UNIVERSITY OF CALIFORNIA SAN DIEGO

Anti-predator strategies of the western spiny brittle star, *Ophiothrix spiculata*, exposed to reduced pH and increased temperature

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for the degree Master of Science

in

Marine Biology

by

Mia Astbury

Committee in charge:

Professor Jennifer Taylor, Chair
Professor Deirdre Lyons
Professor Gregory Rouse

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The Thesis of Mia Astbury is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Anti-predator strategies of the western spiny brittle star, *Ophiothrix spiculata,* exposed to reduced pH and increased temperature

by

Mia Astbury

Master of Science in Marine Biology

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Professor Jennifer Taylor, Chair

Echinoderms are important members of diverse marine ecosystems worldwide, necessitating that many species possess the physiological plasticity to adapt to highly variable environments. Yet, echinoderms generally exhibit strong sensitivity to changes in seawater pCO_2/pH associated with ocean acidification (OA) and increases in seawater temperature associated with ocean warming (OW), often emerging in physiological changes that can alter their ecological interactions. The Western spiny brittle star, *Ophiothrix spiculata,* is one of the most abundant echinoderm species in the highly variable Southern California Bight, where they rely on limb autotomy and mobility to evade their many fish and invertebrate predators. The goal of this study was to determine the effects of climate change stressors on the anti-predator behaviors of *O. spiculata*, with the hypothesis that OA and OW would have measurable, contrasting effects on brittle star mobility. Brittle stars were exposed one of four treatments (N=12 each): control (pH 8.0/12°C), OA (pH 7.7/12°C), OW (pH 8.0/15°C), and OW+OA (pH 7.7/15°C). Following 29 days of exposure, kinematics of the righting, escape, and arm retraction responses were analyzed, along with arm flexibility. We found that brittle stars exposed to the combined OW+OA treatment had a shorter lag time for initiation of the escape response, but none of the other kinematics nor arm flexibility were affected by OA and OW. Under the combined OW+OA conditions used in this study, brittle stars appear to be more reactive, but otherwise the performance of their various anti-predator behaviors remain unaltered by experimental pH and temperature. Overall, *O. spiculata* exhibits little sensitivity to OA and OW conditions in regard to these specific ecologically important behaviors.

INTRODUCTION

Marine organisms inhabiting the Southern California Bight contend with a dynamic environment characterized by seasonal upwelling and El Nino, as well as anthropogenically driven eutrophication (Kessouri et al., 2021), oxygen loss (Kessouri et al., 2021), and heat waves (Fumo et al., 2020). Climate change is further altering this environment through ocean acidification (OA), at a rate of -0.0015 pH units per year (Wolfe et al., 2023), and ocean warming (OW), at a rate of +0.12°C per decade (Fumo et al., 2020). Though organisms in this region have adapted to a certain range of environmental variability, the rapid and ongoing changes associated with OA and OW can affect their physiology in ways that alter their fitness and ecological interactions. This is true for a diversity of species inhabiting the northern part of the California Current Marine Ecosystem (CCME), where they exhibit high sensitivity to changes in pH and temperature despite the dynamic environment (Sunday et al., 2022, Checkley and Lindegren 2014). Fewer species at the southern end of the CCME, in the Southern California Bight, have been assessed for their vulnerabilities to near-future projections for OA and OW. Studies focus mostly on crustaceans, some of which respond to OA and OW conditions with measurable morphological or behavioral responses (Lowder et al., 2022, Moretto and Taylor 2023), but other groups of organisms, such as echinoderms, likely show different vulnerabilities that should be examined to build a better assessment of how climate change may affect local ecosystems.

Echinoderm sensitivity to OW and OA

Warmer sea water temperature (OW) tends to increase the rates of physiological processes in echinoderms, whereas increased pCO_2/reduced pH (OA) can have variable effects on different aspects of physiology. Though OW typically increases rates of growth, metabolism, consumption, and movement, these increases are not necessarily beneficial and survival rates may still decline (Sunday et al., 2021). In contrast, echinoderms generally exhibit high sensitivity to OA conditions, with disruption to some of the early developmental stages of the organism as well as overall negative impacts on fitness, however, responses are species-specific (DuPont et al., 2010). The most deleterious effects of OA appear to occur in the early life stages of echinoderms, where near-future pH conditions (-0.4 pH units, IPCC, 2023) increase the development of abnormal and asymmetric morphologies, causing high mortality rates (DuPont et al., 2008). For example, exposure to a pH of 7.7 for one week resulted in decreased size of larval sea urchins and an 80% mortality rate in larval brittle stars (Chan et al., 2016).

Later life stages are also sensitive to OA conditions, but typically with sublethal effects on their physiology. Growth may be slowed, leading to smaller body sizes of brittle stars and sea stars (Byrne and Fitzer, 2019), but exposure to OA conditions most notably manifest in the skeletal system. The ossicles comprising the skeleton are hardened with highly soluble Mg-calcite, which can readily dissolve in acidified environments (Shirayama et al., 2005). Sea urchin tests, for example, become demineralized and weaker when exposed to elevated pCO_2/reduced pH (Holtmann et al., 2013). Loss of biominerals leads to an increase in test porosity in sea urchins (Byrne and Fitzer, 2019), undermining the mechanical integrity of their skeleton. In the Southern California Bight, sea urchins collected from a range of depths were found to have greater porosity and weaker tests with increasing depth, and this was attributed to a combination of reduced oxygen and pH conditions (Sato et al., 2017). Sea urchin spines may be the most sensitive component, and they can become more brittle when exposed to OA conditions (pH 7.7 and 7.4) for as little as five weeks (Basse et al., 2013). In the sea urchin *Tripneustes ventricosus*, spine fracture force was reduced by 16 to 35% using a two-point bending measuring system (Dery et al., 2017). Such skeleton impairments may not only affect movement, but also predator defenses. For other echinoderms, like brittle stars in particular, these effects may be more pronounced given their high mobility.

Brittle star ecology

Brittle stars are the base of ecosystems around the world. As suspension feeders and scavengers, they play a vital role in the food chain, bringing previously discarded nutrients back into circulation. The Western spiny brittle star, *Ophiothrix spiculata,* is abundant in the Southern California Bight, and gets its name from its arms that are covered in hundreds of micro-spines interspersed within the tube feet. *O. spiculata* consume plankton and detritus using their spines and the mucus that covers their arms, but are also capable of killing larger organisms, like copepods, if they are so inclined. *O. spiculata* tend to dwell in large beds in areas of high productivity, such as kelp forests (Summers et al., 2000). They rely on diurnal feeding patterns, with decreased activity during the day and increased activity at night. This is a trade-off between food acquisition and avoiding predation by fish, large crustaceans, birds, and other echinoderms including sea stars (Lin et al., 2022). Photoreceptors that run up and down *O. spiculata* arms allows them to detect changes in light. While not actively feeding, brittle stars are often buried in the sandy bottom, holding the tips of their arms at the sedi2ment-water interface to monitor for any changing conditions (Rosenberg et al., 2004).

Anti-predatory behaviors

Brittle stars rely primarily on their agility to avoid predators, but they also have the remarkable ability to autotomize their arms. If a predator grabs hold of a brittle star arm, it can be autotomized at any joint along its length, leaving the rest of the organism unscathed and able to locomote away. Connective tissues controlled by the central nervous system cause the arm to split at articulation points near the stimulus, a process that can occur in as little as 0.4 seconds (Wilkie, 1978). Spontaneous autotomy can be induced by stress, such a temperature and salinity extremes (Turner, 1980; Jobson et al., 2024), and subsequent arm regeneration can be slowed under OA conditions (Wood et al., 2011; Wood et al., 2010; Hu et al., 2014) while hastened under OW (Wood et al., 2011). Arm autotomy and regeneration are costly, and brittle stars often react to initial contact with a predator or other physical disturbance with arm retraction (Riedel et al., 2008). When the tip of the arm is physically stimulated, it immediately bends at multiple points, rapidly pulling the tip close to the central disk. This behavior reduces the chance of a predator grabbing hold of an arm and thus the need to autotomize.

Should a predator manage to dislodge a brittle star from the substrate and alter its orientation, it will rapidly right itself upon contact with the substrate before crawling away. Like other echinoderms, being inverted makes them more prone to predation, so this behavior also occurs when animals become overturned by turbulence. This behavior is known as a righting response, and it is simply the reorientation of the animal to a position with its aboral side facing up. Unlike sea stars that use their tube feet to right themselves, brittle stars anchor with one arm while the remaining arms assist in the rotation of the central disk (Kleitman, 1941). Brittle stars can right themselves faster than sea stars, in as little as 5-6 seconds compared to 27 seconds respectively (Kleitman, 1941). The righting response is so fundamental to echinoderms and sensitive that it has long been used as an indicator of animal stress (Lawrence et al., 1996), whereby the greater the magnitude of the stress, the longer the righting time (Kleitman, 1941). Several studies have demonstrated that the righting response is sensitive to temperature (e.g., Ardor et al., 2019; Ubaldo et al., 2007), and speeds up with increased temperature, until reaching a thermal limit where it can either slow or stop altogether (Janecki et al., 2010; Ardor et al., 2019). Fewer studies have examined the effects of OA on the righting response, but in a study on juvenile sea urchins, it was found that elevated pCO_2 shortened the time it took for animals to right themselves (Manríquez et al., 2017). Reflecting the complex interaction between pH and temperature, elevated pCO_2 and elevated temperature each appear to speed up the righting response, but when combined, they have mitigating rather than additive effects on juvenile sea urchins (Manríquez et al., 2017).

Once brittle stars have righted themselves, they immediately crawl away from the threat. When locomoting, brittle stars use slightly different strategies than other echinoderms that rely heavily on their tube feet. Instead, brittle stars use their arms to exert forces onto the substrate. Coordinating five arms is complex and animals tend to move in one of two ways, either by rowing or reverse rowing (Astley, 2012). Rowing occurs when the front arm leads and pulls the rest of the body along behind it, which is how they move approximately 75% of the time. The rest of the time they use reverse rowing, in which the front arm trails behind the rest of the body and the other four arms do the bulk of the propulsion work. Brittle stars can reach crawling speeds of 1.3 cm s^{-1} during rowing (Astley, 2012), making them one of the fastest echinoderms. One of the key factors facilitating the speed and agility of brittle stars is their arm sinuosity, or the ability to curve the arms (Goharimanesh et al., 2023). Arm flexibility is critical to proper functioning during locomotion, as well as other anti-predator behaviors.

Arm mechanics

Brittle star defenses are entirely dependent on the biomechanics of the arms, whose structure permits flexibility and rapid movements. Each arm is built from a series of segments comprised of calcified ossicles supported by muscles and connective tissue. The main vertebral ossicles are centralized and surrounded by four smaller ossicle plates on the dorsal, ventral and lateral sides. Four intervertebral muscles attach to each vertebra and their contraction permits horizontal bending only. Additionally, adjacent segments of the arm are connected by mutable collagenous tissue (MCT), which makes up the tendons of the four intervertebral muscles, the intervertebral ligaments, and the ligaments connecting the non-vertebral ossicle plates (Clark et al., 2019). MCT is present in all echinoderms and is a unique viscoelastic material that can quickly change its mechanical stiffness and strength through neural control. It is made up of a collection of collagen fibrils bound together with a glycoprotein and the rapid changes that occur are due to alterations in interfibrillar cohesion rather than in the collagen fibrils themselves (Wilkie, 2021). Properties of MCT can change on the order of 1 sec to a few minutes (Wilkie, 2005) and works with muscles to facilitate locomotion as well as permit animals to maintain posture for extended periods of time. By stiffening, MCT can help transmit the forces of muscle contraction and increase locomotor speed and efficiency. This stiffening also holds posture without the use of active muscle contraction, which is important for suspension feeding because it reduces the energetic costs of holding arms up in the water column.

MCT is sensitive to seawater ions and protons because its component collagen is stabilized by internal ionic interactions (Hidaka, 1982). Collagen within MCT has a triple-helix structure that can deform when exposed to a more acidic pH. In sea urchins, the MCT at the base of the spines decreased in both stiffness and strength as pH decreases (Hidaka, 1982). Temperature has also been shown to influence MCT functioning. In two studies on sea cucumbers, one showed that an extreme heat exposure of 80° C for 4 hours caused expression of proteins that affect stiffness of MCT (Huang et al., 2023), while the other one described strong effects of temperature on the mechanical properties of the body wall through direct effects on the morphology as well as indirect neural effects (Motokawa et al., 2003). Both temperature and pH can affect the stiffening of MCT through multiple mechanisms, possibly altering its functioning with muscles in brittle star arms in ways that limit their effectiveness in locomotion and food capture.

Objective

The western spiny brittle star, *Ophiothrix spiculata*, inhabits the highly variable environment in the Southern California Bight, but like other echinoderms, it might still be vulnerable to OA and OW conditions. These climate change stressors are known to affect the morphology and behavior of other marine invertebrates. For brittle stars, sensitivity to these stressors could alter arm functioning and anti-predator behaviors. The objective of this study was to determine if OA and OW conditions affect the anti-predator behaviors of *O. spiculata*. It was hypothesized that OA conditions would decrease mobility and arm flexibility, while OW would have the opposite effects.

MATERIALS AND METHODS

Animal collection and maintenance

Spiny brittle stars, *Ophiothrix spiculata,* were collected over a period of several months from waters around the Scripps Institution of Oceanography (SIO) Pier and maintained in the Experimental Aquarium at SIO. Brittle stars were held in a communal touch tank with other local intertidal organisms, mainly mussels, *Mytilus californianus,* sea stars, *Asterias rubens,* purple sea urchins, *Strongylocentrotus purpuratus*, and sea cucumbers, *Parastichopus parvimensis.* This touch tank received flow-through seawater from the SIO Pier (3–4 m depth, 300 m offshore) at ambient temperature (12˚C), pH (8.1), and salinity (33.56) at the start of the experiment. Organisms in the touch tank were fed weekly with fish flakes and approximately every other week with fresh giant kelp *Macrocystis pyrifera,* but they could obtain additional food by scavenging on detritus in the touch tank. During the experimental exposure, brittle stars were fed a consistent diet of fish food (Aqueon Pro Sinking Pellet Fish Food, Revitanew Formula, Franklin WI, USA) twice a week. Pellets were approximately 1 mm in diameter and roughly circular. Uneaten food was removed from the tank twice per week just prior to the addition of new food. Tanks were cleaned of excess detritus every day.

Forty-eight brittle stars were available for the experiment, and due to the aggregate housing, the time individuals were held in the touch tank was unknown. For each brittle star, the diameter of the central disk was measured with digital calipers and used as the metric for body size. Disk diameter ranged from 5.84 to 10.41 mm (mean 8.21 mm ± 0.01 mm). Brittle stars were semi-randomly assigned to treatments such that body size was roughly equivalent.

Some brittle stars used in the experiment had arm abnormalities, such as partial or total amputation, on a single arm. For the control treatment, there were no individuals with recent signs of autotomy, but there were two individuals with partial autotomy in the OA treatment, three with partial autotomy in the OW treatment, two with partial and one with complete autotomy in the OW treatment, and one with partial autotomy in the OW+OA treatment. Autotomy does not affect crawling kinematics of brittle stars (Price et al., 2014), so we anticipated that it would not be an issue in this experiment.

Experimental design

Brittle stars were assigned to four pH and temperature treatments using an experimental ocean acidification system. This system consisted of four header tanks (81 L) that each received filtered seawater pumped from the SIO Pier at ambient conditions and fed water directly to 48 plastic tanks (1.6 L) that housed individual brittle stars. At the start of the experiment, ambient pH was 8.1, salinity was 33 ppt, and temperature was 12°C.

One header tank was maintained at ambient pH (8.1) and temperature (12°C) to serve as the control treatment, while the other header tanks were adjusted for the following treatment conditions: reduced pH/ambient temperature (OA, 7.7/12°C), ambient pH/elevated temperature (OW, 8.1/15°C), and reduced pH/elevated temperature (OW+OA, 7.7/15°C). The reduced pH level of 7.7 was chosen to reflect the lowest pH that occurs during local upwelling (7.69, Kekuewa et al., 2022) as well as the projected 0.3-0.4 unit decline in global surface ocean pH by the year 2100 (IPCC, 2014, Jiang et al., 2023). The target elevated temperature was chosen to be 3°C above ambient conditions, in line with projections for ocean warming (IPCC, 2014), which is warmer than the mean winter temperature at the time of the experiment, but not the mean high experienced during summer (17°C, Kekuewa et al., 2022). Experimental pH conditions were achieved by adding 100% CO_2 gas to the respective header tanks. Increased temperatures were achieved by adding aquarium heaters (Titanium, 500 W, Finnex, Chicago, IL, USA) to the respective header tanks.

Experimental conditions were controlled and monitored with an Apex Lite aquarium controller equipped with Apex Neptune temperature and pH probes (0.01 pH accuracy; 0.1°C temperature accuracy; Neptune Systems, Morgan Hill, CA, USA). Both the pH and temperature of each header tank were logged every 10 minutes throughout the experiment. Apex probes were calibrated prior to the start of the experiment using NBS buffer solutions (Fisher Scientific, Fair Lawn, NJ, USA). Temperature and pH were gradually adjusted over 5 days to minimize animal stress, and after target values were achieved, the was run for 29 days.

Daily readings of pH and temperature were taken from each header tank and each brittle star tank using a portable probe (HQ40d, probe PHC201, precision 0.01 pH, 0.1°C temperature, Hach, Loveland, CO, USA). This probe was calibrated prior to the start of the experiment with NBS buffer solutions (Fisher Scientific, Fair Lawn, NJ, USA) and checked for accuracy with certified reference material (CRM) from the Dickson lab at SIO. On the 10th day of the experiment, the portable pH meter was accidentally damaged and was replaced with a new one (TK295PLUS, with Neptune probes, 0.01 pH accuracy; 0.1°C temperature accuracy; TeckcoPlus Ltd.,Hong Kong, China), resulting in a loss of portable probe data for five days. Apex probe data was still taken every 10 minutes from the header tanks throughout this lapse. The new probe was also calibrated with the same NBS buffers and checked for accuracy using CRM.

Additionally, water samples (N=8) were collected once during the experiment, one from each header tank and one random brittle star tank from each treatment using standard operating procedures (Dickson, 2007). These water samples were submitted to the Dickson lab for analysis of pH and total alkalinity; however, the water chemistry data were not available at the time of this thesis submission. Thus, the pH values presented in these results have not been corrected using the water chemistry, and none of the other carbonate parameters could be calculated.

Anti-predator behaviors

Following the exposure period, each brittle star was assessed using a series of behavioral metrics. We focused on three key anti-predator behaviors of brittle stars: 1. Righting response, 2. Escape response, and 3. Arm retraction. A righting response occurs when brittle stars get flipped over and they use one or more of their arms to right themselves so that their aboral disk faces upward. An escape response involves rapid locomotion away from the threat and often follows a righting response. Lastly, arm retraction occurs when the tip of one of the arms encounters an unpleasant mechanical stimulus, causing the brittle start to quickly recoil the arm inward toward the central disk. Each of these behaviors can be readily stimulated and recorded.

Behavioral assessments of individual brittle stars were carried out in an acrylic aquarium (38.5 cm x 24.5 cm) filled to approximately 19 cm with the animal's treatment water. A 2.5 mm grid card was affixed to the bottom center of the aquarium and used for calibration. Behavior trials were videorecorded in 4K at 30 fps using an iPhone 14 positioned above the aquarium. The three behaviors described above were recorded in the following sequence. First, the righting response was initiated by placing a brittle star on the bottom center of the aquarium with the top of its central disk flush against the bottom surface of the tank. The brittle star was immediately released and the time for it to right itself was recorded. Brittle stars were considered fully righted when all their arms and the central disk were flush with the bottom of the tank, aboral side up. Second, the escape was recorded. Most of the time brittle stars immediately locomoted away following righting. If this did not occur, then we waited one minute before resetting them to the original location in the center of the tank and releasing, which incited an escape response. The end of the escape response was determined by the brittle star either stopping or reaching the side of the aquarium. Lastly, brittle stars were returned to the center of the aquarium and while they remained stationary, arm retraction was induced by gently squeezing the tip of one of the arms with forceps. This process consistently caused brittle stars to quickly recoil the arm towards the central disk.

Kinematics

All kinematics were determined from the recordings and processed as .mov videos using the open-source motion analysis software Tracker v6 (Brown et al., 2024) and the 2.5 mm grid card for calibration. Righting response was calculated as the duration, in seconds, between the first frame in which the animal was released until the frame in which it was completely righted, as defined above. For the escape response, multiple kinematic variables were determined. First, lag time was calculated as the time between the frame at completion of the righting response and the frame at which the central disk moved upon initiation of locomotion. Other kinematics were calculated using the autotracking feature of the Tracker software. Briefly, we tracked the movements of the brittle stars using the central disk as the origin point Velocity and acceleration were calculated over 20-frame increments throughout the recording. Distance was calculated from the frame in which the central disk-initiated movement to the frame in which movement of the central disk stopped. Velocity was calculated as the mean velocity over the entire escape distance while acceleration was calculated from the latter half of the escape response, where it peaked.

For arm retraction, the central disk tended to be pulled as the arm contracted. To account for this, we measured the length of the arm prior to retraction and subtracted from it the length of the arm (linear distance from base to tip) after retraction. Velocity of arm retraction was calculated over the entire retraction while acceleration was calculated over the latter half of retraction, where it peaked.

Arm flexibility

Following the behavioral assays, each brittle star was tested for arm flexibility in air. Individual, live animals were placed on a 2.5 mm grid card with the central disk held in place by hand. Arms with no signs of autotomy and no visible signs of damage were measured for flexibility. A single arm was bent beginning at a distance from the central disk that was approximately 5% of the total arm length. From this point, the arm was coiled into the tightest circle that could be formed without applying significant stress. With the arm in the coiled position, a photograph was taken from above using an iPhone 14 phone camera with a resolution of 48 megapixels. From these images, the radius of curvature (ROC) was calculated using *ImageJ* software, Java 8 (Schneider et al., 2012). Briefly, ROC was calculated as the diameter of the smallest circle that fit the arc of the arm coil. After living measurements were taken, brittle stars were anesthetized and euthanized via placement in a -20°C freezer. The following day, frozen brittle stars were defrosted, and the ROC was measured as described above. Measurements on dead individuals were made to assess the contribution of muscle contraction and MCT in arm flexibility.

Statistical analysis

All statistical analyses were carried out in R (R Core Team, 2023). Disk percent growth, righting response time, escape response (lag time, distance, velocity, acceleration), arm retraction (distance, velocity, acceleration), and arm flexibility (ROC) were all tested for normality and homogeneity of variance using Shapiro-Wilk and Bartlett tests, respectively. Data that did not pass normality assumptions (i.e., lag time, escape velocity and acceleration, arm retraction velocity and acceleration, ROC) were log-transformed. Each measured variable was compared across treatments using ANCOVA, with initial disk size as a covariate, and Tukey post-hoc tests when appropriate. ROC was compared between live and dead specimens for each treatment using paired T-tests.

RESULTS

Water chemistry

Ambient pH remained stable over a 24-hr period, showing no diurnal fluctuations, while target pH levels fluctuated hourly (± < 0.1 pH units) due to solenoid cycling (Fig. 1A). Both ambient and experimental temperatures remained stable, showing no diurnal fluctuations, though the OW+OA treatment was more variable, sometimes fluctuating by > 1°C (Fig. 1B). Over the course of the 29-day experiment, pH continued to fluctuate slightly and disruption in the CO$_2$ gas cylinder on February 7 caused the OA and OW+OA treatments to temporarily reach ambient pH for three days (Fig. 2A). On the other hand, ambient and experimental temperatures were stable over the duration of the experiment (Fig. 2B).

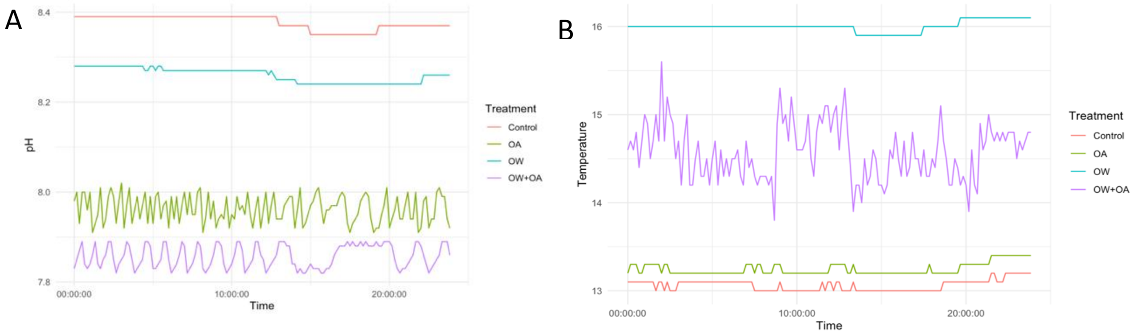


Figure 1. Representative example of pH (A) and temperature (B) over the course of a 24-hr period. Measurements were taken from the header tanks very 20 minutes. Neither pH nor temperature exhibited diurnal fluctuations. pH values are uncorrected.

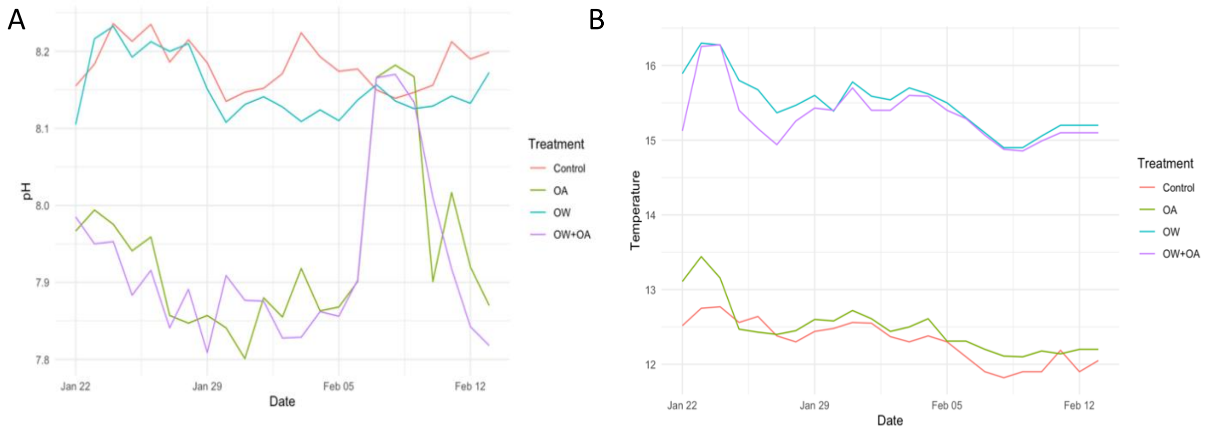


Figure 2. Variation of pH (A) and temperature (B) throughout the duration of the experiment. pH values from the OA and OW+OA treatments reached ambient February 7-9 due to disruption of the CO_2 gas cylinder. pH values are uncorrected.

Mortality and growth

Consistent levels of mortality occurred across all treatments during the experiment, leading to survival rates of 66.7% in control, 75% in OA, 66.7% in OW, and 66.67% in OW+OA treatments. All brittle stars that survived the duration of the experiment (N=32) exhibited growth in disk size (Table 1), but there was no difference in the percent growth between treatments (ANOVA, df = 3, F = 676.4, p = 0.113) (Fig. 3).

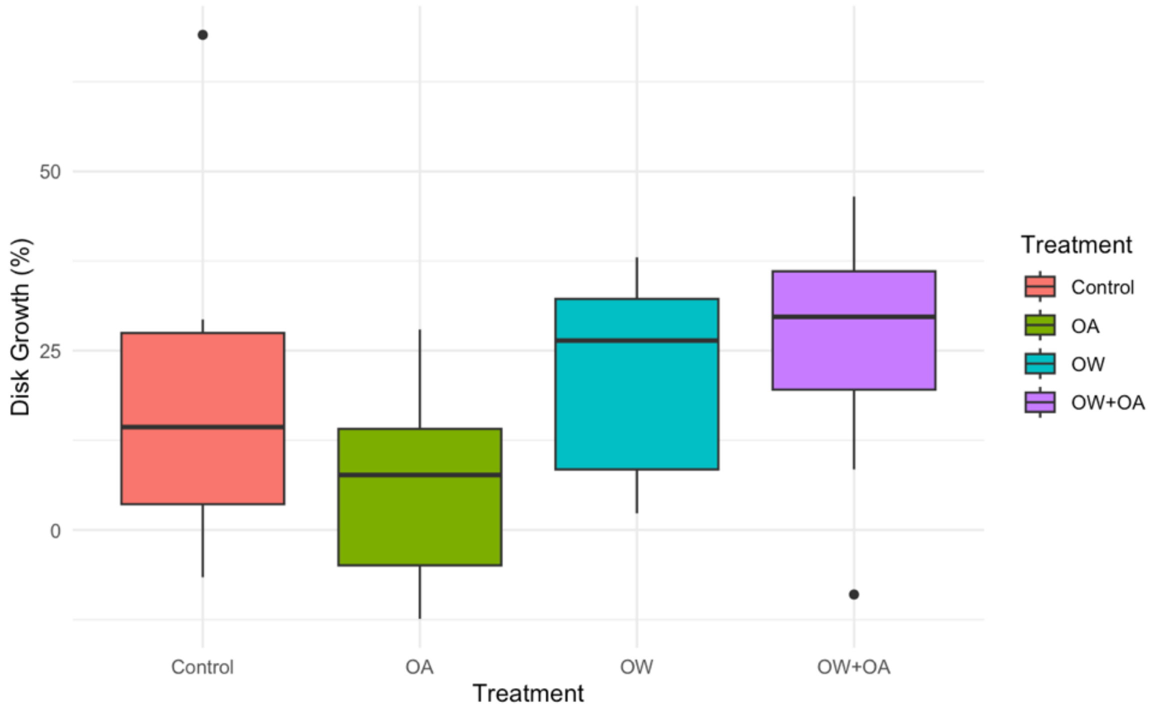


Figure 3. Percent growth of the central disk was the same for all treatments. Box boundaries are the first and third quartiles, whiskers are 1.5 times the interquartile range, the center line is the median, and outliers are solid dots.

Righting response

Brittle stars consistently performed a righting response that took from 4 to 24 seconds (Table 1). The time to complete the righting response did not differ among treatments (ANCOVA, df = 3, F = 0.786, p = 0.514; Fig. 4) and there was no effect of disk size (F = 2.209, p = 0.151).

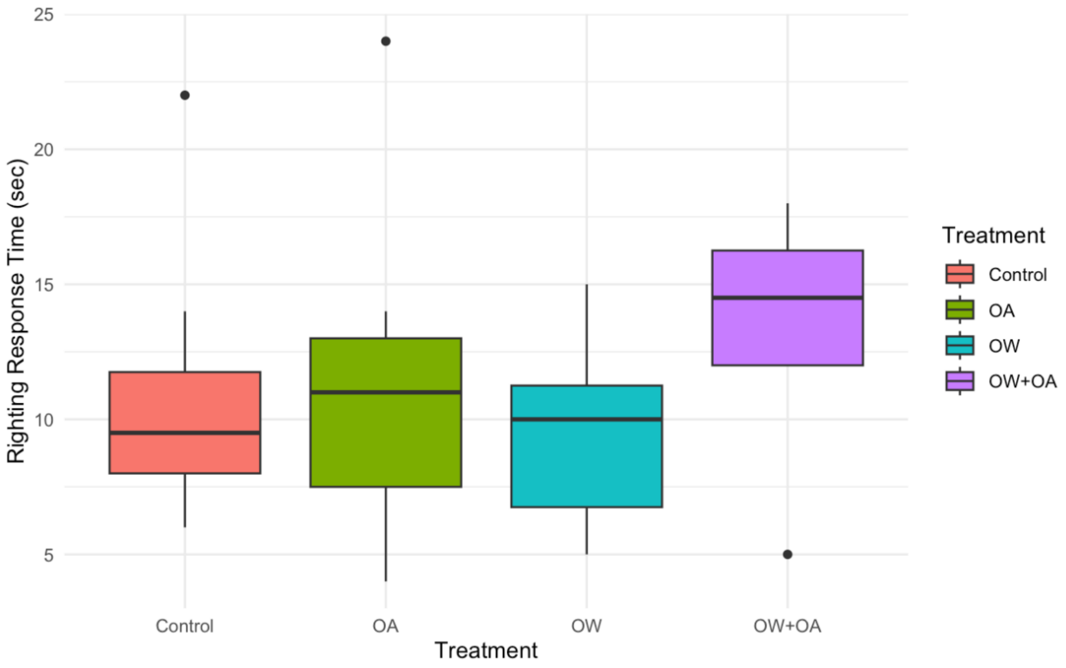


Figure 4. Righting response time did not differ among treatments. Box boundaries are the first and third quartiles, whiskers are 1.5 times the interquartile range, the center line is the median, and outliers are solid dots.

Escape response

Lag time for the initiation of an escape response ranged from 0 to 106 seconds and differed between treatments (ANCOVA, F = 5.024, p = 0.011; Table 1; Fig. 5). Brittle stars in the OW+OA treatment had a significantly lower lag time, meaning they initiated the escape more quickly, than those in the control (p = 0.030) and OA (p = 0.007) treatments but not in the OW treatment (p = 0.057). There was no effect on disk size in any of the treatments (F = 0.004, p = 0.949).

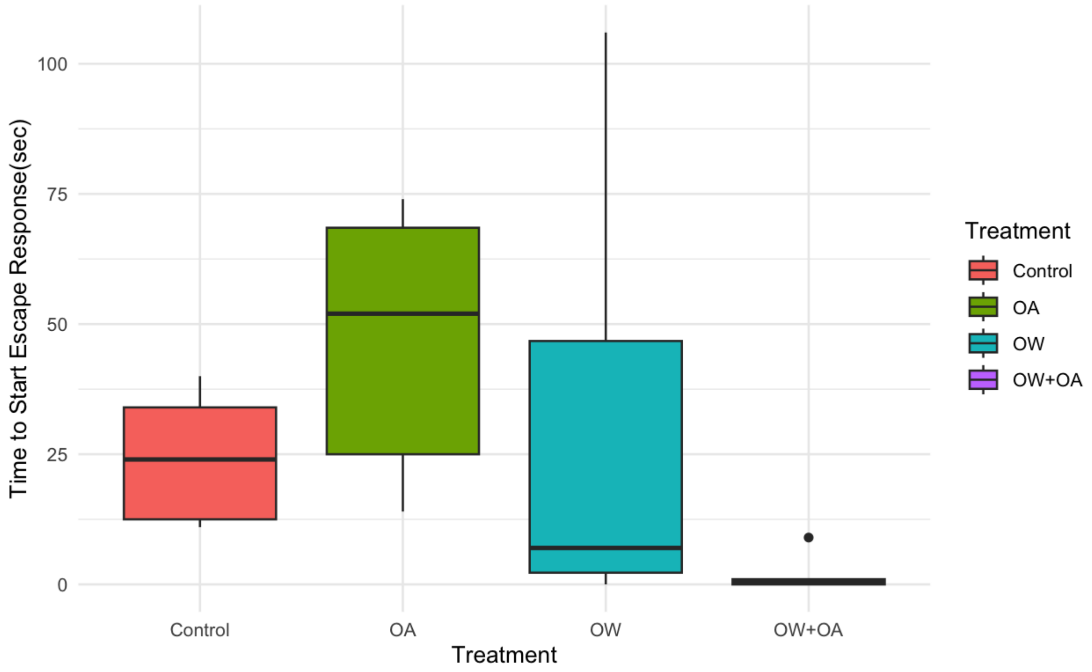


Figure 5. The time brittle stars took to start the escape response, or lag time, was significantly shorter in the OW+OA treatment compared to the Control and OA treatments. Box boundaries are the first and third quartiles, whiskers are 1.5 times the interquartile range, the center line is the median, and outliers are solid dots.

Brittle stars consistently performed an escape response, but with no general pattern. Some individuals crawled away from the starting point in a direct path while others took a more circuitous path. Regardless of the path, all brittle stars headed to the edges or corners of the tank, marking an endpoint for the escape response. The total distance traveled during the escape response was not statistically different between treatments (ANCOVA, df = 3, F = 1.858, p = 0.187; Fig. 6) and there was no effect of disk size (F = 1.858, p = 0.187).

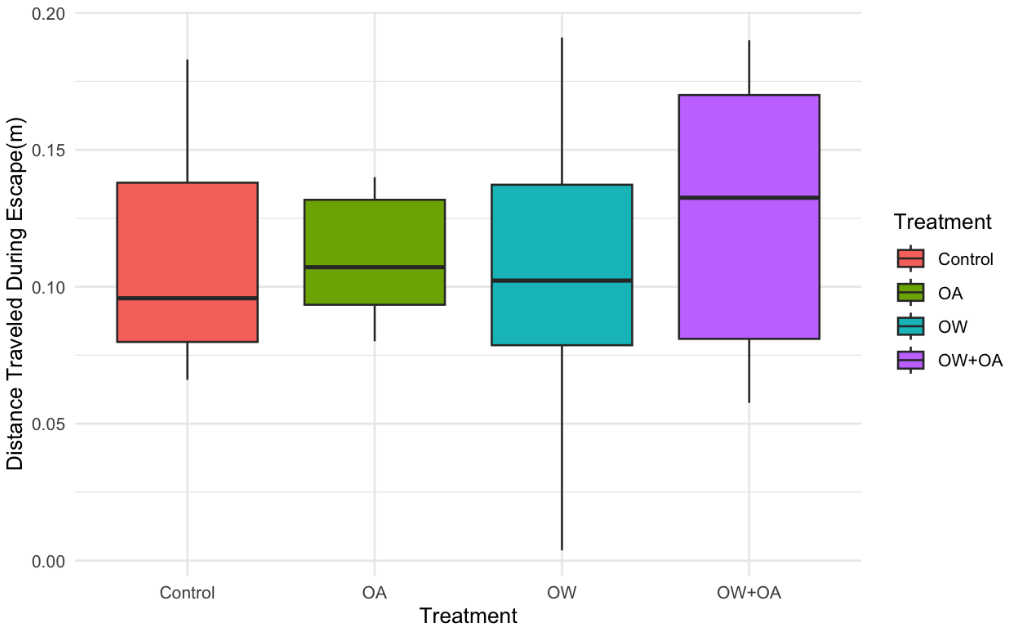


Figure 6. The total distance traveled during the escape response did not differ between treatments. Box boundaries are the first and third quartiles, whiskers are 1.5 times the interquartile range, the center line is the median, and outliers are solid dots.

Escape velocity was not different between treatments (ANCOVA, df = 3, F = 0.827, p = 0.493; Fig. 7A) and disk diameter had no effect on the velocity of the escape response (F = 1.684, p = 0.493). Neither did escape acceleration differ between treatments (ANCOVA, F = 0.672, p = 0.579; Fig. 7B). Disk diameter had no effect on acceleration (F = 4.087, p = 0.056).

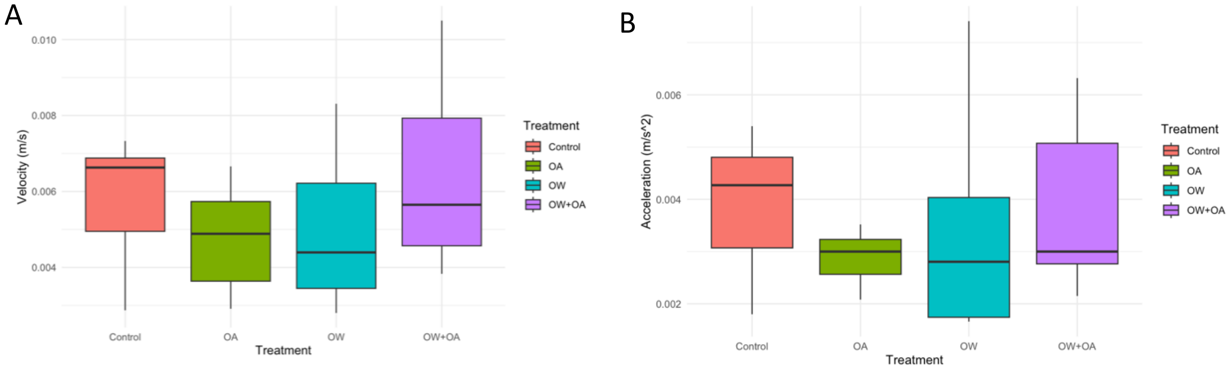


Figure 7. Neither escape velocity (A) nor acceleration (B) differed between treatments. Box boundaries are the first and third quartiles, whiskers are 1.5 times the interquartile range, the center line is the median, and outliers are solid dots.

Arm retraction

Arm retraction distance did not differ between treatment (ANCOVA, df = 3, F = 2.513, p = 0.0849; Fig. 8) and disk diameter had no effect (F = 0.002, p = 0.9681). Neither arm retraction velocity (ANCOVA, df = 3, F = 1.503, p = 0.241; disk size F = 0.138, p = 0.713; Fig. 9A) nor acceleration (ANCOVA, df = 3, F = 1.179, p = 0.34; disk size F = 3.102, p = 0.092; Fig. 9B) differed between treatments.

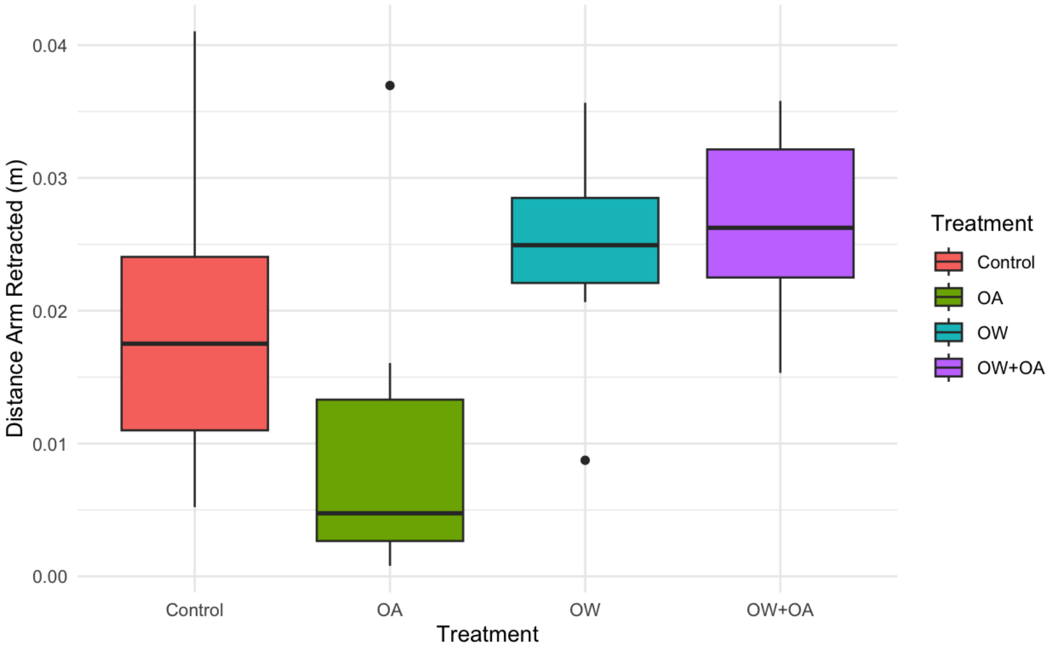


Figure 8. Arm retraction distance was the same across treatments. Box boundaries are the first and third quartiles, whiskers are 1.5 times the interquartile range, the center line is the median, and outliers are solid dots.

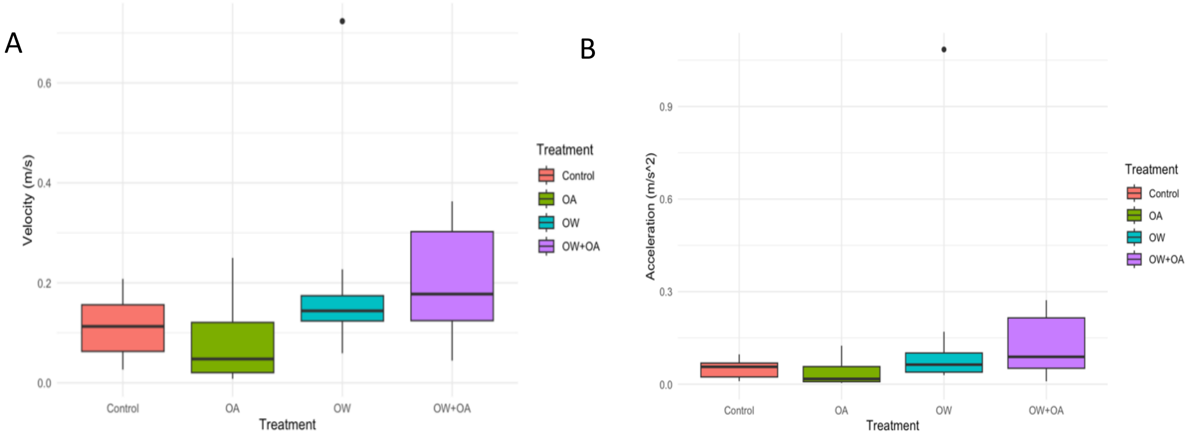


Figure 9. Arm retraction velocity (A) and acceleration (B) was the same across treatments. Box boundaries are the first and third quartiles, whiskers are 1.5 times the interquartile range, the center line is the median, and outliers are solid dots.

Arm flexibility

Arm flexibility, measured as ROC, of live brittle stars did not differ between treatments (ANCOVA, df = 3, F = 1.982, p = 0.148; Fig. 10), and there was no effect of disk diameter (F = 0.389, p = 0.54). However, ROC was higher, meaning less flexibility, in live compared to dead brittle stars exposed to all treatments, except OA (T-test, control: df = 7, t = 3.6969, p = 0.008; OW: p = 0.039; OA: df = 7, t = 2.2487, p = 0.059; OW+OA: df = 7, t = 3.0059, p = 0.020; Fig. 10).

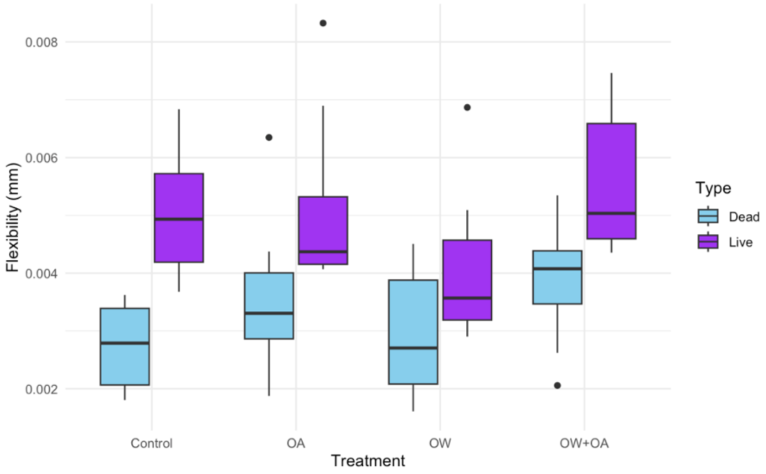
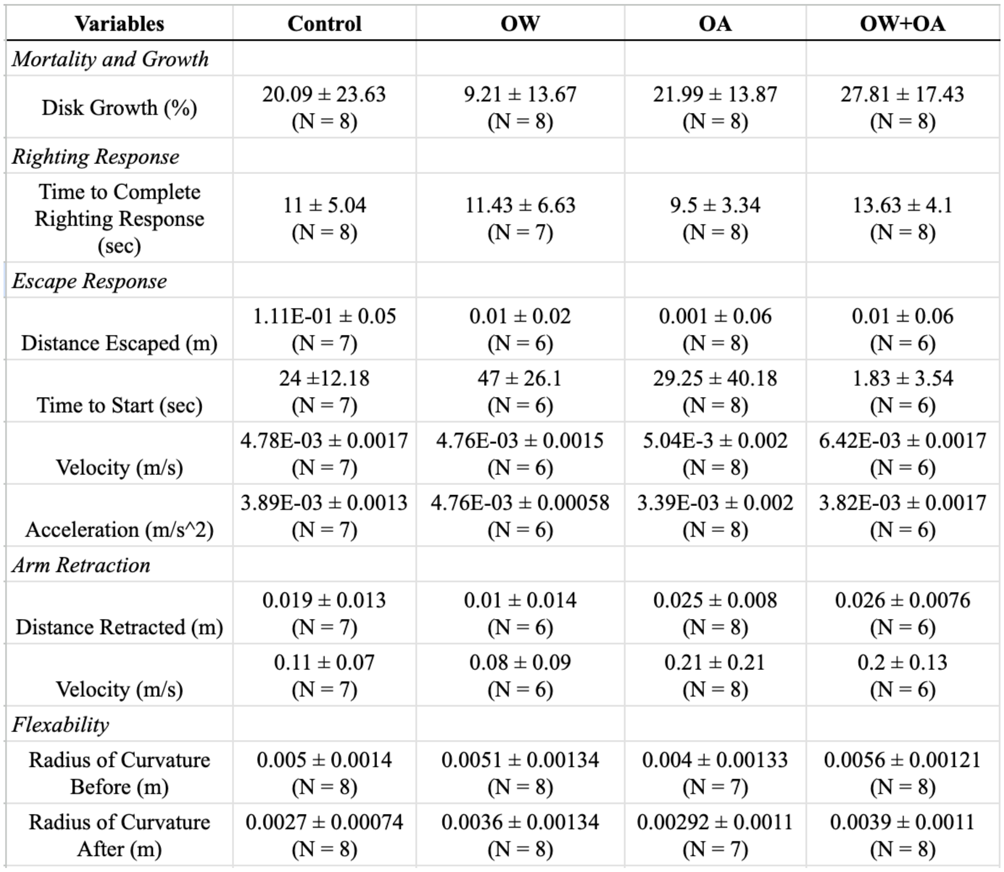


Figure 10. Flexibility (ROC) of brittle star arms in both live and dead condition. Treatment had no effect on arm ROC. There was a significant decrease in ROC after death in all treatments except OA. Box boundaries are the first and third quartiles, whiskers are 1.5 times the interquartile range, the center line is the median, and outliers are solid dots.

Table 1. Summary of all variables measured as mean ± standard deviation. N = sample size



Variables	**Control**	**OW**	**OA**	**OW+OA**
Mortality and Growth				
Disk Growth (%)	20.09 ± 23.63 (N = 8)	9.21 ± 13.67 (N = 8)	21.99 ± 13.87 (N = 8)	27.81 ± 17.43 (N = 8)
Righting Response				
Time to Complete Righting Response (sec)	11 ± 5.04 (N = 8)	11.43 ± 6.63 (N = 7)	9.5 ± 3.34 (N = 8)	13.63 ± 4.1 (N = 8)
Escape Response				
Distance Escaped (m)	1.11E-01 ± 0.05 (N = 7)	0.01 ± 0.02 (N = 6)	0.001 ± 0.06 (N = 8)	0.01 ± 0.06 (N = 6)
Time to Start (sec)	24 ±12.18 (N = 7)	47 ± 26.1 (N = 6)	29.25 ± 40.18 (N = 8)	1.83 ± 3.54 (N = 6)
Velocity (m/s)	4.78E-03 ± 0.0017 (N = 7)	4.76E-03 ± 0.0015 (N = 6)	5.04E-3 ± 0.002 (N = 8)	6.42E-03 ± 0.0017 (N = 6)
Acceleration (m/s^2)	3.89E-03 ± 0.0013 (N = 7)	4.76E-03 ± 0.00058 (N = 6)	3.39E-03 ± 0.002 (N = 8)	3.82E-03 ± 0.0017 (N = 6)
Arm Retraction				
Distance Retracted (m)	0.019 ± 0.013 (N = 7)	0.01 ± 0.014 (N = 6)	0.025 ± 0.008 (N = 8)	0.026 ± 0.0076 (N = 6)
Velocity (m/s)	0.11 ± 0.07 (N = 7)	0.08 ± 0.09 (N = 6)	0.21 ± 0.21 (N = 8)	0.2 ± 0.13 (N = 6)
Flexability				
Radius of Curvature Before (m)	0.005 ± 0.0014 (N = 8)	0.0051 ± 0.00134 (N = 8)	0.004 ± 0.00133 (N = 7)	0.0056 ± 0.00121 (N = 8)
Radius of Curvature After (m)	0.0027 ± 0.00074 (N = 8)	0.0036 ± 0.00134 (N = 8)	0.00292 ± 0.0011 (N = 7)	0.0039 ± 0.0011 (N = 8)

DISCUSSION

Overview

Echinoderms are generally sensitive to ocean acidification and ocean warming conditions, including species that are adapted to environmental variability. These sensitivities can manifest in multiple aspects of physiology, from growth and metabolism to morphology and behavior. In this study, we examined how the western spiny brittle star, *O. spiculata*, responds to OA and OW stressors in their dynamic environment in the Southern California Bight. Given that environmental changes in temperature and/or pH have been shown to alter metabolic rates, neural system functioning, and morphology, we hypothesized that anti-predator behaviors, which depend on each of these physiological parameters, may be altered by OA and OW. Our results reveal that none of *O. spiculata's* anti-predator behaviors, including righting, escape, and arm retraction responses, nor arm flexibility, which is critical to these behaviors, were affected by experimental OA and OW conditions. Only one metric, the lag time in initiating an escape response, was altered by the combination of OW+OA stressors, whereby brittle stars-initiated escapes more quickly under these conditions. Overall, our hypothesis was not supported and *O. spiculata* mostly exhibited no sensitivity, in terms of its mobility and ability to evade predators, to near-future changes on ocean pH and temperature.

Combined stressors reduce escape lag time

In this study, *O. spiculata* exhibited a significantly shorter lag time for its escape response under combined OW+OA conditions compared to the control and OA, meaning that animals initiated an escape response more quickly. It is likely the warmer temperature that is driving this quicker response given that the lag time was not significantly different from the OW treatment, which trended lower, and OA alone had no effect. It is sensible that warmer temperature can speed animal reactivity because metabolic rates of echinoderms tend to increase under OW conditions (Lang et al., 2023), and higher metabolic rates can speed up biological signaling pathways (Glazier, 2015). Furthermore, temperature directly affects the nervous system in ectotherms, with warmer temperatures increasing synaptic transmission, postsynaptic integration, and conduction velocity (Montgomery et al., 1990). On the other hand, our prediction that OA would increase lag time did not hold. While many echinoderms have decreased metabolic rates under OA conditions (Collard et at., 2013) that could slow reaction times, that might not be the case for *O. spiculata* because of species-specific sensitivities. Also, the brittle star nervous system may not be as sensitive to OA as it is for other organisms such as pteropods (Moya et al., 2016) and fish (Tresguerres et al., 2017) but this warrants further study. Nonetheless, our data show that *O. spiculata* may get a head start from predators with their faster escape reaction times under warmer conditions.

Anti-predator behaviors

Once a brittle star initiates an escape response, it is important to accelerate quickly and crawl fast to put distance between the potential predators. Neither OA nor OW conditions influenced the willingness of *O. spiculata* to perform an escape response nor did they affect the kinematics of the escape, including acceleration, velocity and distance. This is somewhat surprising since warmer temperatures in general tend to increase locomotion kinematics in organisms, and specifically it increased movement speed in the brittle star *Ophiura ophiura* (Wood et al., 2010). However, a meta-analysis on echinoderm responses to OW indicate that elevated temperature had no general effect on movement, which was inclusive of righting behavior, locomotion speed, and foraging time (Lang et al., 2023). Given that thermal sensitivities are species-specific, it would be informative to establish the thermal performance curve and acclimation capacity for *O. spiculata* when trying to understand their kinematic response to OW. Similarly, to understand why *O. spiculata* showed no sensitivity to OA conditions, it would be fruitful to determine their acclimation capacity, or physiological plasticity, because that is key to responding to changing conditions (Stillman, 2003).

The distance that brittle stars cover in their escape is not dependent on velocity and acceleration, but can reflect fatigue, the proximity to a shelter, or the pursuit of a predator. We found no effect of OA or OW conditions on escape distance, but this could be due to limitations of the experimental arena. Most brittle stars crawled to the edge of the aquarium where they nestled into the corner, restricting their escape distance to < 20 cm. Over this distance, brittle stars may not experience fatigue, but perhaps over longer distances, the metabolic effects of OA and/or OW might be appreciable enough to affect escape duration and distance. Proximity to a refuge, such as rocks, kelp holdfasts, or beds of conspecifics, influence the behavior of brittle stars (Sköld, 1998) and is another important element of anti-predator behavior not accounted for in this study.

Predators tend to attack brittle stars at the arm tips (Sköld et al., 1998), stimulating the arm retraction response. Pinching the tips of the arms simulates predation and was effective in consistently inducing this response in our study on *O. spiculata,* but neither OA or OW affected the frequency or kinematics of this behavior. Compared to the escape response, arm retraction is less energetically costly, as it requires limited movement of a single arm. It is also a quick reflexive action that may rely more on MCT than locomotion does, though this has not been tested. The fact that we did not detect measurable differences in escape or arm retraction kinematics in our experimental treatments suggest that the musculoskeletal system, including MCT, of *O. spiculata,* is robust to OA and OW conditions.

Righting response suggests no stress

The righting response is a good indicator of echinoderm stress and is known to be sensitive to environmental temperature. Sea urchins, sea stars, and brittle stars all tend to decrease their righting response time as temperature increases (Kleitman, 1941), yet in our study, temperature had no effect on the righting response time of *O. spiculata*. This may be due to the relatively small temperature increment used (+3°C), which appears to be insufficient to elicit stress and changes in the righting response. In another study, a temperature increases of 6°C was sufficient to speed up the righting response in sea stars (Ardor et al., 2019). Unfortunately, the thermal performance curve is not known for *O. spiculata*, so it is not evident what temperature threshold may be necessary to induce stress sufficient to affect the righting response. With seasonal temperature variations in La Jolla, CA ranging from 12 to 26°C (Keukawa et al., 2023), upper thermal limits are likely higher. Similarly, the experimental pH of 7.7 did not affect the righting response times of *O. spiculata*, and while this might suggest that brittle stars were not stressed, it is unclear if the righting response is a good stress indicator of pCO_2/pH. In juvenile sea urchins, elevated pCO_2/reduced pH had no effect on the righting response while temperature did (Manríquez et al., 2017). Nonetheless, the consistency of the righting response of *O. spiculata* is an indication that the OA and OW conditions used in this study did not induce sufficient stress, which may explain why we observed no effects on most behavioral metrics analyzed.

Arm flexibility

Arm flexibility was measured because it is central to all movements of brittle stars. We predicted that flexibility would be affected by OA and OW because of ossicle degradation and disruption to neural and musculoskeletal action, but instead neither stressor had an impact.

Flexibility is determined by primarily by ossicle morphology and skeletal organization, such that the bending permitted at each joint determines the radius of curvature for the entire arm (Tomholt et al., 2020; Clark et al., 2018). If the ossicles, which are known to decalcify under OA (Walker et al., 2013), become less dense and more brittle, flexibility might increase supposing that muscle contraction and MCT do not limit or compensate. We attempted to assess the contribution of muscle and MCT activity by comparing flexibility between live and dead animals in each treatment and found that live brittle stars had less flexibility (larger radius of curvature) than dead ones in all but the OA treatment. It is unclear why animals in the OA treatment had the same flexibility before and after death when the animals in other treatments didn't, and this deserves more attention. Overall, this comparison shows that muscle action and MCT tend to restrict flexibility in live animals, because neither can be activated once the organism is deceased.

Body size independence

Typically, the kinematics of movement correlate with body size of an animal, but we found no effect of disk diameter on any kinematic variable measured in this study. In multiple species of sea stars, animals with a larger disk size take longer to right themselves compared to ones with a smaller disk size (Pollis et al., 1975; Burdi, 2012), partly because it takes more energy to flip their larger body. Likewise, it takes more energy to accelerate and move a larger body underwater due to higher drag forces. In some sea star species, larger animals crawl with greater speeds (Montgomery et al., 2012), yet for others, body size and crawling speed are negatively correlated (Domenici et al., 2003; Mueller et al., 2011). For brittle stars, the central disk is relatively small compared to the length of the arms, but it positively correlates with crawling speed in two other species of brittle stars (*Ophiocoma echinate* and *Ophioderma* *appressum*; Price et al., 2014). The independence of body size in our study on *O. spiculata* is an indication that we may not have elicited maximum responses in our testing.

Methodical considerations

Throughout the course of this experiment, we encountered a few problems when it came to maintaining the desired pH of the system. There was a notable dip in the pH of all treatments on January 29th due to a large storm in the area, reflecting the challenges of using seawater pumped from the SIO pier. In addition, a leak occurred in the CO_2 tank around February 8th, which caused the pH of the OA and OW+OA treatments to reach ambient levels for three days. This disruption was relatively brief for a 29-day experiment, but effects on the brittle stars cannot be ruled out.

We carried out all behavioral assays in a bare, plexiglass aquarium so that we could consistently stimulate and record brittle stars, but this does not mimic the natural, sandy bottom environment that *O. spiculata* live in. We performed preliminary tests using different substrates, including sand and stones, but they produced poor quality videos and inconsistent brittle star behaviors. We did not determine if the smooth plexiglass substrate influenced locomotion, but all treatment animals were tested using the same protocol.

Lastly, our experiment was run for approximately one month, which could either be long enough for acclimation to occur or too short for effects to manifest. The average duration of OA experiments that revealed impacts on echinoderms is less than 50 days, though studies range from 1 day to 5 months (Bednaršek et al., 2021). Due to species variability, it is difficult to determine the appropriate time frame to measure the effects of exposure to OA and OW.

Conclusions

Our study revealed that most anti-predator behaviors of the western spiny brittle star are not affected by OA (pH 7.7) and OW (15°C) conditions over the short-term (one month). Lag time in the escape response was the only parameter that was altered, and it was primarily sped up by warmer temperature. It was surprising that more behavior kinematics were unaffected by temperature because of the common positive correlation between biological processes and temperature, but such a relationship might appear at higher experimental temperatures. Nonetheless, at these low temperature and pH conditions, brittle stars can maintain arm flexibility and perform their key anti-predator behaviors without any detriment to their efficacy.

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