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Ocean acidification modulates the impact of fluoxetine on larval behaviors of non-target organisms

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by

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Abstract
Emerging pollutants, such as pharmaceuticals from human waste, are continuously released into aquatic systems. Although pharmaceuticals alone can adversely impact marine organisms, the bioavailability of many pharmaceuticals are dependent on ambient physical conditions, like pH. As few studies have considered the interactive effects of pharmaceutical pollution and anthropogenic ocean acidification, this study investigated the behavioral response of larval sea urchins (*Heliocidaris crassispina*) and ascidians (*Styela plicata*) to environmentally-relevant concentrations of fluoxetine (10 and 100 ng L^{-1}) under ambient (pH 8.0) and acidified conditions (pH 7.7). Larval ascidians reared at pH 8.0 exhibited swam in slower, more directed paths with increasing fluoxetine. Interestingly, this effect was absent at pH 7.7. On the other hand, I only observed independent effects of fluoxetine and acidification on urchin swimming behavior. My findings highlight the importance of using behavioral endpoints when assessing the realistic sub-lethal organismal and ecological impacts of anthropogenic stressors, and that considering differences in species traits may allow for the generation of more realistic predictions of the impact of emerging pollutants under future climate scenarios.

1. Introduction

[1]Increasing human interference of aquatic ecosystems continues to threaten marine biodiversity. Modern anthropogenic stressors range from ocean warming and acidification caused by increasing carbon emissions (Caldeira & Wickett 2005; Ricke & Caldeira 2014) to contamination with regulated pollutants (Förstner & Wittmann 1981; van Grinsven *et al.* 2012) and emerging pollutants with yet-to-be determined impacts (Deblonde *et al.* 2011; Geissen *et al.* 2015). Within the long list of emergent pollutants, many chemical compounds have the potential to interact with physical stressors to create non-linear impacts on marine organisms (Bryant *et al.* 1985; Stockdale *et al.* 2016; Almeida *et al.* 2018). Understanding the likelihood, magnitude, and mechanisms of such interactions are crucial for determining management goals and strategies (Boyd *et al.* 2018; Sarà *et al.* 2018).

[1] Data presented here is a part of larger collaboration with H.K. Lo from the Hong Kong University of Science and Technology. The resulting manuscript is in review in the journal *Science of the Total Environment.*

Environmental exposure to pharmaceuticals, a class of emergent pollutants, is of urgent concern due to their potential to adversely affect non-target organisms via ubiquitous biological pathways (Gaw *et al.* 2014; Fabbri & Franzellitti 2016). Most pharmaceuticals are only partially absorbed by target organisms (Monteiro & Boxall 2010), leaving trace concentrations in wastewater effluent that are not fully removed by treatment (Kot-Wasik *et al.* 2016). One such pharmaceutical is fluoxetine (FX), a selective serotonin reuptake inhibitor (SSRI) that increases signal transmission across serotonergic synapses and is the active compound in many antidepressant medications (Song *et al.* 1993). Fluoxetine HCl (tradename Prozac® or Sarafem®) is considered to be one of the most acutely toxic pharmaceuticals for non-target organisms, as evidenced by its low EC_{50} and LC_{50} values (Fent *et al.* 2006).

Given that serotonergic pathways are conserved across a wide array of taxonomic groups and are essential for regulating locomotion and development (Hen 1993), it is not surprising that FX exposure is associated with negative behavioral impacts. At concentrations up to 3.45 mg L^{-1}, FX has been reported to decrease feeding in worms (Hird *et al.* 2016), decrease crawling speed in snails (Fong *et al.* 2015), and impair sand digging behavior in cuttlefish hatchlings (Di Poi *et al.* 2013). FX also increased locomotion in estuarine crabs (Peters *et al.* 2017) and increased photosensitivity and geotaxis in amphipods (Guler & Ford 2010). Apart from affecting behavior, serotonin also plays a crucial role in early embryogenesis (Buznikov *et al.* 2001). FX can also affect early juveniles and adults, as evidenced by delayed maturity in polychaetes (Méndez & Barata 2015) and induced spawning in mussels (Lazzara *et al.* 2012). At environmentally-relevant concentrations, zebrafish were able to biotransform FX into its

metabolite norfluoxetine, reducing the overall bioaccumulation of FX (Zindler *et al.* 2020). However, these studies did not account for the possible interaction between FX and other physical ocean conditions, such as temperature or pH.

FX is relatively stable in aquatic environments, with a half-life exceeding 100 days (Kwon & Armbrust 2006). Additionally, its high sorption coefficient suggests it can be readily transported and accumulated in soil and sediment (Yamamoto *et al.* 2005), increasing its ability to persist in aquatic ecosystems in direct contact with wastewater effluent, where FX concentrations can range from 12 to 540 ng L^{-1} (Weston *et al.* 2001; Kolpin *et al.* 2002). For example, recent coastal measurements near the Asian megacity of Hong Kong revealed FX concentrations of up to 97.2 ng L^{-1} (Lo 2020). Coastal regions are hotspots for pharmaceutical occurrence due to their proximity to human activities (Gulkowska *et al.* 2007; Singh *et al.* 2010; Fenet *et al.* 2014). Further, the rising usage of relatively stable pharmaceuticals like FX (Mojtabai & Olfson 2014), combined with the continuous discharge of wastewater into coastal ecosystems, can lead to the long-term "pseudo-persistence" of these contaminants, posing harmful effects to non-target organisms if left unregulated (Gelatti *et al.* 2013; Polverino *et al.* 2021).

The pH for maximizing FX bioavailability is alkaline at 13.7, where over 99.99% exists in its original form; however, below pH 5.6, more than 99.99% of FX is ionized (norfluoxetine), yielding the lowest bioavailability (Brooks *et al.* 2003). Pharmaceutical bioavailability is especially important to consider in the context of ocean acidification (OA), where increasing anthropogenic carbon emissions drive changes in ocean carbonate chemistry, resulting in a predicted pH drop of 0.2 to 0.4 units by the end of the century (Caldeira & Wickett 2005). In addition, coastal regions experience frequent and

large fluctuations in pH that can reach end-of-century pH even at present-day (Hofmann *et al.* 2011; Pecquet *et al.* 2017). For instance, the Pearl River Delta, which includes Hong Kong, experienced a pH drop from 8.2 to 7.7 in the last two decades (Wang *et al.* 2016). Since the bioavailability—and thus potential impact on non-target organisms—of pharmaceuticals, like FX, is modulated by ambient pH, studying the interactive effects of these two anthropogenic stressors will help inform future performance predictions and the management of coastal communities.

To investigate the hypothesis that FX-induced biological impacts would be mitigated by OA due to decreased bioavailability, I exposed the sea urchin *Heliocidaris crassispina* and the ascidian *Styela plicata* to environmentally-relevant concentrations of FX under near-future OA conditions. Sea urchins and ascidians are two ecologically and commercially important model organisms generally found in coastal regions. *H. crassispina* are grazers commonly found in the shallow, rocky habitats along the coasts of East Asia (Chiu 1990; Pearse 2006; Agatsuma & Lawrence 2013). Sea urchins serve as prime model organisms for developmental or neurobiological studies (Epel *et al.* 2006; Aluigi *et al.* 2010, 2012). For example, the purple sea urchin *Strongylocentrotus purpuratus*, whose genome has been fully sequenced (Burke *et al.* 2006), has homologs for serotonin transporters (SERT), as well as Type 1, 2, and 7 serotonin receptors and the γ-aminobutyric acid type A ($GABA_A$) receptor, all of which are involved in FX target pathways (Tunnicliff *et al.* 1999; Fabbri & Franzellitti 2016).

S. plicata is a fouling species with a wide-ranging distribution (Pineda *et al.* 2011; Aldred & Clare 2014), often found in coastal waters with large environmental fluctuations and human disturbance (Naranjo *et al.* 1996). Ascidians in general are

evolutionarily close to vertebrates with a relatively simple central nervous system (Lemaire *et al.* 2008). The ascidian *Ciona intestinalis* also has a fully sequenced genome (Dehal *et al.* 2002) and a well-documented developmental cycle (Satoh 1994), making ascidians prime model organisms for studies in neurobiology (Dahlberg *et al.* 2009), developmental and evolutionary biology (Corbo *et al.* 2001), and ecotoxicology (Gallo & Tosti 2015). Previous studies have observed SERT-expressing coronet cells in the *Ciona* sensory vesicle, as well as genes related to the $GABA_A$ receptor and putative serotonin receptors (Okamura *et al.* 2005; Horie *et al.* 2009; Razy-Krajka *et al.* 2012).

Planktonic larvae are generally more susceptible to environmental stressors than other life stages (Pechenik 1999). Additionally, comparing the larval stages of two non-target organisms provides an informative framework to investigate species-specific sensitivity to combined climate change stressors (Byrne & Przeslawski 2013). Unlike ascidians, many sea urchin larvae are highly calcifying. Calcifiers are suggested to be highly sensitive to OA due to the dissolution of calcified structures (Bednaršek *et al.* 2012), increased metabolic costs of calcification (Stumpp *et al.* 2012), and/or decreased availability of carbonate at low pH (Hofmann *et al.* 2010). Urchin larvae beat cilia to regulate their position in the water column (Grünbaum & Strathmann 2003), whereas ascidian tadpoles are undulatory muscular swimmers (McHenry 2005). Urchin larvae also use these ciliary bands to feed (Strathmann & Grünbaum 2006) while *S. plicata* larvae are non-feeding (David *et al.* 2010), possibly suggesting differential exposure opportunities between the two species.

In the present study, I analyzed the swimming behavior of *H. crassispina* and *S. plicata* larvae to test if combined exposure to OA and FX interactively affect organismal

performance. While most ecotoxicological assays rely on mortality and other physiological markers, behavioral endpoints are integrative, whole-organism responses that provide nuanced information about the sublethal impacts of environmentally-relevant stressors (Gerhardt 2007; Ford *et al.* 2021). Additionally, alterations in larval swimming behavior hold strong implications for dispersal dynamics and population connectivity (Cowen & Sponaugle 2009). My findings reveal multiple behavioral impacts of the non-linear interaction between OA and FX exposure in ascidian larval swimming, as well as altered urchin behavior resulting from independent effects of the two stressors.

2. Materials and methods

2.1 Animal collection and spawning

Adult sea urchins (*Heliocidaris crassispina*) were procured from a fish farm in Hong Kong (22.39° N, 114.11° E) and transported to the HKUST Coastal Marine Laboratory. Adults were kept in 21 ± 1°C, 200 μm-filtered seawater upon arrival and fed a diet of dried kelp. After three months, spawning was induced by injecting 0.5-1 mL of 0.5 M KCl into the intracoelomic cavity (Strathmann 1987). Eggs from three females were collected in separate beakers with MBL artificial seawater (ASW, Cavanaugh 1956, pH_T = 8.007, 20.1°C), followed by washing through a 125-μm mesh to remove large debris. Sperm from three males were collected dry on ice before use. Eggs were fertilized with a sperm concentration of 1000 mL^{-1} (3 females x 3 males). Fertilization success (> 95%) was confirmed under a microscope, with the fertilization envelope observable at 5 minutes post-fertilization (mpf). Fertilized eggs were washed with ASW to remove excess sperm, then mixed and allocated within 30 mpf to 1.5 L glass jars (2.5 individuals

mL^{-1}) at their respective treatments (20 ± 1 °C).

Adult *Styela plicata* were collected by detaching them from ropes on a mariculture raft (Yung Shue Au, Hong Kong; 22.39° N, 114.11° E) and also transported to the HKUST Coastal Marine Laboratory. Prior to dissection, *S. plicata* were maintained at 21 ± 1 °C for one week using free-flowing seawater. Gametes were collected by cutting through the tunics of one individual to expose the hermaphroditic gonads. Oocytes were stripped from the gonad and immediately rinsed with ASW (pH_T = 8.10, 19.6°C) through a 250-µm, then 45-µm mesh filter to remove large particles. Sperm were collected from another 3 individuals by stripping the whitish spermiduct and testes, after which they were observed under a microscope to confirm active swimming. Gametes were combined at a 4:1 sperm-to-oocyte ratio. At 5 mpf, eggs were washed with ASW three times before filtering through a 45-µm mesh, then allocated into 400-mL jars (2.5 individuals mL^{-1}) at their corresponding treatments within 15 mpf (20 ± 1 °C).

2.2 Experimental design

Larvae were exposed to a full factorial design of three nominal FX concentrations (control = 0 ng L^{-1}; low = 10 ng L^{-1}; high = 100 ng L^{-1}) and two pH levels (ambient control = 8.0; OA = 7.7), yielding 6 treatments with 3 replicates per treatment. The low and high FX concentrations reflect present-day pre- and post-typhoon measurements in Hong Kong (Lo, 2020). Notably, the high FX value is conservative relative to the concerning environmental prediction of 370 ng L^{-1} in UK drinking water (Webb 2004). The pH of the OA condition reflects the predicted reduction in global surface ocean pH

by the end of the century (Caldeira & Wickett 2005), and is observed within present-day pH fluctuations in Hong Kong waters (Wang *et al.* 2016; Pecquet *et al.* 2017).

ASW was used throughout the entire experiment to ensure that the nominal FX concentrations would not be affected by potential traces of FX in natural seawater. Prepared ASW was vigorously aerated overnight with compressed air filtered through a 0.22 μm membrane filter before use. Urchin larvae were fed with 5000 cells mL^{-1} *Rhodomonas salina*. Algal concentration was verified with a Coulter Counter (Multisizer 4e, Beckman Coulter, United States), and a complete water change was performed every 2 days. Due to the short-lived and non-feeding larval duration of *S. plicata*, ascidian larvae remained undisturbed until behavioral analysis.

2.3 Seawater carbonate and fluoxetine chemistry

Filtered ASW in each jar was continuously and gently bubbled with air passed through a 0.22-μm filter. Jars at nominal pH_T 7.7 were connected to pH computers (Aqua Medic, Germany) to control pH through the addition of pure CO_2. Temperature, voltage, and pH_{NBS} were monitored daily with a Metrohm 913 pH meter and unitrode with Pt 1000 (Herisau, Switzerland). Salinity was measured with a handheld refractometer. To convert measured pH to total scale pH (pH_T), daily measurements were made on a Tris/HCl buffer solution (salinity = 33) provided by the Dickson Lab.

Total alkalinity (A_T) and dissolved inorganic carbon (DIC) were measured for each jar and the newly prepared ASW of corresponding pH during each water change. Water samples for A_T measurement were filtered through a 0.22 μm syringe filter, while DIC samples were collected in air-free vials without filtration. All water samples were

stored at 4 °C before analysis. A_T was quantified using a computer-driven titrator (905 Titrando, Metrohm, Switzerland) mounted with a glass electrode (Unitrode with Pt1000, Metrohm, Switzerland). DIC was quantified by LiCor Mass Spectrometry (AS-C3, Apollo Technology Solutions LLC, USA). Calibration was performed using the standard seawater provided by the Dickson Lab (Batch 151). Carbonate system parameters (pCO_2, Ω_{ar}, and Ω_{ca}) were calculated from these measurements in CO_2SYS using the dissociation constants from Mehrbach *et al.* (1973) as refitted by Dickson & Millero (1987).

Standard fluoxetine HCl (PHR1394, Sigma-Aldrich) was use to prepare a working standard of 40 µg L^{-1} and stored in darkness at 4 °C. The nominal concentration of working standard was verified with liquid chromatography with electron spray and tandem mass spectrometry (LC-ES-MS/MS) using a modified version of the methods described by Chu & Metcalfe (2007). Given the quantification of FX at the ng L^{-1} level requires at least one liter of water sample, an end-point FX concentration reading from the rearing jar (1.5 L volume) could not be obtained. However, since identical glassware were used and the same amount of algal cells (>300 µg dry mass) were added across all treatments, the loss, if any, would be comparable across all treatments.

2.4 Swimming behavior in response to OA and FX exposure

Larval swimming behavior was recorded at 7 dpf for urchins and 16 hpf for ascidians. Duplicate 10 mL subsamples (≈15-20 larvae) from each replicate jar were each placed in a well of a 6-well plate. After two hours of acclimation, the plate was moved onto the base of a dissecting microscope (MZ6, Leica) mounted with an eyepiece camera (AM4023X, Dino-Lite, Taiwan). The field of view covered the only center portion of the

well to avoid wall effect. After an additional 15 minutes, each well was videotaped for 3 min at 15 fps (at least 7 individuals in view). The process was repeated for all 6 treatments. Formalin-fixed larvae were also filmed for 3 min to account for potential drifting. Room temperature was maintained at 20 ± 1 °C, and water temperature during recording was monitored with a thermocouple.

Videos were processed in FOSICA (Wallingford Imaging, WA), first by removing background and thresholding for size and brightness, then converting the positions of moving particles into pixel coordinates. I used the in-house MATLAB program Tracker3D (Chan *et al.* 2011) for path assembly (Fig. 1a, b). Larval swimming speed was measured by gross and net speed. Gross speed was calculated by applying a smoothing spline to the paths to remove frame rate noise and taking the time derivatives of the splines. Net speed was calculated as the displacement between the first and last coordinates over time. I also computed net-to-gross displacement ratio (NGDR) and mean squared displacement (MSD). NGDR was calculated by dividing the net displacement of a path by the gross displacement. This metric can range from 0 to 1, with low values indicating convoluted trajectories and high values indicating straightforward trajectories (True *et al.* 2015). Similarly, MSD was calculated as:

$$MSD_{path} = \frac{1}{N-j}\sum_{i=1+j}^{N}\left|r_i - r_{i-j}\right|^2$$

where N is the number of datapoints collected from the path, j is the chosen time interval, and r is the physical position of the larva. The MSD of each path was interpreted as the distance explored through Brownian motion, indicating the level of the trajectory's convolution. All swimming metrics were calculated in MATLAB.

Beyond speed and trajectory, I also investigated the effect of combined FX and

OA on discrete larval behaviors. Orientations of urchin larvae (ventral, dorsal, animal, vegetal, left, or right; Fig. 1c) were recorded for all visible larvae during the first, middle, and last frame of each video. Orientations were observed as the side facing the camera (bird's eye view from above the water surface) in a given frame. Ethograms of ascidian movement were created by recording which behavior was being executed for the first 900 frames (1 min) of all videos. Ascidian movement was placed into one of six categories (Fig. 1d). "Still" ascidian larvae did not move their tails or trunks between the current frame and the next frame. "Swimming" larvae moved their tails in the classic undulatory motion, resulting in trunk-directed displacement between frames. "Cruising" larvae also exhibited trunk-directed displacement, but moved without any visible tail manipulation. Larvae were considered "spasming" if there was observable tail movement compared to the previous and/or next frame, but no trunk-directed displacement. "Twitching" larvae exhibited unilateral contraction and relaxation of the tail (i.e., a straight larva bending into a C-shape, then returning to its original position). Finally, "spinning" larvae rapidly contracted their tails resulting in clockwise or counterclockwise rotation around the base of the trunk. As the field of view only excluded the edges of the well, individuals were visible in the field of view for varying amounts of time. Thus, behaviors were calculated as the percent of total time spent by all individuals in a well conducting each behavior.

2.5 Statistical analysis

To determine the effect of OA and FX exposure on larval swimming behaviors (speeds, trajectory, frequency of urchin orientation, and percent time ascidian larvae spent conducting categorical behaviors), I employed a 2-way ANOVA with pH and FX

as fixed factors. All data were checked with a Shapiro-Wilks test for normality and Levene's test for homogeneity of variance. Ascidian ethogram data were arcsine square root transformed before statistical analysis. In the cases where data did not fit normality and homogeneity assumptions, I analyzed the data using an aligned rank transform, non-parametric ANOVA (i.e., ARTool; Wobbrock *et al.* 2011) and significant *post hoc* effects were analyzed with a difference-of-differences contrast. The level of significance was set to $p < 0.05$. All statistical tests were performed in R (R Core Team Version 4.0.2).

3. Results

3.1 Seawater carbonate and fluoxetine chemistry

Rearing conditions for urchin larvae at ambient pH 8.0 and in OA pH 7.7 were stable over the course of the experiment for both urchins and ascidians ($F_{1,58} = 0.66$, $p > 0.5$ and $F_{1,62} < 10$, $p > 0.05$, respectively; Table 1). pH in the ambient control was significantly different from pH in the OA condition ($F_{1,58} = 972$, $p < 0.0001$ and $F_{1,62} < 200$, $p < 0.05$ for urchins and ascidians, respectively). The average measured FX concentration in the working standard was 39.15 μg L^{-1} (≈97.9% of 40 μg L^{-1}) for the urchin experiment and 40.09 μg L^{-1} (≈100.23% of 40 μg L^{-1}) for the ascidian experiment.

3.2 Larval urchin swimming behavior

OA alone ($F_{1,19} = 11.8$, $p < 0.01$; Fig. 2a) significantly slowed urchin gross swimming speed at all FX concentrations. FX exposure alone marginally affected gross speed ($F_{2,19} = 3.85$, $p = 0.051$), with the low concentration reducing gross speed more than control and high concentrations. Neither net speed nor NGDR of larval urchins was

significantly affected by OA, FX, or their interaction (Fig. 2b, c). However, I did observe a slight increase in NGDR when exposed to FX and OA independently. MSD of larvae in pH 8.0 was significantly higher than larvae reared in OA ($F_{1,19} = 7.44$, $p < 0.05$) regardless of FX exposure (Fig. 2d). Additionally, I observed a significant interaction of OA and FX concentration on the frequency of left-side-up larvae ($F_{2,20} = 8.71$, $p < 0.05$; Fig. 3a). At low FX concentrations, there were less left-side-up larvae in reduced pH; this pattern was reversed at high FX concentrations ($F_{1,12} = 8.71$, $p < 0.05$). Larvae were also oriented dorsal-side-up more often in increased FX concentrations, though this effect was only marginally significant ($F_{2,20} = 3.72$, $p = 0.056$; Fig. 3b). There were no significant effects of pH, FX, or their interaction on any of the other four orientations measured.

3.2 Larval ascidian swimming behavior

Both OA ($F_{1,11} = 13.0$, $p < 0.005$) and FX ($F_{2,11} = 5.03$, $p < 0.05$) independently affected median ascidian net speed. I also observed a significant interactive effect ($F_{2,11} = 12.3$, $p < 0.005$), wherein larval ascidians reared in ambient pH had steeper decreases in net speed at low ($F_{1,11} = 9.97$, $p < 0.05$) and high FX ($F_{1,11} = 23.7$, $p < 0.005$) from control FX (Fig. 4b). However, ascidian larvae exposed to OA exhibited no significant differences in net speed across FX. In other words, ascidian net speeds decreased with increasing FX at ambient pH, whereas this trend was absent in OA. While not statistically significant, this pattern is reflected in the NGDR (Fig. 4d). Additionally, there was no significant effect of pH, FX, or their interaction on gross speeds or MSD (Fig 4a, c).

I did not observe a significant difference in the percent of overall time spent by ascidians conducting any of the 6 categorical movement behaviors. However, analysis of

behavior combinations revealed that ambient pH ascidians reared in control FX (14 ± 7.1%; mean ± SD) spent a longer, but non-significant, proportion of time conducting translating behaviors ("swim" + "cruise") than those at high FX (2.1 ± 1.3%; mean ± SD) (Fig. 3c). Concordantly, ascidians at ambient pH spent more time being still at high FX (96 ± 0.4%; mean ± SD) than at control FX (84 ± 6.1%; mean ± SD). However, this FX-dependent pattern is absent under OA. There is a significant interaction between pH and FX on the number of individuals that remained still for the entire recording ($F_{2,12} = 4.94$, $p < 0.05$), the number of long-term still individuals peaks in low FX ($F_{1,12} = 6.59$, $p < 0.05$) under OA but in high FX ($F_{1,12} = 8.14$, $p < 0.05$) in control (Fig. 3d).

4. Discussion

While antagonistic, synergistic, and additive interactions have been reported between pH and other pollutants (Freitas *et al.* 2016; Dorey *et al.* 2018), little is known about the extent and patterns of interactive effects between pharmaceutical pollution and OA on non-target marine organisms. I investigated whether FX-induced impacts are modulated by pH levels relevant to end-of-century OA predictions. By exposing early developmental stages of the urchin *H. crassispina* and the ascidian *S. plicata* to environmentally-relevant pH and FX levels, this study reveals species-specific non-linear impacts on behavior. Rearing ascidians in OA mitigated the reductions in net speed induced by FX exposure; however, OA and FX acted independently on the speed and trajectory of urchin larvae. Thus, recreating realistic field conditions is crucial for predicting the non-linear and species-specific impacts of emergent pollutants on non-target marine organisms in future ocean scenarios.

4.1 OA modulates FX-induced impacts on ascidian swimming

I observed an interactive effect between pH and FX exposure on larval ascidian swimming behaviors. At ambient pH 8.0, net speeds, NGDR, and the time spent exhibiting directed movement decreased with increasing FX concentration; however, this effect was not observed in OA conditions. This pH-dependent modulation of FX impacts could be a result of reduced bioavailability of FX under reduced pH. Within the narrow range of pH investigated, pKa predictions show that at pH 8.3, only 3% of FX remains unionized, further reducing to 0.78% at pH 7.7 (Lo 2020). Previous studies suggest that norfluoxetine is more soluble and has a lower chance of bioaccumulation and reduced bioavailability (Brooks *et al.* 2003). Indeed, reduced FX bioavailability has been observed at pH 7.0 compared to pH 8.0 and 9.0, resulting in reduced acute toxicity and bioaccumulation in Japanese medaka tissues (Nakamura *et al.* 2008).

Another possible driver for pH-dependent responses to FX exposure could be a shift in energy allocation due to increased physiological stress. A companion study to this thesis found that *S. plicata* larvae reared in ambient pH 8.0 had higher levels of DNA damage with increasing FX exposure (Lo 2020). Ascidians rely on apoptosis to defend against pollutant-induced oxidative stress (Barbosa *et al.* 2018). Given that apoptosis is energy-dependent (Eguchi *et al.* 1997; Elmore 2007), it may be the case that at higher levels of stress (i.e., high concentrations of bioavailable FX), ascidians shift energy allocation to prioritize maintenance over swimming, which can also be energetically expensive (Bennett & Marshall 2005). Such trade-offs have been observed in other aquatic invertebrates under environmental stress (Sokolova *et al.* 2012). Relatedly, *S. plicata* larvae did not experience a change in respiration rate when exposed to OA and

FX conditions (Lo 2020). This observation is not surprising given that ascidian larvae are non-feeding and must rely on limited maternal provisions to complete their larval duration (Berrill & Watson 1935).

The observed reduction in net speed and NGDR suggest that in ambient pH, ascidians swim with more convoluted paths than in high FX concentrations. Razy-Krajka *et al.* (2012) showed that high concentrations of FX decreased spontaneous swimming of *Ciona* by acting on the SERT-containing cells in the sensory vesicle, which are involved in sensoreception. However, this study only measured tail beat frequency, and did not account for other metrics of swimming kinematics (e.g. differences in yaw), which may reveal effects of FX on swimming trajectory (McHenry 2005). Additionally, the exact mechanism behind FX action is still unclear, as SERT-containing cells in ascidians are multi-functional and may facilitate transport of other neurotransmitters like dopamine (Razy-Krajka *et al.* 2012). Rudolf *et al.* (2019) also showed that modafinil, a selective a dopamine transporter inhibitor, increased swimming speed of *Ciona*. Similarly, dexmedetomidine, an ADRα2 dopamine receptor agonist, also increased spontaneous swimming in ascidian larvae (Razy-Krajka *et al.* 2012).

Interestingly, ascidians exposed to OA exhibited relatively low net speeds and NGDR regardless of FX concentration. OA has been hypothesized to reverse GABAergic signaling by affecting ion gradients (Nilsson *et al.* 2012). Inhibition of GABAergic signaling has been observed to increase the frequency and duration of spontaneous swimming events in *Ciona* larvae (Brown *et al.* 2005); however, it has also been observed to reduce negative geotaxis in response to light (Bostwick *et al.* 2020). The latter behavior refers to the “light-off response”, where the dimming of visible light

induces negative geotaxis and a rapid increase in swimming, followed by recovery after visible light exposure resumes (Nakagawa *et al.* 1999; Tsuda *et al.* 2003; Zega *et al.* 2006). While I demonstrated that OA and FX affect ascidian swimming behavior in an interactive manner, further studies on the complex sensorimotor mechanisms involved in the light-off response will provide insight into how combined climate change stressors affect neuroethology in a more ecologically relevant context.

One possible approach is to analyze ascidian swimming in response to light change, using cuvettes that allow for geotactic movement (Fig. 5). As my results provide insight on the impact of rearing ascidian embryos in combined stressors, acute exposure assays may help tease apart the effect of developmental abnormalities and resulting behavior. Preliminary data suggest that ascidian larvae exposed to combined OA and FX still exhibit increased swimming responses to light dimming; however, the presence of an interaction requires further in-depth analysis (Chua, unpub. data). I hypothesize that OA may antagonistically interact with FX due to increased serotonergic signaling from the sensory vesicle, depending on where GABA reversal acts on the ascidian neural system. Results supporting this hypothesis would suggest that the change in behaviors associated with OA- and FX-exposure has a neurological basis.

4.2 OA and FX independently affect larval urchin swimming

In contrast with the interactive effect on ascidian behavior, I observed an FX-independent reduction in gross swimming speed and MSD of urchin larvae in OA compared to those at ambient pH. Other echinoid larvae maintained swimming speed under acidified conditions (e.g. *Dendraster excentricus*, Chan *et al.* 2011;

Strongylocentrotus droebachiensis, Chan *et al.* 2015a; *S. purprtaus*, Chan *et al.* 2015b). However, brittle star larvae swim slower at OA-relevant pH levels (Chan *et al.* 2015b), suggesting interspecific variation in swimming responses to acidification within echinoderms. The variation across studies may also be explained by differences in observation approach. Earlier studies focus on movement along the X-Z plane (vertical swimming), while the current study observed swimming in shallow (80 mm deep) well plates through the X-Y plane (horizontal swimming). For example, larval *D. excentricus* exposed to reductions in temperature experience reductions in horizontal speed to compensate for the maintenance of vertical movement (Chan & Grünbaum 2010).

Changes in larval morphology induced by pH stress could have also contributed to the observed difference in speeds. A companion study to this thesis found that larval bodies of *H. crassispina* were longer overall at ambient pH than in OA (Lo *et al.* in review). Smaller larvae may have relatively shorter ciliated bands to generate lift, thus reducing speed (Grünbaum & Strathmann 2003). Acidified echinoderm larvae were observed to have shorter arms and more U-shaped bodies compared to control, which affected swimming biomechanics, such as through increased stability in vertical shear (Chan *et al.* 2011; Chan & Tong 2020). We also observed changes in larval urchin arm symmetry under OA (Lo *et al.* in review), which may affect the larval weight distribution, and hence, swimming (Chan *et al.* 2011). Variation in arm symmetry may also explain the observed differences in left-side up orientation across pH treatments.

Aside from morphological differences, behavioral change could also be induced by low pH, such as through the hypothesized reversal of GABAergic signals in OA may modulate ciliary beating in urchin larvae (Katow *et al.* 2013). Previous studies have also

reported that larval *D. excentricus* avoided unfavorable environments, e.g., low pH (Maboloc *et al.* 2020) or high temperature (Chan & Grünbaum 2010). Here, larvae exposed to FX have a higher incidence of dorsal-side-up orientation at pH 8.0 than at 7.7. Though this difference is not statistically significant, increased downward swimming could be interpreted as an escape response. Given that I analyzed larval swimming in shallow dishes, future observations using free-swimming larvae in a larger volume may provide more insight on the implications of my observed changes in the field (Roy *et al.* 2012; Fuchs & Gerbi 2016).

I also observed that urchin larval gross speed was the slowest at low FX concentrations compared to control and high concentrations. Interestingly, I only observed a marginally significant effect of FX on larval gross speed, despite the regulatory role of serotonin in ciliary beating of sea urchin larvae (Mogami *et al.* 1992; Wada *et al.* 1997). While the negative effects of FX exposure on locomotion are well-documented in other organisms, these studies were conducted at concentrations several orders of magnitude higher than the current study (Hamilton *et al.* 2016; Ford *et al.* 2018). Further, it is possible for FX-induced behavioral changes to manifest over longer time scales than used in the current study, especially at lower concentrations (Di Poi *et al.* 2014; Peters & Granek 2016). Conducting whole life cycle analyses with environmentally-relevant FX levels, combined with future ocean conditions, would allow for more accurate ecological predictions of organismal responses.

4.3 Interspecific variations in OA and FX responses

Overall, FX impacts on larval behavior can be modulated by reduced pH, though

this effect is clearer in ascidians compared to urchins. This variation in impact may be due to species-specific traits. For example, the ascidian larval duration is limited by maternal provisions, and thus may be more pressed to reallocate energy and prioritize essential processes than urchin larvae, which may be able to meet energetic demands through food intake (Sokolova *et al.* 2012). Differential sensitivities to FX exposure have been observed in the polychaete *Hediste diversicolor* depending on feeding mode (Hird *et al.* 2016). Rather than the predicted modulation effect, I observed independent effects of OA and FX on larval urchin swimming. FX inhibits urchin toxicant defenses, e.g., expression of efflux transporters and cytochrome P450 (Lynch & Price 2007; Smith *et al.* 2012). However, reduced transcription of toxicant defense genes in OA (Todgham & Hofmann 2009) may be paired with the hypothesized reduced bioavailability of FX in OA, resulting in a similar extent of FX impact across different pH levels.

Additionally, species-specific differences in the neurophysiological control of swimming may help explain the observed variation in responses. The read-across hypothesis suggests that pharmaceuticals will affect non-target organisms as long as the molecular target is conserved (Rand-Weaver *et al.* 2013), though they may have different outcomes depending on the pathway the molecular target is involved in (Hird *et al.* 2016). SERT and other serotonergic receptors are involved in the swimming pathways of both urchins and ascidians; however, while serotonergic signaling plays a role in ciliary manipulation in urchin larvae, SERT is mainly involved in sensory reception in ascidian larvae. Further, while I did not observe a significant interaction of FX and OA on urchin swimming speed, a companion study found interactive effects on other physiological and

morphological metrics in urchin larvae (Lo *et al.* in review), supporting species-specific modes of action of FX.

5. Conclusion

Exposure to environmentally-relevant levels of FX and pH altered behavioral responses in early life stages of the urchin *H. crassispina* and the ascidian *S. plicata*. However, while the two stressors independently impacted larval urchin responses, exposure to OA antagonistically modulated FX-induced impacts on larval ascidians. Changes in larval swimming behavior have implications for dispersal dynamics (Minchinton & Scheibling 1991) and survivorship (Chan & Grünbaum 2010; Chan *et al.* 2018). Previous research suggests that coastal organisms like ascidians and urchins may be more resilient to OA due to pre-exposure to low pH waters (Hofmann *et al.* 2010). My observations thus highlight the importance of using behavioral metrics as a sensitive tool when investigating sub-lethal impacts of climate change stressors. Future research should emphasize the use of multiple stressors, such as pharmaceutical mixtures (Backhaus 2014), as well as environmentally-relevant stressor levels to more accurately predict organismal responses to future ocean environments.

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Table 1. Carbonate chemistry parameters of artificial seawater used to rear larval urchins (7 dpf) and ascidians (16 hpf). Temperature, total scale pH (pH_T), and total alkalinity (A_T) were measured, and dissolved inorganic carbon (DIC), partial pressure of CO2 (pCO_2), and aragonite and calcite saturation were calculated in CO_2SYS. Values are reported as mean ± SD.

		Measured parameters				Calculated parameters		
	Nominal pH	Temp (°C)	pH_T	A_T (μmol kg^{-1})	DIC (μmol kg^{-1})	pCO_2 (μatm)	Ω_{ca}	Ω_{ar}
Urchin	8.0	19.5 ± 0.1	8.09 ± 0.03	2143 ± 8.2	2092 ± 37.6	375 ± 111	1.9 ± 0.6	1.3 ± 0.4
expt.	7.7	19.6 ± 0.1	7.70 ± 0.07	2166 ± 7.3	2169 ± 20.9	1181 ± 417	0.7 ± 0.2	0.5 ± 0.2
Ascidian	8.0	19.7 ± 0.2	8.11 ± 0.12	2181± 52.2	N.A.	349.6 ± 46	4.6 ± 1.0	2.9 ± 0.6
expt.	7.7	19.6 ± 0.2	7.76 ± .04	2178± 17.8	N.A.	822.2± 40	2.3 ± 0.2	1.4 ± 0.1

Figures

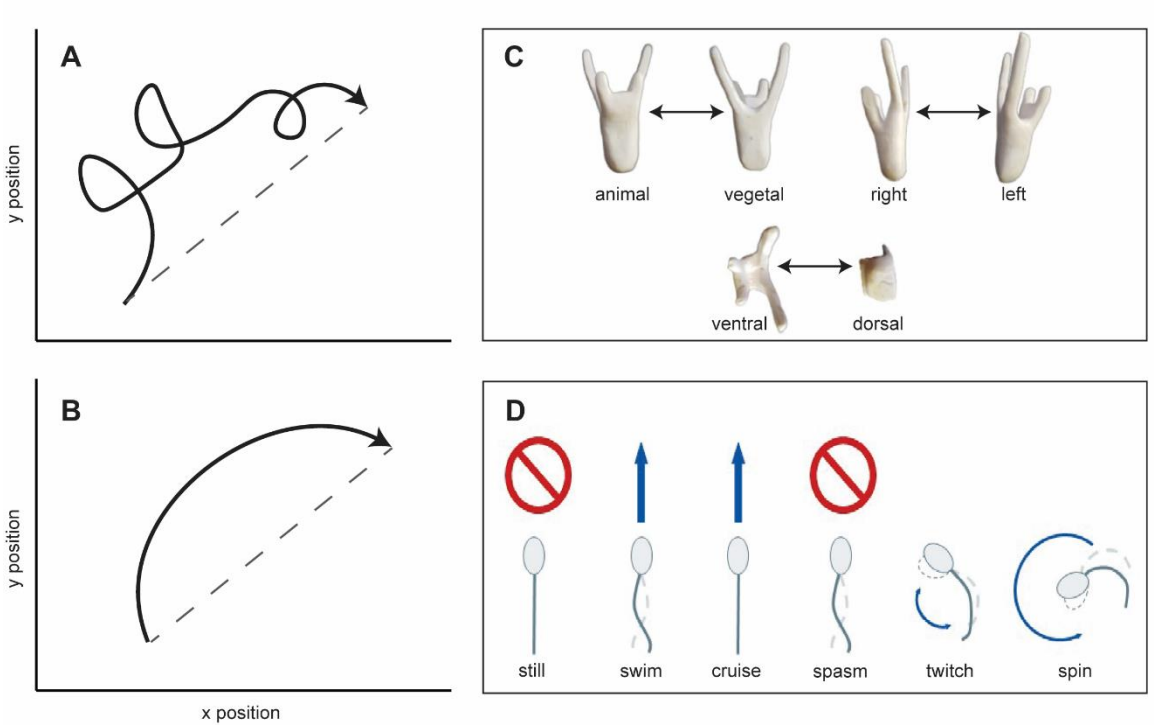

Figure 1. Larval swimming behavior of *H. crassispina* urchins (7 dpf) and *S. plicata* ascidians (16 hpf) was quantified by calculating gross and net speeds, trajectory metrics net-to-gross displacement ratio (NGDR) and mean squared displacement (MSD), as well as discrete movement behaviors. **(A)** Diagram depicting a relatively convoluted path, characterized by low NGDR and high MSD. **(B)** Diagram depicting a relatively straightforward path, characterized by high NGDR and low MSD. Solid black lines indicate total distance traveled as used to calculate gross speed. Dashed lines indicate net displacement traveled as used to calculate net speed. **(C)** A 3D model of a pluteus larva at six discrete urchin larvae swimming orientations, which were used as a proxy to determine swimming direction. **(D)** Ascidian swimming behavior was broken down into six discrete movement categories. Red stop symbols represent no change in displacement over time. Heavy blue arrows represent change in displacement over time (i.e. "translating movement").

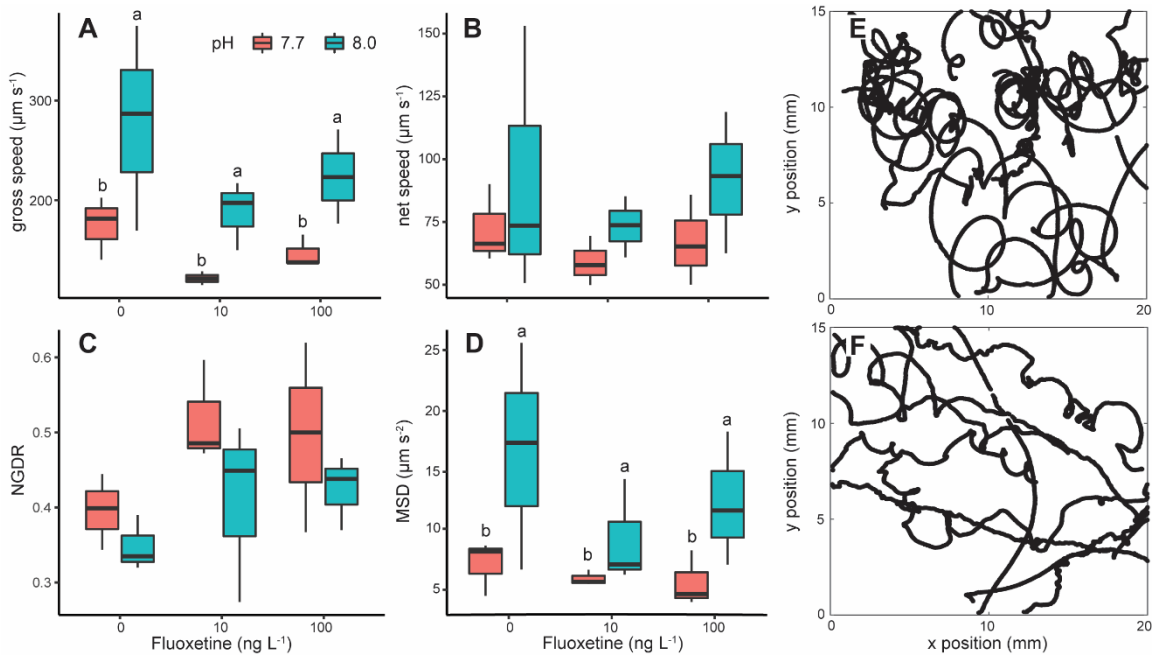

Figure 2. Ocean acidification affects larval urchin swimming behavior. **(A)** Gross speed, **(B)** net speed, **(C)** net-to-gross displacement ratio (NGDR), and **(D)** mean squared displacement (MSD) were used to quantify the effect of ocean acidification (pH 7.7; red boxplots) and increasing fluoxetine concentrations compared to control pH 8.0 (blue boxplots). **(E)** Representative urchin paths at pH 8.0, characterized by high MSD and high gross speed. **(F)** Representative urchin paths at pH 7.7, characterized by low MSD and low gross speed. Letters indicate significantly different groups ($p < 0.05$).

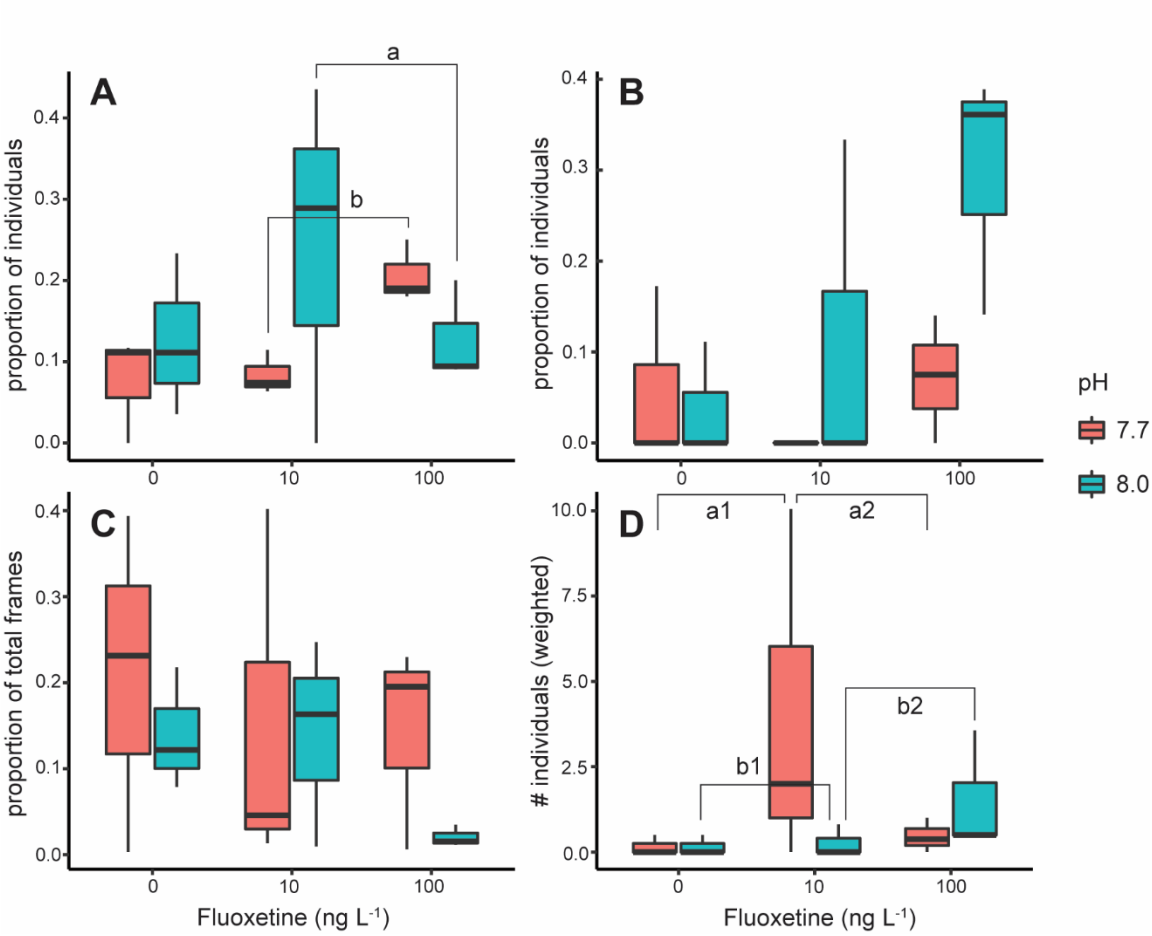

Figure 3. Ocean acidification and fluoxetine interact to affect larval urchin and ascidian behavior. **(A)** The proportion of larval urchins observed at left and **(B)** dorsal side-up orientations were recorded in response to acidification (pH 7.7; red boxplots) and increasing fluoxetine exposure compared to control pH 8.0 (blue boxplots). **(C)** The proportion of time (frames) larval ascidians spent conducting translating behaviors (swim + cruise; see Fig. 1) after exposure to acidification and increasing fluoxetine. **(D)** The number of ascidian larvae counted as completely still throughout the entire duration of the video (~3 min), weighted by the number of frames. Letters represent significantly different differences between groups ($p < 0.05$), with subsequent numbers representing the groups being compared.

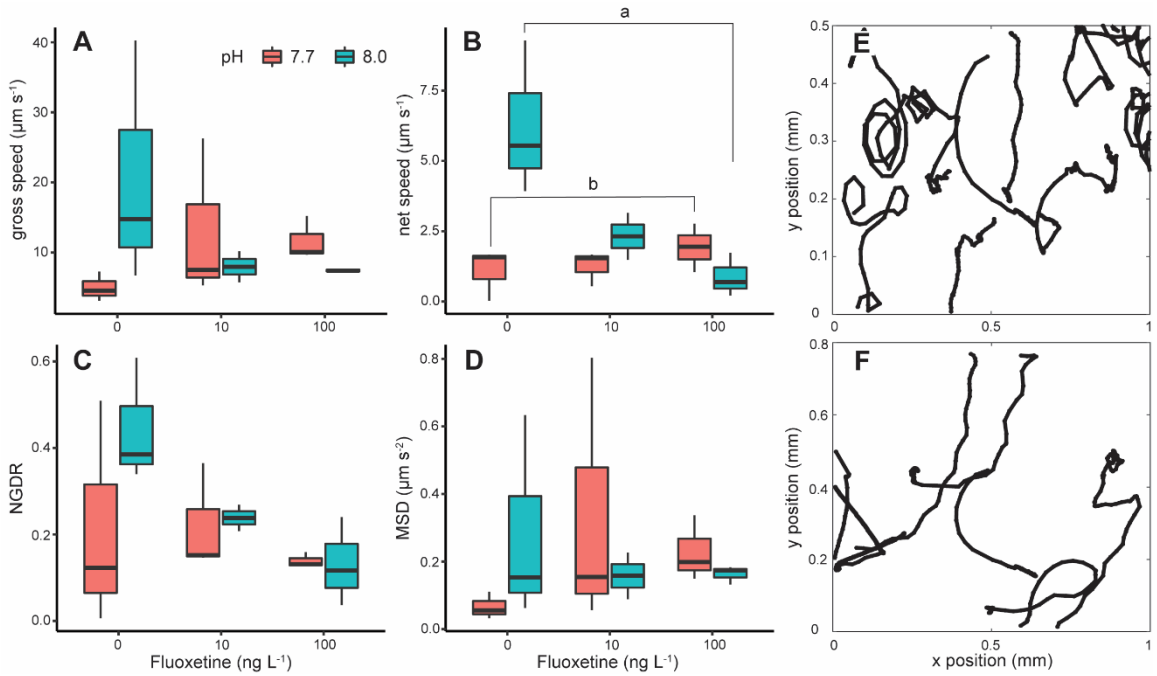

Figure 4. Ocean acidification mitigates fluoxetine-induced impacts on larval ascidian swimming behavior. **(A)** Gross speed, **(B)** net speed, **(C)** net-to-gross displacement ratio (NGDR), and **(D)** mean squared displacement (MSD) were used to quantify the effect of ocean acidification (pH 7.7; red boxplots) and increasing fluoxetine concentrations compared to control pH 8.0 (blue boxplots). **(E)** Representative urchin paths at pH 8.0 without fluoxetine, characterized by relatively high net speed and NGDR. **(F)** Representative urchin paths at pH 7.7 and 8.0 with fluoxetine, characterized by relatively low net speed and NGDR. Letters indicate significantly different differences between groups ($p < 0.05$).

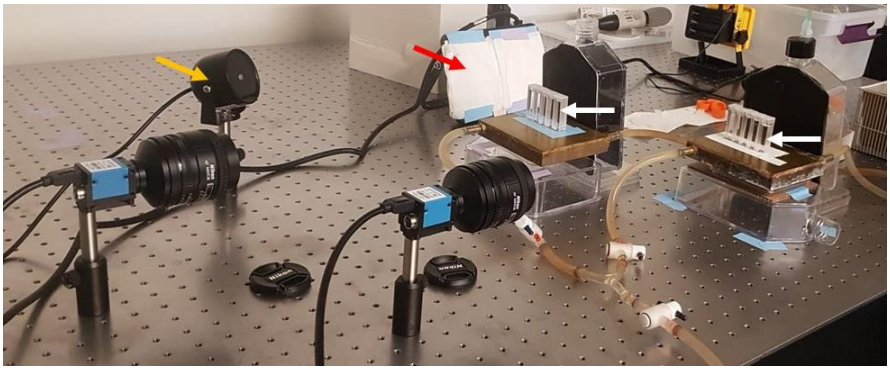

Figure 5. Video analysis of larval ascidian geotactic response to light dimming (i.e., "light-off" or "shadow" response). Two cameras fitted with 35 mm Nikon lenses recorded larval ascidians acutely exposed to combined fluoxetine (0, 10, and 100 ng L^{-1}) and pH conditions (pH 8.0 and 7.7). Vertical cuvettes (white arrows) were used to allow for geotactic response to dimming of soft LED light (red arrow; ≈10 to 0 ppf). Cuvettes were constantly illuminated by IR light (yellow arrow). Cuvette temperature was maintained at 16 ºC with cooling plates.

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