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ECM collagen reduction affects regeneration but not mouth opening kinematics in *Hydra* that successfully regenerate

Vivien Zheng[1], Hengyu Li[1], Hannah Poon[1], Rebecca T. Somach[1], Christina Rabeler[1], Eva-Maria S. Collins[1,2§]

[1]Swarthmore College, Swarthmore, PA, United States

[2]University of Pennsylvania, Philadelphia, PA, United States

[§]To whom correspondence should be addressed: ecollin3@swarthmore.edu

Abstract

Hydra mouth opening relies on ectodermal actomyosin contractions whose forces are transmitted to the endodermal layer via the extracellular matrix (ECM). How regeneration and ECM properties affect mouth opening is unknown. We compared mouth opening in 72-hour regenerated and adult *Hydra* and found differences. Additionally, we studied 72-hour regenerated *Hydra* treated with 2-2' dipyridyl to reduce ECM collagen deposition. Mouth opening kinematics in dipyridyl-treated heads that regenerated tentacles did not differ from untreated regenerated heads. This suggests that collagen levels sufficient for regeneration enable normal mouth opening. Mouth opening in dipyridyl-treated *Hydra* without regenerated tentacles could not be studied.

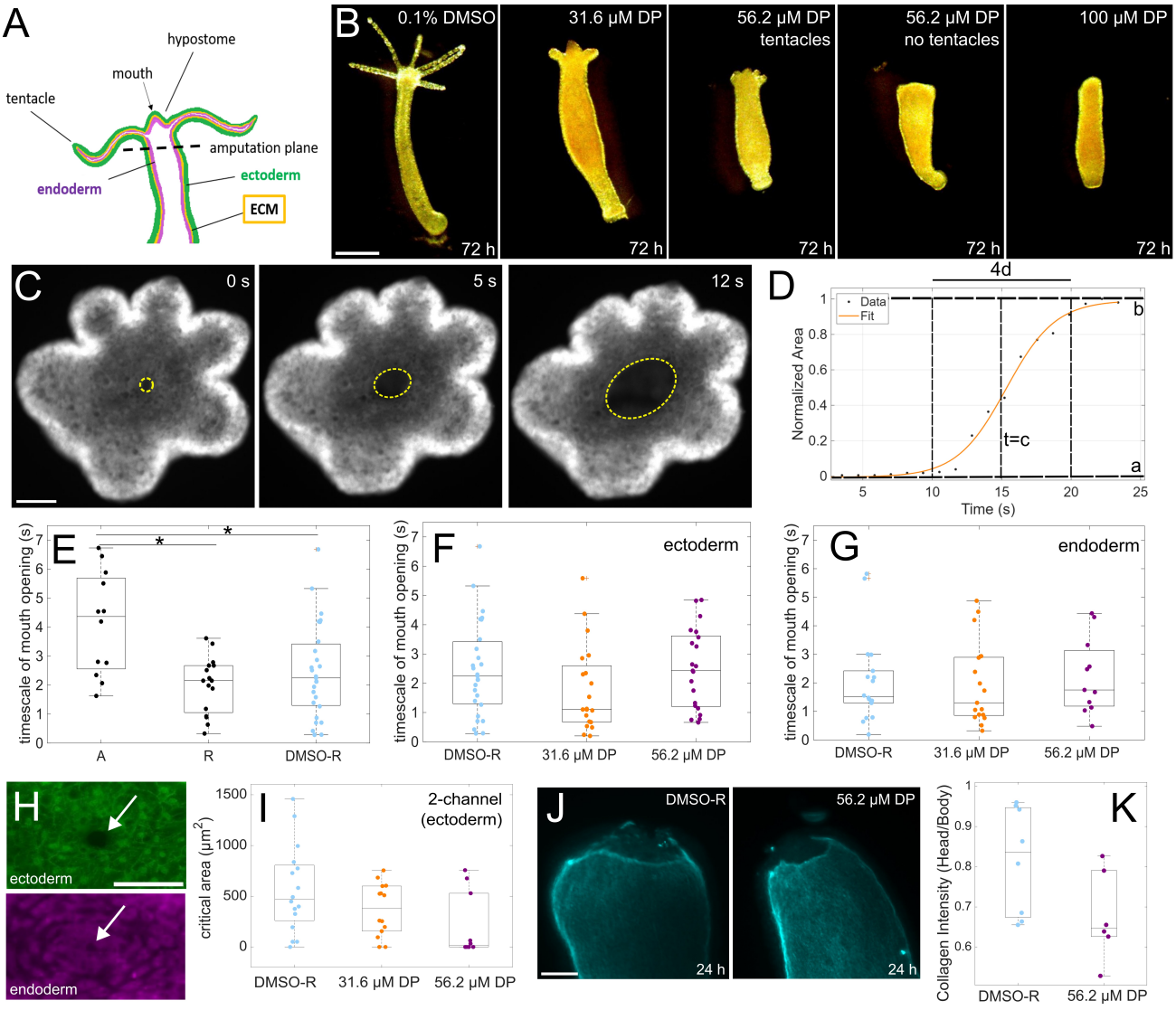


Figure 1. The effect of 2-2' dipyridyl treatment on head regeneration and mouth opening:

Decapitated *Hydra vulgaris* were exposed for 72 h to dipyridyl in 0.1% (v/v) DMSO or only 0.1% (v/v) DMSO and assessed for phenotype, collagen I distribution, and mouth opening. (A) Schematic of *Hydra* head region, showing

epithelial tissues, ECM, and the amputation plane. (B) At 72 h of regeneration, all decapitated *Hydra* exposed to 0.1% DMSO had regenerated a head with tentacles. 73/108 exposed to 31.6 µM dipyridyl regenerated with shortened tentacles. When exposed to 56.2 µM dipyridyl, 74/196 regenerated with stubby tentacles and 122/196 with no tentacles. No tentacles were observed at 100 µM dipyridyl (N=0/7). Scale bar: 1 mm. (C) Still images of the ectoderm layer showing a mouth opening event in a 31.6 µM dipyridyl-treated watermelon (GFP-ectoderm/DsRed2-endoderm; (Glauber et al., 2013)) *Hydra* head. Scale bar: 0.1 mm. (D) Mouth opening vs. time follows a sigmoidal curve that was fit (orange line) as described in the Methods to extract the time scale of opening ('d'). (E) Mouth opening in untreated regenerating *Hydra* heads ('R', N=16) occurs faster than in intact adult heads ('A', N=14). Mouth opening kinematics in 0.1% DMSO-R controls (N=27) is indistinguishable from untreated regenerating *Hydra* (N=16). p=8.12e-3 for groups A and R, p= 9.41e-3 for groups A and 0.1% DMSO-R, p=0.97 for groups R and 0.1%DMSO-R; Kruskal-Wallis test with Benjamini-Hochberg correction. (F-G) The timescale of mouth opening does not differ between 0.1% DMSO control heads or dipyridyl-treated heads, for (F) ectoderm (DMSO-R N=27, 31.6 µM N=20 and 56.2 µM dipyridyl N=21) and (G) endoderm (DMSO-R N=18, 31.6 µM N=17 and 56.2 µM dipyridyl N=11). (H) Example still image of a 31.6 µM dipyridyl-treated *Hydra* head showing the critical ectoderm opening area (see arrow), defined as the area when the endoderm begins opening (see arrow). Scale bar: 0.1 mm. (I) There was no significant difference in critical area between 0.1% DMSO controls and dipyridyl-treated animals (DMSO-R N=16, 31.6 µM N=14 and 56.2 µM dipyridyl N=10). The critical area was set to zero for cases where the endoderm appeared to open before or with the ectoderm, which can be attributed to the imaging delay between the channels of ~0.5 sec. (J) Collagen I antibody staining in regenerating A10 *Hydra* at 24 h in controls and 56.2 µM dipyridyl-treated animals. (K) Collagen I levels were measured by summing the pixel intensity values in a fixed area in the body column and regenerating head tissue, respectively. The pixel ratios are plotted for each *Hydra*. Values were higher in 0.1% DMSO (median [IQR]: 0.836 [0.674–0.947], N=8) than in 56.2 µM dipyridyl-treated animals (median [IQR]: 0.647 [0.632–0.768]; N=6) (Wilcoxon rank-sum test, p = 0.029).

Description

Mouth opening in the freshwater polyp *Hydra vulgaris* is being used as an *in vivo* model to study extreme epithelial deformations (Carter et al., 2016; Goel et al., 2024). *Hydra*'s body is a hollow cylinder, consisting of two epithelial layers connected by extracellular matrix (ECM). *Hydra*'s mouth is located on the head end, at the tip of the hypostome, surrounded by tentacles (**Figure 1A**). Upon neuronal activation, radially oriented supracellular actomyosin filaments in the outer ectodermal cell layer pull the mouth at the center of the hypostome open (Goel et al., 2024). For full opening to occur, ectodermal opening forces must be transmitted to the inner endodermal cell layer through the micrometer-thin ECM (Goel et al., 2024). How ECM composition affects mouth opening has not yet been studied.

The ECM consists of two outer layers of collagen IV and laminin, and an inner layer of collagen I (Shimizu et al., 2008). We hypothesized that reduced collagen deposition would thin the ECM and transmit ectodermal contractile forces to the endoderm more effectively, leading to faster mouth opening. Alternatively, it is also possible that changes to ECM composition would soften the ECM, which may hinder force transmission. Because exposure to 100 µM 2-2' dipyridyl has been reported to reduce collagen levels in regenerating *Hydra* heads (Cox et al., 2026), we used dipyridyl treatment to reduce ECM collagen levels in regenerating *Hydra*. We first confirmed the effectiveness of the dipyridyl treatment. At 72 h post-amputation, all animals treated with 0.1% (v/v) DMSO (vehicle control) showed normal head morphology while 100 µM dipyridyl treatment caused severe regeneration defects with no tentacle formation (**Figure 1B**), as previously described in (Cox et al., 2026). Lower dipyridyl concentrations yielded intermediate phenotypes, i.e. shortened tentacles at 31.6 µM and stubby or absent tentacles at 56.2 µM (**Figure 1B**). Only 0.1% DMSO controls and dipyridyl-treated *Hydra* with tentacles were used for mouth opening experiments (**Figure 1C**); *Hydra* with more severe regeneration defects were too fragile for use in mouth opening experiments.

Previous studies of mouth opening kinematics have focused on adult *Hydra*. We therefore first examined whether mouth opening could be induced in regenerated heads at 72 hours post-decapitation, and with similar kinematics as adults. Mouth opening was successfully induced, and the normalized mouth area as a function of time was measured. The resulting sigmoidal opening curves were fit with a logistic equation to extract the timescale of mouth opening, 'd' (**Figure 1D**) (Carter et al., 2016; Goel et al., 2024). Regenerating heads at 72 hours post-decapitation opened their mouths significantly faster than adults (**Figure 1E**). Mouth opening in 0.1% DMSO control regenerating heads was comparable to that of untreated regenerating *Hydra* (**Figure 1E**). The difference in mouth opening time scales between adult and regenerating *Hydra* are unlikely due to size differences as previous work has shown that heads from buds and from adult *Hydra* that differ in size open with similar rates (Carter et al., 2016). Instead, these differences may indicate that 72 hours of regeneration are insufficient to fully restore adult tissue function.

Next, we compared mouth opening kinematics between DMSO control and dipyridyl-treated regenerated *Hydra*. We did not find a statistically significant difference in mouth opening times between DMSO-R control (N=27), 31.6 µM dipyridyl (N=20), and 56.2 µM dipyridyl (N=21) treated regenerating *Hydra* heads (p=0.14, Kruskal-Wallis test; **Figure 1F**). Because the ectodermal opening forces get transmitted to the endoderm via the ECM, we also quantified mouth opening of the endoderm. Again, we did not observe a statistically significant difference between DMSO-R control (N=18), 31.6

µM DP (N=17), and 56.2 µM DP (N=11) dipyridyl-treated *Hydra* (p=0.69, Kruskal-Wallis test; **Figure 1G**). Finally, we imaged both ectoderm and endoderm layers simultaneously (see Methods) to evaluate the critical ectoderm mouth opening area (**Figure 1H**), which is the area of the ectoderm opening when the endoderm opening is first visible. This corresponds to the point at which force transmission through the ECM is sufficient to initiate endoderm opening (Carter et al., 2016). Several of the 56.2 µM dipyridyl-treated *Hydra* opened the endodermal layer at a smaller critical ectoderm area than controls but the difference was not statistically significant (p=0.05, Kruskal-Wallis test; **Figure 1I**). To test how behavior correlated with ECM collagen deposition, we analyzed collagen I levels in DMSO-R controls and in 56.2 µM dipyridyl-treated *Hydra* at 24 hours (**Figure 1J**), prior to tentacle development, similar to (Cox et al., 2026). There was a significant difference between the two groups (Wilcoxon rank-sum test, p = 0.029). However, 3/6 of the 56.2 µM dipyridyl-treated *Hydra* had collagen I levels similar to DMSO-R animals (**Figure 1K**), comparable to the ~40% 56.2 µM dipyridyl-treated *Hydra* that regenerated heads with tentacle stubs (**Figure 1B**). Notably, collagen staining and mouth opening data were generated from separate groups of animals. Because we could not study collagen levels of the specimen used for mouth opening experiments, our conclusions on the relationship of collagen levels and mouth opening kinematics are preliminary. Nevertheless, these data suggest a threshold effect: collagen I levels sufficient for head and tentacle regeneration are also sufficient for mouth function.

Methods

Hydra lines and care

Hydra vulgaris were maintained in bulk (Hyland & DeSantis, 2022) in *Hydra* medium (HM; https://openhydra.org/) in food-safe containers (Amazon) at 18°C (MIR-554-PA Incubator, Panasonic, Tokyo, Japan). The transgenic watermelon line (GFP-ectoderm/DsRed2-endoderm) (Glauber et al., 2013) was used for mouth opening experiments and the non-fluorescent A10 line (Shimizu et al., 2004) was used for antibody staining. *Hydra* were fed 2-3 times per week with *Artemia* nauplii (Brine Shrimp Direct, Ogden, UT) and used for experiments the day after feeding.

2-2' dipyridyl treatment

Dipyridyl treatment was adapted from (Naik et al., 2020). Stock solutions of 0.1 M dipyridyl (Thermo Fisher Scientific, Waltham, MA) were prepared in 100% DMSO (Sigma-Aldrich, St. Louis, MO), aliquoted, and stored at -20°C for 1-2 weeks until use and thawed a maximum of 3 times. Dipyridyl working solutions (0 µM, 31.6 µM, 56.2 µM, and 100 µM, each containing 0.1% (v/v) DMSO) were made fresh daily. *Hydra* were placed into a ~5cm diameter glass dish containing 5-8ml of 1mM linalool (Sigma-Aldrich cat. no. L2602) in HM and allowed to relax for 5-10 minutes before amputation. We used both, amputation right below the tentacles and body column bisection (**Figure 1A**). Bisection led to a stronger regeneration phenotype and yielded smaller animals. For antibody staining, only bisected animals were used as smaller animals have better antibody penetration. For dipyridyl exposure, headless body columns were washed at least once in fresh HM after amputation, then placed into wells of an untreated 12-well or 24-well plate (Genesee Scientific, El Cajon, CA) with 500µL solution/*Hydra*. Plates were stored in the dark at 18°C and solutions were renewed every 24 hours up to 72 h.

Whole mount collagen I staining:

Collagen I antibody staining was performed following the protocol in (Tran et al., 2017), with specific modifications noted here. *Hydra* were washed briefly in HM, then relaxed for 2-5 minutes in 2% (w/v) urethane (Sigma-Aldrich) in HM before fixation in 4% paraformaldehyde (Ted Pella) in 1x PBS (MP Biomedicals), for 15-30 minutes. Samples were washed 3 times for 10 minutes each in 1x PBS, then permeabilized by a 15-minute incubation in 0.5% Triton X-100 (Sigma-Aldrich, cat. no. T8787) in 1x PBS. Following washes and permeabilization, samples were blocked (10% FBS (Sigma-Aldrich), 0.1% Triton X-100 and 1% w/v BSA (Fisher Scientific) in 1x PBS) for 3-4 hours and then incubated overnight at 4°C in monoclonal Collagen I antibody (mAb39.1, 1:200 dilution in block) (Sarras et al., 1993). They were washed three times, for 10 minutes each, with 1% BSA/0.5% Tween-20 in 1x PBS before an overnight incubation in goat anti-mouse Alexa Fluor™ 546-conjugated antibody (1:1000 dilution in block; Thermo Fisher) at 4°C in the dark. After a final set of washes, stained samples were mounted in tunnel slides (Hackler et al., 2025), and imaged on an Olympus IX81 microscope (Olympus Corporation) using Slidebook 2025 software (Intelligent Imaging Innovations). All samples within an experiment were imaged using the same settings. Z-stacks were collected and a maximum intensity projection was created in Image J (https://imagej.net/software/fiji/). Pixel intensity was quantified as in (Cox et al., 2026) and a Wilcoxon rank-sum test was performed using MATLAB (R2024b, Mathworks).

Mouth opening data acquisition and analysis

Experiments on adults were performed similar to (Goel et al., 2024). For regenerating animals, only *Hydra* that had developed tentacles were used. *Hydra* were decapitated (**Figure 1A**) with a sterile scalpel, allowed to heal for at least 1 hour in HM at 18-20°C, then placed in HM on a tunnel slide and imaged top-down at 1 frame per second using an EVOS FL Auto microscope (Thermo Fisher Scientific). Mouth opening was induced using 100 µl of 0.5 mM quinine hydrochloride in HM (Sigma-Aldrich), made fresh weekly, as in (Goel et al., 2024). Regenerating heads were smaller and

more fragile than adults, limiting the amount of compression during mounting and making focus in both layers more challenging for 2-channel imaging. This was particularly true for regenerated dipyridyl-treated animals, resulting in a lower success rate and greater variability.

Mouth opening image sequences were analyzed using ImageJ. Image sequences were cropped to isolate the mouth area, image thresholding and contrast was applied, as described in (Hackler et al., 2025). Only events starting from a closed or minimally open state (1–2 cell-width), reaching an opening diameter exceeding one third of the head size, and either closing or maintaining maximum opening until the end of recording were included in the analysis. Movies for which automatic thresholding did not work were measured manually in ImageJ using the polygon or freehand tracing tools. All endoderm movies were analyzed manually. The critical ectoderm opening area, defined as the ectoderm area at which the endoderm first opens (Carter et al., 2016), was determined by independent manual analysis by at least two researchers, with consensus decisions recorded. Further data analysis was performed in MATLAB (R2025b and R2026a, Mathworks), with mouth opening area normalized to the maximum opening area of each event. The curve-fitting toolbox was used to fit the normalized mouth opening area and extract the timescale of mouth opening, d, according to (Carter et al., 2016):

$A(t) = a + b / (1 + e^{-(t-c)/d})$

The other fit parameters correspond to the normalized initial mouth opening area (a), the normalized maximum area (b), and the time to reach half maximum (c). Statistical analysis was performed in Google Colab with Python. To determine statistical significance ($p<0.05$), a Kruskal-Wallis test was performed followed by a Benjamini-Hochberg correction.

Artificial intelligence

Claude Sonnet 5.5 (Anthropic) was used to assist in writing MATLAB code for collagen staining quantification, Chat GPT 5.5 (OpenAI) was used to assist in writing MATLAB code for plotting, and Google Colab with Gemini 2.5 Flash was used to write Python code for statistical analysis of mouth opening kinematics. All code was reviewed and validated by the authors and is available via Github (https://github.com/Collinslab-swat/Hydra-Mouth-Opening). All scientific content, interpretations, and conclusions are solely the authors.

Reagents

Reagent	**Description**	**Product #**	**Supplier/Reference**
Hydra vulgaris (watermelon strain)	GFP-labeled ectoderm and DsRed2-labeled endoderm	N/A	(Glauber et al., 2013)
Hydra vulgaris (A10 strain)	Non-fluorescent strain	N/A	(Shimizu et al., 2004)
2-2’ dipyridyl	CAS # 366-18-7	117500100	Thermo Fisher
quinine HCl dihydrate	CAS # 6119-47-7	22630-10G-F	Sigma-Aldrich
dimethyl sulfoxide (DMSO)	CAS # 67-68-5	31727	Sigma-Aldrich
urethane	CAS # 51-79-6	U2500	Sigma-Aldrich
linalool	CAS # 78-70-6	L2602	Sigma-Aldrich
collagen I antibody	Mouse monoclonal antibody	mAb39.1	(Sarras et al, 1993)
goat anti-mouse antibody	Alexa Fluor™ 546-conjugated	A-11003	Thermo Fisher

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