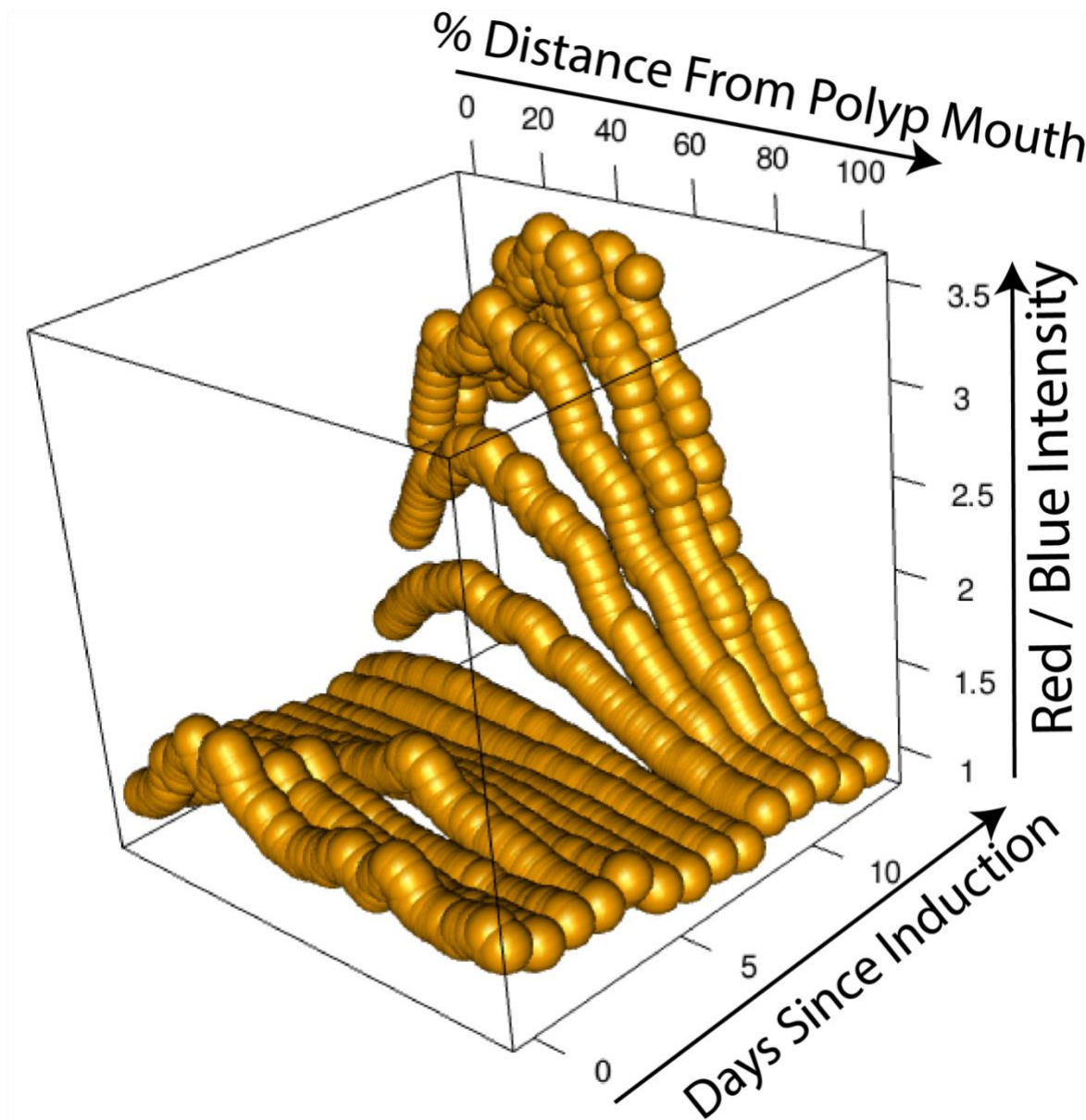


Figure 36 - Changes in ratio of Red and Blue intensities along strobila body column throughout wildtype strobilation

Spheres represent the average ratio of Red/Blue for 1% of the animal's body length at a given time, averaged across individuals ($N = 19$). 0% corresponds to the oral disc of the polyp and 100% corresponds to the foot. The tissue of segments undergoing pigmentation sees a strong increase in Red relative to Blue, while the tissue at the foot of the animal, which retains its polyp identity and coloration, remains at roughly $R/B = 1$.

Modeling pigment propagation

The distance pigmentation propagated from the mouth in monopolar animals was calculated as the farthest distance along the raw length of the transect that reached an R/B threshold. To limit the impact of noise, a cubic spline from a Generalized Additive Model was fitted to each transect using the *ggplot2* command *geom_smooth(method = "gam")* (Wickham, 2016), when the transect was plotted as (x, R/B) where x = distance from the mouth and R/B = scaled red/scaled blue intensity ratio at that point. The maximum value of the first day where the spline met or exceeded 1.5 was used as a threshold value to identify a pigmented state (see Fig 37). The distance traveled by pigmentation was defined as the point furthest from the mouth where the spline met or exceeded this threshold. This dynamic cutoff was used to prevent local R/B maxima displacing the x(t) position of pigmentation too far forward of the main “wave” of color at time of first appearance. In this experiment, an R/B value of 1.5 corresponds to the tan-brown typical of the early onset of metamorphosis (similar to hexcode #bd9c63 or unscaled RGB of roughly (189, 159, 99)).

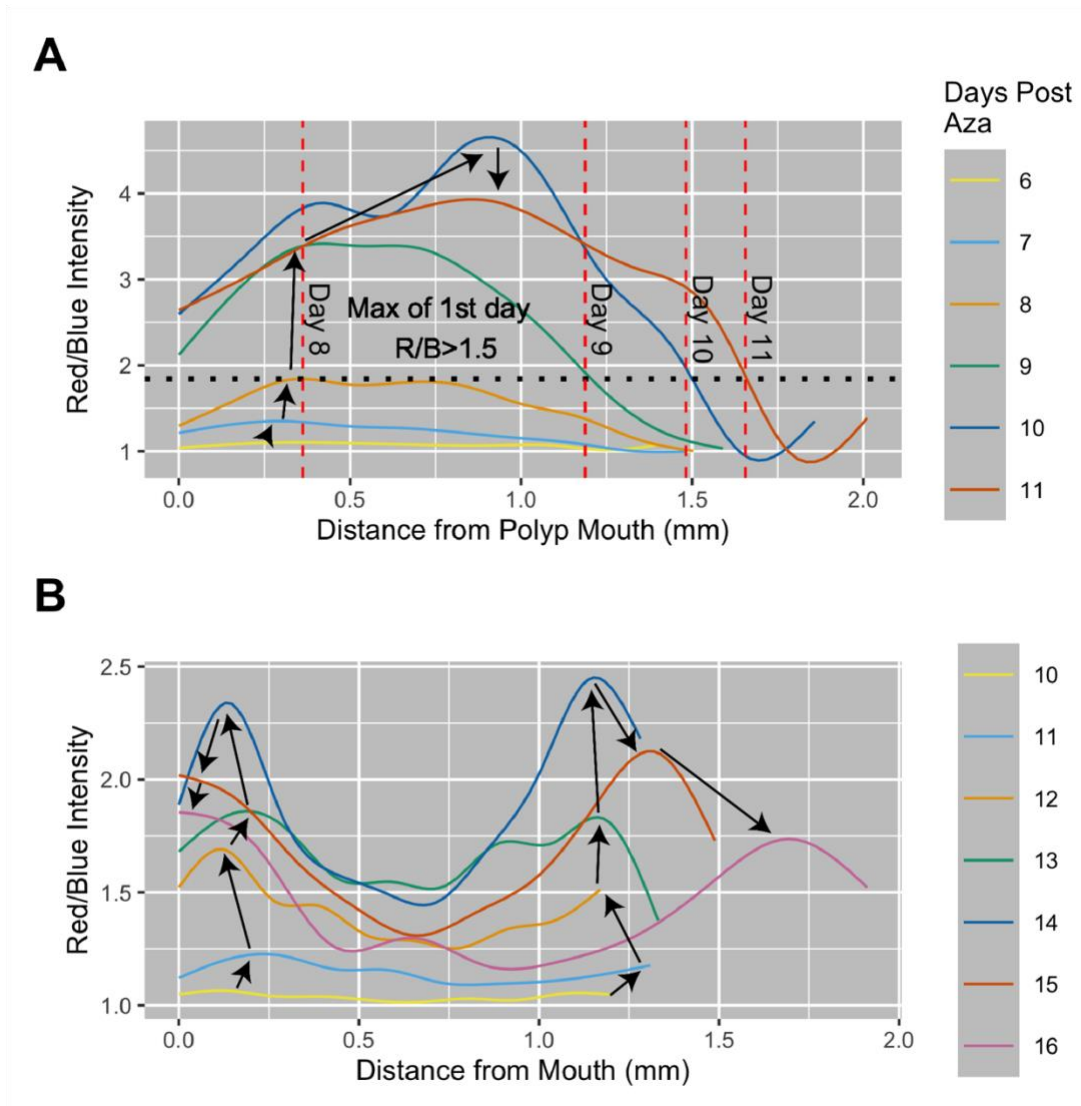


Figure 37 - Tracking propagation of pigment wave in monopolar and bipolar individuals

Lines show the cubic shrinkage spline of a generalized additive model fitted to the daily R/B profile of an individual. Daily maxima R/B values are indicated by black arrows **A)** - A control strobila displays typical monopolar pigment expansion. Day 8 was the first day where $R/B > 1.5$, so the day 8 maximum was used as the threshold to call the progress of pigmentation down the body column for this individual. Tissue reached this threshold value in a nonlinear fashion with respect to the distance from the original oral pole, with the distance traveled by the wavefront between day 8 & 9 exceeding that of the distance traveled on days 9-11. Similarly, the location of the maximum R/B ratio changes in a nonlinear fashion, with minimal change occurring in days 6-9, and a large jump occurring between day 9 and 10. **B)** - Example bipolar pigmentation profile showing two R/B maxima corresponding with the original oral pole and the secondary oral pole formed from the foot. Note that in both A and B the maximum R/B value declines in the last few days (A- day 10 to 11, B-day 14-16), demonstrating that R/B values do not increase monotonically during pigmentation.

With these distances, we used the packages *lme4* (Bates et al., 2015) and *lmerTest* (Kuznetsova et al., 2017) to construct and analyze a Linear Mixed Model (LMM) for the rate of propagation (mm/day) of pigmentation and segmentation along the body column of strobila with a single oral pole (monopolar). We treat the body axis as a 1-D axis and the process of segmentation and metamorphosis as the visible processes of invisible morphogenetic changes which progress along the body column from the mouth ($x = 0$) toward the foot. Our LMM consisted of the formula (*Distance from Mouth*) $\sim$ *Total Body Length* + $P * \text{experimental condition} * t + (1 + P * t \mid \text{individual})$, where P corresponds to if the measurement is for pigmentation or segmentation and t is the time since the first sign of the process was observed for that individual. Variables outside the parentheses represent fixed effects, while variables inside them are random effects, and interactions between variables are denoted by a “*”. Using this model, the packages *lmerTest* and *emmeans* (Kuznetsova et al., 2017; Piaskowski, 2026) were used to calculate the average slopes for each combination of $P * \text{experimental condition}$ and conduct pairwise comparisons between these averages.

For calculations of rate, the relative time since the start of the process was assigned to each individual and process. The time of first sign of pigmentation was defined as the day when any point along the spline estimate of that transect had $R/B > 1.5$. This was defined as pig-day 1. The first sign of segmentation was defined as the day morphological changes associated with the onset of strobilation were observed. Such signs of strobilation are - swelling at the base of the perradial and interradian tentacles, elongation of the body column, and the changing of the calyx of the animal from a conical to a more cylindrical shape. The day when these signs were observed was designated as seg-day 0. For individuals where signs of strobilation were not seen prior to the observation of the first segment(s), the day the first segment was observed was considered to be day 1 of segmentation (seg-day1).

RESULTS

Body length comparisons

Animals across all conditions began with an oral disc to foot length of around 1.7 mm. Control animals expanded along the body column such that at the end of strobilation, the oral disc of the uppermost ephyra was around 2.2 mm from the foot. The ending lengths of Aza Day 1, 3, 4 and 10 animals were not significantly different from the control, however Aza Day 0 animals did not grow in body length and were consequently statistically significant from the control (Dunnett Test $p < 1e-5$, see Fig 38).

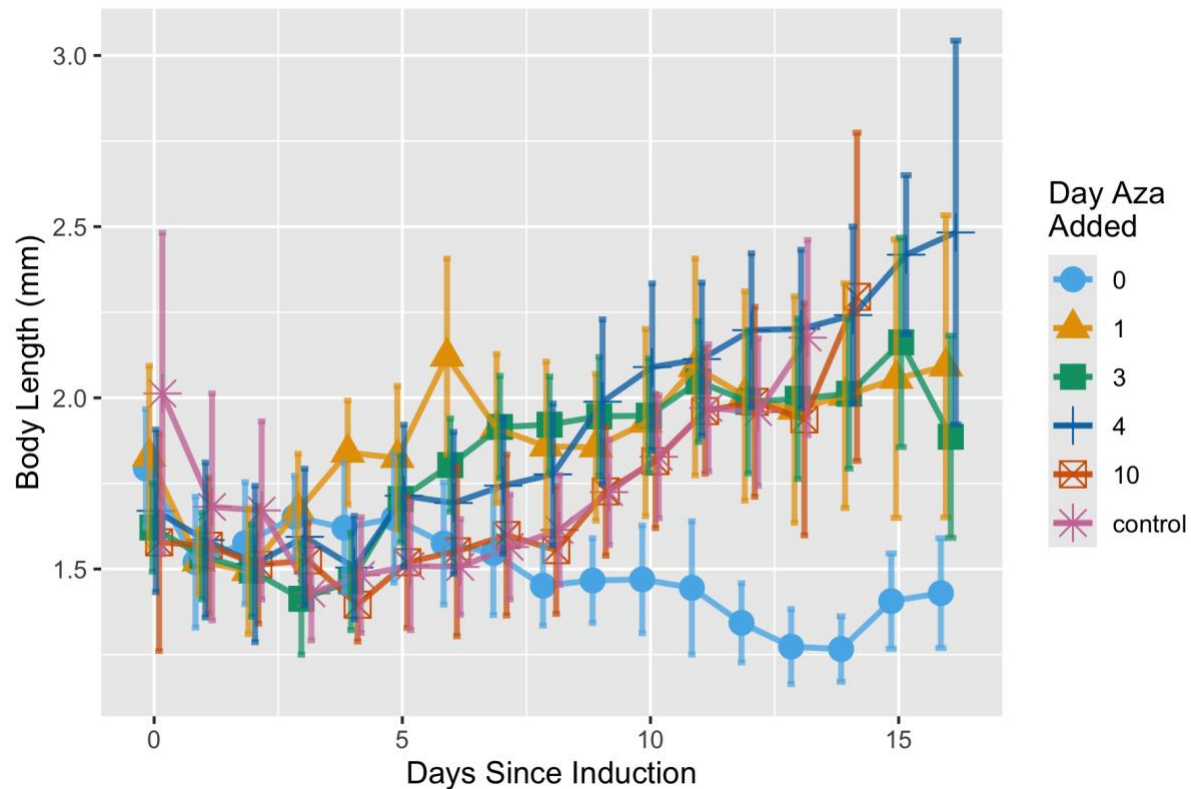


Figure 38 - Change in body length through strobilation by Day of Aza exposure

Aza Day 0 animals somewhat declined in length through the experiment, while control and Aza Day 1-10 increased in length. Error bars show 95% confidence interval of mean for sample at that date.

Impact of Aza addition on segment dimensions

Segments formed post Aza addition were significantly larger than segments formed prior to Aza exposure. The segments formed from the oral disc (primary segments) of Aza Day 0 & 1 animals had oral-aboral axes ~ 3 times the size of control primary segments (Fig 39). The diameter of the segments were also significantly different, at ~1.25 times the size of the control diameters (Fig 40). Comparing segments further down the body column show that Aza Day 3 & 4 animals produced lower segments with significantly elongated primary body axes than control strobila, but that did not significantly differ from control animals when considering segment diameter (see Table 9 & 10).

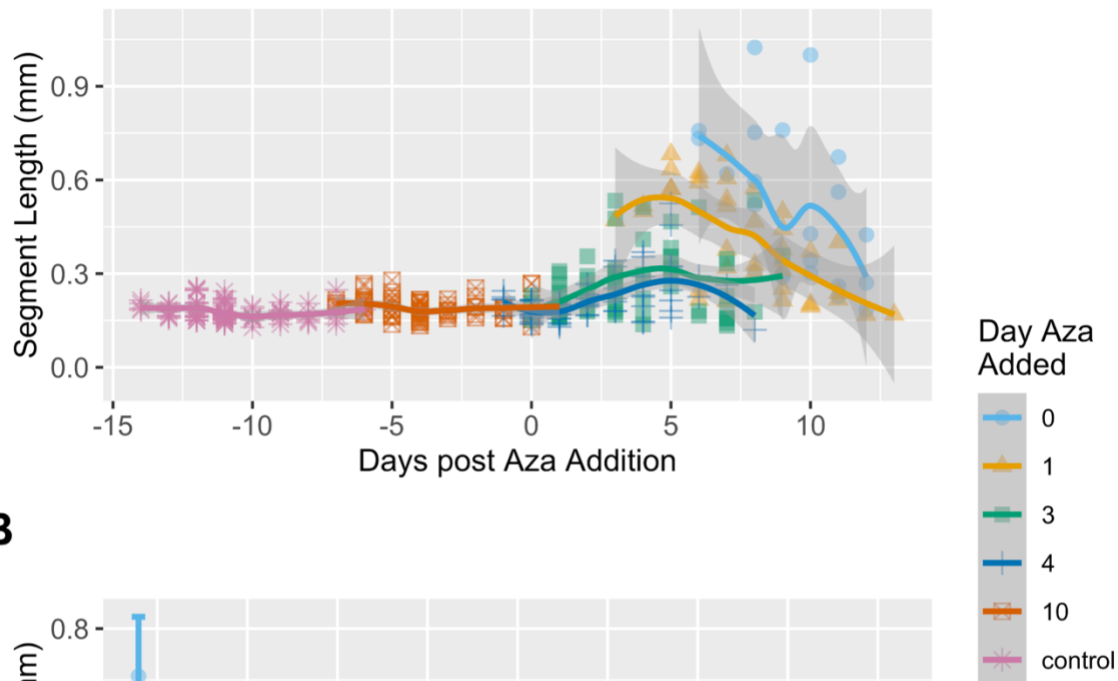
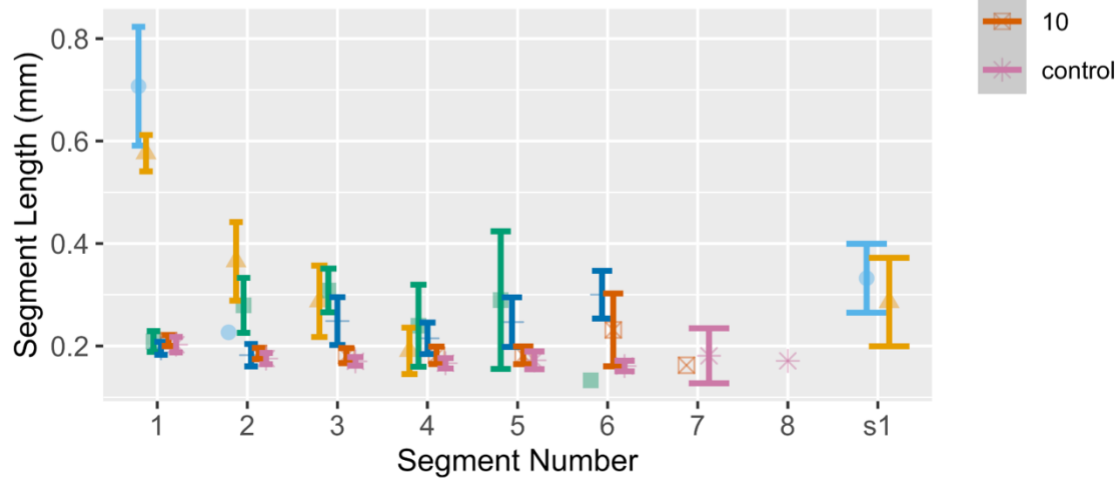
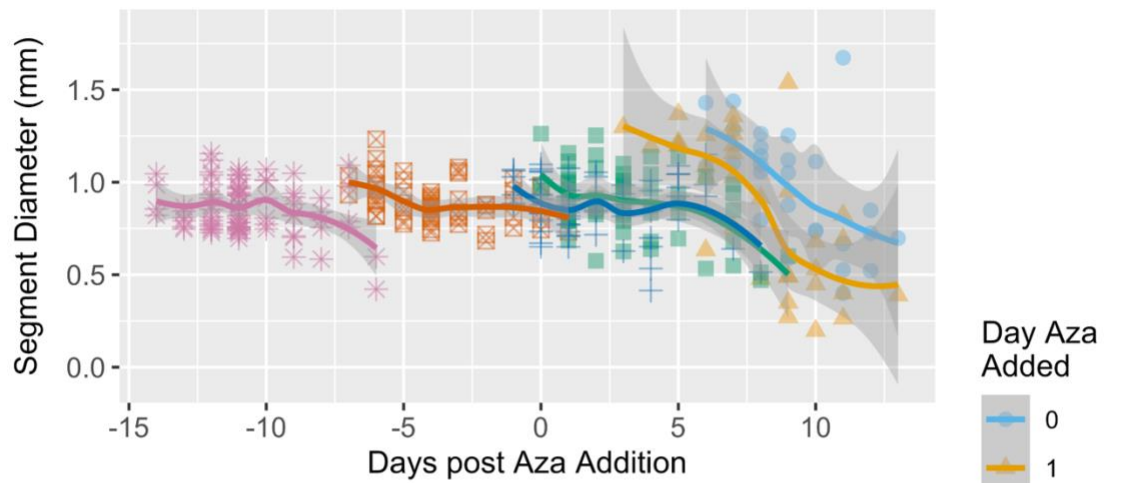
A**B**

Figure 39 - Segment Oral-Aboral length relative to time of Aza addition and segment number

A) Under control conditions (represented as adding Aza on day 17 out of 16), the oral-aboral length of segments was consistent at ~ 0.2 mm. In experimental samples, segments which formed several days after Aza addition were significantly larger than control animals. **B)** Aza Day 0 & 1 animals formed extremely enlarged segments at the top of the strobila stack. There is some delay between time of exposure to Aza and the effect on the oral-aboral axis. See Table 9 for a full report of test results. Trendlines in A show loess regressions. Error bars in B show 95% CI.

A



B

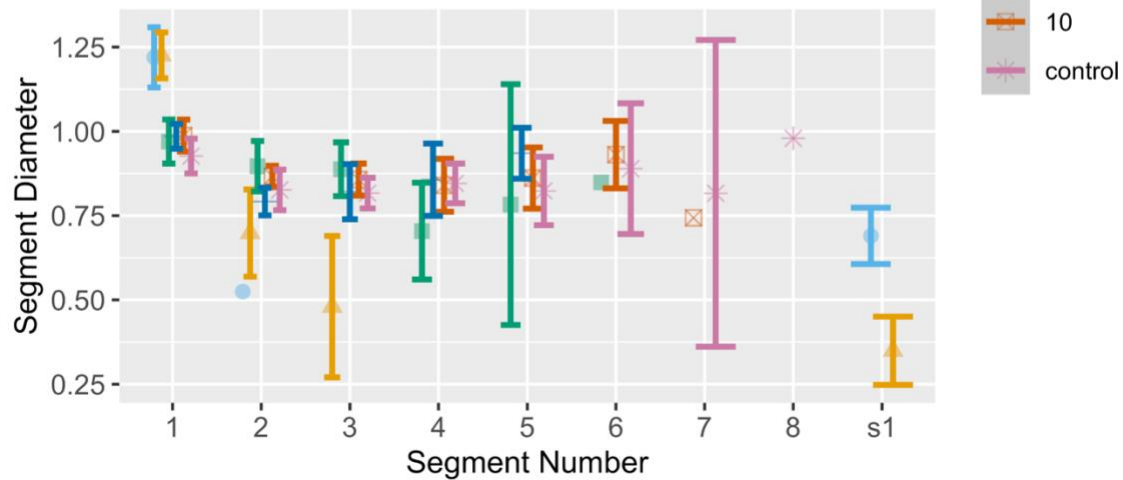


Figure 40 - Segment Diameter relative to time of Aza incubation and segment number

A) All conditions showed a decrease in segment diameter between early and late forming segments, although the difference is most profound in Aza Day 0 & 1 animals. **B)** Only Aza Day 0 & 1 segments significantly differed from those of the control (both $p < 1e-5$ for Dunnett test). See Table 10 for a full report of Dunnett tests for comparison between experimental and control groups from B. Trendlines in A show loess regressions. Error bars in B show 95% CI.

Table 9 - Segment Length comparisons to Control

Dunnett Test p-values shown that were corrected for multiple comparisons between segments of different conditions, but not for total number of comparisons. Bolded p-values pass a bonferroni corrected threshold for a Family Wise Error Rate of 0.05. Aza incubation impacts

segments along the body column in a time dependent manner, with segments formed prior to incubation being unaffected. Bolded p-values differ significantly from control

Comparison	Seg1	Seg2	Seg3	Seg4	Seg5	Seg6	Seg7
D0-control	0.000	0.992	NA	NA	NA	NA	NA
D1-control	0.000	0.000	0.000	0.705	NA	NA	NA
D3-control	0.994	0.000	0.000	0.018	0.004	0.864	NA
D4-control	0.999	0.999	0.026	0.070	0.005	0.001	NA
D10-control	0.973	0.932	0.878	0.854	0.978	0.074	0.632

Table 10 - Segment Width comparisons to Control

Dunnett Test conducted as in Table 1. Unlike segment length, width was significantly impacted only for D0's first segment and D1's segments. Bolded p-values differ significantly from control when subject to Bonferroni correction at 0.05 FWER.

Condition	Seg1	Seg2	Seg3	Seg4	Seg5	Seg6	Seg7
D0-control	0.000	0.094	NA	NA	NA	NA	NA
D1-control	0.000	0.007	0.000	NA	NA	NA	NA
D3-control	0.652	0.978	0.996	0.085	1.000	NA	NA
D4-control	0.306	0.841	0.942	0.977	0.683	0.821	NA
D10-control	0.592	1.000	1.000	0.984	1.000	0.973	0.730

Changes in average R/B throughout control strobilation

Wildtype strobilation displays three phases of coloration (see Fig 36 and 41). The initial phase (days 0-4) sees the pink-orange color of well fed polyps fade to the off-white typical of starved polyp tissues. This time corresponds with the induction of strobilation and early segmentation. The intermediate phase (days 5-8) shows relatively uniform coloration along the body column as segmentation begins. The third and final phase corresponds to metamorphosis (days 9-12) with a rapid increase in R/B in the tissue of the strobila stack, driven by the rapid decline of blue intensity (Fig S47). Throughout this process, the foot tissue maintains its polyp coloration with high intensity in all channels. Despite strobilation resulting from an oral based signal, the uppermost segment did not show the maximal R/B values, instead maxima are seen in the region beneath the upper most segment (Fig 41a, d-f).

R/B profile comparisons across experimental conditions

Aza incubation appears to result in lower R/B maxima, and its effect on polarity is visible in R/B profiles. Aza Day 0 animals consistently developed secondary mouths from their foot tissues, and these mouths underwent pigmentation during strobilation. These secondary mouths created peaks of R/B at the foot of Aza Day 0 animals (Fig 41b). Around half (10/19) of Aza Day 1 animals formed secondary mouths from their foot, however such secondary poles were only barely starting to change in color by the end of the experiment (shown as a slight uptick in Fig 41c at 100% length). In the monopolar control and Aza Day 1-10 animals, the foot remained around 1 R/B throughout strobilation.

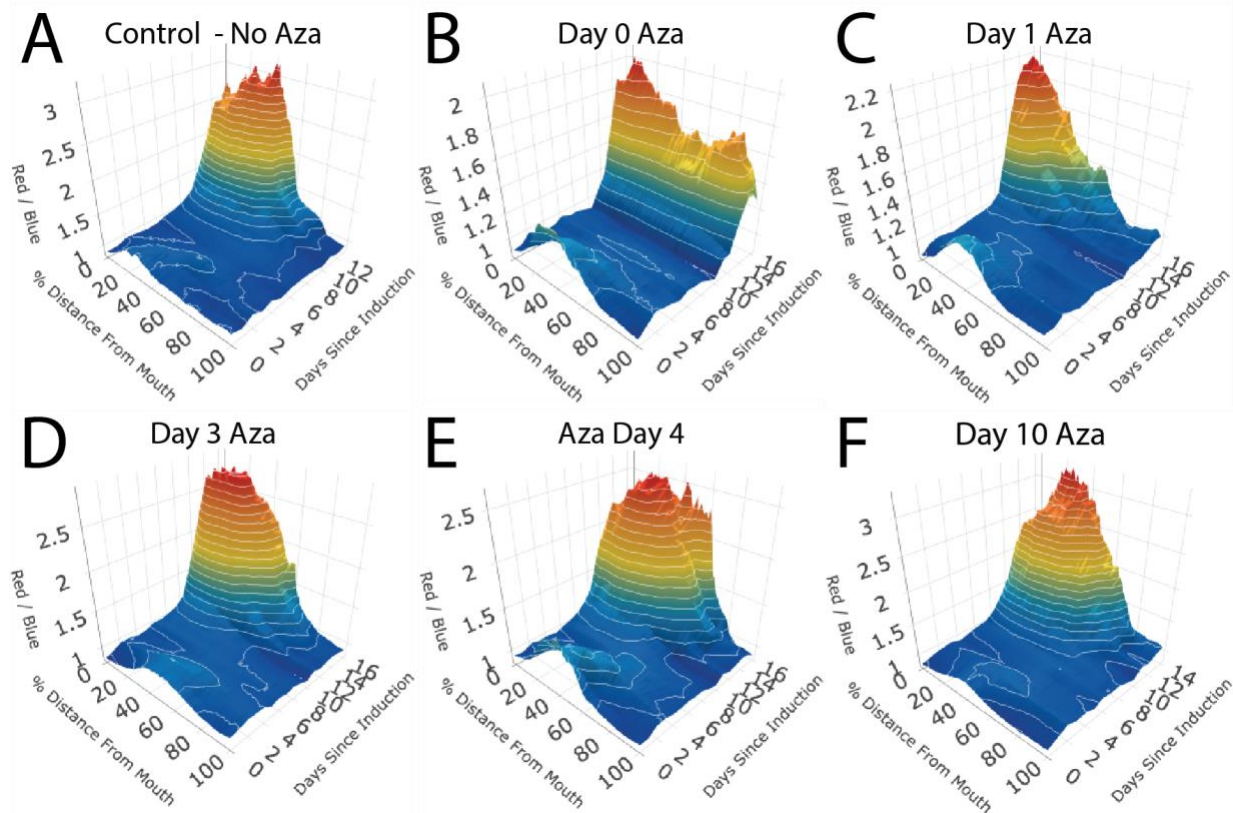


Figure 41- Red/Blue Intensity 3-D surface graphs by Day of Aza Addition

Height on the z-axis indicates a higher Red/Blue ratio, and white contour lines correspond to intervals of 0.1 on the z-axis along the surface. Changes in pigmentation consist of three general phases-phase 1: an initial peak around ~20-40% down the body column rapidly fades over ~3 days, phase 2: a period of near uniform whitish color ($R/B \sim 1$) along the body column, and phase 3: the rapid spread of pigmentation from the mouth, down the body column. Averaged Control and Aza Day 1-10 animals exhibit unipolar pigmentation (A, C-F), while Day 0 animals show bipolar pigmentation with peaks in the value of R/B at the original oral and aboral poles. Control animals show a relatively clear boundary between the segment stack and foot of the strobila, as shown by the steepness of the R/B curve on the final day of the experiment. Aza Day 1 & 3 animals show shallower R/B slopes, indicating that Aza treatment may impede the propagation of pigmentation (a reflection of the metamorphic signal) from the mouth to the foot.

Pigmentation and segmentation propagation during strobilation

The response of bipolar strobila to the treatment was dependent on the time of Aza addition. Aza Day 0 animals generally had two points of maximal R/B signal, one corresponding to each mouth (Fig 37b and 41b). However, in bipolar Aza Day 1 animals, the R/B score of the secondary pole lagged significantly behind that of the original mouth. Additionally, Aza Day 0

animals achieved a near universal level of elevated R/B along their tissue, while Aza Day 1 bipolar animals still had significant portions of the body where $R/B < 1.2$ (Fig 42a)

To limit the confounding effect of multiple sources of developmental signals on pigmentation progress, animals which formed secondary oral pols in AZA addition day 0 and 1 were not included in analyses; only monopolar animals were used to determine rates of progression. Pigmentation progressed in a nonlinear manner, with high R/B signal spreading rapidly in the region below the primary segment and above the foot (Fig 37a). Segmentation in control animals progressed at an average of 0.135 mm/day (see Table S11) and control pigmentation progressed at an average of 0.304 mm/day. The rate of these processes significantly differed from each other in the control. While rates of segmentation propagation did not significantly differ across experimental conditions, early Aza addition significantly decreased pigmentation propagation relative to the control, lowering it to 0.138 mm/day in Aza Day 1, which did not significantly differ from the rate of segmentation propagation (see Fig 43 and Table S12 for all pairwise comparisons).

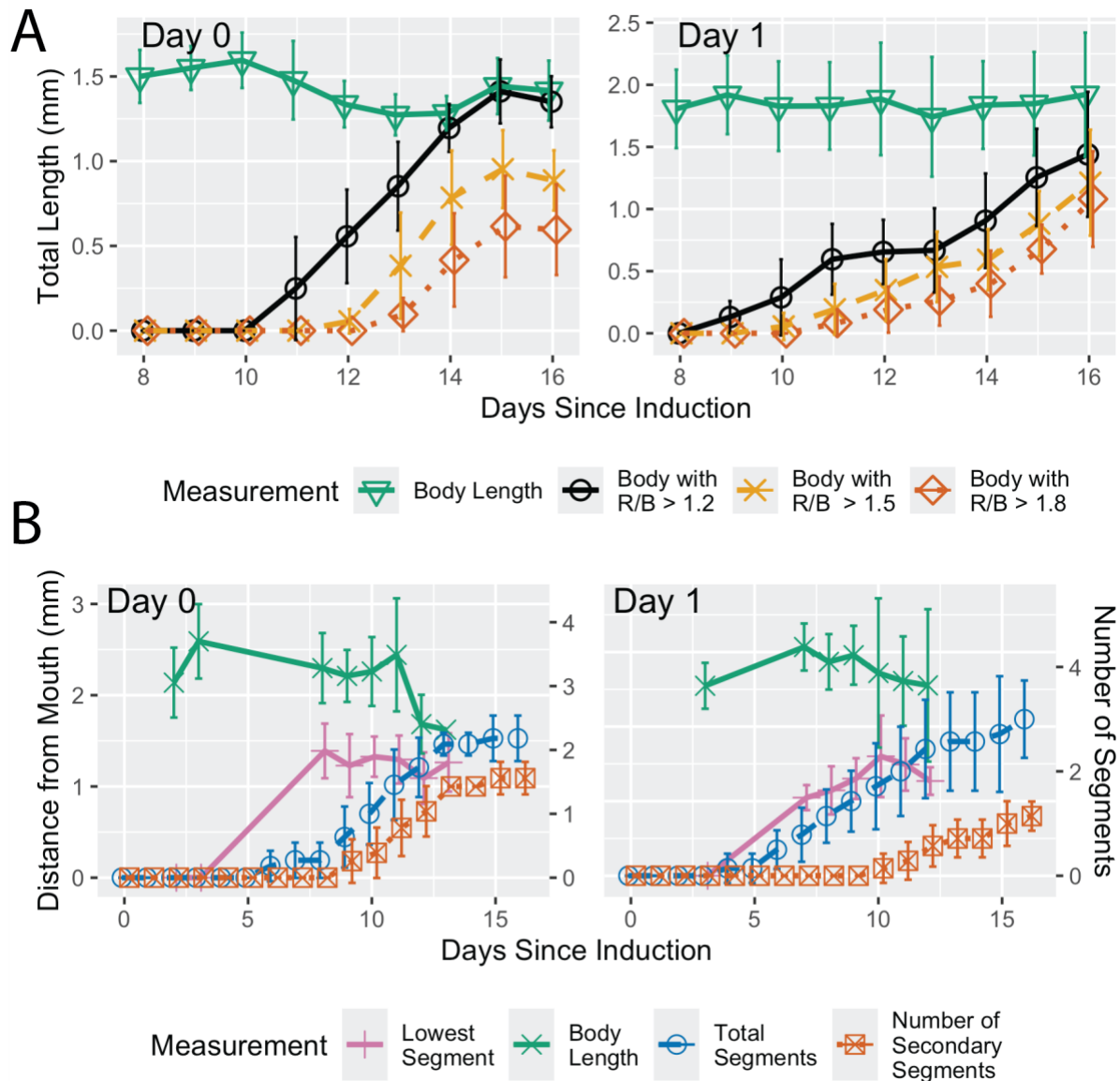


Figure 42 - Segmentation and total length of pigmented body column in bipolar animal

Note that in **A**) the y axis shows the average total length of tissue along the body column, rather than the average location along the body as shown in **B**). **A**) Aza Day 0 animals saw a global increase in pigmentation, with almost all tissue exceeding a 1.2 R/B ratio. Bipolar Aza Day 1 animals had a level of pigmentation along their body, but not to the level seen in Aza Day 0 animals. **B**) Aza Day 0 animals tended to form two segments, one associated with the original mouth (primary segment) and another from the former foot which has assumed an oral identity (secondary segment). The offset between total number of segments and number of secondary segments in Aza Day 1 bipolar animals reflects the fact that Aza Day 1 animals tended to form multiple segments from the tissue associated with the primary mouth.

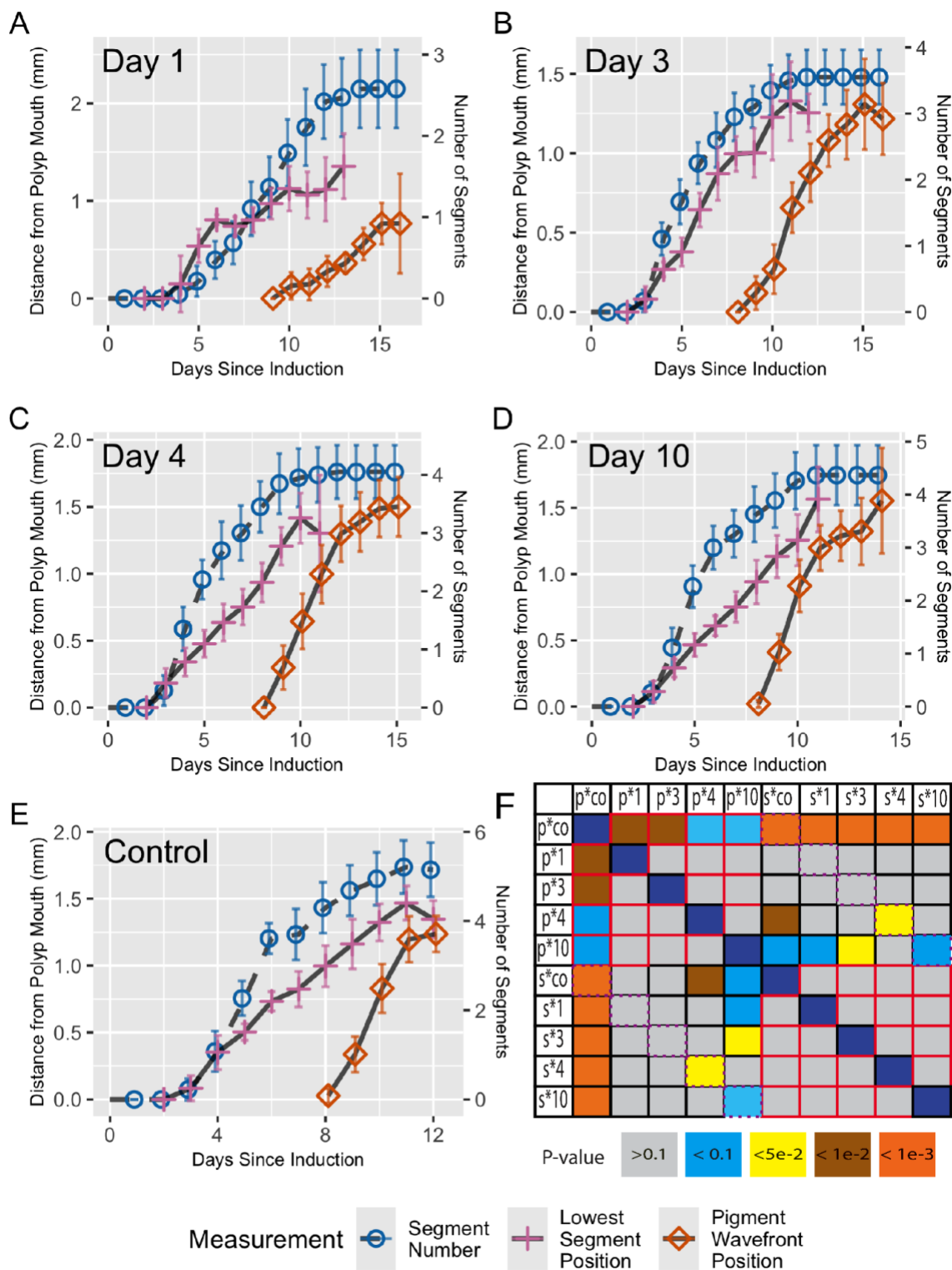


Figure 43 - Comparison of monopolar Segmentation and Pigmentation along the body column

A-E) The “pigmentation front” in Control, as well as Aza Day 3-10 and monopolar Aza Day 1 animals initiated and moved down the body column, even as segments were still being added. **F)** Pairwise comparisons of modeled slopes for combinations of condition and pigmentation vs. segmentation are designated by a “p” for pigmentation or “s” for segmentation, a “*” and then “co” for control or the number of the day when Aza was added to the condition. For ease of comparison, cells outlined in red correspond to comparisons within segmentation or within pigmentation (i.e. between conditions). Cells outlined in a broken purple line correspond to a comparison of segmentation to pigmentation within the same condition. Color coding is based on the Tukey corrected p-value for the comparison. Adding Aza on day 1 or 3 post strobilation induction significantly slows the progress of pigmentation down the body column relative to control conditions. However, adding Aza at any timepoint does not significantly affect the progression of segmentation relative to control conditions. When comparing processes within the same condition, pigmentation and segmentation normally have significantly different rates of progression. However, Aza addition slows the progression of pigmentation down the body column to the point where there is no significant difference between the rate of progression for segmentation and pigmentation.

Overall N = 88, with $n_{Control} = 19$, $n_1 = 10$, $n_3 = 20$, $n_4 = 20$, $n_{10} = 19$. See Table 11 for a summary of slopes and Table 12 for significance estimates.

DISCUSSION

Disconnect between segmentation and pigmentation propagation

While the traveling front-like nature of segmentation propagation has been demonstrated previously in *Aurelia* (Fuchs et al., 2014; Kroiher et al., 2003; Wang, 2013), the propagation of pigmentation along the strobila body column has not been as well studied. It has been suggested that metamorphosis is directly linked to pigmentation intensity via the conversion of tyrosine to the pigment melanin (Berking et al., 2002), however the mechanics of the relationship to the signaling pathways that control strobilation are unclear. Nevertheless there appears to be a relationship between pigmentation and metamorphic processes, as experimentally disrupting retinoic acid signaling during strobilation yields malformed, pale colored ephyrae (Jensen et al., 2025). In our work Aza’s reduction of pigmentation propagation from a mean 95% confidence interval (CI) of 0.252-0.356 mm/day in the control to 0.079-0.196 mm/day in Aza Day 1 animals suggests that GSK-3 β inhibition and/or hyperactivation of Wnt/ β -catenin signaling impedes the movement of the pigmentation signal. The lack of change in segmentation propagation between the control (CI of 0.115 - 0.154 mm/day) and Aza Day 1

animals (CI of 0.976-0.157 mm/day) suggests that GSK-3 β inhibition/cWnt hyperactivation does not affect the rate the segmentation signal progresses down the body column, but alters the signaling thresholds necessary for furrows between segments to form. These differences, and the nonlinear nature of pigmentation under control conditions, suggest that the signal pathways governing metamorphosis and segmentation are different. It is also clear that the processes of strobilation share a common inductive starting point. However, the specifics governing these differences in the signalling cascade that necessarily manifest during development remain to be identified.

Evaluation of the R/B method

The R/B method allows one to measure spatial and temporal changes in the pigmentation of live strobila using a digital camera, a dissecting microscope and computer with *imageJ* or similar image analysis software installed. By visually tracking the process of pigmentation we can approximate metamorphic progress in a noninvasive, nonlethal manner. This should allow further work on the life history of an individual prior to destructive sampling methods such as in whole mount *in situ* RNA hybridization or transcriptomics, providing a more complete developmental history of individual organisms and better understanding of how experimental conditions disrupt metamorphosis.

This approach may be able to fill in where transgenic expression studies are not possible. Adaptations of this method should be viable for many organisms that undergo a localized change in coloration between developmental stages. Such adaptations could incorporate other channels or means of encoding color such as CMYK (Cyan, Magenta, Yellow, Black) rather than RGB, depending on the color-shift involved. Similar work could be pursued in other Scyphozoa, and in the Cubozoa and Staurozoa, whose species' metamorphosis appears to be a modification of strobilation (Straehler-Pohl and Jarms, 2022, 2005; Toshino et al.,

2015). Some of these taxa also exhibit color differences in metamorphosis, for instance, the tissue of the cubozoan *Morbakka virulens* appears to change from a white in the polyp to a grey-green in the juvenile medusae (see Fig 35 of Toshino et al., 2015). The only Medusozoan species with currently established transgenic lines that we are aware of are in the Hydrozoa, so approaches similar to what we have done here may allow the spatiotemporal dynamics of Acraspedan (the clade of Scyphozoa, Cubozoans, and Staurozoa) species to be better explored and compared.

Our LMM estimates the linear average rate of change in position (slope) throughout the process, even though pigmentation progress clearly slows at the base of the stack. This was done to facilitate easier comparisons between segmentation and pigmentation, as comparing linear and nonlinear processes can be difficult. Further modeling could be done to incorporate the nonlinear nature of both these processes, as both segmentation and pigmentation do not advance past the lowest furrow which separates the foot from the strobila stack.

Examining pigmentation propagation in bipolar animals could provide further evidence for the effect of the foot on metamorphosis, however this would require a more complex model as it would require two sources of pigmentation. As seen in Fig 37b, the second pole can be a secondary R/B maxima. It is probably inappropriate to consider each pole separately, as the sources of signal at opposite ends of the body column could initiate at different times, rendering R/B dependent on both poles rather than a single traveling front. This would be particularly relevant for Aza Day 0 animals, as they have incomplete segmentation between their oral poles, and are significantly shorter than Aza Day 1 animals.

Evolutionary implications-segmentation modification generates bell diversity

The relationship of segmentation and metamorphosis to one another could offer a developmental/phenocopy mechanism for the origin and evolution of medusa body shapes amongst the Acraspeda. Acraspedan medusae are the result of partial or complete conversion of polyp tissue into medusa tissue, proceeding from the mouth and progressing aborally (Straehler-Pohl and Jarms, 2022). Scyphozoans are the only members of Acraspeda considered to consistently segment their body columns, however some metamorphosing Cubozoan medusae have been observed to leave behind an aboral tissue remnant which subsequently regenerates into a polyp (Toshino et al., 2015), this could plausibly be considered segmentation or tagmatization. While Staurozoa have not been reported to segment their body column, their medusae show distinctly more medusa-like morphology at the oral end of their body column and retain polyp-like structures at the aboral end (Miranda et al., 2016). This difference in fates between the oral and aboral poles across the Acraspedans suggests that segmentation serves to precisely divide medusa and polyp fates along the body column.

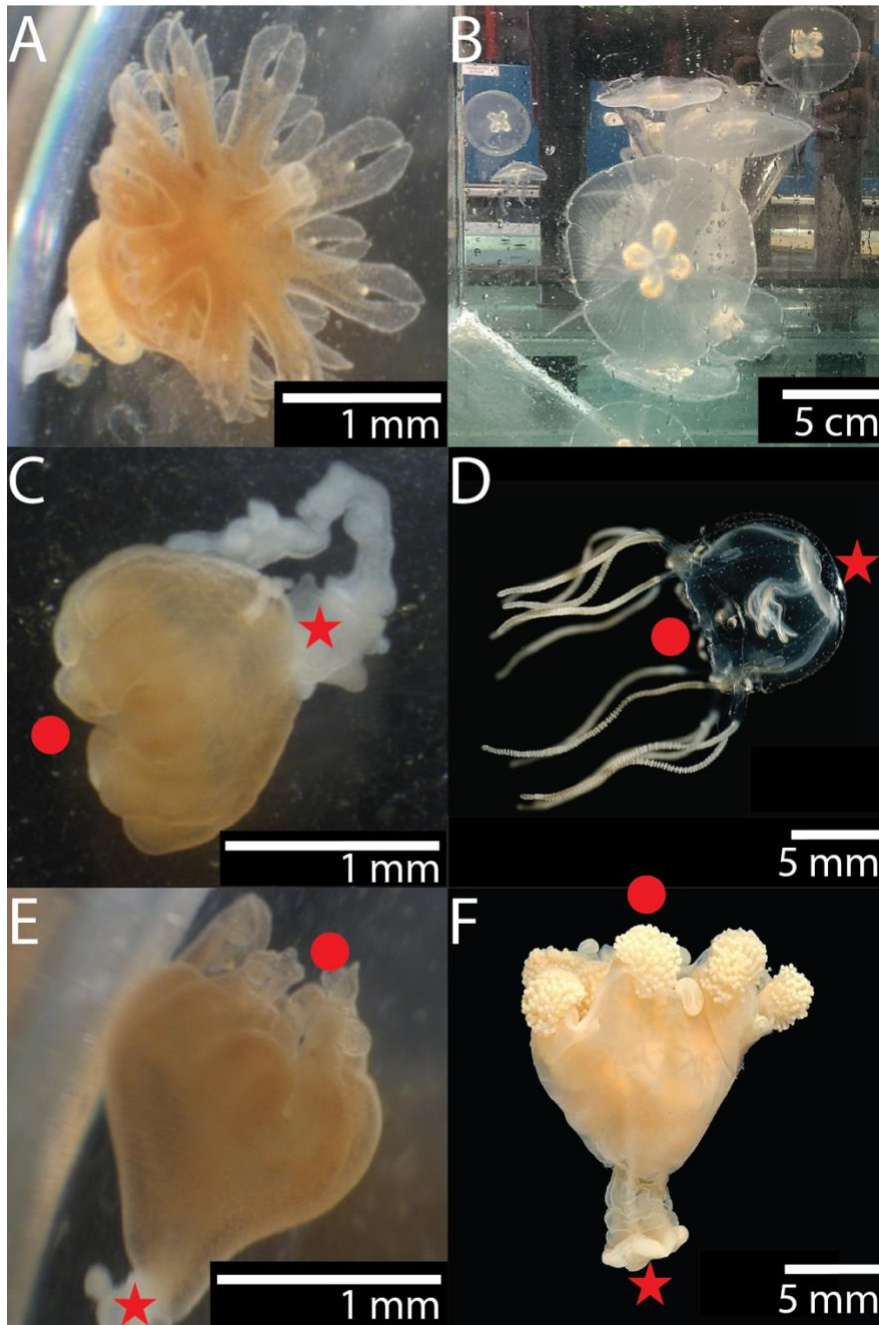


Figure 44 - Comparison of phenocopies and body plans across Acraspeda

A) control strobila which is ready to release ephyrae **B)** Adult *Aurelia coerulea* medusae. In **C-F** the oral pole of the animal is indicated by a red circle, and the aboral pole is indicated by a red star. **C)** Aza Day 1 strobila with elongated bell and sunken manubrium. Whitish tissue is the strobila and polyp tissue **D)** *Trypedalia cystophora* medusa, a Cubozoan **E)** Aza Day 1 animal with peaked bell, small amount of whitish polyp tissue can be seen at aboral end **F)** *Haliclystus auricular* medusa, a Staurozoan. D & F from Wikimedia Commons, originally from (Bielecki et al., 2014) and Yale Peabody Invertebrate Collection (Accession IZ.029402: *Haliclystus auricular*, photo by Lazo-Wasem, E. A., 2016)

The amount of polyp tissue committed to an individual medusa appears to provide a relatively straightforward path to alter the geometry of medusa bells. See Fig 44 for a comparison of medusa bells and phenocopies. Early Aza incubation suppresses segmentation but does not prevent tissue metamorphosis, resulting in ephyra with extremely elongated aboral poles (Fig S45 monopolar case). This bell has a shape similar to that of stalked jellies, where the aboral pole of the medusa is significantly narrower than the oral pole, while the oral pole develops structures derived from structures of the polyp oral disc, such as the manubrium and rhopalia/tentaculocysts. The experimental alteration of GSK-3 β and other cWnt signaling components in Staurozoans and Cubozoans could yield interesting comparative results, particularly if cWnt inhibition/GSK-3 β hyperactivation shortens the oral-aboral axes of medusae or induces segment formation. This could also help determine when the developmental cassette for segmentation originated and if it was secondarily lost in some clades. Modifications of monodisc strobilating Scyphozoans like *Cassiopea* present an opportunity to investigate the evolution of monodisc and polydisc strobilation and confirm the applicability of GSK-3 β inhibition across Scyphozoa.

Medusa bell geometries directly impact their hydrodynamics and swimming ability, making them less suitable for different ecological niches. Shallow bells move a greater volume of water, but do not have the muscle surface area to subumbrellar volume ratio necessary to move the water rapidly, making them slow but efficient swimmers (Colin and Costello, 2002; Costello et al., 2008). Peaked bells have a smaller subumbrellar volume, but a correspondingly higher muscle surface area to subumbrellar volume ratio, and are better able to constrict the outflow of water from their subumbrellar cavity (Costello et al., 2008; Dabiri et al., 2010). This allows the animal to move faster with higher drag and lower energy efficiency. Additionally, the geometries of the bells are viable at different sizes of animals, with peaked bell shapes being

much less viable for large animals than small animals (Costello et al., 2008). Altering the oral-aboral axis of medusae can therefore shift the suitability of its body for a constant slow swimming and filter feeding lifestyle, to an intermittent fast swimming and ambush lifestyle.

Aza incubation has distinct effects on the strobilation subprocesses of segmentation and metamorphosis, indicating that the two processes are transmitted along the body column of *Aurelia coerulea* strobila via different means. While not altering the rate at which the segmentation front propagates along the body column, Aza incubation suppresses segment formation, and simultaneously slows the propagation of the pigmentation front. This indicates that GSK-3 β inhibition extends the body column of forming strobila segments, resulting in ephyrae with peaked umbrellar surfaces, greatly differing in geometry from the flat ephyrae produced by wildtype strobilation. These results indicate that modification of GSK-3 β and cWnt signaling may have facilitated the evolution of diverse umbrellar shapes in Scyphozoa and its cubozoan and staurozoan relatives.

SUPPLEMENTARY TABLES

Table 11 SUPPLEMENTARY - Slope of Pigmentation and Segmentation progress

Under control conditions, pigmentation progresses down the animal at an average of 0.304 mm/day, while segmentation progresses on average 0.135 mm/day. Early addition of Aza following induction slows pigmentation progress to the point that there is no significant difference in the rate of progress between the different processes. As Aza is added later, pigmentation returns to its normal rate of progression.

Process	Condition (day Aza added)	Slope (mm/day)	SE	df	lower.CL	upper.CL
Pigmentation	control	3.04E-01	2.63E-02	1.67E+02	2.52E-01	3.56E-01
Segmentation	control	1.35E-01	9.72E-03	8.07E+01	1.15E-01	1.54E-01
Pigmentation	1	1.38E-01	2.93E-02	6.92E+01	7.94E-02	1.96E-01
Segmentation	1	1.27E-01	1.48E-02	7.34E+01	9.76E-02	1.57E-01
Pigmentation	3	1.73E-01	1.92E-02	4.83E+01	1.34E-01	2.11E-01
Segmentation	3	1.22E-01	9.44E-03	7.32E+01	1.04E-01	1.41E-01
Pigmentation	4	2.04E-01	1.87E-02	4.99E+01	1.66E-01	2.41E-01
Segmentation	4	1.41E-01	9.54E-03	8.49E+01	1.22E-01	1.60E-01
Pigmentation	10	2.04E-01	2.07E-02	6.33E+01	1.63E-01	2.46E-01
Segmentation	10	1.41E-01	9.62E-03	9.44E+01	1.22E-01	1.60E-01

Table 12 SUPPLEMENTARY - Pairwise comparison matrix of p-values of modeled slopes

Boxed p-values signify pairwise comparisons of the same process (segmentation or pigmentation) across different conditions (day of Aza addition). Bolded p-values indicate a comparison of segmentation to pigmentation within the same condition. Values in red pass a Tukey corrected level of significance at an alpha of 0.05

	p*co	p*1	p*3	p*4	p*10	s*co	s*1	s*3	s*4	s*10
p*co	NA									
p*1	2.19E-03	NA								
p*3	4.13E-03	9.91E-01	NA							
p*4	6.85E-02	6.79E-01	9.77E-01	NA						
p*10	9.54E-02	7.06E-01	9.82E-01	1.00E+00	NA					
s*co	3.94E-08	1.00E+00	7.46E-01	4.76E-02	8.37E-02	NA				
s*1	7.36E-07	1.00E+00	6.78E-01	5.52E-02	8.67E-02	1.00E+00	NA			
s*3	2.57E-08	1.00E+00	2.23E-01	8.02E-03	1.77E-02	9.96E-01	1.00E+00	NA		
s*4	8.98E-07	1.00E+00	8.94E-01	4.31E-02	1.59E-01	1.00E+00	9.99E-01	9.22E-01	NA	
s*10	9.39E-07	1.00E+00	8.96E-01	1.03E-01	8.35E-02	1.00E+00	9.99E-01	9.22E-01	1.00E+00	NA

SUPPLEMENTARY FIGURES

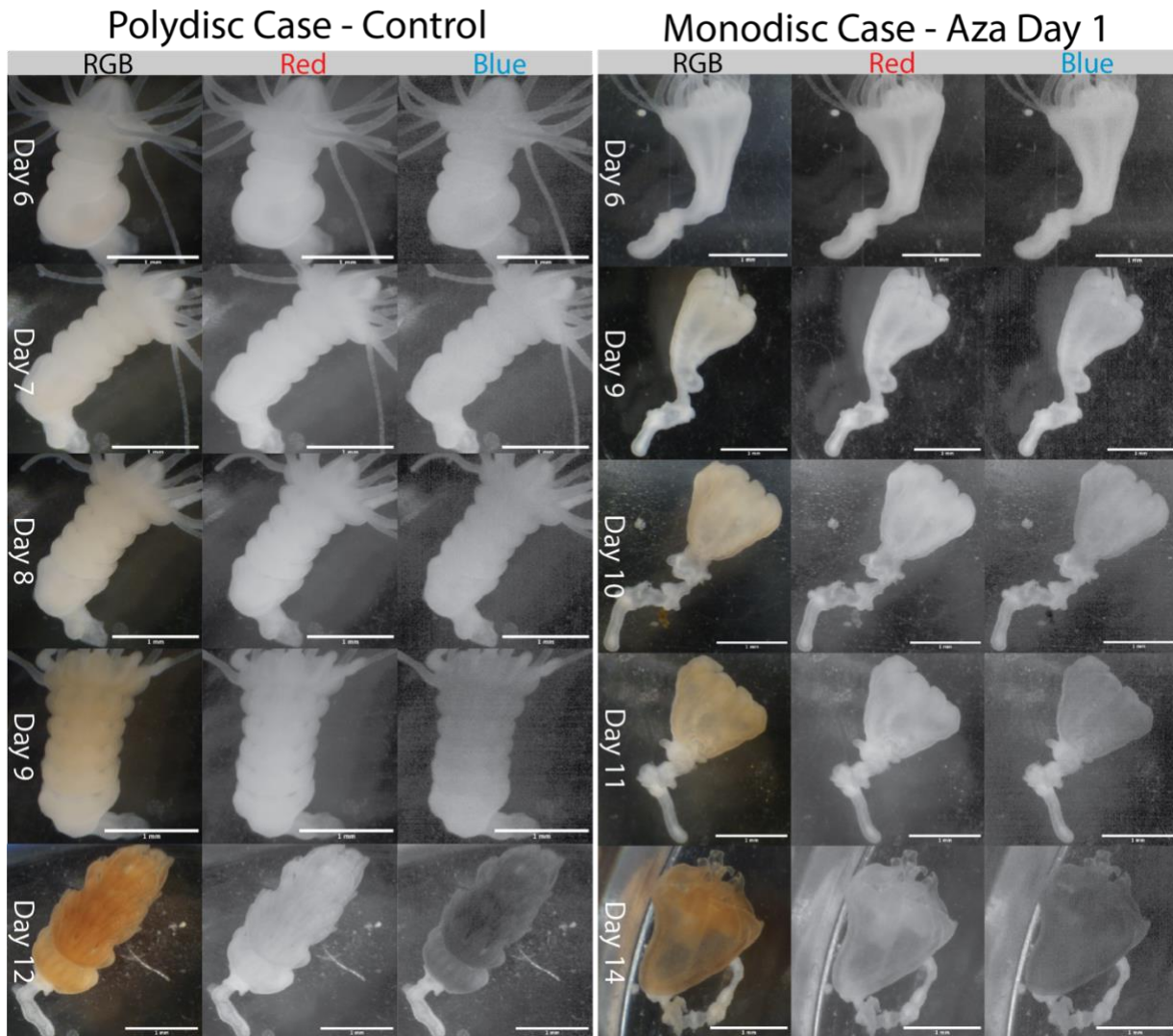


Figure 45 SUPPLEMENTARY - Gradation of pigment during strobilation

A visual demonstration of the change in Red and Blue intensity along the body column of strobila. Changes in intensity can be seen along the body column starting in day 7 (for the polydisc case) or day 9 (for the monodisc case). A subtle gradient of color can be seen along the oral-aboral axis of the polydisc animal in day 8 and 9. The monodisc case shows a distinctly enlarged peaked umbrellar surface, similar in appearance to those of juvenile medusae formed by strobilation reported in Cubozoa (Toshino et al., 2015). Note the line of tissue (likely one of the septa) passing along the midline of the monopolar segment and its impact on intensity readings. The impact of tissue thickness on Red and Blue Intensity can also be seen when examining the aboral-most tissue of the monodisc animal's umbrella. The folded tissue shows as a V-region of increased Red and decreased Blue intensity.

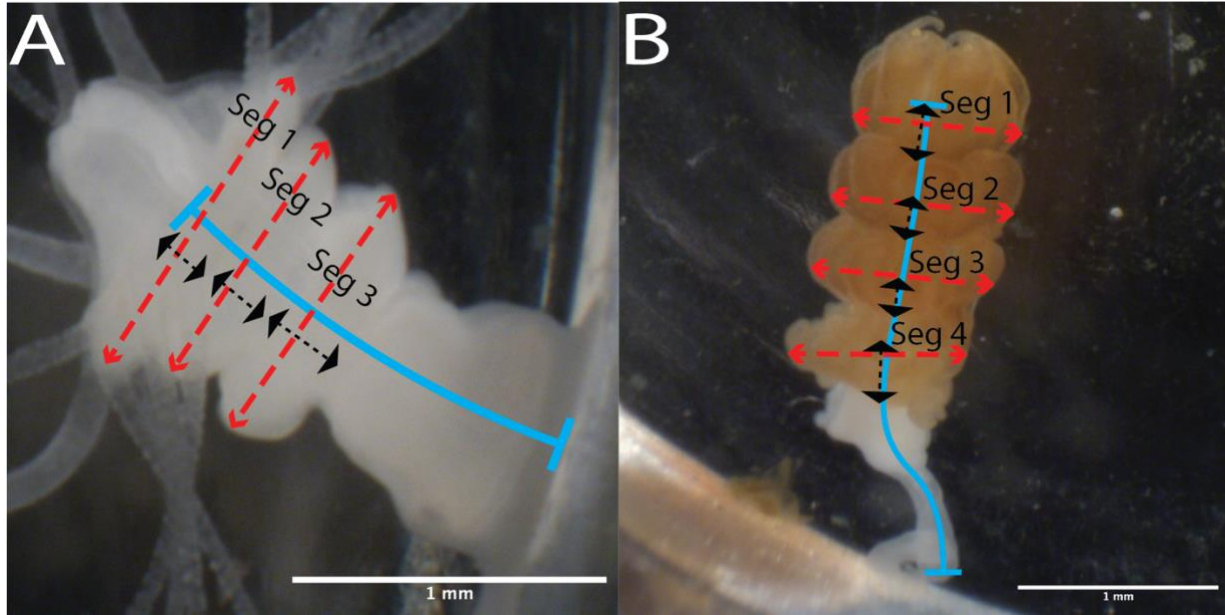


Figure 46 SUPPLEMENTARY - Example linear measurements of strobila

Different linear measurements correspond to different colors and lintypes in the figure. The blue line with bars shows the total body length of the animal, beginning at the upper layer of the oral disc and reaching to the point of attachment to the surface. The red dashed line with edge shaped arrows indicates the diameter of the segment. The black dotted line with triangular arrows shows the length of the oral-aboral axis of each segment, running from the oral surface to the peak of the umbrellar surface. **A)** shows an Aza Day 4 individual as an early strobila when segmentation is ongoing while **B)** shows the same individual as a late strobila when segmentation has ceased, but the spread of pigmentation is ongoing. Note the disruption to bodily organization in segment 4 of **B**, a typical outcome for segments affected by Aza incubation.

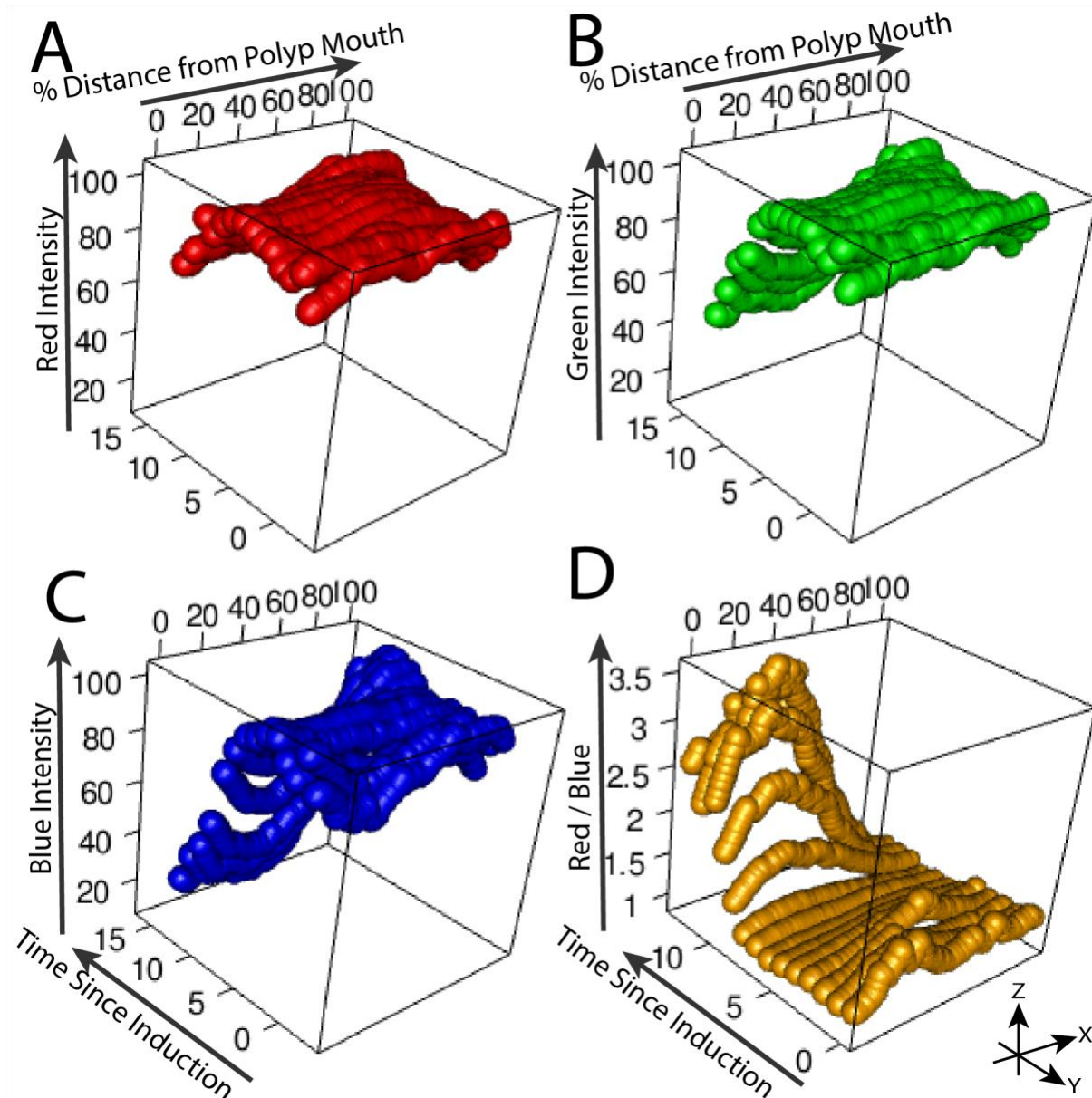


Figure 47 SUPPLEMENTARY - Comparison of average color intensities through time along control animal body axis

Z-axis - scaled white-balanced intensity for **A)** Red, **B)** Green and **C)** Blue while **D)** shows the ratio of Red and Blue intensity. X-axis shows the percent distance of the measurement from the polyp oral pole (0%) to the end of the foot (100%). Y-axis shows time since induction of strobilation by 5-methoxy-2-methylindole addition on day 0. Intensity of all channels decreases along the strobila stack as segments mature into ephyrae, however the decline is most severely shown in the Blue channel. As the Red channel sees the lowest decline in intensity, the ratio of Red/Blue intensities strongly increases as ephyra/segments mature.

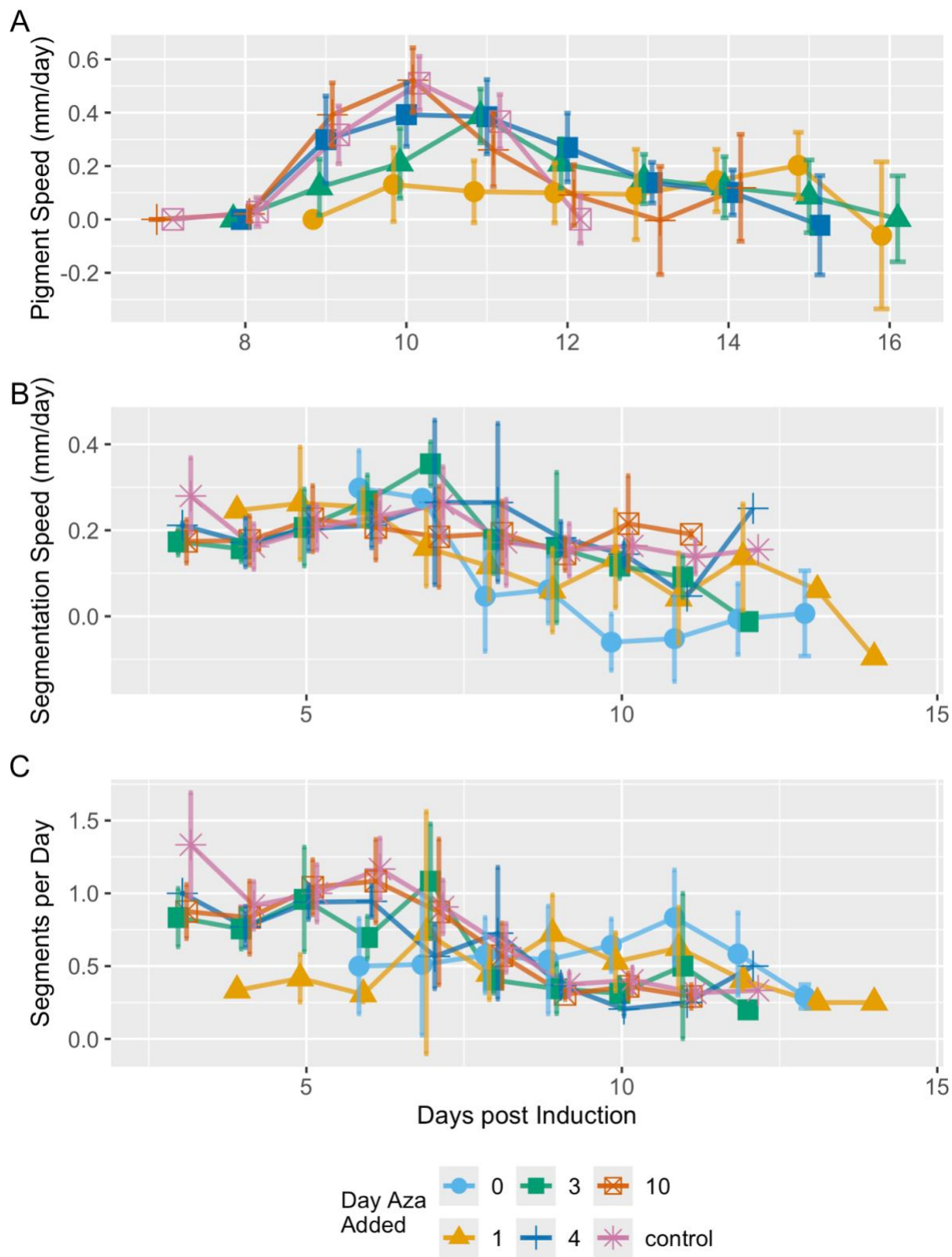


Figure 48 SUPPLEMENTARY - Average rates of progress of Pigmentation and Segmentation

Colored bars correspond to 95% confidence interval for that condition on that day. **A)** Speed of pigment progress down the body column peaks early in strobilation, followed by a sustained period of slow expansion in the lowest segments. **B) & C)** Similarly, segments are added more quickly early in segmentation, resulting in faster progress of the lowest segment down the body column. Pigmentation provides a measure of metamorphic wave progression while the lowest segment formed allows us to judge the progress of any segmentation signal. their oral disc (primary segments), with the size of later segments quickly declining. The delay between Aza incubation and enlarged segments can be seen by comparing Segment 1, (Aza Day 3 & 4 do not differ significantly from the control), Segment 2 (Aza Day 3 differs from control, Aza Day 4 does not) and Segment 3&4 (Both Aza Day 3 & 4 differ from the control). Significance shown by Dunnett Test p-values in Table 11 & 12. Error bars show 95% confidence intervals of the mean.

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