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Reproductive and regenerative performance of symbiotic *Hydra viridissima* under variable thermal conditions

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Abstract:

Hydra viridissima, a freshwater cnidarian, are present in many bodies of water across each continent aside from Antarctica. As ecosystems around the world are impacted by the fluctuating temperatures of climate change, more information is needed regarding how animals and plants will respond to these shifts in the near future. This study aims to investigate the effects of global climate change on the physiology of Green Hydra (*Hydra viridissima*). We measure the regenerative and reproductive capabilities of these hydra across different temperatures. Individual hydra were randomly placed within three different baths, encompassing hot and cold treatments as well as an ambient temperature control treatment. From our investigation, we found that the hydra in our experiment did not reproduce at all in the cold treatment, while there was reproduction in both the ambient and hot treatments. Additionally, hydra in warmer temperatures exhibited more regeneration compared to both cold and ambient temperatures. From these results, we conclude that the reproductive ability of *Hydra viridissima* are inhibited in colder temperatures, while the regenerative ability of this species is more potent in higher temperatures. This implies that green hydra would be able to adapt well to warmer temperatures caused by climate change but may struggle to survive in colder extremes due to their inability to asexually reproduce.

Introduction*:*

The Green Hydra (*Hydra viridissima*) is a sessile, freshwater cnidarian found in the freshwater ponds of Europe and other parts of the world aside from Antarctica (Kaliszewicz 2010). They are diploblastic organisms with tissue composed of an outer ectoderm and an inner endoderm connected by a mesoglea layer which comprises their hydrostatic skeleton (Rahat 1991). Well fed hydra prefer to reproduce asexually by forming buds that detach from the parent polyp (Tomczyk et. al 2014). Under stressful conditions such as cold temperatures or starvation, hydra polyps can reproduce sexually by releasing gametes from their gonads (Ghaskadbi 2020). Despite their minute size (typically between 0.8-3mm), hydra are carnivores and are able to consume animals roughly the volume of their own bodies, feeding on a variety of different insects, crustaceans, and annelids (Massaro & Rocha 2008). During times of starvation, hydra will rely on their zoochlorellae, which are classified as a genus of algal symbionts that provide energy to their host through photosynthesis (Hamada et. al 2018).

Perhaps their most notable feature, hydra are non-senescent (resistant to aging), and they are able to regenerate from multiple regions along their bodies with only 5% of their living cells intact (Bode 2003). These phenomena drove extensive studies on regeneration and anti-senescence properties of hydra, leading scientists to discover that hydra regenerate from just below their hypostomes (mouth region), and they have the capacity to fully regenerate within three days (Bode 2003). However, this regeneration is dependent on ideal

temperature and little is understood about this generation under temperatures outside of their optimal warmth (Bode 2003). Similarly, hydra can asexually reproduce in high frequency forming dense cultures, however, this behavior is contingent on a consistent feeding regimen and being maintained under ideal temperature conditions (Lenhoff and Brown 1970). Over the years, researchers have extensively studied the reproduction and regeneration of hydra, yet very few have considered their adaptability in the context of global climate change as sea temperatures continue to rise (Ye et. al 2019). Considering that climate change will affect a plethora of aquatic organisms in the near future, it is crucial to examine the physiological responses (reproduction and regeneration in this case) of model organisms in relation to the ramifications of warming freshwater bodies. Additionally, with cold extremes also occurring under the effects of climate change (IUCN), the investigation of physiological responses to lower temperatures is also a factor that must be considered when investigating how organisms respond. There is somewhat of a gap in the primary literature regarding the role of temperature in hydra reproduction and regeneration mechanisms, though other studies have found that hydra thermotolerance is strongly host dependent with aposymbiotic hydra having noticeably higher thermotolerance than hydra hosting algal symbionts (Ye et. al 2019).

The manipulation of temperature is necessary to understand how hydra will adapt, survive, and reproduce as their habitats are changed. Here, we test the effect of three temperature range treatments at different extremes on regenerating individuals and reproductive pairs to better understand how climate change will impact the survival and proliferation of symbiotic Green Hydra (*Hydra viridissima*) in the future. We hypothesize that the hydra will not be as sensitive to temperature changes due to their largely unknown albeit widespread geographic distribution and the seasonal as well as daily temperature fluctuations in the waters they inhabit (Stanford 1982). We also predict hydra that experience higher temperatures are likely to bud more often than individuals in the cold treatment due to their increased tendency to reproduce asexually in warm conditions ≥ 20°C (Kaliszewicz 2010). Subsequently, we expect that individuals experiencing colder temperatures are more likely to sexually reproduce due to thermal stress, though it is unlikely sexual reproduction will be observed at all given that hydra in the reproduction treatments will be fed on a regular basis throughout the experiment, thus controlling for the potential effects of starvation induced stress (Ghaskadbi 2020). For our regeneration trials, individual hydra exposed to warmer temperatures are expected to fully regenerate more successfully because their metabolic rates will increase with the steady rise in temperature, however, the opposite result is possible (wherein hydra in colder waters regenerate more successfully) given increased feeding demands coupled with the surge in metabolism that is characteristic of greater heat exposure (Kaliszewicz 2011). Together, this work will provide a superficial baseline understanding of symbiotic hydra response to thermal stress across the metabolic processes of reproduction and regeneration. The knowledge obtained by conducting this study may inform regeneration studies in other hydra species and possibly garner interest in cnidarians as a model organism for anti-senescence studies regarding aging in human beings.

Materials and Methods:

Quantifying Reproductive Success in Hydra viridissima Under Different Temperature Regimes

Green hydra (*Hydra viridissima*) were ordered from the company Carolina Biologicals. To test the effect of temperature on reproduction rates, green hydra were incubated in one of three temperature treatments (cold, ambient, warm). Pairs of green hydra maintained in jars at room temperature were introduced into 6-well plates with each containing two hydra per well (Fig. 1). Once arranged, the plates were sealed and placed in their respective temperature baths; ambient averaged approximately 19℃, cold decreased about 1℃ each day from 17℃, and warm averaged 24℃ +/- 3℃ (Fig. 3). Every 12 hours for 12 days uninterrupted, plates were observed for reproduction, and the number of hydra present in each well were recorded. Wells that contained a single budding hydra were indicated with a value of 2.5, whereas wells with mortality or successful reproduction were indicated with a total count of whole hydra present. After each round of data collection, plates were aerated, measured for salinity (using a refractometer) to check for the presence of saltwater contamination in each well, and placed back into their respective temperature treatments. Hydra were fed *Artemia* nauplii (which were rinsed with deionized water) every 4 days to avoid the effects of starvation on reproduction.

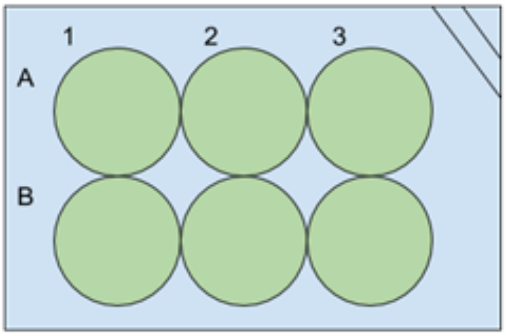

Figure 1 | 6-Well Plate Used for Green Hydra Reproduction Experiment. Each green circle represents a well where 2 intact Green Hydra (*Hydra viridissima*) were placed in deionized water (0 PSU) and monitored for budding and sexual reproduction.

Categorizing Regeneration in Hydra viridissima Under Different Temperature Regimes

Green hydra (*Hydra viridissima*) were acquired from the same company as those used in the reproduction experiment: Carolina Biologicals. Trisected hydra along with uncut counterparts (serving as positive controls) were placed in 48-well plates and incubated in one of three saltwater baths shared with the reproduction trials corresponding to cold, ambient, and warn temperatures in order to test the effect of temperature on regenerative ability. Prior to placement within 48-well plates, individual hydra were pipetted onto glass slides with minimal water and trisected under a dissecting microscope using dissection scalpels. The first three rows were occupied with six (6) cut individual hydra, each body part occupying an unshared well and organized into three respective rows: head, midsection, and foot. In addition, a fourth row was occupied by 6 whole individual hydra to serve as positive controls throughout the experiment (Fig. 2). After pipetting hydra into the 48-well plates, wells were topped off approximately halfway with

room temperature deionized water. Plates were sealed shut and floated in large water baths, which were maintained at their distinctive temperature treatments. Once the tubs were occupied with their respective plates, overhead lights were left turned on for 12 hours each day (~50 PAR) to maintain photosynthesis in the hydra zoochlorellae. Wells on the outer perimeter of each plate were filled with deionized water to test for presence of saltwater contamination and act as a buffer should any leaks occur while plates were floating in their respective baths.

Two trials of regeneration experiments were carried out with each trial representing a unique range of experimental temperatures within both hot and cold treatments. Meanwhile, the temperature of the ambient control tub averaged 18.75℃ consistently with a standard deviation of 0.367℃ throughout both experiments. Trial 1 commenced six days before Trial 2. The warm treatment for Trial 1 began at a temperature of 21℃ then gradually increased approximately 3℃ whereas Trial 2 averaged a more constant 25.5℃ ± 0.75℃. The cold treatment was steadily decreased throughout the experiment, dropping roughly 1℃ per day, thereby leaving Trial 2 to begin at approximately 12℃, while Trial 1 began at 17℃ (Fig. 3).

Trial 1 hydra were initially collected from jars maintained at room temperature; each water-bath began within 2℃ of room temperature, thus about equal to the ambient temperature control. All subsequent hydra used in Trial Two were collected from jars incubating in their respective water-treatments to avoid temperature-shocking samples; Trial Two began with experimental temperatures dissimilar from room temperature. Hydra in the regeneration experiment were only fed before the beginning of each trial and not during.

Each 48-well plate was aerated daily and monitored for regenerative growth. Regeneration values were assessed by photographing and categorizing each hydra every 24 hours, timed to follow each temperature change by 10 hours. An assigned numerical value of 1 indicated little to no regeneration progress, while 2 indicated approximately half of the hydra had regrown, and 3 indicated that the hydra had fully regenerated. Dead hydra with visible green symbionts were categorized as -1 while hydra that could not be seen or observed were categorized as 0 and were assumed to have died and disintegrated.

Data processing and analysis

All data collected were sorted into Microsoft Excel sheets and converted into comma separated value files for processing and statistics in R. To avoid confusion, plates and data corresponding to both cold bath treatments were labeled and referred to as “freezing,” while plates and data from the warm treatments were labeled “hot.” Following suit, the plates and data recorded from the ambient temperature treatment were labeled “control.” Stacked histograms were created for both regeneration trials to visualize the frequency that certain regenerative states were observed. Multiple chi-square tests were also performed to assess the significance of the relationship between temperature and reproductive success within each treatment as well as Tukey tests to determine significance between each individual temperature treatment regenerative success. A Tukey test was also run between reproduction in the freezing treatment versus reproduction in the hot and control treatments. We recognized statistical significance at a p-value ≤ 0.05.

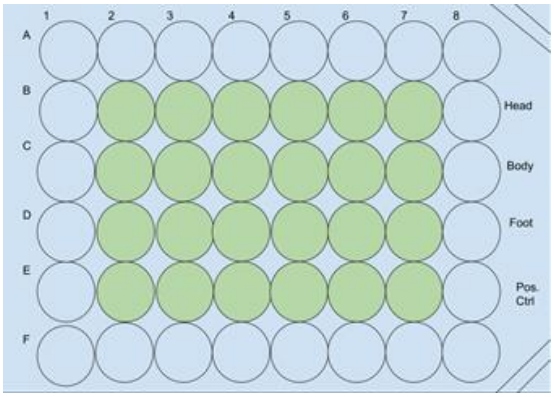

Figure 2 | 48-Well Plate Used for Hydra Regeneration Study. Each green circle represents a well where singular living hydra were placed, whereas blue circles represent wells where only deionized water was placed. Row B wells correspond to the head from each cut hydra, C to isolated midsections, D to each foot from the cut hydra, and E to whole, unmanipulated hydra which served as a positive control.

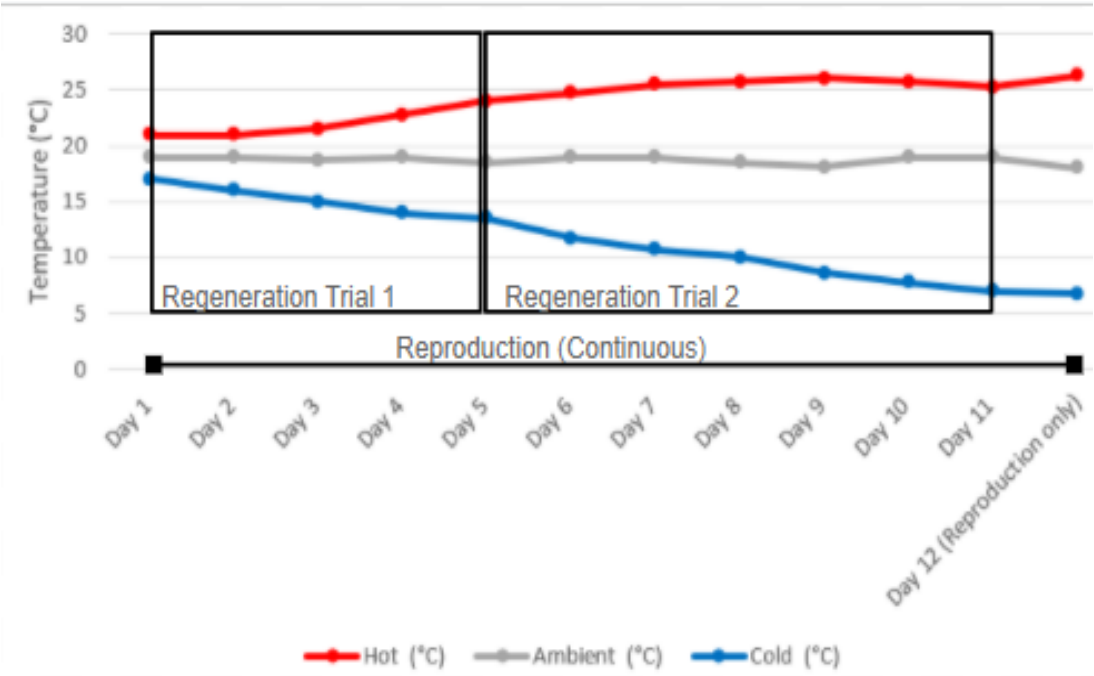

Figure 3 | Average Temperatures in Degrees Celsius Per Treatment Over the Duration of the Experiment. The hot treatment is labeled in red (highest values), ambient treatment in grey (middle values), and cold treatment in blue (lowest values). Regeneration Trial 1 spans days 1-5, as is visualized by the first box, and Regeneration Trial 2 spans days 6-11, as is visualized by the second box. The sole reproduction experiment spans the full 12 days

Results:

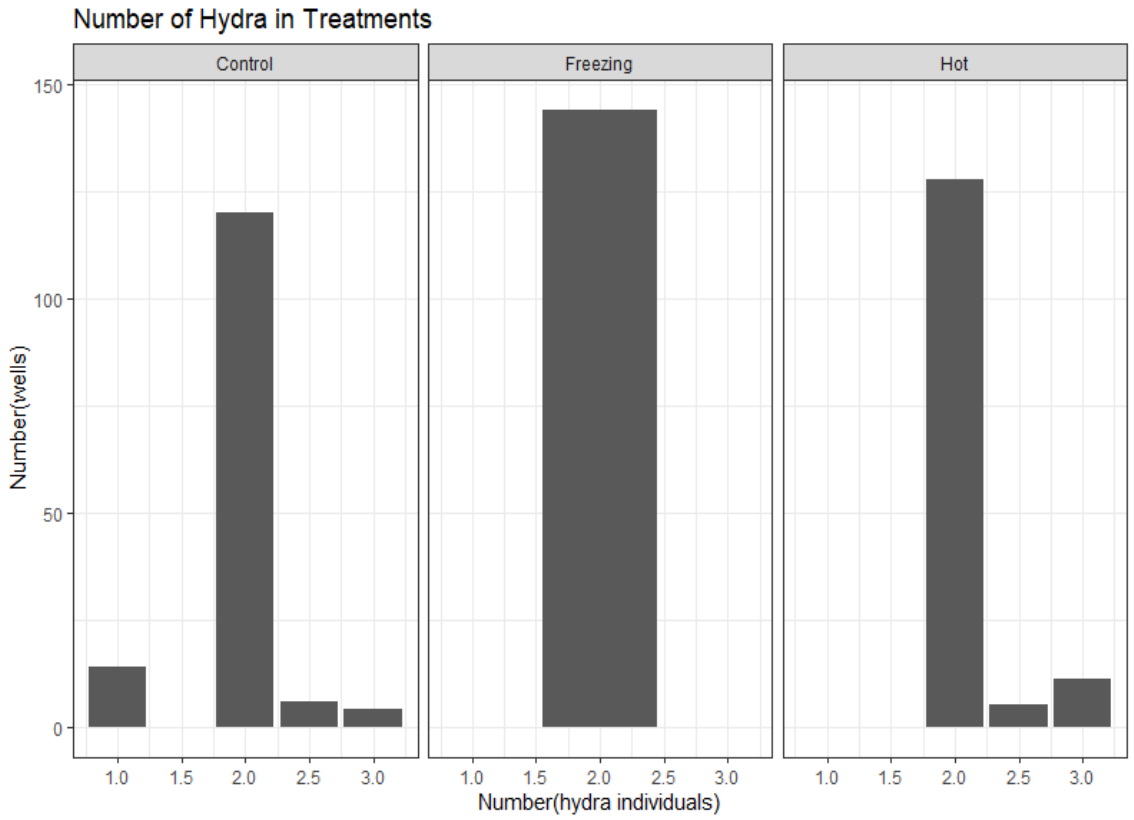

Figure 4 | Frequency of counts for each treatment in reproduction. Numbers above 2 signify that reproduction had occurred (2.5 representing one of the two hydra budding), and numbers equal to or less than 2 show that no reproduction occurred. A chi-squared test reveals that there is a significant relationship between treatment and whether or not reproduction occurred (p-value=0.0003264) and a Tukey test shows a significant difference between reproduction in the freezing treatment and both the control and hot treatments (p-values of 0.03269 and 0.0002 when compared with control and hot respectively). Meanwhile, there was no significant difference between control and hot treatments (p-value = 0.2873).

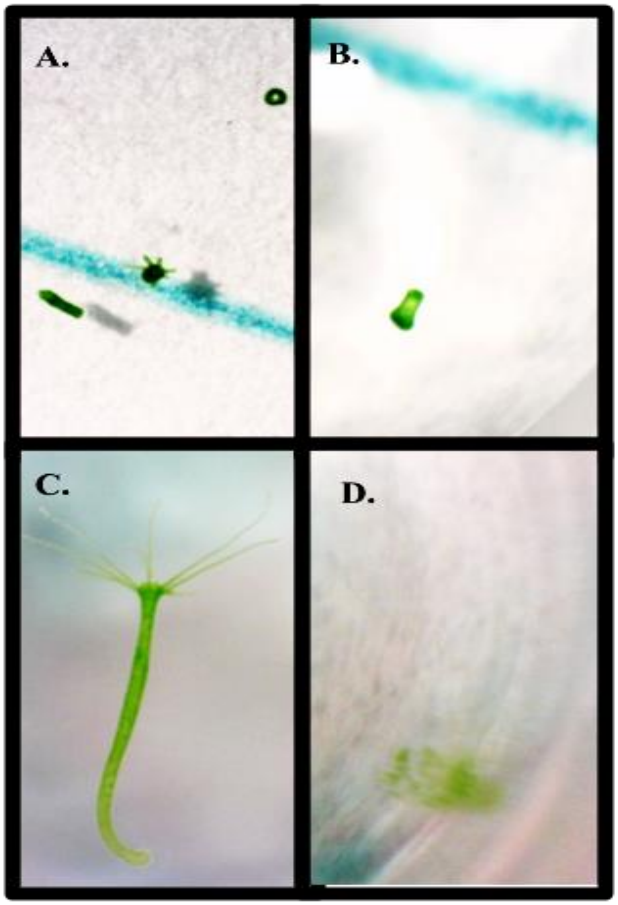

Figure 5 | Stages of Regeneration for Green Hydra under Ambient Conditions. (A) Green Hydra immediately after trisection: top right, foot; center, head; bottom left, midsection. (B) Green Hydra midsection 12h after slicing (photo taken 11/26/2020). (C) Uncut Green Hydra, which is comparable to a fully regenerated sample. (D) Dead Green Hydra with all of its algal symbionts still vibrant green (death occurred 24h after slicing on 11/30/2020).

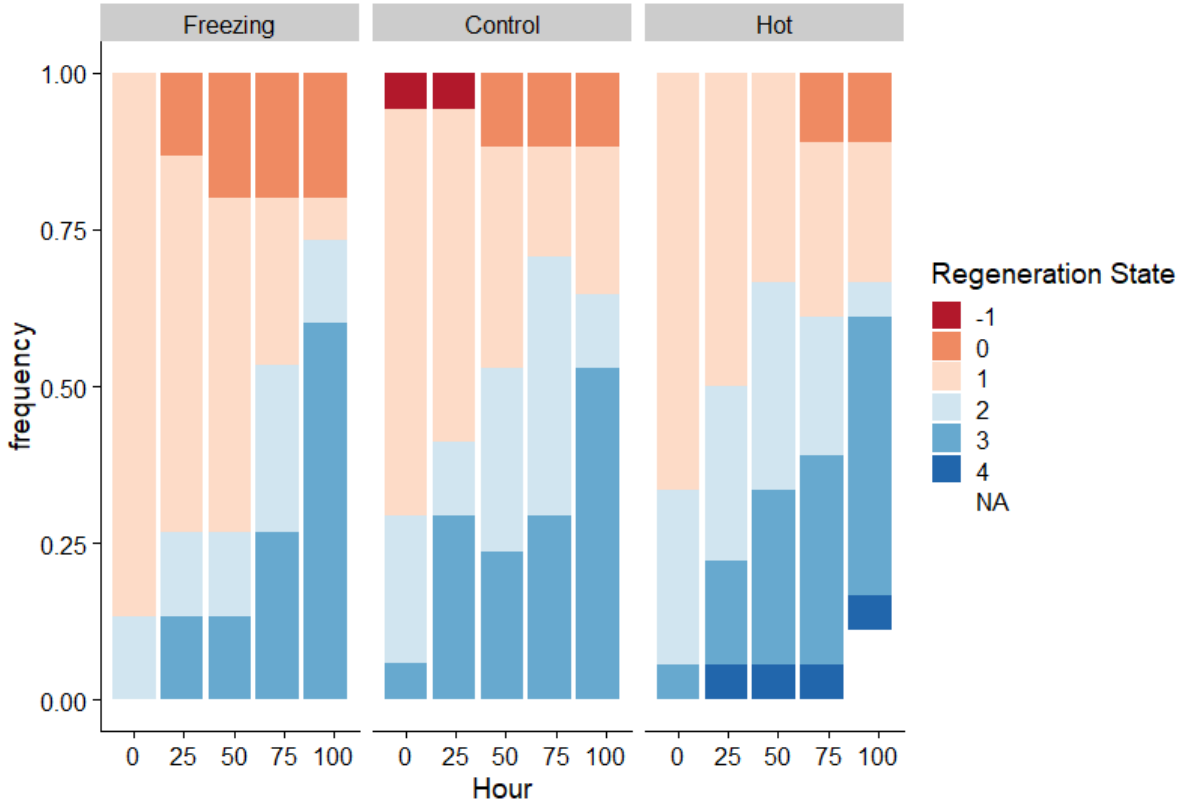

Figure 6 | Frequency of regeneration observations for Trial 1 on a timescale of 100 hours. Regeneration categorizations go as follows: a value of -1 indicates hydra mortality with visibly healthy zoochlorellae; a value of 0 indicates that nothing was observed in a given well; a value of 1 indicates little to no growth; a value of 2 indicates that a given hydra had grown back to about half size; a value of 3 indicates full unhindered regeneration; a value of 4 indicates the presence of another hydra due to reproduction (only asexual budding was observed). Values 2 or above were categorized as successful regeneration, while values below represented no regeneration. There were no statistical differences across treatments regarding regeneration success (p-values > 0.05).

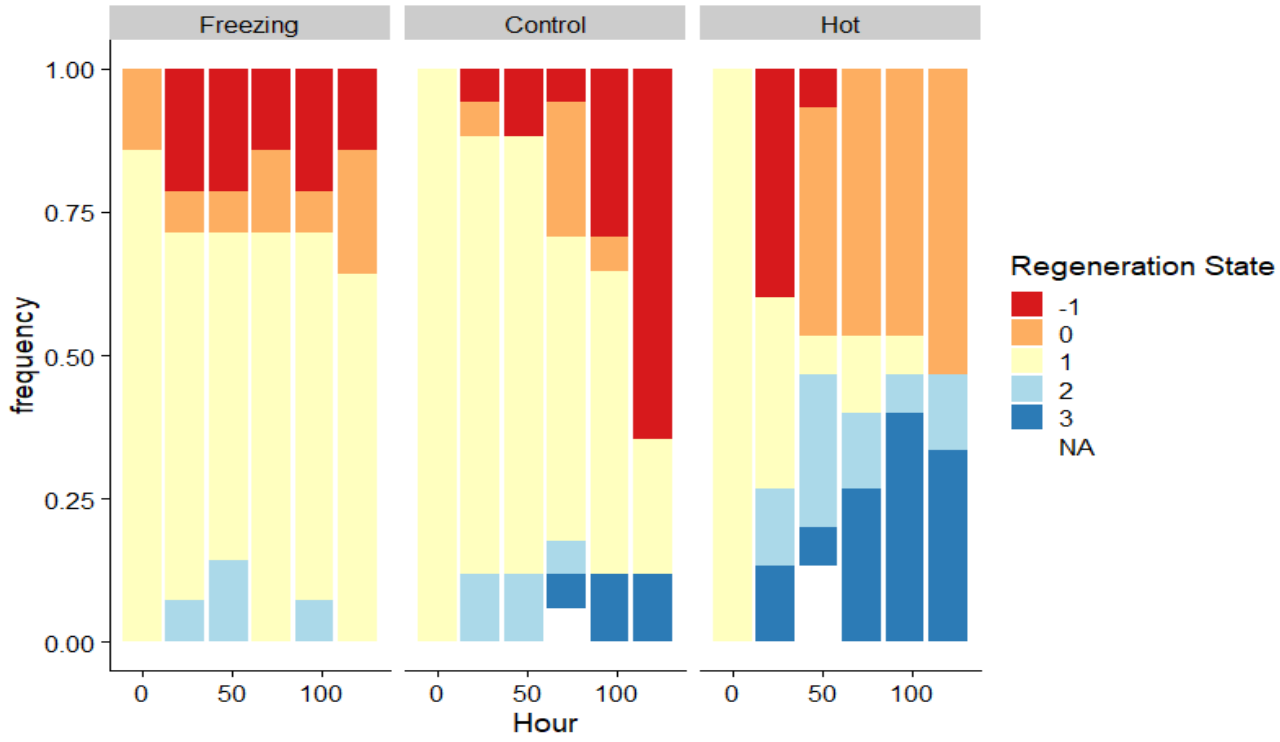

Figure 7 | Frequency of regeneration observations for Trial 2 on a timescale of 120 hours. Regeneration categorizations go as follows: a value of -1 indicates hydra mortality with visibly healthy zoochlorellae; a value of 0 indicates that nothing was observed in a given well; a value of 1 indicates little to no growth; a value of 2 indicates that a given hydra had grown back to about half size; a value of 3 indicates full unhindered regeneration. Values 2 or above were categorized as successful regeneration, while values below represented no regeneration. While there was no statistical difference between cold and ambient treatments (p-value = 0.5732), the hot treatment was statistically significant compared to both ambient and cold treatments (p-values of 0.0000218 and 0.0000006 respectively).

Regeneration Success Rates Across Temperatures

Throughout the full duration of Trial 1, 100% of the uncut hot treatment hydra survived, 83.3% of the uncut control hydra survived (one death), and 83.3% of the uncut cold treatment hydra survived (one death). Throughout the entire duration of Trial 2, 100% of the uncut hot treatment hydra survived, 100% of the uncut control hydra survived, and 33.3% of the uncut cold treatment hydra survived. Therefore, we are inclined to believe temperature is a driving factor only in the second trial, representing the lowest cold temperatures. Otherwise, temperature as a factor is considered to impact regeneration uniquely, with statistical success determined via chi-square tests and Tukey tests, recognizing statistical significance at a p-value p-value ≤ 0.05 for each chi-square test and a 95% family-wise confidence level for each Tukey test.

Trial 1 temperature treatments were statistically insignificant when compared one-by-one against each other, such that no one temperature treatment influenced regeneration rates significantly relative to any others (with all p-values > 0.05). Both Trial 1 and Trial 2 were evaluated for

regenerative success versus failure, with any regenerative stage showing growth considered a success (identified with a 2, 3, or 4 in Fig. 6 and Fig. 7). The hot treatment in Trial 1 had a 54.54% regeneration success rate, which is significantly different when compared against its own failure rate, irrelevant of cut-type (CHI = 0.72727, df = 1, p-value = 0.3938). The ambient treatment in Trial 1 yielded a 51.76% regeneration success rate, which is statistically insignificant when compared to its own failure rate (CHI = 0.10588, df = 1, p-value = 0.7449). The cold treatment in Trial 1 had a regeneration success rate of only 38.67%, which is significantly different from its failure rate (CHI = 3.8533, df = 1, p-value = 0.04965). When comparing Trial 1 hot treatment failure rate to cold treatment failure rate, values were not significantly different, thus implying neither hot nor cold treatments were more detrimental to the health and regeneration abilities of green hydra (CHI = 0.42587, df = 1, p-value = 0.514).

Trial 2 hot temperature treatment had significantly different success rates when compared against the Trial 2 cold temperature treatment and the Trial 2 ambient temperature treatment, respectively. Neither cold nor ambient temperature treatments in Trial 2 yielded significant rates when compared against each other (p-values = 0.5732). Yielding a success rate of 4.76%, the cold treatment for Trial 2 was significantly different when comparing individual failure to success rates (CHI = 68.762, df = 1, p-value < 0.0000). Similarly, Trial 2 ambient temperature treatment yielded a success rate of 9.90% and was statistically significant with regards to individual failure versus success rates (CHI = 64.96, df = 1, p-value < 0.0000. Finally, Trial 2 hot treatment also yielded a significantly different success rate compared to individual failure rate (CHI = 10.227, df = 1, p-value = 0.001384) with a success rate of 32.95%.

While addressing success rates between Trial 1 ambient temperature treatment and Trial 2 ambient temperature treatment, a Tukey test was used to evaluate significant difference. In theory, the experimental controls should yield statistically similar results as no variables were noted to have changed from one trial to the other. The Tukey test found a large significant difference, thereby adding reasonable doubt regarding the consistency of methods and controlled variables between Trial 1 experimental temperature treatments and Trial 2 experimental treatments. As such, Trial 1 and Trial 2 cannot confidently nor accurately be compared against the other.

Discussion:

Individual Hydra viridissima reproduce more in warmer temperatures

Out of the three treatments in our experiment, only the hot and control treatments exhibited asexual reproduction through budding, while none of the hydra in the freezing treatment experienced reproduction in any form (Fig. 4). Similar conclusions have been reported in previous studies concerning hydra reproduction (Kaliszewicz 2010). Increased temperatures do not seem to increase reproductive ability within the hydra as there was no significant difference between reproduction within the ambient and hot treatments. However, the reproduction within the cold treatment was statistically significant from both the ambient and hot treatments (Fig. 4), suggesting that colder temperatures inhibit asexual reproductive capabilities within hydra.

Hydra viridissima exhibit more regeneration in warmer temperatures

For both regeneration trials, our findings were consistent with the tentative hypothesis we formulated surrounding the impact of warmer temperatures on the increase of metabolic rate (Kaliszewicz 2011). When looking at Trial 1, all of the treatments had similar regeneration success and there were no statistical differences between any of the treatments. This can be explained by the fact that Trial 1 was conducted when the temperatures across treatments were close to each other. However, in Trial 2, where there were more extreme temperatures, hydra within the hot treatment had more individuals in regeneration states that were 2 or above (Fig. 7), being significantly different from any of the other two treatments. Meanwhile, there was no significant difference between cold and ambient treatments in Trial 2. In general, it appears that hydra within warmer temperatures have an increased regeneration ability, while hydra in colder temperatures don't seem to have their regeneration significantly affected.

Experimental Design Flaws

There were several limitations that were encountered within the duration of this study. This experiment was conducted in tandem within the same system as two other experiments that worked with saltwater invertebrates including *Exaiptasia pallida* and *Crepidula fornicata*. Because of these circumstances, we had to use well plates to house our freshwater hydra and float them over the treatment tubs that were continuously set to different temperatures. The increased buoyancy characteristic of salt water may have aided in preventing leaks but made the plates slower to reacclimate when placed back into the tubs after recording measurements. Unfortunately, we were not able to establish a larger sample size due to time constraints and the tedious nature of trisecting individual hydra. Because the hydra were prone to contracting on the microscope slides for a short period of time when agitated, precisely equal cuts were difficult to obtain. For this reason, we categorized cut hydra into regenerative states as opposed to measuring their length. The amount of time the hydra spent out of the treatments for cutting, measurement, and photographic purposes may have also caused the hydra to experience different temperatures outside of our experimental design due to acclimation to room temperature. The separation of the specimens into the wells of each plate, containing relatively miniscule volumes of deionized water, created unique artificial microenvironments where water chemistry likely varied, if only slightly. We did not dose additional nutrients that are present in the wild to limit the introduction of additional variables, however the possible inconsistencies in water chemistry were probably exacerbated in the reproduction experiment within wells where there were residual food particles left over from feeding due to some hydra having the tendency to eat or expel more food than others. Unfortunately, we were also unable to regularly change the water within the wells in the experiment on a regular basis and simply used the same water for the duration of the experiment. While we did aerate the samples daily to prevent buildup of metabolic byproducts and introduce fresh oxygen, this method may have been less effective compared to having constant agitation in the water.

Further Research

If this study were to be repeated, we would conduct the experiment with

specimens contained in larger volumes of water incubated at the target temperatures of each treatment. We would also increase the sample size and examine the rate at which regeneration occurs by taking quantitative measurements of physical attributes.

Future studies could expand upon this one by examining the internal gene expression and metabolic mechanisms regulating reproduction and regeneration in response to various gradual temperature changes in *Hydra viridissima.* Additionally, the regenerative capabilities of different sections of the hydra in addition to rate of regeneration could also be evaluated. While our experiment did not account for measuring these characteristics, we noticed that certain body parts appeared to regenerate quicker than others. In the reproduction part of the experiment, we also noticed that individuals within the cold treatment tended to shrivel up, while those in the hot treatment were extended to a great extent. The impact of these physiological responses on our experiment is unknown. Only data on asexual reproduction of hydra was obtained in this experiment, and the effect that temperature has on sexual reproduction is absent in this study. Therefore, further research on sexual reproduction is needed to accurately conclude how climate change affects reproduction.

Conclusion

From our experiment, we can conclude that climate change will have some impact on *Hydra viridissima*. Reproduction is inhibited by colder temperatures and relatively unaffected by hotter temperatures while regeneration success rates are increased by hotter temperatures but not negatively influenced by colder temperatures. A possible prediction from these results is that the effect climate change has on warming bodies of water is unlikely to negatively impact the success and general health of wild hydra populations. However, unexpected cold-snaps worsened by climate change may be likely to negatively impact hydra populations due to a reduced asexual reproduction capability in cold temperatures. On the other hand, the effect on reproduction in general cannot be concluded from this study due to a lack of data regarding sexual reproduction.

Regeneration success was expected to increase as temperatures increased due to their physiological response of increasing their metabolic rate. This study projected that warming water temperatures will increase hydra's ability to quickly regenerate and survive the regenerative process, but other temperature manipulations will not negatively impact regeneration. All in all, this experiment is a good preliminary study for hydra, but there are several other factors that may be able to influence hydra survival as climate change progresses. Due to hotter temperatures increasing hydra regenerative success and not negatively impacting reproduction, climate change may ultimately benefit *Hydra viridissima*.

Acknowledgements

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