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A Comparison of the Effects of Melatonin and Bisphenol A On The Behavior Of Motile Zooxanthellae

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ABSTRACT

Bisphenol A (BPA) is classified as an organic chemical pollutant and has received global concern due to its widespread presence in the world's ocean at relevant environmental concentrations as well as its various negative toxicological and reproductive effects. Endocrine disruption suggests that BPA may compete with hormone receptors involved in cell communication.

Melatonin is an indoleamine produced by many cnidarian species as well as some dinoflagellates. It is a natural neurotransmitter involved in processes such as movement, sexual maturation, metamorphosis, and reproduction.

Research by Ann Tarrant and others suggest that both substances play a role in cell signaling and communication and could be influencing the symbiosis between corals and zooxanthellae. It also proposes that melatonin and BPA share a competitive relationship in vertebrates, with melatonin lowering rates of BPA-induced proliferation of breast cancer cells.

This study aimed to examine the roles and relationships of BPA and melatonin in zooxanthellae, to better understand cell signaling and behavior in dinoflagellates, and explore the similarities and differences in these processes to other marine invertebrates as well as terrestrial vertebrates.

It found negative behavior in zooxanthellae at 100 parts per million BPA and determined that the endocrine-like signal transduction in some dinoflagellates and vertebrates is alike because melatonin and BPA share a competitive relationship in both. It is possible that BPA could be interfering with inter- and intraspecies signaling in marine organisms. Because of these findings, the presence of BPA in high concentrations in the ocean could be devastating to the world's coral reefs.

Introduction

Corals, especially those of reef-building genera such as *Acropora*, are an incredibly important part of the ocean's network of ecosystems. They provide the foundation for coral reefs, which not only provide sustenance and shelter for an estimated 25% of all marine life, but also support around one billion people through food resources and tourism. They belong to the Phylum Cnidaria, along with anemones and sea jellies, and are a colony of organisms: polyps, rather than a single life form.

Cnidarians are distributed among four classes: Hydrozoa (including siphonophores such as the man-of-war, hydroids, and fire corals), Scyphozoa (including true sea jellies), Cubozoa (including the box jelly), and Anthozoa (including corals and anemones). Each has slightly different modes of reproduction and stages of maturation. Organisms of classes Scyphozoa, Hydrozoa, and Cubozoa pass through medusa and polyp forms (How Corals Reproduce., n.d.). Medusae reproduce asexually to produce larvae that go through metamorphosis

to become polyps. Polyps then reproduce asexually to produce medusae, which repeat the cycle (How Corals Reproduce., n.d.).

Most species of corals reproduce sexually externally via broadcast spawning. Because they are hermaphrodites, corals possess both female and male sex organs. In one synchronous event, corals of a certain geographic area release sperm and eggs into the water column, and the gametes that join form plannulae, which are carried by ocean currents to start or expand reefs (NOAA, 2011.; Fautin, D.G., 2002). Once plannulae have settled in a suitable location, they enter metamorphosis and develop into polyps. Internal fertilization is also possible. Eggs are brooded by the mother polyp anywhere from a few days to many weeks. Developed larvae will then be released into the water column where they will settle close to the parent colony and become plannulae (NOAA, 2011.; How Corals Reproduce, n.d.).

Corals can also undergo asexual reproduction through two methods: budding, and fragmentation, although they are not as common as sexual reproduction through broadcast spawning. Budding involves the expulsion of single polyps to start new colonies. Fragmentation occurs when a piece of the entire colony is broken off and carried to a new location (How Corals Reproduce, n.d.; NOAA, 2011.).

Coral reefs are havens for thousands of species of fish, from the cleaner wrasse to the Caribbean reef shark. Corals are keystone species, and thus the balance of the entire ecosystem is dependent on their health. The incredible biodiversity of reefs is credited with a future of providing us with medicines in the next century. These medicines could cure cancer and fight viruses. The National Marine Fisheries Service estimates that coral reefs provide U.S. fisheries with $100 million in food resources. Economies also benefit from coral reef tourism, with diving trips, fishing tours, and other activities bringing in billions of dollars (U.S. Department of Commerce, 2013). Because of emerging research and discoveries regarding coral and coral reefs, It is of utmost importance that they are protected. Not only are they beautiful; they keep the ocean, as well as humankind, alive.

Cnidarians and Zooxanthellae

Corals, as well as other cnidarians, are dependent on zooxanthellae (belonging to the genus *Symbiodinium*): symbiotic, photosynthetic, single-celled dinoflagellates that live in the surface tissues of coral polyps. Both organisms benefit from a mutual relationship: zooxanthellae receive carbon dioxide and water as byproducts of cellular respiration from their host that they then use to carry out photosynthesis (Davy, S. K., et. al., 2012; US Department of Commerce, 2013). They also obtain a stable and safe position in the water column where they can avoid predation and have access to a sufficient amount of light. In return, they aid the coral in metabolism and reproduction as well as provide it with its color (a “bleached” reef is one in which the zooxanthellae have been expelled from the coral polyp in response to stress), and the required nutrients for skeletogenesis (Davy, S. K., et. al., 2012; US Department of Commerce, 2013). Typically, a coral will remain with its symbionts throughout its life cycle, only gaining them from the water column in early life or after a bleaching event. Although it is possible for zooxanthellae to live independently from their host, corals rely on their algal symbionts to sustain them and thus cannot survive on their own (Davy, S. K., et. al., 2012; US Department of Commerce, 2013).

Because populations of free-living zooxanthellae are sparse in the surrounding waters of reefs, corals would benefit greatly from some kind of mechanism of attraction. A study by researchers from multiple countries examined the effect of fluorescent light emitted by corals in response to exposure to blue or ultraviolet light on the behavior of zooxanthellae. It published the first evidence that this emission may be to attract free-living zooxanthellae. This process was suspected to be related to their phototaxis, the natural ability to move toward or away from light-related stimuli (Aihara, Y., et. al., 2019). However, because sunlight is present everywhere on most reefs, it is unlikely that this is the only method of attraction. The existence of chemical signaling as an additional means of communication would provide corals with many more benefits than what could

be seen with the use of phototaxis alone. A chemoattractant would allow for the intake of free-living zooxanthellae during the night and when light alone is unreliable, even when populations are sparse.

Cnidarians and Indoleamines

Research suggests that the behavior of both cnidarians and dinoflagellates is regulated by indoleamines, a group of monoamine neurotransmitters derived from tryptophan that includes serotonin and melatonin as well as others. The literature indicates that these substances, synthesized by the coral's nervous system, have been linked to the stimulation of bell contraction (movement), spawning, sexual maturation, and metamorphosis in many kinds of cnidarians, and are found naturally in their tissues (Tarrant, 2007; Zou, 2010). Although melatonin has long been associated with control of sleep in humans, it also exists as a neurotransmitter in many organisms and is produced naturally by their nervous systems. In cnidarians, although they only have primitive networks of nerves, melatonin and other indoleamines work to aid cells in their communication. Specific binding sites have even been identified for melatonin and other types of indoleamines (Tarrant, 2007) in cnidarians and dinoflagellates, strongly suggesting a purpose of these substances in inter-species symbiosis and communication. Because of this, scientists claim these indoleamines could be involved in the communication between zooxanthellae and corals (Tarrant, A.M., 2005).

Cnidarians and Bisphenol A

The mechanisms of action and targeted processes seen in indoleamines are noticeably similar to those of bisphenol A (BPA), an organic compound commonly used in polycarbonate plastics and resins because of its structure. In 2013, it was the highest volume chemical produced in the entire world (Gowder, S., 2013). BPA can be found in water bottles, food containers, and the epoxies used to seal the hulls of boats. In the ocean, ultraviolet radiation from the sun combines with the salt of the water to break these materials down and release BPA. With 8 million metric tons of plastic waste entering oceans around the world every year, it is no surprise that there is a significant amount of BPA in these bodies of water. In 2010, a report analyzed sea and sand samples from 200 sites in 20 countries and found numbers ranging from 0.01 to 50 parts per million (American Chemical Society, 2010). In addition to plastics and resins, BPA has also been found in dust, flame retardants, protective coatings, car lenses, construction materials, optical lenses, thermal paper, paper coatings, cans and jar caps, food packaging, kitchenware, windows, adhesives, as a developer in dyes, in encapsulation of electrical and electronic parts, in some dental sealants, and even in children's toys (Gowder, S., 2014; Konieczna, A., et. al., 2015).

BPA exhibits cytotoxic and mutagenic qualities, commonly causing damage and death to cells as well as changing genetic material, causing mutations (Michałowicz, J., 2014; Konieczna A., et. al., 2015; Gowder, S., 2014; Ma, Y., et. al., 2019). These effects can be seen in aquatic and marine organisms, terrestrial animals, and humans. Some studies provide evidence of immunotoxicity, neurotoxicity, and adverse developmental and reproductive effects, reporting cases of decreased locomotor activity, sedation, lethargy, and arched backs in adult animals after short-term exposure at extremely high doses Ma, Y., et. al., 2019). Changes in biochemical and neurotransmitter gene expression in brain tissues was also found. Exposure to less than 5 mg/kg BPA has caused various neurodevelopmental effects in the offspring of vertebrates in a variety of research. Some studies suggest possible effects of early life exposure on various sexual behaviors, changes in maternal behaviors, aggression and anxiety, and learning ability (Michałowicz, J., 2014; Konieczna A., et. al., 2015).

Not only is BPA generally toxic, it also acts as an endocrine disruptor, meaning it disrupts the chemical signaling in cells by binding to an estrogenic receptor. In invertebrates such as cnidarians, this means BPA can interfere with sexual and asexual reproduction (Tarrant, 2007). In vertebrates, BPA contributes to the proliferation of hormone-related cancer cells, often resulting in breast cancer (Wang, et. al., 2018). A 2018 study sought

to determine the effect of an indoleamine: melatonin, on this process, and found that it significantly reduced BPA-induced cell proliferation by targeting a steroid receptor coactivator, SRC-1 (Wang, et. al., 2018). This study suggests the possibility that these same effects may be seen in invertebrates. Melatonin appears to be cancelling the effect of BPA, which could have huge implications if translated to symbiosis in cnidarians and dinoflagellates.

Ever since SRC-1 was first discovered in 1995, it has remained at the center of bioregulatory, endocrine, and endocrine-related cancer research (Yao, T. P., et. al., 1996; Walsh, C. A., et. al., 2012). This coactivator is a member of a larger family of similar molecules involved in interacting with hormone-bound receptors (Yao, T. P., et. al., 1996). It has the ability to interact with and co-activate other nuclear receptors in the presence of hormones . Its versatile nature allows it to bind to many different transcription factors and activate expression of target genes (Walsh, C. A., et. al., 2012). Because of this, it is often involved in complex cellular reactions.

Because melatonin appears to be affecting the signaling regulated by SRC-1 and neutralizing the effects of BPA in vertebrates, it could also be having similar effects in invertebrates.

Cnidarians and Tributyltin

There are many organic chemicals that are capable of affecting reproduction or sexual maturation in cnidarians. Although research has not yet determined whether they have any effect on symbiosis, pollutants such as alcohols, thiophenes, and crude oil prevent induced metamorphosis in some hydroids (Anthozoa, n.d.).

For 40 years, the compound tributyltin (TBT) was used as an antifouling agent on the hulls of ships to keep sea snails and other creatures from clinging to them. After it was found to have devastating impacts on the environment, causing females from 200 species of gastropods to grow male sex organs, preventing them from releasing their eggs, it was banned (Oberdörster, Eva, and Patricia Mcclellan-Green, 2002; Bettin, C., Oehlmann, J., & Stroben, E., 1996). The development of large vas deferens, the duct that carries sperm in many male animals, blocked the ability to release their eggs, ultimately causing them to explode (Diep, F., 2021). Falling populations of many marine invertebrates was also linked to the use and release of TBT in the oceans. The chemical was banned from use on boats in 2008, but is still used occasionally to keep things from growing on wood and fabric (Diep, F., 2021; Oberdörster, Eva, and Patricia MclellanGreen, 2002).

A study tested the effect of TBT on the anemone Aiptasia pallida and found that it resulted in the expulsion of its zooxanthellae symbionts (Anthozoa, n.d.). This is extremely significant, because, like BPA, TBT targeted reproductive processes and resulted in disruption of coral-zooxanthellae symbiosis. While no study specifically examined the effect that the widespread use of this chemical had on coral reefs, a study did find that reef-building coral species of genus Acropora exposed to 40 mg/kg TBT were all killed within 38 hours (Smith, L. D., et. al., 2003).

The use and environmental effects of and the response to TBT can all serve as an example of a situation in which a very dangerous chemical that possesses the ability to affect reproduction and was applied to the hulls of boats affected cnidarian-zooxanthellae symbiosis via direct expulsion of symbionts. This research provides proof that there are other molecules that possess the capability to disrupt the symbiosis between corals and zooxanthellae.

Analysis of the Literature

Based on the literature outlined in this section, it was hypothesized that BPA would hinder the ability for zooxanthellae to maintain symbiosis with their host. There is a possibility that BPA and indoleamines such as melatonin and serotonin could be operating by similar mechanisms in cnidarians, dinoflagellates, and some

vertebrates. This study will examine the roles of melatonin and BPA in zooxanthellae-coral symbiosis and compare them to determine if BPA and/or indoleamines may be capable of affecting the symbiosis between zooxanthellae and their host. The results of this study will provide direction for future research on the ecotoxicological and estrogenic effects of BPA and the natural roles of indoleamines in the reef ecosystem as well as expand on the current literature on cell signaling.

Methods

In this study, a culture of isolated, free-living zooxanthellae was exposed to melatonin and bisphenol A (BPA).

This study did not use corals and focused only on analysis of the behavior of zooxanthellae. Because corals cannot survive without their microscopic symbionts, their health begins with zooxanthellae. Additionally, other limitations made the use of corals unrealistic. Proper results did not require the use of them either, as a chemotaxis (the movement of zooxanthellae to or away from a chosen substance) provides enough indication of an induced response.

BPA used in this study was obtained from Sigma-Aldrich and dissolved in water for a 100 ppm solution. 5 mg melatonin tablets were crushed and dissolved in water to create a 100 ppm solution. The tablets also contained dicalcium phosphate, cellulose (of plant origin), croscarmellose, and less than two percent of cellulose coating, natural palm leaf glaze, silica, and vegetable magnesium stearate, which, when mixed to create the solution, gathered at the bottom. Only the surface of the solution was used for experimentation. However, some of this material could have been introduced to the gels. It should not have affected the results, but future research should use pure melatonin. A culture of zooxanthellae was purchased from AlgaGen (PhytoPlasm) and contained zooxanthellae, *Symbiodinium sp*., from clades CCMP827 and a University of Miami isolate from the early 2000s, as well as other culture media. The culture was kept in a refrigerator while not in use. Because motility in free-living zooxanthellae peaks after several hours of exposure to natural light, when in use, a vial with 12mL culture media was manually agitated and set out for 2.5 hours to allow the cells to become active and clump at the bottom. Once settled, the top liquid was pulled off and discarded to leave approximately 40 microliters of concentrated cells at the bottom. The remaining solution was agitated again and 8 microliters were pulled and placed on each microscope slide under a cover slip for study.

Agarose gels were used to store substances and disperse them throughout the concentrated culture. They were prepared using a 10 cm x 7 cm x 3 mm mold. 0.16 grams agarose was dissolved in 20 mL of Tris/Borate/EDTA buffer and microwaved for 30 seconds. The mixture was then allowed to cool and 10 mL was poured into the mold. Surplus was stored in a refrigerator for later experimentation. Gel slices used were no more than 2 mm thick. Gels were cut and placed into containers filled with a solution and then allowed to infuse for one full day. They were then placed up against the cover slip and left for 15 minutes in order to let the substance diffuse from the gel and create a gradient within the solution under the slip.

Following this, a 40x microscope lens was placed up against the gel and aimed at a single, set location along a vertical axis. Once set, the number of zooxanthellae found within that field of view was noted, and the lens was shifted 0.5 mm away from the gel to another field of view (either left or right, depending on the placement of the slide), and the cells there were manually counted. This process was repeated four more times, for a total of six fields of view per slide examined. Statistical analysis was performed using a linear dependence correlation coefficient test between the relative distance of fields of view from infused gels and the number of individual zooxanthellae found. This allowed for proper analysis of chemically-induced taxis. A control was used, in which no substance had been infused into the agarose gels. A test with 100 ppm BPA was used, and another with 100 ppm melatonin was used.

Results

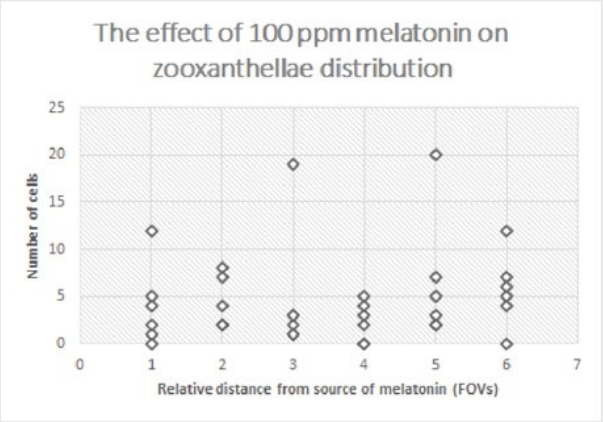


Figure 1. The effect of 100 ppm BPA on zooxanthellae distribution. A significant positive correlation existed in the relationship between relative distance from the source of BPA and the number of cells (r=0.381 with critical value of 0.349).

After 15 minutes, more cells were found in the fields of view further from the agarose gel than in the ones close to it, indicating that cells were repelled by the presence of 100 ppm BPA.

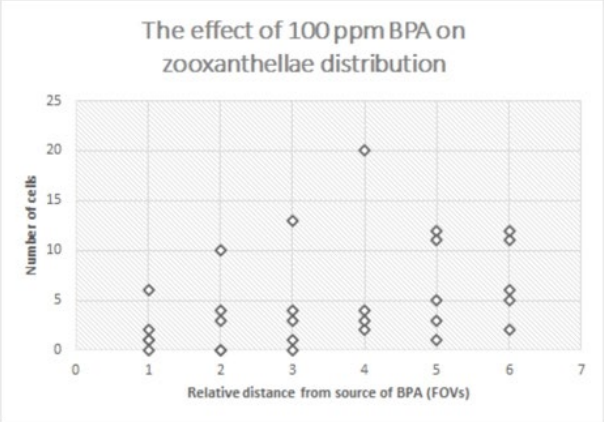


Figure 2. The effect of 100 ppm melatonin on zooxanthellae distribution. No significant correlation between relative distance from the source of melatonin and the number of cells (r=0.178 with critical value 0.33).

There was no significant correlation between cell count and distance from the source and the behavior of the cells remained unchanged. This indicates that cells exposed to 100 ppm melatonin for 15 minutes were not affected.

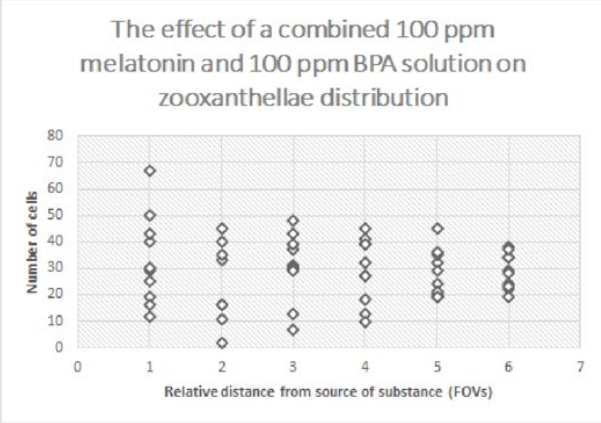
Figure 3. The effect of a combined 100 ppm BPA-melatonin solution on zooxanthellae distribution. A significant positive correlation existed in the relationship between relative distance from the source of BPA and the number of cells (r=0.381 with critical value of 0.349).

Cells exposed to a solution containing both 100 ppm BPA and 100 ppm melatonin for 15 minutes also resulted in a lack of movement.

Discussion

The purpose of the research described in this report was to expand on the current literature on cell signaling by examining the effects of BPA and melatonin on observed zooxanthellae behavior, and question the similarity of these substances' effects to those on cell communication in vertebrates. The research approach was to view and count individual cells as they moved to or away from a source of a substance to indicate a chemotaxis. A significant negative taxis was found when using 100 ppm BPA, and no taxis was found using 50 ppm melatonin or a combined solution of 100 ppm BPA and 100 ppm melatonin.

Because of these findings, it appears that bisphenol A does impact zooxanthellae, but only at concentrations of 100 ppm and above. This could be simply due to general toxicity of the compound, or it could be



due to its estrogenic effects. Regardless, at its current concentrations, BPA does not seem to pose a significant threat to coral reefs and the marine ecosystem or the natural neurotransmission and cell communication process. However, we should always be wary of our impacts on the ocean. If more plastic debris enters the oceanic system, there will be more BPA in our waters as a result. Zooxanthellae-coral symbiosis could be threatened by BPA in the oceans of tomorrow, and further study must explore this possibility to aid in the avoidance of serious issues.

The absence of effect of BPA in the presence of melatonin suggests that the behavioral stimulus incited by BPA was canceled because of melatonin. Because melatonin inhibits cell proliferation incited by BPA in vertebrates and melatonin cancels the negative behavioral effect of BPA in zooxanthellae shown by this study, it can be assumed that the endocrine-like signal transduction pathways in some dinoflagellates and vertebrates are alike in that BPA and melatonin share a competitive relationship in both. However, we must be cautious when assuming bioregulation is the same in every organism. Substances that affect reproductive and hormonal processes in vertebrates may have different effects in invertebrates. It is possible that melatonin binds to a receptor similar to that of BPA for a slightly different purpose, outcompeting it and preventing what was seen in BPA alone. This would offer an explanation of the differences in the two substances' effects when isolated with zooxanthellae. However, more research must be performed to confirm or deny these hypotheses.

The results of this study emerged from a methodology with limitations. Modification of experimental procedures from the literature was necessary due to limited resources. The lack of appropriate cell-counting and video technology, such as flow cytometry, led to the need to manually count individual organisms through a microscope, which could have left some cells unaccounted for. In response to any substance, cells had the choice to move omnidirectionally, whereas the researcher only accounted for the relative left and right fields of view. While the researcher could see the occasional zooxanthellae moving across a field of view or using its flagella to rotate, in general, the zooxanthellae did not move at an observable rate. The cultures obtained were less than 50% motile, according to the culture's seller, which meant the researcher had to rely on the few motile ones to achieve results. This could have likely skewed findings, and thus, more research is necessary. Additionally, although the cultures were maintained and transferred to the site of experimentation in the same way and under the same circumstances, there was a high degree of variability present within the data, with cell numbers ranging from 1 to 149 in each field of view, which resulted in frequent outliers.

Melatonin was the chosen indoleamine for study because previous research indicates that it is capable of inhibiting BPA-elevated steroid receptor coactivators and that seasonal peaks in melatonin are linked with the first stages of sexual maturation in cnidarians (Wang, et. al., 2018; Tarrant, A.M., 2005). Although serotonin and other indoleamines have produced similar effects on corals and zooxanthellae, it was excluded from this experiment due to its acute toxicity. Further research could explore the impacts of serotonin and other indoleamines, but this study used only melatonin.

It is important to note that all solutions were kept at room temperature and were not held in a special environment safe from sunlight or other natural elements. Degradation of substances could have occurred before experimentation and altered findings. Continuation of this research with this error corrected will help ensure that results were accurate.

The use of 100 ppm as a concentration is justified because it is twice the maximum wild-found concentrations of BPA in the marine environment, according to a report proposed to the American Chemical Society. Seeing as many cells did not move, it is highly unlikely that using a 50 ppm concentration would yield significant results. However, this is something that could be explored in future research. The use of the same concentrations of melatonin is justified because it is consistent with the maximum recorded wild concentrations of BPA. However, the range of concentrations of melatonin within cnidarian and dinoflagellate systems was not found in the research, and so natural concentrations may vary.

This study provides a greater understanding of cell signaling in dinoflagellates and of how these processes are similar in other organisms. Further research should explore the effects of indoleamines and BPA on the direct symbiosis and between corals and zooxanthellae, perhaps by using live corals. It should also examine the specific transduction pathways through which these signals are taking place. Additionally, it could re-evaluate the current levels of BPA in the world's oceans. Because the maximum recorded wild concentration of 50 ppm was recorded and published in 2010, since then, more BPA could have entered the world's oceans as a result of the continued discharge of plastic waste. Expansion of the literature in the subject area of cell-signaling between cnidarians and dinoflagellates will provide significant advancements in our understanding of the dangers of BPA and the potential measures required to combat its effects. This study expands the current knowledge of marine ecotoxicology and cell-signaling by proposing that similar signaling pathways and receptors may be utilized in both vertebrates and some dinoflagellates, shown by the discovery that melatonin and BPA share a competitive relationship in both organisms. Because corals cannot survive without zooxanthellae, and the ocean is dependent on corals, it is critical that the integrity of their symbiosis remains strong. It is of paramount importance that we continue to seek understanding of zooxanthellae-coral symbiosis. If BPA interferes and the zooxanthellae are expelled, then the communication between coral polyps and zooxanthellae could be disrupted, to the detriment of coral reefs, our oceans, and humankind

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