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Serotonin and Its Effects on the Chronic Stress Response in Crayfish Following Nonylphenol Exposure

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Serotonin and Its Effects on the Chronic Stress Response in Crayfish Following Nonylphenol Exposure

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The signatories of the committee below indicate that they have read and approved the thesis of Tara Olen in partial fulfillment of the requirements for the degree of Master of Health Science.

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Abstract

Current research suggests that stress in both vertebrates and invertebrates is modulated in part by the neurotransmitter serotonin. It has been shown that crayfish can function as a good neurophysiological model, as they have less complex neurological systems than vertebrates, so mechanistic causes for stress can be more readily studied and understood. One potential stressor for aquatic species such as crayfish would be nonylphenol, a hydrophobic chemical used in agricultural products that can make its way into the water supply due to agricultural runoff. Nonylphenol can lead to physiological and behavioral impairments in crayfish and these impairments likely induce stress in crayfish. Long-term exposure to nonylphenol may therefore induce a chronic stress response in crayfish. One potential target for the treatment of stress is serotonin. Previous research demonstrating that serotonin will increase stress in crayfish has been performed but was conducted using acute stressors, therefore lacking the effects of serotonin on the chronic stress response. Many have also performed behavioral analyses to determine anxiety rather than quantifying stress. Therefore, studies specifically looking at chronic stress may be necessary to give a more in-depth description of the stress response after treatment with serotonin to further the knowledge base on the effects of serotonin on the stress response in crayfish. To determine the relationship between chronic stress and serotonin, chronic stress will be induced via long-term nonylphenol exposure to crayfish, and then we will determine how these stress levels change after exposure to serotonin or a serotonin antagonist.

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Chapter 1: Introduction

Introduction

Stress is due to a feeling of a perceived threat that causes mental or emotional tension, and usually anxiety. Stress can be behavioral, biological, or psychological, and every living species experiences a form of stress. One type of stress is acute stress, in which the stress only lasts a short period of time and is defined by the activation of the fight or flight response and further activation of the adrenomedullary and glucocorticoid pathways. The activation of these pathways in the fight or flight response leads to an increase in heart rate, ventilation, blood pressure, and gluconeogenesis. Another type of stress is chronic stress, in which the stress is consistent and may persist over a significant period of time. Though not universally defined, chronic stress is stress that persists for several weeks and may lead to low energy, decreased appetite, emotional withdrawal, or social isolation. Similar to the increases in glucose seen during an acute stress response, chronic stress can elicit chronic hyperglycemia. Repeated or chronic stress can lead to physical impacts on the body including chronic hypertension, emotional impacts such as anxiety or depression (McGonagle & Kessler, 1990), or a weakened immune system (Landmann et al., 1984). Therefore, discovering causes and potential inhibitors of stress is critical for maintaining proper health.

Recently, it has been shown that crayfish can function as a good neurophysiological model of stress and show stress that can be measured and observed (Abreu et al., 2020). They have less complex neurological systems than vertebrates, so mechanistic causes for stress can be more readily studied and understood. Crayfish have very basic neural development compared to humans but may still act as a good model to further understand human or other mammalian neural circuits (Perry & Baciadonna, 2017). Using crayfish to study neurophysiological

responses may also provide an evolutionary perspective into neurophysiological responses such as the stress responses of mammals, including humans, who may share similar underlying mechanisms for their stress responses (Fosset et al., 2015). Crustacean hyperglycemic hormone (cHH) and hemolymph glucose are both used to measure stress in crayfish, as they mimic the function of glucose in the human stress response. During acute stress, glucocorticoids will function to temporarily increase the levels of glucose within the body. Similarly, chronic stress can elicit hyperglycemic effects. One function of cHH is to raise hemolymph glucose during stress response (Lorenzon, et al., 2004). During a stress response, the body will increase glucose levels to prepare the body with readily available energy. Measuring cHH in the hemolymph will mimic the increase in glucose levels in the human stress response. The results of this study may prove useful for crayfish aquaculture management, either for food production or in a natural ecosystem. Moreover, elucidating chronic stress in crayfish may have translational value to other organisms that experience chronic stress, including humans.

One potential stressor for aquatic species such as crayfish would be nonylphenol, which is a hydrophobic chemical used in agricultural products such as fertilizers, laundry detergents, and several other products that can make their way into the water supply due to agricultural runoff or wastewater. Due to its hydrophobic nature and long half-life, nonylphenol can remain within the sediment for years. Nonylphenol is a known endocrine disruptor in aquatic species, which may lead to disrupted hormone signaling and altered estrogen levels (Kassotis et al., 2020). Decreased male fertility, feminization, and a decrease in survival for aquatic species may also result from increased nonylphenol levels in aquatic environments. Due to its presence in water, nonylphenol can be absorbed and ingested by aquatic organisms such as crayfish, and therefore moved into and along the food web as a result.

Serotonin is a monoamine neurotransmitter known to be associated with mood stabilization, sleep, feeding, and learning. Serotonin is well known to regulate mood in humans and is frequently used in drugs for treating depression. Specifically, serotonin reuptake inhibitors (SSRIs) are prescribed to individuals with depression to increase the amount of serotonin available. In humans, increased serotonin via SSRIs has been used to treat many other disorders such as generalized anxiety disorder, obsessive-compulsive disorder, and panic disorders (Roberts et al.,2020). Previous research showing that serotonin will increase stress in crayfish has been performed (Fossat et al., 2014), but a majority of the research was conducted using acute stressors, therefore not examining the effects of serotonin on the chronic stress response. Many have also performed behavioral analyses to determine anxiety levels rather than quantifying stress. Therefore, studies looking at cHH and chronic stress may be necessary to give a more in-depth description of the stress response after exposure to serotonin to further the knowledge base on the effects of serotonin on the stress response in crayfish. This study will also provide insight into the effects of nonylphenol in crayfish, therefore potentially affecting the environment in which these crayfish live.

Purpose

Observing the effects of serotonin or a serotonin antagonist on chronic stress in crayfish as measured by cHH will provide insight into the effects of serotonin on chronic stress in invertebrates. Chronic stress in invertebrates may affect the environments in which they live and may apply to other species and potentially humans. This study will also demonstrate the impact of nonylphenol on the health of crayfish by determining the stress nonylphenol induces in these invertebrates, and how nonylphenol may affect the aquatic environment in which crayfish reside.

Scope

A total of 50 crayfish, 22 males and 28 females from the species *Faxonius* (formerly *Orconectes) spp.* were exposed to nonylphenol for 35 days. Therefore, the scope of this study is limited to *Faxonius spp.* species. After the chronic nonylphenol exposure, crayfish were exposed to serotonin, a serotonin antagonist (Serotonin-Cinanserin Hydrochloride), or saline control. Hemolymph draws were completed on the crayfish to determine cHH concentrations as a measurement of stress before experimental treatment and following experimental treatment. The exposure period was selected to observe the chronic effects that current research is lacking while remaining within the time restraints of the project.

Assumptions

We assumed that the crayfish in this study would be unstressed prior to stress exposure. We also assumed that a crayfish would show a characteristic stress response and that cHH would accurately measure stress. We assumed that measuring stress variables will elicit additional stress, but this stress will be minimal and equal among experimental and control groups.

Research Questions:

1. What are the effects of chronic nonylphenol exposure on crayfish stress as indicated by CHH concentrations within the hemolymph?
2. How does the treatment of nonylphenol-induced chronic stress with serotonin or a serotonin antagonist affect stress levels in crayfish?

Hypotheses:

1. Chronic nonylphenol exposure will elicit a chronic stress response as defined by high concentrations of cHH in the crayfish hemolymph.
2. Treatment of nonylphenol-induced chronic stress with serotonin will increase stress levels experienced by crayfish as defined by high concentrations of cHH in the hemolymph.
3. Treatment of nonylphenol-induced chronic stress with a serotonin antagonist will decrease the stress levels experienced by crayfish as defined by decreased concentrations of cHH in the hemolymph.

Significance

This study will provide insight into the impacts of chronic stress via nonylphenol exposure. It will also provide information on the stress response via the serotoninergic nervous system's role in modifying stress levels. This will also add to the current literature on the impact of serotonin on stress and may give insight into how serotonin affects stress in other species, including humans by providing a better understanding of the evolutionary basis of the stress response. Lastly, this study will demonstrate the effects that nonylphenol can have on the ecosystem. This will provide an insight into the effects that nonylphenol can have on aquatic organisms such as crayfish, and how it may affect other aquatic organisms within the food web.

Chapter 2: Review of Literature

Definition of Stress and Variations in the Stress Response

Stress is due to a feeling of a perceived threat that causes mental or emotional tension, and usually anxiety. The definition of stress is highly variable, whether it is biological, behavioral, or psychological (Fink, 2016). There are many different forms of stress, and individuals will respond in a variety of ways to different types of stress. One type of stress is acute stress, which involves temporary behavioral and physiological changes by activating two different hormonal pathways: the adrenomedullary pathway and the glucocorticoid pathway. These pathways are considered a part of the fight or flight response. The adrenomedullary pathway, which is typically activated within seconds of the stressor, involves epinephrine and norepinephrine, leading to increases in heart rate, blood pressure, and ventilation. The glucocorticoid pathway in which cortisol is released may impact the immune system and the reproductive system by suppressing body systems not needed in the fight or flight response, as well as raising glucose levels for readily available energy. (Fischer & Romero, 2019).

If acute stress can be defined by its impact on the body, then chronic stress can be defined in this same manner. Chronic stress can be defined as consistent stress that may persist over a significant period of time. According to Yale Medicine (2021), chronic stress is defined as a "consistent sense of feeling pressured and overwhelmed over a long period of time." Some of the symptoms of chronic stress noted in humans are low energy, change in social behavior (more isolation), change in appetite, and emotional withdrawal. If an individual was to experience stress and any combination of these symptoms for a significant period of time, it could be considered chronic stress. Overall, there is not a set amount of time that has been universally

agreed upon for defining chronic stress, but when stress lasts for several weeks and begins to lead to physiological problems is when it can be considered chronic stress.

Overall, previous research has shown that repeated or chronic stress can lead to physical impacts on the body including hypertension, emotional impacts such as anxiety or depression, or a weakened immune system. Specifically looking at animals, Fischer and Romero (2019) noted that the overexposure to stress that animals typically encounter in captivity can lead to many physiological problems, such as a decrease in reproduction, a loss of weight, and decreased immune system. This response is very similar to that of humans when chronically stressed. Acute stress functions to maintain homeostasis (a stable internal environment despite changes to the external environment), while chronic stress impacts allostasis, or the body's ability to maintain homeostasis through physiological or behavioral changes as the conditions change. During acute stress, the hypothalamic-pituitary-adrenal (HPA) axis functions to maintain homeostasis by releasing hormones such as cortisol. Cortisol will have several effects on the body including an increase in gluconeogenesis, temporarily increasing brain functioning as well as memory formation, and decreasing overall inflammation **(figure 1).** Cortisol will contribute to the negative feedback system of the HPA axis, ultimately bringing the body back to a homeostatic level. If stress is prolonged (i.e., chronic), these hormones will continuously be released, altering the HPA axis, and therefore potentially leading to a semi-permanent shift in the quantity of hormone released. This semi-permanent shift in the HPA axis to meet the demands of the body is allostasis. Chronic stress, as a result, can lead to increases in inflammation and an overall decrease in brain function as well as memory formation. To achieve homeostasis or allostasis, the acute stress response and chronic stress response both show an increase in gluconeogenesis. Acute stress increases blood pressure while chronic stress can lead to chronic hypertension.

Acute stress and chronic stress also both show an increase in energy mobilization or the shifting of the body's energy to meet the demands of the body (Shenassa et al., 2021). Therefore, a way we can define acute stress is based on the impacts on the body following hormonal changes following the release of epinephrine, norepinephrine, and cortisol, such as increases in gluconeogenesis, blood pressure, and increase in memory formation. As a result, prolonged stress can lead to allosteric changes to these systems, and therefore chronic stress may lead to a decrease in brain function and memory formation and chronic hypertension. Importantly, both acute and chronic stress lead to an increase in gluconeogenesis.

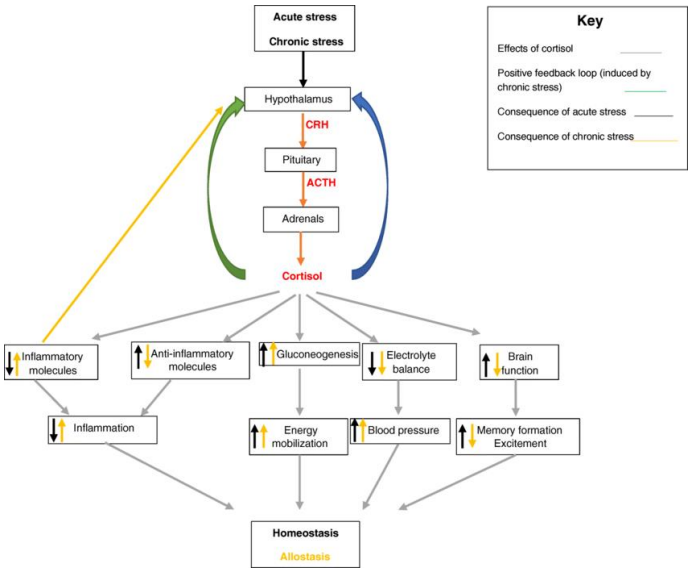

Figure 1: HPA axis response to acute vs. chronic stress (Shenassa et al., 2021)

Research performed by Singh et al., 1999 determined the variation in human stress responses to both physical and psychological stress. Individuals could be classified as high responders to stress if they demonstrated significant increases in adrenocorticotropic hormone (ACTH) following a physical stressor, such as exercise. Those who showed a high response to physical stress also tended to show high responses to psychological stress as well. Those who had a low ACTH response to physical stress also tended to show low responses to psychological stress. This verifies that the stress response does vary on an individual basis.

Many potential factors may determine the variation in stress response per individual. One potential factor for variation in stress response is the binding of the stress hormone, such as

cortisol. Cortisol, a hormone that is frequently associated with stress (acute and chronic), is a steroid hormone that can be measured in blood or saliva. Steroid hormones are typically found bound to a protein such as a corticosteroid-binding globulin. Hormones that are bound are inactive due to the inability to penetrate cell membranes until they are unbound or free. Only when unbound can steroids bind to their target receptors and exert their effects such as anti-inflammatory responses or increase glucose levels within the body. Normally only a small fraction of cortisol in the body is free and is considered active. Individuals will have variations in the number of receptor levels, indicating variations in levels of active cortisol in the body. (Foley, Kirschbaum, 2010). Further confirming this, previous research has shown that the cortisol response to psychosocial stress is correlated to their receptor level of corticosteroid-binding globulin which is a protein that binds cortisol (Kumsta et al, 2007). Cortisol stress responses can also depend on food intake, specifically glucose. Glucose is correlated with increases in cortisol response. Individuals with high levels of glucose may have higher stress responses as a result. Genetics may also play a role in stress responses as well, in that psychosocial stress and cortisol levels have been found to be heritable (Federenko et al., 2004). It has also been previously shown that stress exhaustion, which is a potential result of chronic stress leading to fatigue and a decrease in mental energy, affects the habituation of psychosocial stress, increasing the sensitivity to stress and showing a larger response as a result. Those who do not have exhaustion likely show habituation to the same stressor repeatedly, indicating a decreased stress response (Kudielka et al., 2006).

Repeated or chronic stress may lead to a variety of physical impairments in the body. Acute stress and glucocorticoids released due to stress have previously influenced cognitive functioning such as memory and attention. Specifically, the number of glucocorticoids released and the form

of cognition will vary in this relationship (Vedhara, 2000). There has been an association determined between stress and depressive symptoms, in that chronic stress is more strongly correlated with depressive systems than acute stress is. The emotional effects as a result of acute stressors may also be reduced if chronic stress is also experienced, suggesting that anticipating stress or reevaluating stress may reduce the overall stress response experienced (McGonagle & Kessler, 1990).

Mammalian Stress Models and Results

There are several different coping strategies that animals use to deal with stressors. One type of coping strategy is active coping, which includes confrontational interactions such as fighting or escaping. The other type of coping strategy is passive coping, in which the animal will decrease their responsiveness or immobility altogether (Kaey & Bandler, 2001). Kay and Bandler, 2001 looked further into these stress coping strategies of mammals to determine brain regions associated and effects on the body. They found the periaqueductal gray (PAG) was the region of activation as a result of stress, and the region of the PAG activated determined the coping strategy applied. They determined that both tachycardia and hypertension were induced by stress. Hypertension and tachycardia in the rostral PAG resulted in decreased blood flow to the skeletal muscles and viscera, but an increase in extracranial flow, and was found to induce the passive mechanism. The caudal PAG resulted in increased blood flow to the skeletal muscles and induced the active mechanism. This suggests that animals, including humans, have 2 distinct stress response mechanisms, active or passive, and the region of the PAG that is activated will not only determine the coping mechanism used, but also the type of stressor present (psychological or physical).

Animal models have also been used to determine the function of serotonin and stress. There are over 14 serotonin receptors that have been previously identified (Hannan and Hoyer, 2008). It has been previously determined that serotonin and serotonin receptors play a crucial role in regulating mood, anxiety, and cognition, and drugs that activate this receptor have led to antidepressant responses (Hannan & Hoyer, 2008). There has been a significant amount of research on stress responses in mice. Using a serotonin receptor (HT4R) knockout mice model, Karayol et al. 2021 discovered that losing this serotonin receptor in the hippocampal region of the brain was found to elevate anxiety. The mice that lacked this 5HT4R receptor also show a dysregulation of specific genes that play a role in plasticity as well as neural functioning. Specifically, they found that this 5HT4R receptor regulates both mood and anxiety in mice.

Crayfish as a Model

Recently, invertebrates have been shown to exhibit emotional responses that were thought to have only existed in humans and some mammals. Crayfish have very basic neural development compared to humans, and simpler ways to regulate emotions, yet they may still prove to be useful to further understand human or other mammalian neural circuits (Perry & Baciadonna, 2017). Using crayfish to study neurophysiological responses may provide an evolutionary perspective into the emotional responses of mammals and potentially humans who may also share these basic underlying mechanisms of emotion due to similar homologies between species (Fosset et al., 2015). Several aquatic species, such as crayfish or zebrafish, have been shown to have very similar homologies to mammals, both genetically and physiologically, and show behavioral and physiological responses to stress. In humans, several behaviors can be influenced by emotional states, and many of these behaviors are seen in many other species. These behaviors that can be influenced by emotional states include memory, decision-making,

attention, and many others. (Mathews and MacLeod, 1994; Lerner and Kelter, 2000; Baracchi et al., 2017). Zebrafish and crayfish have previously been shown to be sensitive to drugs that modulate stress as well (Abreu et al., 2020). Overall, previous studies have shown that crayfish are a good model for human emotion, and studying the basic pathways of emotion in these invertebrates may provide an evolutionary perspective into our human emotional responses. Crayfish have natural behaviors which they exhibit during social reactions, such as aggression or avoidance, which may provide strategies for studying anxiety and aggression in these species (Abreu et al., 2020). They also have been shown to exhibit anxiety effects as a result of psychological harassment such as retreating, much like humans or other mammals (Perry & Baciadonna, 2017).

Like humans and many mammals, invertebrates such as crayfish have been shown to have biogenic amines, such as serotonin, dopamine, and noradrenaline, and these biogenic amines have been proven to influence emotion (Bateson et at., 2011). Bateson et al., 2011 also discussed that the serotonin neural system may have been preserved evolutionarily to help regulate emotions, such as anxiety. Because of the potential preservation of this serotonin system, pharmacological manipulation of invertebrates may be a good model for human diseases or disorders that may be linked to emotion (Perry & Baciadonna, 2017). Crayfish are a great laboratory model for many other reasons as well, such as their high reproductive capabilities, easy laboratory maintenance, and manipulation and survival out of water for long-term manipulation (Abreu et al., 2020). Crayfish also experience bioaccumulation; in that they absorb chemicals such as pesticides at a rate faster than it is eliminated via catabolism or excretion. This suggests that crayfish may be a good model to observe the effects of agricultural chemicals on aquatic organisms.

Crayfish Anatomy and Functional Morphology

Crayfish are arthropods, and therefore possess an exoskeleton composed of chitin that functions to protect their internal anatomy. As an arthropod, crayfish will go through stages of molting and replace their exoskeleton as they grow (Duffy and Theil, 2007). As a decapod, crayfish possess 5 pairs of appendages. These appendages serve several functions including locomotion, food handling, sensation, or defense. Crayfish can shed these appendages in response to injury and regenerate these appendages (Gherardi et al., 2010). Like other crustaceans, crayfish possess an abdomen and a cephalothorax, or a joined head and thorax region covered by a carapace. Within the cephalothorax region, decapods possess antennae as well as antennules that play a role in sensation **(figure 2).** Furthermore, the cephalothorax region also features moveable eyestalks on the anterior portion with a compound eye (Gherardi et al., 2010). The base of the eyestalk contains the sinus gland, or the master gland, which is similar to the pituitary gland in vertebrates **(figure 3).** The sinus gland in crustaceans plays a large neuroendocrine role, as it produces many hormones that function to regulate molting, growth, and metabolism (Ruvina et al., In Prep). Similar to the pituitary gland

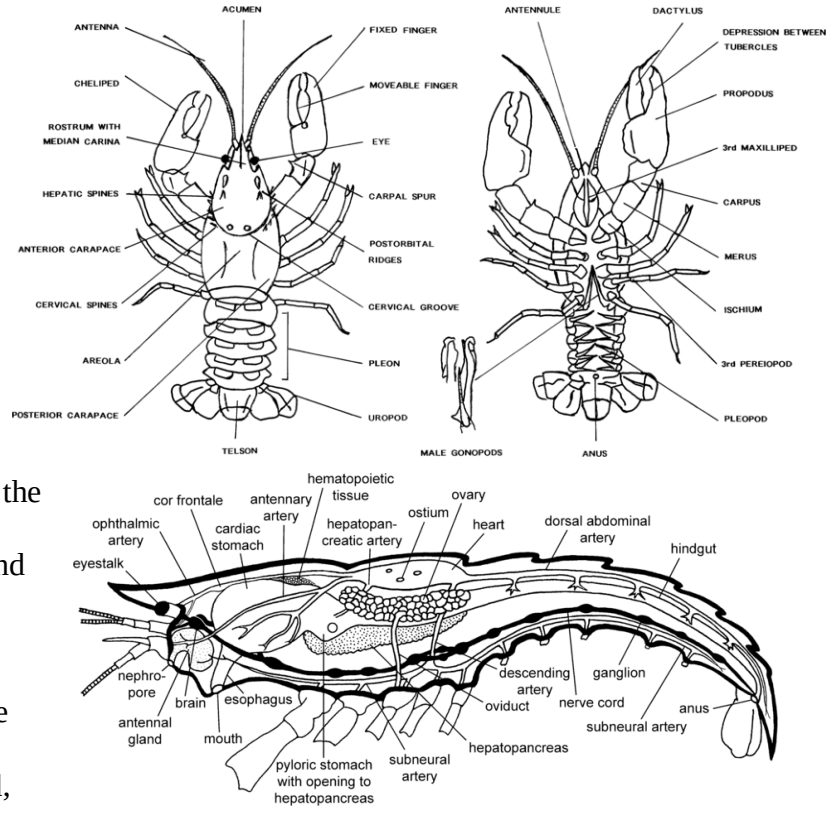

Figure 2: Crayfish Anatomy (Gherardi et al., 2010)

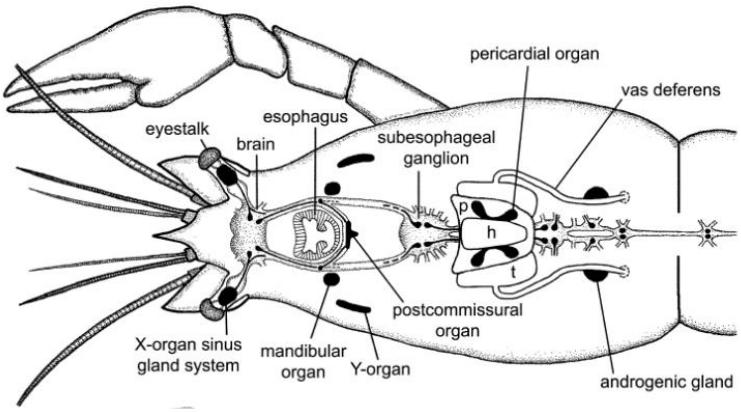

Figure 3: Endocrine System in Crayfish (Gherardi et al., 2010)

in humans, the sinus gland is responsible for synthesizing and secreting several effector hormones (hormones acting on target tissues) and glandotropic hormones (regulating the release of other hormones). A few of the hormones that are released from the sinus gland are the crustacean hyperglycemic hormone (cHH), molt inhibiting hormone (MIH), or mandibular organ inhibiting hormone (MOIH). cHH functions to increase hemolymph glucose levels when in emergency situations or during periods of high levels of activity (Fanjul-Moles, 2006). Neurotransmitters such as GABA and serotonin are known to modulate the release of cHH, and cHH is thought to be regulated by a negative feedback system that is modulated by glucose levels within the hemolymph (Gherardi et al., 2010. This suggests that stress does illicit an increase in cHH, as well as an increase in glucose within the hemolymph of crayfish. Of note, the most common disruptor of the endocrine system of crayfish are environmental disruptors. Exposure to endocrine-disrupting chemicals such as pollutants or pesticides may cause significant complications to the health of crayfish as well as the environment in which they live (Rodriguez et al., 2007).

Crayfish have open circulatory systems and therefore use hemolymph to distribute nutrients and hormones rather than blood within a closed circulatory system. Hemolymph makes up about 27% of the body volume of crayfish, and its concentration varies based on activity, season, molting, or reproduction stage (Gherardi et al, 2010). Of note, the most variable substances within the hemolymph in crayfish are glucose and lipid concentrations.

Assessing Stress in Crayfish - Crustacean Hyperglycemic Hormone (cHH)

Using crayfish as a neurophysiological model, there are several ways in which stress can be assessed in these invertebrates. One common method of measuring stress is by measuring crustacean hyperglycemic hormone, or cHH. This endocrine neuropeptide is known to increase glycolysis for more free glucose (leading to hyperglycemia), lipolysis in the hepatopancreas, and increase glucose utilization (Li et al., 2017; Lin et al., 2013; Chen et al., 2020). Generally, cHH has been determined to be the hyperglycemic hormone. Previously determined was that cHH is released from the eyestalks, specifically the sinus gland of crustaceans, and the study performed by Escamilla-Chimal et al., 2002 further determined the location of secretion of cHH. Using juveniles and adult crayfish, they determined that both adults and juveniles secreted cHH, specifically that juveniles secrete higher amounts, possibly due to developmental stages. Both adult and juvenile crustaceans will release cHH from the eyestalk region, and they noted that cHH is a good measure of hyperglycemic effects.

A study performed by Chang et al, 1998 determined the function of cHH and its capability to measure stress. Using lobsters in their study, they specifically determined the cHH levels following a variety of acute environmental stressors such as emersion (hypoxia), high temperatures, and salinity alterations. In this study, lobsters were exposed to emersion (removal from water) for 4 hours and a significant increase in cHH was detected after only 20 minutes. For the thermal stress, lobsters were exposed to various temperatures, both elevated and lowered compared to the 13°C baseline temperature, which was maintained for 24 hours, at which they found a significant increase after 4 hours for elevated temperatures. Lobsters were exposed to 50% and 150% salinity stress for 6 hours, and there was a slightly significant increase in cHH after 2 hours. The effects of these stressors for a chronic period of time and their effects on cHH

were not measured. To further confirm that cHH is a good measure of stress, research performed by Lorenzon et al., 2004 looked at variations in cHH in the hemolymph produced by the eyestalk of shrimp exposed to stressors such as heavy metals (Copper and Mercury) and lipopolysaccharides. The cHH was measured immediately following exposure, after 0.5, 1, 2, and 3 hours. They found a significant link between exposure to metal or lipopolysaccharides and the release of cHH from the sinus gland into the hemolymph, which was then followed by an increase in blood glucose. This suggests that cHH can be measured directly from the sinus glands located in the eyestalks as well as in the hemolymph. The data showed a significant increase in the following lethal exposure to copper after 2 hours and remained high throughout the experiment. The level of cHH was not quantified following the 3-hour mark. They also found that as the metal concentration increased, so did the rate and amount of cHH released. It was determined in this study as well as several others that there is a basal level of cHH released prior to any stress. This overall shows that cHH has been proven to be released as a result of stress and can be measured in the hemolymph to determine the stress level of a crustacean by the amount of cHH released.

Assessing Stress in Crayfish – Anxiety-like Behaviors

Besides the quantitative assessment of stress using cHH or glucose in the hemolymph, another way previous studies analyzed stress was qualitatively via behavior assessments. One way to assess stress by observing behavior is to use a light/dark maze. This light/dark maze was pioneered by Fosset et al.,2015 and the research they performed to determine anxiety-like behavior in crayfish. Crayfish are known to hide in the dark when they sense danger, which can be seen as an anxiety-like behavior. Based on these natural behaviors, this research group designed a light/dark maze in which the maze was a plus shape with 2 dark protective arms and 2

light aversive arms. For their experiment, they stressed crayfish via an electric shock then placed them into the light/dark maze to observe where the crayfish would spend the most time. Crayfish that were stressed spent the most time in the dark arms, indicating anxiety-like behaviors. Unstressed crayfish spent time exploring both arms of the maze. Adding to this study, they injected the stressed crayfish with an anxiolytic and observed the crayfish exploring both light and dark arms, further indicating this light/dark maze to be effective in determining anxiety-like behavior in crayfish.

Further confirming this light/dark maze's accuracy in measuring anxiety-like behavior, a study performed by Bacque-Cazenave et al., 2017 used this maze to measure anxiety-like behavior following social harassment. In this study, crayfish were placed in tanks for 20 minutes and fought until there was a loser (escaped) and a winner (the harasser) identified. The winner usually would continue to display these aggressive actions even after winning. Then to determine anxiety, the crayfish were placed in the light/dark maze following the social interaction. It was found that losers expressed anxiety-like behaviors by remaining in the dark arms of the maze and this anxiety correlated with the stress intensity of the harassment they experienced. They were also injected with an anxiolytic and found a decrease in anxiety-like behavior. Together these studies suggest that the light/dark maze is a consistent way to measure behavioral anxiety following stress, allowing the researchers to observe acute stress response behaviors.

Based on the previous research by Kaey and Bandler (2001), there are several coping strategies that animals use when stressed, and therefore hiding behaviors may not be the only characteristic behavior of stress. One type of coping strategy is active coping mechanisms, which include confrontational interactions such as fighting or escaping. The other type of coping strategy is passive coping, in which the animal will decrease their responsiveness or immobility altogether.

Therefore, due to the potential variations in stress responses, there are several variables that may be observed in crayfish when stressed, such as frequency of fights, tail flip escape responses, time spent immobile, responsiveness to olfactory stimuli, in addition to hiding behaviors.

Chronic Stress in Crayfish

Chronic stress was defined as stress that is consistent and may persist for a significant period of time and leads to physiological problems such as low energy, change in social behavior (more isolation), change in appetite, and emotional withdrawal. As previously discussed, stress exhaustion, which is a potential result of chronic stress, increases the sensitivity to stress and leads to a larger response as a result. (Kudielka et al., 2006). Crayfish have been shown to function as a model for studying chronic stress, which can be observed in the study performed by Bao, et al., 2020, in which chronic copper exposure was analyzed. In this study, the effects of both acute and sub-chronic toxicity of copper and its effects on respiratory metabolism as well as copper accumulation were evaluated. They determined that acute exposure was after 96 hours of exposure, and sub-chronic exposure was after 14 days. The results determined that both acute and chronic copper exposure leads to an inhibition of oxygen consumption rate as well as the rate of ammonium excretion. It was also determined that for copper accumulation, there was an accumulation of copper within the hepatopancreas and the muscles, which were highest after 14 days and were significantly more than the control crayfish had. This study suggests that like acute exposure to a stressor, chronic exposure also can have significant impacts on the body of these invertebrates. Research performed by Chabera et al., 2021 also focuses on chronic stress and its effects on crayfish. In this study, crayfish were exposed to chloridazon, a herbicide, or its metabolite, chloridazon-desphenyl. There was a 10-day acclimation period, followed by exposure to chloridazon or chloridazon-desphenyl at varying concentrations, with the exposure

persisting over 30 days. Following the 30-day exposure, the crayfish experienced a 15-day recovery period in which the crayfish were housed in chloridazon or chloridazon-desphenyl free environments. This study found that chronic exposure to chloridazon, as well as chloridazon-desphenyl, were associated with increased levels of glucose in the hemolymph, and increased glucose levels are known to be an indicator of stress. This study suggests that chronic exposure to a stressor, such as a herbicide, does elicit long-term effects on crayfish, such as increased glucose levels in the hemolymph. It also suggests that chronic stress can be manifested by the same changes in hemolymph glucose levels as acute stress, and likely increases in cHH as well, but this relationship has not been investigated.

Serotonin

Serotonin is a monoamine neurotransmitter known for its role in mood, cognition, social responses, and learning. It is thought to play a role in several behaviors, such as locomotion, increased aggression, sleep-wake cycles, feeding, mood, learning, and anxiety-like behavior. Serotonin is a complex neurotransmitter, with over 14 known sub receptors, most of which are inhibitory G-protein coupled receptors (Roberts et al., 2020). Because of this large variation in receptors and signaling networks, the effects of serotonin are very species-dependent, based on genetics, sociability, or even stress (Kiser et al., 2012). The environment in which the animal lives may also have effects of serotonin. In mammals, it has been determined that the dorsal raphe is active during uncertainty or surprise and that stress has been found to activate serotonin. A study by Carhart-Harris & Nutt, 2017 suggests that increasing serotonin under extreme conditions such as stress leads to an increase in plasticity and regulations of stress mechanisms to deal with these conditions. Another application of serotonin to stress is found in a study performed by Fosset et al., 2014, which suggests that there is species-specific avoidance, and this

study specifically indicates risk assessment in crayfish, which can be enhanced when injected with serotonin. This indicates that serotonin may play a role in risk assessment as well. So overall, the relationship between serotonin and stress is very complex due to many factors, specifically the vast number of sub-receptors for serotonin. It has been shown that serotonin does play a role in stress, uncertainty, and surprise, in that serotonin will increase behavior modifications, learning systems, and neuroendocrine function as a response to stressful situations.

Serotonin is also known for its role in treating depression. As previously mentioned, serotonin is known for its role in mood regulation. Serotonin reuptake inhibitors (SSRIs), which block the reuptake of serotonin leading to an increase in serotonin within the synaptic cleft, are used frequently as a treatment for depression. There also seems to be a relationship between major depressive disorder and stress, in that many of the brain systems frequently involved in depression are commonly associated with regulation of mood, anxiety, fear, and stress (Price & Drevets, 2012). Together, this suggests that there may be overlap in mechanisms found in depression and stress, indicating that there may be a relationship between the 2 conditions, and this may be beneficial for the treatment of stress or depression.

Serotonin and its Effects on Stress and Anxiety

There has been a great amount of research discussing serotonin and its many effects on the body, including serotonin's function on mood and anxiety. Research performed by Karayol et al., 2021 used a serotonin receptor knockout mouse to determine the role of serotonin on mood and anxiety in mice. They determined that losing this serotonin receptor in specific brain regions, such as the hippocampus, led to not only an antidepressant response, but also increased anxiety

as well, and these regions typically showed decreased neuroplasticity and less excitability as a result. Furthermore, this study also evaluated the role of serotonin reuptake inhibitors (SSRIs) on depression and anxiety. They found that chronic use of SSRIs led to increased anxiety (Baek et al. 2015), possibly due to decreased binding of these serotonin receptors and a decrease in cAMP second messenger signaling and therefore excitability (Vidal et al., 2009). This sprouts the idea that potentially long-term increases in serotonin may lead to increased anxiety as well.

A study performed by Fosset et al., 2015, who studied the role of serotonin as well as dopamine on the crayfish stress response, looked at anxiety-like behavior and serotonin in crayfish. They determined that serotonin concentration was associated with increased anxiety-like behavior, which was observed using a light/dark maze and determining the amount of time spent in the dark arms compared to the illuminated arms. The degree of anxiety-like behavior was found to be strictly associated with serotonin levels, and not dopamine levels. They believed this was due to differential metabolic pathways following stress, even though both dopamine and serotonin have similar biosynthesis. Importantly, this study shows the stress response in crayfish, much like mammals, involves neuroendocrine function. There are also serotonin antagonists, which function to decrease the action of serotonin by binding to serotonin receptors and preventing serotonin from binding or decreasing the amount of serotonin available, therefore inhibiting the effects of serotonin on the body. Therefore, if an increase in serotonin leads to anxiety-like behavior, then a serotonin antagonist will likely decrease this anxiety, but there is a lack of research in this area.

Research performed by Lorenzon et al., 2005 further looked at the role of biogenic amines, specifically serotonin and dopamine, to determine their effects on the hyperglycemic stress response. Looking specifically at the association between serotonin and stress in crayfish,

it has been determined that serotonin leads to increased release of cHH from the eyestalk into the hemolymph, which was then followed by hyperglycemia. This association was not seen after the dopamine injection, as it did not significantly alter cHH or glycemic levels. This research also looked at serotonin levels in the hemolymph following stress exposure and determined that more serotonin was released following an increase in stress intensity (determined using an increased concentration of copper contamination). Another interesting find in this study was that following copper contamination, there was an increase in serotonin from the eyestalk which induced both a cHH increase and hyperglycemia, but this was only seen in crayfish with intact eyestalks, indicating the significance of the eyestalk for cHH and glycemic levels. Building off this idea was a study by Escamilla-Chimal et al., 2002, which further confirmed that cHH is released specifically from the eyestalk and retinal cells and is primed by serotonin using both juvenile and adult crayfish. They found that increases in cHH following increases in serotonin were age-dependent, in that juveniles showed higher cHH secretion following serotonin than adults showed, indicating that serotonin sensitivity may be related to cell maturation and development.

Nonylphenol

Nonylphenol is an alkylphenol chemical that is known to be an endocrine disruptor, as its structure is very similar to that of the endocrine hormone estradiol. It has been found in a variety of aquatic environments, mainly in the soil. Nonylphenol can get into the water supply through several routes such as agricultural runoff, pesticide runoff, fertilizers, livestock feedings, or wastewater. Industrially, nonylphenol has been used by many industries as a non-ionic surfactant and has been used in many products such as personal care products, cleaning

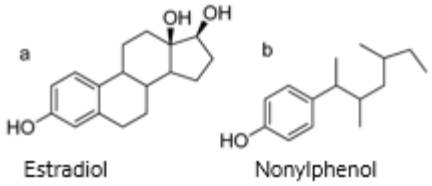

Figure 4: Structure of nonylphenol and its similarity to estradiol.

products, metalworking, and several others (De Bruin, et al., 2019). Structurally, nonylphenol is composed of a phenol ring and a nine-carbon chain **(figure 4),** making it a very hydrophobic chemical with low solubility and is semi-volatile. Due to its hydrophobic nature, nonylphenol has a significantly longer half-life and will persist in the soil for years. Because of its long duration and toxicity to many organisms, determining nonylphenol's effects on the body, such as on the neurological, immunological, or reproductive system is essential. It is also crucial to monitor nonylphenol levels within the environment because aquatic organisms are consistently exposed to this chemical in their environment, and therefore have the potential to bioaccumulate within organisms (Mackay et al., 2017). As stated previously, nonylphenol is a known endocrine disrupter, which can therefore affect endocrine functioning such as hormone signaling \and lead to disease (Kassotis et al., 2020). These endocrine disruptors can also influence estrogen activity as well (Gingrich et al., 2020). Endocrine disruptors such as nonylphenol can be found in food, food packaging, medical supplies, and pesticides which then can make their way into the environment, ultimately leading to impacts not only on the environment but also on aquatic organisms and human health. (Khan et al., 2020).

There are several aversive effects of nonylphenol on both human health as well as on the environment and aquatic organisms. One aversive effect nonylphenol has on human health is cancer, in which nonylphenol exposure has been positively associated with cancer (Noorimotlagh et al., 2020). Specifically, this research found that nonylphenol induced breast cancer progression, and found a positive association between nonylphenol and many other types of cancer, including ovarian, uterine, pituitary, and testicular. This is likely due to nonylphenol being a known endocrine disruptor. Looking specifically at aquatic organisms, nonylphenol has been shown to lead to feminization, reduced male fertility, and a decrease in overall survival

among juveniles, even at low concentrations (Soares et al., 2008, Yang et al., 2020). Research performed by Hong et al., 2020 discussed the effects of nonylphenol on invertebrates, such as crayfish, and determined that nonylphenol has substantial acute toxicity to these invertebrates. Due to nonylphenol getting into the water supply, fish and other aquatic organisms take in this chemical, and bioaccumulation occurs, and therefore it gets passed along the food chain to other organisms.

Looking further into the effects of nonylphenol on crayfish, a study performed by Swift et al. (in press) determined how nonylphenol exposure affects the survival as well as orientation behavior of crayfish. Crayfish were exposed to varying concentrations as well as durations of nonylphenol. Half of the crayfish were exposed to nonylphenol for 24 hours which represented an acute exposure while the other half were exposed for 33 days, representing a long-term exposure. This study determined that even small doses of nonylphenol lead to the lethality of crayfish. At sublethal levels, nonylphenol exposure led to an impaired orientation to locate food. The crayfish were exposed to a lower concentration of nonylphenol during the long-term duration, as this would represent a runoff event. Similar to the acute exposure, they found that long-term exposure also led to disorientation, specifically at the beginning of the exposure, which would decrease as the exposure continued. Following the exposure periods, the crayfish experienced a 2-week recovery period of no nonylphenol exposure, and interestingly they found that the crayfish still showed a decrease in food-finding ability. Overall, this study indicates that even at moderately low nonylphenol concentrations this chemical can be lethal to crayfish, and at lower levels, nonylphenol can lead to both physiological and behavioral impairments.

As previously discussed, nonylphenol can lead to physiological and behavioral impairments, such as orientation behavior and survival of crayfish. These impairments likely will increase the

stress experienced by a crayfish. Long-term exposure to sub-lethal levels of nonylphenol may therefore induce a chronic stress response in crayfish following an exposure. Environmentally, this is significant in that crayfish are a keystone species, and therefore many other species rely on the presence of crayfish for survival. Economically, the health of crayfish is essential for aquaculture. If the health of crayfish were to decline, this could lead to detriments in food production not only for crayfish but also for other species that consume crayfish. This research investigates the chronic stress response induced by long-term exposure to nonylphenol, and the effects of serotonin or a serotonin antagonist on this chronic stress response following nonylphenol exposure in crayfish.

Chapter 3: Methodology

Experimental Group Selection

A total of 50 crayfish, *Faxonius* (formerly *Orconectes) spp.*, consisting of 22 males and 28 females of varying adult ages and sizes, were collected from the Grand River watershed in Michigan and used for experimental trials. Immediately following collection, crayfish were separated based on sex and kept in large aquariums until assigned to a group. Crayfish were weighed, measured, and kept isolated in 1L tanks and fed a normalized diet of one rabbit pellet once a week. The average weight of the crayfish in the study was 6.95g and the average length of 6.17cm. The crayfish were kept at a constant temperature (22-23°C) to avoid thermal stress, and a light: dark cycle of 14 hr:10hr. Isolation tanks were cleaned weekly with 100% water changes. After separating the crayfish based on sex, crayfish were randomly assigned to one of four groups as shown in **table 1**, E1 began with 40 crayfish, E2 and E3 began with 15 crayfish, and E4 began with 14 crayfish, which are necessary due to nonylphenol's potential to cause death even below EPA acceptable levels (EPA, 2014; Swift et al., In Prep).

Exposure set-up

Nonylphenol was purchased from Sigma-Aldrich. Acetone was used as a vehicle due to nonylphenol's viscous and hydrophobic nature. The exterior of the pipette in which the nonylphenol was added to the acetone was cleaned with acetone to ensure no additional nonylphenol will be added to the solution. The negative control tanks received vehicle acetone with water into their tank due to acetone being added to the tanks receiving nonylphenol to keep the set-up consistent. The acetone concentration for all tanks was 2.0 mL per 1L tank. Previous

work has demonstrated that acetone at this level does not negatively impact crayfish behavior (Swift et al., In Prep).

Each group contained half males and half females, which were randomly assigned to a group as shown in **table 1**. The groups receiving nonylphenol received a sublethal dose of 0.15 µl/L of nonylphenol mixed with 2.0 mL of acetone stock solution. This concentration of nonylphenol was determined to be a sub-lethal concentration that still led to effects by a previous study performed by Swift et al. (In Prep).

Table 1: Experimental Groups

Group	**Number of Crayfish***	**Nonylphenol Exposure (Y/N)**	**Experimental Manipulation**
E1: Negative Control	23 (9 male, 14 female)	No	Ringer's Saline
E2: Positive Control	7 (4male, 3 female)	Yes	Ringer's Saline
E3: Experimental Group 1	11 (6 male, 5 female)	Yes	Serotonin
E4: Experimental Group 2	9 (3 male, 6 female)	Yes	Serotonin-Cinanserin Hydrochloride

Table 1: Experimental Groups: *Group 1 began with 40 crayfish (20 male, 20 female), Group 2 began with 15 crayfish (8 male, 7 female), Group 3 began with 15 crayfish (8 male, 7 female), and group 4 began with 14 crayfish (7 male, 7 female). Groups 2-4 began with fewer crayfish due to time constraints and limited space within the lab. Numbers displayed in the table represent the number of crayfish in each group at the end of the experiments.

Drug Compound Selection

As described by the study performed by Reisinger, et al., 2021, each compound will be added to the 1L isolation tanks at an environmentally realistic concentration of 0.5µg/L.

Drug Exposure Protocol

Serotonin or a serotonin antagonist was administered directly to the 1L isolation tanks where the crayfish were housed throughout the experiment to investigate the effects of serotonin on the chronic stress levels of crayfish following the nonylphenol exposure. The experimental group E3 crayfish received serotonin, while the experimental group E4 crayfish received serotonin-cinanserin hydrochloride, a serotonin antagonist. Ringer's saline was administered to crayfish which were randomly assigned to the control groups. Crayfish were treated with serotonin, serotonin-cinanserin hydrochloride, or saline at an environmentally realistic dose of 0.5µg/L, per the study conducted by Reisinger et al, 2021.

To accurately add 0.5µg/L to each isolation tank, a stock solution was produced for each drug (serotonin and serotonin-cinanserin hydrochloride). For the antagonist, serotonin-cinanserin hydrochloride, the quantity of 0.1mg of the drug was diluted to .100L, therefore 1mg of serotonin-cinanserin hydrochloride per liter would give 1000ug/L. This functions as the stock solution. From this stock solution, 0.5mL of the stock solution was added to the 1L isolation tanks, giving a final concentration of 0.5µg/L.

For the measurement of serotonin, the quantity of 25mg was diluted in 1 liter of water, therefore 25mg/L, or 25,000µg/L as the stock solution. From this stock solution, 0.02mL was added to the 1L isolation tanks, giving a final concentration of 0.5 µg/L.

In previous studies (Huber and Delago 1998; Bacque-Cazenave et al. 2018) which examined the effects of injected serotonin on stress and behavior in crayfish, it was discovered that altering serotonin levels increased aggression and boldness. In the study performed by Reisinger et al., 2021 study, similar outcomes were observed in crayfish behavior when serotonin-modifying drugs were added to the water of the crayfish at an environmentally realistic concentration,

indicating that water exposure to the drug can produce outcomes similar to those of direct injection.

Because it takes time for a drug to accumulate within an organism, impacts from the drug are not immediately apparent, therefore, hemolymph samples were obtained 48 hours after the crayfish are delivered the drug via water (Reisinger et al., 2021).

Experiment 1: Nonylphenol-Induced Stress Analysis

To analyze the effects of nonylphenol on chronic stress in crayfish, crayfish were acclimated in isolation tanks for 1 week. Following acclimation, crayfish in the positive control tanks and the two experimental group tanks were exposed to 0.15 µl/L nonylphenol with 2.0mL per 1L water. The negative control tanks were exposed to 2.0 mL of acetone per 1L water. Crayfish were held at these exposure levels for 35 consecutive days representing chronic exposure, as defined by the EPA (2014). Water was changed every 7 days to ensure constant concentrations of nonylphenol throughout the experiment, as well as to ensure a clean environment for the crayfish. After 35 days, crayfish were assessed for their stress level via hemolymph draws following chronic exposure to the stressor.

cHH Collection and Analysis

To determine cHH levels within the hemolymph of crayfish, 0.05mL hemolymph was extracted from the sterna sinus of the crayfish via a 25-gauge hypodermic needle as described by Caldari-Torres et al. (2018). Previous research by Lorenzon et al., 2004 determined that cHH is released via the sinus gland and released into the hemolymph, and therefore cHH can be measured via hemolymph. Due to the size of the crayfish and the availability of hemolymph, roughly 5

crayfish hemolymph samples per group were combined per vial for a total of 0.25mL hemolymph per vial. Initial hemolymph draws were collected prior to nonylphenol exposure and then again after 35 days, representing a chronic exposure to a stressor. Hemolymph was also collected after exposure serotonin, Serotonin-Cinanserin Hydrochloride, or Ringer's saline to determine the effects of these drugs on stress levels in crayfish. Levels of cHH were tested via an ELISA assay kit designed to measure the concentration of cHH within the hemolymph of crayfish. The cHH ELISA was run as a 48-well plate, in which 50µl of hemolymph per vial was used per well. This cHH ELISA kit detected cHH concentrations ranging from 31.2pg/ml – 1000pg/ml, and contained 6 standards (31.5 pg/ml, 62.5 pg/ml,125 pg/ml,250 pg/ml,500 pg/ml, and1000 pg/ml) to create a standard curve at an absorbance of 450nm, allowing us to assess the concentration of cHH per vial and therefore the average cHH concentration per crayfish **(figure 5).**

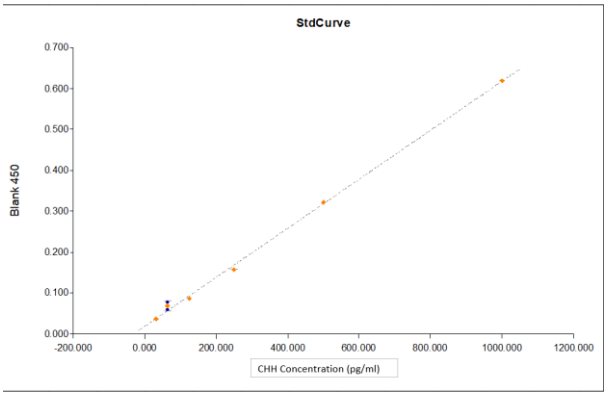

Figure 5: Standard Curve for CHH ELISA at an absorbance of 450nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	BLK	STD1 31.2	STD2 62.5	STD2 62.5	STD3 125	STD4 250	STD4 250	STD5 500	STD6 1000	SPL1	SPL2
B	SPL3	SPL4	SPL5	SPL6	SPL7	SPL8	SPL9	SPL10	SPL11	SPL12	SPL13	SPL14
C	SPL15	SPL16	SPL17	SPL18	SPL19	SPL20	SPL21	SPL22	SPL23	SPL24	SPL25	SPL26
D	SPL27	SPL28	SPL29	SPL30	SPL31	SPL32	SPL33	SPL34	SPL35	SPL36	SPL37	SPL38
E												
F												
G												
H												

Figure 6: cHH ELISA Plate Layout

There was a total of 38 hemolymph samples, 6 standards, and 2 blank wells set up on the plate, with each sample running as a singlet **(figure 6).** The 50µl standards and 50µl hemolymph samples were loaded into the 48-well plate, and 100 µl of HRP-Conjugate reagent was added to each well except the 2 blank wells. After this addition of HRP-Conjugate reagent, the plate was covered with a closure plate membrane and incubated for 60 minutes at 37℃. Following the incubation period, the wells were washed 4 times, and then 50µl of chromogen solution A

and 50µl of chromogen solution B were added to each well, mixed gently, and incubated for 15 minutes at 37℃. After the incubation period, there were noticeable color changes from clear to blue in the wells, with the standards showing a proper gradient of blue coloring and each of the sample wells showing a blue gradient within the ranges of the standards. Then 50µl of stop solution was added to each well and there was another color change to yellow in each well **(figure 7).** The plate was read at an absorbance of 450nm. Statistical analysis was performed using Prism. A two-way ANOVA was performed to determine the statistical significance of the hemolymph samples. The alpha factor for the two-way ANOVA was $\alpha = 0.05$.

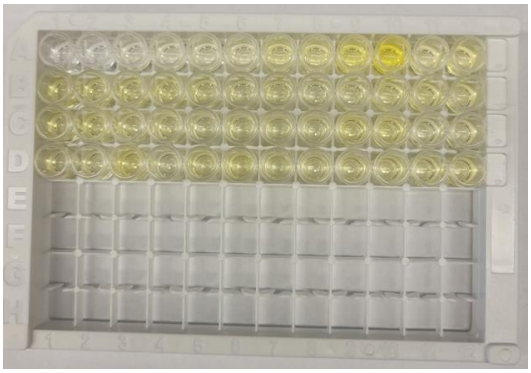

Figure 7: cHH ELISA plate after stop solution was added. A1-A2 were blanks, A3-A10 were standards.

Experiment 2: Serotonin and Stress Analysis

To analyze the effects of serotonin on the chronic stress levels of crayfish, crayfish were exposed via water to serotonin, Serotonin-Cinanserin Hydrochloride, or Ringer's Saline following the 35 days of chronic nonylphenol exposure. After the remeasurement of stress variables following the initial nonylphenol exposure, crayfish were exposed to serotonin, Serotonin-Cinanserin Hydrochloride, or saline, and stress variables were remeasured after 48 hours.

Chapter 4: Results

cHH Analysis:

To determine the average cHH concentration per crayfish, each group had multiple vials of 0.25ml hemolymph, with each vial containing roughly 5 crayfish hemolymph samples (0.05ml per crayfish). The negative control group (E1), for example, at baseline contained 8 vials (4 female vials, 4 male vials), after nonylphenol exposure had 6 vials (4 female vials, 2 male vials), and after the drug exposure contained 6 vials (4 female vials, 2 male vials). Since there were multiple vials per group, and multiple crayfish per vial, the average cHH concentration per crayfish in each vial was calculated (Ex. vial 1 had a cHH concentration of 194.479pg/ml (determined by ELISA output as shown in **table 2**), and was a combination of 5 crayfish hemolymph samples, therefore the average cHH concentration per crayfish in that vial was 38.896pg/ml). Then we averaged the total vials per group at each time point to determine the concentration of cHH per crayfish, per group, as depicted in **table 6.**

Table 2: ELISA Assay Data

Well ID	Name	Well	Conc/Dil	Blank 450	[Concentration]	Count	Mean	Std Dev	CV (%)
BLK		A1		0	<0.000	0	?????	?????	?????
		A2		0	<0.000				
SPL1		A11		0.135	194.479	1	194.479	?????	?????
SPL2		A12		0.158	231.882	1	231.882	?????	?????
SPL3		B1		0.179	267.114	1	267.114	?????	?????
SPL4		B2		0.17	252.087	1	252.087	?????	?????
SPL5		B3		0.158	233.051	1	233.051	?????	?????
SPL6		B4		0.174	259.767	1	259.767	?????	?????
SPL7		B5		0.122	171.604	1	171.604	?????	?????
SPL8		B6		0.1	134.869	1	134.869	?????	?????
SPL9		B7		0.13	185.797	1	185.797	?????	?????
SPL10		B8		0.038	132.531	1	132.531	?????	?????
SPL11		B9		0.122	171.771	1	171.771	?????	?????
SPL12		B10		0.156	228.543	1	228.543	?????	?????
SPL13		B11		0.16	235.556	1	235.556	?????	?????
SPL14		B12		0.151	220.361	1	220.361	?????	?????
SPL15		C1		0.153	223.533	1	223.533	?????	?????
SPL16		C2		0.178	265.779	1	265.779	?????	?????
SPL17		C3		0.125	177.114	1	177.114	?????	?????
SPL18		C4		0.139	199.823	1	199.823	?????	?????
SPL19		C5		0.099	134.368	1	134.368	?????	?????
SPL20		C6		0.122	171.938	1	171.938	?????	?????
SPL21		C7		0.101	137.039	1	137.039	?????	?????
SPL22		C8		0.115	161.251	1	161.251	?????	?????
SPL23		C9		0.184	276.131	1	276.131	?????	?????
SPL24		C10		0.188	282.476	1	282.476	?????	?????
SPL25		C11		0.105	144.386	1	144.386	?????	?????
SPL26		C12		0.119	167.93	1	167.93	?????	?????
SPL27		D1		0.138	199.489	1	199.489	?????	?????
SPL28		D2		0.123	173.44	1	173.44	?????	?????
SPL29		D3		0.211	320.547	1	320.547	?????	?????
SPL30		D4		0.085	110.824	1	110.824	?????	?????
SPL31		D5		0.152	222.031	1	222.031	?????	?????
SPL32		D6		0.169	250.584	1	250.584	?????	?????
SPL33		D7		0.116	163.088	1	163.088	?????	?????
SPL34		D8		0.11	151.393	1	151.393	?????	?????
SPL35		D9		0.122	172.272	1	172.272	?????	?????
SPL36		D10		0.138	198.388	1	198.388	?????	?????
SPL37		D11		0.132	188.468	1	188.468	?????	?????
SPL38		D12		0.162	239.73	1	239.73	?????	?????
STD1		A3	31.2	0.038	31.176	1	31.176	?????	?????
STD2		A4	62.5	0.06	63.08	2	84.191	21.371	25.384
		A5	62.5	0.078	99.303				
STD3		A6	125	0.087	113.83	1	113.83	?????	?????
STD4		A7	250	0.158	232.55	2	232.467	0.118	0.051
		A8	250	0.158	232.383				
STD5		A9	500	0.321	505.224	1	505.224	?????	?????
STD6		A10	1000	0.619	1001.813	1	1001.81	?????	?????

Table 2**:** Data generated from cHH ELISA Assay. Std 2 and Std 4 were run as duplicates, all others were run as singles on the 48 well plate. Concentration units are pg/ml.

Table 3: Results from Two-way ANOVA

2way ANOVA ANOVA results					
Table Analyzed	Avg. CHH Level (total)				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significant?**	
Interaction	33.69	<0.0001	****	Yes	
Row Factor	5.163	0.0014	**	Yes	
Column Factor	14.60	<0.0001	****	Yes	
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	21791	6	3632	F (6, 173) = 17.70	P<0.0001
Row Factor	3340	3	1113	F (3, 173) = 5.424	P=0.0014
Column Factor	9441	2	4721	F (2, 173) = 23.00	P<0.0001
Residual	35506	173	205.2		
Data summary					
Number of columns (Column Factor	3				
Number of rows (Row Factor)	4				
Number of values	185				

Table 3: Two-way ANOVA results from analysis performed on Prism.

Table 4: Within Group Results

Within each row, compare columns (simple effects within rows					
Number of families	4				
Number of comparisons per family	3				
Alpha	0.05				
Tukey's multiple comparisons test	**Predicted (LS) mean diff.**	**95.00% CI of diff.**	**Below threshold?**	**Summary**	**Adjusted P Value**
E1 (- Control)					
Baseline vs. Post Nonyl Exp	13.84	5.463 to 22.21	Yes	***	0.0004
Baseline vs. Post Drug Exp	5.743	-3.298 to 14.78	No	ns	0.2926
Post Nonyl Exp vs. Post Drug Exp	-8.092	-17.48 to 1.294	No	ns	0.1062
E2 (+ Control)					
Baseline vs. Post Nonyl Exp	-22.00	-37.22 to -6.784	Yes	**	0.0023
Baseline vs. Post Drug Exp	-39.11	-54.99 to -23.23	Yes	****	<0.0001
Post Nonyl Exp vs. Post Drug Exp	-17.11	-34.63 to 0.4222	No	ns	0.0575
E3 (Serotonin)					
Baseline vs. Post Nonyl Exp	-7.245	-20.80 to 6.313	No	ns	0.4179
Baseline vs. Post Drug Exp	-14.86	-28.73 to -0.9833	Yes	*	0.0326
Post Nonyl Exp vs. Post Drug Exp	-7.613	-21.75 to 6.524	No	ns	0.4123
E4 (Serotonin Ant)					
Baseline vs. Post Nonyl Exp	-48.26	-62.73 to -33.79	Yes	****	<0.0001
Baseline vs. Post Drug Exp	-16.75	-31.22 to -2.283	Yes	*	0.0187
Post Nonyl Exp vs. Post Drug Exp	31.50	15.54 to 47.47	Yes	****	<0.0001

Test details	**Predicted (LS) mean 1**	**Predicted (LS) mean 2**	**Predicted (LS) mean**	**SE of diff.**	**N1**	**N2**	**q**	**DF**
E1 (- Control)								
Baseline vs. Post Nonyl Exp	54.00	40.17	13.84	3.541	36	30	5.525	173.0
Baseline vs. Post Drug Exp	54.00	48.26	5.743	3.824	36	23	2.124	173.0
Post Nonyl Exp vs. Post Drug Exp	40.17	48.26	-8.092	3.970	30	23	2.882	173.0
E2 (+ Control)								
Baseline vs. Post Nonyl Exp	24.41	46.41	-22.00	6.438	13	8	4.834	173.0
Baseline vs. Post Drug Exp	24.41	63.51	-39.11	6.716	13	7	8.235	173.0
Post Nonyl Exp vs. Post Drug Exp	46.41	63.51	-17.11	7.414	8	7	3.263	173.0
E3 (Serotonin)								
Baseline vs. Post Nonyl Exp	28.70	35.95	-7.245	5.735	13	12	1.787	173.0
Baseline vs. Post Drug Exp	28.70	43.56	-14.86	5.869	13	11	3.580	173.0
Post Nonyl Exp vs. Post Drug Exp	35.95	43.56	-7.613	5.980	12	11	1.800	173.0
E4 (Serotonin Ant)								
Baseline vs. Post Nonyl Exp	21.31	69.56	-48.26	6.121	14	9	11.15	173.0
Baseline vs. Post Drug Exp	21.31	38.06	-16.75	6.121	14	9	3.871	173.0
Post Nonyl Exp vs. Post Drug Exp	69.56	38.06	31.50	6.753	9	9	6.597	173.0

Table 4: Within-group comparison using the 2-way ANOVA performed on Prism. Determined differences within each group at the 3 different time intervals in which hemolymph was drawn. Concentration units are pg/ml.

Table 5: Between Group Results

Within each column, compare rows (simple effects within columns					
Number of families	3				
Number of comparisons per family	6				
Alpha	0.05				
Tukey's multiple comparisons test	**Predicted (LS) mean diff.**	**95.00% CI of diff.**	**Below threshold?**	**Summary**	**Adjusted P Value**
Baseline					
E1 (- Control) vs. E2 (+ Control)	29.60	17.57 to 41.62	Yes	****	<0.0001
E1 (- Control) vs. E3 (Serotonin)	25.30	13.27 to 37.32	Yes	****	<0.0001
E1 (- Control) vs. E4 (Serotonin Ant)	32.70	20.99 to 44.40	Yes	****	<0.0001
E2 (+ Control) vs. E3 (Serotonin)	-4.298	-18.88 to 10.28	No	ns	0.8701
E2 (+ Control) vs. E4 (Serotonin Ant)	3.099	-11.22 to 17.41	No	ns	0.9432
E3 (Serotonin) vs. E4 (Serotonin Ant)	7.397	-6.917 to 21.71	No	ns	0.5385
Post Nonyl Exp					
E1 (- Control) vs. E2 (+ Control)	-6.242	-21.03 to 8.546	No	ns	0.6931
E1 (- Control) vs. E3 (Serotonin)	4.218	-8.476 to 16.91	No	ns	0.8244
E1 (- Control) vs. E4 (Serotonin Ant)	-29.40	-43.52 to -15.27	Yes	****	<0.0001
E2 (+ Control) vs. E3 (Serotonin)	10.46	-6.503 to 27.42	No	ns	0.3815
E2 (+ Control) vs. E4 (Serotonin Ant)	-23.15	-41.21 to -5.095	Yes	**	0.0059
E3 (Serotonin) vs. E4 (Serotonin Ant)	-33.61	-50.00 to -17.23	Yes	****	<0.0001
Post Drug Exp					
E1 (- Control) vs. E2 (+ Control)	-15.26	-31.30 to 0.7865	No	ns	0.0688
E1 (- Control) vs. E3 (Serotonin)	4.697	-8.927 to 18.32	No	ns	0.8078
E1 (- Control) vs. E4 (Serotonin Ant)	10.20	-4.413 to 24.81	No	ns	0.2718
E2 (+ Control) vs. E3 (Serotonin)	19.95	1.984 to 37.92	Yes	*	0.0230
E2 (+ Control) vs. E4 (Serotonin Ant)	25.46	6.726 to 44.18	Yes	**	0.0030
E3 (Serotonin) vs. E4 (Serotonin Ant)	5.502	-11.20 to 22.21	No	ns	0.8282

Test details	**Predicted (LS) mean 1**	**Predicted (LS) mean 2**	**Predicted (LS) mean diff.**	**SE of diff.**	**N1**	**N2**	**q**	**DF**
Baseline								
E1 (- Control) vs. E2 (+ Control)	54.00	24.41	29.60	4.636	36	13	9.029	173.0
E1 (- Control) vs. E3 (Serotonin)	54.00	28.70	25.30	4.636	36	13	7.718	173.0
E1 (- Control) vs. E4 (Serotonin Ant)	54.00	21.31	32.70	4.512	36	14	10.25	173.0
E2 (+ Control) vs. E3 (Serotonin)	24.41	28.70	-4.298	5.619	13	13	1.082	173.0
E2 (+ Control) vs. E4 (Serotonin Ant)	24.41	21.31	3.099	5.518	13	14	0.7943	173.0
E3 (Serotonin) vs. E4 (Serotonin Ant)	28.70	21.31	7.397	5.518	13	14	1.896	173.0
Post Nonyl Exp								
E1 (- Control) vs. E2 (+ Control)	40.17	46.41	-6.242	5.700	30	8	1.549	173.0
E1 (- Control) vs. E3 (Serotonin)	40.17	35.95	4.218	4.893	30	12	1.219	173.0
E1 (- Control) vs. E4 (Serotonin Ant)	40.17	69.56	-29.40	5.445	30	9	7.635	173.0
E2 (+ Control) vs. E3 (Serotonin)	46.41	35.95	10.46	6.539	8	12	2.262	173.0
E2 (+ Control) vs. E4 (Serotonin Ant)	46.41	69.56	-23.15	6.961	8	9	4.704	173.0
E3 (Serotonin) vs. E4 (Serotonin Ant)	35.95	69.56	-33.61	6.317	12	9	7.525	173.0
Post Drug Exp								
E1 (- Control) vs. E2 (+ Control)	48.26	63.51	-15.26	6.184	23	7	3.489	173.0
E1 (- Control) vs. E3 (Serotonin)	48.26	43.56	4.697	5.252	23	11	1.265	173.0
E1 (- Control) vs. E4 (Serotonin Ant)	48.26	38.06	10.20	5.633	23	9	2.561	173.0
E2 (+ Control) vs. E3 (Serotonin)	63.51	43.56	19.95	6.927	7	11	4.074	173.0
E2 (+ Control) vs. E4 (Serotonin Ant)	63.51	38.06	25.46	7.220	7	9	4.986	173.0
E3 (Serotonin) vs. E4 (Serotonin Ant)	43.56	38.06	5.502	6.439	11	9	1.208	173.0

Table 5: Between-group comparison using the 2-way ANOVA performed on Prism. Determined differences between each group at the 3 different time intervals in which hemolymph was drawn. Concentration units are pg/ml.

Table 6: Average cHH Concentrations

Table format: Grouped	Group A			Group B			Group C		
	Baseline			Post Nonylphenol Exposure			Post Drug Exposure		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
E1 (- Control)	54.001	11.236	36	40.166	12.554	30	48.258	15.928	23
E2 (+ Control)	24.405	3.927	13	46.408	4.723	8	63.514	23.188	7
E3 (Serotonin)	28.703	0.289	13	35.948	24.716	12	43.561	9.271	11
E4 (Serotonin Ant	21.306	2.446	14	69.562	31.795	9	38.059	14.241	9

Table 6: The mean cHH concentration (pg/ml) in the hemolymph per crayfish in each of the 4 groups, as well as at the 3 time intervals in which hemolymph draws were completed.

Within-Group Comparison of cHH Concentrations:

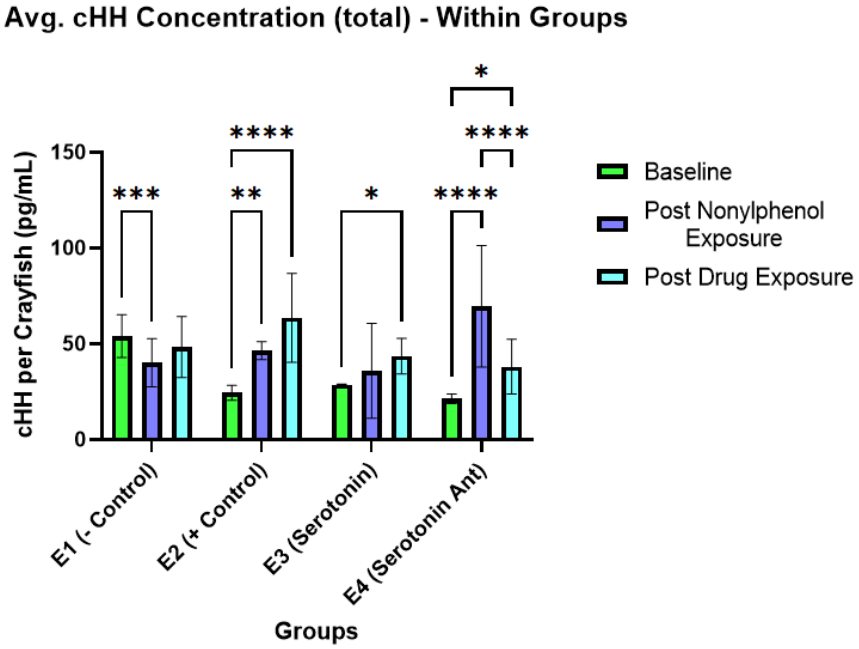

Figure 8: Within-group comparison of the average cHH concentration within the hemolymph for each of the 4 groups.

E1: Within-group comparison (Negative control):

When analyzing the average cHH concentration within the negative control group to establish a baseline, it was found that the mean concentration of cHH in the hemolymph per crayfish was 40.17pg/ml (± 12.55pg/ml) following the nonylphenol exposure period, which was significantly ($p<0.05$) lower than the mean concentration at baseline 54.00pg/ml (± 11.24pg/ml). Though statistically this result was significant, there were large error bars as noted. Also, this group was the first group to receive hemolymph draws, so therefore additional stress may have been caused at baseline due to hemolymph draw errors or redraws. As the negative control, this group did not receive nonylphenol during the exposure period and hence, no stress exposure. Importantly, this overall depicts no significant changes in cHH levels, as expected for the negative control. This suggests our negative control group accurately depicted the stress level of crayfish with no chronic exposure to a stressor **(figure 8).**

E2: Within-group comparison (positive control):

When analyzing the average cHH concentration within the positive control group to establish the effect of chronic nonylphenol exposure on stress, we found that the mean concentration of cHH in the hemolymph was 46.41pg/ml (± 4.72pg/ml) following the nonylphenol exposure period, which is significantly higher ($p<0.05$) than the mean concentration of cHH in the hemolymph at baseline which was 24.41pg/ml (±3.93pg/ml). As the positive control, this group did receive nonylphenol at an environmentally realistic concentration of 0.15 µl/L, therefore, this indicates that chronic nonylphenol exposure did increase the stress experienced by the crayfish. Within this group, it was also found that the mean concentration of cHH in the hemolymph for the post-drug exposure was 63.51pg/ml (±23.19pg/ml), which is significantly higher ($p<0.0001$) than the mean cHH concentration at baseline which was 24.41pg/ml (± 3.93pg/ml). As the positive control, this group did not receive any exposure to a drug, just continuous nonylphenol exposure, therefore, nonylphenol was still present and may have led to this increase in cHH concentration/stress for the crayfish in this group during this additional period of exposure to the stressor **(figure 8).**

E3: Within-group comparison (serotonin):

When analyzing the average cHH concentration within the group receiving the serotonin exposure following the stress exposure, we found that the mean concentration of cHH in the hemolymph following the exposure to serotonin was 43.56pg/ml (±9.27pg/ml), which was significantly higher($p<0.05$) than the mean cHH concentration of 28.70pg/ml (± 0.29) at baseline. Therefore, we suspect that serotonin may have led to this increase in stress experienced

by the crayfish following the exposure to serotonin. Due to the limited number of crayfish and lack of significant data produced for this group, this warrants more trials in the future to further investigate this relationship **(figure 8).**

E4: Within-group comparison (serotonin antagonist):

When analyzing the average cHH concentration within the group receiving the serotonin antagonist exposure, we found that the mean concentration of cHH in the hemolymph following the nonylphenol exposure was 69.56pg/ml (± 31.80pg/ml), which was significantly higher ($p<0.0001$) than the mean cHH concentration within the hemolymph at baseline which was 21.31pg/ml (±2.45pg/ml). This further confirms our findings that chronic nonylphenol exposure increases the stress experienced by the crayfish. We also found that the mean concentration of cHH in the hemolymph following the exposure to the serotonin antagonist was 38.06pg/ml (±14.24pg/ml), which was significantly higher ($p<0.05$) than the mean cHH concentration in the hemolymph at baseline, which was 21.31pg/ml (±2.45pg/ml). Lastly, the mean concentration of cHH in the hemolymph following the serotonin antagonist exposure was 38.06pg/ml (±14.24pg/ml), which was significantly lower ($p<0.0001$) than the mean concentration of cHH in the hemolymph following the exposure to nonylphenol, which was 69.56pg./ml (31.80pg/ml). Importantly, the concentration of cHH was significantly lower after the exposure to the serotonin antagonist following the chronic stress period with nonylphenol, which suggests that nonylphenol increases stress experienced by the crayfish and that the serotonin antagonist led to a dramatic drop in these stress levels **(figure 8).**

Between-Group Comparison of cHH Concentrations:

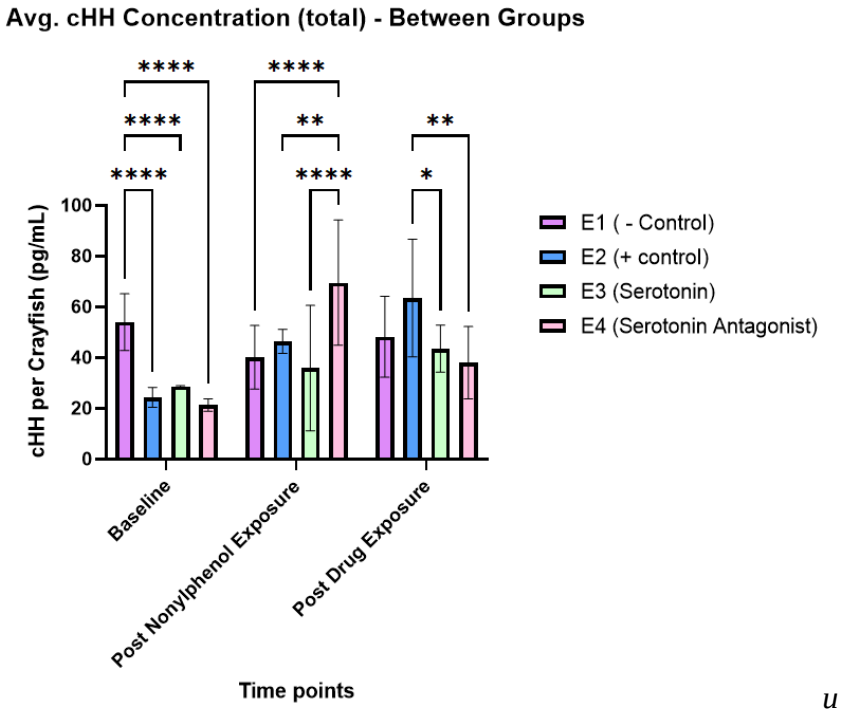

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Figure 9: Between-group comparison of the average cHH concentration within the hemolymph for each of the 4 groups at the 3 different time intervals.

Between-group comparison - Baseline mean cHH concentrations:

To establish if there were any differences in cHH concentrations at the baseline between groups, we found the mean concentration at baseline for the negative control (E1) was 54.00pg/ml (±11.24pg/ml), which was significantly higher ($p<0.0001$, $p<0.0001$ $P<0.0001$) than the mean concentration of cHH in the hemolymph for the positive control group (24.41 ±3.93pg/ml), serotonin group (28.41 ±0.289pg/ml), and the serotonin antagonist group (±21.31pg/ml). This indicates that the negative control group experienced higher levels of stress prior to beginning the experimental manipulations. This may be due to the increased number of crayfish in this group compared to the other groups. The negative control group had 36 crayfish at the first hemolymph draw, while the remaining three groups had around 14/15 crayfish per group. As

mentioned above, this higher level of cHH at the baseline for this group may also be due to our handling of crayfish during hemolymph draws, as this was the first group to receive draws **(figure 9).**

Between group comparison – Post Nonylphenol Exposure mean cHH concentrations:

Following the chronic exposure to nonylphenol, the mean concentration of cHH for the serotonin antagonist group was 69.56pg/ml (± 31.79pg/ml), which was significantly higher ($p<0.0001$, $p<0.05$, $p<0.0001$) than the mean cHH concentration for the negative control group (40.17pg/ml ± 12.55pg/ml), positive control group (46.41pg/ml ±4.72pg/ml), and serotonin group (35.95pg/ml±24.72pg/ml). This suggests that the serotonin antagonist group experienced a higher level of stress following the nonylphenol exposure compared to the other groups, which is expected when compared to the negative control group. This increase in cHH concentrations for this group compared to the other 2 groups who received nonylphenol may be due to experimental manipulation error when adding the nonylphenol **(figure 9).**

Between group comparison – Post Drug Exposure mean cHH concentrations:

Following the drug exposure period, the mean concentration of cHH for the positive control group was 63.51pg/ml (±23.19pg/ml), which was significantly higher ($p<0.05$, $p<0.05$) than the mean concentration of cHH for the group receiving serotonin (43.56pg/ml ± 9.27pg/ml) and for the group receiving the serotonin antagonist (38.06pg/ml ± 14.24pg/ml). This further confirms that nonylphenol increases the cHH concentration, and therefore stress, experienced by the crayfish **(figure 9).**

Chapter 5: Discussion

Overall, our results indicate that chronic exposure to nonylphenol did act as a chronic stressor to crayfish, which was confirmed by the increase in cHH within the hemolymph for the crayfish exposed to nonylphenol that was not seen in the negative control group. This was mainly observed in the positive control group, which received nonylphenol exposure and no drug exposure. This confirms our 1st hypothesis that nonylphenol did elicit a chronic stress response in crayfish. Crayfish were exposed to nonylphenol at a concentration of 0.15 µl/L for 35 days, representing a chronic low level, as described by Swift et al., in press. As previously discussed, nonylphenol is a known endocrine disruptor (Kassotis et al., 2020), and has led to feminization, reduced male fertility, and overall survival (Soares et al., 2008, Yang et al., 2020), as well as other physiological and behavioral impairments such as food finding ability (Swift et al., in press) in crayfish. Adding together all of these potential effects on the health of crayfish, it is expected that nonylphenol would increase stress levels experienced by crayfish, especially for long-term exposure to a stressor, which is typically what would be found in our environment as agricultural runoff. These results suggest that as an environmental pollutant, nonylphenol may increase the stress and therefore the overall health of crayfish within an environment when exposed to this chemical chronically. The health of crayfish is an important part of the environment that surrounds us, as crayfish are a keystone species, and therefore, many species rely upon them for survival. Chronic stress in crayfish as a result of continuous nonylphenol exposure may also affect aquaculture in our community, as chronic stress may lead to reduced overall health in crayfish captured for food supply. Prolonged periods of stress can result in a decrease in ability to cope with stress, as they are overwhelming their physiological systems (Selye, 1975, Schreck & Tort, 2016). Our study confirms this expectation in that chronic

exposure to nonylphenol elicited chronic stress in crayfish as determined by the elevated cHH levels following 35-day exposure to nonylphenol. As a future analysis of this study, we would like to use the hemolymph samples taken to determine the concentration of nonylphenol within the crayfish hemolymph to determine the relationship between the bioaccumulation of nonylphenol within the hemolymph with the level of cHH and therefore stress experienced by the crayfish.

When determining the effects of serotonin and a serotonin antagonist on chronic stress, we found that serotonin tended to increase stress levels, while the antagonist decreased stress levels following chronic exposure to stress. Previous research has confirmed that serotonin increases anxiety-like behavior (Fosset et al., 2014), and that serotonin increases the release of cHH from the eyestalk of crayfish, and ultimately into the hemolymph (Escamilla-Chimal et al., 2002), but most previous studies looked specifically at acute stressors such as an electric shock (Fosset et al.,2015) or hours of stress exposure (Lorenzon et al., 2004, Chang et al, 1998), therefore, there was a lack in data looking into chronic stress. Research performed by Chabera et al., 2021 did investigate the relationship between chronic exposure to stress via an herbicide and glucose levels and did find an increase in glucose levels following acute and chronic stress exposure, however, they did not investigate the effects on cHH levels, nor did they investigate ways to reduce this stress. Our study represents a preliminary study investigating the relationship between serotonin and chronic stress in crayfish, and, we did find that serotonin tended to increase chronic stress levels while the serotonin antagonist tended to decrease chronic stress levels, confirming our 2nd and 3rd hypotheses that chronic stress levels can be modulated by serotonin agonists and antagonists. This relationship is to be further investigated, as our number of crayfish per group was relatively small, and therefore warrants further investigation into these

relationships. Also, our study allowed us to further investigate the effects of serotonin on chronic stress, which may be applicable to humans, as crayfish have evolutionarily basic neurological development (Perry & Baciadonna, 2017), and may provide an evolutionary perspective into the emotional responses of mammals and potentially humans who may also share these basic underlying mechanisms (Fosset et al., 2015).

There are also several limitations to this study that need to be considered as well. Due to difficulties with measurements for our addition of drugs, crayfish in groups E2, E3, and E4 received an additional week of nonylphenol exposure compared to the "exposure" period for E1, which did not receive nonylphenol during this exposure period (negative control). The chronic exposure period for E1 was 28 days, while the chronic exposure period for E2, E3, and E4 was 35 days.

When performing the final hemolymph draws following the drug exposure, E1 crayfish hemolymph draws were completed 24 hours after the chronic exposure period was completed. Due to weather and access to the lab, final hemolymph draws for E2 , E3, and E4 were completed 48 hours following the chronic exposure period.

Due to crayfish mortality, each group contained fewer crayfish than originally anticipated. Originally, there were to be 40 crayfish per group, and due to the inevitable mortality throughout the study, there would still be a significant number per group. Due to space in the lab, the mortality of crayfish, and time constraints, there was a lower than anticipated number of crayfish per group. The E1 group was the only group that began with 40 crayfish. The E2 and E3 group began with 15 crayfish, and E4 began with 14 crayfish. With the lower number of crayfish used in this study, further investigation into the relationships discussed between chronic stress and serotonin using a greater number of crayfish to confirm these relationships should be performed.

In regard to the future direction of this study, glucose levels can be analyzed using the hemolymph samples taken during this study via glucose strips and a glucose meter, or a high-sensitivity glucose assay kit to assess the relationship between cHH, glucose, and stress. The function of cHH, or crustacean hyperglycemic hormone, is to increase glucose levels within the hemolymph (Fanjul-Moles, 2006), and therefore glucose levels increase as cHH levels increase. Previous research by Chang et al., 1998 as well as Lorenzon et al., 2004 determined that following acute stress, cHH levels increase, as did hemolymph glucose levels. Therefore, measuring glucose levels at the time points within this study that cHH levels were measured could further confirm the relationship between cHH and glucose, as well as confirm the actions of nonylphenol, serotonin, and serotonin antagonists on chronic stress.

Another future direction for this study would be further analysis of the data based on sex of the crayfish. Each group was composed of half female and half male, and while performing hemolymph draws, our samples were separated by sex. Therefore, our samples have the capability to be further analyzed to investigate if there is a relationship between stress, the modulation of stress by serotonin, and the sex of crayfish.

Lastly, as a future direction, we could investigate the addition of pharmaceutical runoff on stress and ultimately the environment as well. Direct addition of serotonin and serotonin-cinanserin hydrochloride to the water in the isolation tanks has been demonstrated to provide results that are comparable to those of drug injection (Reisinger et al., 2021). This exposure to the drugs via water mimics that of pharmaceutical runoff that ends up in aquatic habitats (Kolpin et al. 2002, Writer et al. 2013), therefore, our study also could allow us to investigate the effects of pharmaceutical runoff on crayfish stress levels, as well as the environment.

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