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Effects of Synthetic Estrogen (17α-Ethinyl Estradiol) on Male Fiddler Crab Aggression

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A**bstract**

Pharmaceuticals, including hormones and antibiotics, are considered contaminants due to their widespread use and release into the environment. Hormones, like the synthetic estrogen used in oral contraceptives (17α-ethinylestradiol), are present in freshwater and marine systems, but with relatively unknown effects on the organisms that live there. Ethinylestradiol (EE2) accumulates in waterlogged soil (sediment) with potential to harm sediment-dwelling animals. For example, fiddler crabs (*Uca pugilator*) are vital members of salt marsh communities. Their burrowing adds oxygen to sediments and cycles nutrients, and they are an important food resource to birds and raccoons. Male fiddler crabs are territorial, aggressively defending their burrows from intruders. Given that synthetic estrogen reduces aggression in fish, I hypothesized that EE2 affects male fiddler crab aggression. Male crabs exposed to EE2 were expected to retreat from threats instead of attacking more often than untreated animals. Aggression was measured as responses to threats (fleeing, attacking, no response) after exposure to sediment without (control) or with added EE2 (0.5 mg L^{-1}). There was a trend toward EE2 treated crabs fleeing from a fight more often than control crabs which could lend support to the previous prediction. Another trend observed was the EE2-treated males exhibiting no responses to the simulated foreign threats. None of the other responses differed between treatments. The lack of significant responses was likely due to the measured concentration of EE2 in the treated sediment being two orders of magnitude less than the nominal concentration. Therefore, while these findings indicate the possibility that EE2-exposed males may have difficulty protecting themselves, their burrows, or their mate from predators, higher EE2 concentrations with larger sample sizes need to be tested for verification.

Introduction

In the United States, more than 100,000 chemicals have been permitted for commercial use (Sutton et al. 2017). Regulatory agencies typically do not mandate environmental monitoring of many of these chemicals for potential risks because they are present at very low concentrations (in the ng-μg L^{-1} range) in the environment (Sutton et al. 2017, Patel et al. 2020). The immense number of potential contaminants makes it difficult to create guidelines for each one (Geissen et al. 2015). Furthermore, the relevance of each chemical as a potential contaminant (the amount produced and the severity of its effects) fluctuates over time due to changes in synthesis, use and disposal (Geissen et al. 2015). While the chemicals are currently present at low environmental concentrations, as the world population increases, release of unregulated chemicals into the environment will likely increase, amplifying their potential for environmental effects (Patel et al. 2020). Therefore, these chemicals have been categorized as contaminants of emerging concern (CECs) or emerging contaminants (ECs) (Sutton et al. 2017).

Emerging contaminants (ECs) can be manufactured versions of natural compounds or synthetic organic compounds that are not easily monitored, and have the potential to enter the environment and/or have relatively unknown effects on wildlife (Vines et al. 2012, Patel et al. 2020). They are defined as “emerging” because production of new chemicals or changes in use and disposal of existing chemicals can generate new sources of contamination (Geissen et al. 2015). In some cases, the release of ECs into the environment has occurred for a long time but they were not recognized as contaminants until methods were developed to detect their presence (Geissen et al. 2015). Emerging contaminants include chemicals in personal care products (e.g. fragrances, shampoos, soaps, toothpaste, deodorants, etc.), pesticides, phthalates, flame retardants, perfluorinated compounds, nanomaterials and pharmaceuticals. Pharmaceuticals such

as hormones, analgesics, beta-blockers, antibiotics, antiepileptics, antidepressants, and lipid-lowering drugs have been detected in surface waters globally, and may inflict damage to organisms at environmental concentrations (Vines et al. 2012).

Pharmaceuticals, such as hormones used to treat animals and humans, are excreted, ultimately reaching waterways via effluent from wastewater treatment plants, and runoff from agriculture and landfills (Shore et al. 2003; Vines et al. 2012). Some hormones are peptides, made up of amino acids, which are rapidly destroyed due to high solubility in water (Ophardt 2003; Shore et al. 2003). Peptide hormones remain in the blood for a few minutes before being broken down by blood and tissue proteases (Goodman 2021). In contrast, steroid hormones (estradiol, estrone, ethinylestradiol, progesterone, testosterone) are chemically stable, lipophilic and poorly soluble in water (hydrophobic) making them less susceptible to degradation (Shore et al. 2003). Although these chemicals typically enter the environment in water, hormones have the tendency to sorb to and accumulate in sediment due to their hydrophobicity (Sangster et al. 2015). Hormones in sediment can come into contact with aquatic organisms as free hormones via desorption from the sediments and/or by direct contact between aquatic organisms and the sediment-bound hormone (Ellis 2006, Sangster et al. 2015). Exposure to low concentrations ($<0.001\ \mu g\ L^{-1}$) of steroid estrogens cause negative effects including decreased fertility, feminization, and hermaphroditism in fish (Sangster et al. 2015).

Synthetic estrogen, 17α-ethinylestradiol (EE2), is a female steroid hormone classified as an EC with potential for contamination of the environment and adverse effects on aquatic organisms (Barreiros et al. 2016). Often used in combination oral contraceptives, EE2 mimics natural estrogen to inhibit ovulation and prevent irregular shedding of the endometrium (lining of the uterus) (Rivera et al. 1999; Wisniewska and Szaniawska 2015). Synthetic estrogen is an

endocrine disrupting chemical (EDC) that interferes with hormonal regulation and the endocrine system, affecting health and reproduction in animals and humans (Casals-Casas and Desvergne 2011). Endocrine disrupting chemicals can imitate or disturb the production, release, metabolism, and elimination of natural hormones (Casals-Casas and Desvergne 2011).

With a growing world population, the prevalence of estrogens in the environment is increasing (Barreiros et al. 2016). Approximately 17% of the human female population in Western countries take contraceptive pills regularly, resulting in discharge of ~4.4 kg per year per million inhabitants (Barreiros et al. 2016). Synthetic estrogen is also used to improve productivity in livestock by enhancing growth, and treating reproductive disorders (Aris et al. 2014). The level of estrogen excreted by livestock is similar to humans, or may exceed the daily per human contribution by more than an order of magnitude (Aris et al. 2014). Thus, synthetic estrogen is often detected in sewage treatment plant effluent (<1 - 42 ng L^{-1}), and agricultural (livestock) runoff and surface water (< 5 to 831 ng L^{-1}) (Braga et al. 2005; Chen et al. 2010; Lee et al. 2014). Estrogens also have high affinity for the organic carbon in sediment and tend to accumulate due to their slow degradation in low-oxygen environments and limited mobility (Braga et al. 2005; Lima et al. 2012). Sediment concentrations of EE2 can range from < 0.04 to 133.64 ng g^{-1} (Aris et al. 2014). Ethinylestradiol is considered a threat to aquatic systems due to its hydrophobic nature and resistance to degradation promoting environmental persistence and bioavailability (Blewett et al. 2013). These characteristics allow EE2 to have potential adverse effects on organisms, even at low levels (ng L^{-1}) (Barreiros et al. 2016).

Ethinylestradiol adversely affects the physiology and development of organisms in aquatic environments at low (ng L^{-1}) concentrations (Vines et al. 2012; Barreiros et al. 2016). For example, EE2 has been linked to fish feminization via synthesis and secretion of vitellogenin

in male spined stickleback (*Gasterosteus aculeatus*) (Andersson et al. 2007) and reduced male fertility in rainbow trout (*Oncorhynchus mykiss*) (Schultz et al. 2003). Ethinylestradiol also affects invertebrates; male amphipod crustaceans (*Hyalella azteca*) experienced disrupted gonadal development at 0.1–10 μg L^{-1} (Segner et al. 2003). Additionally, delayed hatching of egg masses (EE2: 1000 ng L^{-1}) and deformations of developing juveniles (EE2: 100 - 1000 ng L^{-1}) have been reported in the great pond snail (*Lymnaea stagnalis*) (Segner et al. 2003).

In addition to reproductive defects, EE2 can cause behavioral changes in aquatic organisms (Aris et al. 2014). Contamination with EE2 induces behavioral changes that can have negative effects on reproductive success of the organisms (Aris et al. 2014). For example, exposure to 50-100 ng L^{-1} EE2 suppressed spawning and reproductive behavior (i.e. dancing and copulation) in brackish medaka, *Oryzias melastigma*, and the number of spawned eggs decreased by 84% (Lee et al. 2014). Male zebrafish (*Danio rerio)* exhibited decreased courtship and aggressive behavior as a result of treatment with 0.5 ng L^{-1} EE2 (Colman et al. 2009). This impairment of aggression can affect a male's ability to compete and acquire territories from other males as seen in a study conducted by Majewski et al. (2002) on fathead minnows (*Pimephales promelas*).

Male-male aggression, which is important for communication and establishing a hierarchy, can be altered by EE2 (Frommen 2020). Animals may fight over food, potential mates or high-quality territories which are resources often facilitated by a high social rank attained and sustained through aggression (Frommen 2020). When exposed to three EE2 concentrations (0.5, 5.0, and 50 ng L^{-1}), male zebrafish exhibited reduced aggressive nipping behavior towards control males which led to a shift in social dominance (Colman et al. 2009). Bell (2001) found that the male three-spined stickleback (*Gasterosteus aculeatus*) displayed decreased rates of aggressive biting towards other males post-exposure to EE2 concentrations between 10 and 50

ng L^{-1}. While these studies indicate that EE2 affects aggressive behavior in pelagic organisms, effects may be more severe for benthic organisms interacting with the sediment where EE2 accumulation occurs (Aris et al. 2014).

Ethinylestradiol may adversely affect behavior of the Atlantic sand fiddler crab, *Uca pugilator,* that feeds on and burrows in salt marsh sediments on the east coast of the United States (Weis et al. 1987). Fiddler crabs are ecologically important, providing food to predators such as other crab species, fish, shore birds, and raccoons (Bergey and Weis 2008). Their burrowing activity increases smooth cordgrass (*Spartina alterniflora*) abundance by oxygenating the soil and boosting drainage, soil oxidation-reduction potential, and decomposition of underground plant debris (Hoffman et al 1984; Bertness 1985). Fiddler crabs have potential to come into contact with EE2 because concentrations in salt marsh sediments can be quite high; EE2 concentration was 86.3 ng g^{-1} in organic sediments of an urbanized estuary in Brazil (Pusceddu et al. 2019) compared to 0.05 to 0.5 ng g^{-1} in low-organic sandy marine sediments in Australia (Braga et al. 2005). In addition, fiddler crab activity is susceptible to disruption from EDCs. Tributyltin (a biocide in anti-fouling paint and known EDC; McGinnis and Crivello, 2011) reduced burrowing activity in both sexes of sand-dwelling fiddler crabs (*U. pugilator*) (Weis and Perlmutter 1987). Ethinylestradiol accumulation in salt marsh sediments, coupled with tributyltin's similar mode of action, indicate potential for EE2 to disrupt the behavior essential to success of fiddler crab populations.

The objective of this study was to determine whether synthetic estrogen affects male fiddler crab behavior, specifically aggression. Male fiddler crabs have one large claw that they use to push, pinch and swing at an opponent when threatened or defending their territory (Pratt et. al, 2003). They also wave this large claw to attract the attention of a potential mate to their

breeding burrow (McLain and Pratt 2007). Males use their claws to engage in contests with other males to establish control of breeding burrows and access to females (McLain and Pratt 2007). Females tend to choose males that have larger claws, indicating viability and strength, and are bolder (more aggressive) (Pratt et al 2005; McLain and Pratt 2007). I hypothesized that EE2 affects male fiddler crab aggression toward competitors or potential threats. Male crabs exposed to EE2 were expected to have reduced aggression, retreating from threats instead of attacking more often than control animals. Thus, if EE2-exposed males have difficulty protecting themselves, their burrows, or their mate from predators or other crabs, lifespan and reproductive success may be reduced.

Methods

Crab collection, maintenance and handling

Male and female fiddler crabs (*Uca pugilator*) were collected by hand during low tide from a salt marsh in Savannah, Georgia (31.9475 N, -81.0673 W). Adult crabs with a carapace width of 15 – 20 mm (Weis and Perlmutter 1987; Bergey and Weis 2008) were brought to the Biological Sciences Aquarium Facility at Georgia Southern University, Statesboro, Georgia. Crabs were initially housed in rectangular, plastic terrariums (L×W×H: 29.85 cm × 17.14 cm × 20.32 cm), at a density of 6 crabs per tank in a 2:1 female to male ratio (Weis 1978). Sediment from the collection site was manipulated to form a steady incline ending in a raised plateau (height, approx. 15 cm) at the one end of the terrarium according to the methods of Reichmuth et al. (2009) (Figure 1). Water from the collection site was added to a depth of 3-5 cm at the lower end of the sediment incline to simulate low tide. This allowed the crabs to have access to water and prevent desiccation of both the crabs and the sediment. The crabs were fed commercial food pellets (Hikari Crab Cuisine® Crustacean Food) twice a week (Weis 1978). During feeding periods, salinity was checked with a refractometer and adjusted as needed using deionized water. Crabs were given at least 48 hrs to acclimate to laboratory conditions and dig burrows prior to behavioral measurements (Reichmuth et al 2009). All trials were recorded with a video camera (Canon PowerShot SX540 HS) (Reichmuth et al 2011).

1. Pilot Study – Observing Fiddler Crab Behavior

The goal of the pilot study was to ensure reliable and repeatable measurements could be taken to quantify male crab aggressive behavior under uncontaminated conditions. Therefore, no chemicals were added to the sediment used in the pilot male aggression assays. During these trials, agonistic behaviors (push, pinch, and swing) from the literature were verified. This aided in the determination of which behaviors were classified as an attack during the experiment.

2. Experiment: Effects of Ethinylestradiol on *Uca pugilator* Behavior

The goal of the experiment was to examine changes in male crab aggressive behavior after exposure to EE2 contamination. Crabs were randomly assigned to one of two groups: experimental (exposure to EE2) or control (no exposure to EE2). Crabs were housed in 37.85 L rectangular, glass tanks (L×W×H: 58.1 cm × 32.3 cm × 35.3 cm), at a density of 12 crabs per tank in a 2:1 female to male ratio (Weis 1978). Glass enclosures were used to avoid having hydrophobic organic compounds adsorb to plastic (Lu et al. 2020). There were 8 male crabs per treatment group that were split evenly into two tanks at random (4 crabs per tank). Sediment was manipulated to form a steady incline ending in a raised plateau (height, approx. 15 cm) at the one end of the tank according to the methods of Reichmuth et al. (2009) (Figure 1). The experimental tanks contained sediment that was treated with EE2 while the control tanks contained uncontaminated sediment from the collection site. Treatment exposures occurred over a period of 2 weeks (Weis and Perlmutter 1987). The EE2 treatment was created by dissolving 50 mg of EE2 (CAS 57-63-6, 98% purity, Acros Organics) in 1 mL of ethanol (95% purity, Pharmco-Aaper) then adding it to 1L of seawater from collection site (Colman et al. 2009; Dussault et al. 2009). The stock solution was thoroughly mixed for 4 hours until homogenized. The

concentrated stock solution was then diluted in 10L of seawater to a nominal concentration of 0.5 mg L^{-1}. The dilute solution was incorporated into the sediment by thoroughly mixing with a trowel for even distribution (Weis and Perlmutter 1987; Lee et al. 2014). This was to simulate delivery of EE2 to sediment from the water at high tide (Yang et al. 2018). Nominal concentrations of EE2 in the sediment from the EE2 exposure treatment were verified at the CAIS Lab for Environmental Analysis (University of Georgia, Athens, GA). Samples were prepared for analysis by extracting the sediment with an organic solvent (acetone/hexane mixture) (Braga et al. 2005; Hassan 2021). The extracted EE2 was analyzed using Gas Chromatography (Braga et al. 2005; Hassan 2021). The analysis revealed that the sediment from the collection site had a baseline EE2 concentration of 0.00186 mg L^{-1}. Though the EE2 concentration of the contaminated sediment was expected to be 0.5 mg L^{-1}, the final concentration was 0.00222 mg L^{-1}. The EE2 experienced negligible levels of degradation over the 2-week exposure period (from 0.00222 mg L^{-1} to 0.00203 mg L^{-1}).

Measurement of Male Aggression

Part 1: Individual Males Exposed to Foreign Threats

To observe male behavioral responses to threats, individual male crabs from each treatment (n=8) were placed in an opaque plastic box (45.7 cm × 33.8 cm× 12.2 cm high) (Weis and Perlmutter 1987) with a thin layer of sediment (approx. 2 cm) covering the bottom of the box to allow traction. Crabs were observed one at a time. After a 2 min acclimation period, each crab was provoked to attack with a rubber stopper attached to a dowel (54 cm) a total of 20 - 24 times per trial for a total of three trials. This stopper represented foreign threats such as predators or other species. The dowel was lowered into the tray at a diagonal angle where the researcher was crouched down at the side of the box and out of the crab's field of vision. During each trial, the

crab received 4 prods five to six times, with a 10 sec interval between each round followed by a 2 min rest period between trials (Reichmuth et al 2011). This method was chosen to produce replicable results that were representative of each crab. Attacks were defined by a lunge, pinch, swing and/or push (Pratt et al. 2003). Aggressive and non-aggressive behaviors (attack, flee, no response) for each crab were tallied.

Part 2: Male Responses to Threats from Same Species

A second measure of male aggression was responses to another male of the same species, one from each treatment (n=8). Control and experimental males were differentiated through color-coded markings with nail polish on their carapace (Miller et al. 2007). One male from each group (with similar carapace width and claw size), was placed in the same plastic box previously described and the behavioral interactions recorded for 3 min. After each trial, one of the crabs was randomly chosen to be removed, then reintroduced for a new trial. This eliminated bias and ensured that no crab had an advantage over the other. This process was repeated for a total of 3 trials for each pair, and 8 pairs total. Each pairing was fixed (each crab had only one rival for all three trials and crabs were not used in multiple pairings). The number of times each crab approached their opponent, initiated a fight by attacking the opponent (lunge, pinch, swing and/or push) and fled from a fight were recorded (Pratt et al. 2003). Additionally, the time each crab actively engaged in attacking the opponent and the duration each crab was attacked by the other and did not retaliate were determined (Pratt et al. 2003).

Statistical Analyses

Data were tested for parametric test assumptions of normality using the Shapiro-Wilk *W* test and for homogeneity of variances using Levene's test. Differences in individual behaviors (attack, flee, no response) of crabs between treatments were analyzed using t-tests. Male-male aggression (time spent attacking opponent, time being attacked without retaliation, fight initiations, flees, approaches) as a result of EE2 exposure were also evaluated with t-tests. All statistical analyses were performed using JMP® Software, V.14 (SAS Institute Inc., Cary, NC).

Results

Part 1: Individual Males Exposed to Foreign Threats

There were no differences in the means between the two treatments (with or without EE2) (Attack: t= -0.83, p=0.42; Flee: t= -0.36, p=0.72; No Response: t=1.65, p=0.12; Fig.2). However, there was a trend of EE2-exposed males not responding to foreign threats (represented by the dowel). In the EE2 group, no response occurred 23% of the time, compared to 10% in the control group (Table 1A). Regardless of EE2 treatment, attacking was the most common response to threats (50-60%) (Table 1A).

Part 2: Male Responses to Threats of the Same Species

There were no significant differences in the means between the two treatments (Flees: t=1.63, p=0.13; Fight initiations: t=0.34, p=0.74; Approaches: t=-0.60, p=0.56; Fig.3A). However, there was a trend of EE2 treated males fleeing more often from threats of the same species. The exposed crabs fled 46% of the time, compared to the 26% in the control group (Table 1B). Fight initiations occurred 14% of the time in both treatment groups (Table 1B). There was only a 12% difference between the number of flees and fight initiations in the control group (Table 1B). However, in the EE2 group, the number of flees was more than triple that of

the fight initiations with a difference of 32% (Table 1B). In both groups, there were more than twice as many approaches (40-60%) than there were initiations (14%) (Table 1B). In comparison to the responses of the crabs when faced with a dowel, exposed crabs flee more often when up against the same species rather than a foreign threat. Also, when individual crabs were provoked with the stopper, there were more attacks than when put in pairs.

Between the treatment groups (with or without EE2), the time spent attacking the opponent ($t=0.41$, $p=0.69$) and the time being attacked without retaliation ($t=0.06$, $p=0.95$) did not differ (Fig.3B). However, regardless of EE2 exposure, crabs spent more time attacking their opponents than they did enduring attacks without reciprocating. For the majority of the time (88-89%), crabs did not interact with each other (Table 1B).

Discussion

The hypothesis that EE2 affects male fiddler crab aggression toward competitors or potential threats was not supported by the statistics. However, there were some compelling patterns that could lend support to the prediction that EE2-exposed males would flee more often than control males. Control males initiate fights with the same species with similar frequency as they flee from the fight, but EE2-exposed males flee more than 3 times as often as they initiate fights. These patterns could indicate the possibility that EE2-exposed males may have difficulty protecting themselves, their burrows, or their mate from predators.

When provoked, crabs tend to attack with a higher frequency than when in the presence of the same species. It is possible that crabs did not interact much with their opponent because fights are energetically costly (Jennions and Backwell 1996). Unless the fight is beneficial in some way, they choose not to initiate the encounter (Jennions and Backwell 1996). During trials, burrows were not present, and food was not provided, therefore, crabs had nothing to gain by

fighting. Additionally, without enough sediment to burrow, individual males that were provoked with the stopper, had nowhere to hide. Therefore, it was common for a male to stand his ground (Reichmuth et al. 2011).

There were no differences between EE2-exposed males and control males for all variables tested. This may be due to the preexisting amount of ethinylestradiol (0.00186 mg L^{-1}) found in sediment from the collection site. It is possible that the crabs developed a tolerance to the chemical. This is, however, highly unlikely due to the low adaptive potential of EE2 (Marques da Cunha et al. 2019a). Inherited tolerance to EE2 was tested in brown trout (*Salmo trutta*) offspring with findings indicating that there was no additive genetic variance for tolerance to EE2 (Marques da Cunha et al. 2019b). This means that there was no significant genetic variation that would allow the population to adapt to EE2 (Marques da Cunha et al. 2019a). With the low possibility of crab adaptations, another potential explanation for the results is the low exposure concentration of ethinylestradiol.

The lack of significant response was likely due to the measured concentration of EE2 (0.00222 mg L^{-1}) in the treated sediment being two orders of magnitude less than the nominal concentration (0.5 mg L^{-1}). The lower concentration may be due to the rate of sorption of EE2 in the sediment. Characteristics like pH, particle size and organic matter content can influence sorption (Khunjar and Love 2011). There is a positive correlation between organic matter content and the sorption of EE2 (Sun et al. 2012). Therefore, it is possible that the sediment from the collection site did not have an adequate amount of organic matter for the quick and complete sorption of the added EE2. In future studies, characteristics of the sediment can be identified and utilized as a guide to ensure efficient sorption of EE2.

It is also likely that the EE2 concentration was too low to have effects on fiddler crabs. Various concentrations of EE2 have different effects on organisms depending on the species (Aris et al. 2014). This may be the case for the fiddler crabs as without the complete sorption of EE2, the resulting low concentration seemed insufficient to see a significant change in aggressive behavior. The crabs tested were adults, which may be a reason for their low sensitivity to the EE2 concentration as juvenile aquatic organisms are more sensitive to estrogen (Aris et al. 2014). Another factor that may play a part in the insignificance of the data is the period of chemical exposure. Organisms that are chronically exposed to EE2 can show adverse effects but when the same level of exposure is experienced acutely, no effects may be seen (Nash et al. 2004). Therefore, higher EE2 concentrations with larger sample sizes need to be tested for verification.

Literature Cited

Andersson, C., Katsiadaki, I., Lundstedt-Enkela, K., Örberga, J. (2007) Effects of 17α-ethynylestradiol on EROD activity, spiggin and vitellogenin in three-spined stickleback (*Gasterosteus aculeatus*). *Aquatic Toxicology* 83(1): 33–42

Aris, A.Z., Shamsuddin, A.S., Praveena, S.M. (2014) Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: a review,*Environment International* 69: 104–119.

Barreiros, L., Queiroz, J.F., Magalhães, L.M., Silva, A.M.T., Segundo, M.A. (2016) Analysis of 17-β-estradiol and 17-α-ethinylestradiol in biological and environmental matrices: A review. *Microchemical Journal* 126: 243–262

Bell, A. (2001) Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined stickleback, *Gasterosteus aculeatus. Animal Behaviour* 62 (4): 775–780

Bergey, L. and Weis, J. (2008) Aspects of population ecology in two populations of fiddler crabs, *Uca pugnax*. *Marine Biology* 154: 435–442

Bertness, M.D. (1985) Fiddler crab regulation of *Spartina alterniflora* production on a New England salt marsh. *Ecology* 66 (3): 1042–1055

Blewett, T.A., Robertson, L.M., MacLatchy, D.L.,Wood, C.M. (2013) Impact of environmental oxygen, exercise, salinity, and metabolic rate on the uptake and tissue-specific distribution of 17α-ethynylestradiol in the euryhaline teleost *Fundulus heteroclitus*. *Aquatic Toxicology* 138–139: 43–51

Braga, O., Smythe, G.A., Schäfer, A.I., Feitz, A.J. (2005) Steroid estrogens in ocean sediments. *Chemosphere* 61: 827–833

Casals-Casas, C. and Desvergne, B. (2011) Endocrine disruptors: from endocrine to metabolic disruption. *Annual Review of Physiology* 73:135–162

Chen, T., Chen, T., Yeh, K.C., Chao, J., Liaw, E., Hsieh, C., Chen, K., Hsieh, L., Yeh, Y. (2010) High estrogen concentrations in receiving river discharge from a concentrated livestock feedlot. *Science of The Total Environment* 408 (16): 3223–3230

Colman, J.R., Baldwin, D., Johnson, L.L., Scholz, N. L. (2009) Effects of the synthetic estrogen, 17α-ethinylestradiol, on aggression and courtship behavior in male zebrafish (*Danio rerio*). *Aquatic Toxicology* 91: 346–354

Dussault, E.B., Balakrishnan, V.K., Borgmann, U., Solomon, K.R, Sibley P.K. (2009) Bioaccumulation of the synthetic hormone 17α-ethinylestradiol in the benthic invertebrates *Chironomus tentans* and *Hyalella azteca*, *Ecotoxicology and Environmental Safety*, 72 (6): 1635–1641

Ellis, J.B. (2006) Pharmaceutical and personal care products (PPCPs) in urban receiving waters, *Environmental Pollution* 144 (1): 184–189

Frommen, J.G. (2020) Aggressive communication in aquatic environments. *Functional Ecol*ogy 34: 364–380

Geissen, V., Mol H., Klumpp, E., Umlauf, G., Nadal, M., Ploeg, M., Zee, S. E.A.T.M., Ritsema, C. J. (2015) Emerging pollutants in the environment: A challenge for water resource management. *International Soil and Water Conservation Research* 3 (1): 57–65

Goodman, S. (2021) General modes of intercellular signaling. *Goodman's Medical Cell Biology* 4: 249–270

Hassan, S. (2021) Organic compound characterization & quantification. Center for Applied Isotope Studies. Retrieved from https://cais.uga.edu/service/organic-compound-characterization-and-quantification/

Hoffman, J.A., Katz, J., Bertness, M.D. (1984) Fiddler crab deposit-feeding and meiofaunal abundance in salt marsh habitats. *Journal of Experimental Marine Biology and Ecology* 82(2-3): 161–174

Jennions, M.D. and Backwell, P.R.Y. (1996) Residency and size affect fight duration and outcome in the fiddler crab *Uca annulipes*. *Biological Journal of the Linnean Society* 57 (4): 293–306

Khunjar, W.O. and Love, N.G. (2011) Sorption of carbamazepine, 17α-ethinylestradiol, iopromide and trimethoprim to biomass involves interactions with exocellular polymeric substances. *Chemosphere* 82 (6): 917–922

Lee, P., Lin, C., Chen, T. (2014) Environmentally relevant exposure of 17α-ethinylestradiol impairs spawning and reproductive behavior in the brackish medaka *Oryzias melastigma. Marine Pollution Bulletin*. 85 (2): 338–343

Lima, D., Schneider, R., Esteves, V. (2012) Sorption behavior of EE2 on soils subjected to different long-term organic amendments. *Science of The Total Environment* 423: 120–124

Lu, J., Wu, J., Wu, J., Zhang, C., Luo, Y. (2020). Adsorption and desorption of steroid hormones by microplastics in seawater. *Bulletin of Environmental Contamination and Toxicology.* https://doi.org/10.1007/s00128-020-02784-2

Marques da Cunha, L., Maitre, D., Wedekind, C. (2019a) Low adaptive potential for tolerance to ethynylestradiol, but also low toxicity, in a grayling population (*Thymallus thymallus*). *BMC Evolutionary Biology* 19: 227 https://doi.org/10.1186/s12862-019-1558-1

Marques da Cunha, L, Uppal, A, Seddon, E, Nusbaumer, D, Vermeirssen, ELM, Wedekind, C. (2019b) No additive genetic variance for tolerance to ethynylestradiol exposure in natural populations of brown trout (*Salmo trutta*). *Evolutionary Applications* 12: 940–950. https://doi.org/10.1111/eva.12767

Majewski, A.R., Blanchfield, P.J., Palace, V.P., Wautier, K. (2002) Waterborne 17α-ethynylestradiol affects aggressive behaviour of male fathead minnows (*Pimephales promelas*) under artificial spawning conditions. *Water Quality Research Journal* 37 (4): 697–710

McGinnis, C.L., Crivello, J.F. (2011) Elucidating the mechanism of action of tributyltin (TBT) in zebrafish. *Aquatic Toxicology* 103 (1-2): 25–31

McLain, D.K. and Pratt, A.E. (2007) Approach of females to magnified reflections indicates that claw size of waving fiddler crabs correlates with signaling effectiveness. *Journal of Experimental Marine Biology and Ecology* 343: 227–238

Nash, J.P., Kime, D.E., Van der Vem, L.T.M., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R. (2004) Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environmental Health Perspectives* 112: 1725–1733

Ophardt, C.E. (2003) Amino acids. *Virtual Chembook*. Elmhurst College. Retrieved from http://chemistry.elmhurst.edu/vchembook/560aminoacids.html

Patel, N., Khan, M., Shahane, S., Rai, D., Chauhan, D., Kant, C., Chaudhary, V. (2020) Emerging pollutants in aquatic environment: source, effect, and challenges in biomonitoring and bioremediation- A review. *Pollution*, 6 (1): 99–113. doi: 10.22059/poll.2019.285116.646

Pratt, A., McLain, K., Berry, A. (2005) Variation in the boldness of courting sand fiddler crabs. *Ethology* 111: 63–76

Pratt, A., McLain, K., Lathrop, G. (2003) The assessment game in sand fiddler crab contests for breeding burrows. *Animal Behaviour* 65: 945–955

Pusceddu, F.H., Sugauara, L.E, Rodrigues de Marchi, M., Choueri, R.B., Castro, I.B. (2019) Estrogen levels in surface sediments from a multi-impacted Brazilian estuarine system, *Marine Pollution Bulletin* 142: 576–580

Reichmuth, J., MacDonald, J., Ramirez, J., Weis, J. (2011) Fight or flight: an investigation of aggressive behavior and predator avoidance in two populations of blue crabs (*Callinectes sapidus* Rathbun) in New Jersey. *Hydrobiologia* 658: 173–182

Reichmuth, J., Roudez, R., Glover, T., Weis, J. (2009) Differences in prey capture behavior in populations of blue crabs (*Callinectes sapidus* Rathbun) from contaminated and clean estuaries in New Jersey. *Estuaries and Coasts* 32: 298–308

Rivera, R., Yacobson, I., Grimes, D. (1999) The mechanism of action of hormonal contraceptives and intrauterine contraceptive devices. *American Journal of Obstetrics and Gynecology* 181 (5): 1263–1269

Sangster, J.L., Oke, H., Zhang, Y., Bartelt-Hunt, S.L. (2015) The effect of particle size on sorption of estrogens, androgens and progestagens in aquatic sediment. *Journal of Hazardous Materials* 299: 112–121

Segner, H., Caroll, K., Fenske, M., Janssen, C.R., Maack, G., Pascoe, D., Schäfers, C., Vandenbergh, G.F., Watts, M., Wenzel, A. (2003) Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. *Ecotoxicology and Environmental Safety* 54 (3): 302–314

Schultz, I.R., Skillman, A., Nicolas, J.M., Cyr, D.G., Nagler, J.J. (2003) Short-term exposure to 17α-ethinylestradiol decreases the fertility of sexually maturing male rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry* 22 (6): 1272–1280

Shore, L. S. and Shemesh, M. (2003) Naturally produced steroid hormones and their release into the environment. *Pure and Applied Chemistry* 75 (11-12): 1859–1871

Sun, K., Jin, J., Gao, B., Zhang, Z., Wang, Z., Pan, Z., Xu, D., Zhao,Y. (2012) Sorption of 17α-ethinyl estradiol, bisphenol A and phenanthrene to different size fractions of soil and sediment. *Chemosphere* 88 (5): 577–583

Sutton, R., Sedlak, M., Sun, J., Lin, D. (2017) Contaminants of emerging concern in San Francisco Bay: A strategy for future investigations. *San Francisco Estuary Institute Regional Monitoring Program Contribution* #815: 2

Vines, C. and Cherr, G. (2012) Ecology, conservation, and restoration of Tidal Marshes: the San Francisco estuary. Edited by Palaima, A. *University of California Press* pp 68–86

Weis, J. and Perlmutter, J. (1987) Effects of Tributyltin on Activity and Burrowing Behavior of the Fiddler Crab, *Uca pugilator*. *Estuaries* 10 (4): 342–346

Weis, J. (1978) Interactions of methylmercury, cadmium, and salinity on regeneration in fiddler crabs *Uca pugilator, Uca pugnax* and *Uca minax. Marine Biology* 49 (2):119–124

Weis, J., Smith, G., Zhou, T., Santiago-Bass, C., Weis, P. (2001) Effects of contaminants on behavior: biochemical mechanisms and ecological consequences: killifish from a contaminated site are slow to capture prey and escape predators; altered neurotransmitters and thyroid may be responsible for this behavior, which may produce population changes in the fish and their major prey, the grass shrimp, *BioScience* 51 (3): 209–217

Wisniewska, M. and Szaniawska, A. (2015) Effect of 17α-ethinylestradiol on the time needed for males and females of *Gammarus tigrinus* Sexton, 1939 to re-couple. *Journal of Environmental Science and Engineering* 4: 419–425

Yang, L., Xiao, S., Luan, T., Tam, N.F.Y. (2018) Overproduction of microbial extracellular polymeric substances in subtropical intertidal sediments in response to endocrine disrupting chemicals. Science of The Total Environment 624: 673–682

Table 1. (A) Mean percentage of crab responses exhibited per behavior (attacks, flees, no response) (B) Mean percentage of crab responses exhibited per behavior (flees, fight initiations, approaches) and mean percentage of time spent performing behaviors (attacking opponent, being attacked without retaliation, no interaction) after 2 weeks of exposure to either control or EE2 sediment.

A. Individual Males

Group	Attacks	Flees	No Response
Control	60%	30%	10%
Experimental	50%	27%	23%

B. Male-Male Interactions

Group	Flees	Fight Initiations	Approaches	Time Spent Attacking Opponent	Time being attacked without retaliation	No Interaction
Control	26%	14%	60%	8%	4%	88%
Experimental	46%	14%	40%	8%	3%	89%

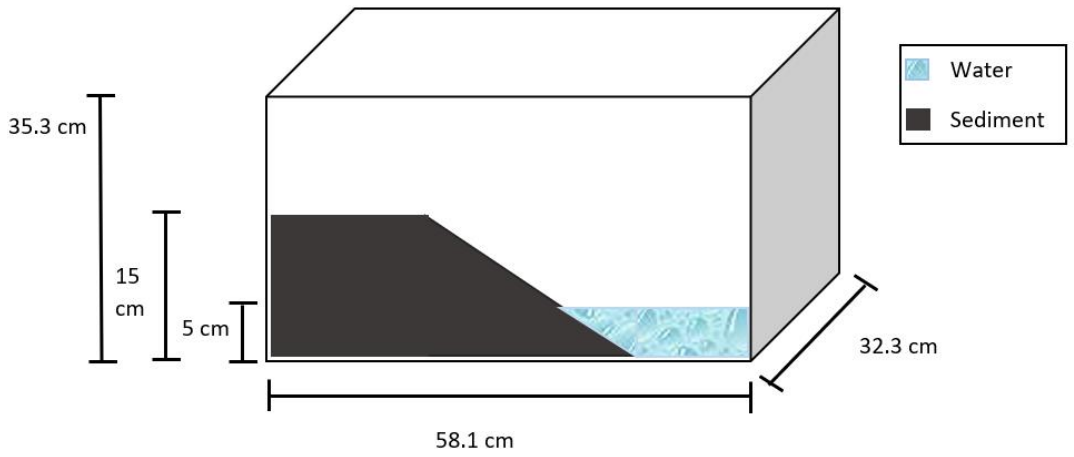


Figure 1. Tank set- up for fiddler crabs during the 2-week exposure period. Sediment (with or without EE2 added) was manipulated to form a steady incline ending in a raised plateau (height, approx. 15 cm) at the one end of the tank. Water from the collection site was added to a depth of 3-5 cm at the lower end of the sediment incline to simulate low tide.

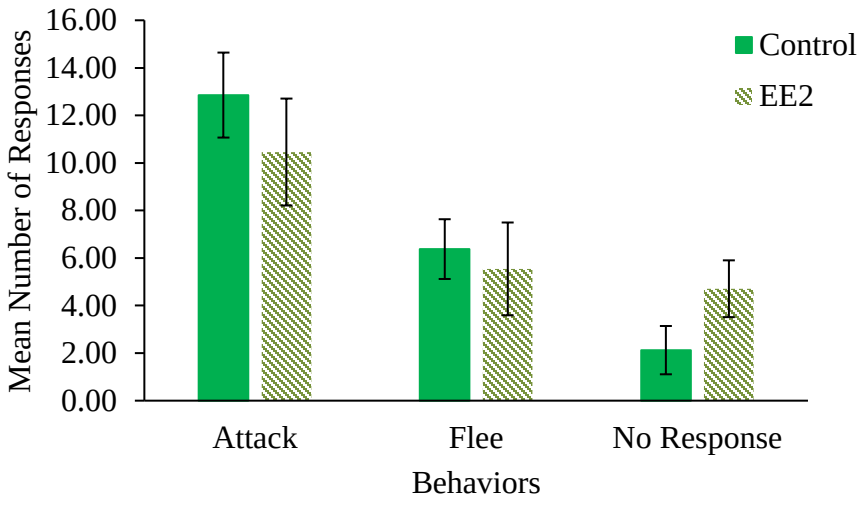
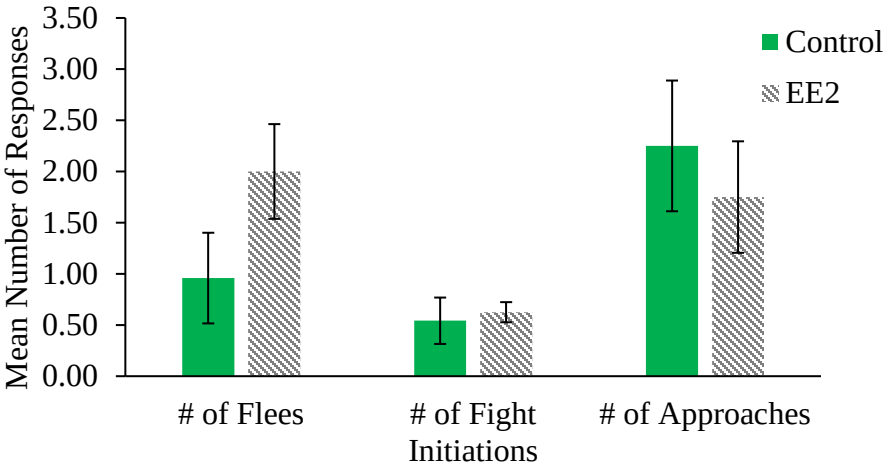


Figure 2. Mean number of crab responses exhibited per behavior after 2 weeks of exposure to either control or EE2 sediment. Error bars are ± one standard error of the mean (SEM) and n=8.

A.



B.

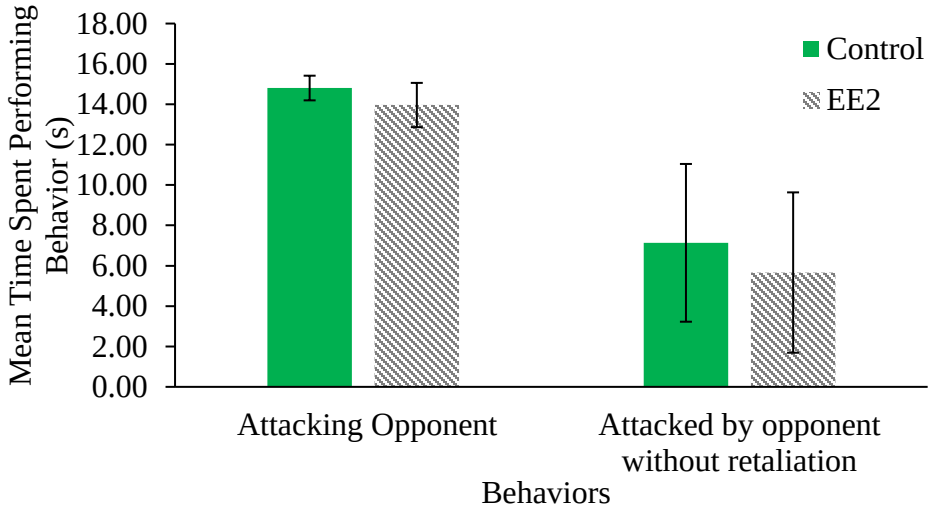


Figure 3. (A) Mean number of crab responses exhibited per behavior (flees, fight initiations, approaches) and (B) mean time spent performing behaviors (attacking opponent, being attacked without retaliation) after 2 weeks of exposure to either control or EE2 sediment. Error bars are ± one standard error of the mean (SEM) and n=8.