Druggin' Jellies:

The Effects of Common Wastewater Pollutants on *Aurelia aurita* Polyps

by

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Abstract

Aurelia aurita jellyfish blooms have been occurring globally more frequently and in larger magnitudes than previously observed, with disastrous consequences ranging from the disruption of fisheries to the shutdown of power plants. These blooms have been observed to occur at the highest frequency and largest scale along coastlines that are chemically rich from wastewater runoff. Despite this, there is a significant lack of published research regarding the effects of wastewater chemicals, such as caffeine and estradiol, on *A. aurita*. This study aimed to determine whether caffeine and estradiol affect the polyp life stage of *A. aurita*. We hypothesized that estradiol would decrease polyp reproduction and strobilation due to hormonal disruption, caffeine would increase polyp reproduction and strobilation due to increased enzyme activity, and combined caffeine and estradiol would increase polyp reproduction and strobilation because caffeine is present in a much higher concentration, so its positive effects will overshadow any negative effects of estradiol. We exposed *A. aurita* polyps to caffeine, estradiol, and combined caffeine and estradiol over a twelve day period. To monitor polyp strobilation and reproduction, the number of elongated polyps, ruffled polyps, asexual buds, and the total number of polyps in each treatment were counted. Treatment response variables were compared to a control through GAMLSS models, and Spearman's Rank-Order test of correlation was used to determine the effect of time on all response variables. We found that estradiol had no effect on *A. aurita* polyps, caffeine caused an increase in *A. aurita* polyp strobilation, and the combination of caffeine and estradiol caused an increase in both polyp strobilation and reproduction. This indicates that caffeine, and the combination of caffeine and estradiol, may play a role in the harmful *A. aurita* blooms that occur along polluted coastlines worldwide.

Keywords

Jellyfish Blooms, Environmental Toxicology, Strobilation, Caffeine, Estradiol, Interactive Effects

1. Introduction

Globally, jellyfish blooms have been occurring more frequently and in larger magnitudes than previously observed (Mills et al., 2001). These blooms are harmful to marine ecosystems and humans alike, with their catastrophic effects ranging from the disruption of fisheries to the temporary shutdown of power plants worldwide due to their capacity to clog fishnets and seawater intake screens (Shimomura, 1959; Matsueda, 1969). An abundant species that is frequently responsible for immense blooms is *Aurelia aurita* (Linneaus, 1758), commonly known as the moon jellyfish (Mills et al., 2001).

A. aurita is a member of the phylum Cnidaria and is found throughout almost all oceans

globally (Lucas, 2001; Ruppert et al., 2004). Adult *A. aurita* medusa (Fig. 1A) release gametes that form planula larvae (Fig. 1B), which then develop into small, long-lived benthic polyps (Fig. 1C). These polyps can reproduce asexually via budding to create more polyps, or undergo a process known as strobilation (Fig. 1D) to produce pelagic ephyra (Fig. 1E) (Ruppert et al., 2004). The amount of asexual reproduction occurring corresponds with the number of polyps present, and through strobiliation each polyp can produce many ephyra that then develop into large adult medusae. This means that the magnitude of asexual reproduction and strobilation directly impact the scale of adult medusa *A. aurita* blooms (Fuchs et al., 2014). As such, factors impacting polyp reproduction and strobilation have strong ecological relevance (Wang et al., 2020).

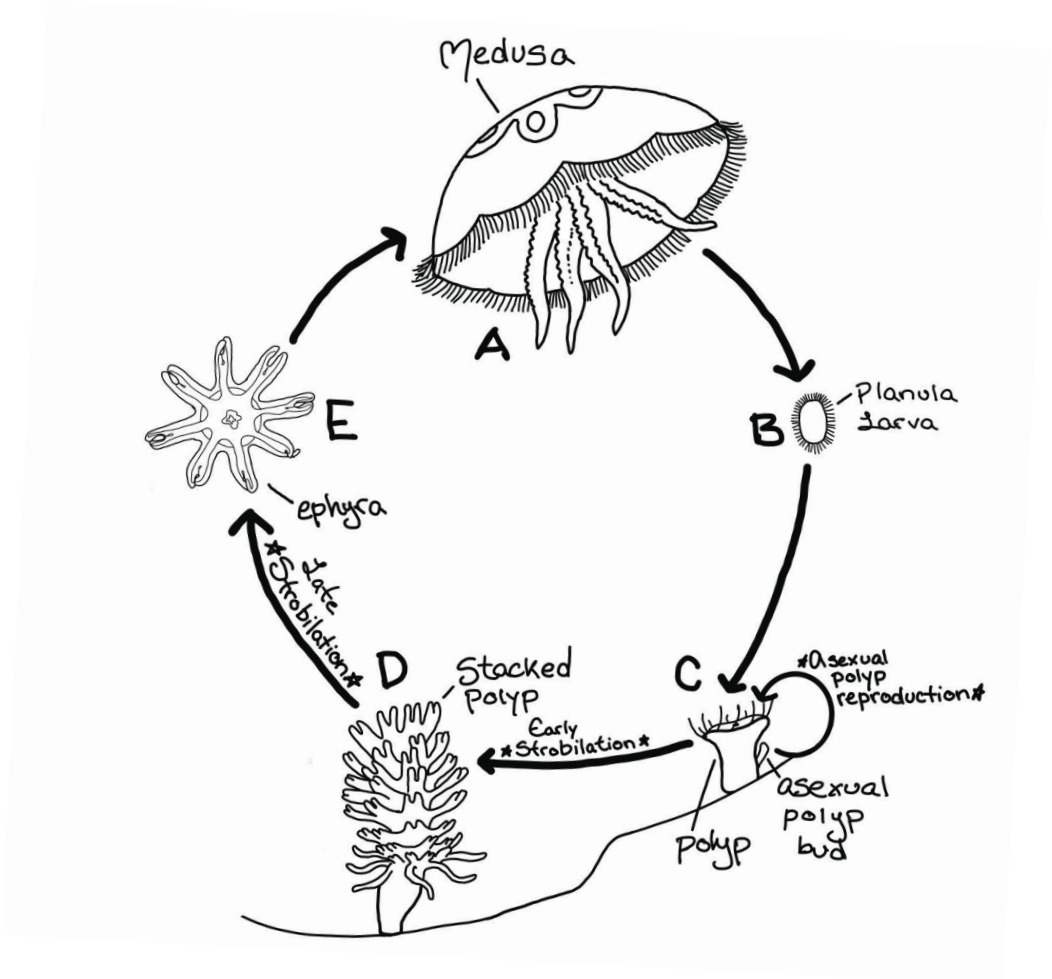

Figure 1. The *Aurelia aurita* life cycle. Adult medusae (A) release eggs and sperm to form planula larvae (B) that develop into benthic polyps (C). These polyps can form buds that reproduce asexually or form transverse segments and stacks in early strobilation (D). Stacked polyps undergo late stage strobilation to produce juvenile ephyra (E) that grow into adult medusae.

Despite its importance, little is known about the mechanisms behind strobilation in *A. aurita* (Fuchs et al., 2014). Exposure to some chemicals, such as Lugol's iodine (Spangenberg et al., 1967), acetylcholine chloride, 5-methoxy-2-methylindole or indomethacin (Helm & Dunn, 2017), has been found to induce strobiliation (Ishii et al., 2008; Wang et al., 2020). Furthermore, *A. aurita* blooms have been observed to occur at the highest frequency and largest scale along coastlines that are chemically rich from wastewater runoff (Mills et al., 2001), such as Tokyo Bay in Japan (Mills et al., 2001), Elefsis Bay in Greece (Papathanassiou et al., 1987) and the Saanich Inlet in Canada (Ishii & Tanaka, 2001). However, there is a significant lack of published research regarding the effects of wastewater chemicals on the strobilation and reproduction of *A. aurita*.

There are many anthropogenic pollutants that enter marine environments through wastewater runoff (Garcia et al., 2013). One such pollutant is estrogen, which is found very frequently in nearshore marine environments (Atkinson et al., 2003). Estrogens end up in the environment through municipal sewage treatment plants and agricultural practices, and it is estimated 30,700 kg of estrogen are released into the environment each year from birth control alone (Adeel et al., 2017). Excess environmental estrogens are thought to disrupt the metabolic, reproductive (Janer & Porte, 2007) and endocrine function (deFur et al., 1999) of some marine invertebrates. Estradiol is the most biologically active form of estrogen in wastewater (Atkinson et al., 2003) and is known to be involved in some cnidarian reproduction, such as coral spawning (Tarrant et al., 2004). Additionally, the use of hormones to regulate life stage transitions in other marine organisms is well documented (Fuchs et al., 2014). However, the effects of estradiol on the reproduction and polyp-to-ephyra transition of *A. aurita* are largely unknown.

Caffeine is another common anthropogenic pollutant found in wastewater (Krogh et al., 2017) and the marine environments where blooms are commonly observed (Papathanassiou et al., 1987). Like estradiol, wastewater treatment systems cannot effectively remove caffeine, making it extremely widespread in aquatic systems and present in high concentrations (Moore et al., 2008). For example, a 2017 literature review showed that of twenty-two seawater samples collected worldwide, fifteen contained caffeine and six had high enough concentrations to have potential long term effects on marine organisms (Dafouz et al., 2017). The effects of these high concentrations of caffeine on marine organisms vary from increased biotransformation enzyme

activity in the clam *Corbicula fluminea* (Aguirre-Martinez et al., 2015) to small effects on the reproduction of a freshwater invertebrate species (Moore et. al, 2008). However, like estradiol, the effects of caffeine have not been studied in most marine organisms, including *A. aurita*. Considering the prominence of *A. aurita* blooms in coastal areas with high wastewater runoff, it is important to consider the effects of this chemical on *A. aurita* strobilation and asexual reproduction.

Both estradiol and caffeine are present in wastewater, with caffeine presence typically being 4.4 thousand times more concentrated than estradiol presence (Heffron et al., 2016; Krogh et al., 2017). As a result, the effects of caffeine may be more prominent in organisms exposed to wastewater than the effects of estradiol, simply because of their relative concentrations. Alternatively, a study by Silva et al. (2002) determined that combining weak estrogenic chemicals resulted in significant "mixture effects", which are observed effects that cannot be accounted for through the sum of individual component effects. This phenomenon indicates that there may be unpredictable interactions when estradiol and caffeine are combined. However, there is scarce published literature discussing estradiol and caffeine in combination, so it is difficult to predict the effect this chemical interaction will have on *A. aurita* polyp strobilation and reproduction.

Thus, the objective of our study was to determine if caffeine and estradiol affect strobilation and reproduction in the polyp life stage of *A. aurita*. To do this, we tested the effects of caffeine and estradiol on polyps separately. We also tested the two chemicals together, allowing us to monitor for any combined effects of these pollutants since both are likely to be present simultaneously in wastewater. Firstly, we hypothesize that estradiol will decrease polyp reproduction and strobilation due to hormonal disruption (Janer & Porte, 2007). We predict that fewer polyps will exhibit elongation, ruffling or budding when exposed to estradiol than in the control treatment. We also predict that estradiol exposure will result in a smaller increase in the total number of polyps than in the control. Secondly, we hypothesize that caffeine will increase polyp reproduction and strobilation due to increased enzyme activity (Aguirre-Martinez et al., 2015). We predict that more elongation, ruffling and budding will be observed in caffeinated polyps than in the control treatment. We also predict that caffeine exposure will result in a greater increase in the total number of polyps than in the control. Finally, we hypothesize that combined

caffeine and estradiol will result in an increase in polyp reproduction and strobilation because caffeine is present in a much higher concentration, so its positive effects will overshadow any negative effects of estradiol. Here, we predict that more polyps will exhibit elongation, ruffling or budding when exposed to both chemicals than in the control. We also predict combined chemical exposure will result in a greater increase in the total number of polyps than in the control treatment.

2. Materials and Methods

2.1. Study Organism and Husbandry

Aurelia aurita polyps (N = 180 polyps) were collected from the Shaw Centre for the Salish Sea. We randomly separated the polyps into groups of 15 and placed them on watch glasses to settle. We placed the twelve experimental watch glasses into individual mason jars (N = 12 jars), which were then placed into a flow-through water table for temperature regulation. We inserted an airline into each jar to ensure adequate oxygen. The polyps remained in these closed systems for over two weeks to acclimatize, and we fed them twice daily (after some investigation to determine the feeding frequency that polyps appeared most successful at) with *Artemia spp.* nauplii. Prior to feeding, we filtered out the *Artemia* from the seawater to prevent dilution of the chemical concentration within the treatments.

Every three days (during data collection), we conducted water changes using seawater that contained the corresponding chemical concentrations (specified below) for each treatment. We removed 500 mL of water, and replaced it with 500 mL of the appropriate seawater and chemical solution to all jars to maintain consistent volume. The seawater used for water changes was approximately 10°C.

2.2. Experimental design

The night prior to the experiment, we induced strobilation in the polyps so that potential negative effects on polyp strobilation could be monitored. To do so, we raised the ambient temperature of the polyps to room temperature (approximately 20°C) and treated them with a 20 drops/L solution of Lugol's iodine overnight (Spangenberg, 1967).

We then haphazardly separated the jars into four treatment groups: control, caffeine,

estradiol, and combined caffeine and estradiol. Each treatment contained three jars with 15 polyps per jar (N = 45 polyps per treatment) and we maintained the treatments for twelve days to allow for adequate strobiliation time (Wang et al. 2020).

The caffeine treatment consisted of seawater with 22,330 umol/L of caffeine, following recommendations from Krogh et al. (2017) for an ecologically relevant concentration. The estradiol treatment consisted of seawater with 5 umol/L of estradiol, following recommendations from Heffron et al. (2016) for an ecologically relevant concentration. The combined caffeine and estradiol treatment consisted of seawater with 22,330 umol/L of caffeine and 5 umol/L of estradiol. This final treatment allowed us to monitor interactive effects of these pollutants since both are likely to be present at the same time in wastewater, and is henceforth referred to as the combination treatment.

We collected data every three days, beginning on the first day of the experiment prior to the addition of chemicals. We monitored the number of elongated and ruffled polyps, as elongation and ruffling are signs of strobilation (Wang et al., 2020). Ruffled polyps were those with clear divots and/or folding on their sides (Fig. 2A) and elongated polyps were those with a triangular or rectangular shape (Fig. 2B). To minimize subjective variation in these measurements, one researcher always counted elongation and the other counted ruffling. We also monitored the polyps for transverse segments (clear horizontal lines forming across the polyp; Fig 2D), stacking (complete segmentation of the polyp into immature, still-connected ephyra; Fig 2E) and ephyra production, as these are also signs of strobilation (Wang et al., 2020). Additionally, we counted the number of polyp buds and the total number of polyps in each jar to monitor for asexual polyp reproduction (Ruppert et al., 2004). A polyp bud was considered a protrusion from the main body of a polyp that had not yet split off or developed its own tentacles (Fig. 2C).

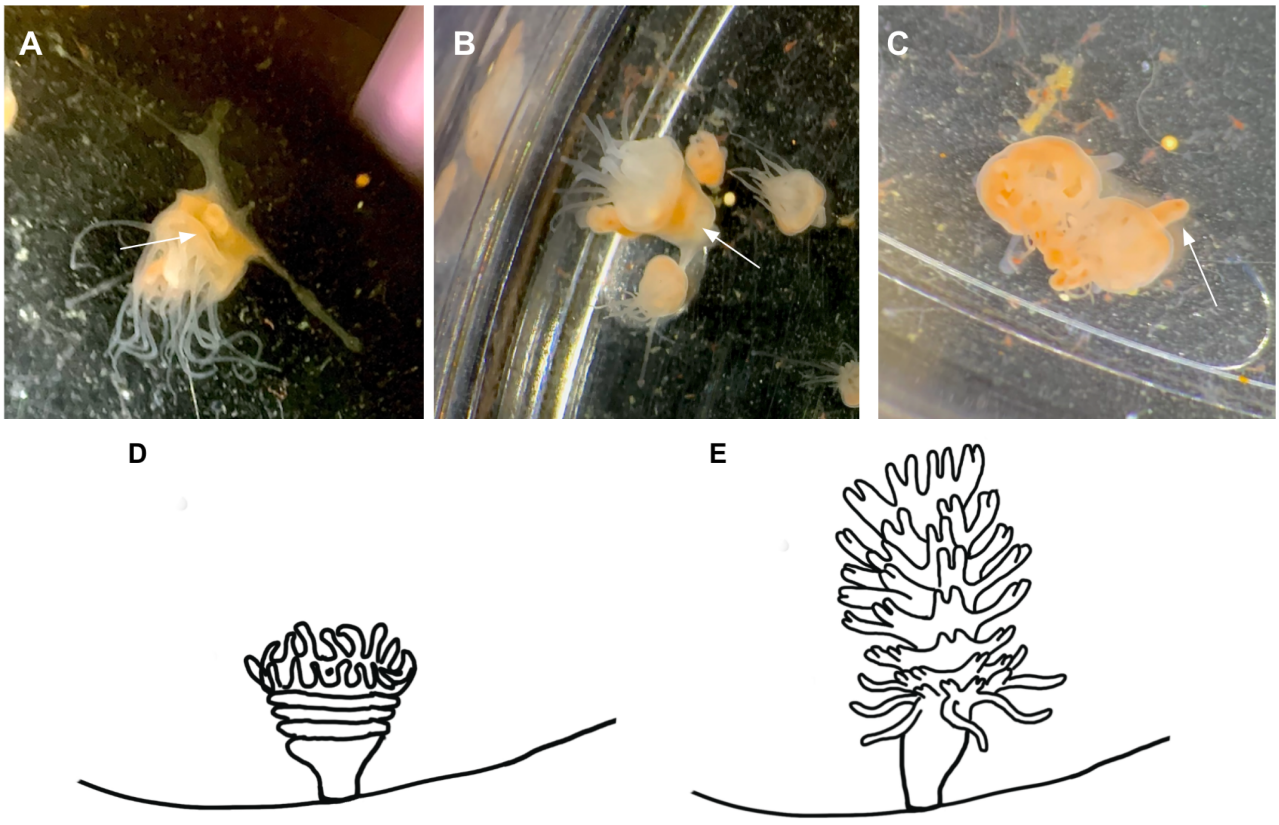

Figure 2. The early stages of *Aurelia aurita* polyp strobilation (ruffling [A], elongation [B] and budding [C]) observed under a dissection microscope at 2x magnification, and the later stages of strobilation (transverse segmentation [D] and stacking [E]) drawn for reference.

2.3. Statistical Analyses

We ran a fit of univariate distributions to non-censored data using the *fitDist* function from the gamlss package to determine the best distribution fit for the dataset of each individual response variable (Rigby et al., 2005). The ruffling data was fit to the Weibull distribution (Weibull, 1951), elongation and budding were fit to the Negative Binomial Type II distribution (Simon, L., 1962) and the total number of polyps was fit to the Delaporte distribution (Delaporte, 1960; Table S1). Using these distributions, two sets of Generalized Additive Models for Location Scale and Shape (GAMLSS) were built to test our treatment response variables against our control (Rigby et al., 2005; Table 1). Jar was included as a random effect in all models to account for variation within the experimental container of each replicate. The second set of models included polyp total as a random effect in ruffling, elongation and budding to account for changing polyp total due to asexual reproduction. A Generalized Akaike Information Criterion

test (Akaike, 1974) was then completed to compare the two sets of models. The test indicated that models containing polyp total as a random effect were more representative of the data, so the results of these models were analyzed. A Spearman's Rank-Order Correlation test (Spearman, 1904) was completed on all response variables to determine the correlation between the variables and time. All statistical analyses above were completed in RStudio (R versions 4.0.3 and 4.1.1).

3. Results

3.1. The Effect Of Time

The models (Table S1) suggest that time causes an increase in the number of ruffled *Aurelia aurita* polyps (p = 2.84e-11; Fig. 3A), the number of elongated polyps (p = 6.74e-05; Fig. 3B), the number of polyp buds (p = 4.48e-07; Fig. 3C) and total number of polyps (p = 0.0111; Fig. 3D; Table S2). The Spearman's Rank-Order Correlation test also indicates that all variables are strongly correlated with time (Table S3).

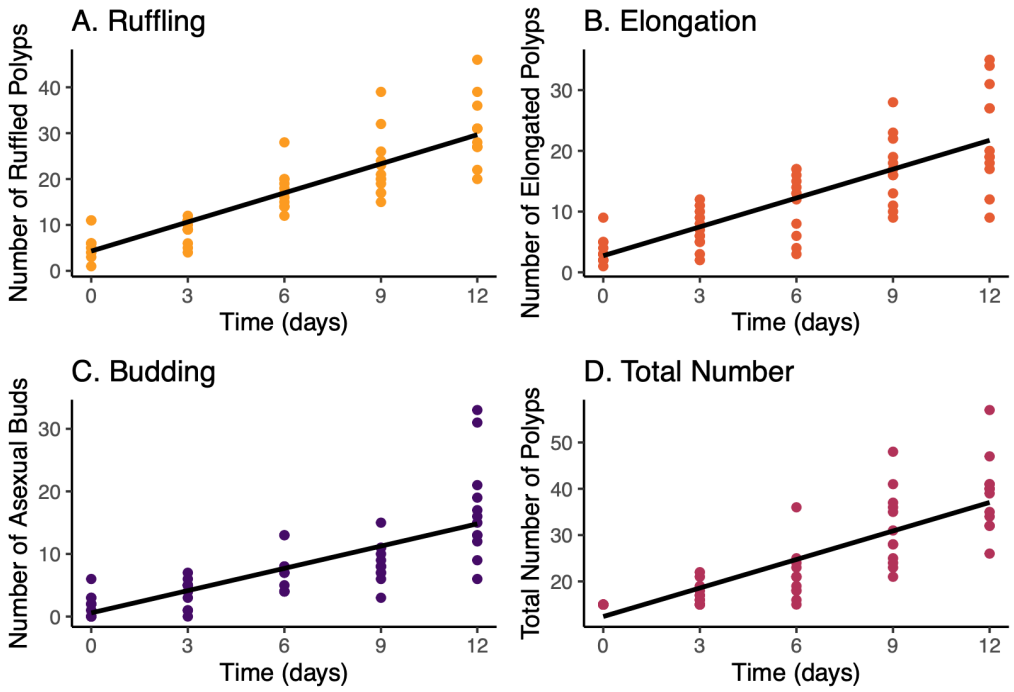

Figure 3. Experimental measures over time in *Aurelia aurita*. (A) The number of ruffled *Aurelia aurita* polyps (p = 2.84e-11, Spearman's rho = 0.910); (B) the number of elongated *A. aurita* polyps (p = 6.74e-05, Spearman's rho = 0.830); (C) the number of *A. aurita* polyp buds (p = 4.48e-07, Spearman's rho = 0.845); and (D) the total number of *A. aurita* polyps (p = 0.0111, Spearman's rho = 0.899), all over time.

3.2. Polyp Ruffling

The ruffling model (Table S1) indicates that there is an overall increase in the number of ruffled *A. aurita* polyps in the caffeine (p = 0.0278) and combination (p = 0.0298) treatments (Fig. 4; Table S2). In fact, comparing the caffeine treatment to the combination treatment indicates that there is no significant difference between these two treatments (p = 0.8767; Table S2). The model also suggests that there is no overall effect on polyp ruffling in the estradiol treatment (p = 0.665; Table S2). Furthermore, there is no interaction of time on the effect on polyp ruffling in the caffeine (p = 0.330), estradiol (p = 0.990) or combination (p = 0.435) treatments (Fig. 5B; Table S2).

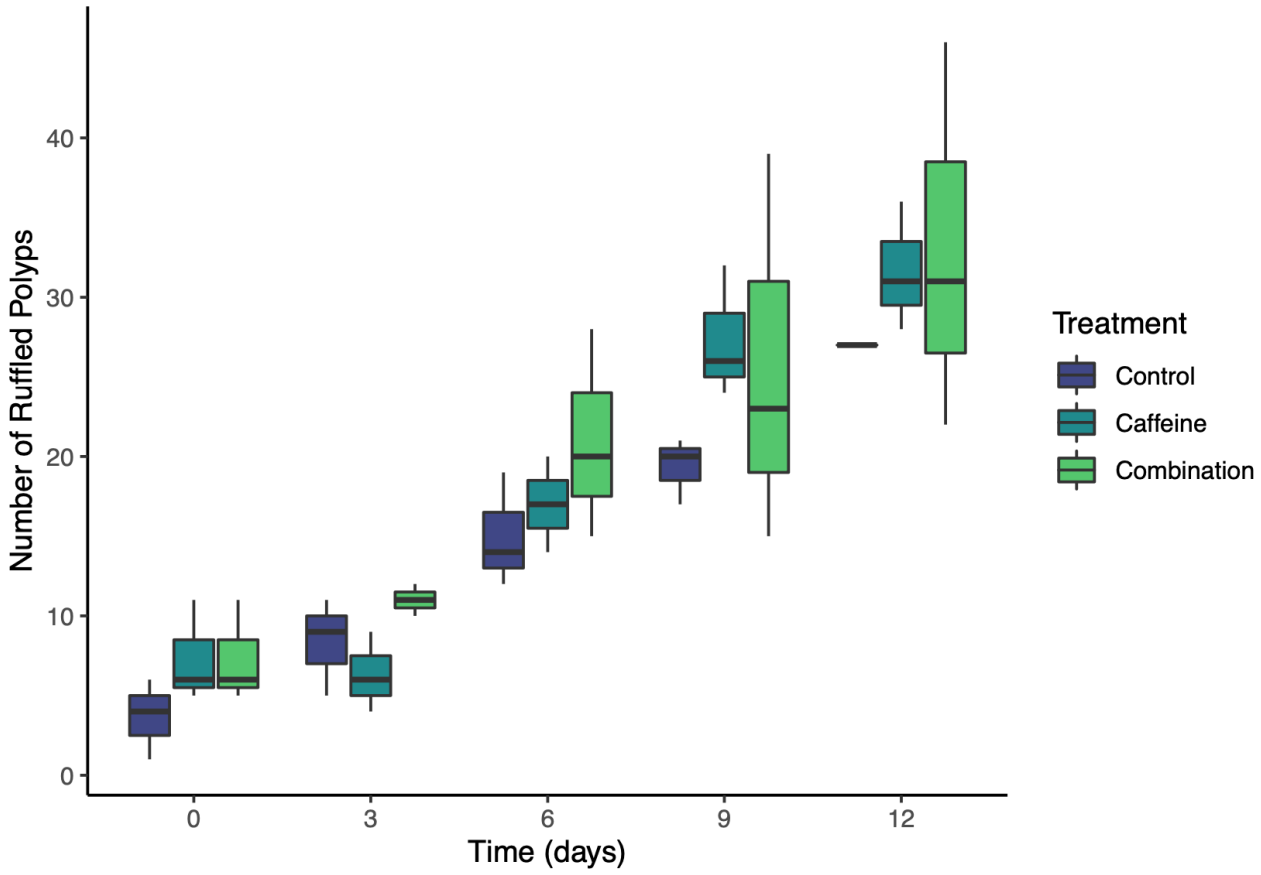

Figure 4. The number of ruffled *Aurelia aurita* polyps in the caffeine (p = 0.0278) and combination (p = 0.0298) treatments, in relation to the control, counted every third day. The caffeine treatment contained 22,330 umol/L of caffeine in seawater, while the combination treatment contained 5 umol/L of estradiol and 22,330 umol/L of caffeine in seawater.

3.3. Polyp Elongation

Our elongation model (Table S1) shows that there is no overall effect on polyp elongation in the caffeine (p = 0.647), estradiol (p = 0.935) or combination (p = 0.757) treatments (Fig. 5A; Table S2). There is also no interaction of time on the effect of polyp elongation in the caffeine (p = 0.234), estradiol (p = 0.266) and combination (p = 0.128) treatments (Fig. 5B; Table S2).

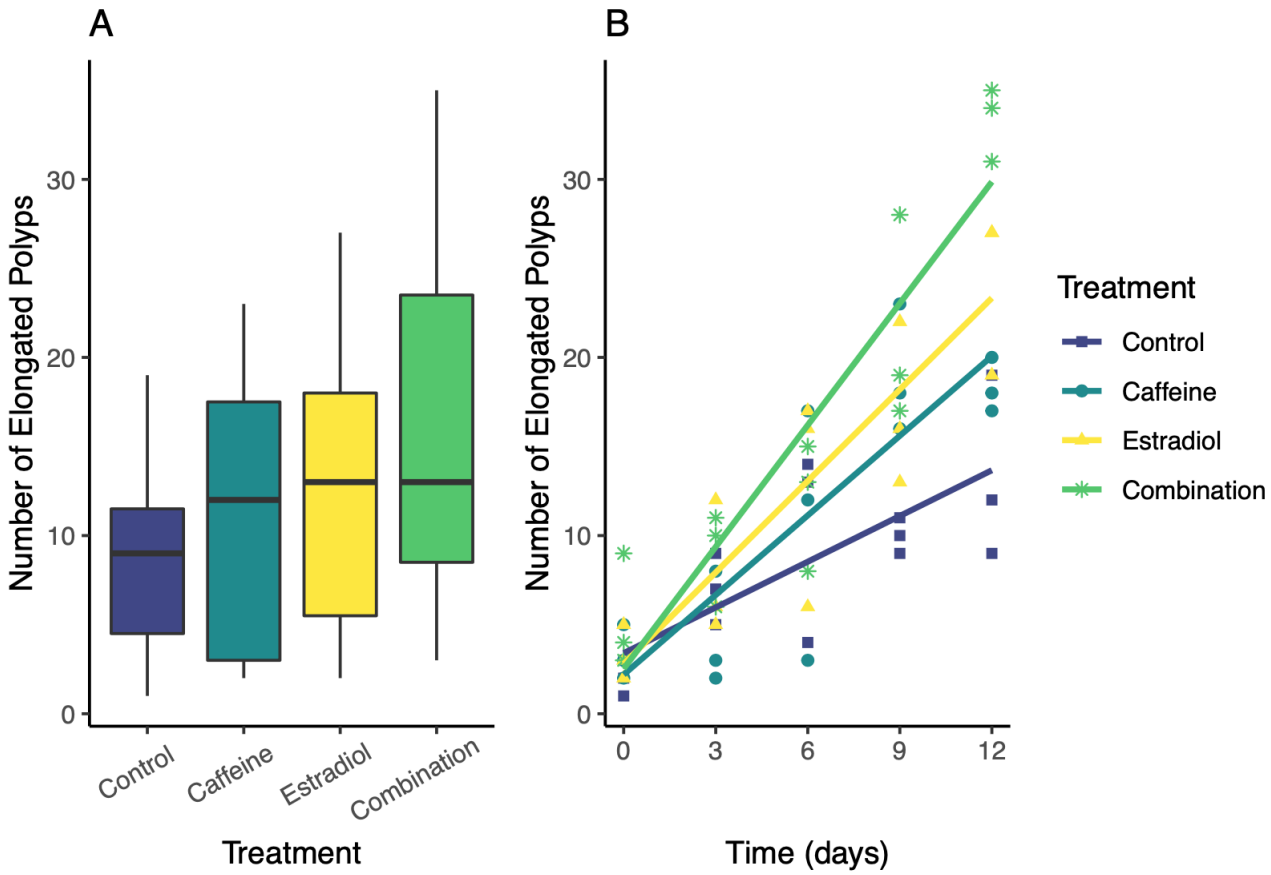

Fig. 5. (A) Overall number of elongated *Aurelia aurita* polyps in the caffeine (p = 0.647), estradiol (p = 0.935) and combination (p = 0.757) treatments compared to the control. (B) The number of elongated polyps in the caffeine (p = 0.234, Spearman's rho = 0.823), estradiol (p = 0.266, Spearman's rho = 0.914) and combination (p = 0.128, Spearman's rho = 0.927) treatments over time, compared to the control (Spearman's rho = 0.745). The caffeine treatment contained 22,330 umol/L of caffeine in seawater, the estradiol treatment contained 5 umol/L of estradiol in seawater, and the combination treatment contained 22,330 umol/L of caffeine and 5 umol/L of estradiol in seawater.

3.4. Polyp Budding

The budding model (Table S1) suggests that there is an overall increase in the number of polyp buds in the combination treatment (p = 0.00201; Fig. 6A; Table S2). The model further indicates that there is an interaction of time on the effect of polyp budding in the combination treatment (p = 0.0392; Fig. 6B; Table S2). However, there is no overall effect on polyp budding in the caffeine (p = 0.153) and estradiol (p = 0.760) treatments (Table S2). There is also no interaction of time on the effect on polyp budding under the caffeine (p = 0.540) and estradiol (p = 0.651) treatments (Table S2). Finally, there is a significant difference between the caffeine and the combination treatments when compared directly to each other (p = 0.0405; Table S2).

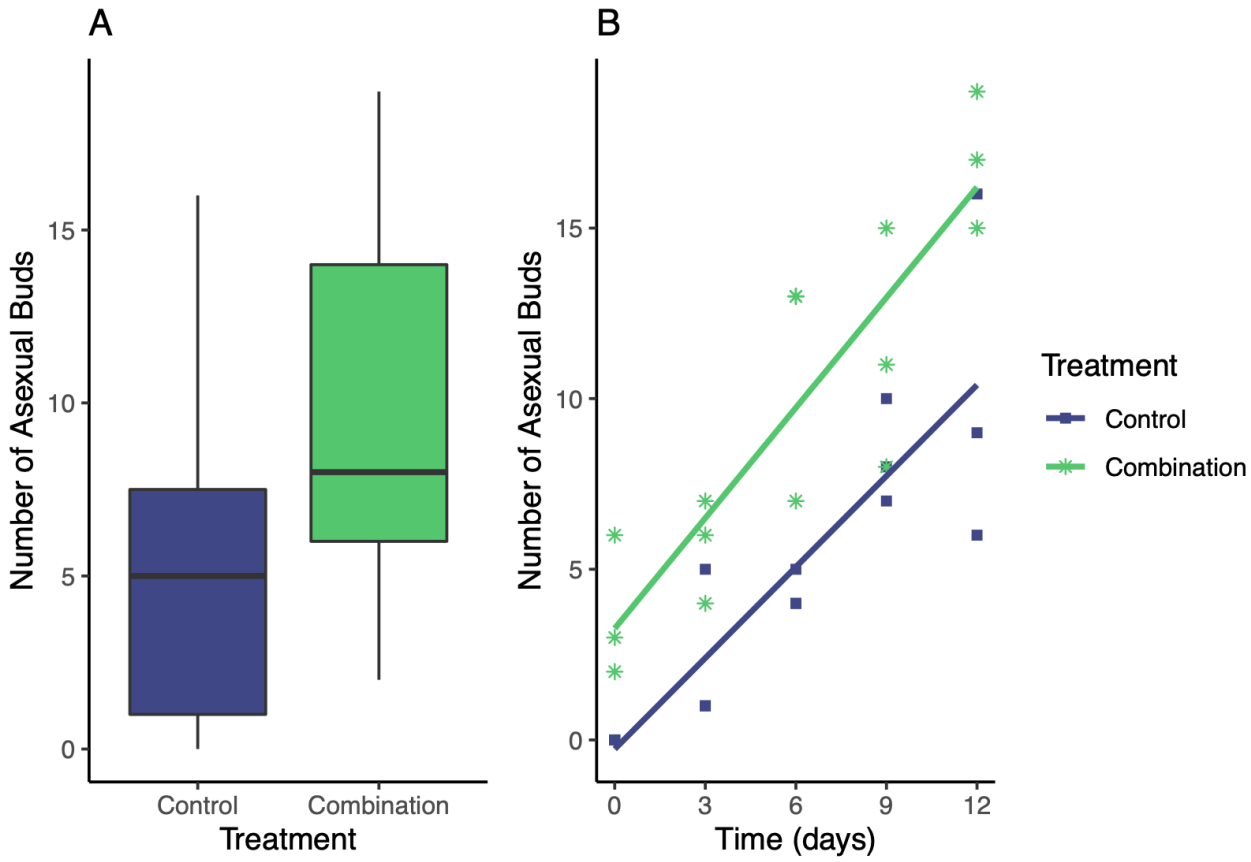

Figure 6. (A) The overall number of asexual *Aurelia aurita* polyp buds in the combination treatment (p = 0.0392), compared to the control. (B) The number of asexual polyp buds in the combination treatment (p = 0.00201, Spearman's rho = 0.914) over time, compared to the control (Spearman's rho = 0.917). The combination treatment contained 22,330 umol/L of caffeine and 5 umol/L of estradiol in seawater.

3.5. Total Number of Polyps

Our model for the total number of polyps (Table S1) suggests that there is no overall effect on polyp total in the caffeine (p = 0.698), estradiol (p = 0.961) or combination (p = 0.514) treatments (Fig. 7A; Table S2). There is also no significant interaction of time on the effect on polyp total within the caffeine (p = 0.983), estradiol (p = 0.991) or combination (p = 0.911) treatments (Fig. 7B; Table S2).

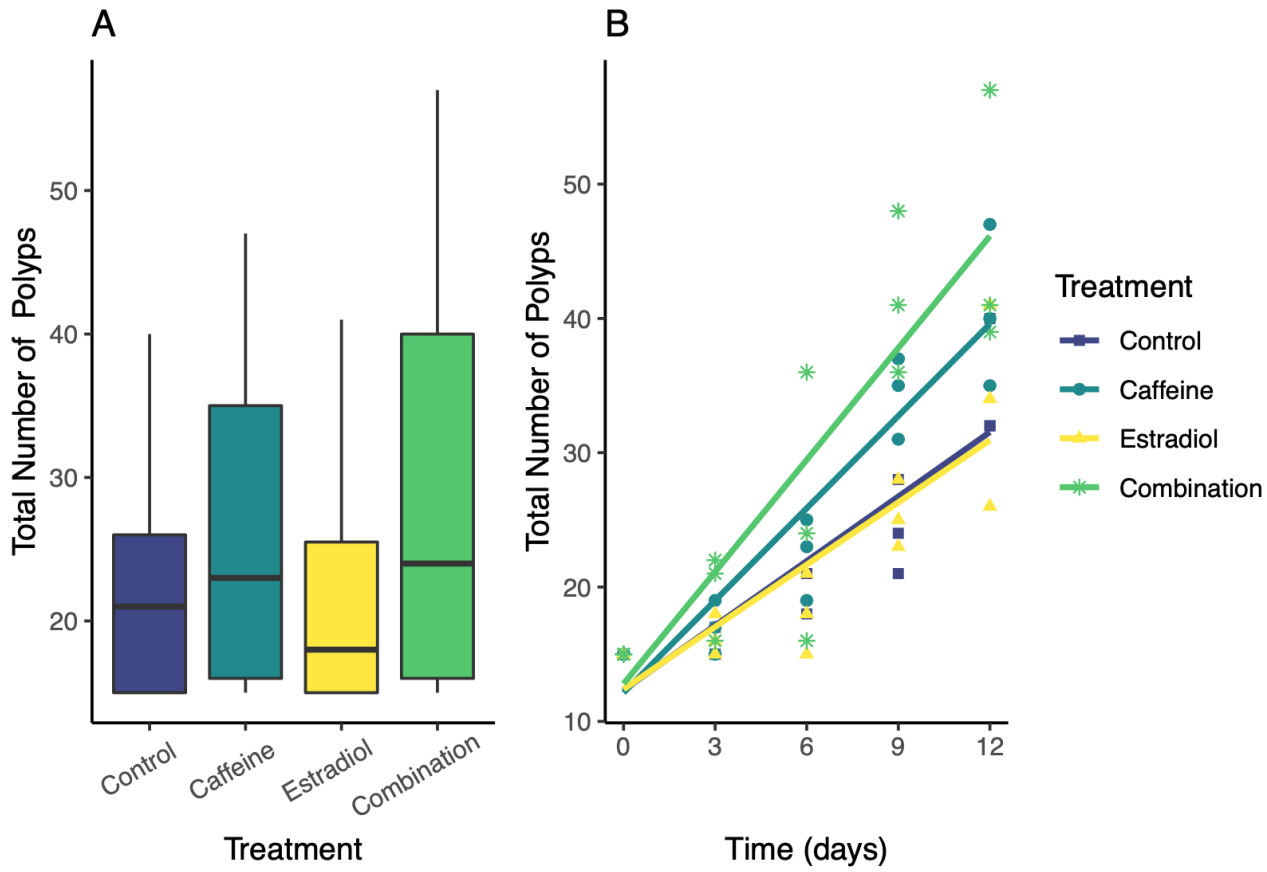

Figure 7. (A) The overall total number of *Aurelia aurita* polyps in the caffeine (p = 0.698), estradiol (p = 0.961) and combination (p = 0.514) treatments, compared to the control. (B) The total number of polyps in the caffeine (p = 0.983, Spearman's rho = 0.954), estradiol (p = 0.991, Spearman's rho = 0.906) and combination (p = 0.911, Spearman's rho = 0.917) treatments over time, compared to the control (Spearman's rho = 0.960). The caffeine treatment contained 22,330 umol/L of caffeine in seawater, the estradiol treatment contained 5 umol/L of estradiol in seawater, and the combination treatment contained 22,330 umol/L of caffeine and 5 umol/L of estradiol in seawater.

3.6. Transverse Segments, Stacking and Ephyra

No transverse segments, polyp stacking or ephyra were observed throughout the duration of the experiment, so the effects of our treatments on these markers of strobilation could not be observed.

4. Discussion

4.1. Overview of Findings

The objective of our study was to determine if caffeine and estradiol affect strobilation and reproduction in the polyp life stage of *Aurelia aurita*. We hypothesized that estradiol would cause a decrease in polyp strobilation and reproduction due to hormonal disruption (Janer & Porte, 2007), while caffeine would cause an increase in polyp strobilation and reproduction due to increased enzyme activity (Aguirre-Martinez et al., 2015). We also hypothesized that combined caffeine and estradiol would cause an increase in polyp strobilation and reproduction because caffeine is present in a much higher concentration, so its positive effects would overshadow any negative effects of estradiol. However, estradiol had no significant effect on any response variable when compared to the control, so our estradiol hypothesis is not supported. In the caffeine treatment we observed a significant increase in polyp ruffling when compared to the control, but there was no significant increase in any other response variables, so our caffeine hypothesis is partially supported. Finally, we observed an increase in polyp ruffling and budding in the combination-exposed polyps compared to the control, so our combination hypothesis is partially supported.

In addition to observations regarding the tested hypotheses, we saw a positive correlation between time and each response variable (polyp elongation, ruffling, budding and the total number of polyps) across all treatments. The polyps were shocked with iodine and temperature prior to chemical exposure, which is a well studied method of inducing strobilation in *A. aurita* (Spanenberg, 1967; Ishii et al., 2008; Helm & Dunn, 2017). Elongation and ruffling are precursors to strobilation, so their increase through time was expected. However, the observed increase in asexual budding and in the total number of polyps was not expected, as these characteristics are typically tied to polyp reproduction, not strobilation. Polyps reproduce via budding to make other polyps, while they strobilate to produce ephyra. The observed increases in

budding and in the total number of polyps may be tied to the temperature and iodine shock, or it may be normal polyp behaviour that would have been observed regardless. However, we did not have a non-shocked treatment to compare back to, so the effects of temperature and iodine shocks on polyp budding are unknown. As a result, the increase in asexual reproduction observed in all polyps over time may have been caused by another unknown mechanism.

Additionally, the effects of a temperature and iodine shock are usually more distinct than we observed; typically full polyp strobilation and ephyra production occur within 14 days (Spanenberg, 1967; Wang et al., 2020). Although some signs of strobilation were observed in the experimental polyps, there were no transverse segments, stacking or ephyra produced by the end of the 12 day experimental period. The absence of these characteristics indicates that the stress induced strobilation mechanism in our polyps was not fully triggered. This may be because the polyps were not sufficiently stressed; in most experiments found in the literature polyps were fed every few days (Spanenberg, 1967; Purcell et al., 1999; Han & Uye, 2010), while we fed our polyps twice a day. It is possible that more frequent feeding allowed the polyps ample nutritional resources for rapid recovery from the temperature and iodine shock, preventing the stress reproduction response from occurring as intensely (Ishii & Watanabe, 2003). As such, future research should investigate the effects of varying feeding procedures post-shock.

In addition to analyzing changes through time, we examined potential variation between treatments to determine the effect of caffeine and estradiol on polyp strobilation and reproduction. In polyps exposed to estradiol, there was no significant difference from the control in any response variable. Since estradiol had no effect on *A. aurita* polyp reproduction or strobilation, our hypothesis is not supported. There is limited published literature regarding the potential effects of estradiol on *A. aurita* strobilation and reproduction, but the transition between life phases is highly regulated by hormones in other invertebrates (Fuchs et al., 2014). Furthermore, steroidal hormones such as estradiol have been shown to be involved in reproduction in crustaceans, echinoderms and molluscs (Janer & Porte, 2007). If estradiol does play a role in regulating polyp reproduction or the polyp-to-ephyra transition, the addition of estradiol through wastewater would likely disrupt this process. Despite this, our results indicate that estradiol had no significant effect on the reproduction or strobilation of *A. aurita* polyps, so it is possible that estradiol does not interfere with the hormonal pathways that regulate the

polyp-to-ephyra transition and asexual budding. However, the fact that strobilation was only partially induced means that we may have missed some effects that estradiol would have on full *A. aurita* strobilation. Furthermore, estradiol may interfere with strobilation and reproduction, but no effect was observed in this study due to the use of low, environmentally relevant concentrations. Regardless, our results suggest that an environmentally relevant concentration of estradiol does not have a positive effect on *A. aurita* polyp strobilation or reproduction, so jellyfish blooms near wastewater runoff areas are not being exacerbated by estradiol's presence in the water.

In polyps exposed to caffeine there was a significant increase in ruffling, but no significant change in any other variables. Since caffeine had no effect on *A. aurita* polyp reproduction and caused an increase in some metrics of strobilation, our hypothesis is partially supported. The discrepancy between the null effect of caffeine on reproduction and its positive effect on strobilation indicates that these processes may be regulated by different mechanisms. However, literature discussing the effects of caffeine on marine invertebrates is sparse, making it difficult to speculate on these mechanisms and highlighting the need for further research.

In contrast, the literature indicates that caffeine can increase enzyme activity in other marine invertebrates, so the increase in polyp ruffling was expected (Aguirre-Martinez et al*.,* 2015). It was unexpected that there was a marked increase in only polyp ruffling and not any other response variables. Ruffling may occur prior to elongation, so these results could indicate that the polyps had insufficient time to transition into elongation from the early ruffling stages of strobilation. More research is needed to confidently determine the sequence of these characteristics. Furthermore, elongation and ruffling were chosen as response variables because they are precursors to the formation of the transverse segments that develop into immature ephyra through strobilation (Ruppert et al*.*, 2004; Wang et al*.,* 2020). However, the use of elongation and ruffling as metrics for strobilation was drawn from only one study (Wang et al*.,* 2020). Further research is also needed to confirm the accuracy of these characteristics as metrics of strobilation, and our results should be interpreted with this in mind. Since there is little published research using these same response variables, or on *A. aurita* polyp strobilation in general, it is difficult to compare our findings to the literature. Regardless, our results indicate that an environmentally relevant concentration of caffeine increases strobilation in *A. aurita*

polyps to some extent, but has no effect on asexual reproduction. As such, *A. aurita* jellyfish blooms may be worsened by the presence of caffeine in areas with high wastewater runoff.

In the combination treatment, which contained both estradiol and caffeine, we saw an increase in polyp ruffling and budding, but not in elongation or total number of polyps. As discussed above, ruffling and elongation are metrics of strobilation, while budding and polyp total are metrics of reproduction. The observed increase in some metrics of polyp reproduction and strobilation partially supports our hypothesis. Our results also show that the caffeine and combination treatments are not significantly different from each other with respect to ruffling, so there do not appear to be any interactive effects of caffeine and estradiol on polyp strobilation. Thus, the effect of the combination treatment on strobilation can be attributed solely to the presence of caffeine. This is consistent with our estradiol results, which indicate that the presence of estradiol has no effect on polyp strobilation.

In contrast, our results indicate that there was significantly more budding in the combination treatment than in the caffeine treatment. This suggests that the chemicals have a different effect on polyp reproduction individually than when combined. There is minimal published literature discussing the interaction between caffeine and estradiol, but a study by Silva et al. (2002) determined that combining weak estrogenic chemicals resulted in significant "mixture effects", which are observed effects that cannot be accounted for through the sum of individual component effects. This phenomenon is consistent with our results, as we observed no change in budding in the caffeine or estradiol treatments, but saw significant change when the chemicals were combined.

The observed discrepancy between the interactive effects on strobilation and those on reproduction indicate that combined chemicals are more unpredictable than simply summing the effects of individual chemicals. As such, we cannot predict what factors are affecting strobilation and reproduction in *A. aurita* polyps near wastewater runoff by looking only at the effects of individual chemicals. Given the potential for interactive chemical effects, wastewater has uncertain and possibly detrimental impacts on jellyfish blooms.

4.2. Limitations and Future Research

A primary limitation in our study is that strobilation was not induced as effectively as anticipated, so we may not have observed the full effect of wastewater pollutants on this process. Future research should therefore focus on the addition of chemicals to already strobilating polyps to ensure that the full range of chemical effects on strobilation can be observed. As mentioned previously, another limitation in our study is that elongation and ruffling are not well established metrics of strobilation, while budding is a clear measure of asexual reproduction. As such, not as much weight can be given to results regarding ruffling and elongation as can be given to results regarding budding. Future research should therefore investigate the effectiveness of ruffling and elongation as metrics of strobilation. Finally, our study is limited in its capacity to link the results to jellyfish blooms in highly chemically rich areas, as there are over a dozen chemicals present in wastewater (Heffron et al., 2016; Krogh et al., 2017) and only two were investigated here. We observed an interactive effect of estradiol and caffeine on reproduction, which suggests it may be interesting to look into the effects of more wastewater chemicals, both in isolation and in combination. Unstudied compounding effects of these chemicals and other abiotic factors, such as the low oxygen levels present in these polluted areas (Ishii et al., 2008), should be researched, as they may also impact *A. aurita* polyps and promote jellyfish blooms.

4.3. Conclusion

We found that estradiol had no effect on *A. aurita* polyps, caffeine increased *A. aurita* polyp strobilation, and the combination of caffeine and estradiol caused an increase in both polyp strobilation and reproduction. The magnitude of polyp strobilation and reproduction are directly linked with the quantity of ephyra and adult medusa present in the water, so more polyp reproduction results in increased pelagic jellyfish presence (Fuchs et al., 2014). This indicates that caffeine, and the combination of caffeine and estradiol may play a role in the vast *A. aurita* medusa blooms that have been observed globally in areas with high chemical presence from wastewater runoff (Papathanassiou et al., 1987; Ishii & Tanaka, 2001; Mills et al., 2001). Despite the catastrophic consequences of jellyfish blooms, such as damaging fishing operations and powerplants (Shimomura, 1959; Matsueda, 1969), the driving mechanisms behind them are largely unknown (Mills et al., 2001). This study contributes a small piece to the mosaic that is

jellyfish life history; using these results in tandem with more research regarding the impact of other abiotic factors on *A. aurita*, we will one day be able to determine the cause of jellyfish blooms and mitigate their disastrous effects.

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Supplementary Materials

Table S1
The model structure (model type, distribution and random effects) used for each *Aurelia aurita* polyp response variable (ruffling, elongation, budding and total). Random effects of the jar account for variation within the experimental container of each replicate. Random effects of the polyp total account for the changing total number of polyps throughout the course of the experiment due to asexual reproduction.

	Response Variable			
	Ruffling	*Elongation*	*Budding*	*Total*
Model Type	GAMLSS	GAMLSS	GAMLSS	GAMLSS
Model Distribution	Weibull	Negative Binomial Type II	Negative Binomial Type II	Delaporte
Random Effects	Jar & Total	Jar & Total	Jar & Total	Jar

Table S2
P-values of the overall effect of treatment (caffeine, estradiol and combination) compared to control for *Aurelia aurita* polyp response variables (ruffling, elongation, budding and total number). P-values of the interaction of time on the effect of treatment on each response variable are also listed. Ruffling in the caffeine and combination treatments are compared against each other. Values are determined by the corresponding GAMLSS models and significant p-values (<0.05) are in bold.

	Response Variable			
	Ruffling	*Elongation*	*Budding*	*Total*
Collection Day (time)	**2.84e-11**	**6.74e-05**	**4.48e-07**	**0.0111**
Caffeine	**0.0278**	0.647	0.153	0.983
Estradiol	0.647	0.935	0.760	0.991
Combination	**0.0298**	0.757	**0.00201**	0.911
Caffeine Through Time	0.330	0.234	0.540	0.698
Estradiol Through Time	0.900	0.266	0.651	0.961
Combination Through Time	0.435	0.128	**0.0392**	0.514
Caffeine vs. Combination	0.8767	-	**0.0405**	-

Table S3
The correlation coefficient (Spearman's rho) of treatment (caffeine, estradiol and combination) with time for each *Aurelia aurita* polyp response variable (ruffling, elongation, budding and total number), as determined by the corresponding Spearman's Rank-Order Correlation test.

	Response Variable			
	Ruffling	*Elongation*	*Budding*	*Total*
Control Through Time	0.745	0.745	0.917	0.960
Caffeine Through Time	0.823	0.823	0.926	0.954
Estradiol Through Time	0.914	0.914	0.856	0.906
Combination Through Time	0.927	0.927	0.914	0.917
All Treatments Through Time	0.910	0.830	0.854	0.899