THE UNIVERSITY OF HULL

The effects of combined stress from pH and microplastic derived odours on *Carcinus maenas.*

being a Thesis submitted for the Degree of

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by

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1. Abstract

In recent years, a large amount of research has been done on climate change, which predicts ocean acidification (OA) will end up causing a significant drop in the pH of the oceans by the end of the 21st century. Research carried out looking at pH reduction in the oceans show that a drop as little as 0.5 units can cause issues with behaviour and detection of cues in crustaceans. This drop in pH (0.5 units) is predicted to have occurred by the end of this century.

Ocean acidification is causing a wide range of problems including, coral bleaching, warming oceans and shell calcification within certain calcifying species. Every marine species is facing slightly different challenges. While there is considerable data looking at the overall impact on calcifying organisms, relatively little research has been undertaken examining the impacting animal sensory systems (olfactory disruption), as it is considered an 'invisible' impact of ocean acidification.

The main objectives of this study are to investigate the impact of reduced pH and microplastic odour on the olfactory capacity of the *Carcinus maenas*, looking in detail at whether the combination of the stressors has a greater impact on olfactory capacity in this crab species.

The European shore crab (*Carcinus maenas*) is a globally invasive species, it originates in European waters but has now spread to foreign waters and can be found globally in places such as the Southeast USA, Australia and South Africa. Like many crustaceans, *Carcinus* utilise chemical cues to detect food and mating partners. The aim of this study is to obtain a greater understanding of how a combination of stressors (pH and microplastic derived odours) may impact the olfactory capacity of the *Carcinus maenas* and whether these

stressors may influence the behaviours exhibited. Plastic odour, food cues and a female produced sex pheromone cue are the chemical cues being used in this study. Males and females will both be used within the study as females may respond to the pheromone for other reasons, such as a food source from a moulting female.

Uridine diphosphate (UDP) and Uridine triphosphate (UTP) make up the pheromone bouquets used, the food cue is made from Glutathione (GSH) and the plastic odour is created from Polyethylene (PE). All cues stated above were made into gels using carboxycellulose powder and then freeze dried. The *Carcinus* were exposed to all gels, in pH 8.2, 7.6 and 7.2.

The results from this study show that *Carcinus maenas* took longer to react to the odours in the reduced pH conditions, confirming low pH causes olfactory disruption. This finding was significant when comparing data from pH 8.2 and pH 7.2 ($p=0.0017$). The *Carcinus maenas* has also shown behaviour (burying etc) that indicates that the combination of low pH with the microplastic odour present worsens this effect on olfactory capacity.

The findings from this study show similar results to other studies carried out on the impacts of ocean acidifications on different marine species, backing up the theories of low pH causing olfactory disruption. Further research in the field would help to determine whether long-term exposure to these low pH levels could lead to adaptations within crustaceans and other marine species. It would also help to determine the long-term impacts of a combination of microplastics and reducing pH within the environment, and how this combination may affect food consumption and predator avoidance.

2. Acknowledgements

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4. Background information for the study

4.1. Human Environmental Impacts, Actions and Consequences.

Over the last few centuries, the human population has rapidly increased, causing many environmental issues. There has been a huge loss of biodiversity, IUCN red list states 900 species have been lost in the last five centuries, including 229 molluscs, 80 fish and 11 crustaceans (The IUCN Red List of Threatened Species, 2022). This impacts the earth's ability to support all life on it (Bradshaw et al., 2021). Ecosystems are changing locally and globally due to this exponential growth of the human population (Ceballos et al., 2015). These changes in ecosystems are leading to more competition between species, survival of the fittest within species due to depleting resources and therefore extinction rates of biodiversity dramatically increase (Ceballos et al., 2015). Overall, 1 million species are already threatened with extinction in the future, including 40% of plants globally, the global biomass of wild mammals is also said to be 25% less since the late Pleistocene (Antonelli et al., 2020, Bar-On et al., 2018, Mora et al., 2011)

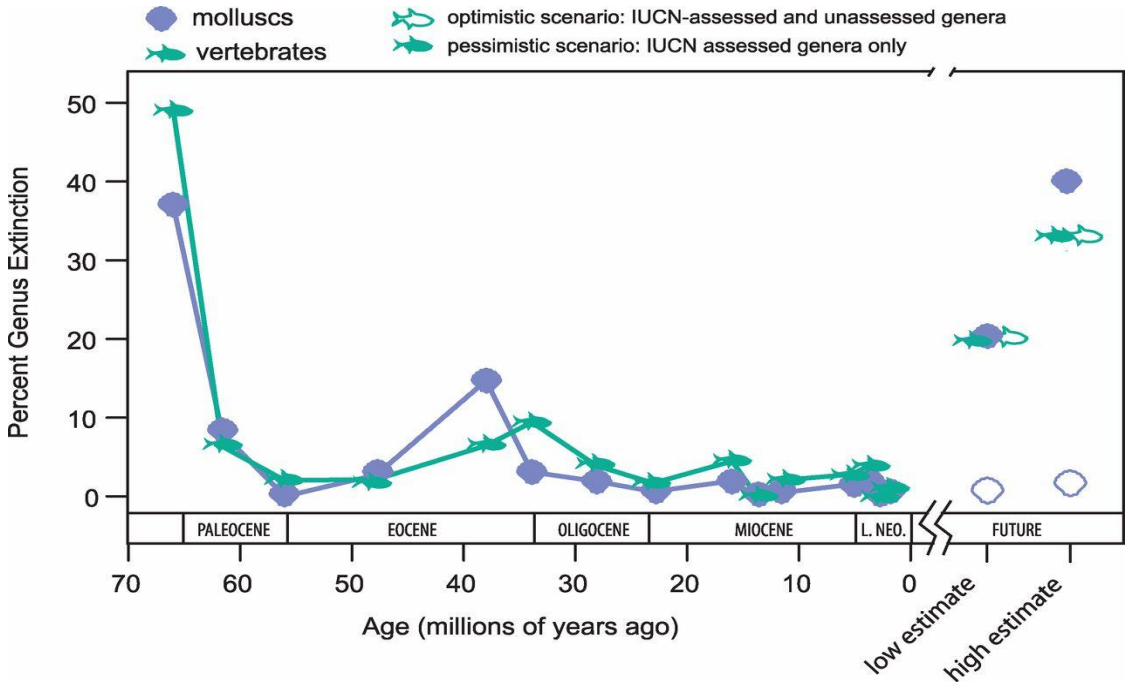

Figure 2. This shows genus extinction over time for marine molluscs and invertebrates. Molluscs and vertebrate numbers rapidly fell during the Palaeocene and have fluctuated since (Payne et al., 2016)

The Marine environment has been used for human consumption for thousands of years playing a key role in global food security, however with the development of technology as the human population expanded, it began to be over- exploited (Coleman and Williams, 2002, (Dobson et al., 2006). Marine species are used as a protein source for the human race, but this fishing pressure has led to a serious depletion of many oceans fishing stocks (Myers and Worm 2003). However, due to fisheries management protocols being implemented, including quotas, some populations are now recovering (Frank et al., 2022, Smith et al., 2022).

The demand for fossil fuel has continued to grow into the 21st century, as the human population requires greater quantities of it (Archer et al., 2009, Bala, 2013), the Figure (2) above shows that atmospheric CO_2 increases from 275ppm (parts per million) 10 thousand years ago, to 420ppm currently, which is a rise of 145ppm in the last 10 thousand years. Predictions are that 1000ppm could still be reached by 2100 (Barry et al., 2010), even with the new approaches at a more sustainable way of living.

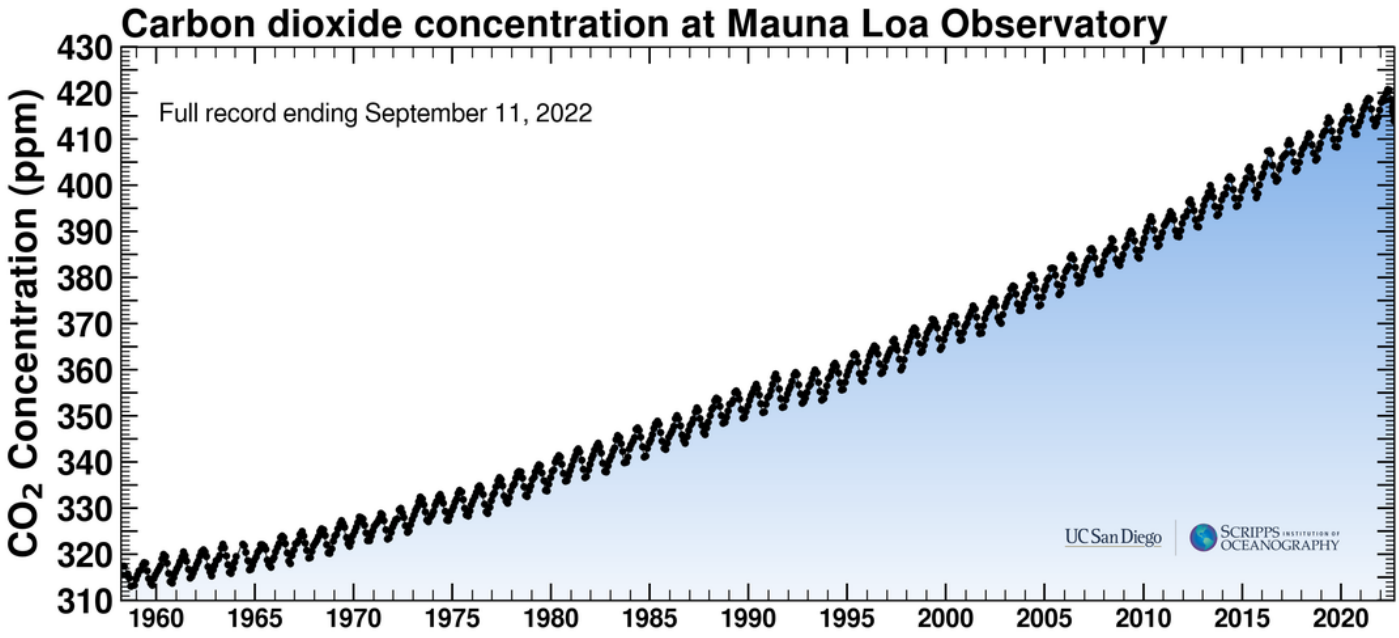

Figure 3. This graph shows atmospheric CO_2 readings since 1960, exponential increase can be seen occurring due to the increase in fossil fuel usage, the latest reading reaching over 420ppm. This image was taken from the Scripps Institution of Oceanography (UCSD 2022).

Climate change can be described as a change to global/regional climate patterns, largely due to increased levels of atmospheric carbon dioxide from increased burning of fossil from the mid-20th century (Perera and Nadeau, 2022). Impacts of climate change include, sea level rise (Nerem et al., 2018), ocean warming (OW) (Rathore et al., 2020), ocean acidification

(OA), hotter temperatures, more unpredictable weather systems and increase in extreme events (Gul et al., 2021).

One of the most known impacts of global warming is the increase in atmospheric CO_2, CO_2 is better known as a "green-house" gas. Alongside other gases, CO_2 is trapping solar rays within the atmosphere, leading to increasing global temperatures. Global surface temperatures recorded in 2018 found it to be the 4th warmest year ever recorded (Hansen et al., 2019).

Land temperatures have warmed twice as much as ocean temperatures, with predictions of 0.8 degrees ocean warming since 1975, and 1.6 degrees land warming (Hansen et al., 2019), as seen in the Figure 4 below.

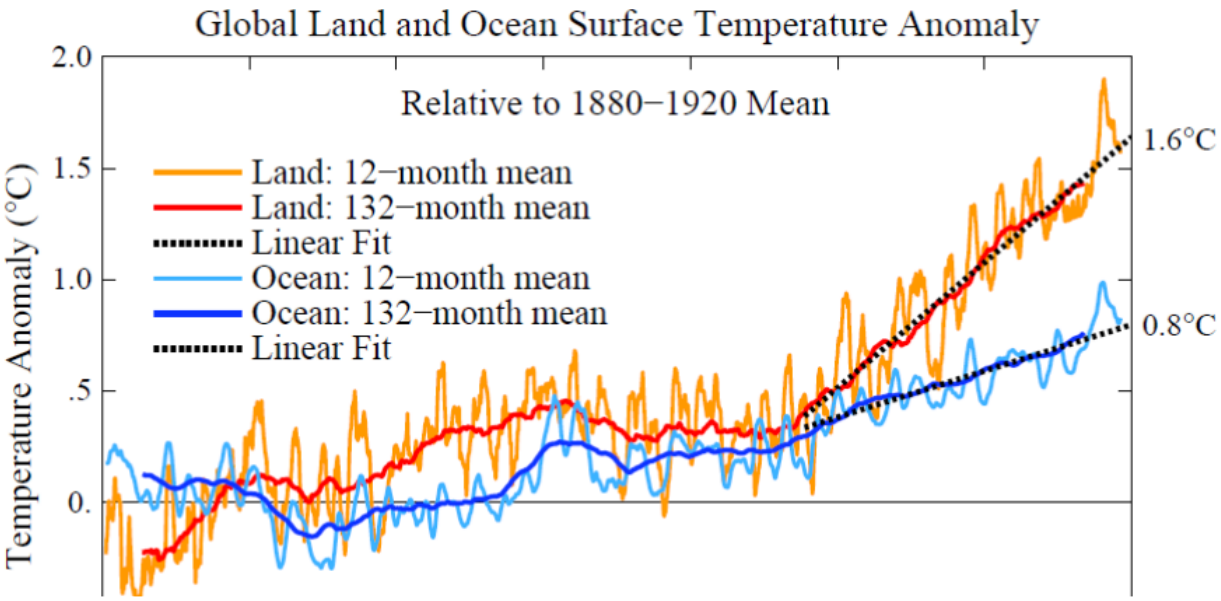

Figure 4. This graph shows temperature anomalies from 1880-2020 for global ocean and global land areas, both areas seeing an increase in temperatures (Hansen et al., 2019).

4.2. What is Ocean Acidification?

An increase in global CO_2 is a very well-known factor of climate change, however another direct impact of increasing CO_2, is ocean acidification (Capstick et al., 2016), a less well-known problem. This phenomenon could substantially alter marine biodiversity, ecosystem services and fisheries. The process of ocean acidification occurs when atmospheric CO_2 dissolves into the oceans and combines with H_2O, it then forms carbonic acid (H_2CO_3). Carbonic acid breaks down into a bicarbonate (HCO_3^-) and a Hydrogen ion (H^+). These hydrogen ions cause the seawater to acidify and pH to decrease (Birchenough, Williamson and Turley, 2017). This process can be seen the Figure 5. The increase in hydrogen ions causes a reduction in carbonate ions, causing issues for calcifying species such as corals and shell building creatures that rely on these carbonate ions. Estuary dwelling marine calcifiers such as the Atlantic mud crab (*Panopeus herbstii*) and the eastern oyster (*Crassostrea virginica*) could be significantly affected, due to naturally lower levels of calcium carbonate in these environments (Waldbusser et al., 2011). Tests carried out on these species (Dodd et al., 2021) showed that both species had lower calcification rates when exposed to ocean acidification conditions, which could cause long-term problems for these species. A study carried out by Kubota et al (2017) studied the impacts of ocean acidification (OA) in the western Northern Pacific Subtropical Gyre, they discovered through boron isotype measurements that OA affects the pH levels in calcification fluids in *Porite* corals.

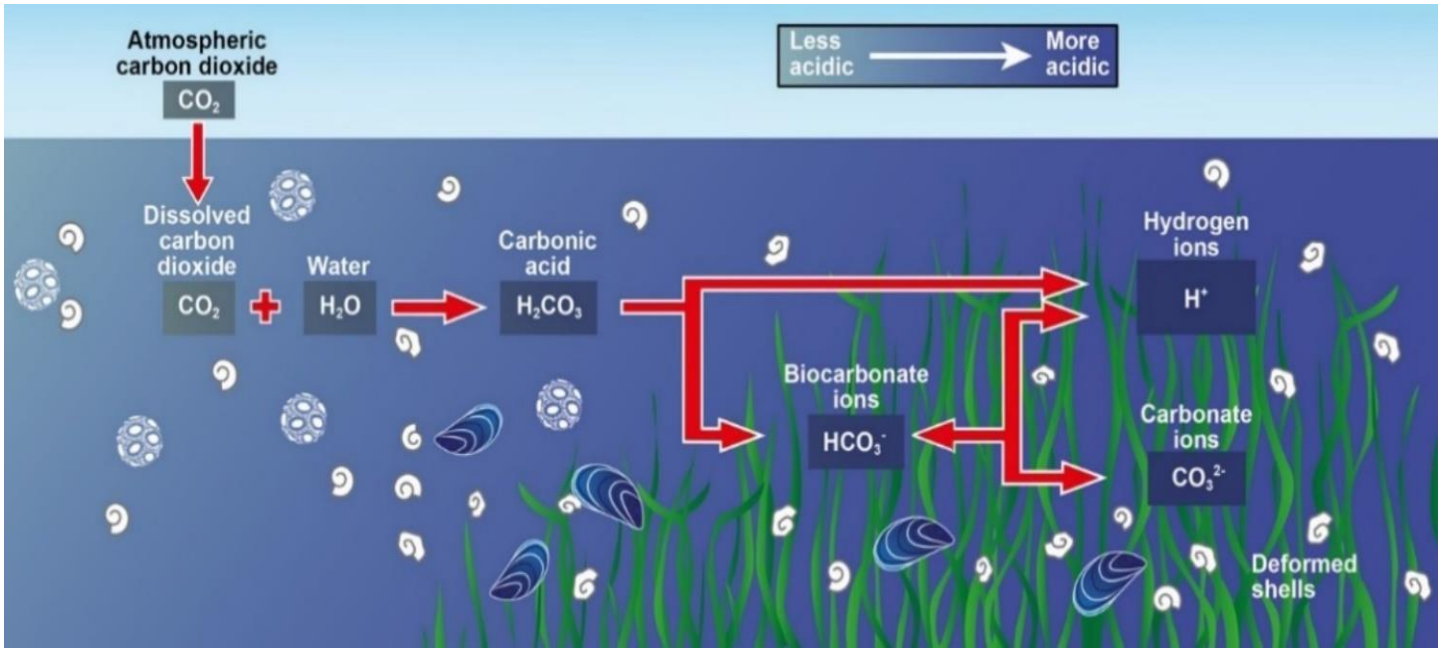

Figure 5. This infographic shows the process of ocean acidification, it starts with atmospheric carbon dioxide being absorbed into the oceans, the dissolved carbo dioxide then mixes with sea water, creating carbonic acid, this then turns to hydrogen ions, carbonate ions and bicarbonate ions, which turns the water more acidic. This image was taken from (Ocean acidification, 2021).

The oceans are the largest carbon sink globally, they absorb over 45% of CO_2 (Sabine et al., 2004, Llyina 2016). One of the concerns with ocean acidification is that even if global CO_2 levels are lowered to target levels rapidly, this process may still continue for up to 100 years, due to stored CO_2 in the environment. This could cause non-reparable environmental damage.

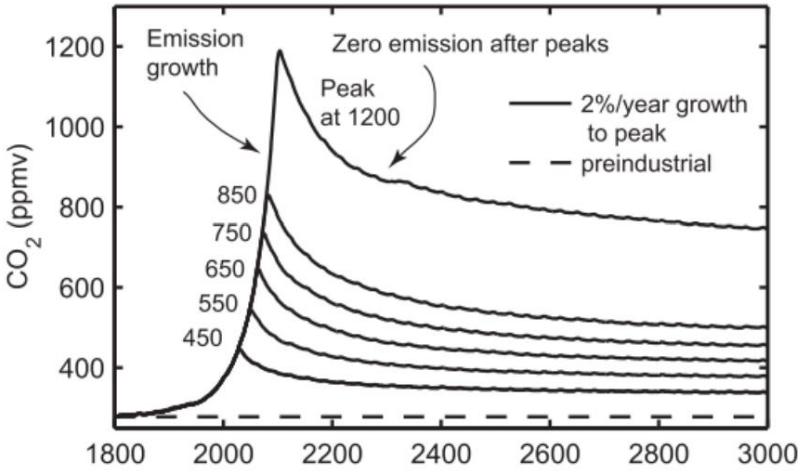

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Figure 6. This image shows the predicted CO_2 levels by century, once levels have peaked at 1200ppm there are then zero emissions. And levels of atmospheric carbon slowly start to drop. Image from Solomon et al., (2009).

which is an acidity increase of more than 100% (Lotterhos, Láruson and Jiang, 2021). Some areas of the marine environment that already naturally have a lower pH, for example areas of natural upwellings may see pH falling to as low as 7.2. Figure 7 shows the pH in the Hawaii Ocean from January 1988- January 2010, a decrease can be seen as the pH values continue to drop. The natural fluctuation in pH within the seasons can also be seen, lower pH levels are seen in the winter months due to CO2 being dissolved more efficiently in lower temperatures.

Research that has been carried out in marine and freshwater environments have found ocean acidification is leading to major problems with the olfactory capacity of fish, crustaceans, molluscs and other macroinvertebrates (Leduc et al., 2013). There has been recorded links between pH change and behavioural shifts in many species including; Polychaetes, Molluscs, Crustacea, Arthropoda and Chordata (Bhuiyan et al., 2021, De Le Haye et al., 2011, Munday et al., 2009, Kate et al., 2012 and Watson et al., 2014).

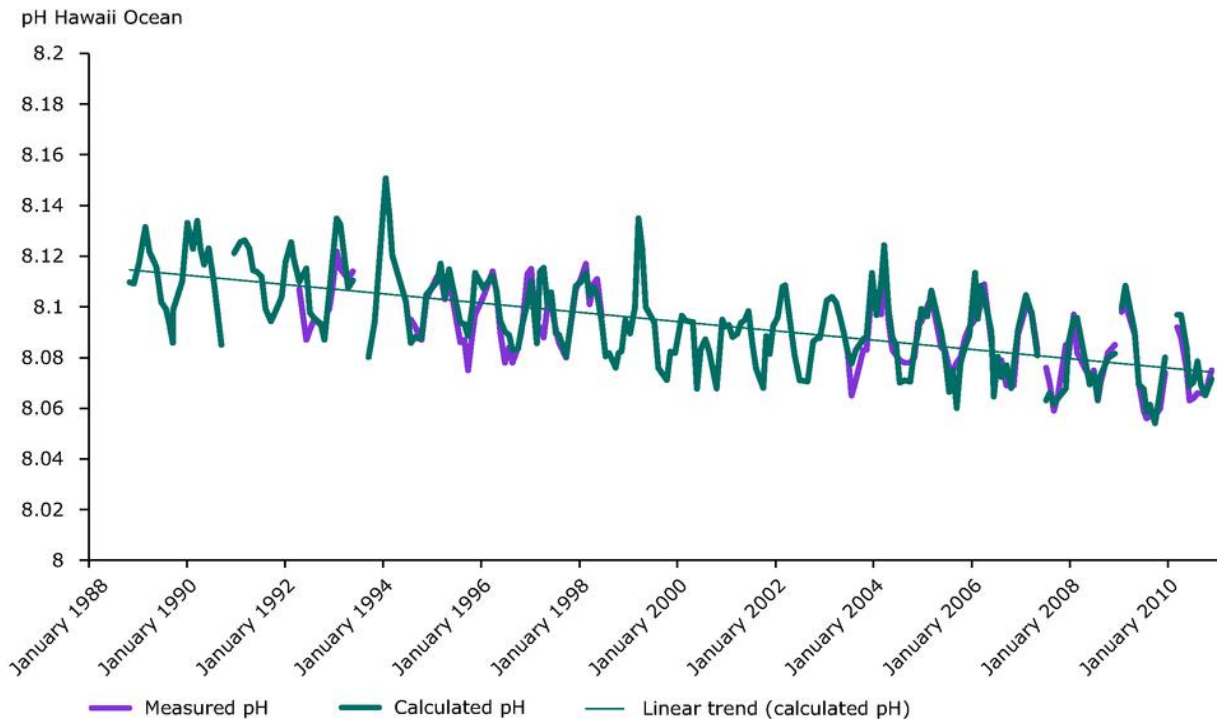


Figure 7. This Figure shows the pH change in the Hawaii ocean from 1998-2010, you can see the negative correlation occurring, you can see that fluctuations within pH occur naturally, but these are occurring now at a lower pH level (Johnson, 2021).

Marine communities are shaped by complex interactions between the environment and the organisms living within. Marine biomes are considered more predictable, in comparison to freshwater and terrestrial biomes in terms of the abiotic factors (Kim et al., 2015). The greatest change in ocean pH is seen at the surfaces, which is the reason most studies examine species that live within these habitats as they are better adapted to deal with fluctuations. Caldeira and Wickett (2003), conducted a study that showed that even a very minute change in pH at the bottom of the oceans could have major consequences on the deep dwelling species that inhabit these areas, as they are much more sensitive to environmental variation.

Studies show many species will be impacted in different ways with low pH, marine calcifiers, such as Molluscs, echinoderms and crustaceans, may struggle to maintain shell structure due to biomineralisation (Chandra Rajan et al., 2021). Biomineralisation is key to coral reef growth, experimental studies carried out that look at the impact of OA on biomineralisation show a general negative response to calcification when exposed to reduced pH (Comeau et al., 2017). Maintaining biomineralisation under OA conditions requires a greater amount of energy, which adversely effects other processes such as growth and reproduction (Meseck et al., 2016, Wood et al., 2008). Studies have been carried out to find the thresholds at which certain species become vulnerable to ocean acidification. Thresholds have been found in echinoderms (Bednarsek et al., 2021), the 8 thresholds within this study shows that OA will negatively impact populations in the long term, five out of eight of these thresholds occur within pH 7.6-7.75 range. Bednarsek et al., (2019) also found thresholds within pteropods, when comparing the two, echinoderms have a significantly lower pH threshold in comparison to the pteropods, making echinoderms more vulnerable to OA.

Other marine groups such as polychaetes may also be affected by these conditions. A study carried out on polychaete worms showed that offspring survival rates and fertilisation success was dramatically impacted when exposed to low pH (Wage et al, 2016). The tube worm

Hydroides elegans will struggle with biomineralisation in lower pH (Lane et al., 2012, Vinn, 2021), which could provide some explanation for other marine worms having less success with fertilisation as less energy is allocated to reproduction and more to biomineralisation. Leung et al (2015), looked at the impact of lower pH on locomotory activity, respiration rates and foraging performance in a gastropod (*Nassarius festivus*). Their results showed lower respiration rates, less effective foraging, and increased hiding (Leung et al., 2015). Pteropods are a group of holoplanktonic gastropods, they are found in most trophic webs and are eaten by a huge range of species, including small fish like Herring (*Clupea harengus*) and Mackerel (*Scomber scombrus*), sea birds, squid and large shrimp . Pteropods have had their shells placed in the predicted OA conditions for the year 2100, and worryingly their shells slowly dissolved over 45 days (Bednaršek et al., 2012).

A similar study considered the role of a change in pH on the olfactory success of predator-prey interactions in *Carcinus maenas* (Richardson et al., 2021). The olfactory cues used in this study were from the Common Cuttle fish (*Sepia officinalis*) as the predator cue and Blue Mussel (*Mytilus edulis*) as the prey cue. Results show detection and response to presence of odour cues from predators goes unchanged in the different pH's, however the ability to respond and detect to prey cues is altered with lowering pH levels. Males detect the prey cues faster than females in reduced pH, this data suggests that the males may be better adapted for future OA conditions.

Coralline algae are another calcifying species, inhabiting rocky habitats found in the marine photic zone globally. Coralline algae are considered to be one of the most susceptible taxa to negative impacts of ocean acidification (Cornwall et al., 2021). A study carried out by Peña et al (2021) revealed large variability in growth, calcification and photosynthesis with the Coralline algae over a range of different pH's. More than half of coralline algae species were lost when OA conditions were introduced (Peña et al., 2021). There are fears that if these

conditions continue then there will be huge loss to biodiversity globally (Talukder et al., 2022).

One of the most widely studied areas of OA is the impact on calcifying species, although there are other problems occurring in different species. Research has been carried out on the clown fish (*Amphiprioninae*), and data shows that lowering pH can reduce their efficiency of detecting predators and can also affect larval clownfish finding a suitable habitat to settle in (Dixson et al., 2010). Fish are affected as the cells take in carbonic acid which alters the pH of the fish's blood, known as respiratory acidosis (Dixson et al., 2010, Esbaugh et al., 2012).

There have been very few studies carried out examining the behaviours of marine animals that are exposed to these low pH levels over extended periods of times, often multiple generations. Nagelkerken et al (2015), have found that often an animals first response to a change within their environment is through modification to their behaviour, often altering ecological processes and species interactions. Higher carbon dioxide levels can alter behaviour in marine organisms by altering the function of neurotransmitters within the brain (Watson et al., 2014)

There are very few studies that found any positive impacts OA on the marine environment; however, certain algae, sea weeds (*Callophyllis lambertii*) and seagrass (*Posidonia oceanica*) actually benefit from higher CO2, as it allows more photosynthesis to occur (Britton et al., 2019, Hendriks et al., 2014). Some studies are looking into this and whether this could be a way in helping to slow down the process of OA on a local scale.

Another paper written by Clements and Hunt (2015) that reviewed published literature on ocean acidification, showed that approximately 10% of animal behaviours actually improved under OA conditions. Increased levels of CO_2 have been found to have positive effects on predator avoidance within juvenile *Concholepas concholepas* (Manriquez et al., 2013), Bibby

et al (2007) also found greater predator avoidance within the intertidal gastropod, *Littorina littorea*.

4.4 Olfactory Disruption

Changes in abiotic factors can influence whether certain functions within an organism will still work effectively (Wingfield, 2013). Many crustaceans rely heavily on chemoreception as their predominant sense. Olfaction is the function which allows chemical signals to be interpreted in the marine ecosystem, via specialised organs, such as antennules on *Carcinus maenas*. Crabs rely heavily on olfaction to explore their environments and make decisions on foraging (Stachowicz et al., 2007; Tierney et al., 1988), predator avoidance (Hazlett, 2011; Mitchell et al., 2017; Stachowicz et al., 2007) and mating (Clark, 2017; Liu, 2020; Stachowicz et al., 2007). Many polychaetes, including the *Nereis* family rely heavily on olfaction due to low visibility habitats, so changes to reception due to pH change could be detrimental for these species (Ivanina and Sokolova, 2015).

Many species rely heavily on olfactory cues, for example the male *Carcinus maenas* may show reproductive behaviours when exposed to female conditioned water from sexually active post and pre moult animals during the summer months. The effect of female sex pheromones is so strong that males reacted to stones that had been exposed to the water from freshly moulted females (Hardege et al., 2002, 2011). The process of olfaction relies on a step-by-step process. It starts with a suitable compound binding onto the olfactory receptor, which opens the ion channels on the receptor cell. This in turn leads to the change in the membrane potential (Breithaupt and Thiel, 2011), which change causes an action potential along the neurons, causing a release of neurotransmitters that move through the bodily fluid towards the brain or the synaptic ganglia. Once the signal reaches the brain/ganglia the signal is processed, and this is when a stereotyped behaviour may be triggered (Breithaupt and Thiel, 2011). Olfactory receptors often detect single amino acids and peptides, size and shape are a factor in signal binding and recognition, which infers that olfactory reception is

vulnerable to structural changes, which can be caused by low pH, change in salinity and temperature (Roggatz et al., 2016, Velez et al., 2019).

The main structures we are examining when considering ocean acidification are peptides, they are defined by their folding shape, bonds and constituent elements, all of these are dictated by the environment (Sadownik and Ulijn, 2010). If structures of said molecules are altered due to dropping pH levels, then this could have dramatic impacts on the species that rely heavily on chemical reception and detection of certain cues (Porteus et al., 2021). Numerous studies have shown that chemical cues play a huge role in the behavioural interactions of marine organisms (Porteus et al., 2021), and in some cases, they are essential for the species survival (Hardege et al., 1998)

Roggatz et al (2016) looked at the effects of ocean acidification and how it may alter chemical communications by changing function and structure of peptide signalling molecules within *Carcinus*. Their results showed peptide signalling cues are susceptible to protonation in reduced pH conditions, altering overall charge and charge distribution over the molecules thus altering the signalling cues. Bioassays showed an impairment to the function of signalling peptides at low pH. Modifications to structure, charge and function of signalling molecules can help explain altered behaviour in *Carcinus* and a range of other signalling systems when exposed to low pH (Roggatz et al., 2022).

4.5 Microplastics in Marine Environment.

Plastic can now be found in nearly every environment, from the poles to the equator. Microplastics are fragmented pieces of plastic that can be found in marine environments, they are plastics <5mm in size (Thompson et al., 2009). In 2018, 6.3 billion metric tonnes of plastic waste was produced globally, it is believed that 79% of this is now in the natural environment or in landfill sites (Ariza-Tarazona et al., 2020). There is a large range of different sources of microplastics, which can be seen in Figure 8. Since microplastic research started, ingestion is now not the only concern.

Microplastics have now been acknowledged as a persistent marine contaminant (Cózar et al., 2014), alongside direct ingestion there are other issues. Microplastics they can absorb hazardous chemicals and persistent organic pollutants (POP's) from the environment. One of the main problems with microplastic is they become a host for biofouling biota, which produce info-chemicals and biofilms which leads to the plastic getting its own chemical signal, which is a reason some species are finding plastic an attractant (Pohnert et al., 2007; Yoch, 2002; Zettler et al., 2013).

A study that has been carried out on the goldfish, *Carassius auratus* (Shi et al., 2021) looks at how microplastic's might impact olfactory-mediated behavioural responses in this species. The microplastic (MP) used in this testing was polystyrene (PS), the results from this showed behavioural responses being significantly hindered after a 28-day exposure to MP's. It is believed exposure to MP's could suppress the expression of genes encoding olfactory protein receptors, inhibiting ATPase transport (Shi et al., 2021). This study was carried out in freshwater, so the different conditions could cause different behaviours if carried out on marine species.

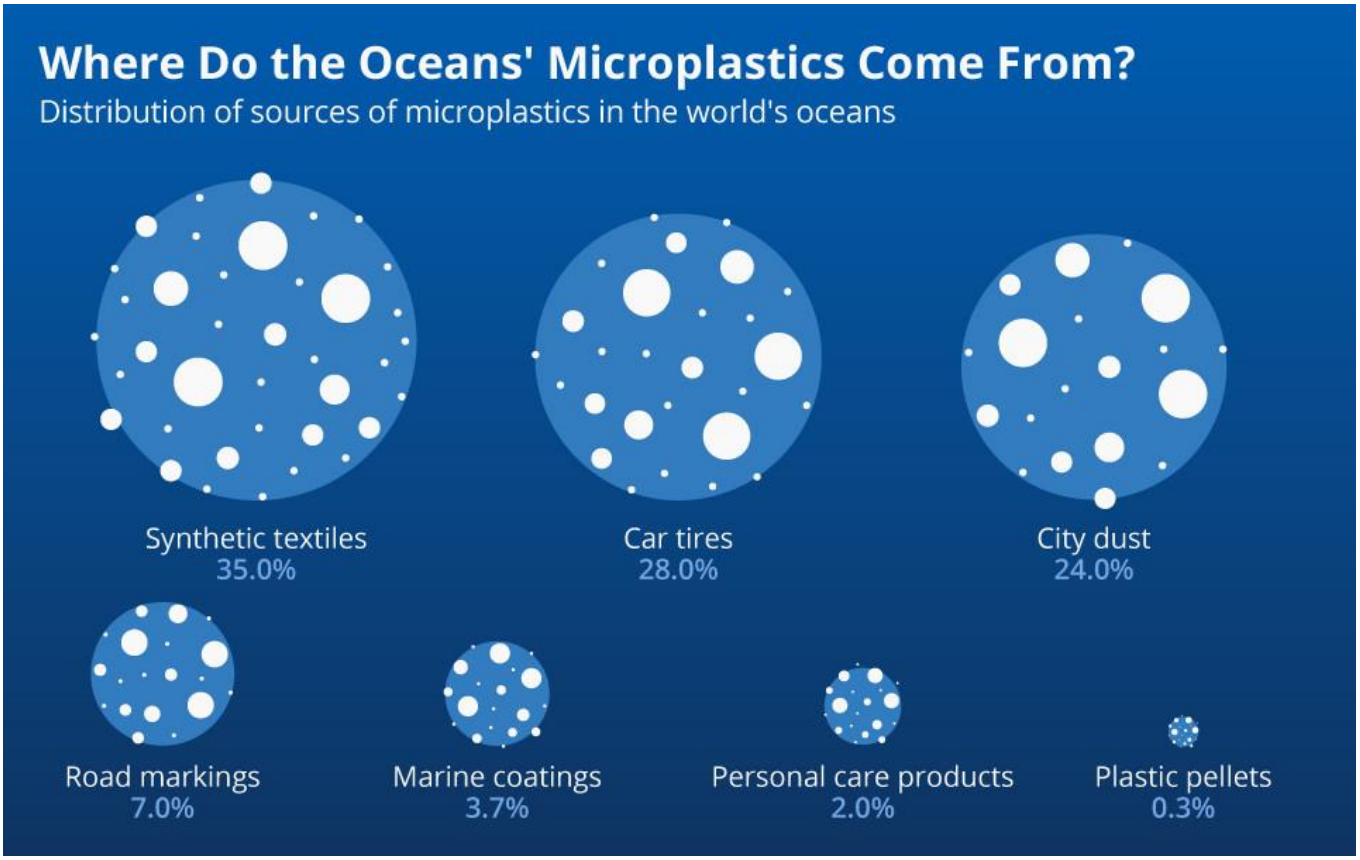


Figure 8. This Figure shows a breakdown of the main types of microplastics found in the oceans, and where each type is coming from, the most common type of microplastic present are synthetic textiles. The least common are plastic pellets. (Stastica, 2022).

4.6 Combination of Stressors.

Few studies have been carried out considering the combined effects of ocean acidification with another stressor (Plastic, Ocean warming etc). We know that low pH is causing a range of problems for a large variety of marine species including fish like the Olive flounder (*Paralichthys olivaceus*), Echinoderms such as the sea urchin, *Paracentrotus lividus* and many other organisms (Bertucci et al., 2022, Lee et al., 2022). But various studies have tested low pH alongside another stressor. There are studies that look into the combined effects of OA and ocean warming (OW), Manríquez et al, 2021, who covered this combination in mussels (*Perumytilus purpuratus*). The mussels were exposed to different temperatures (15 and 20 °C) and different pCO_2 (500 and 1400 μatm) for 10-14 weeks. This study showed that OA conditions led to increased oxygen consumption and OA and OW combined increased ATP demand and use of carbohydrate reserves, showing that more energy is being used for other processes, which long term may negatively impact this species.

Coccolithophores are calcifying phytoplankton, which are found in abundance in the Mediterranean Sea, (D'Amario et al., 2020). Research was carried out on this species, looking at the combination of OA and OW. The findings from this study highlighted that cell abundance dramatically decreases in OW conditions and also under combined OA and OW conditions. This suggest that this species will be impacted by OW, but the impacts may be worse when OA is involved.

Microplastic pollution and ocean acidification are two consequences of anthropogenic activities. Within the natural marine ecosystem, OA and exposure to microplastic pollution simultaneously is becoming a much more likely scenario. Solomon et al, 2009, found that even if current emissions targets are met $1000_{ppm\ pCO2}$ may still occur. We know pH is going

to continue to fall, and the quantity of plastic is still rising within the marine environments. Research carried out on mussels, *Mytilus edulis*, showed OA increases toxicity of microplastics and digestion (Wang et al., 2020), this shows this combination could be more dangerous than just a single stressor.

The effects of OA and OW are mainly being considered in early growth and development within species. Sui et al (2021) examined the combined effect of ocean acidification and MPs on the physiological response and the embryonic development of the larval oyster, *Crassostrea rivularis*, they used two different pH levels, 7.3 and 8.1, and three levels of microplastic (0, 10 and 1000 items L^{-1}). Hatching rate remained unaffected, however deformity rate increased under low pH and MP exposure In the sea urchin, *Paracentrotus lividus*, results show that in low pH (7.6) there is a reduction in larval growth and more deformities are being found (Bertucci and Bellas, 2021). They also confirmed microplastics intensified the effects of OA on the larvae of the sea urchins, the combination of pH 7.6 and MPs significantly reduced larval growth in comparison to the controls. Interestingly this study also looked at ocean warming alongside OA and MP and showed this is an additional stress and did cause a reduction in larvae stomach volume. From these studies it shows that MP's and OA can indeed cause greater problems.

4.7. *Carcinus maenas*:

Carcinus maenas, commonly known as the European shore crab is found in most European waters. It is a very sensitive species, affected by water chemistry, noise, background odours etc, making it ideal for studies like this one. Many factors must be considered when a behavioural study is carried out, including size, sex and colour of the crabs, the crab's stage of moult, its age, appetite, and social status alongside the season that the study is carried out in. *Carcinus maenas* is a complex species, variables such as temperature, light intensity, water chemistry, noise and background odours can all impact the behaviour of this species, (Fusi et al., 2017; Hubert et al., 2018; Liu X, 2020; Souza et al., 2019; Tran, 2015). *C.maenas* are an intertidal species, so are well adapted to changing pH, temperature and salinity changes, due to constant exposure during natural tide cycles.

Figure 1. This image shows the European shore crab, *Carcinus maenas.*

alien species on the global invasive species database (www.upane.it, 2021). It is one of the

most successful marine invasive species found globally (Christie, 2016). *Carcinus maenas* is commonly found in European waters, but has now reached foreign waters surrounding South Africa, Australia and both the West and North coast of the US. Like many other decapod crustaceans, *Carcinus maenas* relies heavily on chemoreception and olfactory cues to survive. Chemical cues in the marine environment allow the crabs to sense predators, source food and find mates (Stachowicz et al., 2007), alongside other behaviours.

Carcinus are often used for behavioural studies, they are an intertidal species, highly studied in ecotoxicology experiments. They are commonly found, easy to measure, sex and mark, making them very suitable for studies (Crothers, 1968; Langhammer et al., 2016; Young & Elliott, 2020). This study is looking at individuality, the shore crabs used in this study ranged in size, weight and shell colour, alongside sex and injuries. Numerous studies have shown behavioural responses of an organism is heavily impacted by an individual's characteristics (Richardson et al., 2021).

The sex of the crab is known to alter is behavioural choices made when certain chemical cues are present. Richardson et al (2021) found sex specific responses to a lowered pH when prey cues were present, there was altered responses under future OA conditions, with delayed responses at pH 7.6. Males detected these cues much more rapidly than the females, showing possible morphological or physiological differences between the sexes. This also suggests males having the potential to acclimate more successfully than females to future OA conditions.

5. Aims and Objectives

The aims for this study are to obtain a better understanding of how the predicted ocean acidification conditions could influence the response of chemical cues within *Carcinus maenas*, and whether a combination of stressors can cause greater problems with olfactory capacity. Within this study there are four broad questions that are being asked. These questions are:

1) Does exposure to low pH affect the behaviour and responses of *Carcinus maenas*.

2) Does exposure to microplastic odour have any impact on the behaviour and reactions of the *Carcinus maenas*?

3) Does a combination of lowered pH and microplastic odour have a greater impact on the behaviour and responses of *Carcinus maenas*?

6. General Methodology

6.1 Animal Husbandry

Animal Collection

When the project began there was already 50 shore crabs in the flow through tank set ups (10/03/2021), with an even mix of males and females. More animals were collected in the first week of May (03/05/2021) this bought the numbers up to 130, with 70 males and 60 females. The animals were collected from Whitby Bay in Yorkshire (Grid reference: NZ 90024 11788). This location was chosen due the guaranteed presence of *Carcinus maenas* in and around the harbour area. The animals were collected using bacon attached to hooks with a large net underneath, to pick up the crabs once they attached to the bait. Once the correct numbers of animals had been caught, they were placed into large freezer boxes, with ice in them to lower the crab's metabolic rate and thus reduce activity during transportation, until they were back at the research facility. The animals were then sexed, and measured so similar sized animals were stored together, males and females separate, this process followed all ethical procedures. The crabs are stored in a flow through artificial sea water system, with pH at 8.2, with 6-8 crabs per tank. Crabs were stored with similar sizes to reduce fighting within the study, as social hierarchies could influence crab behaviour. We kept males and females separate so that no mating pairs could form, as this is another factor that could alter behaviours.

Feeding

The *Carcinus maenas* were fed twice a week during the study duration, with small pieces of commercially available frozen mussel, *Mytilus edulis*, originating from Chile. Frozen mussels were left to defrost in warm water before being cut into small portion sizes. Each crab received one piece of mussel, and any uneaten food was removed from the tanks after feeding to prevent increased nitrate and ammonia levels within the tanks.

6.2. General Experimental Procedures

These all followed the University ethics guidelines, using ethics codes UO 20, approved by the University ethics committee.

Weighing and sizing of Animals

Before the testing started for this project all of the animals were weighed, in order to help determine any trends between size and behaviour. Each crab was dried off before being placed into a plastic container, this container had been weighed and the scales tared to the weight of it before the crabs were placed onto the scales. The weight was recorded in grams.

At the same time as the crabs were weighed, their carapace width was also measured (cm) using a Vernier caliper. The colour of their shell (red/green) and the condition of their bodies (missing legs/claws) were recorded. They were also given a number, which was written on their shells using a coloured nail varnish, blue for males and pink for females (Sally Hansen,

Non-Toxic). These numbers then correlated directly with the details of that specific individual for the duration of the study. The numbers were topped up throughout the study when required.

Preparation and Storage of Chemical Cue Solutions

Multiple chemicals were used in solutions to create the chemical cues used throughout the study, these include the females sex pheromones Uridine-triphosphate (UTP) and Uridine-diphosphate (UTP), glutathione (GSH) which is a food cue and Polyethene (PE), all of these chemicals were placed into gel form. The pheromone gels were created by mixing these two chemicals (UDP and UTP) with carboxycellulose powder and purified water, to create the gels at 10^{-3}M, the GSH gels were created in the same way and were also 10^{-3}M, the blank control gels used were created using purified water and carboxycellulose powder. The PE gel was created slightly differently, the PE came in pellet form, 50g of plastic was put into a blender with 230ml of purified water, this was then blitzed for 5 minutes in the blender. This solution was then stored in the fridge for 48 hours, and was shaken vigorously at intervals during this time. It was then strained using filter paper so the odour was separated from the plastic pellets. This odour was then mixed with carboxycellulose powder to create gels. All of these gels were put into -80 ºC freezer before being freeze dried, then stored in -20 ºC. The decision to freeze dry the gels was based on increasing the longevity and activity within the gel mix, allowing a more effective gel to be created.

7. The Impact of reduced pH upon Crustacea

7.1 Introduction:

Many decapod crustaceans use chemoreception as a predominant sense to explore their environment and make decisions upon key behaviours including foraging (Stachowicz et al., 2007; Tierney et al., 1988), escape behaviours (Hazlett, 2011; Mitchell et al., 2017; Stachowicz et al., 2007) mating (Clark et al., 2017; Liu X et al., 2020) and brood care. Size, weight, colour, condition and sex of crab could impact crab's responses, due to factors such as social and dominant hierarchy (Lord et al., 2021). Growth curves can be used to estimate the time in which crabs reach sexual maturity, which is important to help explain choices made within the study.

This study is examining how a combination of stressors (low pH and microplastic odour) may impact the crab's behaviour, when presented with olfactory cues, such as sex pheromones, MP odour and feeding cues. Environmental factors impact crab behaviour, including; water chemistry, activity level, moulting stage, light intensity, temperature and level of background odour and noise (Fusi et al., 2017; Hubert et al., 2018; Liu X et al., 2020; Souza et al., 2016; Tran 2015; Zimmer-Faust et al., 1996). This needs to be taken into account when looking at trends in results and individual crab's responses, as some crab's may be more sensitive to these environmental factors than others. Some of the smaller crabs that are used may have less visible reactions or slower reaction times to the pheromone gels, as they have not reached sexual maturity, which needs to be taken into account (Hardege et al., 2011).

Chemical cues in the environment:

Chemical communications are a vast part of the marine world, numerous decapod crustaceans rely on chemoreception as their predominant sense (Bublitz et al., 2008). Crabs rely heavily on olfaction to explore their environment and make decisions on foraging (Stachowicz et al., 2007; Tierney et al., 1988), predator avoidance (Hazlett, 2011; Mitchell et al., 2017; Stachowicz et al., 2007) and mating (Clark, 2017; Liu, 2020; Stachowicz et al., 2007). When a crab detects a cue, it will rapidly flick its antennules (Fusi et al., 2017, Velez et al., 2019). Crabs often detect a food odour before physically finding the food source, due to olfaction. They can detect a molecule as soon as it has been released (Kamio et al., 2017; Souza et al., 2016), with preference to the molecule changing throughout the seasons. Olfaction is key to allow crabs to detect predators such as larger decapods, fishes and sea birds in the wild (Klassen et al., 2007), if they have a fast reaction to this olfactory fingerprint of a predator, it will help them avoid predation (Hazlett, 2011; Mitchell et al., 2017; Stachowicz et al., 2007). Responses of crabs towards chemical cues such as pheromones will vary depending on the season, and the stage of moult of the crabs. This means different behaviours will be seen year-round. For example, the crabs' reaction to feeding cues will differ significantly between summer and winter, as they are less attracted to food during mating season (Wimalasiri et al., 2016, Xiao et al., 2004).

Chemical reception in the marine environment can also be altered by the seawater pH. As pH drops it can affect the efficiency of stimulus receptor binding by altering the charge distribution on the stimulus molecules (Tierney and Atema, 1988). Research carried out by Ross and Behringer (2019) looking at the Caribbean spiny lobster (*Panulirus argus*) showed that when these animals were exposed to pH 7.65, they lost all ability to avoid stone crabs (*Menippe mercenaria*) and diseased conspecifics, showing lowered pH alters the chemosensory driven behaviours in this species. Richardson et al (2021) looked at predator

odour cues and ocean acidification. *Carcinus maenas* showed a delayed response to prey cues in pH 7.6, females being worse affected than the males (Richardson et al., 2021). This could cause long term issues within the species if males can adapt better to reduced pH than females.

Seasonality:

Seasonality will have an impact on the crab's hormones, and these will also change with maturation. Moulting and maturation hormones will increase over summer and prior to ecdysis when crab's moult. These hormones are daylength and temperature dependant in every organism (Clark, 2017; Liu, 2020; Stachowicz et al., 2007). The time of year the study is carried out could impact the results. One thing that is often overlooked in studies like this is diel cycles/acute changes that impact animal's behaviour and physiology, *Carcinus maenas* occupy a variety of shoreline types, including estuaries, which means they may get isolated in shallower waters, if these animals were then used in a study, they may have already adapted more to temperature, salinity and light intensity fluctuations than *Carcinus* sourced from open coastlines. Zarrella-Smith et al (2022), looked at the daily and seasonal movements of green crabs in the Webhannet River estuary (Maine, USA), their findings showed that downstream movements of crabs were associated with changes in temperature below 10 °C, regardless of sex. 9 individuals from the study overwintered downstream, potentially in areas of deeper water, whilst the rest remained upstream.

UDP is a female sex pheromone found in the urine of crabs (Hardege et al., 2011) which has been shown by Bublitz et al (2008) to also have bioactivity on a range of crustaceans. Zhang et al., 2009, found UDP and the related UTP to also induce mating in *Lysmata* shrimps again with bioactivity restricted to only the short period of the female moult. In shore crabs, UDP is

also perceived differently between winners and losers of social hierarchy dominance fights with subdominant males responding significantly less frequently and showing a longer time to respond to the nucleotide (Fletcher & Hardege, 2009). Females can also respond to these chemical cues, for a different reason. Female *Carcinus* may associate these odours with a freshly moulted female, which they may view as a potential food source.

This complexity of animal responsiveness to olfactory cues due to seasonality and social interactions is a field yet to be explored in detail.

Effects of ocean acidification on *Carcinus maenas*:

The greatest pH change is seen at the surface of oceans, most OA studies focus on species in these habitats. Even a very slight change in pH at the bottom of the oceans could have major consequences on these deep-sea species as they are much more sensitive to variation (Caldeira and Wickett, 2003; Kim, Taylor, Lovera and Barry, 2015). Very few OA studies have been carried out in areas of upwellings, which have naturally lower pH. Many of these areas are now seeing pH levels fall below thresholds (Bednarsek et al., 2019, 2021), but there is very limited data to show what effects this could have to the organism and communities that live in these areas (Aguilera et al., 2020), which is why more studies need to focus on these very low pH areas.

Accurate measurements of pH can be difficult to obtain within the field (Douglas and Byrne, 2017), pH also varies due to natural shifts in salinity and temperature, these natural shifts occur daily due to changing tides. pH is one of the most used control variables in studies focusing on ocean acidification (Sordo et al., 2016). An increasing number of studies focus mainly on the potential impacts OA can have in altering physiological behaviour of marine life. Leung et al (2015) using a similar approach to this study, looked at the impact of lower

pH on locomotory activity, respiration rates and foraging performance in a gastropod (*Nassarius festivus).* Their results showed lower respiration rates, less effective foraging, and increased hiding (Leung et al., 2015). Refer back to section 4.2. for more information.

Effects of microplastics on marine life:

Plastic debris can now be found in nearly all environments, from the poles to the equator. Microplastics are found in the marine environment and are classified as plastics <5mm (Thompson et al., 2009), refer back to section 4.5. Microplastic research frequently focuses on ingestion, this can occur directly and via trophic transfer. Sucharitakul et al (2021) studied this in jelly fish (*Aurelia coerulea*). *A.coerulea* were exposed to aged microplastics and *Artemis nauplii* that had ingested microbeads, the study found 35 times more microbeads were ingested via trophic transfer. Suggesting ingestion occurs more frequently due to trophic transfer.

Harmful chemicals associated with plastics can be split into three categories, chemicals absorbed from the environment, by-products of manufacturing and ingredients of the plastic material (Groh et al., 2019, Rani et al., 2015). Thus, many plastic derived compounds have to be monitored by government organisations, as many are toxic to organisms (Rochman, 2015).

Ethylene and propylene are plastic monomers that are not considered harmful to the environment (Lither et al., 2011). However, as many polymerization reactions are never fully completed, small oligomers or unreacted monomers can still be released from the plastic (Björnsdotter, 2015). BPA is one of these polymers that is know to disrupt the endocrine functions, styrene and vinyl chloride are both examples of polymers that have shown carcinogenic and mutagenic effects (Bang et al., 2012; Oehlmann et al., 2009; van Wezel et al., 2000). This research shows how harmful certain polymers can be to the environment.

Microplastics can absorb hazardous chemicals and persistent organic pollutants (POP's) including polychlorinated biphenyls and polycyclic aromatic hydrocarbons from the environment due to their large surface to volume ratio and their chemical surface properties (Ariza-Tarazona et al., 2020). There is evidence that shows chemosensory cues could influence the bioavailability of microplastics (Vroom et al., 2017; Savoca et al., 2016; Allen et al., 2017; Procter et al., 2019, Greenshields et al., 2021). Greenshields et al (2021) researched Oleamide, a plastic additive that is a known signalling molecule. Results from the study showed Hermit crabs (*Pagurus bernhardus*) showed attraction to low concentrations of oleamide. Oleamide shares some structural similarities with oleic acid, which is a molecule released by arthropods during decay and decomposition, so this attraction could be due to a mistaken odour of a possible food source (Greenshields et al., 2021).

Harmful substances are being found that are leaching out of microplastics, plastics may contain over 2400 hazardous substances (Wiesinger et al., 2021). Many plastics contain harmful chemicals, solvents and additives (Lithner et al., 2011, Groh et al., 2019). Björnsdotter, 2015 researched leaching from five different virgin plastic pellets. Plastics tested were polyvinyl chloride (PVC), high-density polyethylene (HDPE), polyethylene (PE), polypropylene (PE) and polystyrene (PS). Three out of five (PVC, HDPE and PS) of these leached additives and residual monomers after 24 hours soaking in artificial sea water, including aliphatic hydrocarbons and styrene monomers. Oleamide is another info-chemical found in the marine and terrestrial environment. It is a common slipping agent that shares similarities with a chemical released by decaying organisms, specifically arthropods in this case. Crustacean species may show an attraction towards the odour due to confusion over a possible food source (Schirrmacher et al., 2021).

Microplastics found in the marine environment can become a host for biofouling biota, which can produce info chemicals (chemical compounds carrying information that is used by small organisms that cannot communicate using sound) (Barnes, 2002; Lobelle, 2011). When these organisms colonize microplastics they can produce biofilms, which leads to plastics debris getting its own chemical signal. This signal can then be picked up by organisms that use chemoreception to detect food cues, and may be an attractive cue (Pohnert et al., 2007; Yoch, 2002; Zettler et al., 2013). One of these info-chemicals is dimethyl sulfide (DMS), DMS concentrations range from 1-7nM globally. Research looking at DMS has found many species showing foraging behaviour when it is present, these species include copepods (specifically *Calanus helgolandicus* and *Acartia tonsa*) the European lobster *Homarus gammarus* and the Logger head turtle *Caretta caretta* (Pfaller et al., 2020).

Combination of stressors:

Many OA studies look at a combination of climate change induced stressors, for example OA and rising temperature (OW) (Bell et al., 2018), however very few looks at the combination of microplastic and OA, refer back to section 4.6. Manríquez et al., (2021) showed that in mussels (*Perumytilus purpuratus)*, OA combined with OW increased ATP demand and use of carbohydrate reserves. Walther et al (2009) looked at the impact of anthropogenic ocean acidification on thermal tolerance of the spider crab *Hyas araneus,* their results suggest that there is a narrowing of the thermal window in this species when exposed to moderate increases in CO_2.

In the marine environment ocean acidification and exposure to micro plastics (MP's) may occur simultaneously and is certain to do so more in the future. A study by Solomon et al (2009) shows that even if current emission targets are met from now, $1000_{ppm\ pCO2}$ will still occur, making these studies even more relevant for the future. There isn't a huge amount of data available on this combination of stressors though. One study that looks at mussels (*Mytilus edulis*) and their digestion showed that OA can enhance the toxicity of microplastics, showing the combination of stressors can be worse (Wang et al., 2020). A more recent study by Bertucci and Bellas (2021) looked in the effects of falling pH, increasing temperatures and MPs on the development of the sea urchin *Paracentrotus lividus,* their results demonstrate that MP's may aggravate the effects of falling pH and this combination of stressors threaten the sea urchin population, which will cause long term issues for coastal ecosystems as they struggle to adapt to numerous stressors.

One more commonly studied combination of stressors is OA and ocean warming (OW). The green turtle, *Chelonia mydas* has already been seen to be shifting its distribution, due to shifting ocean warming hotspots. This displacement could lead to alterations in seasonal

residency (Franco et al., 2020). A study carried out on the shrimp, *Palaemon spp*, discovered that when this species is exposed to ocean warming conditions they exhibit riskier behaviour, they foraged more actively for longer periods of time, even with a live predator present (Marangon et al., 2019). This behaviour will lead to long terms problems for this prey species, but will benefit the predators.

There is however some evidence, that marine species can adapt to these changing conditions. Many marine biotas may be more resistant than thought, this is because their ability to acclimate is believed to be more rapid than the changes in sea water chemistry (Hendriks et al., 2010; Munday, 2014). A study looking at the impacts of OA on gastropods (*Nassarius festivus*) showed that the individuals exposed to low pH for long periods of time can recover from exposure (Leung et al., 2015). Ocean acidification and microplastics are both well-studied areas, due to them being complicated problems with impacts on food security and ecosystem as well as human health. As such, the study that we are carrying out will help to fill a major gap in current knowledge - the impact of this combination of these stressors.

7.2 Aims and Hypotheses:

7.2.1 Hypotheses on Ocean acidification impacts:

1) Response and behaviour towards chemical cues related to feeding in *Carcinus maenas* will be significantly impacted by exposure to low pH levels.

2) Response and behaviour towards chemical cues related to reproductive functionality in *Carcinus maenas* will be significantly impacted by exposure to low pH levels.

7.2.2 Hypotheses on combined stressors

3) Exposure to microplastic odour will have an impact on the olfactory capacity and behaviour of *Carcinus maenas*.

4) Exposure to microplastic odour and low pH combined will have a greater impact on *Carcinus maenas* behaviour and olfactory capacity.

These hypotheses will be tested using *Carcinus maenas*, individual crabs will be exposed to feeding, reproductive, control and microplastic odour cues in current (8.2), predicted (7.6) and upwelling (7.2) pH levels. If behaviour and reaction times to the feeding cue and reproductive are not significantly different within the different pH levels then hypotheses one and two must be rejected and the null hypotheses accepted, however if there is a significant difference between the pH levels the hypotheses can be accepted, and this will show that predicted future pH levels will indeed impact the olfactory capacity of *Carcinus maenas*.

If detection and reactions to microplastic in low pH are not significantly different to current pH, hypothesis three will be rejected and the null hypothesis accepted, but if there is a difference to the combination of these two stressors between the pH, then it can be accepted, which would prove that a combination of stressors can sometimes be more damaging than a single stressor. If the combination of stressors on behaviours is significantly different in the three pH levels, and impacts the locomotion of the *Carcinus maenas* then hypothesis four can be accepted and the null rejected.

7.3 Methodology:

This study was carried out using the European shore crab (*Carcinus maenas maenas*), it followed the university guidelines, using ethics code UO 20, which has been approved by the ethics committee at the University of Hull.

Carcinus maenas were collected from Whitby, North East Yorkshire at the end of May, they were stored in tanks of 6-8 other crabs with flow through natural seawater, males and females stored separately, at pH 8.2, 17-20˚C. The crabs were fed twice a week with frozen mussel (*Mytilus edulis*) during the testing period.

This study was carried out with COVID 19 precautions in place, so there could have been a chance to expand further if COVID hadn't prevented that.

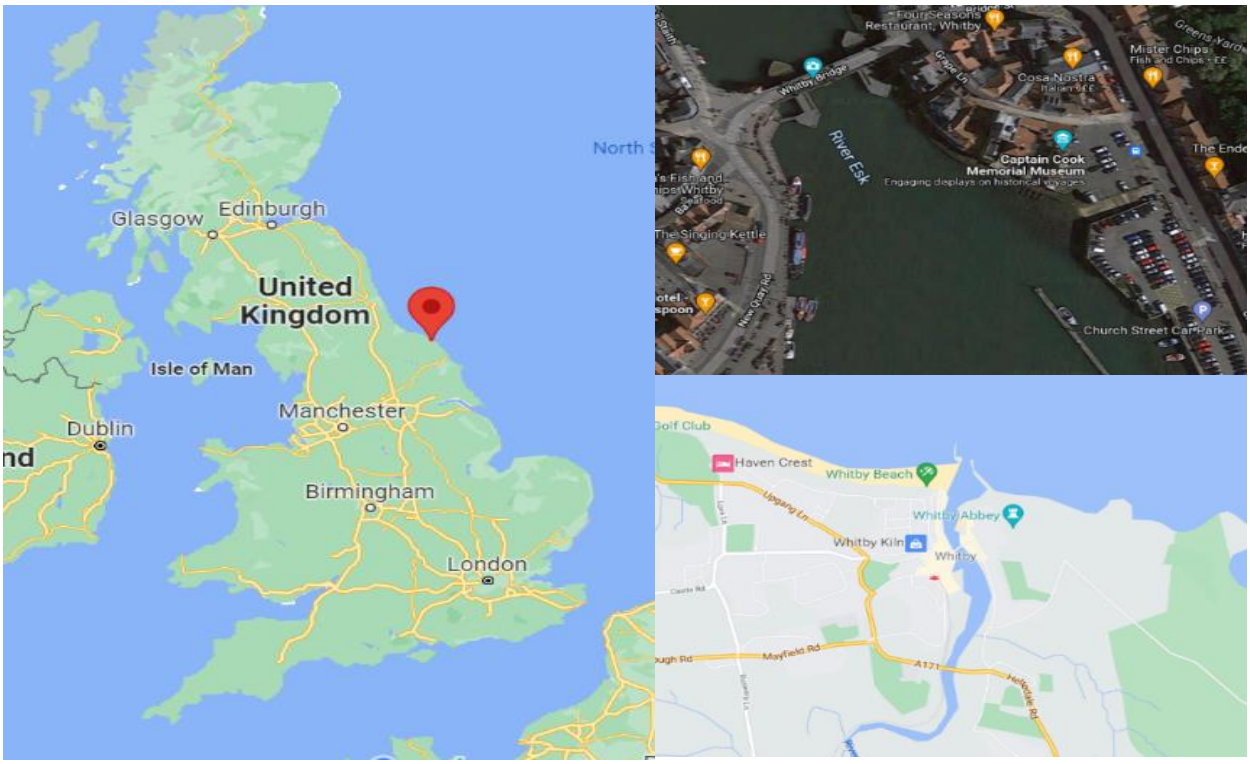


Figure 9. Approximate location of the sampling site on the rocky shore in Whitby, North Yorkshire (Pictures from Google Maps, 2022; Wikipedia contributors, 2022).

Figure 10. Markings on the *Carcinus* to determine individuals made using non-toxic nail varnish.

The individuals were all marked for the duration of the study, to allow individuals to be distinguished. The carapaces were marked using non-toxic coloured nail varnish, each with a unique number and colour depending on sex. Previous studies had shown nail varnish had no impact on mortality rates of *Carcinus* (Lee et al., 2005, Sturm et al., 2006). The markings on the crabs lasted for the duration of the study. They were reapplied if the numbers began wearing off, or if an individual moulted (Figure 10).

7.3.1 Y-shaped olfactometer and Experimental design:

During this study 6 experimental conditions were tested in Y-shaped olfactometers. These conditions were microplastic odour (PE) Vs food cue (glutathione), microplastic odour Vs pheromone (UDP+UTP), food cue Vs pheromone, Microplastic odour Vs control, food cue Vs control and pheromone Vs control. Each condition was tested in the current ocean pH (8.2), the predicted pH by the end of the century (7.6) and the pH in upwelling areas (7.2). Within each condition, 40 repeats were carried out in all three pH's, so 720 bioassays were carried out.

	pH 8.2	**pH 7.6**	**pH 7.2**
Microplastic odour V Control			
Microplastic odour V Food Cue			
Microplastic odour V Pheromone cue			
Food cue V Control			
Food cue V Pheromone cue			
Pheromone cue V Control			

Table 1: This Table shows the conditions tested during this research, in all three pH levels.

This experiment was carried out in the summer (July-September 2021) on summer crabs that were sexually active. Responsiveness to sexual stimuli was tested as positive control by exposing the males to female sex odours (UTP and UDP) and watching for mating behaviour. Once mating behaviour was observed the crabs were ready to be used. The Table below shows behaviours that were looked for with a basic description of what those behaviours look like.

Wafting	This behaviour can be defined by a rapid back and forth movement created by the *Carcinus maenas* mouth pieces.
Grabbing	This behaviour was when the *Carcinus maenas* physically grabbed the tea strainer that the odour was inside of with either claw.
Buried	This behaviour was recorded if the *Carcinus maenas* buried into the sediment, either at the start of the experiment or near a cue.
Non-Visible	This behaviour was recorded if the *Carcinus maenas* didn't show any visible behaviours.
Rapid Antennular Flicking	This behaviour was defined by the *Carcinus maenas* rapidly flicking their antennules.
Cradled cue	This behaviour was recorded when the *Carcinus maenas* showed cradling (Male will cradle a female from behind to allow mating) behaviour towards the cue.
Ran Around Cue	This behaviour was recorded if the *Carcinus maenas* reached the cue then continued to run in a confused manner around it.

Table 2: This Table above describes the behaviours exhibited from the *Carcinus maenas* within the study.

Chemical cues

The chemical cues were made from carboxycellulose gels infused with natural and synthetic odours of food, microplastic odour and the female shore crab's sex pheromones. These carboxycellulose gels were created by mixing carboxycellulose powder with artificial sea water and mixing well, the odour was added in at this stage. The pheromone gels were created using 4:1 uridine diphosphate and uridine triphosphate, at a concentration of 10^{-3} M. The pheromone gels used are not pH stable, due to the UDP/UTP chemical. The control gels used were blanks made from carboxycellulose mixed with purified water, the glutathione

(GSH) gels were made at a concentration of 10^{-3} M, these gels are not pH stable. The plastic odour gels were created by 1/6 plastic odour with carboxycellulose. The chemical structure of these can be seen in the Figure (11) below.

Preliminary testing was carried out to assess the cue dispersal rate and how long an odour lasted until it has entirely diffused and could no longer be detected. To Figure out how long the cues lasted, we added cues to the tanks and recorded results until the *Carcinus maenas* stopped responding, this was carried out multiple times to get the lasting time of the cue. To Figure out the time it took a cue to travel from one end of the tank to the other, we used red food colouring and timed how long it took to reach the end, this was tested at different flow rates. The results (Table 1 and 2 appendix B) showed odours took approximately 5 minutes to diffuse to the other end of the Y-shaped tank and lasted approximately 2 hours before needing replacing.

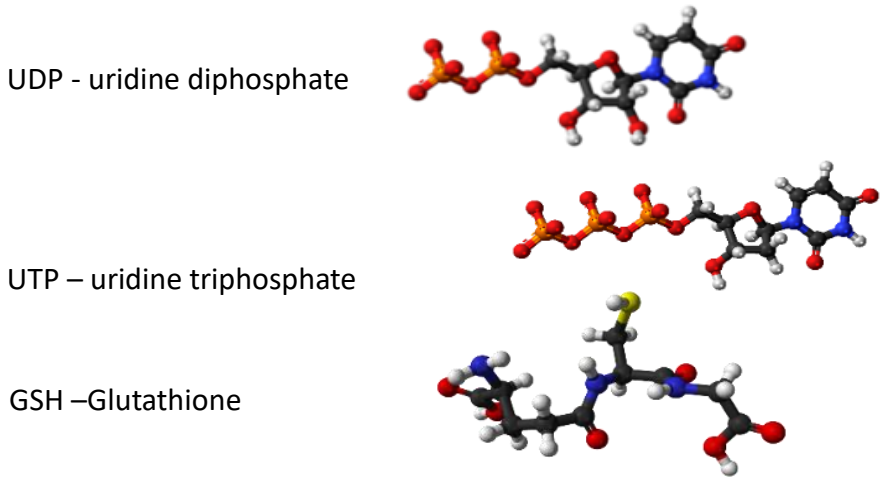


Figure 11. These images show the chemical structures of the three chemical cues being used in the study, the first molecule us UDP, the second is UTP and the third molecule is GSH. File: Glutathione-from-xtal-3D-balls.png - Wikimedia Commons. [cited 12 Mar 2020]. Available: https://commons.wikimedia.org/wiki/File:Glutathione-from-xtal-3D-balls.png

Experimental design:

Two identical Y-shaped olfactometers (Figure 12) were used to carry out the bioassays. The tanks were made out of a grey plastic, to limit reflections, light and shadows while testing.

Artificial sea water created at 35% salinity by mixing 35g of aquarium salt per litre of deionised water and stirring well was used for these systems. The pH of the sea water was dropped to the required levels by bubbling CO_2 through it until it reached the correct levels. This water was put into 114L water butts under the Y-shaped olfactometers. Flow was set up in both tanks, a pump was placed into the waters butts that pumped the artificial seawater (15-20ºC) up into the tanks. It entered the tank at the two tips of the Y and left at the base (Figure 12), the flow rate was set at 1 litre per minute, based on preliminary testing (results in appendix), so 500ml per minute entered through each tip of the tank. This flow rate allowed the chemical cues to diffuse at an equal rate, and allowed the odours from previous testing to be removed from the tanks. The tanks were filled up to 12cm, and the base of the tanks were covered in a layer of marine sediment (2.5cm thick) which was washed thoroughly to remove any odours before adding to the tanks. Both y-shaped olfactometers were set up identically, the only difference was the pH of the sea water. Once tanks were set up and the pH, temperature, light intensity and salinity had been measured, the freeze-dried gels were placed into silicone tea strainers, which were then placed at the tips of the Y-shaped olfactometers with flow running over them, allowing the cues to diffuse. The cues being used were placed blindly at either end of the Y, to eliminate any chances of bias and anomalies that may have occurred.

The crabs that were used were kept in current pH (8.2) and at 15-20ºC, males and females were stored in separate tanks, with up to 8 crabs per tank. The crabs were marked with numbers before testing started, they were weighed, carapace measured, colour of the shell

recorded and overall condition was noted. The crabs used were being fed twice a week with mussels.

Once both tanks were set up, a crab was placed in a holding basket at the base of the tank, then left to acclimatise for 2 minutes. The basket was lifted after two minutes and timer started. The data recorded was initial reaction time (seconds) which was marked by rapid antennular flicking, whether they went to cue/control/neither, reaction at the cue (whether there was no visible reaction, cradled the cue, buried, wafted frequently or ran around it) and how long it took them to reach the cue (seconds). These behaviours are described in Table 2. The crabs were given five minutes to react. This was carried out on 40 crabs, in all three pH's for each experimental condition, creating 720 bioassays. Each day the 40 crabs were rotated from the tanks that housed around 170 crabs, so the crabs had around four days of rest before they were used again.

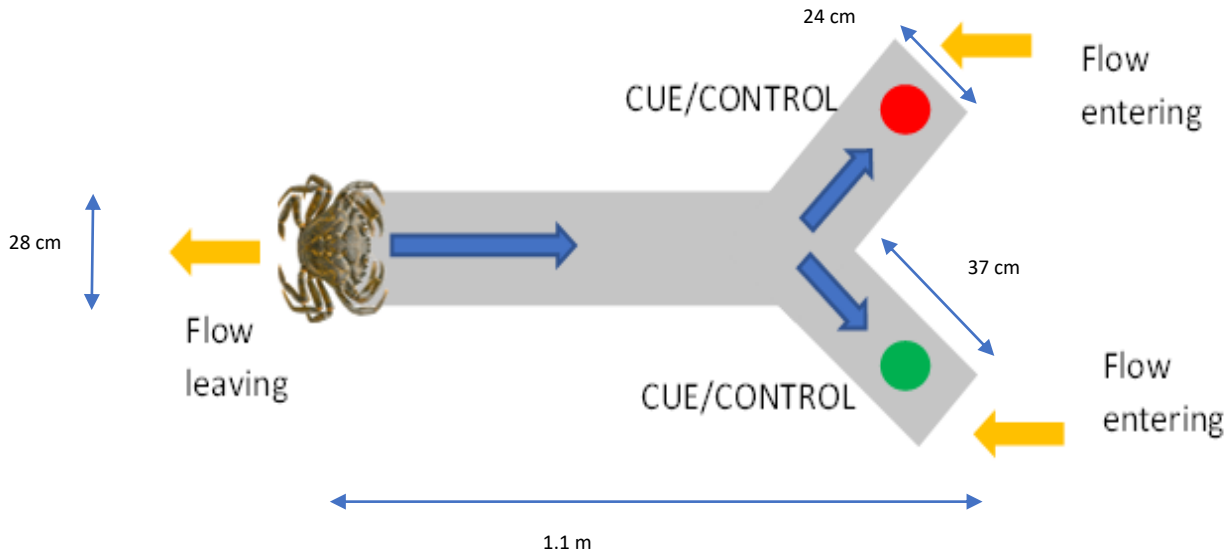


Figure 12. This diagram was created to show the layout of this experiment. It shows the setup of the Y-shaped olfactometer, with the flow entering at the tips of the Y and flow leaving at the base of the Y. It shows the location of the cue/control for the study. The crab is placed at the base before it is released.

7.3.2 Football Pitch Method:

Heatmaps were created using the football pitch method (Figure 13). A large tank was set up (58cm by 31cm), filled with 20 litres of artificial sea water (35% salinity, 19 ºC), and a tripod was placed 83 cm above the tank, where the camera (iPhone) was placed. The plastic odour used was created by blitzing PE pellets and adding to distilled water, the odour was left for 48 hours before filtering. 2ml of odour was placed on 4.25cm round filter paper.

Filter paper containing one of the 2 cues were placed at opposite ends of the bioassay container, the cues were plastic odour and control (distilled water). The crabs were placed into holding baskets within the tank and acclimatised for 20 seconds in centre of tank, allowing cues to diffuse. Crabs were released and time recorded in each area recorded on video for 2 minutes. The plastic odour and the cue were regularly swapped around between tests to reduce bias.

Data collection and video analysis was performed by the same person, recordings of time spent in each zone were treatment blind to remove observational bias. Crabs that stayed in the same place for the video were removed from the data set. Video footage was inputted into the Lolitrack software, once the videos uploaded, tracking analysis steps were carried out which created individual heatmaps. The steps carried out on each video included selecting the tank area followed by the individuals body points, once these were done, the software processed the video and provided the heatmap video.

22 crabs were tested, 11 females and 11 males, in pH 8.2 and 7.6, creating 44 bioassays to be analysed by an automated video tracking system, Lolitrack5^{TM}. The videos were uploaded onto the software, before being calibrated within the software itself, the videos were recorded in pixels and seconds.

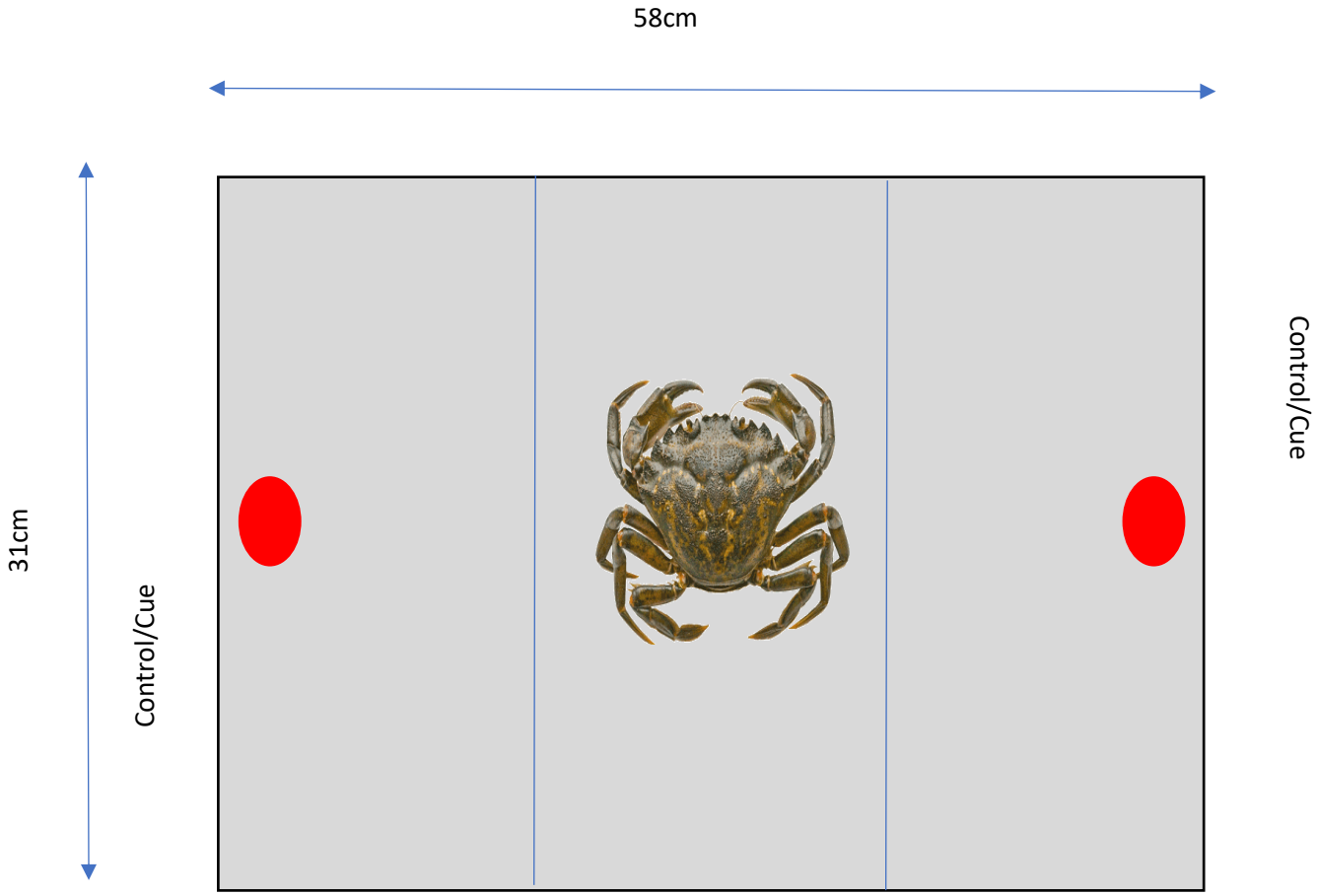


Figure 13. This diagram shows the layout of the tank that we used to create the heatmaps. The crab was placed in the centre of the tank and the video was started. The videos are two minutes long. They were then analysed using the Lolitrack software.

Statistical analysis:

All statistical analysis was carried out in R studio v1.3, data was put into excel spreadsheets with simple columns, it was then saved as a comma separated value file (.csv) which allowed it to be read in R studio and analysis performed. Some of the Figures were created via Microsoft excel. R script can be found in appendages.

All data was tested for normality with the Shapiro wilk test before stats were carried out, the data was not normal $p = <0.05$ and was discontinuous, so non-parametric tests were carried out.

Wilcoxon matched pair tests were performed to compare average initial reaction times in the three different pHs in order to see if pH change caused a significance difference to these results. Kruskal Wallice tests were carried out to test differences in individuality data, to show if certain personality traits within the crabs caused certain results to reoccur.

The heatmaps and video footage was analysed and created on Lolitrack software.

7.4 Results:

7.4.1 Y-Shaped olfactometer:

720 individual behavioural bioassays were completed within this study, under six different experimental conditions, with cues placed in Y-shaped olfactometers. These conditions were PE vs Control, PE vs Food, PE vs Pheromone, Pheromone vs Control, Pheromone vs Food and Food vs Control. All of these conditions were tested in pH 8.2, 7.6 and 7.2.

One of the key findings from this study was that the overall average initial reaction time (Antennular flicking) of the crabs in the lower pH is much higher (Figure 14). Looking at the average initial reaction times in all three pH levels, it starts at 2.79 seconds at pH 8.2, then 6.01 seconds in pH 7.6 and finally 12.69 seconds in pH 7.2, so there is a difference of 9.9 seconds between pH 8.2 and 7.2. A paired T-test was carried out on all initial reaction times in the three pH's, there was significance in mean initial reactions between pH 8.2 and 7.6 $t(11)= -9.045$, $p= .00001$, between pH 8.2 and 7.2 $t(11)= -6.33004$, $p= .0017$and between pH 7.6 and pH 7.2 $t(11)= -3.60281$, $p= .001581$. So all of these show significance of $p < .05$.

The initial reaction times in pH 8.2 and 7.6 vary slightly between conditions, but not noticeably. However, PE v Food and PE v Control have significantly slower initial reactions

time in pH 7.2, compared to the other conditions, this can be seen on the error bars on figure 16. This allows hypothesis three to be accepted, which states there is a greater impact on the *Carcinus maenas* olfactory capacity when stressors are combined, in this case, plastic odour and low pH. The reason for PE v Pheromone not following the trend of the other two conditions using PE may be due to the reproductive season, the males' natural instinct during this season is to be attracted to female sex pheromone over any other chemical cue (Hayden et al., 2009). So expected responses would be males choosing pheromone over food and plastic odour during the reproductive season if the plastic odour represents a food cue type stimulant.

Using a Kruskal Wallis test that is used to determine if there is a statistically significant difference between two or more groups of variables. All of the p values below came from a Kruskal Wallis test carried out on R studio. We found that there is statistical significance between the size of the crab (cm) and its initial reaction time (s), p=0.047 (Table 3, row d). There is also significance for size and decision made (which cue they chose) (p=0.0016) (Table 3, row f). There is statistical significance between decision made and sex of crab (p=0.00012) (Table 3, row g) and between the sex of the crab and its reaction to the chemical cue (p=0.0015) (Table 3, row a). There is statistical significance between weight of the crab (g) and pH (p=0.0146) (Table 3, row l), this shows that pH has a different impact on crabs of different weights, which may explain some of the ranges within results. All of the results from the Kruskal Wallis tests can be seen in Table 3 below, these were carried out via Kruskal Wallis and not by a one-way anova as the measurement values didn't fit the assumption of a one- way anova.

Kruskal Wallis	**χ 2**	**df**	**p-value**
a. Sex x behaviour	19.566	5	0.001507
b. Injury x cue chosen	9.5196	4	0.04935
c. Condition x behaviour	14.243	5	0.01414
d. Initial Reaction x size	36.673	24	0.04716
e. Initial Reaction x colour	4.1635	1	0.0413
f. Cue chosen x size	49.527	24	0.001626
g. Cue chosen x sex	14.727	1	0.0001242
h. Cue chosen x weight	94.908	64	0.007296
i. Time to cue x injury	8.99	1	0.002715
j. behaviour x size	37.75	24	0.03677
k. behaviour x injury	4.2399	1	0.03948
l. pH x weight	91.095	64	0.01467
m. Size x injury	10.187	1	0.001414
n. Weight x injury	6.0947	1	0.01356

Table 3. This Table shows the results of Kruskal Wallis tests carried out on certain variables; the highlighted Figures show significant results from the tests, so there was high significance between cue chosen and size, cue chosen and sex and cue chosen and weight. There was also significance found between initial reaction and size and initial reaction and colour, all of which link to individuality.

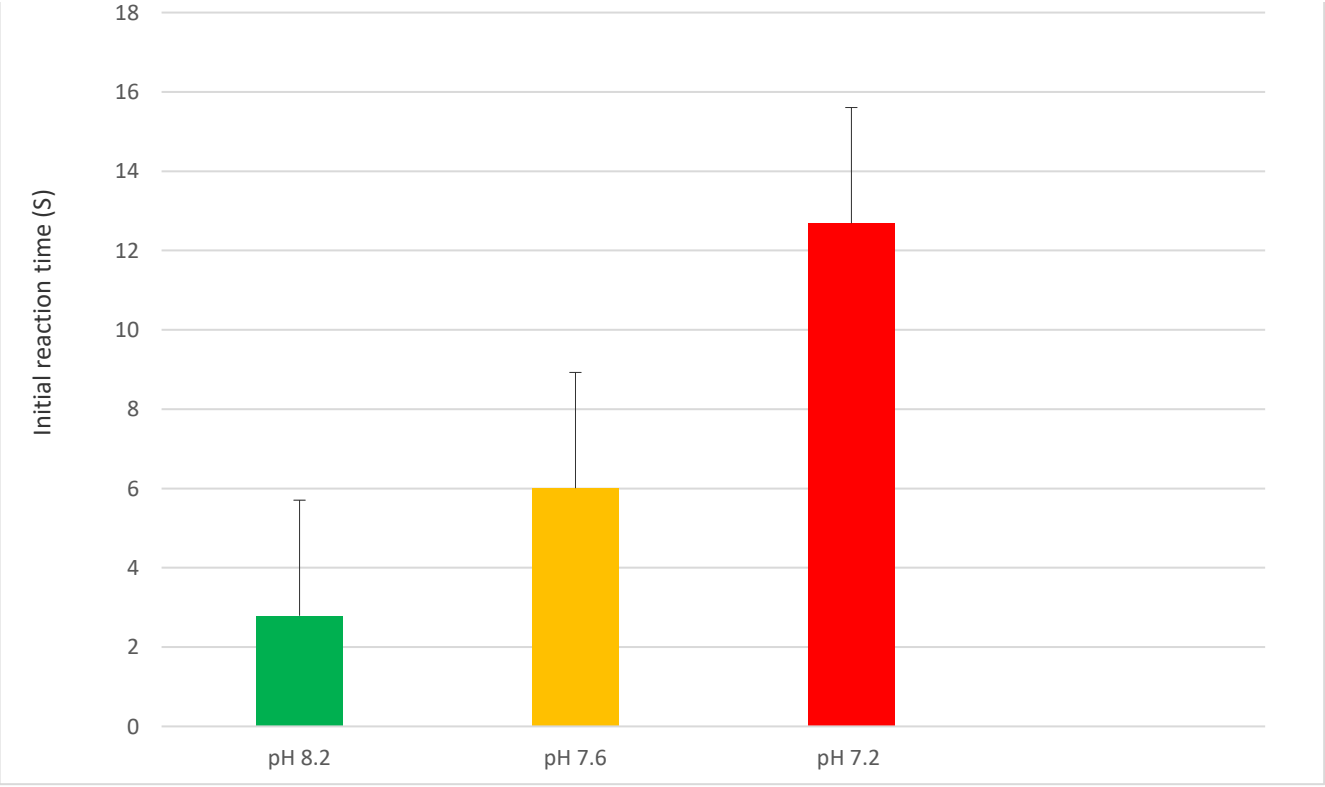


Figure 14. This graph shows the average initial reaction time (s) in three pH levels, 8.2, 7.6 and 7.2. in pH 8.2 it was 2.79 seconds, it then increased to 6.01 seconds in pH 7.6 and then doubled in pH 7.2 to reach 12.69 seconds. Initial reaction time was recorded as rapid antennular flicking. These are the results from all experiments.

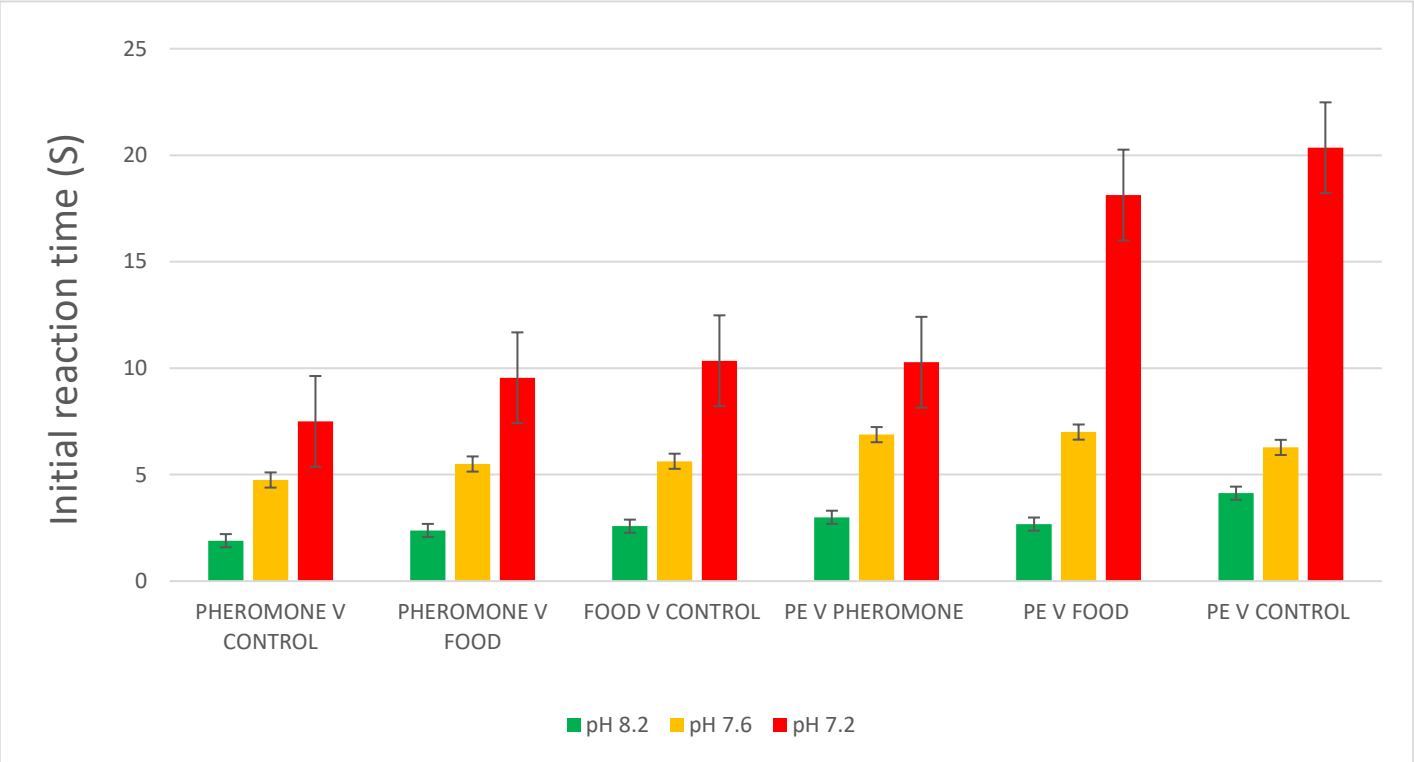


Figure 15. This graph shows the initial reaction times (s) of the crabs in each experimental condition, under three different pH's, 8.2, 7.6 and 7.2. The reaction times are much larger in pH 7.2, due to the crab's showing confusion and taking longer to detect cues. This is prominent in PE v food and PE v control.

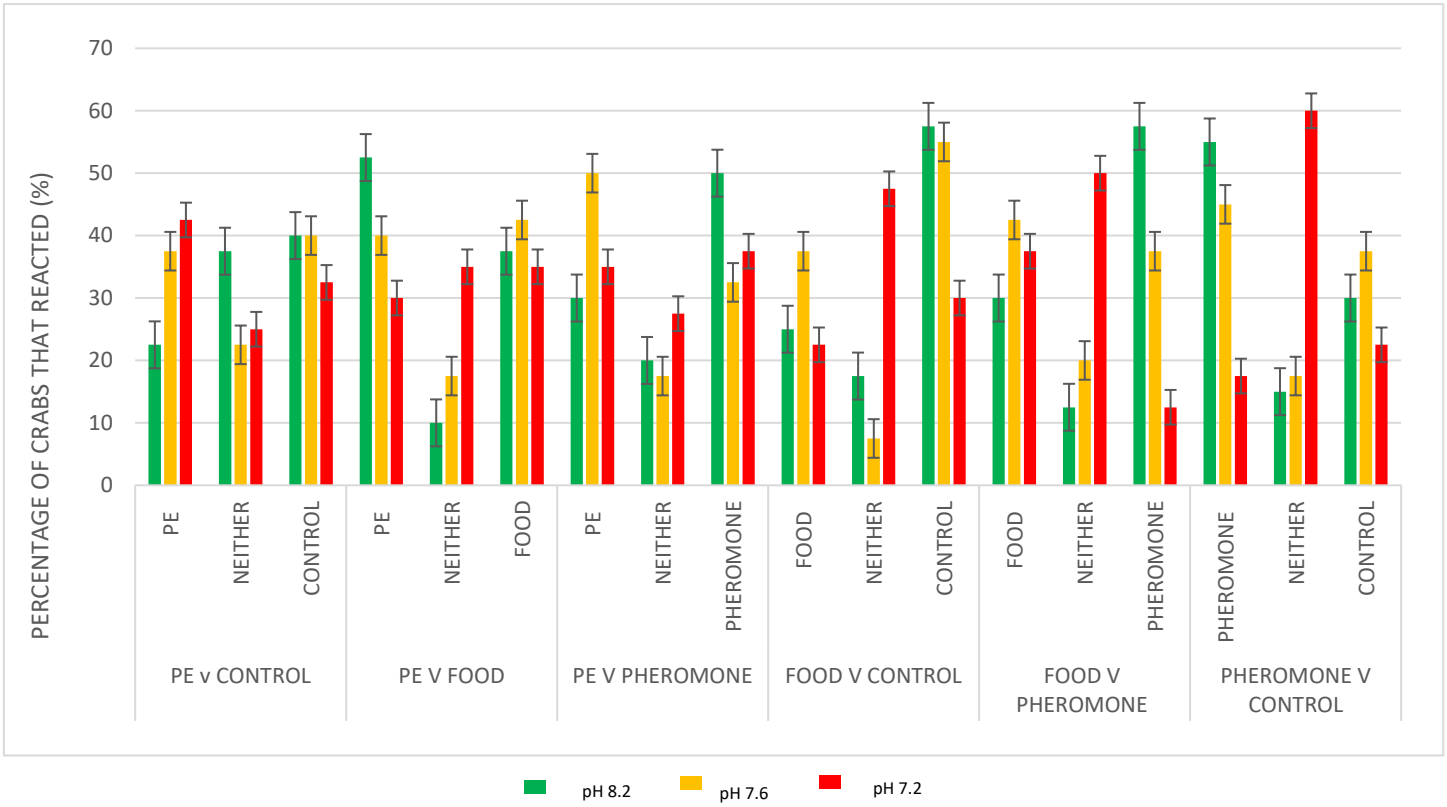


Figure 16. This graph shows the percentage of crabs that reacted to each cue or didn't react at all, in all six of the experimental conditions, looking at pH 8.2, 7.6 and 7.2.

Looking at Figure 16, in PE v Control most the crabs in pH 8.2 chose control, pH 7.6 chose control slightly more over PE and in 7.2 they chose PE more. In PE v Food most the crabs in 8.2 chose PE, in 7.6 they chose Food slightly more than PE and in 7.2 they choose food/neither equally.

In PE v Pheromone the crabs choose pheromone mainly, in 7.6 they choose PE and in 7.2 they pick PE and pheromone. In Food V Control the crabs in 8.2 choose control, in 7.6 control also and then in 7.2 they picked neither. In Food V Pheromone carbs in 8.2 chose pheromone, 7.6 chose food and 7.2 picked neither. Finally in Pheromone v Control, most crabs in 8.2 picked pheromone, in 7.6 pheromone and 7.2 neither. The choice of pheromone is what we would expect testing on males at this time of year (reproductive season). The choice of neither in pH 7.2 shows a sort of odour confusion, with the crabs not being able to

make a decision on the two cues present. The highest levels of indecision can be seen in pH 7.2. Here the crabs either struggle to decide, or failed to correctly detect the cues, presumably due to chemical changes that are occurring at reduced pH levels or due to physiological impacts upon the crabs.

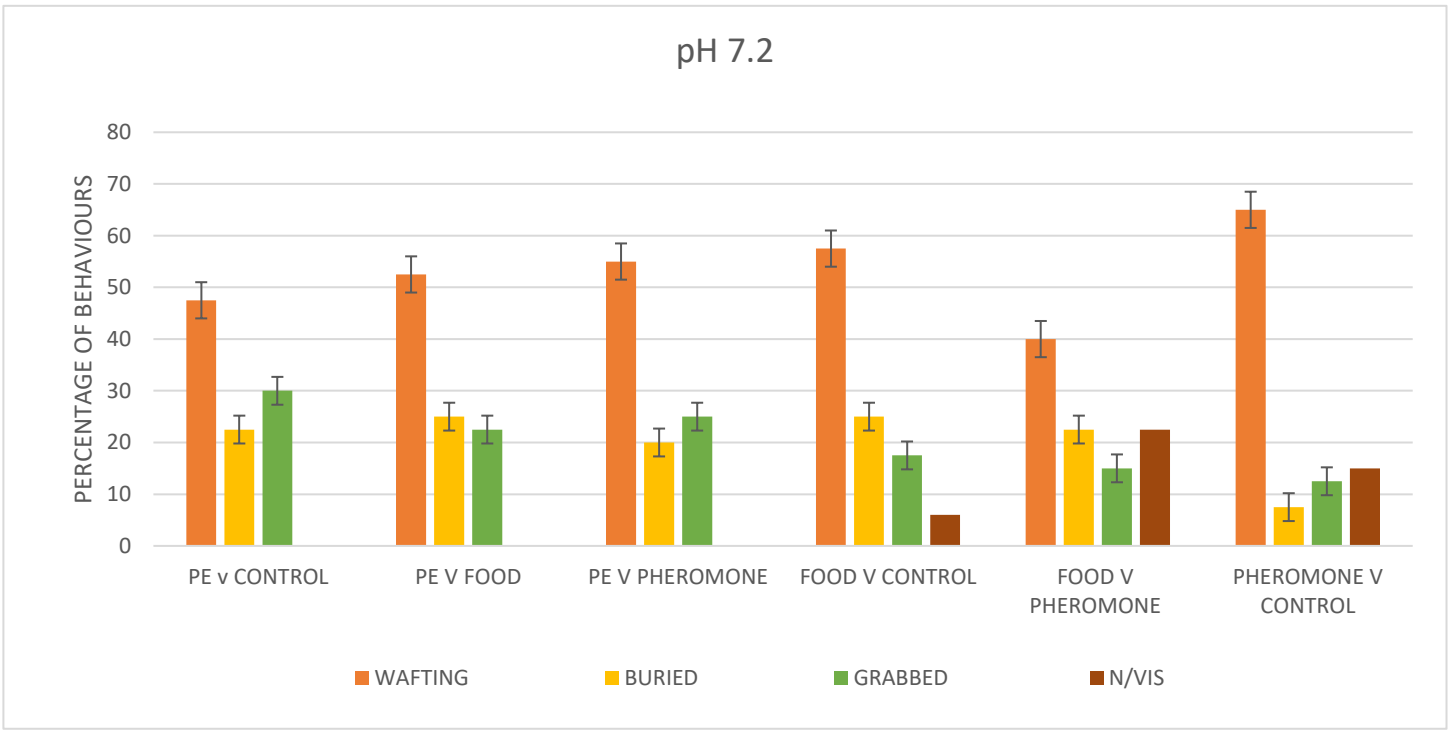


Figure 17. This graph shows the percentage of crabs which exhibited certain behaviours (Table 2). This pH shows the highest levels of burying. It also contains the only non-visible reactions from the crabs.

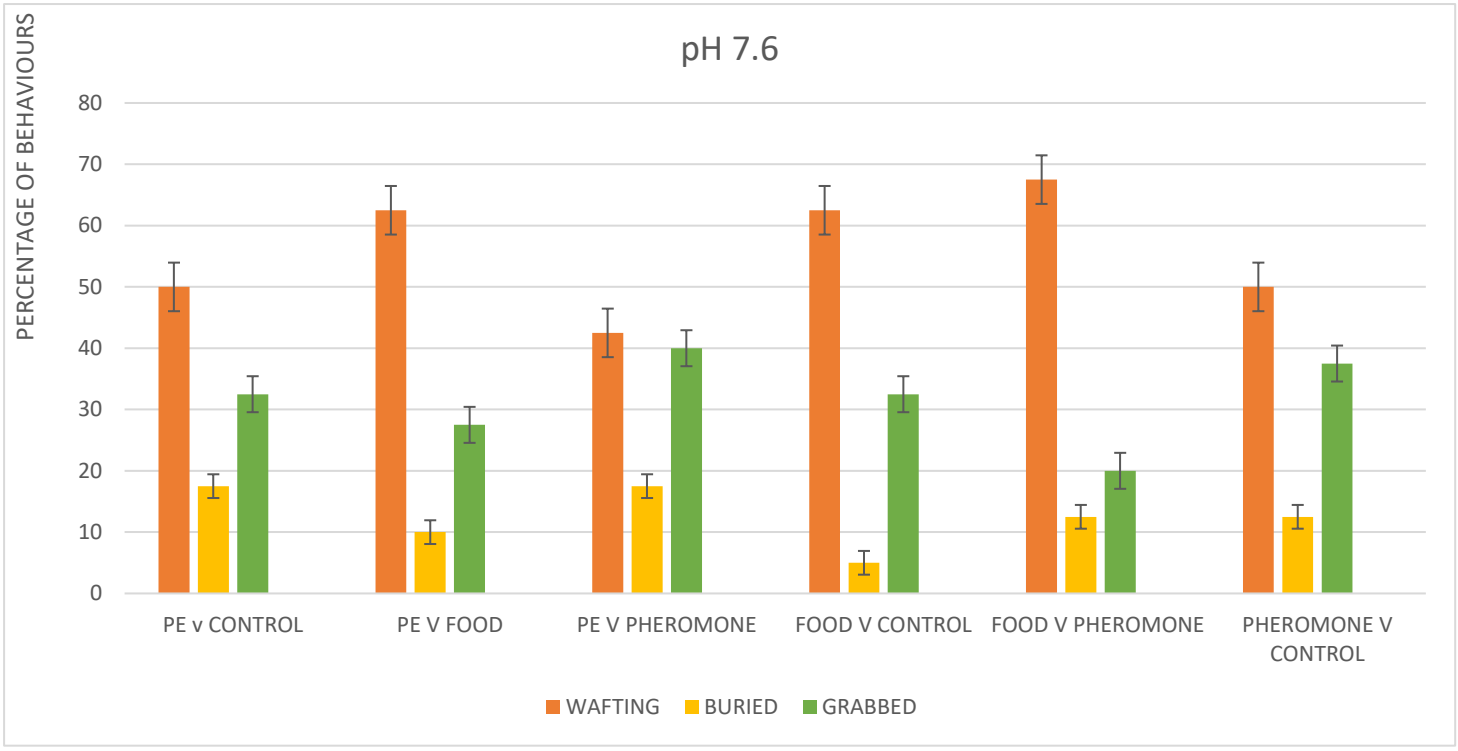


Figure 18. This graph shows the percentage of crabs which exhibited certain behaviours (Table 2). This pH shows higher levels of wafting compared to pH 7.2.

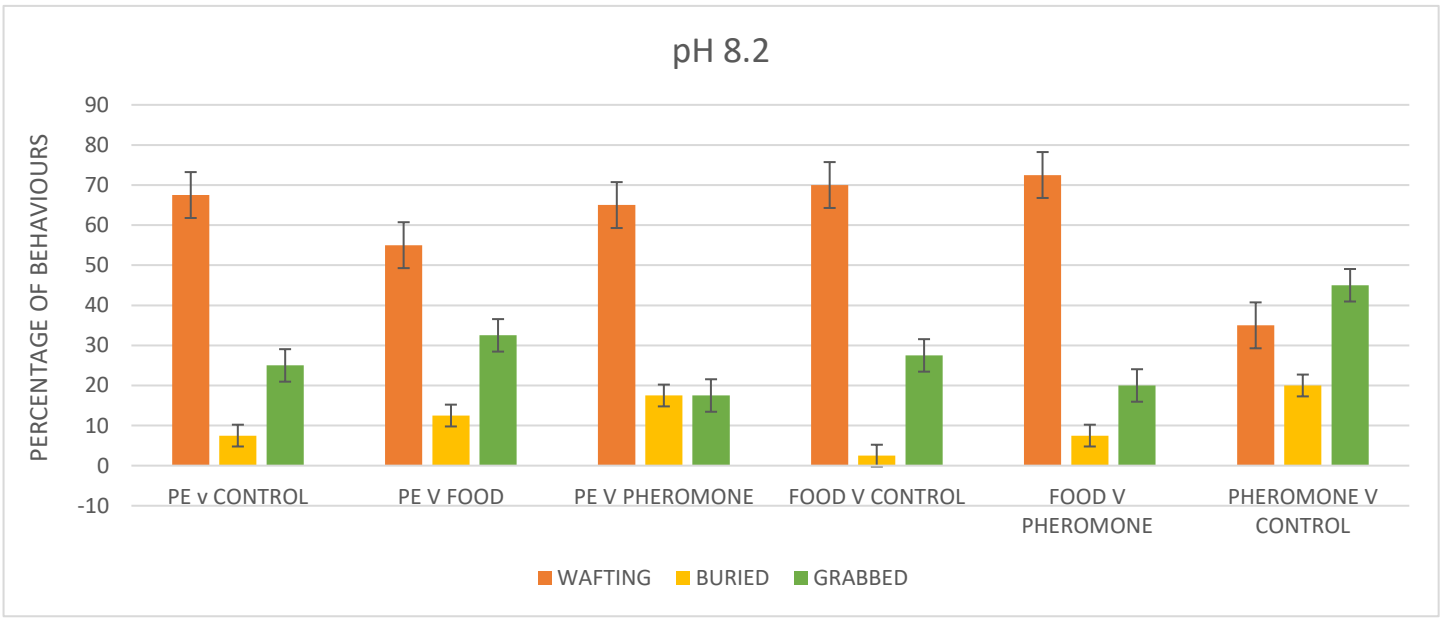


Figure 19. This graph shows the percentage of crabs which exhibited certain behaviours (Table 2). This pH shows the highest level of crabs wafting and the lowest number of crabs burying.

Looking at individual behaviours in Figure 17, the crabs buried themselves more in pH 7.2, compared to the others (Figure 18 and 19), this was also the only pH to have some crabs show no visible reaction to the cues. This trend could show the crabs are very indecisive or stressed in these conditions, causing them to bury for self-protection, the crabs that showed no visible response could have frozen due to the stress of the condition they were in. One interesting trend from this graph is that none of the crabs showed no visible reactions when PE was present, they all wafted, grabbed or buried when they detected the cue.

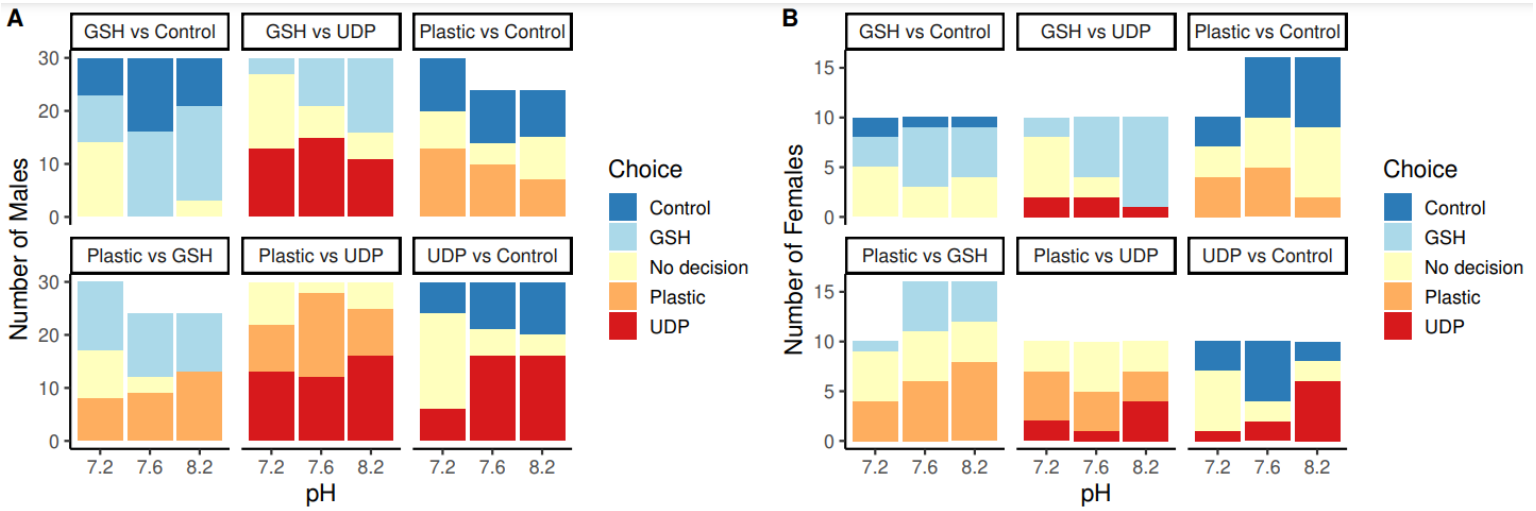


Figure 20. This graph gives a basic breakdown of how many animals chose which cue in each condition, in all three pH's, but taking into account sex. The left Figure looks at males and their reactions and the right Figure looks at the females.

In the plastic v GSH condition (Figure 20) there is a very similar pattern seen between males and females, with the attractiveness of the plastic dropping with the pH. However, in plastic V control, this pattern is reversed, making the PE odour interesting as we can't tell if it's an attractant. An interesting assay to look at is the females in UDP v control, as it is not expected for that many females to have chosen the female sex pheromone.

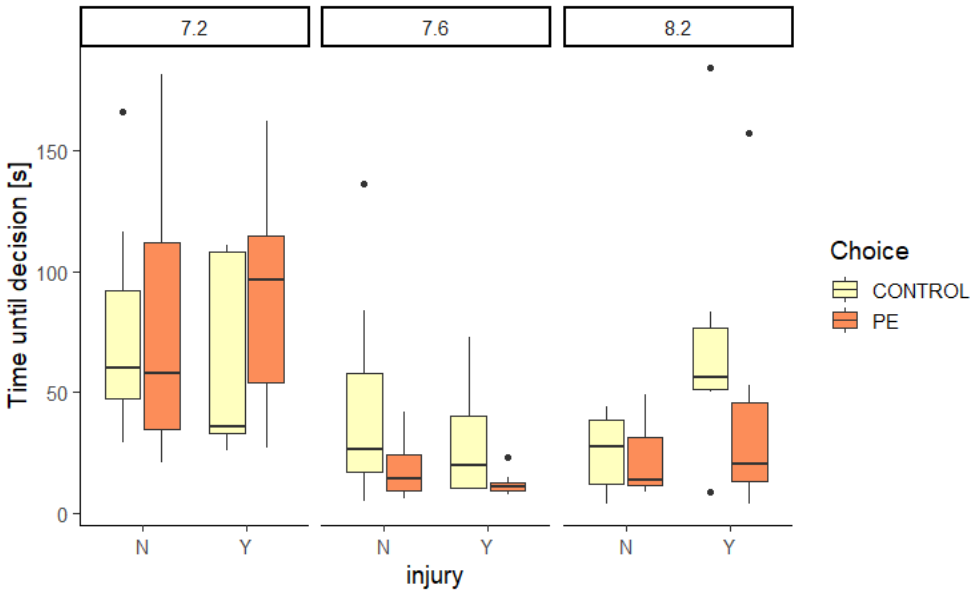


Figure 21. This histogram shows the time it took each crab to make a decision in all three pH's under the control v PE experimental condition.

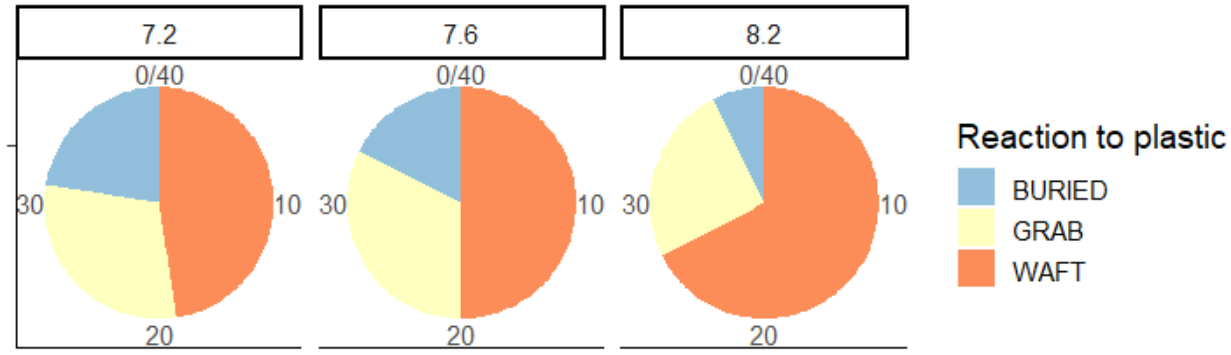


Figure 22. These pie charts show the number of crabs that buried, grabbed the cue or wafted in PE v control. The greatest amount of wafting occurred in pH 8.2, before dropping off in pH 7.6 and 7.2. The most burying can be seen in pH 7.2, probably due to higher levels of stress and confusion within the crabs.

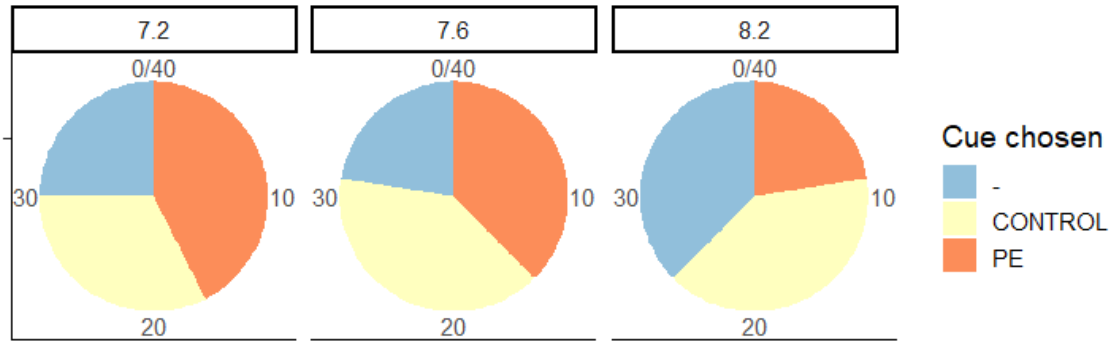


Figure 23. These pie charts show the percentage of crabs that chose the PE cue, control cue or neither in all three pH's.

	GSH V Control			GSH V Pheromone			Pheromone V Control		
	GSH	Control	N/A	GSH	Pheromone	N/A	Pheromone	Control	N/A
8.2	25	57.5	17.5	30	57.5	12.5	55	30	15
7.6	37.5	55	7.5	42.5	37.5	20	45	37.5	37.5
7.2	22.5	30	47.5	37.5	12.5	50	17.5	60	22.5

Table 4. This Table shows the percentage of crabs that chose each cue in all three pH's in GSH v Control, GSH v Pheromone and Pheromone v Control.

pH	**PE V Control**			**PE V GSH**			**PE V Pheromone**		
	PE	Control	N/A	PE	GSH	N/A	PE	Pheromone	N/A
8.2	22.5	40	37.5	52.5	37.5	10	30	50	20
7.6	37.5	40	22.5	40	42.5	17	50	32.5	17.5
7.2	42.5	32.5	25	30	35	35	35	37.5	27.5

Table 5. This Table shows the percentage of crabs that chose each cue in all three pH's in PE v Control, PE v GSH and PE v Pheromone.

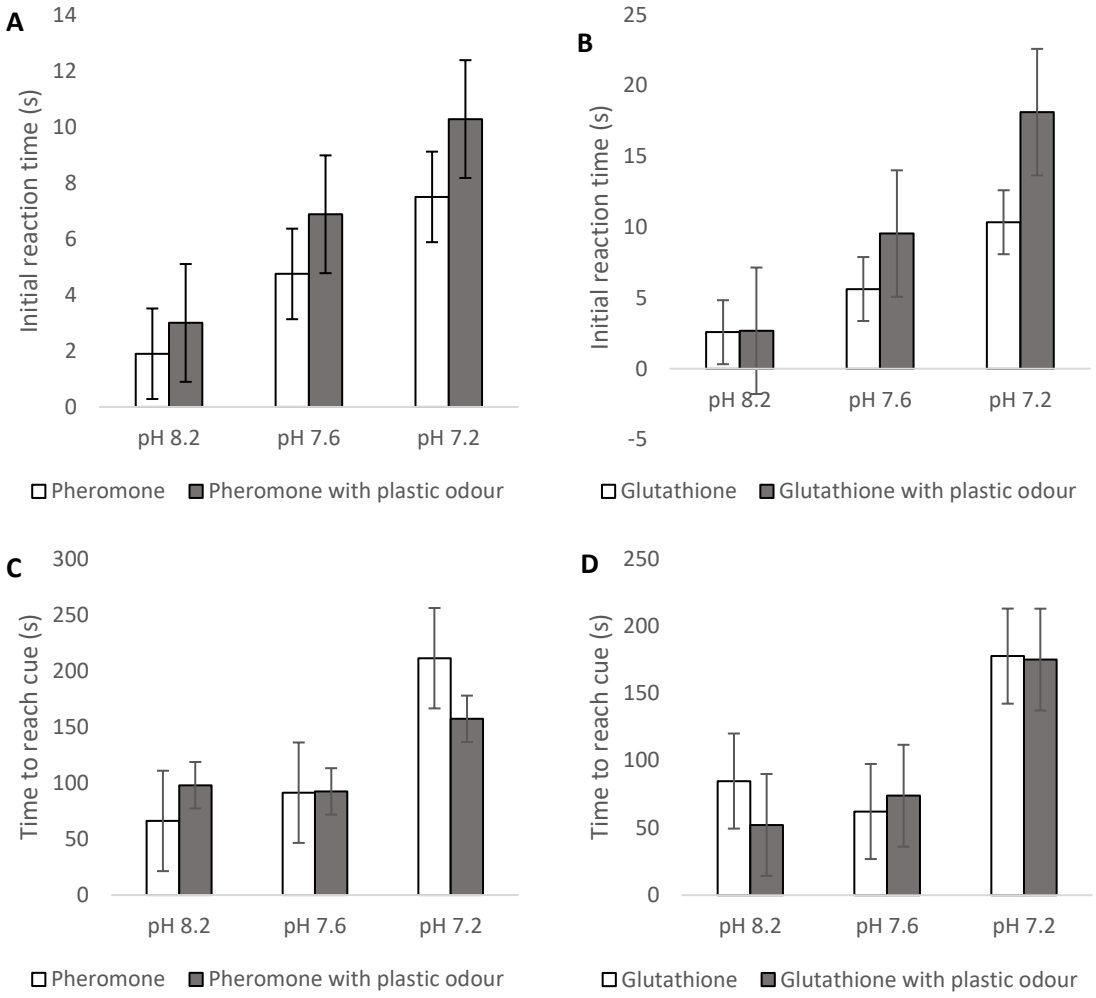


Figure 24. Graph A shows the initial reaction time in all three pH's for pheromone and pheromone with plastic. Graph B shows the initial reaction time in all three pH's for glutathione (GSH) and glutathione with plastic. Graph C shows the time to reach the cue in all three pH's for pheromone and pheromone with plastic and Graph D shows the time to reach the cue in all three pH's for glutathione and glutathione with plastic.

The graphs above (Figure 14 and 15) show initial reaction time increasing at pH falls, but the main pattern is the increase in initial reaction time when plastic odour is present. The plastic odour is causing much more confusion, therefore much slower reaction times. Graph A shows around 1 second delay to the initial response when plastic is present.

7.4.2 Lolitrack:

Statistical analysis was carried out on the behavioural assay videos recorded for the football pitch method. A Wilcoxon test compares two paired groups of data, here we looked at data linking to average distance to the plastic (PE), average distance to plastic was worked out using Lolitrack software and R studio. The Figure 25, below looks at males and females in pH 8.2 and 7.6. The P value for 7.6 was p=0.07 (not significant) and 0.25 (not significant) in pH 8.2. This Figure still clearly shows that males are more attracted to the plastic odour than females, and they are attracted more in the lower pH. This would suggest there is something within the plastic that becomes more attractive to males as the pH falls. Figure 26, seen below, shows heat maps created by the Lolitrack software. The images show where the crabs spent their time during the testing.

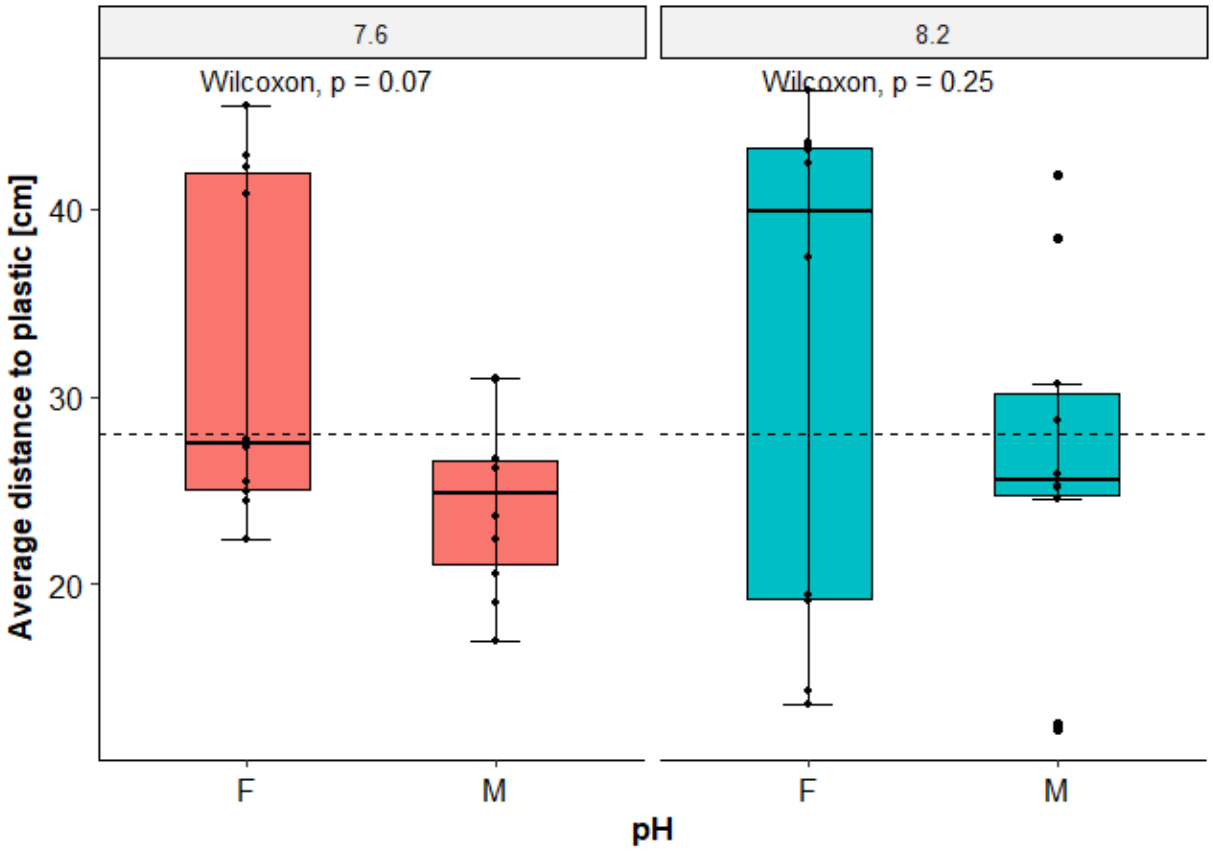
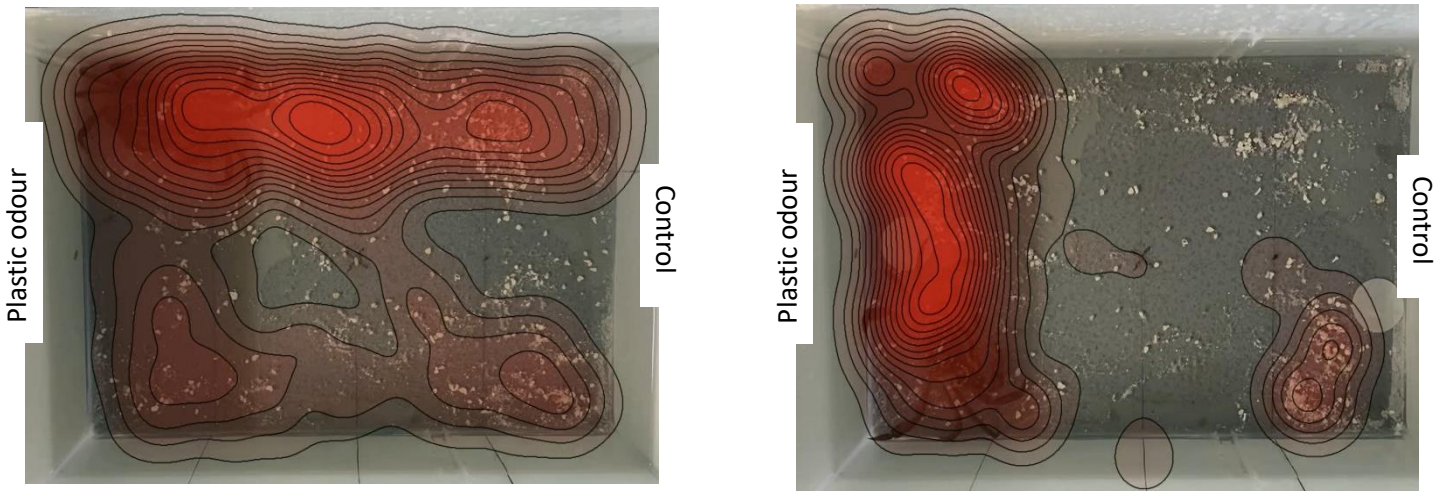


Figure 25. This Figure shows the average distance to the plastic in pH 8.2 and 7.6, within males and females. The values from the Wilcoxon test are p=0.07 in 7.6 and p=0.25 in 8.2, neither of which are statistically significant.



Control male pH 8.2

Male pH 8.2

Figure 26. This Figure shows two different composite heat maps, showing a male exposed to plastic odour in pH 8.2 and a male exposed to a blank control in 8.2. The control image shows what you would expect when no cue is present, very fluid movement and not spending a huge amount of time in either end. The other image shows a male that had a very strong attraction to the plastic odour, spending most its time on the cue side of the tank.

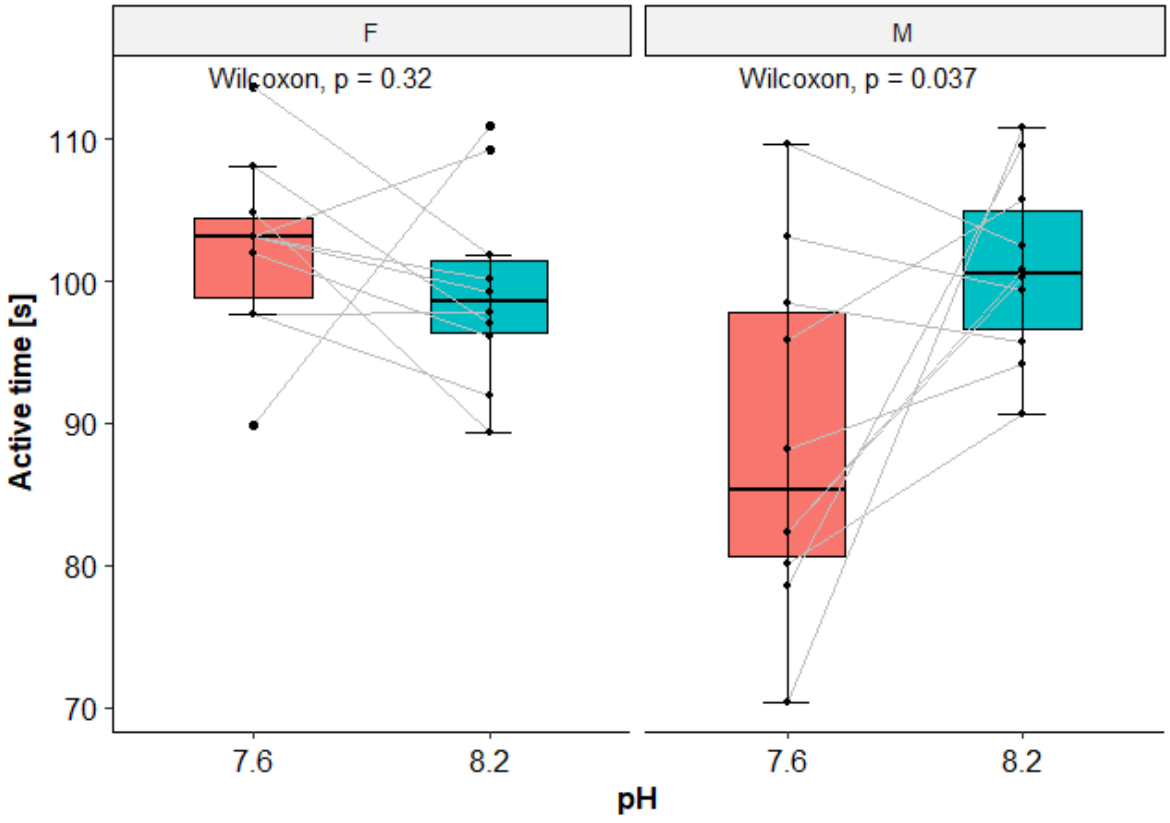


Figure 27: This histogram shows the active time of male and female crabs in pH 8.2 and 7.6, these results are from the Lolitrack footage.

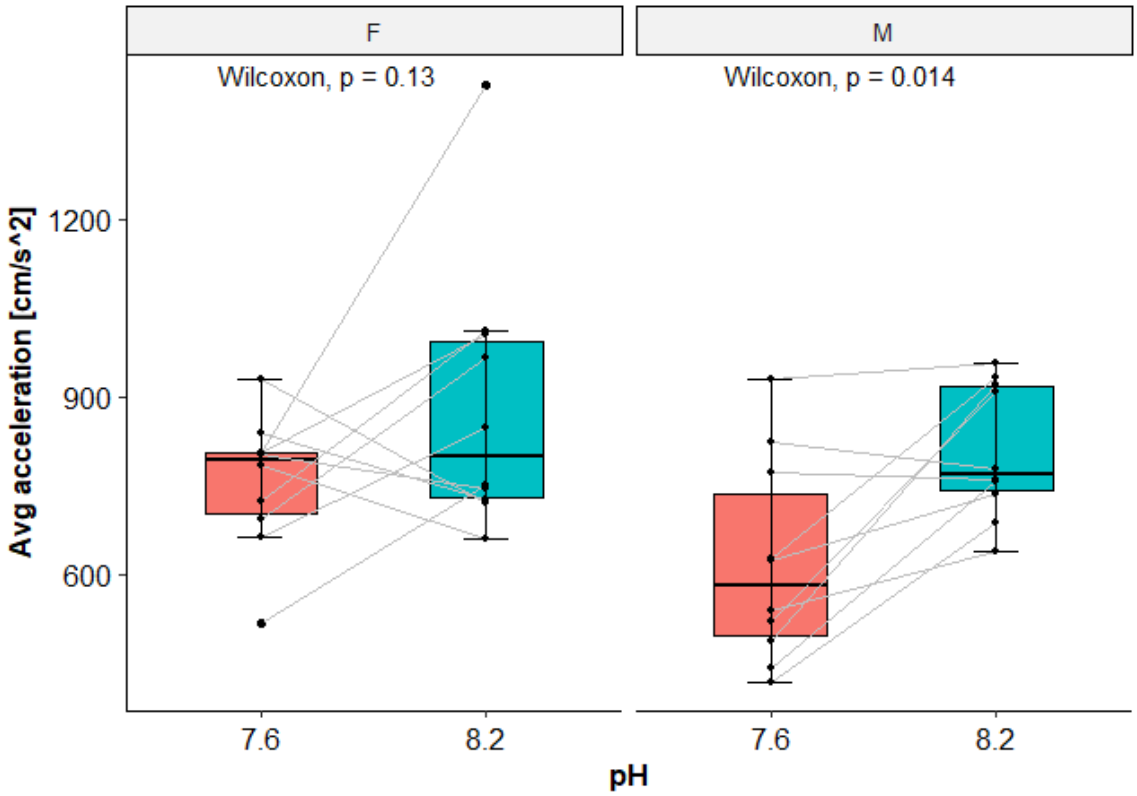


Figure 28: This histogram shows the average acceleration of males and females, in pH 8.2 and 7.6, these results are from the Lolitrack footage.

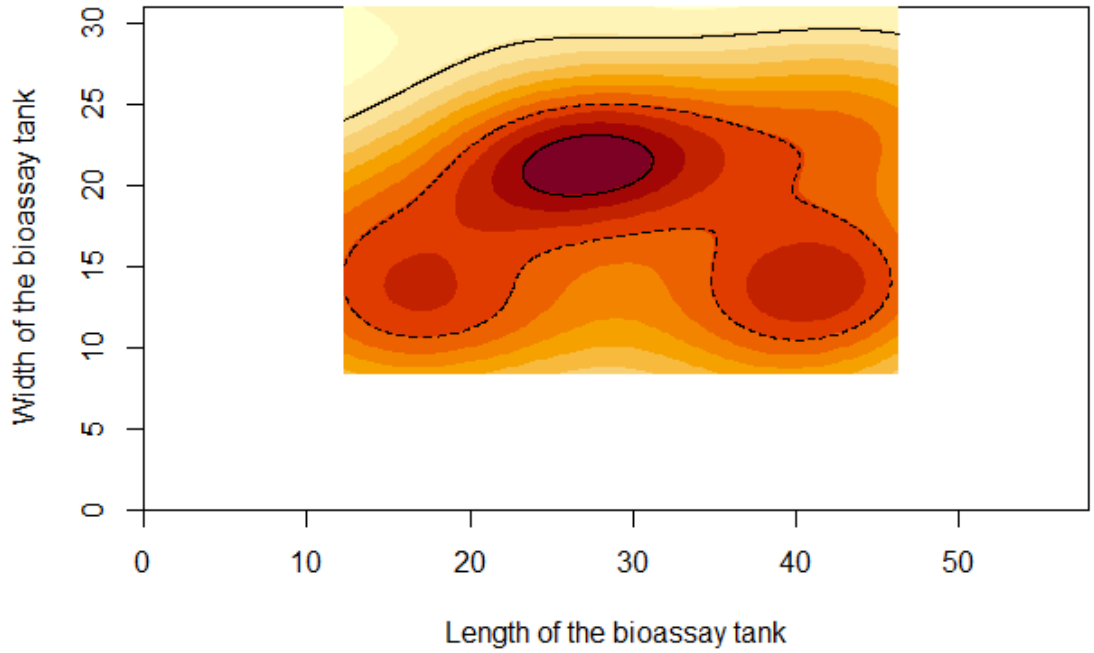


Figure 29: This image is a heatmap, created with all of the Lolitrack footage combined, there are no obvious trends.

7.5 Discussion:

In this study we observed how shore crabs, *Carcinus maenas,* behaviour and responses to odour cues may be impacted when pH is dropped to future OA levels. A combination of OA and MP was hypothesised to create greater issues for *Carcinus*.

Overall, the outcomes from this current study on *Carcinus maenas* indicate that the crab's initial reaction times begin to slow down when exposed to the lower pH conditions (Figure 14 and 15), this may have been caused due to a physiological change within the crab. Initial reactions are impacted by just a 0.5-unit pH drop, which is the predicted drop by the end of the century (Cox et al.,2000). It is significantly impacted by a 1-unit pH drop, which could occur in areas of upwellings and areas with natural pH fluctuations like estuaries and tide pools (Bitter et al., 2021, Hofmann et al., 2011, Richardson et al., 2021).

One of the main aims of this study was to explore whether microplastic odour under altered pH conditions causes greater impacts to *C. maenas* behaviour. The results from the study indicate that in some of the experimental conditions the combination of stressors (low pH and microplastic odour) have a greater impact on the *C.maenas* olfactory capacity. The conditions that show this best are PE v Food and PE v Control. *C.maenas* have significantly slower initial reactions time in pH 7.2, compared to pH 7.6 and 8.2 (Figure 14 and 15).

Our data from this research contributes to the growing amount of climate change research observing the impact of reducing pH on marine life (De la Haye et al., 2012). It confirms that *Carcinus maenas* are a species impacted by falling pH (Richardson et al., 2021), alongside other groups such as; fish, molluscs and polychaetes (Espinel-Velasco et al., 2021, Munari et al., 2018). A key conclusion taken from these results in the current study is; lowered pH impacts the Carcinus maenas olfactory system, and alters behavioural responses. Crabs in lower pH had slower reaction times, reacted more unusually and showed mixed responses to

signals given to them. The consequences of this include more time actively searching for food, less time sheltering, and therefore more time at risk of predation.

Other species are experiencing similar problems when exposed to lower pH levels, the European sea bass (*Dicentrarchus labrax*) had to be 42% closer to odours in order to detect them in OA conditions, lowering their chances of avoiding predators and finding food in the future predicted conditions unless they learn to adapt (Porteus et al., 2018). Manriquez et al (2020) studied the impacts of OA and OW on the shell crushing crab *Acanthocyclus hassleri*, one finding was the OA decreased pinching strength of the crab, which would make them a less effective predator long term. Our outcomes also demonstrate that ocean acidification alone alters the reception of olfactory cues by slowing the detection of these cues, without another stressor present.

Similar works has shown lower pH affects the efficiency of the stimulus receptor binding, by altering the charge distribution on the stimulus molecule which leads to reduced responses in some species, including *Pagurus tanneri* (Kim et al., 2016, Stachowicz et al., 2007, Wu et al., 2017). Kim et al (2016) found that alongside delayed antennular flicking within *Pagurus tanneri*, there was also a reduction in prey detection, which could be catastrophic for the species survival long term. Data from our study showed that antennular flicking (Initial reaction) was delayed as pH fell (Figure 14), indicating that crabs take longer to recognise a cue when present within the environment. If crustacean behaviours are affected by lower pH other marine species may also be having problems.

Crustaceans are not the only group being affected, a study looking at predatory reef fish found elevated CO_2 levels made larval fish more vulnerable to predation. However, Cripps et al (2011) also found predators spending 20% less time in water containing prey odours, this is a positive for prey species as they may be harder to detect. Behavioural changes are being

found in a larger range of marine species (Cripps, Munday and McCormick, 2011), and are a huge threat for the future of certain species. Data from this study showed reduction in initial detection of cues in *Carcinus*, which would link with the findings from Cripps et al (2011), if initial detection is affected by lowered pH/elevated CO_2 the prey detection will be lower, leading to less time within these areas (Richardson et al., 2021).

Alongside pH altering prey detection, it may also be affecting mate detection. Male *Carcinus* rely heavily on chemoreception in order to detect the female pheromone odours as the mating is restricted to the very short period after the female moult (Hardege et al., 2002). Findings of Hayden et al, (2007), demonstrated males react to sex pheromones more frequently than food during the reproductive season (May-October). Our main research period was during the *Carcinus maenas* reproductive season, so male's preference to pheromone was as expected. Velez et al, (2019) studied Sea Bream, their results showed that when a feeding cue is present, amino acid responses only fell slightly and providing a male with a pheromone can act as a deterrent (Hayden et al., 2011), this may also be the case in male *Carcinus maenas*, if this study was repeated in winter.

Male *Carcinus maenas* showed sexual behaviours within the study, this included taking up cradling positions, when UDP and UTP odours were present. These nucleotides are major components of female crab urine (Hardege et al., 2011). Once a male senses a female odour they respond with a searching activity that can lead to a guarding behaviour, which involves cradling the female crab (Eales A.J, 1973). The detection of the pheromones is altered when pH is dropped (Figure 16), if males can't detect the females as accurately, the species could struggle to reproduce, and therefore impact the future of the species.

This study used a unique bouquet of pheromones (Fletcher et al., 2021), which have been made into a pheromone gel using a carboxy-cellulose matrix from which they dispersed.

Pheromone gels are a technique that could be used to help with the invasion of the shore crab to areas of the world including South Africa, Australia and the North coast of America, via integrated pest management (IPM). It could be a hugely effective way to manage populations of *Carcinus maenas*, thus reducing the impacts of *Carcinus maenas* invasion on local ecosystems. This is currently a gap in the field of research, using pheromone bouquets and gels as a way to catch specific aquatic species (Stebbing et al., 2003) and the current study reveals that both, environmental pH and microplastics in the ecosystem could affect trapping efficiency.

Pheromones are a known method for IPM, mainly in terrestrial environments. Insect IPM is much more studied and methodology developed. Species such as palm weevils, fruit flies and armyworms have had success with pheromone gels managing their invasions effectively (Bhagat et al., 2013, El-Wahan et al., 2021, Haenniger et al., 2020).

The pheromone cue and the food cue gels used within this investigation were not pH stable due to the chemicals within them being pH sensitive. This should be investigated for future studies to come up with a pH stable pheromone bouquet variant, as results may be more effective. Gels within this research are freeze dried to make the gels easier to use and more efficient, research has confirmed that freeze drying the gels will not reduce the effectiveness of them, the pheromonal activity will not be altered (Okamura et al., 2018, Xiao et al., 2004). Making these gels an innovative way to control IPM within the field.

During the study crabs were stored in gender specific tanks, with 6-8 crabs per tank (Methods- Experimental design). These communal tanks allowed for animals to interact, so fights could occur, creating social hierarchies. There is a huge range in initial reaction times within the crabs, and this may be linked to social status and the social hierarchy that has been created. Chen et al (2017) and Jiménez et al (2018) studying Crayfish, showed that winners

of fights are expected to respond more often and have a quicker reaction time to chemical cues compared to losers. Fletcher and Hardege (2009) showed that winners/losers in Carcinus react differently to UDP, 100% of dominant males displayed full sexual behaviour after the fights, whereas only 60% of the losers responded after the fight, they also took much longer to respond to cues, showing an increased latency to respond.

Dominant males keep their social status for a long period of time and losers remember this (Jiménez et al, 2018). Losers and winners will be present within this study, we therefore used animal randomisation to keep the testing unbiased. Nevertheless, it may help explain the large variability in the data in terms of reaction times and creates individuality.

The individuality of the crabs within this study could have impacted the results, there was a mix of male and female's crabs, ranging from 2.5cm in size up to 8.5cm, a mix of red and green coloured (shells) crabs (Cheung, 1966) and a range of injuries within the group (missing legs/claws). Statistical analysis of these were carried out by Howard (2022) analysing the size and weight data of the crabs. There was a statistical significance between the size of the crab and its reaction to the cue ($p=0.036$) and the weight of the crab and the decision it made ($p=0.0072$). One interesting result was a significant difference between the initial reaction time and the colour morph of the crab ($p=0.041$), so this is worth looking at in greater detail in the future. There was also statistical significance when it came to looking at data linked to injured animals, these include size of the crab and injuries ($p=0.0014$), weight of the crab and injuries ($p=0.0135$), reaction at the cue and injuries ($p=0.039$), time to reach the cue and injuries ($p=0.0027$) and decision made and injuries ($p=0.049$). These results from Howard (2022) show that individuality within has impacted the results, crabs that have been injured, are much less likely to respond rapidly or they may show more indecisiveness/confusion about the cues presented to them (Howard, 2022).

Microplastics are rapidly accumulating in the world's oceans, maximum concentrations have been found at 100,000 particles m^3 (Wright et al., 2013). Microplastic research often looks at ingestion, ingestion occurs from the bottom of the food web up, starting with species such as zooplankton (Botterell et al., 2019). This will have negative impact for all species above in the food web as microplastics can be ingested via trophic transfer, they can also be directly ingested as a food source (Egbeocha et al., 2018). Within this study microplastic odour was chosen over raw pellets as we didn't want ingestion of microplastic pellets to occur and cause harm to the *Carcinus*.

Alongside ocean acidification and microplastic pollution, ocean warming is also occurring. This study didn't cover ocean warming, but it would be very interesting to repeat this study with predicted ocean warming (+3 °C), to look into the results we may see when another stressor is added. In the Southern Atlantic Ocean strong ocean surface warming hotspots have been observed, Franco et al (2020) studied the green turtle, *Chelonia mydas*, the distribution of *C. mydas* is seen to change in relation to the sea surface temperature. Warming of the water column likely drove this turtle displacement, which could lead to changes in seasonal residency. Carcinus maenas may also see changes in seasonal residences if temperatures in shallower estuaries move above their temperature thresholds, they may start to inhabit deeper ecosystems. A study carried out on the shrimp, *Palaemon spp*, discovered that when this species is exposed to ocean warming conditions they exhibit riskier behaviour, they foraged more actively for longer periods of time, even with a live predator present (Marangon et al., 2019). Such shifts in animal behaviour will lead to long terms problems for prey species, but will benefit the predators (Draper and Weissburg., 2019). If other species are seeing behavioural changes occurring due to OW, *Carcinus* may also be affected, so this should be considered for future studies.

Like ocean warming, changes in ocean salinity due to excess freshwater melt need to be looked into (Ellis et al., 2018), though not directly linked to this study, it is still important to look into the impacts of changing salinities on *Carcinus* in future studies. All of these stressors (OW, OA, microplastic pollution and salinity changes) will become more problematic as climate change worsens (Gao et al., 2019), so a future study repeating this work but including OW and changing salinities may provide crucial information on the future problems *Carcinus* may encounter.

Ocean acidification is causing many problems with *biomineralisation* in calcifying species such as molluscs and larval echinoderms (Byrne et al. 2009; Gazeau et al. 2013; Manriquez et al. 2013), reduced offspring survival rates and fertilisation success in polychaetes and echinoderms and increased deformity rate in larval oysters (Bednarsek et al., 2021, Chandra Rajan et al., 2021, Sui et al., 2021, Wage et al, 2016), alongside behavioural changes.

Ocean acidification is predicted to continue occurring long beyond the end of this century, so long-term adaptability is crucial for the survival of many species (Solomon et al, 2009). Data from this study would show *Carcinus* being at risk unless they learn to adapt. Many marine biotas may be more resistant than we think, this is because their ability to acclimate is believed to be more rapid than the changes in sea water chemistry (Hendriks et al., 2010; Munday, 2014). Carcinus is highly tolerable of high and low temperatures, Tepolt et al (2014) found Carcinus to survive higher temperatures than some native crustaceans in environments they have invaded, showing the ability to adapt to future conditions.

A study looking at the impacts of OA on gastropods showed that the individuals exposed to low pH for long periods of time can recover from exposure (Leung et al., 2015). This does not guarantee survival of this species if it was exposed to these conditions for the foreseeable future, but it does show the ability for acclimation. A study carried out on the California

Grunion (*Leuresthes tenuis*) looked at the genetic capacity and variation. They found their estimates of covariation and genetic variation suggests populations of this fish have the capacity to adapt fairly swiftly to long-term changes in ocean pH. An additional study looked at marine sea snails, they exposed them to high CO_2 levels over multiple generations, the main findings showed that they adapted by building more durable shells, by adjusting parts of their shells, including calcium carbonate crystals (Leung et al., 2020).

Acclimation is a form of phenotypic plasticity which is the capacity for a genotype to express on phenotype according to the environment (Whitman & Agrawal 2009). The effects of ocean acidification are often researched in short term studies; however it is more likely that these changes in ocean chemistry will occur over several generations (Melzner et al. 2009; Welch et al. 2014). Adaptive potential needs to be considered after long term conditioning, the effects of microplastic odour and OA exposure can't be fully assessed without this consideration (Sunday et al. 2014; Gaylord et al. 2014; Foo and Byrne 2016).

Geological location and solar and lunar interactions can impact environmental conditions, salinity, temperature, pH, oxygen and hydrostatic pressure will also fluctuate during the natural tide and diel cycles (Aagaard, 1996; Morris and Taylor 1983). Species such as *Carcinus*, living in areas of fluctuations are often eurythermal and have a greater potential for acclimatisation (Fangue et al., 2006; Hopkin et al., 2006). A study carried out on the Porcelain crab (*Petrolisthes galathinus*) that is found in environments very close to their upper thermal limits showed that this species has struggled to acclimate to any temperatures above this tolerance, whereas species found in cooler temperatures successfully shifted their thermal tolerances (Stillman, 2003; Calosi et al., 2008; Bozinovic et al., 2011).

In *C.maenas*, Tepolt and Somera (2014) researched cold and warmth tolerances alongside acclimatory plasticity. They found this species to be highly tolerant in both temperatures, but

a higher survival rate in higher temperatures. This thermal tolerance may aid invasion of a broader range of habitats for this species (Tepolt and Somera, 2014).

Overall, the significant findings from this study show that *Carcinus* behaviour will be affected by predicted OA conditions and presence of microplastics, however there is plenty to be added to a future study to get a complete idea of the how other factors may affect these results.

7.6 Limitations of Current Study:

Within this study there were limitations. One limitation to the study was that the crabs were stored together in communal tanks, albeit the males and females were stored separately. This would have impacted the results of the study as crabs are known to participate in dominance fights (Sheddon et al., 2000), these fights help determine social hierarchy and social status within the tanks. The winners of these fights are expected to react more often and repeatedly much more rapidly to the chemical cues in comparison to the losers (Fletcher & Hardege, 2009), this helps to explain the range in reaction times within the study.

Another limitation to this study is that it was carried out during the summer months (May-September), which are the reproductive months for the *Carcinus maenas* and therefore allowed tests with the sex pheromones. This study could be replicated in all seasons, to see what trends are found albeit in the winter we would have to use feeding cues only (Clark et al., 2017). Some of the results from this study follow patterns that would be expected, males

should choose a pheromone cue over a food cue from May-October, if this had been repeated from November-April we may have seen males switch to choosing the food cue instead. This pattern is well researched, however the behaviour towards a plastic odour is not, so to get a better understanding of if PE is an attractant or deterrent, it could be tested in all seasons to see if patterns can be seen.

There is a clear result from this study, olfactory capacity of the *Carcinus maenas* is reduced as pH levels fall. This will naturally be occurring within the oceans as OA continues. This could impact *Carcinus maenas* ability to locate food and successfully reproduce in the wild, therefore the species survival long term.

A future adaptation of this study would be to collect urine samples from the crabs being used, these would be analysed throughout the year. UTP and UDP levels could be detected from these samples and would give us a better understanding of the fluctuations that may be occurring (Hardege et al., 2011). These fluctuations that may be found in UDP and UTP may link to seasonality. Future work should be carried out in the other three seasons, to see what trends may differ. As stated in the discussion, we would expect a higher percentage of males to be attracted to food in winter and very few to pheromones, and the reverse in summer. However, we don't know how the response to PE may change through the seasons, so this would be interesting future work.

Testing could also be carried out on *Carcinus* to see if they eat less or grow slower in OA conditions, even if mating becomes less successful for them. Carcinus have a relatively long reproductive season, this could be carried out on another crustacean with a shorter reproductive cycle, like *Gammarus*. This would also allow testing to be carried out on multiple generations.

During preliminary testing, three plastics were considered (Table 3, Appendix B). Polyethene was the plastic selected for this study. Before PE was chosen 2 other plastics were tested, they were PVC and PP. PE had the most crabs showing behavioural responses, hence why it was used. Results from this are in Table 3 in appendix B. Future work could be carried out replicating this study but using the other two types of plastics, to see if the trends found in this study reoccurs. Additional research could also be carried out which looks at the chemicals that are leaching out of the plastic, and these could be analysed via LC-MS-MS to determine a reason for the attractiveness of it, especially in the lower pH.

This study covered two stressors, pH and microplastic odour. In the future, ocean warming could be introduced as another condition during the testing. This study didn't take into account thermal stress, even though ocean warming is occurring alongside ocean acidification. OW and OA are a much more commonly studied combination, this testing could be carried out again, taking into account current ocean temperatures, and predicted. This would give a much broader range of impacts of climate change on the *Carcinus maenas*. The hypothesis of combined stressors lead to greater olfactory problems within the *Carcinus maenas* could still be explored in much greater detail, including looking at OW and other problems such as changing ocean salinity levels.

7.7 Conclusions:

The main conclusions from this study are that a lower pH causes significant problems for the olfactory capacity of the *Carcinus maenas*. Initial reaction times in the lower pH conditions were much slower (Figure 14), due to altered decision making and problems in detection of the chemical cues. Results also showed that a combination of stressors, in this case, low pH and microplastic odour, had a greater impact on the olfactory capacity of the *Carcinus maenas* (Figure 15). Although not statistically proven, there is an obvious attraction to the PE from the males, especially in the low pH, this suggests that something that is released from the PE becomes more potent in the lower pH, causing this attraction. Further investigation on what is leaching from the PE would help to explain the reactions from the males, and why it becomes more potent in low pH.

The overall findings from this study show lowering pH will impact the *Carcinus*, unless they can learn to adapt to future conditions.

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9.Appendices:

Appendix A:

###Lolitack

```
setwd("~/Documents/R_script")

location_tab<-read.table("R_script.csv", header=T, sep = ",", blank.lines.skip = T,
stringsAsFactors = T)

str(location_tab)

location_tab$pH<-as.factor(location_tab$pH)

location_tab1<-subset(location_tab, Condition !="control")

location_tab7<-subset(location_tab1, pH =="7.6")

location_tab8<-subset(location_tab1, pH =="8.2")

library(spatstat)

library(ggplot2)

library(dplyr)

library(RColorBrewer)

library(latticeExtra)

library(graphics)

library(ggpubr)
```

```
library(rstatix)

ggplot(location_tab1, aes(x=Avg_x_pos_corr, y=Avg_y_pos, col=pH))+

geom_point()+

theme_minimal()

###############################################

## drawcontour.R

## Written by J.D. Forester, 17 March 2008

###############################################

##This function draws an approximate density contour based on

##empirical, bivariate data.

##change testit to FALSE if sourcing the file

testit=TRUE

draw.contour<-function(a,alpha=0.95,plot.dens=FALSE, line.width=2, line.type=1,

limits=NULL, density.res=300,spline.smooth=-1,...){

##a is a list or matrix of x and y coordinates (e.g., a=list("x"=rnorm(100),"y"=rnorm(100)))

## if a is a list or dataframe, the components must be labeled "x" and "y"

## if a is a matrix, the first column is assumed to be x, the second y

  ##alpha is the contour level desired

  ##if plot.dens==TRUE, then the joint density of x and y are plotted,

  ##   otherwise the contour is added to the current plot.
```

```
  ##density.res controls the resolution of the density plot
  ##A key assumption of this function is that very little probability mass lies outside the limits
of
  ## the x and y values in "a". This is likely reasonable if the number of observations in a is
large.
  require(MASS)
  require(ks)
  if(length(line.width)!=length(alpha)){
  line.width <- rep(line.width[1],length(alpha))
  if(length(line.type)!=length(alpha)){
  line.type <- rep(line.type[1],length(alpha))
  }
  if(is.matrix(a)){
    a=list("x"=a[,1],"y"=a[,2])
  }
  ##generate approximate density values
  if(is.null(limits)){
    limits=c(range(a$x),range(a$y))
  }
  f1<-kde2d(a$x,a$y,n=density.res,lims=limits)
```

```
##plot empirical density
if(plot.dens) image(f1,...)
if(is.null(dev.list())){
  ##ensure that there is a window in which to draw the contour
  plot(a,type="n",xlim=limits[1:2],ylim=limits[3:4],...)
}
##estimate critical contour value
## assume that density outside of plot is very small
zdens <- rev(sort(f1$z))
Czdens <- cumsum(zdens)
Czdens <- (Czdens/Czdens[length(zdens)])
for(cont.level in 1:length(alpha)){
  ##This loop allows for multiple contour levels
  crit.val <- zdens[max(which(Czdens<=alpha[cont.level]))]
  ##determine coordinates of critical contour
  b.full=contourLines(f1,levels=crit.val)
  for(c in 1:length(b.full)){
    ##This loop is used in case the density is multimodal or if the desired contour
    ##  extends outside the plotting region
    b=list("x"=as.vector(unlist(b.full[[c]][2])),"y"=as.vector(unlist(b.full[[c]][3])))
```

```
    ##plot desired contour
    line.dat<-xspline(b,shape=spline.smooth,open=TRUE,draw=FALSE)
    lines(line.dat,lty=line.type[cont.level],lwd=line.width[cont.level])
   }
  }
##############################
##Test the function
##############################
##generate data
if(testit){
  n=10000
  a<-list("x"=location_tab8$Avg_x_pos_corr,"y"=location_tab8$Avg_y_pos)

draw.contour(a=a,alpha=c(0.95,0.5,0.05),line.width=c(1,1,1),line.type=c(1,2,1),plot.dens=TRUE, xlab="Length of the bioassay tank", ylab="Width of the bioassay tank", xlim=c(0,58), ylim=c(0,31))
}
ggpaired(location_tab1, x = "pH", y= "Avg_acc", fill='pH',
        line.color = "gray", line.size = 0.4, facet.by = 'Sex')+
  stat_boxplot(geom ='errorbar', width=0.2) +
```

```
  #geom_jitter( width=0) +
  stat_compare_means(paired = TRUE)+
  theme_classic(base_size = 28) +
  labs(x=expression(bold('pH')), y=expression(bold("Avg acceleration [cm/s^2]"))) +
  theme_pubr(legend = "none")
ggpaired(location_tab1, x = "pH", y= "Active_time", fill='pH',
         line.color = "gray", line.size = 0.4, facet.by = 'Sex')+
  stat_boxplot(geom ='errorbar', width=0.2) +
  #geom_jitter( width=0) +
  stat_compare_means(paired = TRUE)+
  theme_classic(base_size = 28) +
  labs(x=expression(bold('pH')), y=expression(bold("Active time [s]"))) +
  theme_pubr(legend = "none")
ggpaired(location_tab1, y = "Avg_x_pos_corr", x= "Sex", fill='pH',
         line.color = "gray", line.size = 0.4, facet.by = 'pH')+
  stat_boxplot(geom ='errorbar', width=0.2) +
  #geom_jitter( width=0) +
  geom_hline(yintercept=28, linetype=2)+
  stat_compare_means(paired = F)+
  theme_classic(base_size = 28) +
```

```
  labs(x=expression(bold('pH')), y=expression(bold("Average distance to plastic [cm]"))) +
  theme_pubr(legend = "none")
#### point pattern analysis ####
?as.ppp.data.frame()
ppp7<-as.ppp.data.frame(location_tab7[10:11], W=c(0,58,0,31))
ppp8<-as.ppp.data.frame(location_tab8[10:11], W=c(0,58,0,35))
location_tab7[10:11]
plot(ppp7, main=NULL, cols=rgb(0,0,0,.2), pch=20)
plot(ppp8, main=NULL, cols=rgb(0,0,0,.2), pch=20)
qTest <- quadrat.test(ppp7, nx = 2, ny = 1)
qTest <- quadrat.test(ppp8, nx = 2, ny = 1)
qTest
### subsets for males and females needed but doesn't make much sense with only 10 animals, so will simplify stats

wilcox_test(Avg_x_pos_corr ~ 1, mu=28, data=subset(location_tab7, Sex=='M'))
wilcox_test(Avg_x_pos_corr ~ 1, mu=28, data=subset(location_tab7, Sex=='F'))
wilcox_test(Avg_x_pos_corr ~ 1, mu=28, data=subset(location_tab8, Sex=='M'))
wilcox_test(Avg_x_pos_corr ~ 1, mu=28, data=subset(location_tab8, Sex=='F'))
#### binomial choice ####
```

```
Table(location_tab1$attraction, location_tab1$pH, location_tab1$Sex)

binom.test(c(8,2)) #not significant
```

##Y-Shaped

```
setwd("~/Masters/MRes")

R_script_Yshape<-read. Table("R_scipt_Yshape.csv", header=T, sep = ",", blank.lines.skip = T)

R_script_Yshape <- read_csv("R_script_Yshape.csv")

summary(R_script_Yshape)

str(R_script_Yshape)

R_script_Yshape$pH<-as.factor(R_script_Yshape$pH)

install.packages("plyr")

library(ggplot2)

library(car)

library(FSA)

library(ggsignif)

library(dplyr)

library(plyr)

shapiro.test(R_script_Yshape$size)

shapiro.test(R_script_Yshape$IR)
```

```
shapiro.test(R_script_Yshape$time)

shapiro.test(R_script_Yshape$ID)

shapiro.test(R_script_Yshape$size)

shapiro.test(R_script_Yshape$weight)

shapiro.test(R_script_Yshape$condition)

shapiro.test(R_script_Yshape$decision)

shapiro.test(R_script_Yshape$reaction)

shapiro.test(R_script_Yshape$pH)

shapiro.test(R_script_Yshape$colour)

shapiro.test(R_script_Yshape$injury)
```

```
ggplot(R_script_Yshape, aes(x=condition, y=IR, fill=pH))+

  geom_boxplot()+
```

```
  #geom_signif(y_position = 4, xmin=1, xmax=3, annotation="*", vjust=0.5, textsize = 16,
tip_length = 0.05)+
  ylab('Initial reaction [sec]')+ xlab('condition')+
  theme_classic(base_size = 28)
ggplot(R_script_Yshape, aes(x=condition, y=time, fill=pH))+
  geom_boxplot()+
  ylab('Time to reach cue [sec]')+ xlab('condition')+
  theme_classic(base_size = 28)
ggplot(R_script_Yshape, aes(x=condition, y=time, fill=pH))+
  geom_boxplot()+
  ylab('Time to reach cue [sec]')+ xlab('condition')+
  theme_classic(base_size = 28)
ggplot(R_script_Yshape, aes(x=condition, y=size, fill=pH))+
  geom_boxplot()+
  ylab('Size [cm]')+ xlab('Condition')+
  ylim(0,10)+
  theme_classic(base_size = 28)
kruskal.test(data=R_script_Yshape, condition~injury)
kruskal.test(data=R_script_Yshape, IR~injury)
kruskal.test(data=R_script_Yshape, decision~injury)
```

```
kruskal.test(data=R_script_Yshape, time~injury)

kruskal.test(data=R_script_Yshape, reaction~injury)

kruskal.test(data=R_script_Yshape, pH~injury)

kruskal.test(data=R_script_Yshape, size~injury)

kruskal.test(data=R_script_Yshape, sex~injury)

kruskal.test(data=R_script_Yshape, colour~injury)

kruskal.test(data=R_script_Yshape, weight~injury)

kruskal.test(data=R_script_Yshape, injury~)




dunnTest(IR ~ pH,
         data=R_script_Yshape,
         method="bonferroni")
 R_script_Yshape %>%
  group_by(reaction) %>%
  summarise(no_rows = length(reaction))
count(R_script_Yshape$reaction)
levels(R_script_Yshape$reaction)
count<-count(R_script_Yshape$reaction)
count
```

```
blank_theme <- theme_minimal()+
  theme(
    axis.title.x = element_blank(),
    axis.title.y = element_blank(),
    panel.border = element_blank(),
    panel.grid=element_blank(),
    axis.ticks = element_blank(),
    plot.title=element_text(size=14, face="bold")


library(scales)
install.packages("scales")
ggplot(count, aes(x="", y=freq , fill=x))+
  geom_bar(width = 1, stat = "identity")+
  coord_polar("y", start=0)+
  blank_theme +
  theme(axis.text.x=element_blank()) +
  geom_text(aes(y = freq/3 + c(0, cumsum(freq)[-length(freq)]),
                label = percent(freq/100)), size=5)
#The position of the text is still a bit off ...
#that might be resolved once the number of groups gets smaller
```

```
#it's just a start, we would need two pie charts for each pH anyways, right?

ggplot(count, aes(x="", y=freq , fill=x))+

  geom_bar(width = 1, stat = "identity")+

  coord_polar("y", start=0)+

  theme_minimal()
```

POTRUGAL ABOVE

```
install.packages("readxl")

str(R_script_Yshape)

levels(R_script_Yshape$reaction)#correct spelling of reaction!

levels(R_script_Yshape$reaction)<-c("BURIED", "BURIED", "BURIED",
"BURIED","GRAB", "NV", "WAFT")#cleaning up

R_script_Yshape$date<-as.factor(R_script_Yshape$date)

R_script_Yshape$pH<-as.factor(R_script_Yshape$pH)

R_script_Yshape<-within(R_script_Yshape, subject <- as.factor(paste(ID,sex,
sep='_')))#create individual levels called subject

Table(is.na(R_script_Yshape$decision1), R_script_Yshape$sex,
R_script_Yshape$condition)# sex-specific differences in number of animals that didn't
respond only when UDP is involved - as expected
```

```
Table(is.na(R_script_Yshape$decision1), R_script_Yshape$sex, R_script_Yshape$pH,
R_script_Yshape$condition)



library(ggplot2)

library(RColorBrewer)

library(cowplot)



R_script_Yshape_choice<-as.data.frame(Table(R_script_Yshape$decision1,
R_script_Yshape$condition, R_script_Yshape$pH, R_script_Yshape$sex, useNA='ifany'),
stringsAsFactors = F)

R_script_Yshape_choice[is.na(R_script_Yshape_choice)]<-'No decision'

R_script_Yshape_choice$Var1<-factor(R_script_Yshape_choice$Var1, labels=c("Control",
"GSH", "No decision", "Plastic", "UDP"))

R_script_Yshape_choice$Var2<-factor(R_script_Yshape_choice$Var2, labels=c("GSH vs
Control", "GSH vs UDP", "Plastic vs Control", "Plastic vs GSH", 'Plastic vs UDP', "UDP vs
Control"))

R_script_Yshape_choice$Var3<-as.factor(R_script_Yshape_choice$Var3)

R_script_Yshape_choice$Var4<-factor(R_script_Yshape_choice$Var4, labels=c("female",
"male"))

R_script_Yshape_choice
```

```
plot1<-ggplot(data = subset(R_script_Yshape_choice, Var4=='male'), aes(x = Var3, y = Freq,
fill=Var1)) +
  geom_bar(stat = "identity")+
  scale_fill_brewer(palette="RdYlBu", direction=-1)+
  facet_wrap(~Var2)+
  labs(y='Number of Males', x='pH', fill='Choice')+
  theme_classic(base_size = 14)

plot2<-ggplot(data = subset(R_script_Yshape_choice, Var4=='female'), aes(x = Var3, y =
Freq, fill=Var1)) +
  geom_bar(stat = "identity")+
  scale_fill_brewer(palette="RdYlBu", direction=-1)+
  facet_wrap(~Var2)+
  labs(y='Number of Females', x='pH', fill='Choice')+
  theme_classic(base_size = 14)

plot_grid(plot1, plot2, labels = "AUTO")
```

```
str(R_script_Yshape)

levels(R_script_Yshape$reaction)#correct spelling of reaction!

levels(R_script_Yshape$reaction)<-c("BURIED", "BURIED", "BURIED",
"BURIED","GRAB", "NV", "WAFT")#cleaning up

R_script_Yshape$date<-as.factor(R_script_Yshape$date)# date should be a factor

R_script_Yshape$pH<-as.factor(R_script_Yshape$pH)# pH should be a factor

R_script_Yshape<-within(R_script_Yshape, subject <- as.factor(paste(ID,sex,
sep='_')))#create individual levels called subject


Table(is.na(R_script_Yshape$decision1), R_script_Yshape$sex,
R_script_Yshape$condition)# sex-specific differences in number of animals that didn't
respond only when UDP is involved - as expected

Table(is.na(R_script_Yshape$decision1), R_script_Yshape$pH, R_script_Yshape$sex,
R_script_Yshape$condition)# pH-dependent trend of animals that didn't respond



library(ggplot2)

library(RColorBrewer)

library(cowplot)

library(survival)
```

```
library(survminer)

library(coxme)



#### overview choice plots ####

R_script_Yshape_choice<-as.data.frame(Table(R_script_Yshape$decision1,
R_script_Yshape$condition, R_script_Yshape$pH, R_script_Yshape$sex, useNA='ifany'),
stringsAsFactors = F)

R_script_Yshape_choice[is.na(R_script_Yshape_choice)]<-'No decision'

R_script_Yshape_choice$Var1<-factor(R_script_Yshape_choice$Var1, labels=c("Control",
"GSH", "No decision", "Plastic", "UDP"))

R_script_Yshape_choice$Var2<-factor(R_script_Yshape_choice$Var2, labels=c("GSH vs
Control", "GSH vs UDP", "Plastic vs Control", "Plastic vs GSH", 'Plastic vs UDP', "UDP vs
Control"))

R_script_Yshape_choice$Var3<-as.factor(R_script_Yshape_choice$Var3)

R_script_Yshape_choice$Var4<-factor(R_script_Yshape_choice$Var4, labels=c("female",
"male"))

R_script_Yshape_choice



plot1<-ggplot(data = subset(R_script_Yshape_choice, Var4=='male'), aes(x = Var3, y = Freq,
fill=Var1)) +
```

```
  geom_bar(stat = "identity")+
  scale_fill_brewer(palette="RdYlBu", direction=-1)+
  facet_wrap(~Var2)+
  labs(y='Number of Males', x='pH', fill='Choice')+
  theme_classic(base_size = 14)

plot2<-ggplot(data = subset(R_script_Yshape_choice, Var4=='female'), aes(x = Var3, y =
Freq, fill=Var1)) +
  geom_bar(stat = "identity")+
  scale_fill_brewer(palette="RdYlBu", direction=-1)+
  facet_wrap(~Var2)+
  labs(y='Number of Females', x='pH', fill='Choice')+
  theme_classic(base_size = 14)

plot_grid(plot1, plot2, labels = "AUTO")

#### first time-to-response-analysis for plastic ####
Plastic_tab<-subset(R_script_Yshape, condition =='PvC')
str(Plastic_tab)
```

```
fit<- survfit(Surv(IR,decision=='PE') ~ pH+sex, data = Plastic_tab)#choice of plastic

fit2<- survfit(Surv(time,decision=='CONTROL') ~ pH+sex, data = Plastic_tab)#choice of seawater


ggsurvplot(fit,

        conf.int = F, pval = F, facet.by=rev("sex"))


m1<-coxph(Surv(time, decision=='PE') ~ pH*sex, data = Plastic_tab)#choice of plastic

m2<-coxph(Surv(time, decision=='PE') ~ pH+sex, data = Plastic_tab)#the interaction of pH and sex is not significant (see anova(m1,m2))

m3<-coxph(Surv(time, decision=='PE') ~ pH, data = Plastic_tab)#the additive effect of sex is not significant (see anova(m2,m3))

m4<-coxph(Surv(time, decision=='PE') ~ 1, data = Plastic_tab)#pH alone is not significant (anova(m3,m4))

m5<-coxph(Surv(time, decision=='PE') ~ sex, data = Plastic_tab)#sex alone is not significant (anova)(m5,m4)

anova(m4,m5)

anova(m2) #to check out effect sizes of variables in model
```

```
m1<-coxme(Surv(time, decision=='PE') ~ pH*sex+injury+colour+weight+ (1|subject),
data=Plastic_tab)

m2<-coxme(Surv(IR,decision=='PE') ~ pH+sex+ (1|subject), data=Plastic_tab)

m3<-coxme(Surv(time,decision=='PE') ~ pH+ (1|subject), data=Plastic_tab)

m4<-coxme(Surv(time,decision=='PE') ~ sex+ (1|subject), data=Plastic_tab)

m5<-coxme(Surv(time,decision=='PE') ~ 1+ (1|subject), data=Plastic_tab)


anova(m3,m5)

summary(m1)

anova(m1)


#### some more plot ideas ####


ggplot(data = na.omit(Plastic_tab), aes(x = injury, y =time , fill=decision1)) +
  geom_boxplot()+
  scale_fill_brewer(palette="RdYlBu", direction=-1)+
  facet_wrap(~pH)+
  labs(y='Time until decision [s]', x='injury', fill='Choice')+
  theme_classic(base_size = 14)
```

```
Plastic_tab2<-as.data.frame(Table(Plastic_tab$decision, Plastic_tab$pH, Plastic_tab$sex))

Plastic_tab2

ggplot(Plastic_tab2, aes(x = "", y = Freq, fill =Var1)) +

  geom_col() +

  facet_wrap(~Var2)+

  scale_fill_brewer(palette="RdYlBu", direction=-1)+

  labs(x="", y=", fill='Reaction to plastic')+

  coord_polar(theta = "y")+

  theme_classic(base_size = 14)



Plastic_tab2<-as.data.frame(Table(Plastic_tab$reaction, Plastic_tab$pH, Plastic_tab$sex))

Plastic_tab2

ggplot(Plastic_tab2, aes(x = "", y = Freq, fill =Var1)) +

  geom_col() +

  facet_wrap(~Var2)+

  scale_fill_brewer(palette="RdYlBu", direction=-1)+

  labs(x="", y=", fill='Reaction to plastic')+

  coord_polar(theta = "y")+

  theme_classic(base_size = 14)
```

Appendix B:

Flow Rate	Time to diffuse (Minutes)
0.5 L/Min	9.14
1 L/Min	5.01
1.5 L/Min	3.45
2 L/Min	2.46
2.5 L/Min	2.01
3 L/Min	1.32
3.5 L/min	0.54
4 L/Min	0.36

Table 1: This Table shows the preliminary testing for flow rate and cue defusal using red food colouring, this was tested by adding food colouring to the flumes, and timing how long it took for any trace of it to leave, under each flow rate,

Cue	Average time (Minutes)
Pheromone	120.24
Food	120.004
Plastic	120.01
Control	120.168

Table 2: This Table shows the results from preliminary testing, to see how long the cues lasted for. The results are based off 6 repeats, they are the average number of minutes it took for the cues to start being less effective.

Plastic Odour	Percentage of crabs reacted (%)
PE	75%
PP	55%
PVC	45%

Table 3: This Table shows the preliminary testing carried out to decide which plastic was most effective. 20 crabs were tested in pH 8.2.

pH 7.6	PE v CONTROL	PE V FOOD	PE V PHEROMONE
WAFTING	47.5	52.5	47.5
BURIED	15	22.5	25
GRABBED	37.5	25	27.5
pH 8.2	PE v CONTROL	PE V FOOD	PE V PHEROMONE
WAFTING	40	37.5	60
BURIED	27.5	32.5	25
GRABBED	32.5	30	15
pH 7.2	PE v CONTROL	PE V FOOD	PE V PHEROMONE
WAFTING	55	52.5	30
BURIED	20	20	35
GRABBED	25	27.5	35

Table 4: These Tables show the percentage of the behaviours exhibited by the crabs in three conditions, these tests were carried out in late Autumn, the crabs were winter crabs, and did not respond to pheromones any more.

pH 7.6	PE v CONTROL	PE V FOOD	PE V PHEROMONE	FOOD V CONTROL	FOOD V PHEROMONE	PHEROMONE V CONTROL
WAFTING	50	62.5	42.5	62.5	67.5	50
BURIED	17.5	10	17.5	5	12.5	12.5
GRABBED	32.5	27.5	40	32.5	20	37.5
pH 8.2	PE v CONTROL	PE V FOOD	PE V PHEROMONE	FOOD V CONTROL	FOOD V PHEROMONE	PHEROMONE V CONTROL
WAFTING	67.5	55	65	70	72.5	35
BURIED	7.5	12.5	17.5	2.5	7.5	20
GRABBED	25	32.5	17.5	27.5	20	45
pH 7.2	PE v CONTROL	PE V FOOD	PE V PHEROMONE	FOOD V CONTROL	FOOD V PHEROMONE	PHEROMONE V CONTROL
WAFTING	47.5	52.5	55	57.5	40	65
BURIED	22.5	25	20	25	22.5	7.5
GRABBED	30	22.5	25	17.5	15	12.5

Table 5: This Table shows the percentage of behaviours exhibited by the crabs, in all three pH's and all six conditions, these were the summer crab's results.

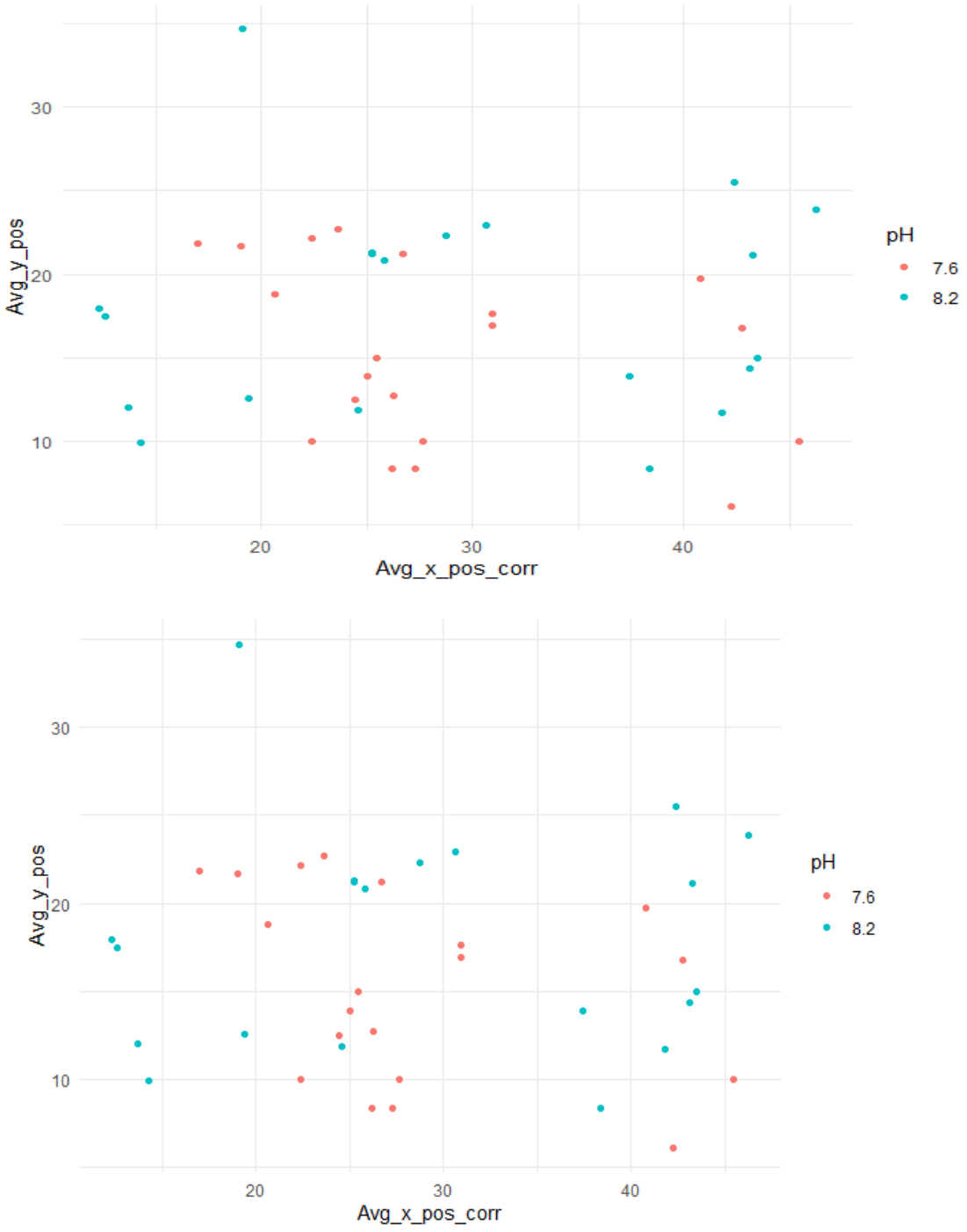

Figure 1: This box plot shows the average positions for all the crabs tested in both pH's during the Lolitrack footage.

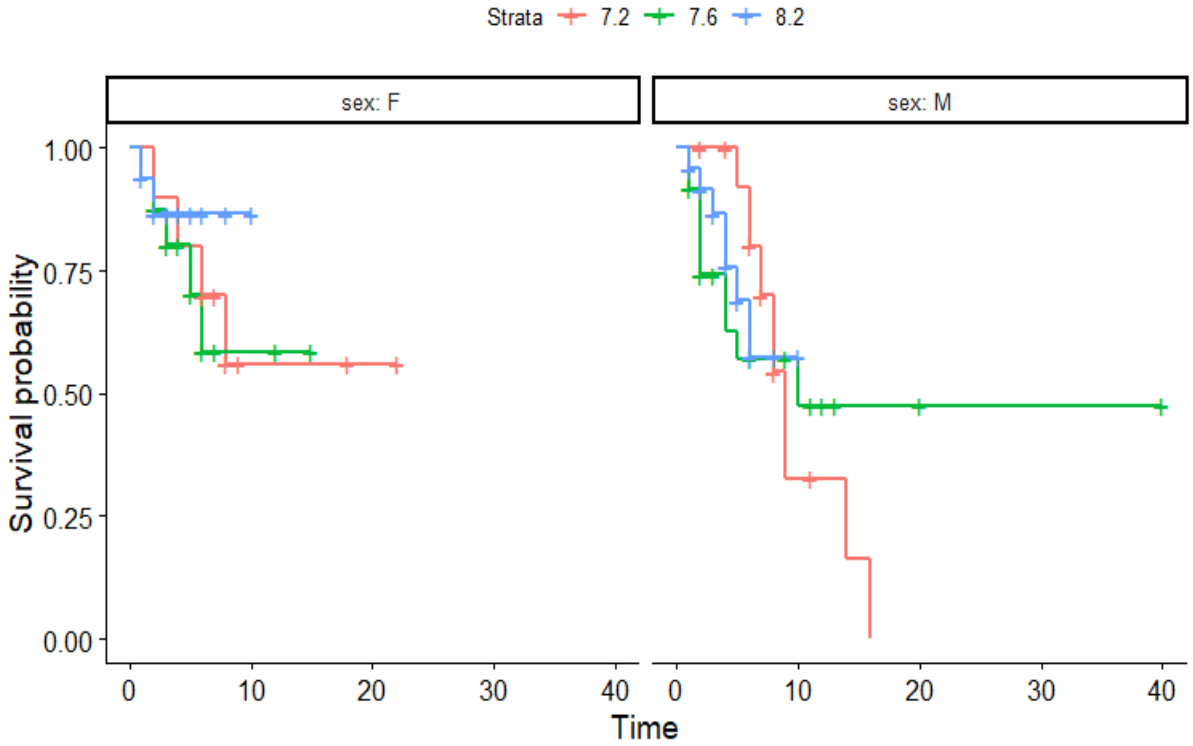

Figure 2: This is a survival plot, created using the data from PE v Control, this Figure was created on R, script found in appendix A.

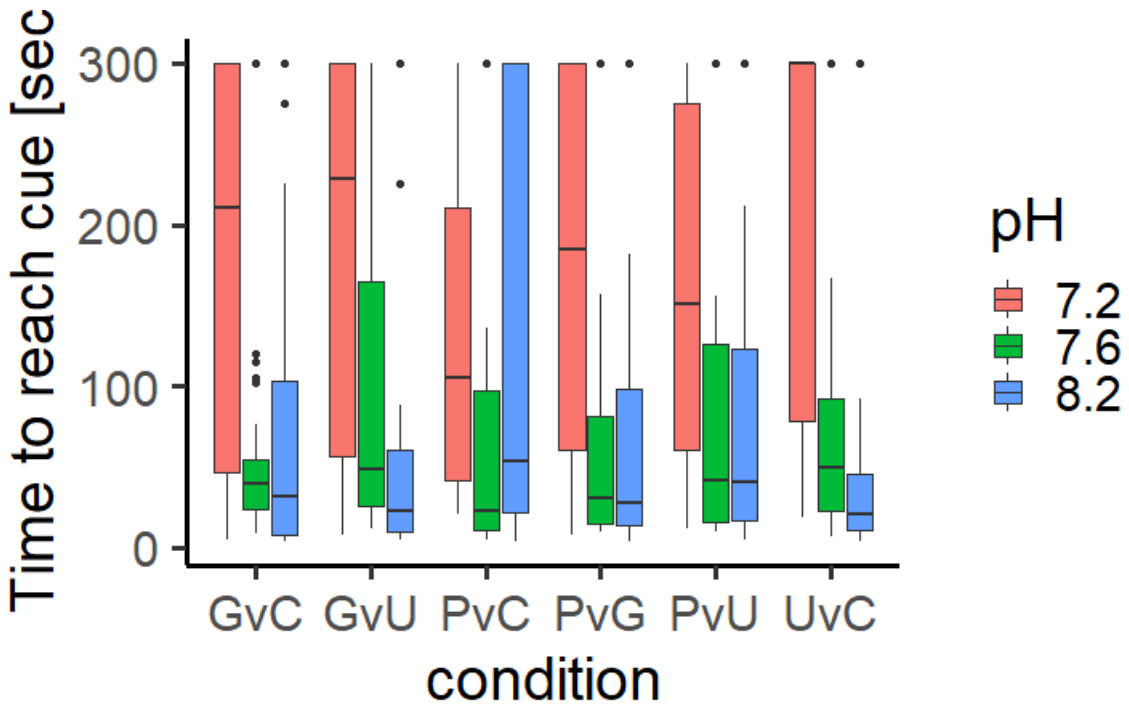

Figure 3: This histogram shows the time to reach the cue, in all three pH's under all six conditions,

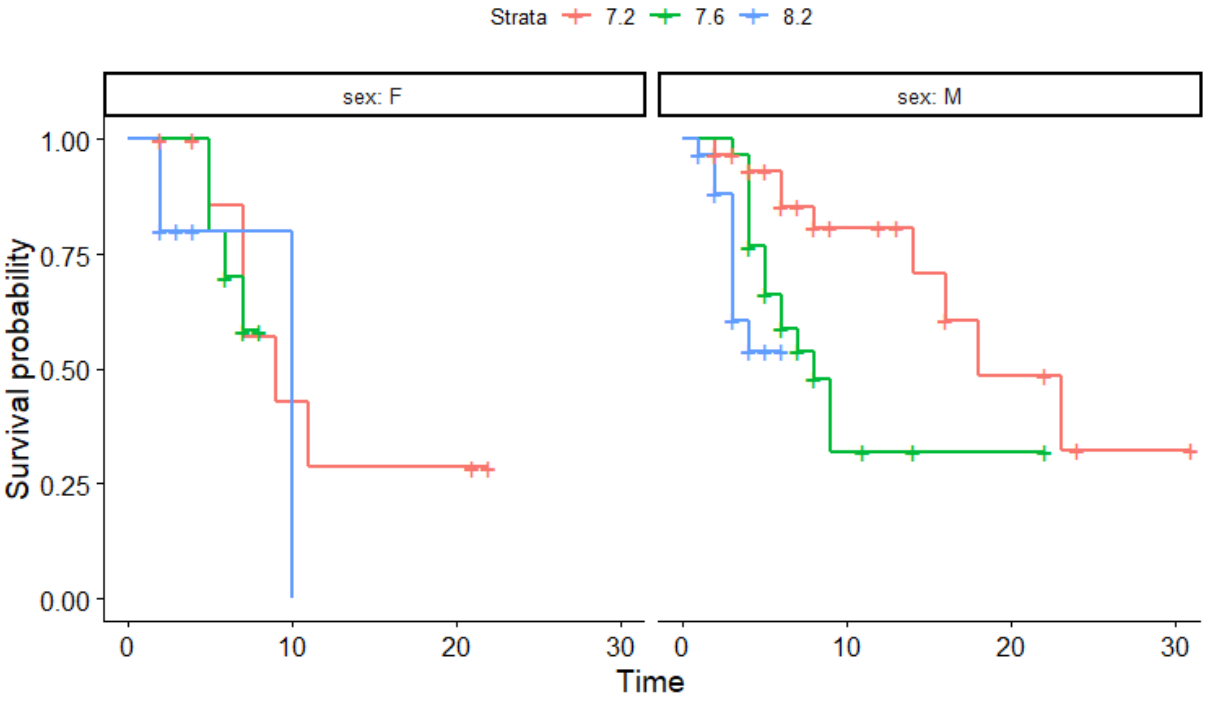

Figure 4: This is a survival plot, created using the data from PE v UDP condition.

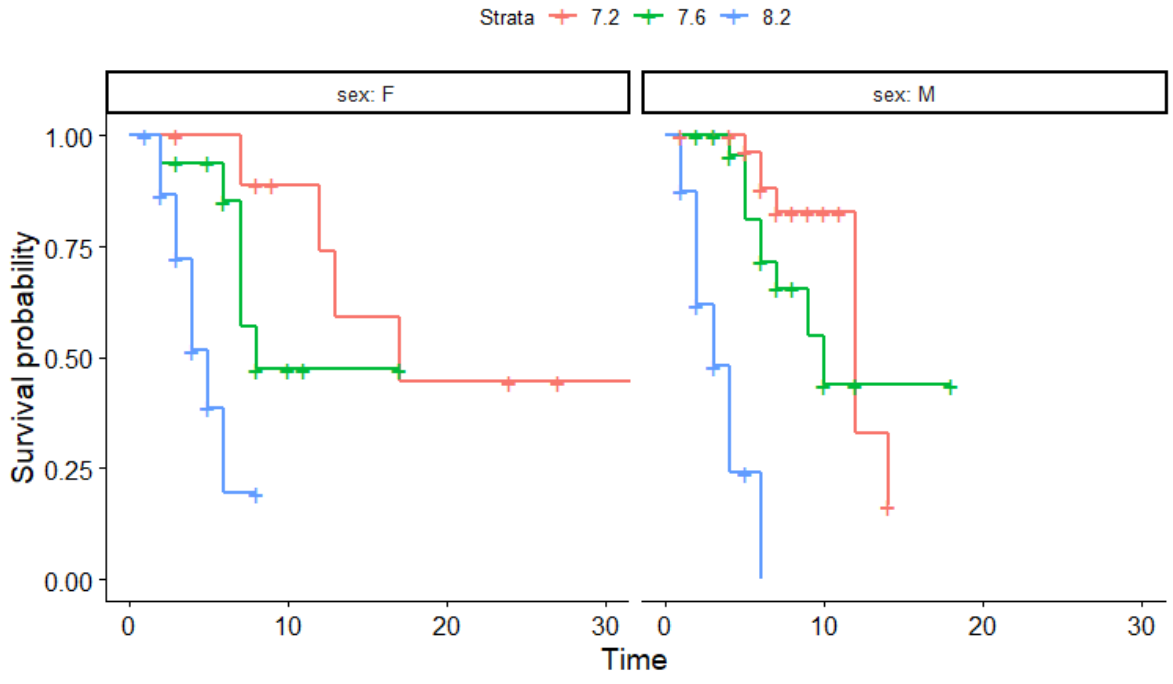

Figure 5: This is a survival plot, created using the data from PE v GSH condition.

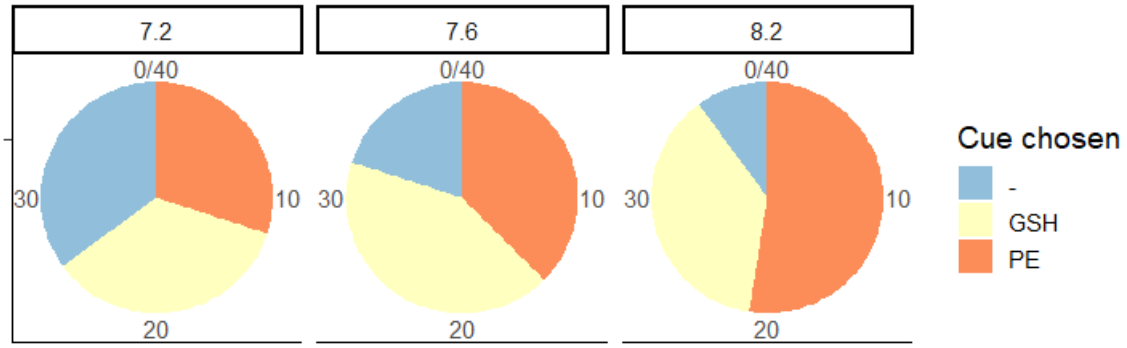

Figure 6: This is a histogram, showing time until decision was made in all three pH conditions, in GSH v PE condition.

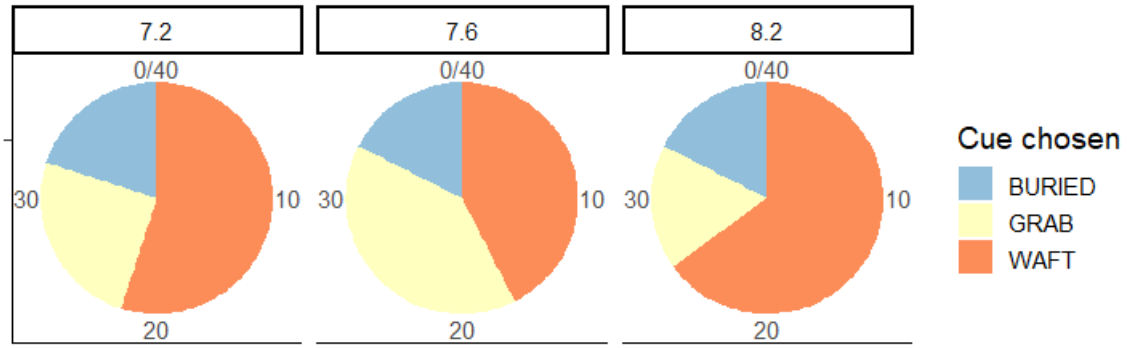

Figure 7: This is a histogram, showing time until decision was made in all three pH conditions, in PHE v PE condition.

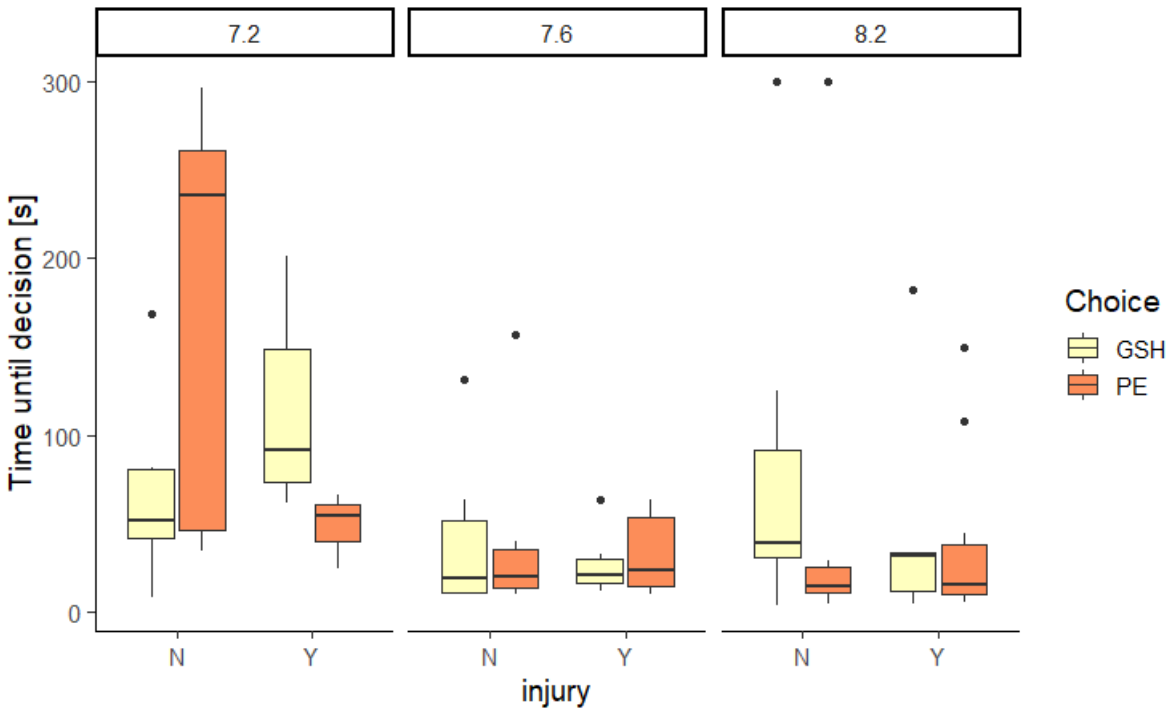

Figure 8: These histograms show the time to make a decision in all three pH's during the GSH v PE condition.

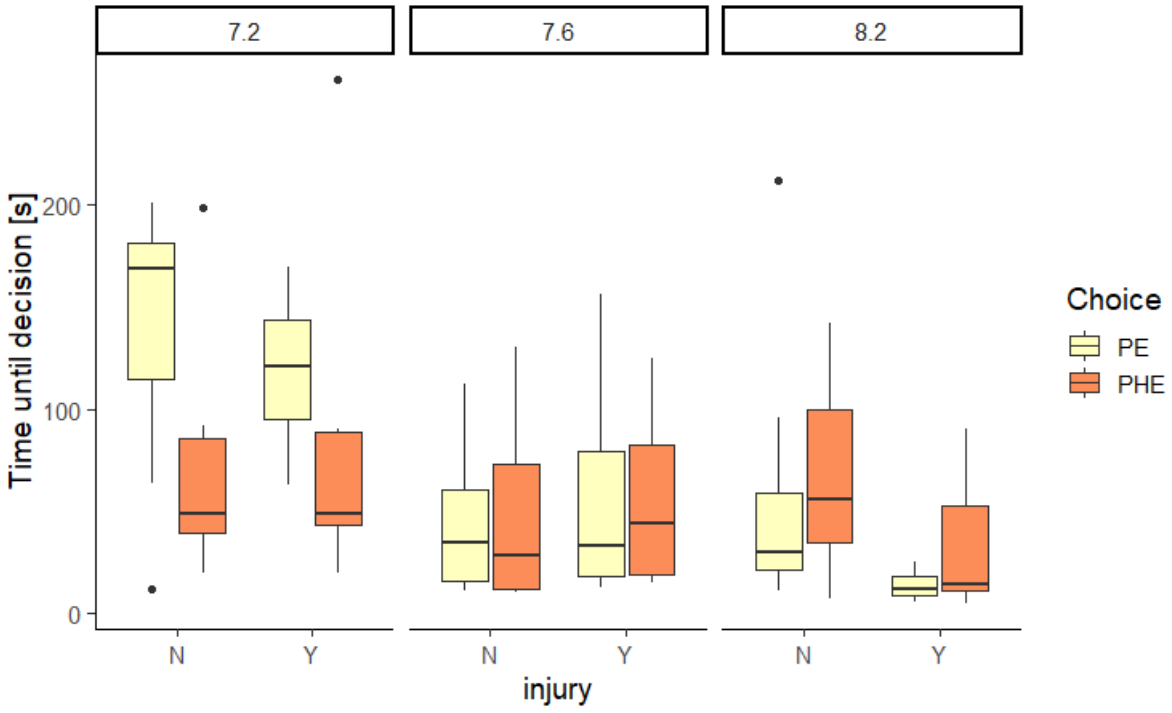

Figure 9: These histograms show the time to make a decision in all three pH's during the Pheromone v PE condition.

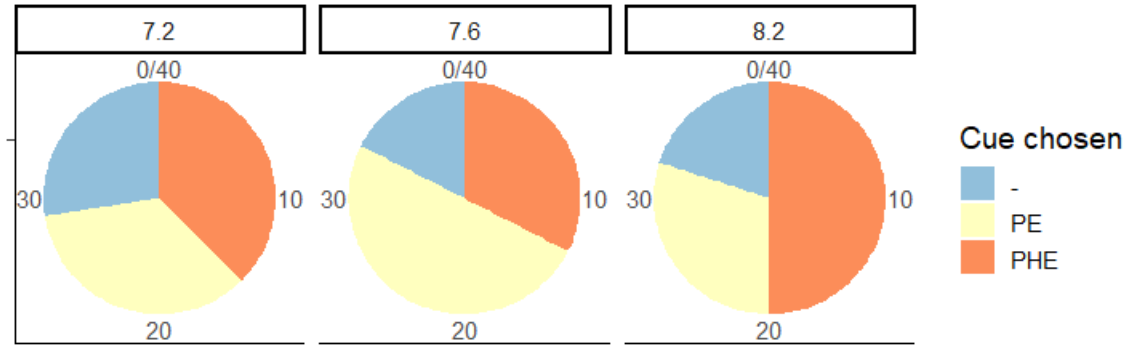

Figure 10: These pie charts show the cues chosen in all three pH's for GSH v PE and the reaction the crabs had.

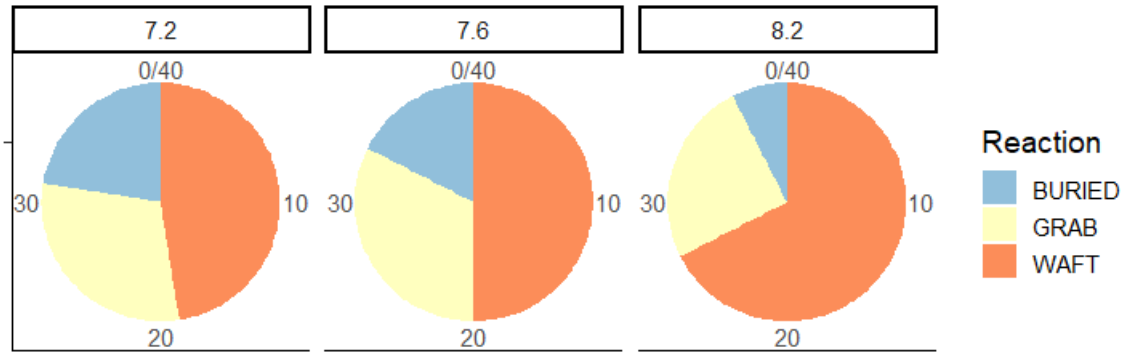

Figure 11: These pie charts show the cues chosen in all three pH's for PHE v PE and the reaction the crabs had.