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Pharmaceutical Contaminants as Stressors on Rocky Intertidal and Estuarine Organisms: a Case Study of Fluoxetine

Joseph Richard Peters
Portland State University

Pharmaceutical Contaminants as Stressors on Rocky Intertidal and Estuarine Organisms: a Case Study of Fuoxetine

By

Joseph Richard Peters

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Thesis Committee:
Elise F. Granek, Chair
Angela L. Strecker
Catherine E. de Rivera

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ABSTRACT

Contaminants such as pharmaceuticals are of increasing concern due to their ubiquitous use and persistence in surface waters worldwide. Limited attention has been paid to the effects of pharmaceuticals on marine life, despite widespread detection of these contaminants in the marine environment. Of the existing studies, the majority assess the negative effects of pharmaceuticals over an exposure period of 30 days or less and focus on cellular and subcellular biomarkers. Longer studies are required to determine if chronic contaminant exposure poses risks to marine life at environmentally relevant concentrations. Also scarce in the literature is examination of whole organism effects to identify potential community-level consequences. Two long-term studies with the antidepressant pharmaceutical, fluoxetine (the active constituent in Prozac®) were conducted to determine whether nominal concentrations detected in estuarine and coastal environments affect organism health and interactions.

First, we measured whole organism metrics in the California mussel, *Mytilus californianus* over a period of 107 days. Specifically, we measured algal clearance rates, growth, and condition indices for both reproductive and overall health. We found that fluoxetine negatively affects all measured characteristics, however many effects are mediated by length of exposure. Perhaps the most notable result was that mussels spiked with fluoxetine cleared less algae after 30 days of exposure. Reduced growth and

condition indices likely are a consequence of improper nutrition among fluoxetine-treated mussels. Any level of fluoxetine significantly affected the gonadosomatic index after 47 days. The results from this study on mussels fill an important data gap, highlighting organism-level effects of chronic exposure periods; such data more explicitly identify the impacts of pharmaceuticals and other contaminants on marine communities and ecosystems.

Fluoxetine has also been documented to affect the behavior of fish and invertebrates, including freshwater and marine bivalves, crustaceans, and fish. Given that other crustaceans exhibited increased activity levels under fluoxetine exposure, we hypothesized that this would subject them to greater predation risk. In our second exposure study, we assessed whether a similar range of fluoxetine concentrations used in the mussel study altered the risk behavior of the Oregon mud crab, *Hemigrapsus oregonensis*, in response to a common predator, the red rock crab, *Cancer productus*. We conducted this study for 60 days, conducting day and night behavioral trials (with and without predators) four times a week. We found that crabs exposed to any amount of fluoxetine (3 or 30 ng/L) had increased activity levels relative to controls; however behaviors of 3 ng/L-spiked crabs were not always significantly different from controls. Among control crabs, day and night trials yielded similar results, where a clear response to the addition of the predator was observed. Crabs dosed with fluoxetine exhibited more foraging and active behaviors in the presence of the predator. Additionally, crabs spiked with fluoxetine at 30 ng/L had the greatest risk of mortality either by predation by red

rock crabs or due to more aggressive behaviors among conspecifics. The results of this study shed light on a particularly unexplored area of contaminants research: how do psychoactive pharmaceuticals affect animal behavior when exposed to the low concentrations persisting in the aquatic environment for a prolonged period of time?

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This project was a venture into unexplored territories of research and was challenging for me to grasp both theoretical and applied aspects of the ecotoxicology discipline. I would like to thank my adviser Dr. Elise F. Granek and my thesis committee members, Dr. Angela L. Strecker and Dr. Catherine E. de Rivera for their guidance and wisdom in developing components of the project. I would also like to acknowledge Dr. William Fish for his input and expertise of toxics into the original project proposal.

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Chapter 1: Introduction

Whole suites of pharmaceuticals and their derivatives routinely enter the aquatic realm through human wastewater effluent, septic systems, and animal waste runoff from agricultural lands (Kolpin et al. 2002; Kinney et al. 2006; Ramirez et al. 2009). While these drugs are often detected at very low concentrations (e.g., ng/L; Fent et al. 2006), these compounds are designed to illicit cellular responses (e.g., enzymes, receptors) and should not be regarded as trivial threats to aquatic organisms (Meredith-Williams et al. 2012; Franzellitti et al. 2014). Additionally, many pharmaceuticals are persistent in aquatic environments, putting aquatic organisms at risk of chronic exposure and bioaccumulation (Ramirez et al. 2009; Meredith-Williams et al. 2012). Numerous studies have documented acute and chronic toxicities of countless pharmaceuticals on aquatic organisms (Trudeau et al. 2005; Corcoran et al. 2010; Brausch et al. 2012); however studies on coastal and marine organisms are lacking (Brooks et al. 2009; Brodin et al. 2014; Gaw et al. 2014). With large and growing human populations along coastlines, much remains to be learned about the effects of pharmaceuticals on marine organisms to better inform best management practices (Seiler et al. 2002; Valbonesi et al. 2003; Regoli and Giuliani 2013).

Despite shared interests in pharmaceuticals as stressors to organisms and use of the same species and similar endpoints, limited cross-citation suggests that the disciplines of ecology and ecotoxicology are growing independently (Brodin et al. 2014). Most

ecotoxicological data are based on acute exposure periods of less than 24 hours with recent studies running up to 30 days (Daugton and Ternes 1999; Brooks et al 2009; Gaw et al. 2014). While useful for assessing how pharmaceuticals may alter cellular activities, this approach is insufficient in length and scope to determine organism- or ecosystem-level chronic effects. Ecologists are interested in how the presence of pharmaceuticals may alter organism behavior (Hazleton et al. 2013; Chen et al. 2015), physiological functioning (Fong 1998; Di et al. 2014), and ultimately how this may shift community and ecosystem dynamics (Brodin et al. 2014; Hazleton et al. 2014). Short term (30 days or less) chronic exposure studies allow for only limited inferences by ecologists about the effects of pharmaceuticals on ecosystem processes. Long-term exposure studies with concentrations that reflect those detected in the environment are required to answer most ecological questions (Gaw et al. 2014, Brodin et al. 2014).

Estuarine and rocky intertidal organisms are particularly at risk from pharmaceuticals as environmental stressors (Fong & Ford 2014), however data from prolonged studies are lacking (Berninger & Brooks 2010; Gaw et al. 2014). Exposure to heavy metals, pesticides, petroleum and other legacy contaminants have also been shown to affect marine organisms by altering habitat preference, shifting migration patterns, or increasing negative species interactions (Fleeger et al. 2003; Khoury et al. 2009; Eades & Waring 2010; Fukunaga et al. 2010). Such alterations to normal behaviors have been linked to reduced fitness, and changes to population structure and ecosystem function (Frid & Dill 2002; Fahrig 2007; Ings et al. 2009). Experiments with pharmaceuticals can

be designed in a similar fashion by substituting the nominal concentrations of the drug or its constituents as the stressor(s), and determining organism- or community-level responses.

Selective serotonin reuptake inhibitor (SSRI) anti-depressants such as fluoxetine hydrochloride (Prozac®) are among the more prevalent categories of pharmaceuticals detected in the marine environment (Kreke and Dietrich 2008; Vasskog et al. 2008; Brodin et al. 2014; Gaw et al. 2014). SSRIs have been developed to delay the reuptake of serotonin, moderating neurotransmission in the human brain. However, serotonin, serotonin analogs, and serotonin-altering drugs have been shown to dramatically affect several marine species (see Fong & Ford 2014 for a recent review). Serotonin is an important neuromodulater in bivalves, regulating gill cilliary activity, oocyte maturation, and the induction of spawning (Gibbons and Castanga 1984). In crustaceans, serotonin is well known to affect behaviors though stimulating the release of hyperglycaemic, neurodepressing, moult-inhibiting, and gonad-stimulating hormones (Fong and Ford 2014).) Several other studies have demonstrated that fluoxetine leads to adverse physiological and behavioral outcomes in aquatic organisms that could alter their functional roles within the community (Perreault et al. 2003; Lynn et al. 2007; Stanley et al. 2007; Mennigen et al. 2010; Schultz et al. 2011; Dzieweczynski & Herbert 2012; Kohlert et al. 2012; Bossus et al. 2013; Barry 2013; Munari et al. 2014; Chen et al. 2015).

The objectives of the fluoxetine case study were to 1) assess whole-organism metrics on the California mussel, *Mytilus californianus* during a long-term exposure

experiment with environmentally relevant concentrations of fluoxetine detected in nearshore marine environments (Chapter 2); and 2) determine if prolonged exposure to similar fluoxetine concentrations affected activity levels, predation risk behavior, and mortality in the Oregon shore crab, *Hemigrapsus oregonensis* with exposure to red rock crabs, *Cancer productus* (Chapter 2). These two studies are critical steps towards addressing how fluoxetine and other pharmaceutical contaminants may affect marine and estuarine species in two very different ways. This work will help fill existing data gaps to better inform best management practices and cradle-to-grave stewardship of pharmaceutical drugs so they can be reduced or eliminated before entering the marine environment.

Chapter 2: Long-term exposure to fluoxetine reduces growth and reproductive potential in the mussel, *Mytilus californianus*

Introduction

Pharmaceuticals are commonly detected in the aquatic environment (Daughton and Ternes 1999; Ankley et al. 2007; Brausch and Rand 2011; Boxall et al. 2012). Due to dilution, absorption, and physical breakdown, most pharmaceuticals are detected at very low concentrations (e.g., ng/L; Fent et al. 2006). However as drugs, these compounds are designed to illicit cellular responses (e.g., enzymes, receptors) and therefore their nominal concentrations should not be regarded as trivial threats to aquatic organisms (Meredith-Williams et al. 2012; Franzellitti et al. 2014). Because of their ubiquitous use, many pharmaceuticals are persistent in aquatic environments, putting aquatic organisms at risk of chronic exposure and bioaccumulation (Ramirez et al. 2009; Meredith-Williams et al. 2012). Numerous studies have documented acute and chronic toxicities of countless pharmaceuticals on aquatic organisms (Trudeau et al. 2005; Corcoran et al. 2010; Brausch et al. 2012); however studies on coastal and marine organisms are fewer (Brooks et al. 2009). With large human populations along coastlines much remains to be learned about the effects of pharmaceuticals on marine organisms to better inform best management practices (Seiler et al. 2002; Valbonesi et al. 2003; Regoli and Giuliani 2013).

There is debate over whether standard ecotoxicological methods are sufficient for determining chronic exposure effects on organisms at environmentally relevant

concentrations (Corcoran et al. 2010; Franzellitti et al. 2014). Most ecotoxicological data are based on acute exposure periods of less than 24 hours with recent studies running up to 30 days (Daugton and Ternes 1999; Brooks et al 2009; Gaw et al. 2014). While useful for assessing how pharmaceuticals may alter cellular activities, this approach is insufficient in length and scope to determine organism- or ecosystem-level chronic effects. Ecologists are interested in how the presence of pharmaceuticals may alter organism behavior (Hazleton et al. 2013; Chen et al. 2015), physiological functioning (Fong 1998; Di et al. 2014), and ultimately how this may shift community and ecosystem dynamics (Hazleton et al. 2014). Depending on the life history of the organism (e.g., long vs. short life span), short term (30 days or less) chronic exposure studies allow for only limited inferences by ecologists about the effects of pharmaceuticals on ecosystem processes. Long-term exposure studies with concentrations that reflect those detected in the environment are required to answer most ecological questions for long-lived species.

Among the more prevalent categories of pharmaceuticals detected in the marine environment are selective serotonin reuptake inhibitor (SSRI) anti-depressants such as fluoxetine hydrochloride (Prozac®) (Kreke and Dietrich 2008; Vasskog et al. 2008). These drugs have been developed to delay the reuptake of serotonin, moderating neurotransmission in the human brain. However, serotonin is also an important neuromodulater in bivalves, regulating gill cilliary activity, oocyte maturation, and the induction of spawning (Gibbons and Castanga 1984; Fong and Ford 2014). Increased serotonin levels in mussels via fluoxetine exposure have been shown to alter several

important cellular activities that lead to reduced health status (Franzellitti et al. 2014; Munari et al. 2014). Fong and Molnar (2008) found that norfluoxetine, the active metabolite of fluoxetine, induced spawning and parturition in both estuarine and freshwater bivalves at high concentrations (e.g. 29.5 mg/L). Several other studies have demonstrated that fluoxetine leads to adverse physiological and behavioral outcomes in marine invertebrates that could alter their functional roles within the community (Stanley et al. 2007; Oakes et al. 2010; Bossus et al. 2013; Munari et al. 2014; Chen et al. 2015).

Bivalves such as mussels and oysters are at risk of chronic exposure to fluoxetine particularly downstream from effluent-dominated coastal waterways (Brooks et al. 2005; Brooks et al. 2006; Kwon and Armburst 2006; Kreke and Dietrich 2008). Oxidative stress was observed in the marine mussel *Mytilus galloprovincialis* after 15 days of exposure to fluoxetine at a concentration of only 75ng/L, a concentration detected in surface waters (Gros et al. 2006; Metcalfe et al. 2003; Gonzalez-Rey and Bebianno 2013). Franzellitti et al. (2014) found that fluoxetine had adverse outcomes on cell signaling and reduced the health status of the marine mussel *M. galloprovincialis* following 7-day exposure to a range of concentrations detected in the marine environment (e.g., 0.03-300 ng/L). But how do marine mussels react to this range of fluoxetine concentrations over a longer exposure period (i.e., over 30 days) and in terms of whole body metrics?

I designed a laboratory experiment to build on the findings of other marine mussel- fluoxetine exposure studies where adverse effects among cellular biomarkers

were identified at high concentrations for short time periods. I hypothesized that whole-mussel metrics would be affected by four low, but environmentally-relevant, fluoxetine concentrations over longer exposure periods. Specifically, I tested whether growth, body and reproductive condition indices, and the rates of algae cleared by *Mytilus californianus* mussels were affected by fluoxetine exposure over time. We measured these variables at 47, 67, and 107 days of exposure. This study addresses two critical gaps in emerging pharmaceutical contaminants research: 1) the impacts to whole organism physiology and function; and 2) the effects of prolonged exposure periods.

Materials and methods

Experimental organisms and holding conditions

M. californianus mussels were collected from a single location on the jetty north of Rockaway Beach, Oregon (45°39'18.4"N, 123°56'31.2"W) on August 1, 2014 and transported in chilled seawater to the laboratory at Portland State University. Upon arrival, mussels were measured and sorted into size classes. From these, 21 mussels were randomly distributed into 25 housing tanks (~ 64 L each) with a mean total biomass of 87.13 ± 1.17 g per tank. Mean length and mass of individual mussels did not differ among treatments (mean length = 32.22 ± SE (0.35) mm; one-way ANOVA, $P = 0.1$; mean mass = 4.19 ± 0.13 g; $P = 0.2$) or tanks (one-way ANOVA, $P > 0.7$ in both cases; See Appendix A for full summary of mussel metrics by treatment group).

Mussels were allowed to acclimate to laboratory conditions for one month before the exposure study began. Each housing tank had an independent water chilling and

filtration system (Aquatic Enterprises). Seawater was prepared using Instant Ocean and deionized water with salinity and temperature maintained at 35 PSU and 15 °C respectively, to replicate conditions at the collection site. We monitored water chemistry (i.e. ammonia, pH, nitrate, and nitrite) every two weeks to ensure levels were appropriate for mussels. To reduce buildup of animal waste products, 20% of the seawater was replaced with fresh seawater every 20 days. Tanks were dosed with fluoxetine following water changes. Light cycle conditions were maintained at 10 h of dark and 14 h of daylight. During the acclimation period, mussel health and condition were monitored. A total of 4 mussels died during acclimation and were immediately replaced with one of the extra mussels from the original collection. During the exposure study, there was no mussel mortality.

Twice weekly, mussels were batch fed Shellfish Diet 1800® (Reed Mariculture) diluted tenfold with seawater. The algae in the Shellfish Diet 1800® is a combination of six marine microalgae *Isochrysis*, *Pavlova*, *Tetraselmis, Chaetocerous calcitrans*, *Thalassiosira weissflogii* and *Thalassiosira pseudonana* with cell diameter sizes ranging from 5 to 16 μm. Mussels were fed algae according to the total biomass in each tank. As mussels were removed from the tanks for subsampling, algae diet was adjusted to the total biomass in each tank (see Appendix A for total biomass of mussels per treatment over the course of the study).

Experimental design

We assigned 21 mussels to each tank representing one of five treatments of fluoxetine ranging from 0 to 300 ng/L, which covers the range detected in the marine environment (Franzellitti et al. 2014). Treatment groups were 0.3, 3, 30, and 300 ng/L of fluoxetine and a control with no fluoxetine (Figure 2.1). Our experimental units were the individual tanks (n=25) with 5 replicate tanks nested within each treatment group. Each tank was subsampled on days 47, 67, and 107 with 6, 6, and 9 mussels sacrificed respectively. With three sample periods the total number of observations was 75. A set of 5 tanks with no mussels was used to determine a baseline for algae removed by the tank filtration system during algal clearance trials. We note that one of the no-mussel tanks malfunctioned after 20 days into the experiment and was excluded from further analyses, reducing no-mussel tank replicates to 4.

Before each dosing period, fluoxetine solutions were prepared using a stock solution of 1.0 mg/mL fluoxetine hydrochloride (Sigma-Aldrich) dissolved in nanopure water. Each treatment concentration (0.3, 3.0, 30.0, and 300.0ng/L) was prepared using separate dosing solutions, which were prepared through serial dilution of the original stock solution. Every 10 days, the tanks were dosed by adding 193µL of the appropriate fluoxetine dosing solution into each tank. Controls without fluoxetine received 193µL of nanopure water on dosing days.

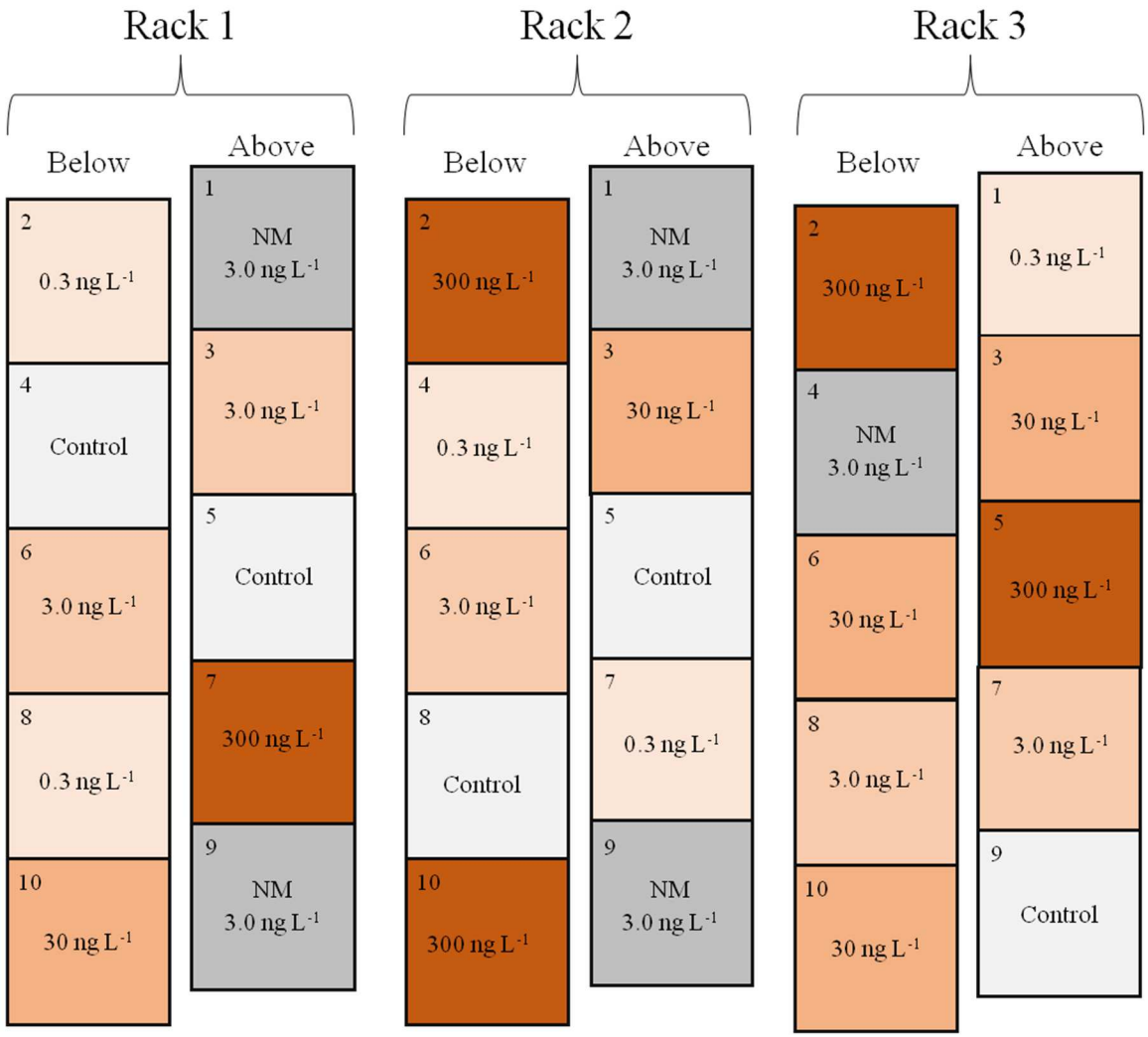

Figure 2.1 Schematic of aquarium set up, with the order of treatment tanks randomized. Treatment groups included: Control, 0.3 ng L^{-1}, 3.0 ng L^{-1}, 3.0 ng L^{-1}, 30 ng L^{-1}, 300 ng L^{-1}, No Mussel (NM) 3.0 ng L^{-1}.

Fluoxetine exposure study

1. Algal clearance

On feeding days, a 10 mL seawater sample was obtained within 5 minute after the algae mixture was added to each of the tanks to allow for thorough mixing. Mussels were allowed to feed for 3 hours before an additional 10mL sample was extracted. These samples served as initial and final concentrations, respectively. From each sample, we counted algal cells in three 0.5 mL aliquots using a Beckman Coulter Counter (model Z1, 100 μm aperture) and determined the mean initial and final concentrations within each tank. We collected a total of 11 samples over the course of the 107-day study.

Filtering rates were estimated from the rate of change in suspended particle concentrations. Following Coughlan (1969), we based filtering rates on four assumptions: a) the reduction in the concentration of particles is due to filtration by the animal, and to settling, b) mussel pumping rate is constant, c) particle retention is 100% efficient and d) there is homogenous suspension of particles. A set of identical tanks without mussels (n=4) served as blanks for feeding trials. For each of the 11 sample dates, clearance rates for each mussel were calculated using the following formula (Coughlin 1969):

$$CR = (M/n) [\ln (C_0/C_t)/t] - [\ln (C_0\text{blank}/C_t\text{blank})/t]$$

where CR = clearance rate ($\text{cells}^{-1}\text{mL}^{-1}\text{min}^{-1}$); M = volume of seawater in each tank (mL); n = number of mussels in tank; t = feeding time (min); C_0 = initial concentration of particles in tank; C_t = final concentration of particles in tank. C_0blank = initial

concentration of particles in tank without mussels; $C_t$blank = final concentration of particles in tank without mussels

2. Mussel growth

Shell length was measured from the umbo to the ventral margin using digital calipers (Mitutoyo 500 196-30) with an accuracy of ±0.01mm. Biomass measurements were made by weighing towel-dried mussels on an analytical balance (Southern Laboratories) with an accuracy of ±0.001g. Two mussel growth estimates (increase in shell length and biomass) were determined as the change between final and initial measurements.

3. Body condition

Three mussels from each subsample period (n=225) were dissected to assess condition and gonadosomatic indices. We separated the somatic and gonadal tissues, desiccated each in a drying oven set at 60°C for 48 hours (Quincy Labs), and recorded their respective dry weights (dw). We calculated the gonadosomatic index (GSI) for each mussel using the following equation:

GSI= [gonads dw (mg)/total soft tissue dw (mg)] x 100

Additionally, the condition index (CI) was calculated for each mussel using the equation:

CI= [total tissue dw (mg)/shell length (mm)] x 100

4. Statistical analysis

For each sampling period, we averaged within-tank means for mussel growth, GSI, CI, and algal clearance parameters. Normality and homogeneity of variances were assessed through graphical inspection of the model residuals and respective Shapiro-Wilk's and Levene's tests, which indicated a need for data transformation. Algal clearance data underwent a Box-Cox transformation (Box and Cox 1964) and mussel growth and body condition data were log-transformed. The assumption of sphericity was determined for each parameter using the Mauchly test and adjusted using the Greenhouse-Geisser correction. In order to determine the effects of fluoxetine treatments on measured response variables we ran repeated-measures ANOVAs with fluoxetine treatment and sample date as factors with tanks included as an error term to account for the non-independence between samples. Main effects were considered significant at α=0.05. Mixed-effects models were generated using the lme4 package in R (Bates et al. 2015). Fluoxetine treatment and sample date were treated as fixed factors while the tanks were treated as a random factor. Using the multcomp package in R, I generated post-hoc multiple pairwise comparisons (with a Bonferroni correction) between treatment groups and sample dates for each measured parameter (See Appendices B-E for pairwise comparisons of treatment by sample date). All statistical analyses were performed using R statistical platform (RStudio Version 3.2.2 (2015)).

Results

1. *Algal Clearance*

Mean clearance rates differed among treatment groups (ANOVA: $F_{4, 20} = 34.4$, $P < 0.001$, Figure 2.2), being higher in the controls [mean = 63.0 (SE 3.7) cells $mL^{-1}min^{-1}$] than in the treatment groups [36.3 (SE 4.2) cells $mL^{-1}min^{-1}$; Table 1, Figure 2.2]. Mean clearance rates also differed between sample dates ($F_{10, 20} = 46.0$, $P < 0.001$; Table 2) being higher towards the end of the study [76.7 (SE 4.7) cells $mL^{-1}min^{-1}$] than at 30 days since the start [38.2 (SE 3.1) cells $mL^{-1}min^{-1}$], suggesting that clearance rates were variable with time. There was an interaction between treatment and sample date ($F_{40, 20} = 3.0$, $P < 0.001$), indicating that the effect of treatment was mediated by sample date. In general, clearance rates were inversely proportional to fluoxetine concentrations, where mussels treated with 30 and 300 ng/L cleared algae at a slower rate than the lower treatment groups (0.3 and 3 ng/L) and controls (see Table 2.1 for full list of summary statistics).

Table 2.1. Summary statistics from algal clearance trials: mean, minimum, and maximum values ± SE. Clearance rates were calculated as cleared algal cells $mL^{-1}min^{-1.}$

Treatment	Mean	Min	Max
Control	63.0 ± 3.7	38.8 ± 1.8	119.2 ± 4.3
0.3 ng/L	45.2 ± 3.7	19.8 ± 2.6	93.3 ± 2.7
3 ng/L	41.8 ± 4.2	18.7 ± 3.8	75.1 ± 4.9
30 ng/L	31.9 ± 4.2	18.6 ± 3.4	57.9 ± 8.3
300 ng/L	26.3 ± 4.6	13.2 ± 1.2	47.0 ± 7.8

2. *Mussel growth*

Growth in shell length varied by treatment level (ANOVA: $F_{4, 20} = 22.6$, $P < 0.001$, Table 2, Figure 2.3 A), with faster growth in controls [0.22 (SE 0.04) mm] than in treatment [0.12 (SE 0.02) mm; Figure 2.3 A] groups. While there was also an effect of the sample date ($F_{2, 20} = 18.7$, $P < 0.001$) there was no interaction between treatment and sample date ($F_{8, 20} = 0.7$, $P = 0.3$) indicating that treatment effects are not dependent on sample date. Overall growth in shell length followed a similar pattern over time: lower treatment groups (0.3 and 3 ng/L) grew at a similar rate to controls and were much greater than the 30 and 300 ng/L treatment groups. Post-hoc Tukey tests indicated that controls were significantly different from 3.0 ng/L ($P = 0.04$) but not 0.3 ng/L ($P = 0.08$) treatment groups, while 30 and 300 ng/L were not different from each other ($P = 0.81$).

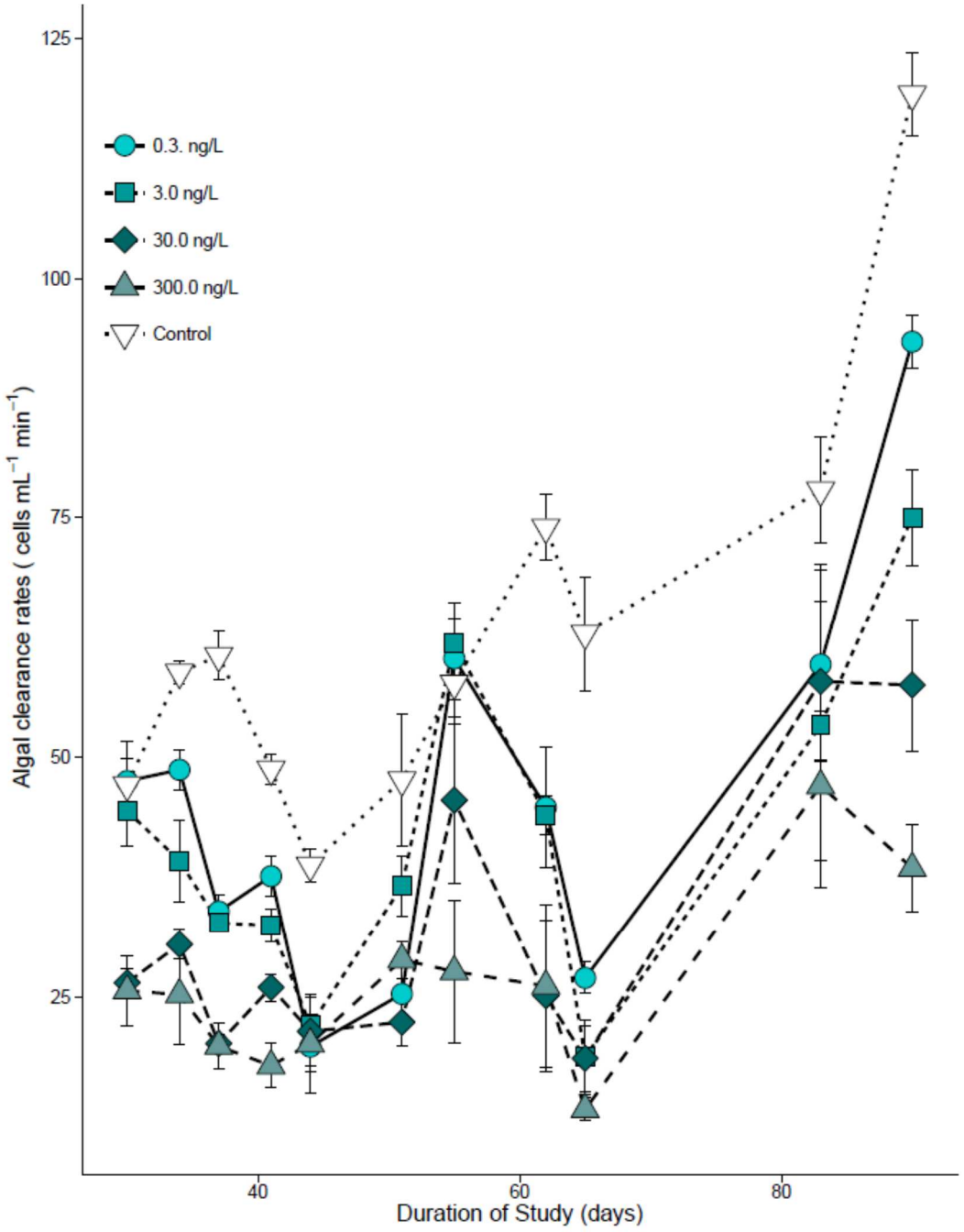

Figure 2.2. Clearance rates of mussels; samples collected during 11 feedings over study duration. Clearance rates were defined as the amount of cells removed per mL per min per individual mussel. Note mussels were removed over the study: 21 mussels (day 0-47), 15 mussels (day 47-67, and 9 mussels (67-107). Error bars reflect the standard error (SE) of the mean.

Growth in biomass also differed among treatment groups (ANOVA: $F_{4, 20} = 5.2$, $P = 0.005$; Table 2), with greater increases in biomass in controls [166.41 (SE 33.32) mg] than treatment [108.81 (SE 14.06) mg; Figure 2.3 B] groups. However, post-hoc Tukey test revealed there was no significant difference between controls and 0.3 ng/L treatment groups ($P = 0.17$). There was an effect of sample date ($F_{2, 20} = 24.5$, $P < 0.001$) and an interaction between sample date and treatment ($F_{8, 20} = 2.4$, $P = 0.04$), indicating the effect of fluoxetine on mussel biomass is dependent on exposure period, specifically with stronger effects after sample date 47 where the group means diverge. There was no difference in biomass change among mussels treated with 3 ng/L fluoxetine and those treated with 30 ng/L ($P = 0.29$) or 300 ng/L ($P = 0.35$).

3. Body condition

The gonadosomatic index (GSI) of mussels in control groups was much higher [28.3 (SE 3.6) GSI] than those treated with any concentration of fluoxetine [8.8 (SE 2.0) GSI; ANOVA: $F_{4, 20} = 24.9$, $P<0.001$, Figure 2.4 A]. There was only a marginal effect of sample date on mussel GSI ($F_{2, 20} = 3.5$, $P= 0.05$) and no 2nd order interactions ($F_{8, 20} = 1.2$, $P= 0.37$). There were no differences in mean GSI among fluoxetine treatment groups. The condition index (CI) was also higher in controls than in treatment groups (ANOVA: $F_{4, 20} = 5.6$, P=0.001, Figure 2.4 B) and there was no difference among fluoxetine treatment groups.

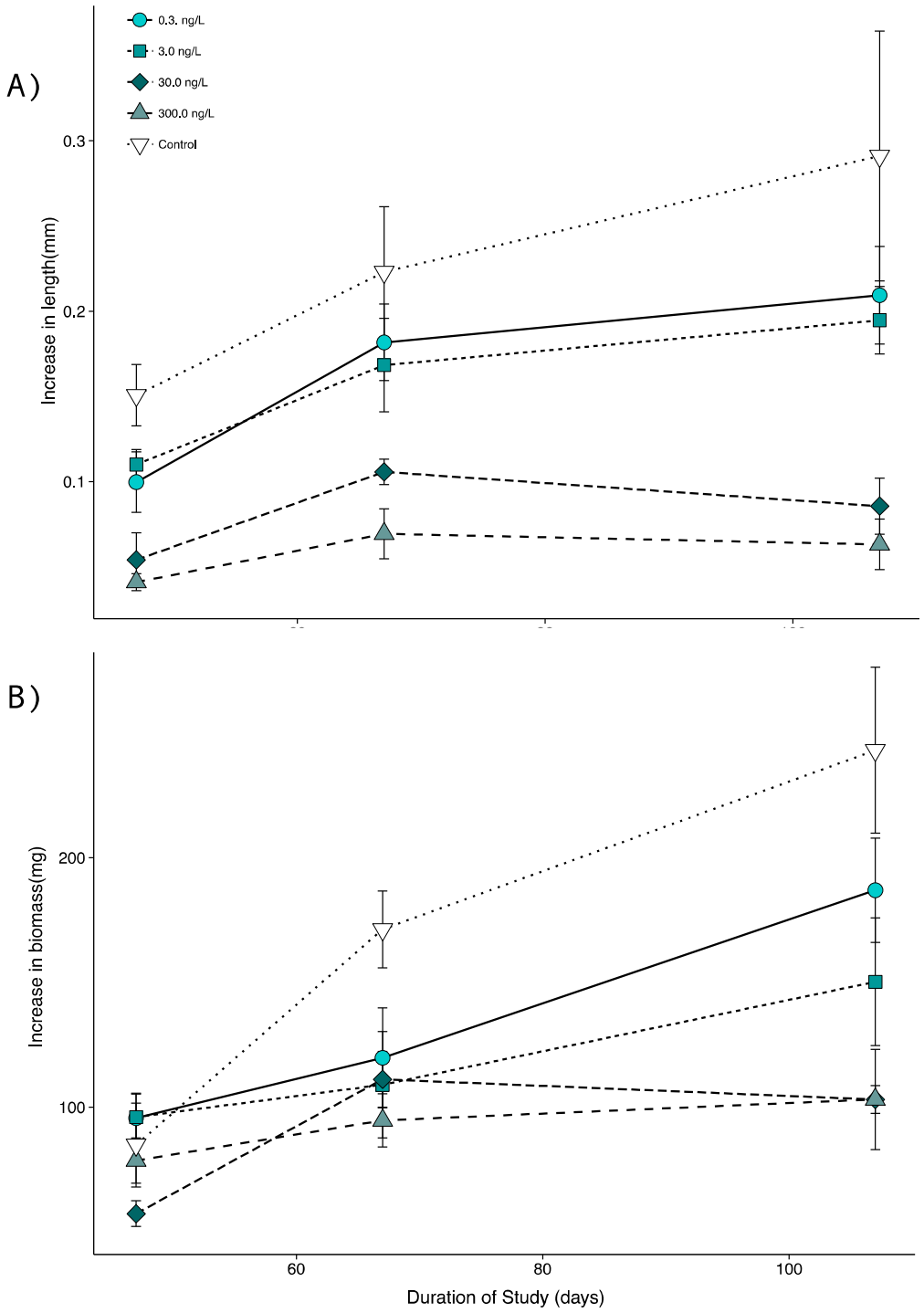

Figure 2.3. Mussel growth as the increase in A) shell length (mm), and B) biomass (mg) across treatment groups over study period. Error bars reflect the standard error (SE) of the mean.

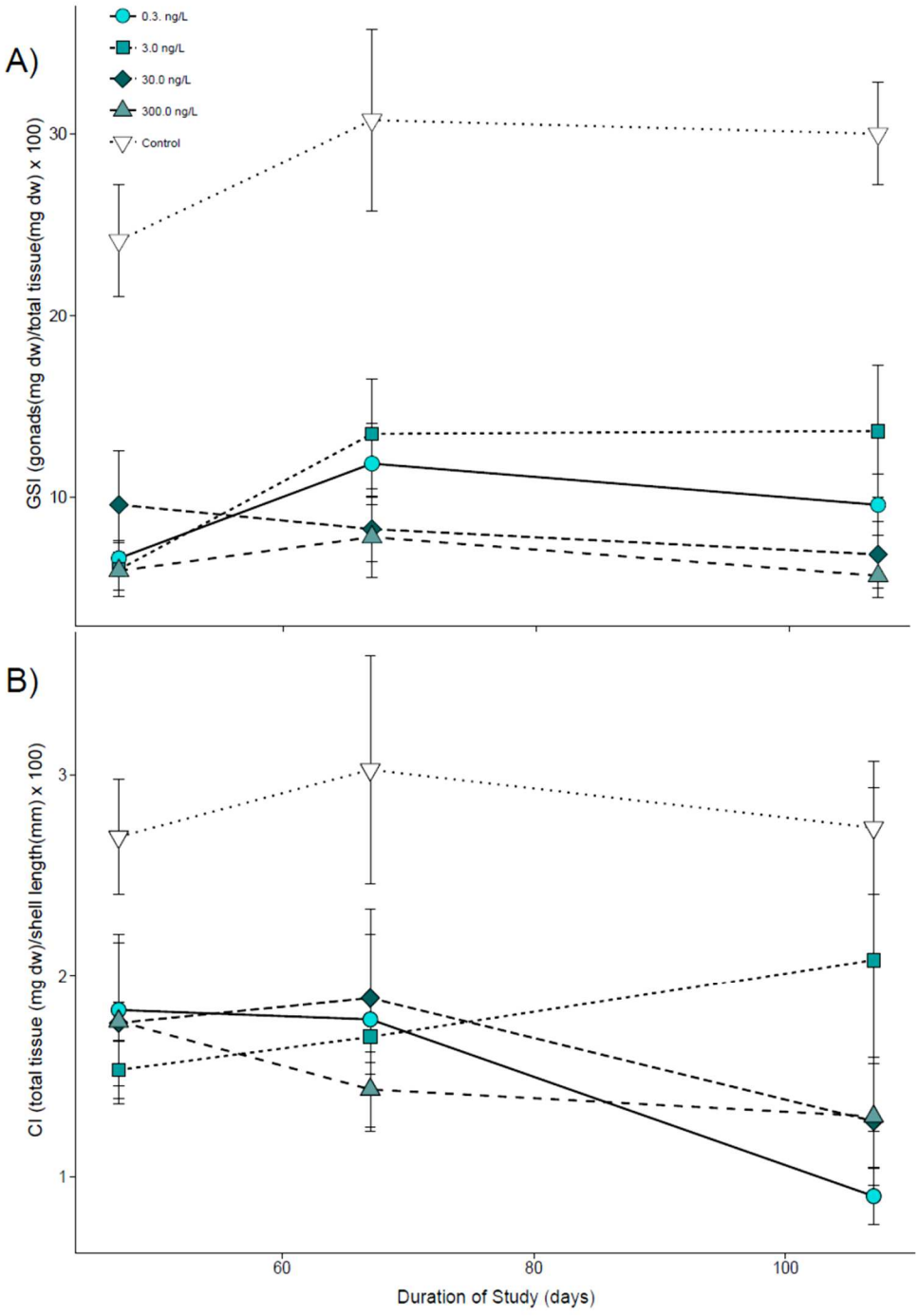

Figure 2.4. A) Gonadosomatic and B) condition indices for mussels on day 47, 67, and 107 of the study. Error bars reflect the standard error (SE) of the mean.

Table 2.2. Results from Repeated measures ANOVAs, including all factors and 2nd order interactions. Clearance rate data underwent a Box-Cox transformation; other response variables were log-transformed. Treatment refers to fluoxetine concentration; sample date refers to subsample group; P-values <0.05 are shown in bold.

Dependent Variable	Factor	SS	MS	df	*F*	*p*
Clearance rate	Treatment	72.43	18.11	4	34.35	**<0.001**
	Sample date	81.85	8.19	10	45.95	**<0.001**
	Treatment*Sample date	21.20	0.53	40	2.96	**<0.001**
	Error among groups	10.54	0.53	20		
	Error within groups	35.63	0.18	200		
Growth (length)	Treatment	18.84	4.71	4	22.64	**<0.001**
	Sample date	4.98	2.49	2	18.70	**<0.001**
	Treatment*Sample date	0.70	0.09	8	0.65	0.73
	Error among groups	4.16	0.21	20		
	Error within groups	5.33	0.13	40		
Growth (mass)	Treatment	3.06	0.76	4	5.19	**0.005**
	Sample date	4.61	2.31	2	24.53	**<0.001**
	Treatment*Sample date	1.77	0.22	8	2.36	**0.04**
	Error among groups	2.94	0.14	20		
	Error within groups	3.76	0.09	40		
Gondosomatic index (GSI)	Treatment	21.22	5.31	4	24.93	**<0.001**
	Sample date	1.49	0.74	2	3.51	0.05
	Treatment*Sample date	2.09	0.26	8	1.23	0.37
	Error among groups	3.45	0.17	20		
	Error within groups	9.32	0.23	40		
Condition index (CI)	Treatment	4.97	1.24	4	5.6	**0.001**
	Sample date	1.35	0.51	2	1.0	0.05
	Treatment*Sample date	4.12	0.14	8	0.7	0.51
	Error among groups	3.56	0.18	20		
	Error within groups	6.23	0.16	40		

Discussion

Our study demonstrates that prolonged exposure to nominal concentrations of fluoxetine impairs mussel physiology and function. We sampled mussels regularly over the exposure study to better understand how they respond to fluoxetine over time. Whole body metrics of fitness like growth in biomass and shell length were only affected over longer time periods. However, for parameters like the GSI and CI, fluoxetine exposure concentrations affected organisms by 47 days, without increasing differentiation over time. Our study builds on previous studies documenting fluoxetine's effects on aquatic organisms by identifying organism-level and chronic exposure effects over several months (e.g., >100 days). By simulating chronic fluoxetine exposure in the laboratory, we offer a snapshot of how this single contaminant may impair mussels along rocky intertidal shorelines in the wild.

Mussels like *M. californianus* regularly clear the water column of algae, suspended particles, and pollutants, improving water quality and providing a critical ecosystem function along coastal zones. At locations where fluoxetine impairs mussel filter feeding, this important ecosystem function may be reduced. Hazleton et al. (2014) conducted a 67-day study with adult freshwater mussels, *Lampsilis fasciola,* exposed to four fluoxetine concentrations (0, 0.5, 2.5, and 22.3 µg/L), and assessed impacts on metabolism, movement, and filtering behavior. They found that mussels dosed with 2.5 or 22.3 µg/L fluoxetine had increased activity levels when compared with controls, suggesting contaminated animals may be more susceptible to predators and reduced

energy storage, at least at higher fluoxetine concentrations. Increased activity levels (such as movement) in *M. californianus* may explain the reduced filtering function and slower growth rates. While we did not quantify movement patterns following Hazleton et al. (2014), we did observe that individual mussels exposed to 30 and 300 ng/L fluoxetine were more dispersed within the tanks and did not cluster as the controls and 0.3-3 ng/L treatment groups did. Hazelton and colleagues (2014) did not find clear differences between algal clearance rates but suggested observing clearance rates over shorter time periods (< 24 h). Our clearance trials were 3 h and we did see clear differences between control and treated groups. Further, after day 67 we observed that water clarity was qualitatively reduced in all 5 of the 300ng/L tanks suggesting that clearance was impaired.

Because fluoxetine exposure impairs mussel clearance rates, it follows that energy storage and mussel growth would also be reduced (Bringolf et al. 2010; Hazelton et al. 2014). Munari et al. (2014) exposed the clam *Ruditapes phillippinarum* to fluoxetine at six concentrations (0, 1, 5, 25, 125, 625 µg/L) for 7 days and found that haemocyte proliferation increased significantly in clams exposed to 25, 125, 625 µg/L, while gill acetylcholinesterase (AChE) activity decreased significantly in clams exposed to 1 or 5 µg/L. Their findings suggest that fluoxetine, at least at higher concentrations, strongly affects immune parameters and neurotransmission in clams. Franzellitti et al. (2014) reported similar effects with even lower concentrations of fluoxetine (e.g., 0.03-300 ng/L), where fluoxetine reduced the health status of mussels in numerous cellular

biomarkers. Specifically, fluoxetine reduced the lysosomal membrane stability in haemocytes and caused accumulation of neutral lipids in the lysosomes of the digestive glands. We believe that the low condition index values of mussels treated with fluoxetine in our study are linked to similar cellular responses, although we quantified only organism-level metrics. Similarly, we observed reduced growth in shell length and biomass in mussels exposed to fluoxetine above 3ng/L. After sample date 67, growth rates decreased for mussels exposed to 30 and 300 ng/L of fluoxetine (see slopes in Figure 2), suggesting that these concentrations have a stronger effect on growth. However these patterns would not be apparent from the typical short-term exposure studies of 30 days or less. The findings demonstrate that responses measured over short time periods may miss the extent to which fluoxetine and other pharmaceutical compounds can affect marine organisms.

In mussels, serotonin is involved in physiological and behavioral functions such as gill ciliary activity, oocyte maturation, and the induction of spawning (Stanley et al. 2007; Bringolf et al. 2010; Fong & Ford 2014). Because fluoxetine regulates the reuptake of serotonin, it is likely the drug increases serotonin levels in mussels (Gibbons & Castagna 1984). Bringolf et al. (2010) found that fluoxetine accumulates in mussel tissues and has the potential to disrupt several aspects of reproduction in freshwater mussels. Despite their strong results, they recommended additional testing to evaluate the effects of long-term exposure to environmentally relevant concentrations. With our long-term testing, the proportion of reproductive tissue to total tissue (GSI) was markedly

affected by fluoxetine after 47 days of exposure, a long time period among chronic exposure studies assessing reproduction. We hypothesize that fluoxetine is concentrated in mussel tissues over time resulting in a reduction of reproductive potential. A similar statement can be made about the CI, an assessment of the mussel's overall health status. Overall, the energy invested into the gonad and somatic tissues was lower in mussels treated with fluoxetine than in controls.

Fluoxetine is one of the most widely used antidepressants in the world (Metcalfe et al. 2010). A robust amount of research has documented its occurrence in freshwater (Kwon and Armburst 2006; Ramirez et al. 2009; Bringolf et al. 2010; Corcoran et al. 2010) and marine (Kreke and Dietrich 2008; Vasskog et al. 2008) environments. Our study conditions mimic fluoxetine entering the environment in pulses, such as flushing from rain events, and organism exposure over time. The findings by Franzellitti et al. (2014) of the numerous adverse outcomes and fluoxetine bioconcentration at 30 and 300 ng/L exposure concentrations corroborate our results. Further, with growing human populations in coastal zones, increasing use of antidepressants like fluoxetine is expected, suggesting higher future concentrations in the marine environment.

The results of this study serve as a foundation to understand how pharmaceuticals and other emerging contaminants are affecting marine species and community interactions. While we found fluoxetine to be a considerable stressor to marine mussels, it is only one of many stressors on marine organisms (Ankley et al. 2007; Boxall et al. 2009). Nearshore flora and fauna are exposed to a cocktail of contaminants, many of which (e.g.,

sertraline (Effexor®; Bossus et al. 2013), carbamazepine (Tegretol®; Martin-Diaz et al. 2009)) have negative effects on freshwater and marine organisms (e.g., Metcalfe et al. 2010; Meredith-Williams 2012). Some studies have assessed pharmaceutical effects on animal behavior and their potential to alter species interactions (Gaworecki and Klaine 2008; Bossus 2013; Hazelton et al. 2013). Yet, long-term studies examining effects of multiple compounds are warranted to understand interactive and cumulative organismal and potential ecosystem level effects (Brausch et al. 2012). To our knowledge no studies have assessed community or ecosystem responses to pharmaceuticals or other emerging contaminants, an important step in understanding how these compounds may influence important inter- and intra-specific interactions. Finally, ecological studies are needed to assess how these compounds affect ecosystems in a changing world, considering interactive effects with ocean acidification and other impacts from climate change.

Chapter 3: Exposure to nominal concentrations of the pharmaceutical fluoxetine increases predation risk in the mud crab, *Hemigrapsus oregonensis*

1. Introduction

Predator-prey behavior dynamics are often regulated by a combination of abiotic and biotic factors (Holt and Lawton 1994; Abrams 2000; Grabowski 2004). Physical factors such as temperature, salinity, and photoperiod often limit where organisms can survive (e.g., fundamental niche), while species interactions such as competition, predation, or facilitation further restrict the spatial and temporal extent of an organism (e.g., realized niche; Hutchinson 1957; Lima & Dill 1990; De Roos et al. 2003; Chase et al. 2009). Animal behaviors are rooted within this realized niche wherein individuals modify their behaviors to balance risks (e.g., predation) with rewards (e.g., access to resources; De Roos et al. 2003, Brown and Kolter 2004). Often these risk-taking behaviors are plastic and change depending on the spatial (Morgan et al. 2006) or temporal (Miller & Morgan 2015) conditions (Snell-Rood 2013). Ecologists are eager to understand animal behaviors to more accurately predict population-, community-, or landscape-level processes (Abrams 2000; Shochat et al. 2006; Sih et al. 2012; Balke et al. 2014).

Yet, there is a growing list of human-driven impacts that alter animal behavior, setting additional boundaries on an animal's realized niche (Barros 2001; Frid & Dill 2002; Fahrig 2007; Dodd et al. 2015). Fisheries have historically removed large

predators, modifying community behaviors through release from predation pressure (Myers & Worm 2003; Catano et al. 2015). Anthropogenic noise pollution in the ocean has been shown to alter the behaviors of numerous marine mammals (Nowacek et al. 2007). Ocean acidification alters development of larval fishes, disrupting their ability to detect predator cues and leading to increased mortality (Munday et al. 2010). Exposure to heavy metals, pesticides, petroleum and other legacy contaminants affect animal behaviors by altering habitat preference, shifting migration patterns, or increasing negative species interactions (Fleeger et al. 2003; Khoury et al. 2009; Eades & Waring 2010; Fukunaga et al. 2010). Such alterations to normal behaviors have been linked to reduced fitness, and changes to population structure and ecosystem function (Frid & Dill 2002; Fahrig 2007; Ings et al. 2009).

Much less studied are the effects of pharmaceuticals and other emerging contaminants on animal behavior, despite frequent detections of these compounds in the marine environment (Boxall et al. 2012; Brausch et al. 2012; Gaw et al. 2014). Pharmaceutical compounds and their derivatives regularly enter estuaries and nearshore coastal ecosystems via transport of contaminated surface and groundwater runoff, suspended river sediments, and untreated sewage effluent (Metcalfe et al. 2010; Bringolf et al. 2010; Khairy et al. 2014). As medical drugs, these compounds are designed to illicit biological responses and could have considerable effects on organism health, despite detections at low concentrations (Seiler 2002; Ankley et al. 2007). Prolonged studies on marine organisms at environmentally relevant concentrations are lacking (Berninger &

Brooks 2010; Gaw et al. 2014). Most pharmaceutical exposure studies are rooted in ecotoxicological methodology focused on adverse outcomes at the cellular or subcellular-level (Boxall et al. 2012). Exposure studies that assess the effects of pharmaceuticals on whole-organism effects, and multi-organism or community-level interactions are needed to improve ecological inferences and predictions (Fleeger et al. 2003; Brooks et al. 2009; Corcoran et al. 2010; Gaw et al. 2015).

Mesocosm and tank experiments are often used to assess animal behavior responses to stressors, particularly when the stressor cannot be controlled in the field. These include studies where different combinations of stressors such as chemical cues, temperature, or pH are manipulated in order to measure the behavioral response (Munday et al. 2009; Dodd et al. 2015). Pharmaceutical contaminants as stressors require a similar approach to determine if detected or projected concentrations affect organism behaviors (Hellou 2011; Mesquita et al. 2011; Lazzara et al. 2012; Maranho et al. 2015). Relatively few studies have assessed how pharmaceuticals affect interspecific behaviors such as predator-prey interactions (Brodin et al. 2014; Gaw et al. 2014). Yet several studies have demonstrated alterations in behavior that could lead to increased predation and mortality (Corcoran 2010; Schultz et al. 2011; Hazelton et al. 2013; Brodin et al. 2014).

Selective serotonin reuptake inhibitor (SSRI) anti-depressants such as fluoxetine hydrochloride (Prozac®) are among the more prevalent categories of pharmaceuticals detected in the marine environment (Kreke and Dietrich 2008; Vasskog et al. 2008; Brodin et al. 2014; Gaw et al. 2014). SSRIs have been developed to delay the reuptake of

serotonin, moderating neurotransmission in the human brain. In crustaceans, serotonin is well known to affect behaviors through stimulating the release of hyperglycaemic, neurodepressing, moult-inhibiting, and gonad-stimulating hormones (Fong and Ford 2014). McPhee and Wilkens (1989) found that *Carcinus maenas* injected with serotonin displayed increased activity levels during the day, whereas normally they are photonegative with increased activity at night. In the same crab species, fluoxetine significantly altered locomotor behaviors at 120 μg/L (Mesquita et al. 2011). Several other studies have demonstrated that fluoxetine leads to adverse physiological and behavioral outcomes in aquatic organisms that could alter their functional roles within the community (Perreault et al. 2003; Lynn et al. 2007; Stanley et al. 2007; Mennigen et al. 2010; Schultz et al. 2011; Dzieweczynski & Herbert 2012; Kohlert et al. 2012; Bossus et al. 2013; Barry 2013; Munari et al. 2014; Chen et al. 2015).

Using a controlled laboratory set up, we designed a study to assess the behavior of the mud crab, *Hemigrapsus oregonensis*, following exposure to the pharmaceutical contaminant, fluoxetine. Fluoxetine has been frequently detected in coastal areas at low concentrations (0.03ng/L -300 ng/L) and is considered toxic to fish and marine invertebrates (Brooks et al. 2003). In our study, aquarium habitats were designed to emulate estuarine conditions to assess alterations of *H. oregonensis* behaviors under the influence of fluoxetine at controlled concentrations (3 and 30ng/L). We conducted diurnal and nocturnal behavior trials to assess whether fluoxetine altered the risk-taking behaviors of *H. oregonensis* in response to a predator, the red rock crab *Cancer*

productus. Our study is one of the few studies to assess how pharmaceutical contaminants may affect risk-taking behavior in marine animals.

2. Material and methods

2.1. Study Animals

The Oregon mud crab, *Hemigrapsus oregonensis* (Dana, 1851; Figure. 1A), is a small intertidal shore crab belonging to the family Grapsidae, and is one of the most common species inhabiting estuarine shorelines between Resurrection Bay, Alaska and Bahia de Todos Santos, Baja California. They forage mostly at night, with a diet consisting primarily of diatoms and green algae, but they will eat carrion and other meat, if available (Lindberg 1980). *H. oregonensis* spend most of their time on, beneath, or near rocks in gravel and fine sediment substrate. To escape predators, *H. oregonensis* often quickly burrow in mud or hide beneath rocks; they also rely on camouflage while remaining motionless (Lindberg 1980). Because *H. oregonensis* inhabit the soft sediments of estuaries, they are likely exposed to contaminants, including fluoxetine; in estuaries, fluoxetine concentrations have been detected as high as 30ng/L (Franzellitti et al. 2014). For this reason, I chose these crabs as a model organism for fluoxetine exposure during behavioral trials.

Red rock crabs, *Cancer productus* (Randall, 1839; Figure 3.1 C), are one of several Cancer species that inhabit the Pacific Coast of North America, occupying a similar range as *H. oregonensis*. They range from sub- to intertidal habitats, but will regularly occur in estuarine habitats during high tide (McGraw 2005). They regularly

prey on barnacles, amphipods, intertidal invertebrates, and smaller crabs, including *Hemigrapsus* spp. I chose *C. productus* as my model predator because of its overlapping range at high tide, whereby the predator may enter an estuary contaminated with fluoxetine and encounter prey species such as *H. oregonensis*.

2.2. Experimental holding conditions

H. oregonensis and *C. productus* crabs were collected from a single location along an estuarine shoreline in Netarts Bay, Oregon (45°24'51.21"N, 123°56'4.38"W) on June 15, 2015. *C. productus* were caught using crab traps deployed at high tide while *H. oregonensis* were hand captured along the edge of the shoreline. Both species were transported in chilled seawater to the laboratory at Portland State University. Upon arrival, *H. oregonensis* (n= 90) were sorted, measured, and randomly distributed into 30 housing tanks (~64 L, 3 crabs in each). *C. productus* (n= 15) were sorted into three designated housing tanks (~120 L, 5 in each) to prevent cross contamination following exposure to fluoxetine during behavioral trials. Each housing tank had an independent water chilling and filtration system (Aquatic Enterprises). Seawater was prepared using Instant Ocean® and deionized water; salinity and temperature were maintained at 35 PSU and 16.0 °C to replicate conditions at the collection site. To reduce buildup of animal waste products, 20% of the seawater was replaced with fresh seawater every 20 days. I monitored water chemistry (i.e., ammonia, pH, nitrate, and nitrite) every two weeks to ensure levels were appropriate for crabs. Water criterion was adequate each time. Light cycle conditions were maintained at 10 h of dark and 14 h of daylight. Animals were

allowed to acclimate to aquarium habitats (Figure 3.1 A and B) and laboratory conditions for 2 weeks before the exposure study began. During the acclimation period, crab health and condition were monitored. A total of 8 *H. oregonensis* died during acclimation and were immediately replaced with one of the extra crabs of the same gender and size class from the original collection. During the exposure study (60 days), 31 crabs perished either through predation by *C. productus* during trials (n=18) or through conflicts between conspecifics (n=13), in which case each was immediately replaced by an individual of the same size class and gender.

Every two days, *H. oregonensis* were fed a diet of either squid or shrimp pieces. In addition, *H. oregonensis* regularly grazed algae from rocks and sediment and filter fed by rapidly beating their third maxillipeds near their mouth. *C. productus* were fed squid every 2 days. At the end of the study I sacrificed all *H. oregonensis*, and quantified the number of appendages lost as a proxy for aggression among conspecifics.

2.3. Experimental design

The experiment followed a repeated measures design in which the tank was the subject measured at each time point (day vs. night periods, with vs. without predators, and multiple times for each period and trial type) and was nested within the between-measures factor, fluoxetine treatment. The fluoxetine treatments consisted of 3 concentrations: 0, 3, and 30 ng/L which are the range detected in estuarine and harbor waters (Kreke and Dietrich 2008; Vasskog et al. 2008). Each treatment group was comprised of 10 replicates. Each fluoxetine treatment concentration (3.0 and 30.0 ng/L)

was reached using separate dosing solutions prepared through serial dilution of the original stock solution of 1.0 mg/mL fluoxetine hydrochloride (Sigma-Aldrich) dissolved in nanopure water. Every 10 days, tanks were dosed by spilling 193µL of the appropriate fluoxetine dosing solution into each tank. Controls without fluoxetine received 193µL of nanopure water on dosing days.

Three *H. oregonensis* were assigned to each tank, with 1 dominant male, 1 subordinate female, and 1 subordinate male. While we recorded the behavior of each animal, our experimental units were the individual tanks (n=30) with 10 replicate tanks nested within each treatment group. Weekly behavioral trials were our observational units, where all animals in each tank were observed for one-hour periods at day and night times, both with and without predators present (4 trial types over 9 weeks, n=36). No-predator trials were used as a reference for assessing behaviors without any perceived threats.

2.4. Behavioral Trials

Housing tanks were designed to simulate the estuarine conditions from which *H. oregonensis* were collected. Each tank was filled with sand (500g) and small pebbles (500) for burrowing substrate and one large rock (600-750g) to hide under (Fig. 1c). Tanks were assembled on 3 racks (10 tanks per rack) with sides between tanks blacked out with plastic lining to maintain behavioral isolation. Each tank contained 3 *H. oregonensis*: 1 large dominant male (mean carapace width (CW) ± SE = 25.54 ± 0.42

mm; mean biomass ± SE = 9.3 ± 1.4 g), 1 small female (CW = 19.25 ± 0.74 mm; 3.6 ± 1.5 g), and 1 small male (21.29 ± 0.65; 4.97 ± 0.97 g). Mean size of crabs did not differ among treatments or tanks (one-way ANOVA, $P > 0.4$ in both cases). This density of crabs (3.0 / 30 cm^2) is within the natural range of *H. oregonensis* densities at the collection site (densities as high as 20 crabs/50 cm^2 were observed). We kept crab densities low to allow enough space for escape from the much larger *C. productus* (range: 100 to 150 mm CW) during predator addition trials.

Hour-long trials were recorded using ethograms with common crab behaviors outlined for each animal. These behaviors were organized by category: Still, active, foraging, aggression, non-aggression, avoidance, and predator avoidance behaviors. Still behaviors included: buried, unmoving, moving mandibles only. Active behaviors included: walking, digging, swimming and moving in place. Foraging behaviors were those where crabs were probing, handling, or eating food. Aggression, non-aggression, and avoidance behaviors were defined as interactions between conspecifics such as fighting, charging, mating, or avoiding one another. Predator avoidance behaviors were interspecific, where *H. oregonensis* displayed escape or non-escape behaviors in the presence of *C. productus*. We also noted the number of *H. oregonensis* captured or killed by *C. productus.*

Figure 3.1. Pictures of A) an *Hemigrapsus oregonensis* in the aquarium habitat, B) example of the tank set up with sides blacked out, C) addition of *Cancer productus* during predator trials, and D) an observer recording crab behavior during a night trial.

Following procedures outlined by Altmann (1974) observers recorded the behavioral acts of all individuals in each tank via instantaneous scan sampling at 5 min intervals for 1 hour. Scans lasted 30 seconds, allowing the observer to record acts of individuals in 10 tanks before returning to the first tank for the next interval. A total of 12 acts were recorded for each animal during the hour period. Day trials were conducted from 10:00-11:00 am and night trials were conducted from 7:00-8:00pm. During night

trials, we used red LED lights to record observations while avoiding the effects of visible light wavelengths on nocturnal behaviors (Figure 3.1.D). No-predator trials for both day and night trials preceded predator trials by 24 hours with 80 hours in between each week's two predator trials to allow crabs to recuperate. All trials were conducted from June 29 to August 27, 2015.

2.5. Statistical Analysis

Ethograms from the trials were analyzed for crab behavior and predation risk. I assessed the effect of fluoxetine treatment on *H. oregonensis* diurnal and nocturnal behaviors by examining the differences in the proportions of active, foraging, agonistic, and predator avoidance behaviors in trials with and without predators. To determine these proportions, I *a priori* divided active behaviors (i.e., walking, digging, and interactions between conspecifics) and non-active behaviors (i.e., remaining still, buried, or just moving mandibles); foraging and non-foraging behaviors; as well as agonistic and non-agonistic behaviors. Predator avoidance behaviors were also *a priori* determined as remaining buried, still, or retreat under rock/elsewhere in tank and non-avoidance behaviors as remaining active, foraging, or interacting without response to the predator.

Within each trial type (no predator/predator) and time of day (day/night) I tested whether the effect of fluoxetine treatment on behavioral proportions varied across crab sex and gender. Specifically, I tested the probability of successfully exhibiting behavioral acts using mixed-effect generalized linear models (GLMM) fitted with a binomial error distribution using the glmer function in the lme4 package (Bates et al., 2015) in R Studio

(R Core Team, 2015). Behavioral proportional data was over-dispersed, indicating a need to fit the logistic regression with a random intercept (the 30 individual housing tanks). The random effect of the tanks accounts for variance structure between observations made on the same animals overtime. A separate GLMM was fitted to each behavior in question (i.e. active, foraging, agonistic, and predator avoidance) to determine if the effect of fluoxetine treatment varied among crab gender and status. In all GLMMs the proportional data underwent logit transformation to ensure normality and homoscedasticity of the residuals.

For behavioral GLMMs, I added components to the null model (i.e., random intercept) stepwise to determine if they improved the model fit based on Akaike information criterion (AIC). Components that significantly benefitted the full model fit included: fluoxetine treatment groups and crab gender and status. In all models, the length of fluoxetine exposure (in weeks) was not significant (likelihood ratio test, LRT, $P > 0.3$ in both cases) so exposure time was not included. Post hoc multiple comparisons of the models were generated using the multcomp package in R (Hothorn et al. 2008).

3. Results

Active behaviors

The effect of fluoxetine on *H. oregonensis* active behaviors varied across trial types and time of day (glmer, likelihood ratio test (LRT), χ^2 (2) = 292.31, $P < 0.0001$, Figure 3.2). A crab exposed to 30 ng/L of fluoxetine had the highest probability of

exhibiting active behaviors when predators were absent at night (predicted probability of active behavior =0.79) and during the day (0.68). When predators were present the probabilities declined for both time periods (0.62, 0.60, respectfully).

During the day when predators were not present, fluoxetine affected *H. oregonensis* (χ^2 (2) = 23.78, $P < 0.001$, Figure 3.2), increasing the proportion of active behaviors when exposed to 30 ng/L of fluoxetine (estimate ± SE; 1.23 ± 0.27, P <0.001). The effect of 3ng/L and control groups on active behaviors were negative (-1.37 ± 0.19 and -0.29 ± 0.27, respectively), indicating that crabs in these treatments spent a greater proportion of their time being still relative to the 30ng/L group.). Crab gender and status did not significantly explain the variation of active behaviors alone (χ^2 (2) = 4.73, P =0.09), however the interaction between fluoxetine treatment and crab gender and status significantly improved the model fit (χ^2 (6) = 72.95, P <0.001). Dominant males were more active than females and subordinate males (estimate 0.44 ± 0.11 vs. -0.09 ± 0.11 and -1.50 ± 0.20, P <0.001 in both cases). However subordinate males were more active when exposed to 30 ng/L of fluoxetine compared with controls (P=0.02).

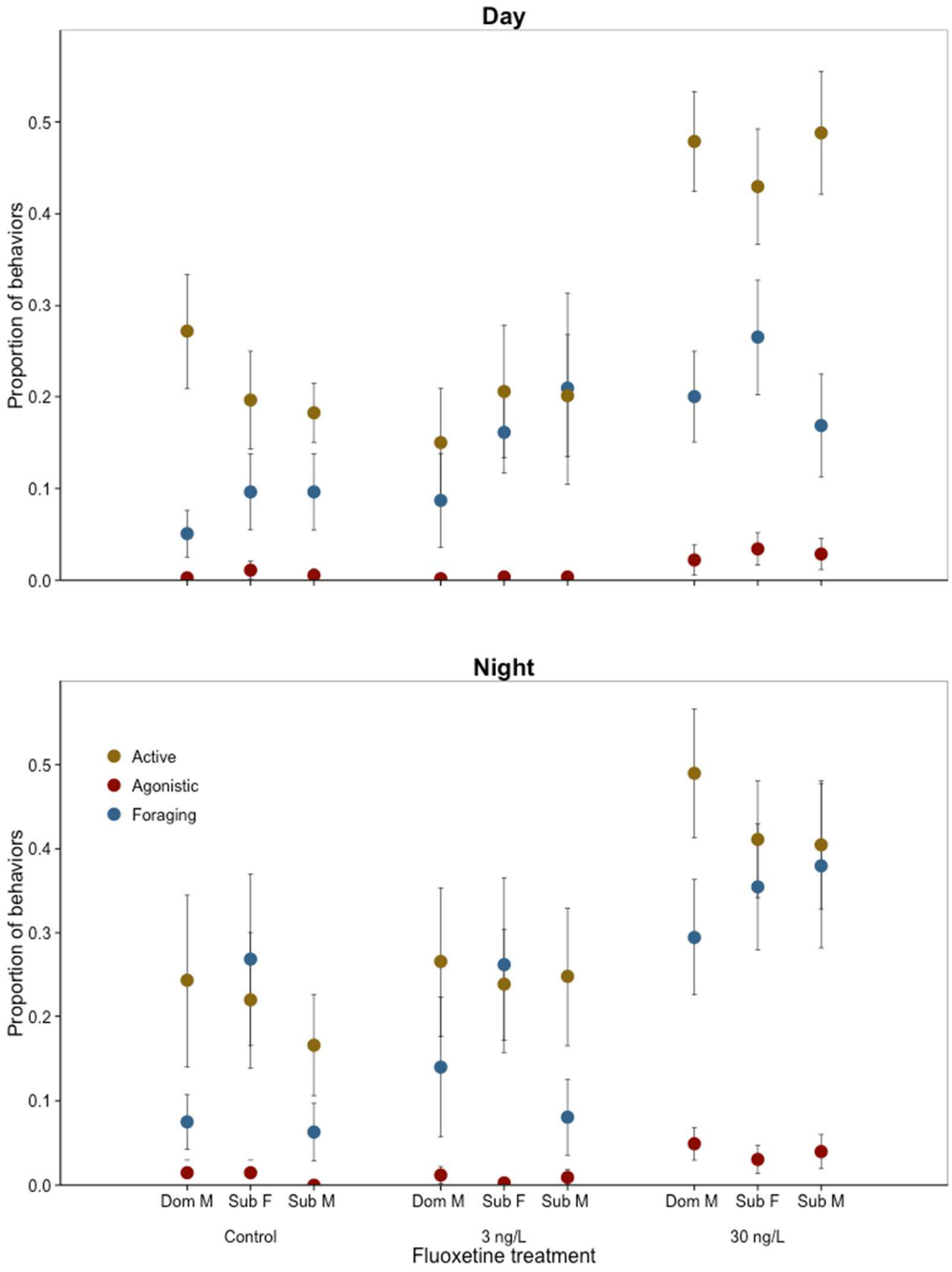

Figure. 3.2. Proportions of *H.oregonensis* day and night behaviors by gender, status, and fluoxetine treatment when no predators are present. Circles indicate proportions (yellow = active, red = agonistic, blue = foraging); genders (M/F); status (Dom=dominant, Sub=subordinate).

Foraging behaviors

Fluoxetine exposure affected foraging behaviors as well (χ^2 (2) = 13.77, *P* =0.001), although this effect was mediated by the gender and status of the crab (χ^2 (6) = 82.68, *P* <0.001). Dominant males and subordinate females in 30ng/L spent a greater proportion of time foraging during the day than their counterparts in control groups (*P*<0.01, in both cases), however subordinate males did not differ significantly (*P*=0.22). Fluoxetine had a strong effect on nocturnal foraging behaviors (χ^2 (2) = 8.21, *P* = 0.02). Both dominant and subordinate males exposed to 30 ng/L of fluoxetine significantly increased their foraging behaviors at night (P<0.001, P=0.001, respectively), however in females there was no treatment effect (P>0.5, in all cases).

Agonistic behaviors

Crab aggression varied across treatment combinations and time of day (χ^2 (8) = 18.63, *P* = 0.002). The proportion of aggressive acts among *H. oregonensis* was low across all treatments (range: 0.008-0.03), but crabs exposed to 30 ng/L fluoxetine were predicted to have the highest probability of aggressive behavior (0.03) compared to 0.3ng/L (0.01) and control crabs (0.008). Post-hoc Tukey contrasts indicated significant differences between controls and 30ng/L treatment groups (P < 0.001 in all cases). Controls and 0.3ng/L groups only differed when the predator was added (P= 0.007), where 0.3ng/L crabs exhibited slightly more aggressive behaviors (0.005) than the controls (0.0002, Figure 3.3). Nocturnal agonistic behaviors were higher than diurnal agonistic behaviors yet were also affected by fluoxetine (χ^2 (2) = 20.27, *P* <0.001).

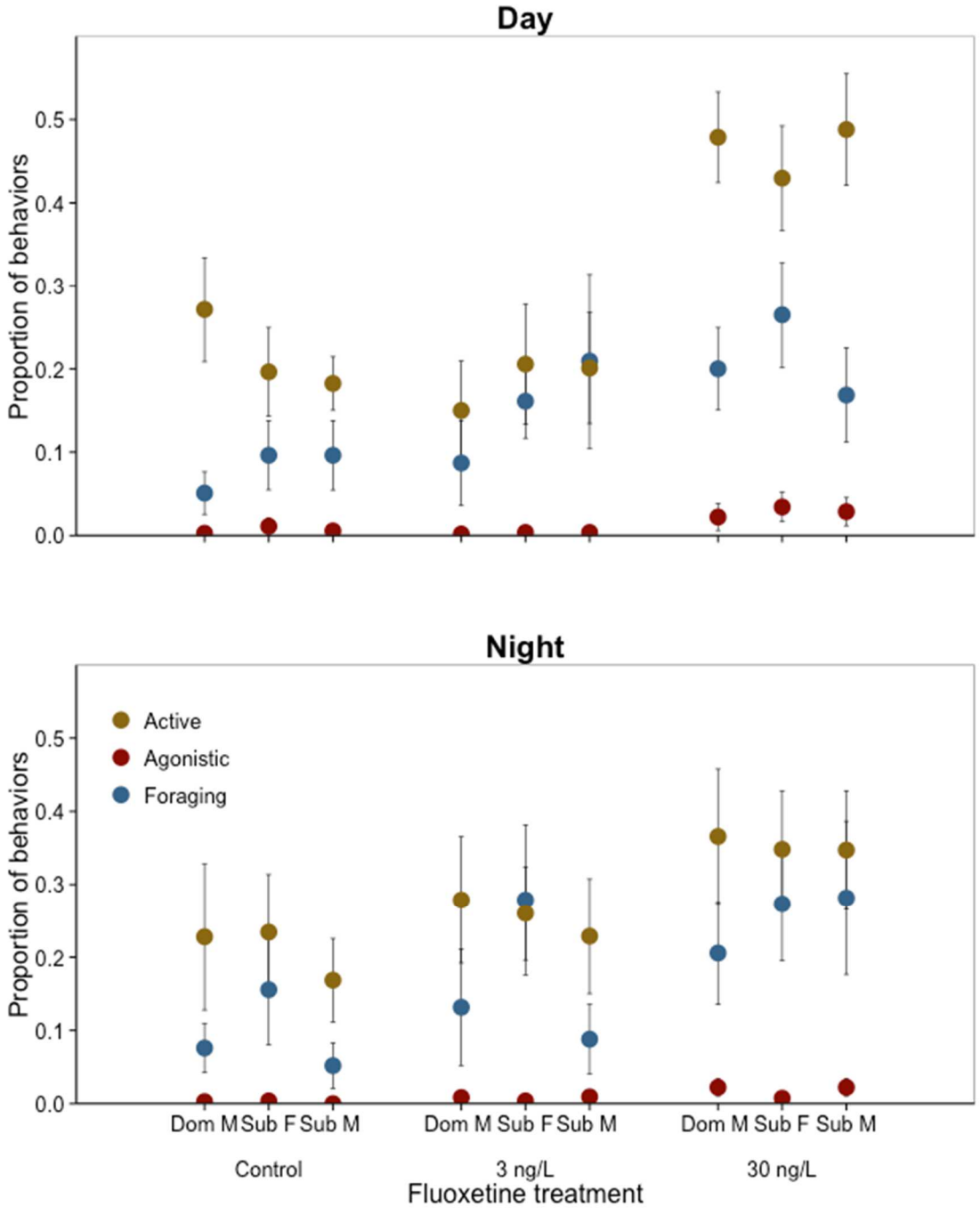

Figure. 3.3. Proportions of *H.oregonensis* day and night behaviors by gender, status, and fluoxetine treatment when predators are present. Circles indicate proportions (yellow = active, red = agonistic, blue = foraging); genders (M/F); status (Dom=dominant, Sub=subordinate).

Predator avoidance behaviors

Predator avoidance behavior also varied across treatments and time of day (χ^2 (8) = 220.17, $P < 0.0001$, Figure 3.3). Control crabs were most likely to avoid predators during the day (predicted probability of predator avoidance = 0.92) and at night (0.75) compared to crabs exposed to 3 ng/L (0.61 during the day; 0.57 at night), or 30 ng/L of fluoxetine (0.37 during the day; 0.40 at night). When predators were added during the day, crabs generally decreased active, foraging, and agonistic behaviors. However, crabs treated with 30 ng/L did not show a significant decrease in these behaviors when compared with 3 ng/L and control groups (χ^2 (2) = 43.78, $P < 0.001$, Figure 3.2). Fluoxetine exposure had a strong negative effect on predator avoidance behaviors despite crab gender or status (LRT, d.f. = 7, 9, $\chi^2 = 51.11$, $P < 0.0001$). However the predator avoidance behaviors of males were more affected by fluoxetine exposure than for females (See Figure 3.2. for a list of proportions of predator avoidance behaviors by gender and status). More crabs were captured and predated upon by *C. productus* in the 30 ng/L treatment group (n=8) than in the control and 3 ng/L groups (n=5 in each). Neither predator avoidance nor active behaviors varied over time (lm, $F_{1,21}$=2.5, $P = 0.23$; $F_{1,21}$=1.5, P=0.23, respectively).

Table. 3.1. Mean proportions of *H. oregonensis* predator avoidance behaviors (± SE) by fluoxetine treatment and time of day. Arranged by crab gender (♂/♀) and status (dominant = Dom/subordinate = Sub).

	Day	**Night**
Control		
Dom ♂	0.91 ± 0.01	0.69 ± 0.04
Sub ♂	0.89 ± 0.02	0.76 ± 0.02
Sub ♀	0.81 ± 0.02	0.61 ± 0.04
3.0 ng/L		
Dom ♂	0.57 ± 0.02	0.57 ± 0.04
Sub ♂	0.64 ± 0.03	0.67 ± 0.02
Sub ♀	0.57 ± 0.03	0.45 ± 0.04
30.0 ng/L		
Dom ♂	0.41 ± 0.03	0.41 ± 0.03
Sub ♂	0.42 ± 0.02	0.36 ± 0.03
Sub ♀	0.41 ± 0.02	0.37 ± 0.03

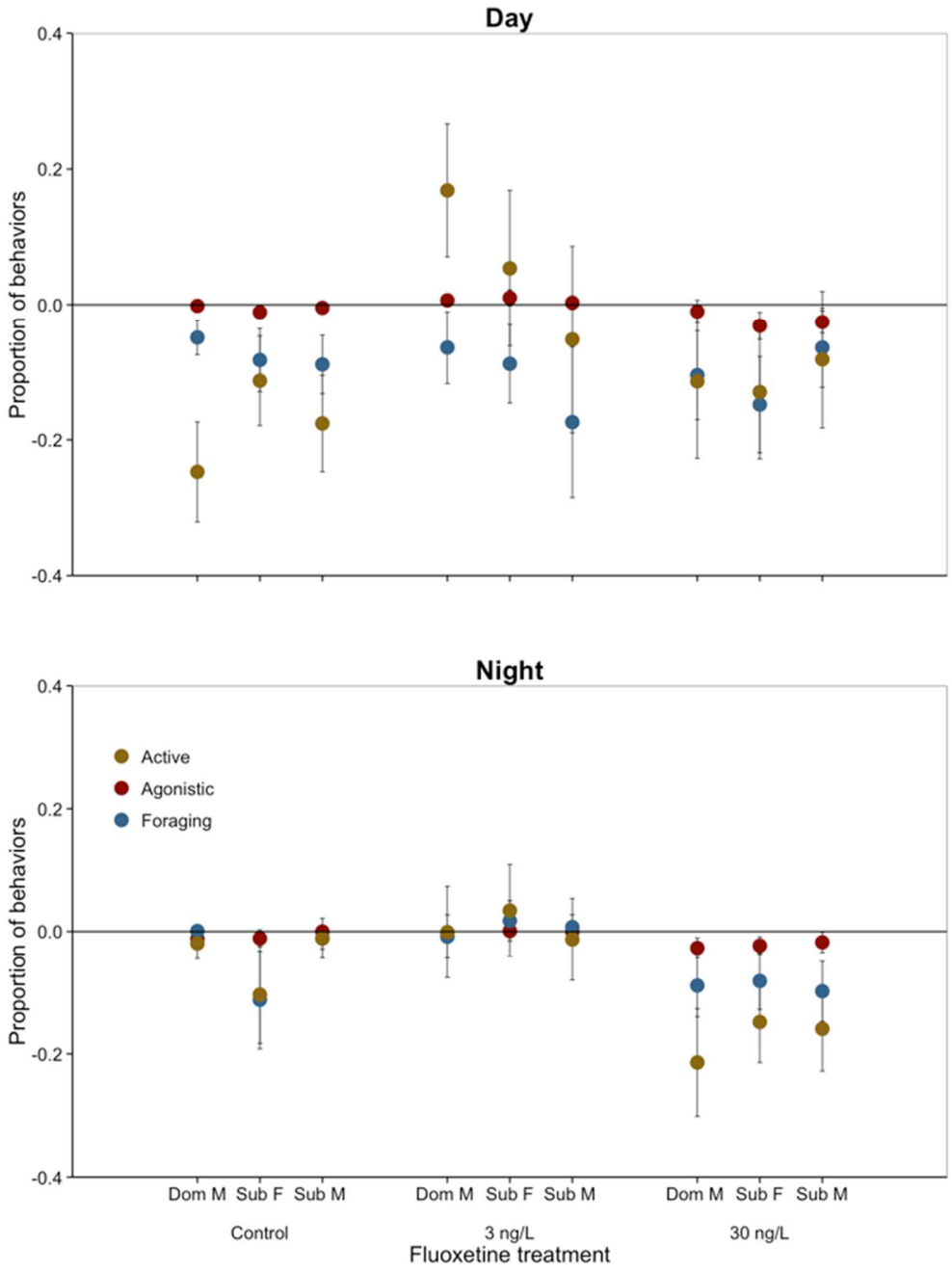

Figure. 3.4. Proportions of *H. oregonensis* day and night behaviors by gender, status, and fluoxetine treatment representing the difference between no predator and predator trials (i.e., the predator effect). Circles indicate proportions (yellow = active, red = agonistic, blue = foraging); genders (M/F); status (Dom=dominant, Sub=subordinate). Points above the line indicate scenarios in which a behavior was greater in the presence of a predator.

4. Discussion

In the presence of predators, prey will often modify their behaviors to balance the risk of mortality with the reward of accessing food, mates, or other resources (Weis 2010; Sih et al. 2012; Snell-Rood 2013; Catano et al. 2015). Prey may reduce their activity levels, utilize defenses, or seek refuge when they perceive the risk to be high (Lindberg 1980, Lima & Dill 1990; Preisser et al., 2007). We assessed whether the risk-taking behaviors of *H. oregonensis* would be altered under the influence of fluoxetine, a pharmaceutical contaminant commonly detected in estuaries and harbor waters (Kwon and Armburst 2006; Kreke and Dietrich 2008). Crabs exposed to the highest level of fluoxetine were more likely to be active and exhibit risk-taking behaviors in the presence of *C. productus*, resulting in a greater probability of predator capture and mortality. In fact, more crabs were captured by *C. productus* in the higher fluoxetine treatment than in the 3 ng/L or control treatments. Crabs in control groups exhibited a greater probability of predator avoidance behaviors because they reduced their activity levels and/or actively sought refuge when the predator was an immediate threat. Our results suggest that fluoxetine stimulates crab activity levels and reduces their inhibition to predator threats. For crabs inhabiting harbors or estuaries contaminated with fluoxetine, the changes to their normal behaviors may place them at greater risk of injury and mortality, with potential community-level effects.

I designed this experiment to simulate estuarine conditions in the laboratory, whereby *H. oregonensis* could reside in a similar habitat while exhibiting somewhat natural behaviors. I controlled between tank variation by maintaining identical abiotic conditions (e.g., light, temperature, salinity) and habitat substrate (e.g., rocks, gravel, and sand) across treatments. Therefore I propose that the differences in crab behavior reported here were attributable to fluoxetine rather than experimental artifacts. The 59 crabs that survived until the end of the trials (60 days) were likely overexposed to crab predators although we did not see a pattern of learned tolerance of predator presence. We believe any learned tolerance was minimal because 1) we allowed for sufficient time between predator trials (i.e., 80 hours); 2) we did not preclude *C. productus* from predating on *H. oregonensis* during the trials; and 3) predator induced mortality did not decline over time. Further, our observed proportions of crab active and predator avoidance behaviors did not change significantly over the length of the study, which we would expect if *H. oregonensis* learned to not perceive *C. productus* as a threat. Rather, the variability in *H. oregonensis* risk-taking behaviors remained fairly low across treatments during predator trials (see mean proportions by week in Figure 3.3).

Our predictive models were best fit by the interactions between treatment combinations and time of day, which suggests that crab behavior was mediated by photoperiod. Like other crabs, *H. oregonensis* are photonegative, increasing activity levels and foraging primarily at night. Assuming crabs in control groups serve as a reference, exhibiting the most 'typical' behaviors, we would expect higher activity

amongst all crabs during night trials. However, crabs exposed to 30 ng/L-spiked water exhibited twice as much active behavior at night as control groups, suggesting a strong effect of fluoxetine. Interestingly, there was little difference between diurnal and nocturnal activity levels in crabs exposed to 3ng/L of fluoxetine. Perhaps photoperiod was not as important for regulating activity in this treatment group, since predator avoidance behaviors were also low. From our observations, crabs in this group appeared to be the least affected by the addition of the crab predator, as evidenced by the lack of behavioral alterations between trial type (Figures 3.2. and 3.3).

Serotonin and serotonin analogs have been shown to alter agonistic behaviors (McPhee & Wilkens 1989; Tierney & Mangiamele 2001) and activity levels (Perez-Campos et al. 2012; Fong & Ford 2014) in crustaceans. Fluoxetine in concentrations equal or greater than 120µg L^{-1} caused a stimulation of locomotor behavior in the crab *Carcinus maenas* (Mesquita et al. 2011). We found similar increases in agonistic behaviors of crabs exposed to 30ng/L of fluoxetine, but with much lower exposure concentrations than in Mesquita et al. (2011). In *Chasmagnathus* crabs, Pedetta et al. (2008) modulated the individual aggressiveness via manipulation of serotonin and octopamine levels, where aggressiveness increased and decreased with the addition of the respective hormone. Perhaps fluoxetine, through modulation of serotonin levels, stimulates crab activity levels and drives the observed aggressive behaviors. Further, our results demonstrate that fluoxetine may inhibit predator avoidance behaviors. The drug's effect on serotonin levels appears to increase boldness and potentially other risk taking

behavior as studies on other species have suggested (Tierney & Mangiamele 2001; Pedetta et al. 2010; Mesquita et al. 2011; Dzieweczynski & Herbert 2012; Fong & Ford 2014).

Fluoxetine is one of the most widely used antidepressants in the world (Metcalfe et al. 2010). A robust amount of research has documented its occurrence in aquatic (Kwon and Armburst 2006; Ramirez et al. 2009; Bringolf et al. 2010; Corcoran et al. 2010) and marine (Kreke and Dietrich 2008; Vasskog et al. 2008) environments. With growing human populations in coastal zones, increasing use of antidepressants like fluoxetine is expected, suggesting higher future concentrations in the marine environment. Our results demonstrate how pharmaceuticals and other emerging contaminants may affect species behaviors and their interactions. Brodin et al. (2014) summarized several ecologically important behavioral traits for assessing sublethal effects of pharmaceutical exposure, and potential direct or indirect ecological effects. These behavioral traits include: activity, aggression, boldness, exploration, and sociality. Each of these behavioral traits lead to direct ecological effects such as cooperation, dispersal/migration, feeding rates, mating success, parental care, and predator avoidance. These direct effects can be linked to differences in community structure, cross-boundary effects, ecosystem function, feedback loops, population dynamics, and trophic cascades. Anthropogenic impacts to coastal systems such as ocean acidification, warming surface water temperatures, and pollution have all been identified as significant environmental stressors, altering much of the aforementioned ecosystem processes (Munday et al. 2009;

Fukunaga et al. 2010; Dodd et al. 2015). Since pharmaceuticals have been shown to affect many of the same processes through similar mechanisms, they warrant consideration as an important environmental stressor in need of further research.

Estuarine and coastal organisms are exposed to whole suites of contaminants, many of which (e.g., sertraline (Effexor®; Bossus et al. 2013), carbamazepine (Tegretol®; Martin-Diaz et al. 2009)) have negative effects on aquatic and marine organisms (e.g., Metcalfe et al. 2010; Meredith-Williams 2012; Gaw et al. 2014; Fong & Ford 2014). Our study and others have assessed the effects of single pharmaceuticals on animal behavior and their potential to alter species interactions (Gaworecki and Klaine 2008; Bossus 2013; Hazelton et al. 2013). Yet, additional studies examining the effects of multiple compounds are warranted to understand interactive and cumulative effects on organisms and ecosystems (Brausch et al. 2012; Brodin et al. 2014). Further, studies that assess how pharmaceuticals interact with lower pH (i.e., ocean acidification conditions) would add to the growing field of multiple stressor research. To our knowledge no studies have assessed community or ecosystem responses to pharmaceuticals or other emerging contaminants, an important step in understanding how these compounds may influence important inter- and intra-specific interactions. Finally, it would be advantageous for both ecology and ecotoxociology to merge components of pharmaceutical contaminants research, as both disciplines use similar species and examine similar endpoints while addressing separate questions. If we are to truly

understand how pharmaceuticals may act as stressors to marine ecosystems, we need to learn from the collective work in this emerging field.

Chapter 4: Conclusions

This case study of fluoxetine builds on previous studies documenting fluoxetine's effects on aquatic organisms by identifying the chronic exposure effects (47-107 days) on organism health, behavior, and functioning. The results from the two different experiments indicate that fluoxetine is a considerable environmental stressor, even at the low concentrations detected in the marine environment. Specifically, nominal concentrations of fluoxetine significantly affect both mussel and crab physiology and behavior, which may negatively affect individual fitness and species interactions. While both studies involve only one or two species, the implications of the results suggest that fluoxetine exposure could affect community- or ecosystem-level processes. By simulating chronic fluoxetine exposure in the laboratory we offer a snapshot of how this single contaminant may serve as an environmental stressor to invertebrates along rocky intertidal and estuarine shorelines in the wild.

Fluoxetine is one of the most widely prescribed antidepressants in the world and a significant amount of research has documented its occurrence and negative effects on organisms in aquatic and marine environments. With growing human populations in coastal zones, increasing use of antidepressants, like fluoxetine, is expected, suggesting higher future concentrations in the marine environment. We have demonstrated that fluoxetine reduces *M. californianus* algal clearance rates, growth, and reproductive potential at very low concentrations. We also found that similar low concentrations

increased predation susceptibility by stimulating activity levels and risk-taking behaviors in *H. oregonensis*. For mussels and crabs inhabiting harbors or estuaries contaminated with fluoxetine, the changes to their normal behaviors and functioning may yield community-level consequences.

The results of this study serve as a foundation to understand how pharmaceuticals may act as emerging environmental stressors, affecting marine species and their interactions. While we found fluoxetine to be a considerable stressor to marine mussels and crabs, it is only one of many stressors on marine organisms. Other studies have assessed how individual pharmaceuticals may affect animal behavior and health, and their potential to alter species interactions. These collectively fill important data gaps with respect to emerging contaminant research. However, long-term studies examining the effects of multiple stressors, such as multiple pharmaceuticals or the combination of pharmaceuticals with ocean acidification or other pollutants, are warranted to understand interactive and cumulative organism and potential ecosystem level effects.

Finally, ecology and ecotoxicology, the two primary disciplines that assess impacts from pharmaceutical as environmental stressors, need to integrate their research. The advantage of combining the findings from these two research fields is evident, as pharmaceuticals in the environment often modify important ecosystem processes. This project attempts to bridge the two fields by providing data from a hybridized methodology that combined standardized ecotoxicology testing with ecological questioning.

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Appendix A. Table of mussel metrics organized by mean ± SE and fluoxetine treatment group. Total biomass was the aggregate wet biomass of mussels per tank (n = number of mussels).

Fluoxetine Treatment	Initial Length (mm)	Initial Mass (g)	Total Biomass (g) *n =21*	Total Biomass (g) *n = 15*	Total Biomass (g) *n = 9*
Control	32.67 ± 0.31	4.39 ± 0.14	90.33 ± 1.26	63.11 ± 1.33	34.02 ± 1.30
0.3 ng/L	32.55 ± 0.44	4.18 ± 0.11	86.95 ± 0.71	59.61 ± 1.29	35.36 ± 0.63
3 ng/L	31.78 ± 0.32	4.02 ± 0.14	84.15 ± 1.14	59.95 ± 1.30	35.28 ± 1.64
30 ng/L	31.58 ± 0.36	4.03 ± 0.12	84.12 ± 1.66	56.38 ± 0.51	33.24 ± 0.98
300 ng/L	32.53 ± 0.32	4.33 ± 0.14	90.15 ± 1.06	63.32 ± 1.00	36.46 ± 1.30

Appendix B. Mussel growth (length) multiple comparison tests by treatment group and sample date. P-values adjusted by Bonferroni correction.

	Pairwise comparisons	Estimate	Standard Errror	Z - value	P (> | Z |)
Growth (length)	Control_67 - 300_67 == 0	1.181	0.252	4.697	**<0.01**
	Control_67 - 30_67 == 0	0.684	0.252	2.720	0.290
	Control_67 - 3_67 == 0	0.257	0.252	1.022	1.000
	Control_67 - 0.3_67 == 0	0.166	0.252	0.660	1.000
	Control_47 - 300_47 == 0	1.303	0.252	5.181	**<0.01**
	Control_47 - 30_47 == 0	1.181	0.252	4.695	**<0.01**
	Control_47 - 3_47 == 0	0.299	0.252	1.190	0.997
	Control_47 - 0.3_47 == 0	0.442	0.252	1.757	0.913
	Control_107 - 300_107 == 0	1.493	0.252	5.938	**<0.01**
	Control_107 - 30_107 == 0	1.166	0.252	4.635	**<0.01**
	Control_107 - 3_107 == 0	0.289	0.252	1.149	0.998
	Control_107 - 0.3_107 == 0	0.234	0.252	0.932	1.000
	300_67 - 30_67 == 0	-0.497	0.252	-1.977	0.810
	300_67 - 3_67 == 0	-0.924	0.252	-3.674	**0.019**
	300_67 - 0.3_67 == 0	-1.015	0.252	-4.037	**<0.01**
	300_47 - 30_47 == 0	-0.122	0.252	-0.486	1
	300_47 - 3_47 == 0	-1.004	0.252	-3.991	**<0.01**
	300_47 - 0.3_47 == 0	-0.861	0.252	-3.424	**0.045**
	300_107 - 30_107 == 0	-0.328	0.252	-1.303	0.994
	300_107 - 3_107 == 0	-1.204	0.252	-4.788	**<0.01**
	300_107 - 0.3_107 == 0	-1.259	0.252	-5.006	**<0.01**
	30_67 - 3_67 == 0	-0.427	0.252	-1.698	0.933
	30_67 - 0.3_67 == 0	-0.518	0.252	-2.060	0.759
	30_47 - 3_47 == 0	-0.881	0.252	-3.504	**0.034**
	30_47 - 0.3_47 == 0	-0.739	0.252	-2.938	0.176
	30_107 - 3_107 == 0	-0.877	0.252	-3.486	**0.037**
	30_107 - 0.3_107 == 0	-0.931	0.252	-3.703	**0.017**
	3_67 - 0.3_67 == 0	-0.091	0.252	-0.362	1.000
	3_47 - 0.3_47 == 0	0.142	0.252	0.566	1.000
	3_107 - 0.3_107 == 0	-0.055	0.252	-0.218	1.000

Appendix C. Mussel growth (mass) multiple comparison tests by treatment group and sample date. P-values adjusted by Bonferroni correction.

	Pairwise comparisons	Estimate	Standard Errror	Z - value	P (> | Z |)
Growth (mass)	Control_67 - 300_67 == 0	1.432	0.292	4.908	**<0.01**
	Control_67 - 30_67 == 0	1.340	0.292	4.591	**<0.01**
	Control_67 - 3_67 == 0	0.870	0.292	2.983	0.160
	Control_67 - 0.3_67 == 0	0.957	0.292	3.279	0.071
	Control_47 - 300_47 == 0	1.428	0.292	4.893	**<0.01**
	Control_47 - 30_47 == 0	1.035	0.292	3.547	**0.031**
	Control_47 - 3_47 == 0	1.442	0.292	4.941	**<0.01**
	Control_47 - 0.3_47 == 0	1.299	0.292	4.452	**<0.01**
	Control_107 - 300_107 == 0	1.741	0.292	5.968	**<0.01**
	Control_107 - 30_107 == 0	1.589	0.292	5.444	**<0.01**
	Control_107 - 3_107 == 0	0.891	0.292	3.054	0.133
	Control_107 - 0.3_107 == 0	1.202	0.292	4.119	<0.01
	300_67 - 30_67 == 0	-0.092	0.292	-0.317	1.000
	300_67 - 3_67 == 0	-0.562	0.292	-1.925	0.841
	300_67 - 0.3_67 == 0	-0.475	0.292	-1.629	0.953
	300_47 - 30_47 == 0	-0.393	0.292	-1.346	0.992
	300_47 - 3_47 == 0	0.014	0.292	0.047	1.000
	300_47 - 0.3_47 == 0	-0.129	0.292	-0.441	1.000
	300_107 - 30_107 == 0	-0.153	0.292	-0.524	1.000
	300_107 - 3_107 == 0	-0.850	0.292	-2.914	0.191
	300_107 - 0.3_107 == 0	-0.539	0.292	-1.849	0.878
	30_67 - 3_67 == 0	-0.469	0.292	-1.609	0.957
	30_67 - 0.3_67 == 0	-0.383	0.292	-1.313	0.993
	30_47 - 3_47 == 0	0.407	0.292	1.394	0.988
	30_47 - 0.3_47 == 0	0.264	0.292	0.905	1.000
	30_107 - 3_107 == 0	-0.698	0.292	-2.391	0.522
	30_107 - 0.3_107 == 0	-0.387	0.292	-1.325	0.993
	3_67 - 0.3_67 == 0	0.086	0.292	0.296	1.000
	3_47 - 0.3_47 == 0	-0.143	0.292	-0.489	1.000
	3_107 - 0.3_107 == 0	0.311	0.292	1.066	0.999

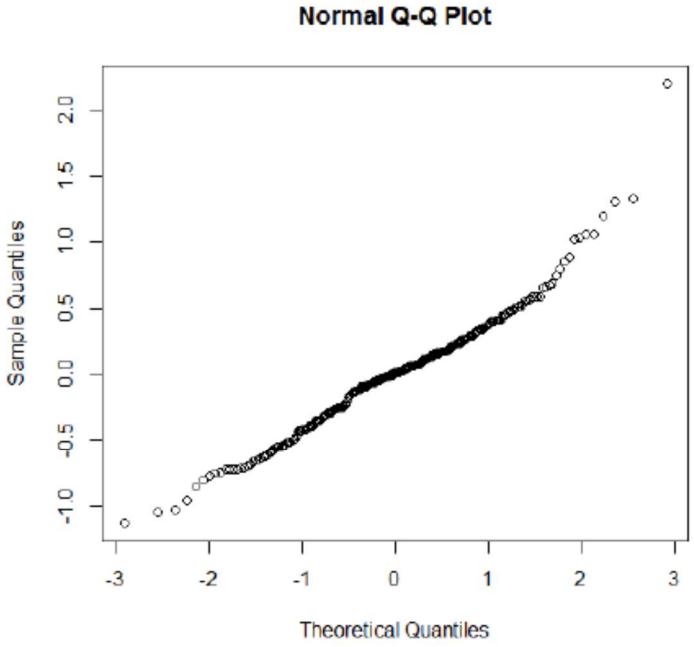
Appendix D. Mussel gonadosomatic index (GSI) multiple comparison tests by treatment group and sample date. P-values adjusted by Bonferroni correction.

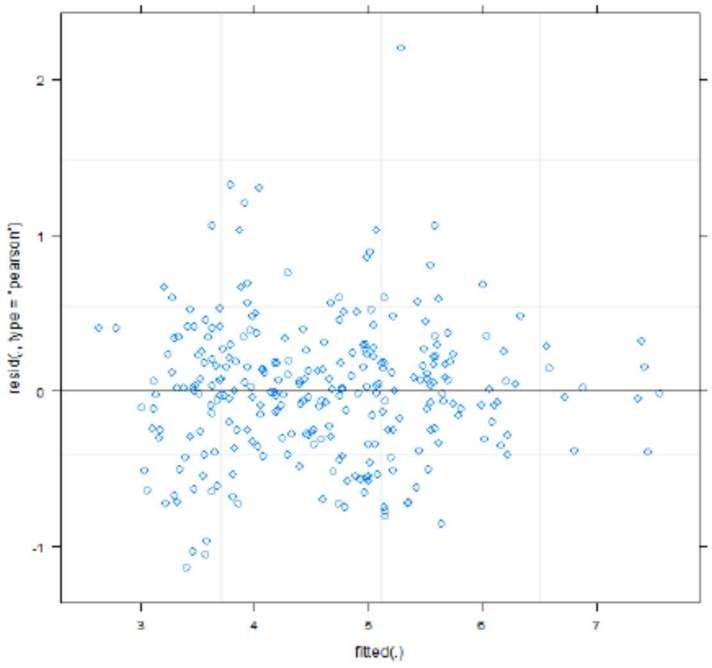
	Pairwise comparisons	Estimate	Standard Errror	Z - value	P (> | Z |)
Gonadosomatic Index (GSI)	Control_67 - 300_67 == 0	1.432	0.292	4.908	**<0.01**
	Control_67 - 30_67 == 0	1.340	0.292	4.591	**<0.01**
	Control_67 - 3_67 == 0	0.870	0.292	2.983	0.160
	Control_67 - 0.3_67 == 0	0.957	0.292	3.279	0.071
	Control_47 - 300_47 == 0	1.428	0.292	4.893	**<0.01**
	Control_47 - 30_47 == 0	1.035	0.292	3.547	**0.031**
	Control_47 - 3_47 == 0	1.442	0.292	4.941	**<0.01**
	Control_47 - 0.3_47 == 0	1.299	0.292	4.452	**<0.01**
	Control_107 - 300_107 == 0	1.741	0.292	5.968	**<0.01**
	Control_107 - 30_107 == 0	1.589	0.292	5.444	**<0.01**
	Control_107 - 3_107 == 0	0.891	0.292	3.054	0.133
	Control_107 - 0.3_107 == 0	1.202	0.292	4.119	**<0.01**
	300_67 - 30_67 == 0	-0.092	0.292	-0.317	1.000
	300_67 - 3_67 == 0	-0.562	0.292	-1.925	0.841
	300_67 - 0.3_67 == 0	-0.475	0.292	-1.629	0.953
	300_47 - 30_47 == 0	-0.393	0.292	-1.346	0.992
	300_47 - 3_47 == 0	0.014	0.292	0.047	1.000
	300_47 - 0.3_47 == 0	-0.129	0.292	-0.441	1.000
	300_107 - 30_107 == 0	-0.153	0.292	-0.524	1.000
	300_107 - 3_107 == 0	-0.850	0.292	-2.914	0.191
	300_107 - 0.3_107 == 0	-0.539	0.292	-1.849	0.878
	30_67 - 3_67 == 0	-0.469	0.292	-1.609	0.957
	30_67 - 0.3_67 == 0	-0.383	0.292	-1.313	0.993
	30_47 - 3_47 == 0	0.407	0.292	1.394	0.988
	30_47 - 0.3_47 == 0	0.264	0.292	0.905	1.000
	30_107 - 3_107 == 0	-0.698	0.292	-2.391	0.522
	30_107 - 0.3_107 == 0	-0.387	0.292	-1.325	0.993
	3_67 - 0.3_67 == 0	0.086	0.292	0.296	1.000
	3_47 - 0.3_47 == 0	-0.143	0.292	-0.489	1.000
	3_107 - 0.3_107 == 0	0.311	0.292	1.066	0.999

Appendix E. Mussel condition index (CI) multiple comparison tests by treatment group and sample date. P-values adjusted by Bonferroni correction.

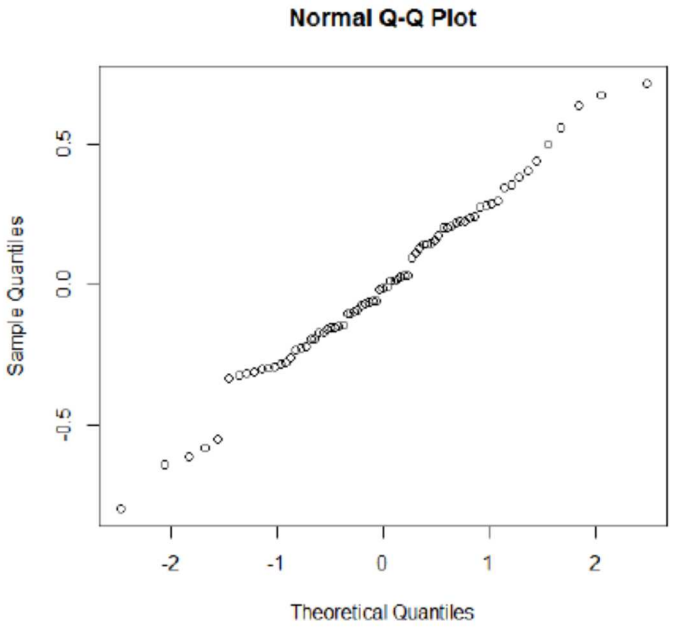
	Pairwise comparisons	Estimate	Standard Errror	Z - value	P (> | Z |)
Condition Index	Control_67 - 300_67 == 0	0.723	0.256	2.821	0.236
	Control_67 - 30_67 == 0	0.473	0.256	1.844	0.880
	Control_67 - 3_67 == 0	0.544	0.256	2.125	0.718
	Control_67 - 0.3_67 == 0	0.633	0.256	2.471	0.460
	Control_47 - 300_47 == 0	0.396	0.256	1.546	0.969
	Control_47 - 30_47 == 0	0.497	0.256	1.941	0.832
	Control_47 - 3_47 == 0	0.556	0.256	2.169	0.686
	Control_47 - 0.3_47 == 0	0.438	0.256	1.708	0.931
	Control_107 - 300_107 == 0	0.782	0.256	3.054	0.133
	Control_107 - 30_107 == 0	0.824	0.256	3.217	0.084
	Control_107 - 3_107 == 0	0.491	0.256	1.917	0.844
	Control_107 - 0.3_107 == 0	1.118	0.256	4.364	**<0.01**
	300_67 - 30_67 == 0	-0.250	0.256	-0.977	1.000
	300_67 - 3_67 == 0	-0.178	0.256	-0.696	1.000
	300_67 - 0.3_67 == 0	-0.090	0.256	-0.349	1.000
	300_47 - 30_47 == 0	0.101	0.256	0.395	1.000
	300_47 - 3_47 == 0	0.160	0.256	0.623	1.000
	300_47 - 0.3_47 == 0	0.042	0.256	0.162	1.000
	300_107 - 30_107 == 0	0.042	0.256	0.163	1.000
	300_107 - 3_107 == 0	-0.291	0.256	-1.137	0.999
	300_107 - 0.3_107 == 0	0.336	0.256	1.31	0.994
	30_67 - 3_67 == 0	0.072	0.256	0.28	1.000
	30_67 - 0.3_67 == 0	0.161	0.256	0.627	1.000
	30_47 - 3_47 == 0	0.059	0.256	0.229	1.000
	30_47 - 0.3_47 == 0	-0.060	0.256	-0.232	1.000
	30_107 - 3_107 == 0	-0.333	0.256	-1.3	0.994
	30_107 - 0.3_107 == 0	0.294	0.256	1.147	0.998
	3_67 - 0.3_67 == 0	0.089	0.256	0.347	1.000
	3_47 - 0.3_47 == 0	-0.118	0.256	-0.461	1.000
	3_107 - 0.3_107 == 0	0.627	0.256	2.447	0.479

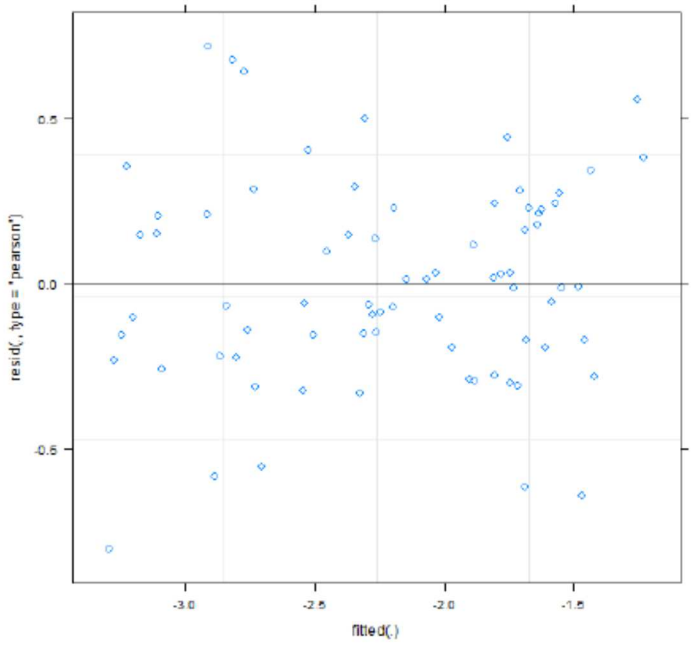
Appendix F. Plots of residuals from clearance rates model (e.g. mussel experiments). Model fit a normal distribution after data underwent Box-Cox transformation.



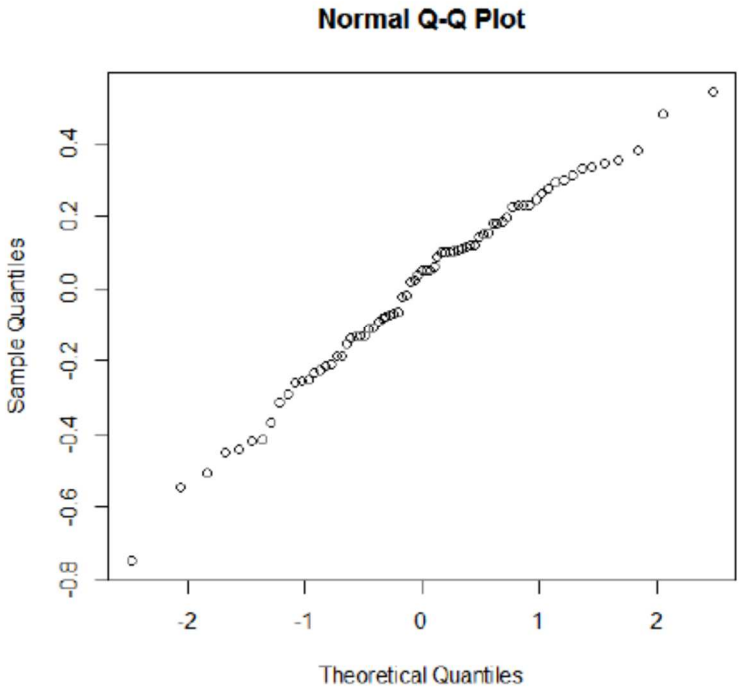


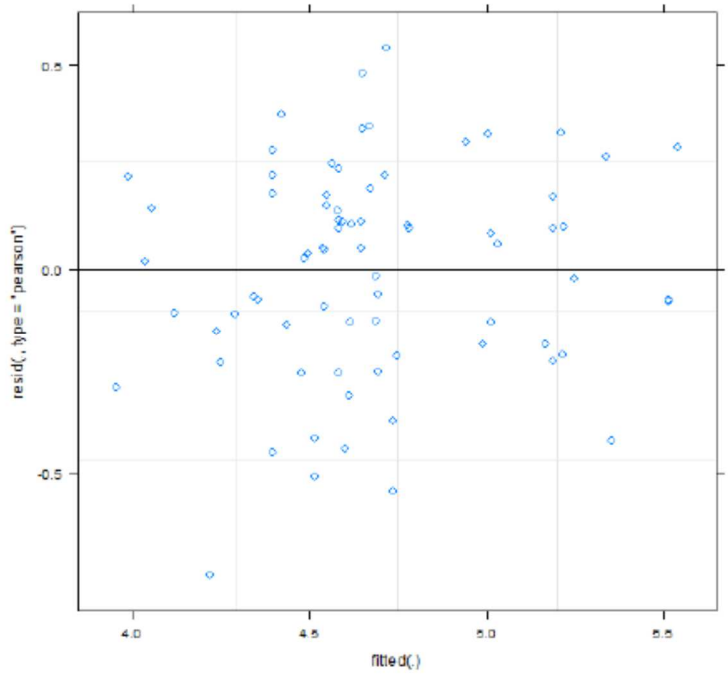
Appendix G. Plots of residuals from mussel growth (length) model. Model fit a normal distribution after data was log-transformed.



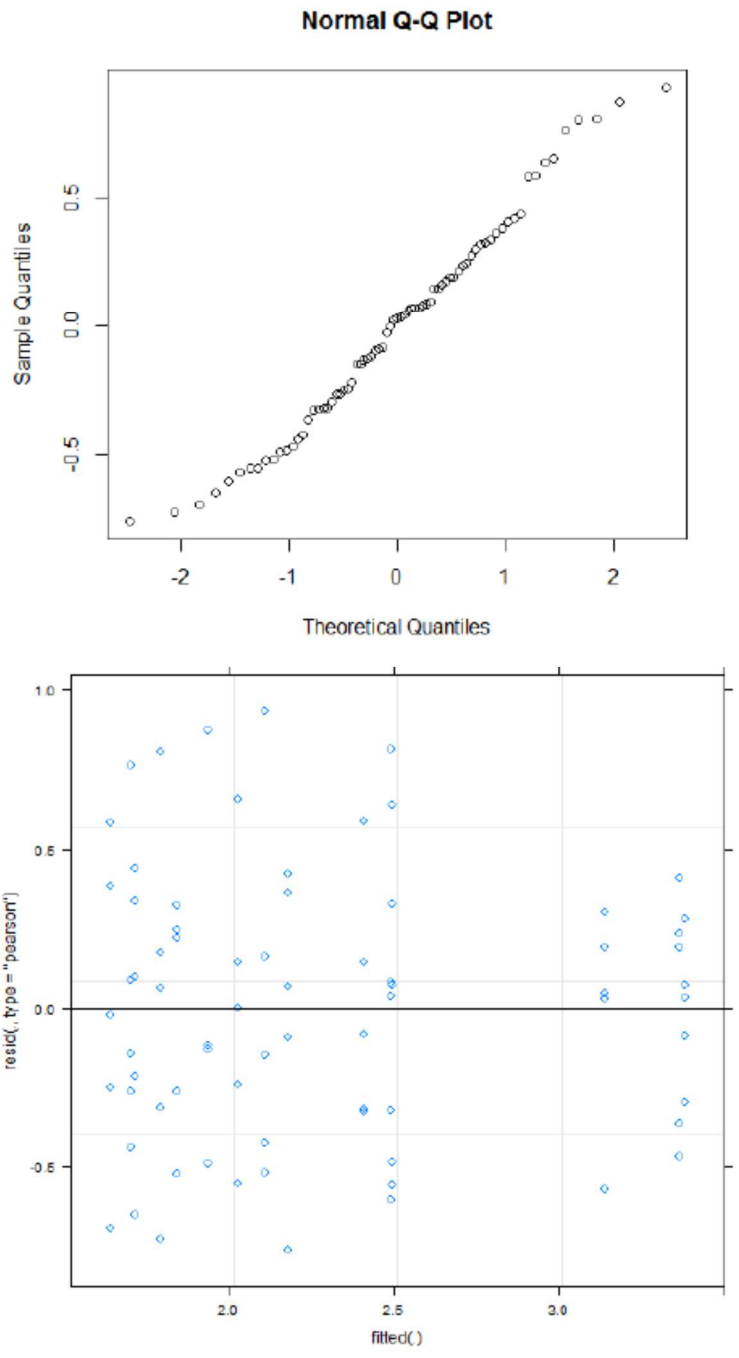


Appendix H. Plots of residuals from mussel growth (mass) model. Model fit a normal distribution after data was log-transformed.

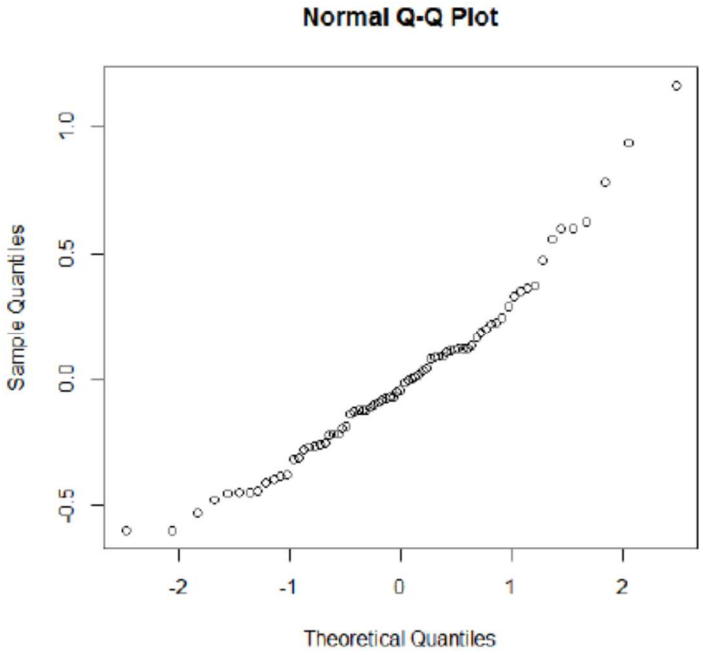


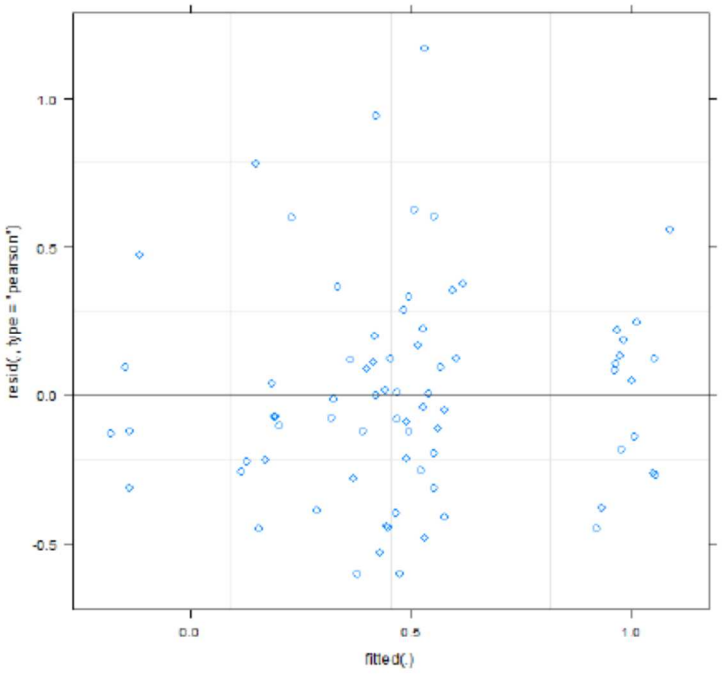


Appendix I. Plots of residuals from mussel gonadosomatic index model. Model fit a normal distribution after data was log-transformed.

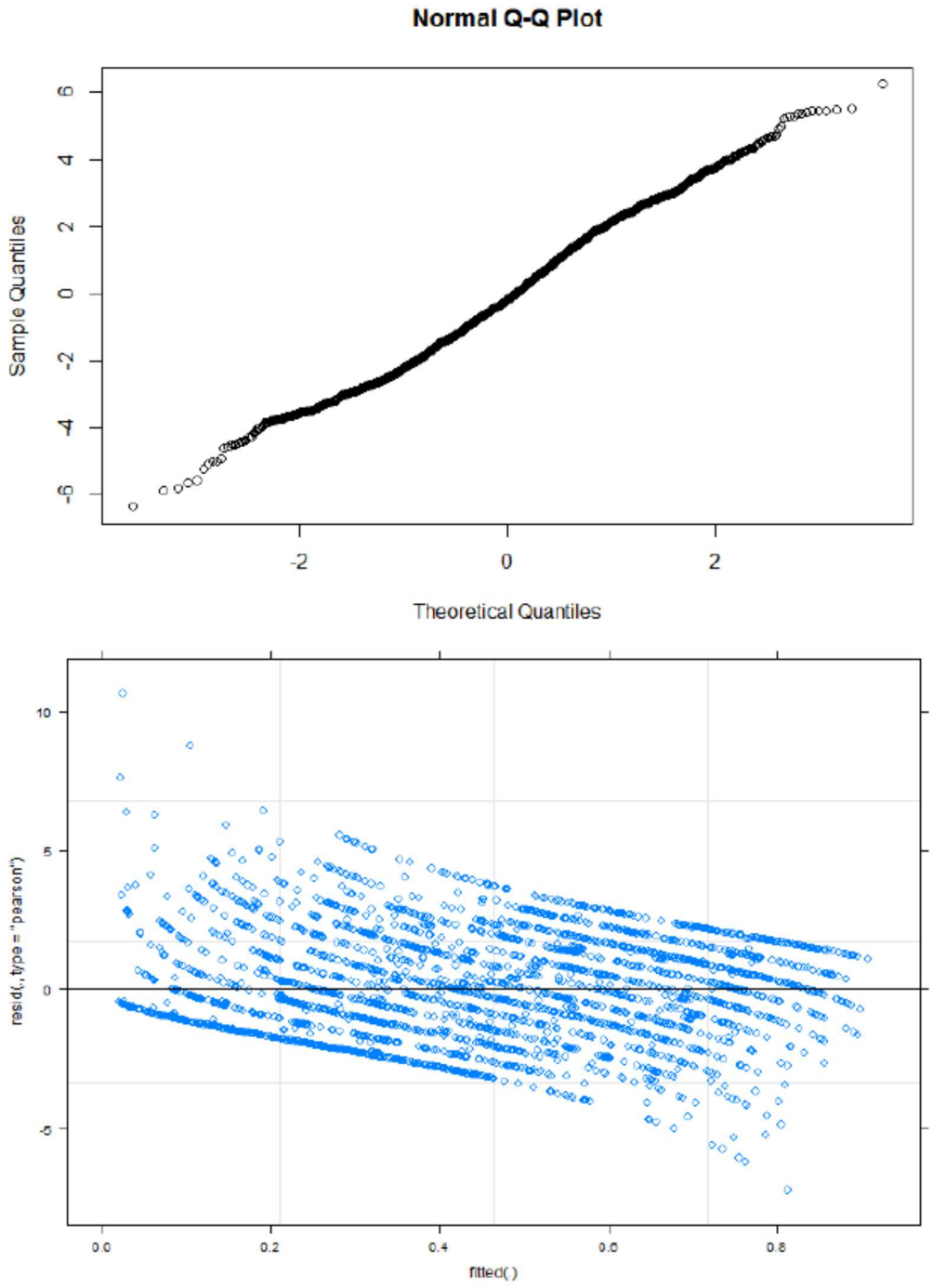


Appendix J. Plots of residuals from mussel condition index model. Model fit a normal distribution after data was log-transformed.

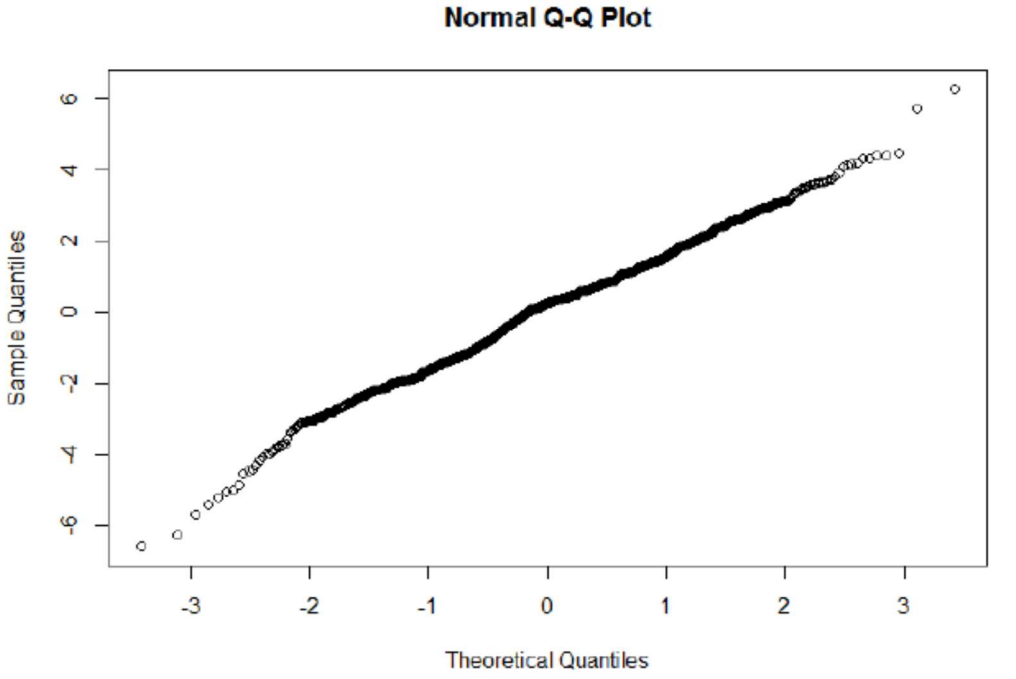


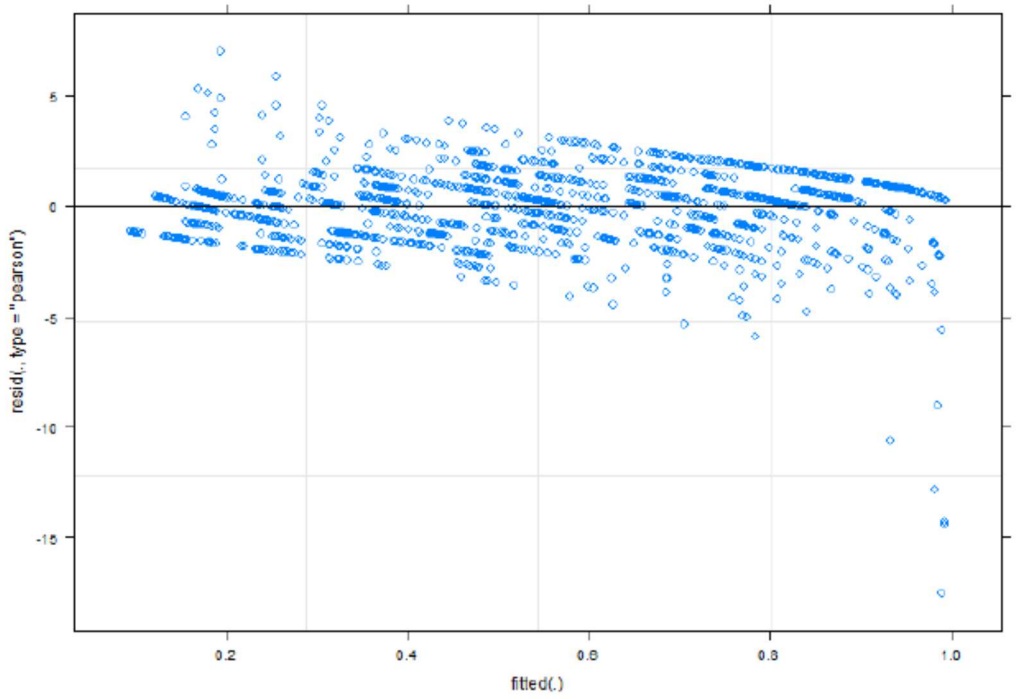


Appendix K. Plots of residuals from crab active behaviors model. Model fit a binomial error distribution.



Appendix L. Plots of residuals from crab predator avoidance behaviors model. Model fit a binomial error distribution.





Appendix M. A record of modification to the initial study proposal

This section provides a detailed record of all modifications made to the study design and methodology in the initial study proposal. A copy of the proposal is provided here for reference. Wherever a modification was made to the study design an endnote was added to provide explanation of the reason for the modification and any other relevant details.

Project Title: *Multiple stressor effects of pharmaceuticals on Oregon's rocky intertidal communities: A case study of fluoxetine and carbamazepine*

Methods

-Laboratory Experiments-

1.Animal Collection, Housing and Husbandry

1.1. Collection and Aquaria conditions

I will collect 525, 2-3 cm, *Mytilus californianus* mussels from the mussel bed at Boiler Bay, Oregon[1]. Collected animals will be housed in 60-liter tanks in Portland State University. Each tank is attached to its own filtration, a biobag filter will be used to filter mussel waste products from the aquaria. Aquarium conditions (e.g., salinity, temperature, light cycle) will reflect in situ conditions at time of mussel collection. Specifically, water salinity will be kept between 32 and 35 ppt, using artificial seawater from Instant Ocean salts. The light cycle will reflect summer daylight hours (14 hours light, 10 hours dark).

Water temperature will be kept between 12-15 °C (55-60°F) and will be regulated using non-toxic glycol-based chillers.

Upon arrival at PSU, mussels will be weighed and measured and will then be divided into three weight classes. From each weight class, 7 mussels will be selected at random and placed into housing tanks for a total of 21 mussels per tank (21mussels/tank X 25 treatment tanks with mussels = 525 mussels total). Mussels will be labeled using different colored acrylic nail polish[2]. To reduce stress on the animals from handling, mussels will be placed onto watch glasses for a total of 7 mussels per watch glass. Mussels will be allowed an acclimation period of 7 days to reattach byssal threads to the watch glass[3]. After 7 days, the mass of each watch glass group will then be measured collectively, and then repeatedly throughout the study, however individual lengths and widths will be measured separately using a small ruler[4].

1.2. Feeding

Mussels will be fed using Shellfish Diet 1800 from Reed Mariculture at amounts per mussel following Rodriguez del Rey et al. (2011). Each mussel will be fed 0.41 mL of shellfish diet at each feeding event, every 5 days. 8.61 mL of shellfish diet (0.41mL X 21 mussels/tank)[5] will be added to each housing tank using a calibrated syringe. To measure feeding rates, water samples will be taken 1 minute after adding the shellfish diet and approximately one hour after, and samples will be collected again. To measure

differences in chlorophyll a concentration, samples will be analyzed using spectrophotometry[6].

1.3. Water changes

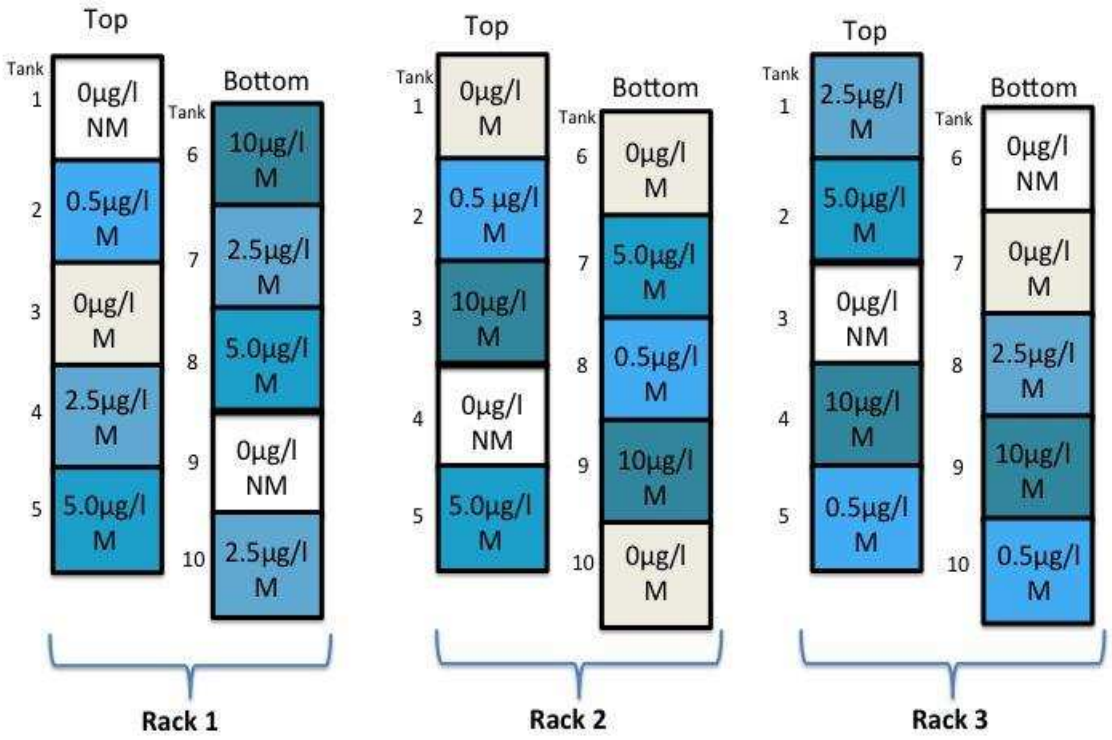
Every 30 days a 75% partial water change will be performed. The biofilter bags will filter the nitrogenous wastes from mussels, however it is important to replace the majority of the water each month. This will be done along the same timeline as the 10-day dosing for each treatment, including adding 0.01% ethanol (EtOH)[7] to the controls tanks with and without mussels. The 0.01% EtOH will be added to account for the fluoxetine treatment reagents, which use the ethanol. Tanks will not be allowed to completely dry because of the risk of damaging bacteria colonies on the biobag filters.

2. Exposure to fluoxetine

2.1. 90-day[8] exposure study design

Mussels will be exposed to one of four fluoxetine levels (0.5, 2.0, 5.0, 10.0µg/l)[9] following environmentally relevant concentrations determined by Choong et al. (2006). Two control treatments, with and without mussels, with no fluoxetine (0µg/l) but with 0.01% EtOH will be used to determine if there is an effect of fluoxetine treatment. Using a block design, there will be 30 tanks with a random assortment of four treatment and two control types with a total n=5 per treatment (Figure 1). Fluoxetine will be added to

treatment tanks on day 1, and then added every 10 days to mimic pulse events of contaminant delivery.



Fluoxetine exposure levels (0, 0.5, 2.5, 5.0, 10 µg/l)
M= Mussels
NM= No Mussels

2.1.1 Measurements

a. Growth rates

Each mussel will be identified using rack, tank, watch glass number, and nail polish color (blue, red, green, purple, yellow, orange, pink) (e.g., 321B = rack 3, tank 2, watch glass 1, blue). Every 10 days mussels[10] will be measured for group wet-weight (per

watchglass) and individual length and width. Shell thickness of individuals will be measured by notching their shells at the beginning of the study. Accretion rate[11] will be based on the amount of measured growth over the time interval between measurements (10 days).

b. Feeding rates

Feeding behavior/rate will be monitored every 10 days[12] while the animals are fed. As outlined above, feeding rates will be measured per tank as a function of the difference in chlorophyll a concentrations at the time of feeding and 60 minutes after. To estimate individual feeding rates, the tank measurement will then be divided by the total number of mussels (n=21).

c. Reproductive function/other physiological responses

Every 30 days[13], 6 random individuals from each group will be sacrificed from each tank to measure gametogenic activity. Here, gametogenic activity is characterized by measuring the gonadosomatic index (weight of gonad/soft tissue weight; GSI), following Gagne et al. 2009).

d. Water samples[14]

To keep a running background of concentrations of fluoxetine for each treatment tank, water samples will be taken on day one, then every 10 days prior to fluoxetine

addition, and frozen until analyzed using protocols adapted from Rodriguez del Rey et al. (2011). Water samples will be collected from the respective tanks in the following order: control without mussels > control with mussels > 0.5µg/L> 2.5µg/L> 5.0µg/L>10µg/L. 50mL of water from each tank will be extracted using a calibrated syringe and then filtered through a centrifuge tube with Whatman glass fiber filters into a 50mL centrifuge tube. These samples will then be frozen until they are ready for preparation and analysis. Samples will be analyzed using a Fluoxetine enzyme-linked immunosorbent assay (ELISA) test kit to detect for the presence of fluoxetine.

-Field Experiments-[15]

1. Study Sites

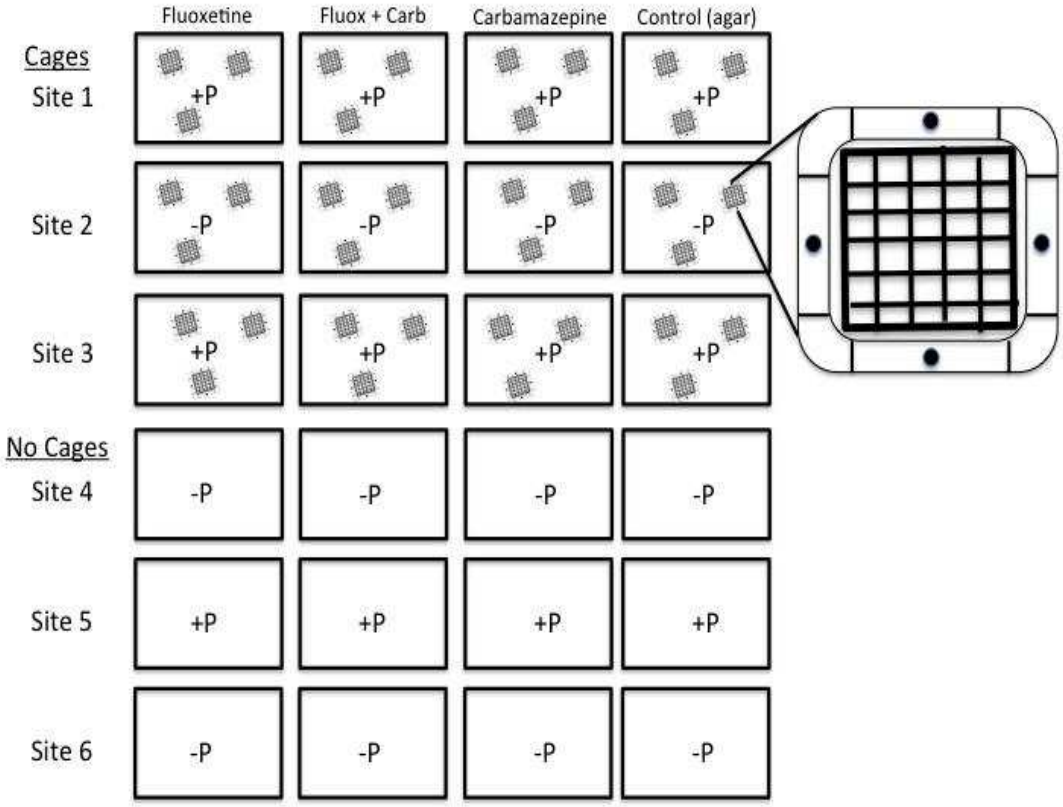
A 16-week long field experiments will be conducted at the same location as mussels collected for laboratory experiments, Boiler Bay, OR (44°83'N, 124°06'W).

2. Exposure to Fluoxetine and Carbamazepine

The pharmaceutical drugs carbamazepine and fluoxetine will be used to test H2 and H3, whether there is a cumulative effect of multiple stressors from these contaminants on mussel growth, byssal thread integrity, and resistance to predation. Six treatments (fluoxetine (2 levels), carbamazepine (2 levels), fluoxetine + carbamazepine (lowest and highest level combinations)) and a control (agar) will be administered using diffusing devices at 4 sites at Yachats (Figure 2). To test for caging effects, cage controls

(no cages) will be used for each treatment. At these cage control sites, *Pisaster* will be removed manually every two weeks.

Because exposure experiments will be used in conjunction with predator manipulation, predator exclusion cages will be outfitted with contaminant diffusing systems (CDS). This will consist of wire cages mounted to the rock wall with bolts and a layer of neoprene to ensure no entry from seastars beneath the wire. The contaminant diffusion system will be secured to this cage by using a previously assembled PVC square with 4, one-inch diameter holes drilled into each arm (see Figure 2 for schematic). Film canisters filled with a set agar gel containing the contaminant (e.g., fluoxetine, carbamazepine, or both) will be secured into these holes for contaminant diffusion. These will then be replaced every 4 weeks with a new canister to ensure chronic, near-constant exposure to mussels within the cages and neighboring mussels. Every 2 weeks, mussel and seawater samples at 0m (within cage), 0.5m, 1m, 2m, and 5m outside of the CDS cages will be collected and analyzed using ELISA kits for presence of contaminants.



a. Mussel Growth

At each exposure level (e.g., distance interval from CDS cage), 10 mussels will be identified using colored nail polish. Every 2 weeks, total length will be measured.

b. Byssal thread integrity

The attachment strength of *Mytilus californianus* will be measured following methods of Harger (1970). Mussels will be hooked onto a spring dynamometer (constructed to record maximum force) with a wire loop. A pulling force will be applied perpendicular to the mussel bed until the hooked mussel is dislodged. This will be done for 5 mussels at each exposure level every two weeks.

c. Predation intensity experiment

I will measure predation intensity following a design similar to Navaratte and Menge (1996). In this case the stressors will the individual contaminants or the combination of the two contaminants and the primary effect will be mussel resistance to predation. As mentioned earlier CDS cages will be placed within either a control or treatment plot. In the cages where seastars are removed (P-) cages will have a roof and four sides. In predator control plots (P+), seastars will have access through cages that have two open sides, to account for potential caging effects. In control areas without contamination, CDS cages (two open sides) will be filled with canisters of agar gel.

Every two weeks cages will be monitored and maintained to ensure predator removal. Small and medium sized seastars, as well as other benthic predators such as crabs are capable of entering cages. Upon each visit, the number of live and dead mussels remaining in cages and controls will be counted.

3. Statisical analyses[16]

Analysis of variance (ANOVA) will be used to determine if there are differences between each treatment and the controls. ANOVA assumptions of independence, normality, and homogeneity of the variances of the residuals will be met by using their appropriate tests. Tank samples will maintain independence by being isolated from one another, including water and filtration. Field samples will maintain independence by having an appropriate amount of space from one another (e.g. >50m). Both field and tank samples will be tested for normality and equal variance by using the Shapiro-Wilk test and F-test respectively. All statistical analyses will be performed using R studio version 2.11.1.

[1.] *M. californianus* mussels were collected from a single location on the jetty north of Rockaway Beach, Oregon (45°39'18.4"N, 123°56'31.2"W)

[2.] Mussels were enumerated using super glue and water proof paper labels. Nail polish flaked off after 2 weeks, during the acclimation period.

[3.] Mussels were acclimated for one month, not 7 days.

[4.] Mussels were weighed individually on 3 sample dates: 47, 67, 107. On days 47 and 67 mussels were weighed and measured and individuals not sacrificed were placed back in tanks.

[5.] We modified feeding based on feedback from Reed Mariculture: Twice weekly, mussels were batch fed Shellfish Diet 1800® (Reed Mariculture) diluted tenfold with seawater. The algae in the Shellfish Diet 1800® is a combination of six marine

microalgae *Isochrysis*, *Pavlova*, *Tetraselmis, Chaetocerous calcitrans*, *Thalassiosira weissflogii* and *Thalassiosira pseudonana* with cell diameter sizes ranging from 5 to 16 µm. Per mussel volume of algae fed was constant throughout the study.

6. Clearance rates were determined using the following modified methods: On feeding days, a 10 mL seawater sample was obtained ~1 minute after the algae mixture was added to each of the tanks. Mussels were allowed to feed for 3 hours before an additional 10mL sample was extracted. These samples served as initial and final concentrations, respectively. From each sample, we counted algal cells in three 0.5 mL aliquots using a Beckman Coulter Counter (model Z1, 100 µm aperture) and determined the mean initial and final concentrations within each tank. We collected a total of 11 samples over the course of the 107 day study.

Filtering rates were estimated from the rate of change in suspended particle concentrations. Following Coughlan (1969), we based filtering rates on four assumptions: a) the reduction in the concentration of particles is due to filtration by the animal, and to settling, b) mussel pumping rate is constant, c) particle retention is 100% efficient and d) there is homogenous suspension of particles. A set of identical tanks without mussels (n=4) served as blanks for feeding trials. Clearance rates for each mussel were calculated using the following formula (Coughlin 1969):

$$CR = (M/n)\,[\ln (C_0/C_t)/t] - [\ln (C_0\text{blank}/C_t\text{blank})/t]$$

where CR = clearance rate (cells^{-1}mL^{-1}min^{-1}); M = volume of seawater in each tank (mL); n = number of mussels in tank; t = feeding time (min); C_0 = initial concentration of particles in tank; C_t = final concentration of particles in tank. C_0blank = initial concentration of particles in tank without mussels; $C_t$blank = final concentration of particles in tank without mussels

[7.] Because fluoxetine is water soluble, we did not use 0.1% EtOH to increase the solubility of the solid. Fluoxetine hydrochloride was dissolved only in nanopure water.

[8.] Study period was 107 days for mussel experiment

[9.] Fluxoetine treatments were 0.3, 3, 30, and 300 ng/L of fluoxetine and a control with no fluoxetine (Figure 2.1). A set of 5 tanks with no mussels were used to determine a baseline for algae removed by the tank filtration systems during algal clearance trials. We note that one of the no-mussel tanks malfunctioned after 20 days into the experiment and was excluded from further analyses, reducing no-mussel tank replicates to 4.

[10.] Made measurements on day 47, 67, and 107

[11.] Did not measure shell accretion rates

[12.] Algal clearance rates were measured twice weekly, except in the event where the Coulter counter machine was not working.

[13.] Made measurments on day 47,67, and 107.

[14.] Bioconcentraion of fluoxetine was transerferred to Dylan Dayrit as an undergraduate thesis project under the direction of Dr. Elise Granek. Samples were preserved in the -80 freezer in the Granek/de Rivera Lab.

[15.] Did not do a field component or use the pharmaceutical carbamazepine. Designed a predator avoidance experiment using Fluoxetine instead.

[16.] Statistical analyses were modified to the following procedures:

Fluoxetine exposure study: *M. californianus:*

For each sampling period, we averaged within-tank means for mussel growth, GSI, CI, and algal clearance parameters. Normality and homogeneity of variances were assessed through graphical inspection of the model residuals and respective Shapiro-Wilk's and Levene's tests, which indicated a need for data transformation. Algal clearance data underwent a Box-Cox transformation (Box and Cox 1964) and mussel growth and body condition data were log-transformed. Separate two-way ANOVAs were run with treatment and sample date as fixed factors and tanks as an error term to account

for non-independence between subsamples. Main effects were considered significant at α=0.5. Post-hoc Tukey HSD tests were used for pairwise comparisons among treatment and sample date means. All statistical analyses were performed using R statistical platform (RStudio Version 3.2.2 (2015)).

Fluoxetine exposure study: *H. oregonensis:*

Ethograms from the trials were analyzed for crab behavior and predation risk. We assessed differences in the proportion of active behaviors among *H. oregonensis* across fluoxetine treatments, time period type (day and night), and trial type (predator/no predator). To determine this proportion, we *a priori* divided active behaviors (i.e., walking, digging, foraging, and interactions between conspecifics) and non-active behaviors (i.e., remaining still, buried, or just moving mandibles). We then tested whether the probability that crabs would exhibit active behaviors varied among the three fluoxetine treatment groups, time periods, and trial type, or a combination of these variables, with a mixed-effect generalized linear model with a binomial error distribution using the glmer function from lme4 package (Bates et al., 2015) in R Studio (R Core Team, 2015). Our mixed-effect model included the crabs, trials, and tanks as random effects to account for non-independence between samples, due to repeatedly observing the same crabs over several trials and because of influences of behavior by individuals within the same tank.

We used a similar modeling approach to assess whether the proportion of predator avoidance behaviors varied by fluoxetine treatment and over time. Using data from

predator trials only, we *a priori* determined predator avoidance behaviors as remaining buried, still, or retreat under rock/elsewhere in tank and non-avoidance behaviors as remaining active, foraging, or interacting without response to the predator. We used a separate mixed-effects generalized linear model with a binomial error distribution to test the probability that crabs would exhibit predator avoidance behaviors differently among the three fluoxetine treatment groups and day/night time periods.

We were also interested in whether aggression among conspecifics varied across fluoxetine treatments. We developed a third generalized mixed effects model with a binomial error distribution that tested whether the proportion of aggressive acts between *H. oregonensis* varied across the fluoxetine treatments, time of day, and trial type.