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Effects of ocean acidification relevant pH on foraging behaviour and response by the hermit crab, *Pagurus bernhardus.*

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Abstract

The current rise of atmospheric CO_2 has led to an increased rate of ocean acidification (OA), a process that results in a decrease in oceanic pH. Forecasts predict the rate of OA to further increase, the largest to be seen in the past 300 million years. This process can consequently have significant implications upon marine life and their ecosystem functions. This study aims to expand on pre-existing knowledge, identifying effects of OA on olfaction by hermit crabs, *Pagurus bernhardus*. Investigations explored the variation in threshold concentrations of chemical foraging cues, required for successful detection by individuals at pH 8.15 and decreased pH levels 7.7 and 7.2. Response personality of individuals was examined through a series of biological assays. Statistical analysis and results suggest detection thresholds for chemical foraging cue, glutathione, are dependent upon and vary with trial individual and pH condition. As a population both reduced pH levels, 7.7 and 7.2, had great impact on abilities to successfully detect and respond to the presence of the chemical foraging cue glutathione. Additionally, response personality of individuals was exhibited across individuals. Some individuals displayed greater frequency in engaging foraging behaviours than conspecifics. This was also evident under subjection to reduced pH conditions. However, small sample size was a major limitation to the power of statistical significance here. Findings suggest, future rates of OA and further reduction in pH amongst coastal areas, will impact the olfactory process of *P. bernhardus* individuals. Future research should focus on long-term and multi-generational studies. These will best consider acclimation and genetic adaptation possibilities, in the event of rapidly accelerating OA related conditions.

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Contents

Chapter One: Introduction

1.1 Atmospheric Rise in CO_2

In recent decades, the concentration of CO_2 within the Earth's atmosphere has significantly increased as a result of anthropogenic activities (Le Quéré et al., 2015). The major source of anthropogenic CO_2 emissions originates from the burning of fossil fuels (Le Quéré et al., 2015). Other contributions include; cement production and land use, cover and management, including deforestation and agricultural expansion (Booth et al., 2017; Houghton, 2003; Le Quéré et al., 2015). Prior to the rapid development of the industrial era in 1750, atmospheric CO_2 concentrations were approximately 277 parts per million (ppm) (Le Quéré et al., 2015). Recently, global atmospheric CO_2 concentrations reside at around 412.30 ppm (NOAA, 2020). By the year 2100 most models predict that the concentration of CO_2 in the atmosphere will average around 985 ppm (Collins et al., 2013). This dramatic rise in atmospheric CO_2 not only has the potential to alter climate conditions via global warming but can also impact oceanic chemistry and further disrupt ecosystem functioning at a dangerously escalating rate (Caldeira & Wickett, 2003; Doney et al., 2009).

1.2 Ocean Acidification (OA)

The ocean acts as the largest, primary carbon sink, absorbing carbon dioxide from the atmosphere (Joos et al., 1996). Currently, the oceans absorb and store more than 40% of atmospheric CO_2 (DeWeerdt, 2017; Raven et al., 2005). Therefore, as the concentration of atmospheric CO_2 increases the concentration absorbed by oceans also increases (Pearson & Palmer, 2000). This exchange of CO_2 between the atmosphere and surface water results in an overall reduction in oceanic pH and an increase in acidity (Caldeira & Wickett, 2003; Doney et al., 2009). The pH level is defined by a logarithmic scale that measures the acidity (<pH7) or alkalinity (>pH 7) of a solution. A drop of 1 unit therefore signifies a substantial increase in acidity or decline in alkalinity (Pörtner, 2008). The process of reduction in oceanic surface pH begins with the formation of carbonic acid (H_2CO_3) when CO_2 in gaseous state is absorbed and reacts with water (Albright et al., 2016). Within the water chemistry, bicarbonate and carbonate ions are then produced as a result of carbonic acid dissociating and releasing hydrogen ions (H^+) (Albright et al., 2016). The concentration of hydrogen ions therefore increases within the water body and leads to the reduction of pH ($pH = - \log[H^+]$) (Doney et al., 2009; Hurd et al., 2020). This process is referred to as ocean acidification (OA) and is indicated by the following chemical equations (DeWeerdt, 2017*;* Doney et al., 2009):

$$CO_{2(atmos)} \rightleftharpoons CO_{2(aq)}$$

$$CO_{2(aq)} + H_2O \rightleftharpoons H_2CO_3$$

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

$$H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-}$$

As a result of current atmospheric CO_2 trends, oceanic pH changes are predicted to be larger than what has ever occurred in the past 300 million years (Caldeira & Wickett, 2003). Current oceanic pH levels reside at around 8.15, roughly a 0.1 unit decline since the pre-industrial era (Esbaugh et al., 2012; Hardege et al., 2011; Hönisch et al., 2012; Orr et al., 2005). Due to OA, future oceanic pH levels are predicted to drop significantly further. Predictions suggest that by the year 2100, oceanic pH will equate to 7.7 (Caldeira & Wickett, 2003). Areas, such as those located nearby coastal upwellings, with pre-existing highly saturated CO_2 water bodies, may experience pH conditions as low as 7.2 (Feely et al., 2008). This may be true for the West Coast of the USA, in Oregon (Feely et al., 2008).

Naturally pH can vary depending on location, as seen for coastal waters. Additionally, variation can correspond with combinations of other factors. For example; location, time of day (day or night), seasonal shifts and composition of marine organisms which inhabit and potentially abundantly dominate, particular coastal ecosystems (Baumann et al., 2014; Carstensen & Duarte, 2019; Cornwall et al., 2013; Kwiatkowski & Orr, 2018; Wootton et al., 2008). Biological interactions largely influence fluctuations in pH levels particularly photosynthetic organisms and respiratory mechanisms (Wootton et al., 2008). Input from land including freshwater runoff and upwelling regions, especially amongst coastal areas, also contribute to such pH variation (Carstensen & Duarte, 2019; Cooper et al., 2016). There is large concern for the future of marine environments particularly considering all of the possible direct and indirect implications of OA. The significance of such research area, therefore requires substantial investigation and study, in attempt to determine how marine life will be impacted.

1.3 Impacts of Ocean Acidification

Existing literature has focused upon the impacts of OA on calcifying organisms such as corals and species with shell structures e.g. periwinkles (*Littorina littorea*) and Mollusca (Bibby et al., 2007; Hardege et al., 2011; Rodolfo-Metalpa et al., 2010). It has been identified that OA negatively causes disruption to both growth and formation of shell and skeletal structures, as the availability of calcium carbonate ($CaCO_3$) ions become limited (Hoegh-Guldberg et al., 2007; Orr et al., 2005). With the

progression of OA and reduction in pH, carbonate chemistry of the water body becomes modified (Orr et al., 2005). Including the saturation state of calcium carbonate and aragonite, which become significantly reduced in concentration (Fabry et al., 2008). Negative effects follow such under-saturated states corresponding to OA, as calcifying organisms rely on the presence of these ions for structural production and formation of biogenic $CaCO_3$ (McDonald et al., 2009; Orr et al., 2005). Previous studies have expressed, under reduced saturation states of $CaCO_3$, *Mytilus edulis* larvae, successfully develop shells. However, upon comparison to control conditions, larvae subjected to reduced pH, developed shells significantly smaller in size (Bechmann et al., 2011). Similarly, planktonic shelled pteropods species, like many other organisms, have been found to be susceptible to under-saturated states of $CaCO_3$, especially as key producers of aragonite (Orr et al., 2005).

Additionally, fully developed shell and skeletal structures are also susceptible to OA related conditions through the effect of corrosion (Rodolfo-Metalpa et al., 2010). Increased dissolution was found to take place to the growing edge of shell apertures of the pteropod, *Clio pyramidata,* when subjected to carbonate saturation levels predicted to exist by the end of the century, for surface waters of southern oceans (Orr et al., 2005). Impairment to such structures which provide support and protection for organisms, could increase vulnerability to predators and competition successes. Further implicating overall survival outcomes (Bibby et al., 2007; Pörtner, 2008). Species subjected to environments that experience CO_2 fluctuations naturally, such as intertidal organisms, may also be potentially negatively affected, as limits of such environments become subject to greater change of a more permanent basis (Landschützer et al., 2018; Pörtner et al., 2004).

Environmental conditions linked to OA have also been noted to negatively disrupt the acid-base regulation of some marine organisms. Inducing hypercapnia and acidosis within internal body compartments (Gutowska et al., 2010; Pörtner et al., 2004). This process naturally requires adaptation of internal compartments as a result of environmental variation experienced. This occurs via transportation of ions throughout plasma and body fluids, in addition to changes in metabolic and respiratory rates (Fabry et al., 2008). Such compensatory mechanisms are energetically costly for individuals and may lead to the impairment of vital biochemical processes (Pörtner et al., 2004). Marine species extremely vulnerable to such issues are squid, Teuthida, as a result of their naturally high metabolism (Gutowska et al., 2010; Pörtner et al., 2004). In such instances, a moderate decline in environmental pH, leads to a decline of blood pH as CO_2 diffuses into intracellular and extracellular compartments. Resulting in intracellular acidification (Gutowska et al., 2010). Binding of oxygen within blood is pH sensitive and therefore has the potential to negatively cause a reduced oxygen capacity for the effected individual (Pörtner et al., 2004).

Sensory systems have the potential to be disrupted as a result of OA. Marine organisms rely on the detection of olfactory cues and their internal transmission, via nerve impulses and neurotransmitters, for the normal functioning of their existence (Clements & Hunt, 2015; Nilsson et al., 2012). Clements and Hunt (2015) review the vulnerability of the signal transduction process, via the key neurotransmitter Gamma-Aminobutyric acid (GABA), to low pH conditions. This molecule is vital for both motor and sensory functioning in individuals. The behaviours therefore, associated with this, are at increased risk to variation. Which may compromise survival success (Clements & Hunt, 2015; Nilsson et al., 2012; Schunter et al., 2019). Nilsson et al. (2012) found evidence to support the hypothesis that increased concentrations of pCO_2 (partial pressure of carbon dioxide – a reflection of the amount of dissolved carbon dioxide) impact transmembrane gradients in some neurons.

The development of depolarized and excitatory $GABA_A$ receptors from inhibitory receptor roles, leads to altered regulation of gradients for Cl^- and HCO_3^- molecules. Which could greatly impact related animal behaviours; olfaction, acid-base regulation, etc. (Fabry et al., 2008; Nilsson et al., 2012). Increased pCO_2 also altered regulation of molecules, resulting in the up-regulation and gene expression of $GABA_A$ receptors. That would otherwise function to normalise levels of Cl^- and HCO_3^- molecules, regulating acid-base balance (Schunter et al., 2019). However high pCO_2 conditions had the opposite effect, resulting in the initiation of a continuous cycle, comprising of the upregulation of depolarised excitatory $GABA_A$ receptors (Schunter et al., 2019). Organisms most susceptible to such impacts are those with higher rates of metabolism and gaseous exchange (Nilsson et al., 2012). OA additionally, negatively impacts auditory senses. Responses of otolith growth show variation in accordance to CO_2 concentration, especially in early life stages (Simpson et al., 2011).

Kinetic mechanisms, swimming ability, speed and activity may also be impaired indirectly as a result of low pH and acidified conditions (Dissanayake & Ishimatsu, 2011). Consequently due to reduced metabolism in individuals subjected to high CO_2 concentrations. In such instances, 'trade-offs' are made to conserve energy for use elsewhere e.g. in the regulation of internal acid-base balance (Dissanayake & Ishimatsu, 2011). This may impact organisms of all life stages, from larvae to adults and gametes (sperm swim speed/motility) (Havenhand et al., 2008; Havenhand & Schlegal, 2009). Swimming activity in adult European sea bass, *Dicentrarchus labrax*, was found to significantly decline, by up to 40%, when individuals were subjected to elevated CO_2 conditions (Porteus et al., 2018). Similar results were obtained in a study by Dissanayake and Ishimatsu (2011), which recorded decreased aerobic scope and swim speed of adult Penaeid, *Metapenaeus joyneri*, individuals. Additionally, the swim speed and motility of sperm, produced by *Heliocidaris erythrogramma*, significantly declined when exposed to high CO_2 conditions (Havenhand et al., 2008). Overall, reduction in swimming speed and motility could lead to reduced fertilisation success

and reduced capability amongst individuals to; escape predators or catch prey (Havenhand et al., 2008). However, conflicting studies found no impact upon swim speed in a range of species (Havenhand & Schlegal, 2009; Melzner et al., 2009; Munday et al., 2009). Proving the emerging picture of implications to be of great diversity.

Coral reef ecosystems are sensitive to changing climates. For example, ocean warming has resulted in mass bleaching events world-wide (Baker et al., 2008). Bleaching of corals occurs as a result of host corals losing symbionts zooxanthellae, which share a mutualistic relationship (Baker et al., 2008). OA now is also considered a substantial threat to corals and their subsequent ecosystems (Hoegh-Guldberg et al., 2017). The combined impacts of warming and OA may lead to the occurrence of a greater frequency of bleaching events and further decline of coral reefs and ecosystem health (Baker et al., 2008). In a recent study, subjection to high CO_2 concentrations resulted in the significant bleaching of crustose coralline algae, *Porolithon onkodes*, and the staghorn coral, *Acropora intermedia* (Anthony et al., 2008). Synergistic effects of OA and warming lead to an increased magnitude of bleaching in massive corals, *Porites lobata*, when subjected to both high temperature and CO_2 concentrations (Anthony et al., 2008). Reef building corals are additionally, prone to erosion in such acidic conditions (Form & Riebesell, 2012). Coral reefs are key components to the suitable habitation of many marine species (Hoegh-Guldberg, 1999). They provide optimum environmental conditions for the growth, survival and reproduction of a large variety of organisms (Hoegh-Guldberg, 1999). The threat that OA poses to coral reefs is prominent and future research must investigate this basis in greater detail for such valuable ecosystems (Hoegh-Guldberg, 1999).

In contrast to many of the negative implications of OA, some organisms may benefit from increased CO_2 concentration within the marine environment. Including algae species and other photosynthetic organisms. Rates of productivity and photosynthesis by such organisms increase in the presence of increased CO_2 concentrations. Resulting in the generations of large blooms (Gobler et al., 2017). Greater productivity amongst photosynthetic organisms can have further impact on other marine species within the approximate environment, both positively and negatively. For example, seagrass meadows provide nursery grounds, shelters and sustainable resources for many species, such as turtles and fish at both juvenile and adult life stages (Guinotte & Fabry, 2008). Greater productivity in these areas would therefore be highly beneficial for the respective ecosystem.

However, greater productivity and larger algal blooms can also result in eutrophication (Gobler et al., 2017; Guinotte & Fabry, 2008). During such events, both light and oxygen become limited resources. This often leads to harsh environmental conditions that could be the cause of fatality in

marine individuals (Gobler et al., 2017). Large productive kelp forests result in natural pH fluctuations, often related to variation in rates of respiration and photosynthesis, influenced by diurnal cycles (Cornwall et al., 2013). Such natural variation is likely to be magnified, creating new extremes in the event of OA (Cornwall et al., 2013). Alternatively, bloom scenarios involving harmful algae species which have the ability to produce toxins and red tides can also negatively impact other species (Gobler et al., 2017; Roggatz et al., 2019). Studies have shown that the production of such toxins increases in quantity under conditions representing OA (Tatters et al., 2012). It has also been proven that the relative toxicity of toxin produced is magnified (Fu et al., 2010; Roggatz et al., 2019). Further verifying the wide range of effects, OA influences and differences between species.

Chemical senses are well developed in marine organisms and chemical signalling is widely used within the marine environment (Carr & Derby, 1986). Signalling requires a pathway of signalling molecules or cues, senders and receptors (Atema, 1995; Roggatz et al., 2016). Chemical molecules disperse through the immediate environment once released by the sender and are transported towards receptors of receiving organisms (Atema, 1995; Roggatz et al., 2016). Such transportation is a result of the dispersal of molecules, further directed by flow within the water body or the involved organisms (Breithaupt, 2001). For example, crayfish create jets of water with use of anterior fan organs. This draws the water body containing cues (e.g. odour stimuli to chemoreceptors) (Breithaupt, 2001; Denissenko et al., 2007).

Coral reef ecosystems especially, have vast diversities of chemical cues and these disperse by water movement e.g. waves, tides and currents (Lecchini et al., 2017). This process ensures relevant information is sent and received from once source to another, whereby an appropriate response can be induced (Atema, 1995; Roggatz et al., 2016). Signalling molecules, or cues, are molecules produced by marine organisms for purposes of signalling functions. Molecules identified as signalling cues exist in many forms (e.g. amino acids and carbohydrates). Each cue has particular biological functional capacities (Hay, 2009; Rittschof, 1990; Wyatt, 2014). In sufficient concentrations (thresholds), chemical stimuli are detectable at distances by chemical sensors of organisms, referred to as chemoreceptors (Kamio & Derby, 2017; Roggatz et al., 2016). Chemoreceptors of crustaceans are located on sensory hairs covering much of the body surface (Carr & Derby, 1986). Reception of chemical stimuli can provide organisms with important environmental information. For example, during foraging; the presence, location and quality of food (Denissenko et al., 2007).

Chemical signals and chemoreception within the marine environment are vital for processes such as; predator avoidance, reproduction, fertilisation, social interactions, foraging and larval settlement

(Wyatt et al., 2014). For example, foraging behaviour amongst invertebrates is enabled by the possession of chemoreceptors, which have the ability to initiate tracking, tasting and selection of food by organisms (Kamio & Derby, 2017). More broadly, such process involves olfaction followed by gustation. Olfaction is classed as a form of chemoreception, simply described as an individual's ability to smell over distances. Larvae of some marine species, use olfaction for the successful detection, orientation, discrimination and location of suitable habitats for settlement (Lecchini et al., 2017; Munday et al., 2009; Porteus et al., 2018). Similarly, adults use an array of olfactory cues for migratory behaviour to such habitats (Devine et al., 2012). Suitable habitats provide protection and sufficient resources for the successful development of larvae and reproduction strategies between adult individuals and are therefore, of high importance (Munday et al., 2009).

Studies have identified larvae also use olfaction to distinguish conspecifics and parents. Preventing mating between developed offspring and parents (Lecchini et al., 2017; Munday et al., 2009). It has been suggested, failure to detect cues and misinterpretation of them could have great detrimental impact upon recruitment and respective adult populations of such species (Lecchini et al., 2017). Lecchini et al. (2017) discovered when crustacean, *Stenopus hispidus,* and fish, *Chromis virdis*, larvae were subjected to OA related conditions they failed to display attraction towards chemical signatures of conspecifics, that would otherwise signal suitable reef habitat. This also occurred when such larvae were exposed to increased suspended sedimentation as a result of turbidity, terrestrial runoff and pesticide pollutants (Lecchini et al., 2017).

Signalling molecules, their interaction with receptor proteins and their transportation are all factors potentially disrupted as a result of OA and can lead to the impairment of chemical communication (Wyatt et al., 2014). OA may disrupt chemical communication by acting on each of the phases within the signalling process (Roggatz et al., 2016). This can include physical damage or alteration to receptors, changing of molecule shape, hydrophobicity and transportation disruption (Hardege et al., 2011; Roggatz et al., 2016). This will lead to a compromised ability for chemoreception and therefore a change in behaviour that deviates away from the norm (Hardege et al., 2011; Roggatz et al., 2016; Wyatt et al., 2014). When deep sea urchins, *Strongylocentrotus fragilis*, were subjected to reduced pH conditions the time taken to forage increased significantly compared to controls (Barry et al., 2014). Authors suggested this was a result of impairment to the chemosensory behaviour of individuals (Barry et al., 2014). Olfactory disruption may consequently lead to a decline in the replenishment of adult populations, reduced individual fitness or survival rate (Munday et al., 2009).

Studies have proven low pH and high CO_2 environments negatively affect olfactory ability in clownfish, *Amphiprion percula* (Munday et al., 2009). When exposed to seawater pH of 7.6,

individuals did not respond to chemical cues presented to them (Munday et al., 2009). During trials of pH 7.8, individuals showed altered response to cues of preferential and non-preferential habitats (Munday et al., 2009). The outcome of choices led individuals to cues associated with less suitable habitats (Munday et al., 2009). Devine et al. (2012) found that when exposed to acidified conditions and released back into the field adult cardinal fishes, *Cheilodipterus quinquelineatus*, had reduced navigating and homing success. Similarly, alternative studies identified the impairment of olfaction in the European sea bass, *Dicentrarchus labrax*, and shore crab, *Carcinus maenas* (Clements & Hunt, 2015; Porteus et al., 2018; Roggatz et al., 2016). Roggatz et al. (2016) additionally suggested higher concentration of signalling cue was required to induce behavioural response amongst low pH conditions. Further proposing this resulted from a reduced binding affinity.

1.4 Biological Variation in Response to Ocean Acidification

OA has the potential to effect marine organisms in a variety of forms and magnitude (Kroeker et al., 2013). Current studies show that there is variation in response and level of impact upon marine individuals when exposed to OA conditions (Clements & Hunt, 2015; de la Haye et al., 2011; Doney et al., 2009; Gutowska et al., 2010). For example, behavioural responses can experienced at ecosystem level, population level, individual level and intra-individual level (White & Briffa, 2017). The level of impact experienced by individuals may lead to some individuals gaining advantage over others (de la Haye et al., 2011). Gutowska et al. (2010) explored effects of OA conditions upon mollusc species and found that the magnitude of impact differed across classes, with some classes demonstrating greater tolerance than others.

Tolerance has also been shown to vary amongst life-stages, from larvae to adults. Both within and between species, under subjection to OA related conditions (Bechmann et al., 2011; Kroeker et al., 2010; Kurihara, 2008; Pörtner, 2008). McDonald et al. (2009) studied the impact of reduced pH on the intertidal barnacle, *Amphibalanus amphitrite.* As a result of chronic exposure, only *some A. amphrite* individuals of discrete life-stage appeared negatively affected. Similarly, elevated CO_2 resulted in a greater variation of behaviours displayed by *Conus marmoreus,* compared to control conditions (Watson et al., 2017). Suggesting tolerance was widely diverse between individuals of the same species (Watson et al., 2017). Geographical differences also impact individuals of the same species in their response to acidified conditions (Briffa et al., 2008; Broadhurst & Morrell, 2018). Studies on the hermit crab, *Pagurus bernhardus*, showed behavioural response to predator cues, varied between individuals collected at different coastal sites within the UK (Briffa et al., 2008). Explanations for the observed variation in response incorporate many theories. Most research explores possibilities of acclimation, adaptation or abilities to act plastically in the event of repeated

or chronic exposure to OA related environmental conditions (Briffa et al., 2008; Donelson et al., 2019; Kroeker et al., 2011).

Acclimation can be defined as an individual's ability to demonstrate phenotypic, plastic responses via physiological, morphological or behavioural change (Donelson et al., 2019; Munday, 2014). Such change contributes to the maintenance of an organisms fitness. Particularly in circumstance of new environments, environmental pressures and constraints (Donelson et al., 2019; Munday, 2014). When subjected to reduced pH conditions, the olfactory ability of deep-sea hermit crabs, *Pagurus tanneri*, became impaired and their metabolic rates increased (Kim et al., 2016). Further, it was determined, pH 7.1 resulted in greater individual variation of behaviours, which enabled quantification of olfactory success (Kim et al., 2016). Measurable variables included speed individuals flicked antennules and detected prey (Kim et al., 2016). Variation between individuals of the same population was observed and described by Kim et al. (2016) to exist as a result of abilities to acclimate to novel environmental conditions.

Acclimation occurs over shorter periods of time (Donelson et al., 2019). Dupont et al. (2013) found mature sea urchins, *Strongylocentrotus droebachiensis*, had abilities to acclimate to increased pCO_2 levels months after continued subjection. This period is relatively short considering the lifespan of *S. droebachiensis.* However, a period of months for alternative species can amount to a significant proportion of their lifetime (Stadniczeñko et al., 2015). Chatzinikolaou et al. (2019) observed acclimation features concerning the gastropod, *Hexaplex trunculus*. Juveniles previously developed and hatched at reduced pH 7.6, displayed a more effective foraging performance compared to fully developed adults, with no known previous rearing experience in low pH (Chatzinikolaou et al., 2019). Acclimation can occur across generations as transgenerational acclimation. Whereby environmental conditions adults experienced previously, natures the reaction norm elicited by offspring (Munday, 2014; Vargas et al., 2017; Welch & Munday, 2017). Nevertheless, transgenerational acclimation may not always be advantageous and could be maladaptive. Particularly, in the event offspring are not subjected to similar conditions as parents (Munday, 2014; Welch & Munday, 2017).

Adaptation is defined by variation of genetic components which allow for the phenotype linked to the fittest trait to be expressed (Donelson et al., 2019; Munday, 2014). Such genetic variation has the potential to be passed from generation to generation (Donelson et al., 2019). The effectiveness of traits however has to be approached with caution, multiple traits resulting from environmental change can co-exist. In such circumstance, it is possible the expression of one can significantly reduce successfulness in performance of another (Laubenstein et al., 2019). Typically, adaptation occurs over a longer period than acclimation. Requiring multiple generations to convey (Munday,

2014). Species with short generation periods are more probable to demonstrate genetic adaptation (Munday et al., 2009). However, heritability is not constant and could vary with level and duration of environmental change (Welch & Munday, 2017). Local adaptation occurs in populations, influenced by conditions of the immediate environment. Genotypes correlated to the highest fitness potentials become fixed, through processes of natural selection. Genotype pools therefore vary in comparison to distant populations in accordance to differences between environments (Sanford & Kelly, 2011). Differences between populations demonstrate variation across spatial scales and life histories (Sanford & Kelly, 2011). Such scenario may especially exist in coastal and intertidal habitats. Whereby organisms within such ecosystems may demonstrate pre-existing adaptive features to reduced pH conditions. Largely as a result of the natural variation within their ecosystem.

Parental phenotype influences the presence of transgenerational plasticity, tolerance and adaptiveness in offspring under OA related conditions (Schunter et al., 2018). Welch and Munday (2017) explored how duration of exposure to increased CO_2 conditions of damselfish, *Acanthochromis polyacanthus*, impacted heritability of tolerance to such conditions. Suggesting, phenotypic variation is reduced with longer periods of offspring exposure. Further, implying non-adaptive plasticity, limits the potential of individual success in harsh, changing environments (Welch & Munday, 2017). Thomsen et al. (2017) described that adaptation had occurred for populations of Baltic mussels, *Mytilus edulis*. Particular populations appeared more tolerant to OA compared to North Sea populations. Great variation was noted in tolerance to high pCO_2 within these populations. Thomsen et al. (2017) express variation was the result of some larvae cohorts having exposure to acidified conditions and some not. pCO_2 monitoring of the respective environments determined great fluctuations. In summary, adaptation poses as both; advantageous and disadvantageous to marine organisms in the instance of OA. Few examples of adaptation currently exist within literature. Future research should focus on such areas, especially synergistic interactions between adaptation, acclimation and plasticity potentials (Fox et al., 2018).

Similar in concept to acclimation, behavioural plasticity is described as the mean level of continuously changing response by individuals subjected to variation (e.g. environmental conditions) (Briffa et al., 2013; Dingemanse et al., 2010). Characteristically, behavioural plasticity is a fast, low cost energetic response. Both; abiotic and biotic environmental factors, can impact behavioral phenotypes of an individual (Fox et al., 2018; Rudin et al., 2019). Phenotypic plasticity is the occurrence of a genotype expressed by different phenotypes, resulting from environmental change (Fox et al., 2018; Hattich et al., 2016). The pattern of such expression is referred to as the reaction norm of a particular environmental context (Hattich et al., 2016).

An organisms ability to act plastically will determine how successfully they acclimate to new environmental conditions such as OA. Under elevated pCO_2 levels, marine organisms that display efficient performances have been noted to exhibit transgenerational phenotypic plasticity (Thomsen et al., 2017). This is considered to be a short-term alleviating method, to achieve increased performance in organisms, in the instance of moderately unfavourable environmental change (Thomsen et al., 2017). It represents a vital driving mechanism necessary for organisms to sustain fitness, individual development and survival in the absence and delayed onset of genetic adaptation (Hattich et al., 2016; Thomsen et al., 2017).

Behavioural plasticity can further result in the migration of organisms, enabling avoidance of unfavourable environmental conditions and allowing maintenance of the same environmentally conditioned niches (Donelson et al., 2019). However, in the presence of OA, a high abundance of habitats will experience a level of environmental change and organisms will be subjected to variation in environmental conditions, difficult to avoid. Plasticity traits that have developed as a result of environmental change can be selected for within the population. Resulting in adaptation (genetic assimilation) features, spanning generations (Donelson et al., 2019; Jarrold & Munday, 2019).

Animal personality is defined as the consistent demonstration of behavioural variation by individuals as a response, across particular settings (Briffa et al., 2008; Rudin et al., 2019; Sih et al., 2004). Individuals can portray both; behavioural plasticity and animal personality (Briffa et al., 2008). For example, this was evident in a study that exposed hermit crabs, *P. bernhardus,* to variation in temperature. Anti-predator behaviour proved to be influenced in such scenario (Briffa et al., 2013). Boldness, exploratory behaviour, aggressiveness, activity and sociability of an individual have all been characterised and linked to behavioural personality (Garcia et al., 2020; Kelleher et al., 2018; Lane & Briffa, 2017; Rudin et al., 2019). Additional studies have investigated the shyness or boldness of hermit crab individuals as a measure of exploratory behaviour, when subjected to a new environment. Alternative observations explore aggressiveness during conflict and fighting encounters (Briffa et al., 2008; Garcia et al., 2020; Lane & Briffa, 2017).

Clibanarius symmetricus, crab individuals, collected from varied location and type of microhabitat substrate, were observed to demonstrate personality traits. Trials examined an individuals ability to assess risk and display exploratory behaviours (Garcia et al., 2020). Bold individuals collected from environments, deemed to be of higher risk, with greater exposure to predators, explored more (Garcia et al., 2020). Under OA related conditions, increased boldness of individuals can lead to negative implications (Nagelkerken & Munday, 2016). For example, bolder individuals may display an impaired ability to assess risk. Reduced risk perception could consequently lead to foraging in

circumstance where there is increased susceptibility to predation, hindering survival chances of respective individuals (Alcaraz et al., 2020; Nagelkerken & Munday, 2016).

1.5 Model Environments to Study Response to Ocean Acidification

Studies have taken place in the approximate locations of CO_2 vents such as in Ischia, Naples (Italy). Such studies investigated ecosystem structures and possibilities of acclimation and adaptation of organisms in such areas. Making attempts to predict responses of organisms and the marine environment to OA (Calosi et al., 2013; Hall-Spencer et al., 2008; Kroeker et al., 2011). Underwater volcanic vents result in the natural CO_2 content of the proximate locations to be high. In some cases CO_2 concentrations appear very similar to oceanic conditions expected as a result of OA by the end of the century (Kroeker et al., 2011). pH levels and carbonate saturation states are significantly reduced in areas of volcanic vents as a result of increased concentrations of dissolved inorganic carbon (DIC) (Kroeker et al., 2011). pH levels of CO_2 vent environments have been recorded amongst sites located in Italy. pH levels of such locations have been noted at approximately 7.8, reaching low extremes between 7.4 and 7.1 at some vent sites (Hall-Spencer & Rodolfo-Metalpa, 2009; Kerrison et al., 2011). These conditions have been seen to influence marine community demographics. Kroeker et al. (2011) found a reduction in taxonomic groups of benthic invertebrate communities inhabiting extremely low pH zones, compared to areas of higher pH levels. Further increase in small sized crustacean abundance was observed. Suggested to be a result of increased availability of macroalgae food sources in extreme low pH regions (Kroeker et al., 2011). Contrastingly, reduction in the abundance and absence of mollusks and decapods was noted within such areas (Kroeker et al., 2011).

Amongst deeper waters (40m), with reduced light penetration at pH levels of 8.2, calcifying organisms are dominant species (Linares et al., 2015). However, at similar depths and naturally low pH levels 7.8 and 7.4, benthic communities are found to vary from this substantially. For example, studies concerning CO_2 vents of the Columbretes Islands Marine Reserve, Spain (Linares et al., 2015). Calcifying organisms are no longer dominant species in here and instead deep-water kelp and fleshy macroalgae species are found in greatest abundance. Such species would otherwise inhabit deeper waters (Linares et al., 2015). Of the few calcifying organisms that do exist in such location, high-magnesium-calcifying organisms appear more sensitive to reduced pH levels. In such scenario by high-aragonite-calcifying species are deemed to thrive (Linares et al., 2015). Alternative studies have also witnessed change in taxa demographic within reduced pH areas of CO_2 vents. Again, including community shifts from calcareous organisms to non-calcifying organisms (particularly, non-native algal species) (Hall-Spencer et al., 2008). Hall-Spencer et al. (2008)

additionally noted the presence of the barnacle *Chthamalus stellatus*. It is suggested, such species tolerate vent environments as a result of adaptive mechanisms. Whereby individuals have the ability to close rostral plates to better maintain internal environments. Overall, altered communities mostly consist of fewer species yet an increase in the number of thriving/specialist individuals (Hall-Spencer et al., 2008).

Mollusks found to inhabit areas of volcanic vents/CO_2 seeps nearby Vulcano Island, Italy, experienced significant changes to shell mineral composition and toughness. Leading to increased vulnerability of disease, infection and predation (Duquette et al., 2017). However, between the four species investigated in this study, structural shell response to reduced pH did vary (Duquette et al., 2017). Calosi et al. (2013) also established different species of sea urchins displayed variation in response at locations of CO_2 vents. Here species showed dissimilarities in distribution densities. This gives further insight, highlighting different species and closely related taxa display variation in tolerance and resilience to acidified conditions (Calosi et al., 2013). However, caution must be taken in assessing impact of high CO_2 vent conditions, as organisms possess abilities to move from unfavourable conditions. Additionally, conditions approximate to vents are extremely variable (Calosi et al., 2013; Hall-Spencer et al., 2008; Kroeker et al., 2011). Results therefore, may not represent the continued subjection of low pH levels, linked to predictions of future OA.

Coastal and intertidal environments may also experience greater variation in environmental conditions (e.g. water chemistry, pH, etc.). They are subjected to not only OA caused by atmospheric CO_2 but additionally; terrestrial run off, freshwater runoff, tide cycles and coastal upwellings (Carstensen & Duarte, 2019; Cooper et al., 2016). Marine species inhabiting such areas are therefore exposed to fluctuating conditions naturally. As a result, coastal communities can experience pH conditions as low as 6.4, which has been noted of waters located on the eastern coast of the USA (Carstensen & Duarte, 2019). Across the west coast of the USA, waters located in approximation to Oregon have great variation in pH. Resulting from seasonal upwellings (Chan et al., 2017; Rose et al., 2020). Here, pH has been recorded to reach 7.43 at particular sites. Almost 20% of all pH recordings in this region have been noted below 7.8 (Chan et al., 2017). Additionally, marine ecosystems in these coastal waters experience rapidly changing rates of pH. Daily fluctuations can vary by 0.8 units. pH changes of 0.3 units hourly have also been recorded (Chan et al., 2017). Similarly, pH recordings from European coasts have found drops in pH to levels of 7.2 in tidal pools (Carstensen & Duarte, 2019). Pre-existing and natural lower limits of pH in marine environments can therefore surpass levels predicted from future OA (Carstensen & Duarte, 2019; Hurd et al., 2011). Vargas et al. (2017) identify the need to subject coastal species to future OA conditions relevant to their habitat. As many current studies, subject such species to pH conditions

predicted for open ocean environments and very few utilise pH fluctuations. Results obtained are therefore considered irrelevant to predicting outcomes of future OA scenarios. Highlighting the need for research in such areas, whilst making coastal and intertidal environments novel habitats in the study of potential implications (Jarrold et al., 2017, Jarrold & Munday, 2019; Kwiatkowski & Orr, 2018; Landschützer et al., 2018).

Seasonal rising of cold nutrient and CO_2 rich water to the surface known as upwellings in coastal regions, expose organisms to acidified seawater (McDonald et al., 2009). Coastal regions encounter such large variation in pH additionally due to abundance of primary producers inhabiting these ecosystems. Great shifts in pH between night and day occur, largely as a result of photosynthetic mechanisms (Hurd et al., 2011). Excess light during the day allows optimal rates of photosynthesis, up-taking CO_2 from the water body (Cornwall et al., 2013; Wootton et al., 2008). Photosynthesis then halts at night due to absence of light. This effect combined with continuation of respiratory activities, results in an increase of CO_2 and reduction in pH (Cornwall et al., 2013; Wootton et al., 2008).

Fluctuations of pH can also be the result of seasonal changes. As sunlight varies between winter, spring, summer and autumn (Vargas et al., 2017). Along the coast of Chile, there is a great variation in pCO_2, as a result of; seasonal differences in phytoplankton productivity (therefore pCO_2 uptake), fresh water runoff, upwelling and river plume areas (Vargas et al., 2017). In such regions, marine organisms are consequently subjected to both short-term and chronic exposure of reduced pH (Vargas et al., 2017). The tolerance of such organisms to OA conditions, may therefore be more substantial in comparison to species located elsewhere (Pörtner et al., 2004). Coastal regions of the Baltic sea are also currently threatened by a multitude of environmental stressors. Ecosystems are currently subjected to high levels of OA in comparison to other coastal areas. As both regional and worldwide changes accumulate more rapidly (Reusch et al., 2018). Mostly resulting from upwelling areas and low buffering capacity of the water body (Reusch et al., 2018).

Examples such as the Baltic Sea pose as environments of opportunity. Which may allow researchers to study outcomes of predicted levels of pH. Additionally, how effective and adaptive management strategies may be at buffering and reversing negative implications to such ecosystems (Reusch et al., 2018). Species inhabiting such environments may help fill current knowledge gaps within research in regards to resilience, acclimation or plasticity (Pörtner et al., 2004). However, Shang et al. (2020) discovered that fluctuating acidification and hypoxia caused less negative effects upon the mussel, *Mytilus coruscus*. Compared to constant subjection of acidification and hypoxia related conditions. Both; the internal environment and growth performance of individuals,

demonstrated stronger resistance to diel fluctuating acidification. Suggesting, impacts of stress can be alleviated if intermittently subjected (Shang et al., 2020). The use of coastal species to predict future outcomes upon ecosystems experiencing constant OA stress, like open ocean environments, may therefore not be appropriate or completely representative.

1.6 Project Outline

OA has the ability to affect production, transmission and detection of chemosensory cues. Consequently, leading to the impairment of key ecological behaviours, for example; foraging and predator-prey interactions (Draper & Weissburg, 2019; Watson et al., 2017). This network of complex interactions regulates ecosystem function, community structure and population dynamics (Doney et al., 2012; Kroeker et al., 2014). The existence of such complex and varying interactions between species within an ecosystem makes it difficult to predict outcomes related to future OA conditions (Kroeker et al., 2014; Watson et al., 2017). It is therefore increasingly important to research foraging behaviours and predator-prey dynamics of marine organisms, in the event of changing environments. To further help develop greater understanding of threats imposed upon marine ecosystems (Dodd et al., 2015; Froehlich & Lord, 2020). At present there is limited literature concerning the impact of OA, upon foraging behaviour of intertidal species, via disruption to olfaction. Research should also focus more greatly upon the mechanisms in which intertidal species have embraced, to cope with such a highly varying natural environment. As this may prove useful in assessing the impact to individuals, populations and ecosystems as a result of future OA conditions. The current study therefore aims to evaluate the potential impacts of OA on foraging behaviours and olfaction. With the use of the intertidal hermit crab *Pagurus bernhardus* as sample species.

Pagurus bernhardus (*P. bernhardus)* is a species of hermit crab native to Europe, and is one of the most common species of hermit crab found along the coastal waters of Britain (Lancaster, 1988). The process of individuals locating food is mostly dependent upon olfaction and gustation. Individuals do not largely depend on sight, in such circumstance (Lancaster, 1988). *P. bernhardus* are opportunistic, omnivorous feeders, consuming a variety of food, from small crustaceans, polychaetes and bivalves to plant matter and algae (Gerlach et al., 1976). Individuals feed via mouthparts, using appendages for grasping and have additionally been considered as filter feeders (Gerlach et al., 1976). To allow appropriate growth, individuals must shed hard exoskeleton structures before the construction of a new one. The exoskeleton acts as a protective structure, however, does not cover the abdomen of individuals (Lancaster, 1988). Individuals claim and inhabit vacant gastropod shells, which amongst other uses, protects their naked abdomens from damage,

primarily caused by predators and other environmental stressors (Hazlett, 1981; Lancaster, 1988). Individuals withdraw into shells under stress induced circumstance, when threatened or startled (Bridger et al., 2015)

Crustaceans are surrounded by a sensory landscape within the domains of their ecosystem including chemical signals (Harzsch & Krieger, 2018). Such chemosensory landscapes are complex, by the array and mixture of chemical cues and signals released by predators, prey and conspecifics. As previously stated, such cues enable organisms to make informed decisions (Harzsch & Krieger, 2018). Experiments have identified individuals display behavioural responses to the presence of chemical cues that represent both; attractive or repulsive stimuli (Broadhurst & Morrell, 2018). In the presence of predatory cues, *P. bernhardus* individuals elicited a response, both at individual and combined presence (Dalesman & Inchley, 2008). This response involved the cessation of feeding behaviour followed by fleeing movement away from the assessed risk of the predator location (Dalesman & Inchley, 2008). Marine environmental stressors, such as OA, have the potential to indirectly change chemosensory landscapes and olfactory cue-scapes. Environmental change can induce modification to ecosystem structuring, community abundance and cues emitted by such organisms (Nagelkerken et al., 2019). Hence, olfactory sensitivity and chemical cue detection are of high importance. Especially for basic and successful biological interactions such as; foraging, predator avoidance and mating.

P. bernhardus, is the chosen species for the proposed study. Particularly as previous literature has established hermit crab to be model species for the study of OA implications upon; chemoreception, olfaction and response personality. Hermit crabs are regularly subjected to harsh conditions and a naturally varying environment. Especially as they inhabit intertidal areas and coastal regions, whereby pH, temperature and resource availability can fluctuate substantially (Bueno-Guerra & Amici, 2018; Pörtner et al., 2004). They further prove to be an interesting species to study to determine how a species that frequently experiences low pH in their natural environment, responds to low pH conditions induced in a laboratory environment. Some studies identify that memories in hermit crabs are only short lasting (Bueno-Guerra & Amici, 2018). For example in recognizing shell quality by information gathered in previous encounters (Bueno-Guerra & Amici, 2018). Additionally, making them an appropriate sample species to conduct laboratory behavioural trials. Crustaceans in general are both of ecological and economic importance, often reflecting the health of the ecosystem they inhabit (Whiteley, 2011). Research delving into potential impacts of climate change and OA on this species is therefore highly valuable (Whiteley, 2011).

To investigate the aims and objectives of the proposed study a series of behavioural assays will be conducted. Assays are a well-developed, quantitative form of assessing the behavioural response of organisms to particular laboratory settings (Carr & Derby, 1986; Wyatt, 2014). Successful behavioural assays will consider; the influence of alternative modes of signalling, negative and positive controls and the use of 'blind' assays. Whereby the observer is not fully aware of all factors within the assay until analysis of results (e.g. individual in trial, etc.) (Hardege et al., 2002). One issue of behavioural assays may be the potential of individuals to learn from previous experiences/assay trials. Responses therefore may not be truly representative of those naturally observed within the field (Hardege et al., 2002). To avoid such outcomes *P. bernhardus* individuals were given rest periods and returned to aquarium tanks. Rest periods were between 3 to 4 days in length (Hardege et al., 2002). *P. bernhardus* individuals are highly cognitive and use a variety of sensory modalities to gain relevant information. Especially regarding their approximate location (Bueno-Guerra & Amici, 2018). Which may include chemical processes of visual, vibrational and/or tactile nature (Bueno-Guerra & Amici, 2018; Wilby et al., 2018). *P. bernhardus* individuals provide substantial opportunity to study the possibility of plasticity and behavioural consistencies, as they demonstrate a variety of quantifiable and measurable behaviours (Briffa et al., 2008; Broadhurst & Morrell, 2018). This amongst other reasoning, is why they are the selected sample species within this study.

The use of behavioural assays will be implemented throughout methods of the current study to quantify the behavioural impact of OA on *P. bernhardus* individuals. Amongst data Chapter Three behavioural assay methods will additionally make use of respirometer apparatus to measure oxygen consumption of individuals with the addition of foraging cues. Pimentel et al. (2014) used respirometer chambers to identify effects of OA upon oxygen consumption and locomotion of dolphinfish (*Coryphaena hippurus*) larvae. Many other previous studies have also made use of measuring oxygen consumption and environmental concentration to investigate the impact of OA related conditions amongst marine organisms (Gao & Zheng, 2010; Miller et al., 2016; Munday et al., 2009). Detection of foraging cues, leads to the active searching and increased locomotion of organisms (Horwitz et al., 2020; Kamio & Derby, 2017). This will therefore further result in increased rates of respiration and oxygen consumption. Increased O_2 consumption will lead to a decrease in the O_2 concentration of the environment. If individuals fail to detect the presence of cue as a result of reduced pH, locomotion will also decrease. Rates of O_2 consumption and therefore oxygen concentration of the environment will display little change (Ashur et al., 2017). Time required to forage by *Stylocheilus striatus* was found to increase with subjection to OA related stressors, whilst locomotion was noted to decrease (Horwitz et al., 2020). Measuring the oxygen concentration of the environment can therefore suggest if an organism has successfully detected the addition of a

foraging cue. The weight of individuals will also be considered amongst investigations as the size of individuals may influence foraging behaviour and response (Hayden et al., 2007; Laidre & Elwood, 2008; Lancaster, 1988).

Many peptides function as signaling molecules within the marine environment. Their wide distribution can relay important information to receiving organisms, such as signaling the presence and quality of food, mates, conspecifics, suitable habitat, etc. (Hay, 2009; Nagelkerken et al., 2019; Rittschof, 1990; Rittschof et al., 1989; Velez et al., 2019). Glutathione (GSH) is a tripeptide found in tissue of marine animals, structured by a combination of amino acids (Hardege et al., 2004; Ide et al., 2006; Zeeck et al., 1998). Such amino acids are also dispersed into seawater from dead and injured organisms (Hay, 2009; Ide et al., 2006). As *P. bernhardus* individuals are considered opportunistic feeders, it is reasonable to state that such stimuli can induce foraging and feeding behavioural response by individuals. Especially if the presence of glutathione is detected via olfaction. Previous studies have found hermit crabs to be attracted to chemical cues of gastropods that were both; decomposing and freshly predated in a study by (Alcaraz et al., 2020).

Additional studies have determined amino acids as stimulants for responses related to reproductive behaviour (Hardege et al., 2004; Zeeck et al., 1998). GSH has previously been labelled as a pheromone precursor. Involved in pheromone bouquets used to initiate reproductive behaviour and gamete release in *Nereis (Alitta) succinea* (Hardege et al., 2004). A study by Welch and Munday (2017), made use of chemical alarm cues, in efforts to determine the implications of increased CO_2 conditions, amongst antipredator behavioural response by *Acanthochromis polyacanthus* offspring. Exploring the use of GSH as a chemical foraging cue would bring greater understanding of the use of such chemical stimuli in experimental procedures. The use of GSH in the current study will also give insight into the effects of OA on chemical cues, olfaction and foraging response of *P. bernhardus* individuals.

Taking into consideration current techniques used within research to investigate the impacts of OA, the structure of the current project is as follows. Chapter Two highlights general methodology, relevant to both data Chapters Three and Four. Data Chapter Three initially seeks to determine the minimum concentration (threshold) of a chemical foraging cue, glutathione (GSH), required to induce a behavioural response amongst trial individuals. Exploring how such threshold concentrations vary between individuals and with reduction in pH. Data Chapter Four further observes the response of individuals amongst alternate behavioural assay settings. Focusing on foraging behaviours and differences between the response of individuals. Finally, Chapter Five concludes such findings.

1.7 Aims and Hypotheses

For the following study, aims and objectives attempt to further expand existing knowledge on the research topic OA. More specifically, investigating the impact of reduced pH levels upon the foraging behaviour and olfactory ability of *P. bernhardus* individuals. Methods will make use of behavioural assays, chemical foraging cues and measurement of O_2 to investigate the following overarching hypothesis: reduced pH will decrease successful detection of foraging cues and foraging response by *P. bernhardus* individuals and such level of impact will vary between individuals.

Chapter Two: General Methodology

2.1 *Pagurus bernhardus* Collection and Husbandry

20 hermit crab individuals, *Pagurus bernhardus*, were collected at low tide from Boggle Hole Scarborough, UK and transported to the University of Hull during November. At the University individuals resided in aquarium tanks, of ambient temperature 15°C ± 1°C. Amongst tanks, individuals were further placed within individual boxes with meshed lids and air holes. Boxes contained vacant shells to enable individuals the opportunity to switch. The shells hermit crabs inhabit offer protection from predators, competition and generate a buffering environment (Briffa et al., 2008). The provision of empty shells therefore, aimed to decrease competition between individuals and reduce likelihoods of cannibalism. Light within aquaria represented natural photoperiods. Individuals were invasively labelled via the use of tippex correction fluid, for purposes of identification and data analysis. The combined wet weight of individuals and their occupied shells was measured. Once individuals vacated and switched shells of occupancy, the sole weight of individuals could be calculated. Using this method, the weight of 13 out of 20 individuals was recorded. Individuals were fed twice weekly, with fragments of *Mytilus edulis,* purchased frozen from a local supermarket. Preparation of feed required defrosting three frozen *M. edulis* by submersion in warm water for two minutes. Once defrosted, *M. edulis* was cut into smaller fragments using scissors. Fragments measured roughly 5.00 mm by 5.00 mm in size. Each individual was fed one fragment of prepared *M. edulis* per feed, via placement inside each individual holding box. Individuals were starved 48 hours prior to experimental trials, in attempts to standardise hunger levels.

2.2 Sample Preparations

For the purpose of both data Chapters Three and Four, a dilution series of the chemical foraging cue glutathione (GSH, 70-18-8, Sigma-Aldrich) was prepared. Resulting in a series of concentrations of GSH available for the addition to experimental environments, as detailed in the following data chapters. To create the dilution series, 1ml of stock GSH solution was diluted with 9ml of distilled water, generating a dilution of 10^{-1}M/l. A further, 1ml of this dilution was then added to another 9ml of distilled water, generating a dilution 10^{-2}M/l. Such methodology was repeated until the production of 10^{-4}M/l, 10^{-5}M/l, 10^{-6}M/l, 10^{-7}M/l and 10^{-8}M/l concentrations. Similar concentrations have previously been used in studies to investigate detection thresholds of amino acids, under acidified conditions (Velez et al., 2019).

Positive control trials required the addition of 'mussel conditioned artificial seawater'. Such sample was created by adding three pieces of frozen *M. edulis,* of combined frozen weight 15g, into a sealed container holding 25ml of artificial seawater (35 PSU, 19 ± 0.5°C and pH 8.15). *M. edulis* was removed from the container after 60 minutes. The use of mussel conditioned artificial seawater is an accurate natural food source representation of *P.* bernhardus individuals. Whilst the same volume of mussel conditioned artificial seawater was added to each trial amongst Chapter Three and Four methods, the exact concentration of the quantity added cannot be confirmed and may have the potential to vary. In attempts to best avoid this occurrence, solutions of mussel conditioned artificial seawater were thoroughly homogenized by mixing pre-use and prepared using the same methods previously exercised. Artificial seawater was chosen over the use of natural seawater for use in experiments as it could assumed that no pre-existing olfactory cues were contained within samples.

Artificial seawater was used for both the solution individuals were held in amongst behavioural experiments and negative control additions in replacement of cues. Artificial seawater measured 35 PSU in salinity and a temperature of 19 ± 0.5°C. Salinity of natural seawater is equivalent to 35 PSU, meaning roughly 35 grams of salts are dissolved for every 1 litre of water. For trials, artificial seawater was therefore created to have salinity level of 35 PSU. Salinity was measured using HI-96822 Refractometer (Hanna Instruments). Temperature was recorded using glass thermometer, however room temperature of laboratory was controlled and set with thermostat to 19°C. pH levels of samples were adjusted as desired by bubbling CO_2 through samples using a pressurised canister. Both before and after trials, pH was recorded using hand-held pH meter (Fisherbrand™ accumet™ AB150 pH Benchtop Meter). Small amounts of CO_2 was added to samples for short periods of time to ensure prevention of overshooting reduction of pH.

During behavioural trials precaution was taken to avoid vibration or shadows from observation. As both have been found to disturb and impact the behaviour of *P. bernhardus* individuals (Bueno-Guerra & Amici, 2018). Levels of light was controlled within the laboratory to simulate natural daylight, however dimmed sufficiently enough to prevent generation of shadows within trial environments.

Chapter Three: Biological Assay Determining Individual Thresholds

3.1 Introduction

Ocean acidification is occurring at a rapid rate and is predicated to cause much stress upon organisms amongst marine environments (DeWeerdt, 2017). More recently, the effect of OA upon chemical communication has been explored and hypothesised. Such suggestions include; disruption to neurotransmitters of organisms, damage to signalling molecules and receptors and additionally impaired chemosensory behaviour of organisms (Nilsson et al., 2012; Roggatz et al., 2016; Scunter et al., 2019). This includes olfactory processes and can therefore lead to a decline in population of ecosystems and individual fitness of marine organisms (Munday et al., 2009). Foraging behaviours of many marine organisms, in particular, will rely heavily on the successfulness of olfaction to detect and engage with food sources (Barry et al., 2014).

As previously stated, the impact of OA on chemical communication has been explored within current research. However, such research mostly consists of the effects upon predator-prey relationships, conspecific recognition and identification of suitable habitat (Lecchini et al., 2017; Munday et al., 2009; Porteus et al., 2018). Fewer studies have focused on the impact of OA to foraging behaviours of intertidal species. Gaps of knowledge therefore exist within current literature regarding the impact of OA related conditions upon foraging response to cues, via olfaction, and the necessary concentration of foraging cue required for successful detection by *P. bernhardus* individuals. Contributions of this study therefore intend to achieve knowledge regarding such area of research. Foraging is a highly important behaviour which can determine the health of individuals and ultimately ecosystem functioning. There is therefore an urgent need amongst research to identify the impact of future OA conditions upon such behaviour.

Chapter Three is the first of two data chapters. Here general methodology from Chapter Two will be utilised alongside additional methods to explore detection threshold concentrations of foraging cue required by *P. bernhardus* individuals in the event of OA related conditions. Detection threshold concentrations are defined within this chapter by: the minimum concentration of foraging cue required to induce a change in respiration rate and stereotyped feeding behavioural response by *P. bernhardus* individuals subjected to pH conditions of 8.15. Two foraging cues and a control addition have been selected for investigations. These are the chemical foraging cue GSH, mussel conditioned artificial seawater and control addition artificial seawater. Determining threshold concentrations of *P. bernhardus* individuals will be achieved via the use of behavioural assays and respirometer apparatus measuring O_2 concentration amongst trial environments and therefore rates

of oxygen consumption and respiration. Measurement of such factors will determine if *P. bernhardus* individuals have successfully detected the addition of foraging cues by olfaction, further exploring the impact of reduced pH in line with future OA related conditions. It is expected that reduced pH level will result in greater concentrations of foraging cue required by individuals for successful detection (Roggatz et al., 2016). The size and therefore weight of marine organisms has previously been suggested to impact foraging behaviour (Hayden et al., 2007; Laidre & Elwood, 2008; Lancaster, 1988). Therefore, Chapter Three investigations will take this into consideration.

3.2 Aims and Objectives

The aims for Chapter Three is to distinguish the potential impacts, of ocean acidification upon olfactory ability of hermit crab, *Pagurus bernhardus*, individuals. Specifically, the effect of reduced pH levels upon threshold concentrations of foraging cue, required by individuals for successful detection. Objectives will also consider the effectiveness of chosen foraging cues for such investigations and if the weight of individuals is influential amongst results.

3.3 Hypotheses

1a) Reduction in pH level from 8.15 to 7.7 and 7.2, will increase detection threshold concentrations for the chemical foraging cue GSH, required by *P. bernhardus* individuals.

1b) Overall drop in oxygen concentration (%) of the trial environment, will decrease with reduced pH level, amongst trials with the addition of GSH and mussel conditioned artificial seawater.

1c) For the trial population and across all pH levels, overall drop in oxygen concentration (%) will be greatest with the addition of mussel conditioned artificial seawater.

1d) The weight of *Pagurus bernhardus* individuals will influence individual detection threshold concentrations for GSH and the overall drop in oxygen concentration (%).

3.4 Methods

Fiber-Optic Oxygen Meter FireStingO_2 PyroScience apparatus (FSO2-4) was used for the conduction of respirometer experiments. Whereby sensors within a closed container measure oxygen content of the internal environment. For set-up of the apparatus, instructions were followed as suggested by the FireStingO_2 user manual (PyroScience, n.d.). To use such equipment the Pyro

Oxygen Logger software was first downloaded and installed onto Windows PC from PyroScience website (https://www.pyroscience.com/en/products/all-meters/fso2-4#downloads), as directed. The chosen sensors used within experiments were sensor spot of sensor code SC7-538-193. Such sensor spot was first removed from packaging and fixed to the inside of the container used during trials. The container described measured 6.5 cm in diameter and 6 cm in height. The lid of such container was fit with septum, penetrable with needle. sensor spot was fixed onto the container wall, as required, using acetic acid based silicone adhesive and left to dry.

On the day of trials, Fiber-Optic Oxygen Meter FireStingO_2 PyroScience apparatus was connected to the PC using USB cable. The fiber spot adapter and cable binder was adjusted to fit around the container and positioned to align with sensor spot. The cable was then inputted into FireStingO_2 Fiber-Optic Oxygen Meter Hub channel. Once appropriately connected and before undertaking experimental procedure, the apparatus was calibrated in accordance to the user manual. Apparatus were calibrated with each day of experimental trials. To calibrate apparatus the first step was to open PyroScience software. Amongst PyroScience settings, parameters including sensor code (SC7-538-193), raw value units, fiber length (1.0 m) were added. Additionally, fixed temperature was selected for, alongside Water (DO) and internal pressure sensor settings. Fixed temperature was recorded to be 19°C, via glass thermometer. Which also corresponded with laboratory room temperature set by thermostat.

Upon completion of setting inputs, the next steps of calibration could commence. This required filling the container with sensor spot attached, with 125 ml of artificial seawater and bubbling oxygen through the solution. This was achieved using pressurized canister for a period of five minutes. The container was then sealed re-connected to the FireStingO_2 hub and PC. After selecting calibrate, on the PC application window, the following settings were then selected: ‘1-point in water or humid air’ and ‘set air’. As directed in the FireStingO_2 User Manual (PyroScience, n.d.). Once the reading for ‘Oxygen (%air sat)’ became stable, ‘set air’ was selected again. This step completed calibration.

A total of 20 individuals were tested individually at each of the three pH conditions of 8.15, 7.7 and 7.2. The same 20 individuals were tested across all pH and trial conditions. In consideration of later statistical analysis this is important to note, as the use of the same individuals amongst all trial conditions may lead not allowing for non-independence of data. For each trial a randomly selected Individual was placed in the sealed container previously described. Using PyroScience apparatus, the oxygen content of the environment (artificial seawater individuals were placed in) was measured for a total of 6 minutes. 125ml of artificial seawater was used for such purposes. The drop in oxygen concentration of the environment and respiration rate of individuals was then calculated. Duration

of trials were short in time so that effects of individuals on environmental seawater pH was negligible for the closed system. The testing procedure started with the first minute, whereby individuals were given time to acclimate to their new environment. Data was then recorded regarding the concentration of oxygen within the environment. To do this the graphics window of any existing plots was cleared by selecting the 'clear graph' function, followed by the 'clear graph and zero time scale' functions. Continuous settings was selected additionally. Sample intervals was set to 00:00:01. This set data point recordings to be every second. Upon completion of these demands the 'log to file' function was selected to choose the location of saved files. By selecting 'start', recording began, as directed within the FireStingO_2 User Manual (PyroScience, n.d.).

0.1ml of the chemical foraging cue GSH was then added, through the septum of lid into the container, using a microlitre syringe and fine hypodermal needle 21G. Primarily, concentrations of cue were low (10^{-8}M/l) increasing in concentration until the addition of higher concentrations (10^{-4} M/l). GSH was added in 60 second increments. This protocol is as seen in Figure 1. Acting as both negative and positive controls, 0.1ml of artificial seawater and mussel conditioned artificial seawater was injected in replacement of GSH. Whereby it was predicted, there will be no significant change in respiratory rate and an increase in respiratory rate, respectively. This can be seen in Figure 2. Upon completion of each trial, the function 'stop' was selected on the PyroScience application window and data points were exported to an Excel data file. The temperature, salinity and pH of artificial seawater samples used in each trial were recorded prior to the trial and again following completion. Having the water temperature as an accurate fixed value was a very important trial factor, as variation in temperature could lead to variation in pressure and pH. Further resulting in significantly inaccurate oxygen concentration recordings. Equipment was thoroughly rinsed and dried between trials with distilled water. Individuals were starved 2 days prior to experiments to standardise hunger levels. Experimental days occurred after 3 day rest periods. Treatment order also varied amongst trials.

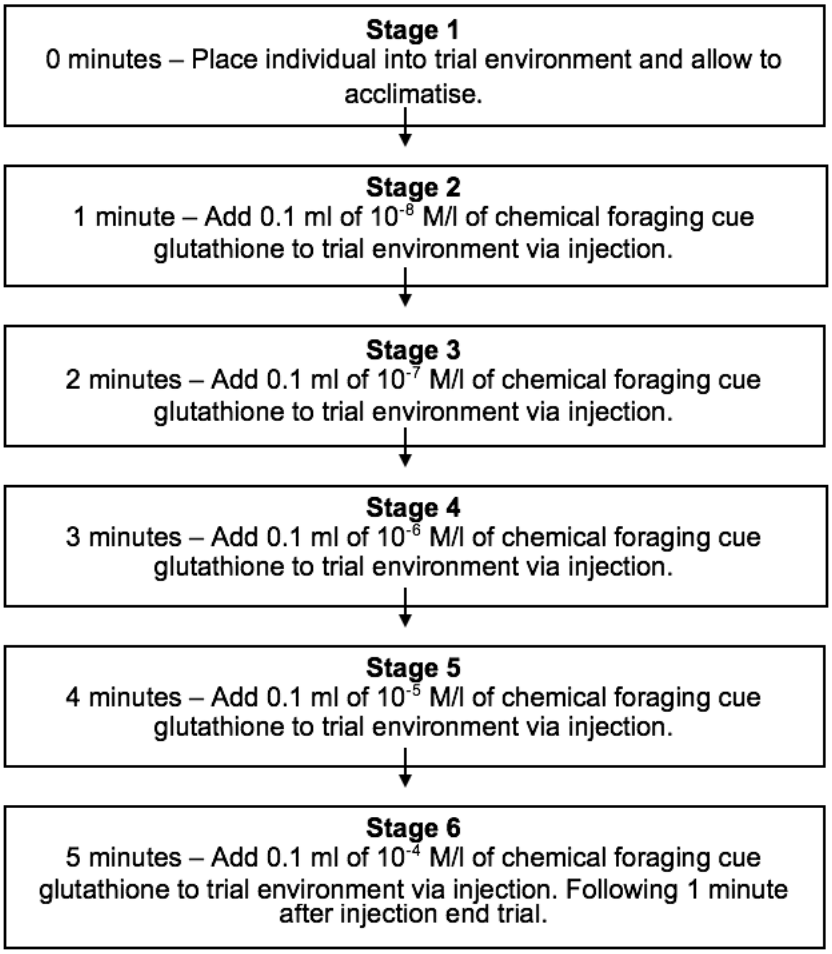

Figure 1: Biological assay flowchart demonstrating stages of experimental protocol.

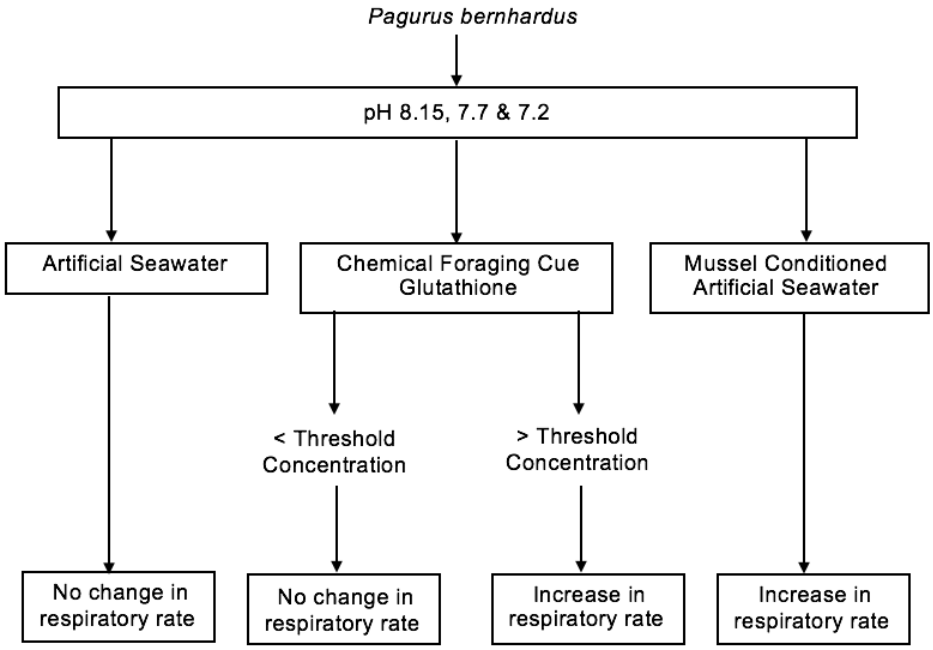

Figure 2: Predicted outcomes and outline of Chapter Three experimental trials using Fiber-Optic Oxygen Meter Firesting O_2 Pyroscience apparatus.

3.5 Statistical Methods

Detection Threshold Concentration of Olfactory Cues

All raw data from Fiber-Optic Oxygen Meter Firesting O_2 apparatus were presented as values of remaining O_2 concentration (%). To evaluate the change in oxygen concentration within the trial environment the drop in oxygen concentration (%) per stage of each trial was calculated using the following equation:

Oxygen concentration drop (%) per stage –

(Stage final O_2 (%) reading ÷ Stage starting O_2 (%) reading) X 100 = B

100 – B = Oxygen concentration drop (%) per stage for respective individual

For example, if the starting oxygen concentration equated to 100% at the beginning of stage 2 and the oxygen concentration equated to 50% at the end of stage 2 (after a total of 1 minute), the equation to calculate the drop in oxygen concentration for stage 2 would be:

(50% ÷ 100%) X 100 = 50%

100 % – 50% = 50% = A drop in oxygen concentration of 50%

Upon the calculation of these values, corresponding statistical analysis comprised of the statistical programme R (version 3.6.0). Whereby an array of additional packages, as seen in appendix, were used. A series of graphical figures were firstly generated to aid in visualising data. Plots incorporated the drop in O_2 concentration for each individual. Detection threshold concentrations for each individual were determined as the point in which the addition of GSH resulted in a greater drop in oxygen concentration, compared to that which occurred during stage 1 (0-1 minute), the acclimation/control phase for each pH level. Upon statistical analysis data was found to be non-normally distributed with application of Shapiro-Wilks normality test. Additionally, data did not follow the assumption of homogeneity of variance, as required to perform a One-Way ANOVA statistical test. This was also true for log-transformed data. The non-parametric statistical equivalent to One-Way ANOVA was therefore selected to determine if there was significant difference between detection threshold concentrations for chemical foraging cue GSH and pH level. After application of Kruskal-Wallis statistical test, the Pairwise Wilcoxon test for comparison was run to determine were significance existed.

Determination of pH and Overall Drop in Oxygen Concentration (%)

Similarly, to evaluate the change in oxygen concentration within the trial environment from start to finish the overall drop in oxygen concentration (%) for the entirety of each trial, was calculated using the following equation:

Overall drop in oxygen concentration (%) –

$$(\text{Final } O_2 \text{ (\%) reading} \div \text{Starting } O_2 \text{ (\%) reading}) \times 100 = A$$

$$100 - A = \text{Overall drop in oxygen concentration (\%) for respective individual}$$

To illustrate the use of such an equation, as before, an example is given. If the starting oxygen concentration of the trial environment equated to 100% and this reduced to 50% at the end of the complete trial (after a total of 6 minutes), the equation to calculate the overall drop in oxygen concentration would be:

$$(50\% \div 100\%) \times 100 = 50\%$$

$$100\% - 50\% = 50\% = \text{An overall drop in oxygen concentration for the entirety of the trial of 50\%}$$

This equation uses both the initial and final data recording of trials using Fiber-Optic Oxygen Meter Firesting O_2 Pyroscience apparatus and software. Therefore it is important to note that although this method gives the overall drop in oxygen concentration for the entire trial, it most likely does not take account of any potential fluctuations in O_2 concentration drop, which may have occurred. Upon calculation of these values, statistical analysis made use of the statistical programme R (3.6.0), as seen in the appendix. To determine if pH impacts the overall drop in oxygen concentration (%) with the addition of chemical foraging cue GSH and mussel conditioned artificial seawater, One-Way ANOVA was applied. This test procedure was also applicable to data concerning trials, with the addition of artificial seawater. Assumptions of this statistical test must be met and again normality was defined via the application of Shapiro-Wilks normality tests and plotting of QQ plots, once fitted to a linear model. Whereby non-normal data undergoes log transformation to generate normal distribution. In the instance data was found to be non-normally distributed after log-transformation, the non-parametric Kruskal-Wallis equivalent was conducted. Additionally, the Levene's test was performed to test One-Way ANOVA assumption of homogeneity in variance of data. Boxplots were additionally generated to allow visual analysis.

Impact of Olfactory Cues and Control Additions on Overall Drop in Oxygen Concentration (%)

Overall drop in oxygen concentration (%) of the trial environment of individuals was calculated in accordance to the equation above. Statistical analysis using programming software R (3.6.0) was used to identify the impact of treatment addition (chemical foraging cue GSH, mussel conditioned artificial seawater and artificial seawater) on overall drop in oxygen concentration (%) across all pH levels. As seen in appendix. The One-Way ANOVA was, again, considered the most appropriate statistical test providing test assumptions are met and data is normally distributed. This was determined via Shapiro-Wilks normality test, QQ plots of normality and Levene's test of variance. Non-normal data was log-transformed. Tukey multiple comparisons of means 95% family-wise confidence level tests were applied to significant results of One-Way ANOVA.

Pagurus bernhardus Weight

To determine if correlation existed bewteen the weight (g) of *P. bernhardus* individuals and both overall drop in oxygen concentration (%) and detection threshold concentration of GSH at pH 8.15, linear regression analysis was performed. Shapiro-Wilks normailty tests confrimed that overall drop in oxygen concentration (%) data was of normal distribution. This did not therefore require log transformation. However, detection threshold concentration data was found to be non-normally distributed and was therefore log transformed. Scatter plots were generated to aid with analysis and intereptation of results. The weight of 13 individuals from 20 was known and used in analysis.

3.6 Results

Detection Threshold Concentration of Olfactory Cues

Figures 3, 4, 5 and 6 demonstrate how the drop in oxygen concentration for the environment of each individual differed with addition of varying chemical foraging cue GSH concentration (M/l) and pH (8.15, 7.7 and 7.2). From the figures it is clear, response to the addition of chemical foraging cue GSH varied between individuals. Detection threshold concentrations for chemical foraging cue GSH were calculated. For each individual, such thresholds were defined as the initial point in which the drop in oxygen concentration (%) was greater than the drop recorded during acclimation/control phase (stage 1). Detection thresholds represent the minimum concentration required for successful initial detection. Table 1 demonstrates detection threshold concentrations for each individual at each pH.

Detection threshold concentration data was non-normally distributed ($P = 6.7x10^{-9}$). This was also evident after log transformation ($P<0.05$). Data also did not meet assumption of homogeneity in

variance, required by One-Way ANOVA parametric test ($F_{2, 57}$ = 7.45, P = 0.0013). Kruskal-Wallis statistical test was therefore selected for analysis. Whereby, results determined pH level influenced detection threshold concentrations for chemical foraging cue GSH ($X^2_{57, 20}$ = 7.2, P = 0.027, Fig: 6). Differences in detection threshold concentration between pH levels 7.7 and 7.2 were greatest, confirmed with Pairwise Wilcoxon comparisons test (P = 0.037). Comparisons between pH levels 8.15 and 7.7 and further 8.15 and 7.2 proved to show no statistical difference (P = 0.56 and 0.07, respectively). Leading to the partial acceptance of hypothesis 1a), as reduction in pH level increased detection threshold concentrations for GSH, however such significance only existed in pH level reduction from 7.7 to 7.2. The median detection threshold concentration for all 20 individuals at pH 8.15 was 10^{-7}M/l, at pH 7.7 this was 10^{-8}M/ and for pH 7.2 equated to 10^{-6}M/l.

Figure 7 shows the impact of pH and the corresponding detection threshold concentration for chemical foraging cue GSH. This varied greatly between individuals. 1 of 20 individuals failed to detect the presence of all concentrations of chemical foraging cue GSH when subjected to pH 7.7. A total of 9 individuals failed to detect the presence of all concentrations of chemical foraging cue GSH when subjected to pH 7.2. pH had no impact on the detection threshold concentration for chemical foraging cue GSH for individual 16. Such individual detected the presence of chemical foraging cue GSH with minimum concentration 10^{-8}M/l in each trial. Individuals 9, 17 and 18 detected chemical foraging cue GSH at increasing concentration ass pH decreased. 4 of 20 individuals required the addition of a greater concentration of chemical foraging cue GSH for detection at 7.7 compared to concentrations required at pH 8.15. Similarly, this was found for 4 individuals when subjected to pH 7.2. A further 5 individuals required a greater detection threshold concentration at pH 7.2 compared to thresholds required by such individuals at pH 7.7.

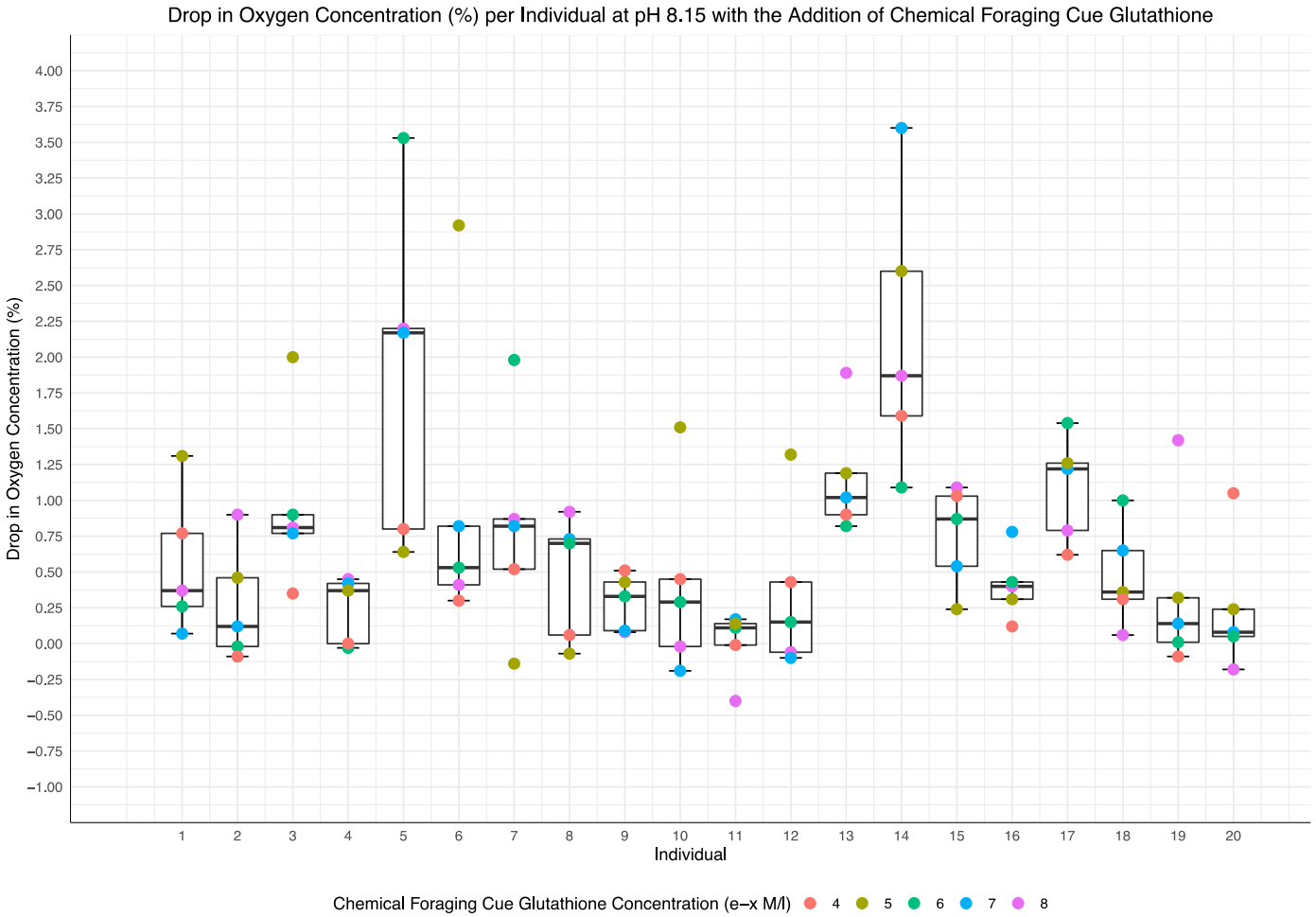

Figure 3: Variation between individuals and the drop in oxygen concentration (%) of their trial environment at pH 8.15, with the addition of chemical foraging cue glutathione of varying concentration. Whiskers signify 95% confidence limits (Cis). Outliers are represented by singular points.

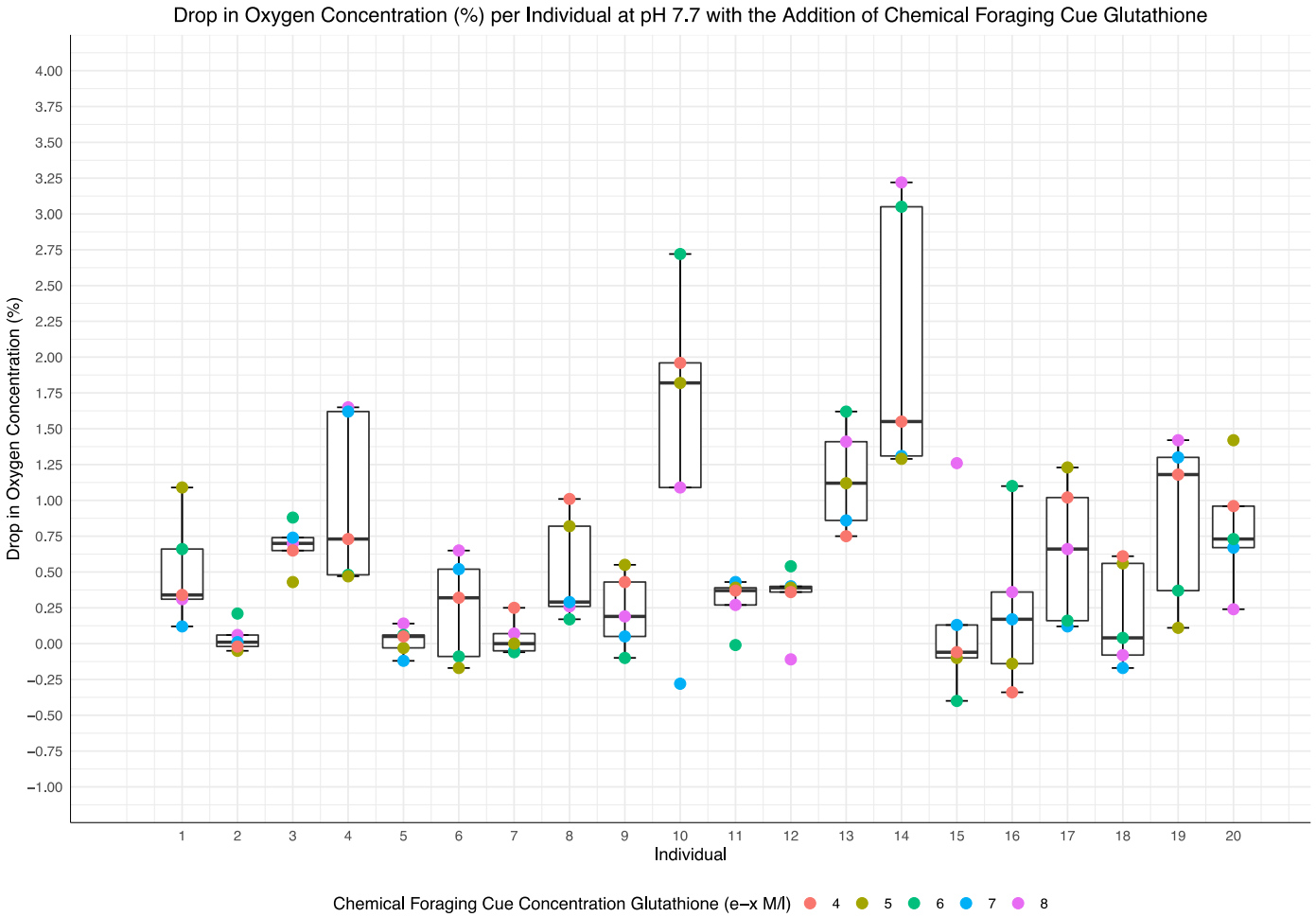

Figure 4: Variation between individuals and the drop in oxygen concentration (%) of their trial environment at pH 7.7, with the addition of chemical foraging cue glutathione of varying concentration Whiskers signify 95% confidence limits (Cis). Outliers are represented by singular points.

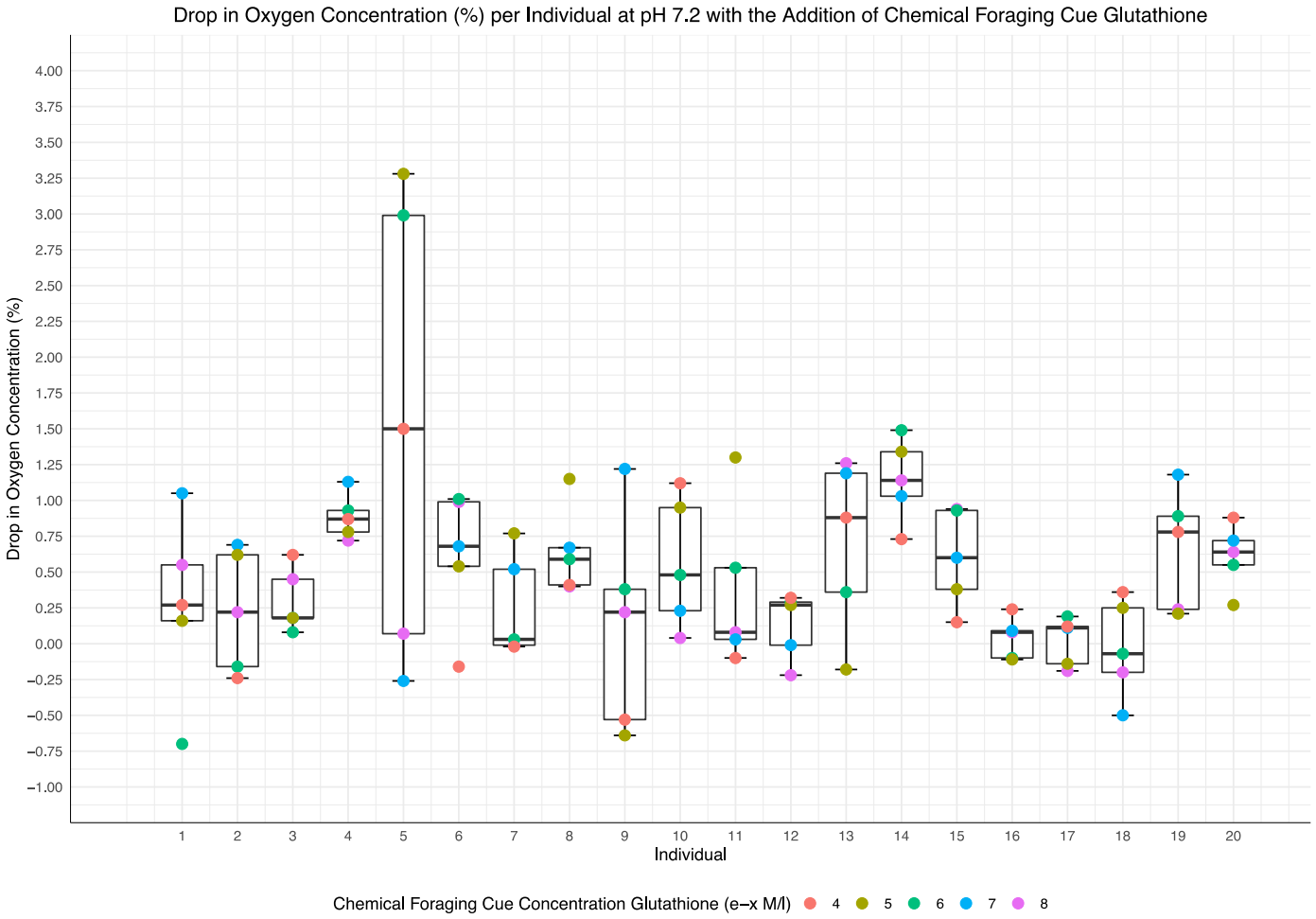

Figure 5: Variation between individuals and the drop in oxygen concentration (%) of their trial environment at pH 7.2, with the addition of chemical foraging cue glutathione of varying concentration Whiskers signify 95% confidence limits (Cis). Outliers are represented by singular points.

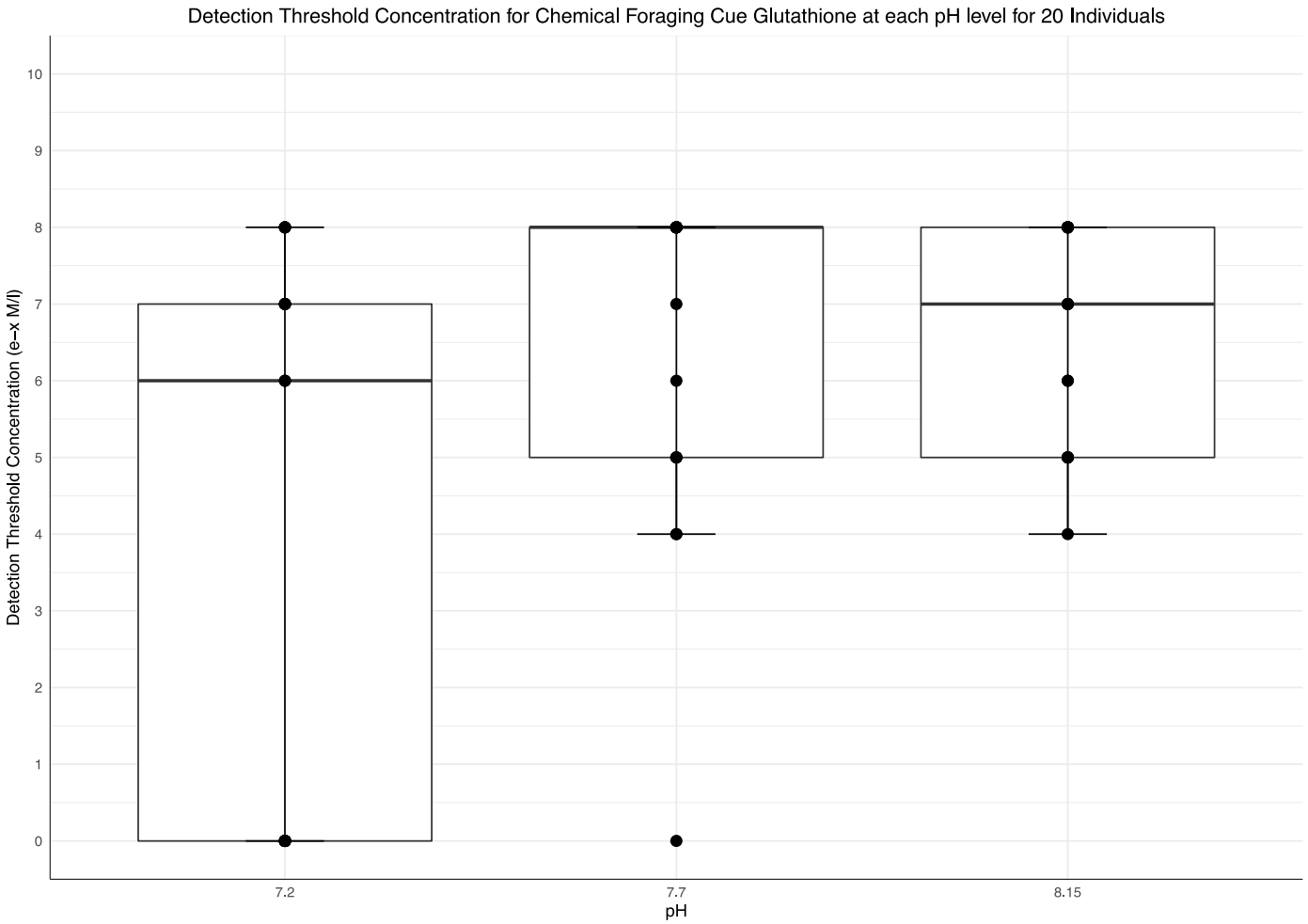

Figure 6: Detection threshold concentration (M/l) for chemical foraging cue glutathione required by 20 individuals at pH levels: 8.15, 7.7 and 7.2. Whiskers signify 95% confidence limits (Cis). Outliers are represented by singular points. Detection threshold concentration e^{-0} M/l indicates instances were cue was not detected at any concentration in trials.

Table 1: Detection threshold concentrations for chemical foraging cue glutathione for each *P. bernhardus* individual at pH levels 8.15, 7.7 and 7.2.

	Detection Threshold Concentration for Chemical Foraging Cue Glutathione (10^{-x} M/l)		
Individual	**pH 8.15**	**pH 7.7**	**pH 7.2**
1	10^{-5}	10^{-8}	10^{-8}
2	10^{-8}	10^{-8}	10^{-7}
3	10^{-5}	No Response	No Response
4	10^{-8}	10^{-8}	No Response
5	10^{-6}	10^{-8}	No Response
6	10^{-7}	10^{-8}	No Response
7	10^{-8}	10^{-4}	10^{-7}
8	10^{-8}	10^{-8}	10^{-7}
9	10^{-5}	10^{-4}	No Response
10	10^{-5}	10^{-6}	10^{-7}
11	10^{-7}	10^{-8}	10^{-6}
12	10^{-5}	10^{-7}	10^{-6}
13	10^{-8}	10^{-8}	No Response
14	10^{-7}	10^{-8}	10^{-8}
15	10^{-8}	10^{-8}	No Response
16	10^{-8}	10^{-8}	10^{-8}
17	10^{-7}	10^{-5}	No Response
18	10^{-6}	10^{-5}	No Response
19	10^{-8}	10^{-8}	10^{-7}
20	10^{-4}	10^{-5}	10^{-8}

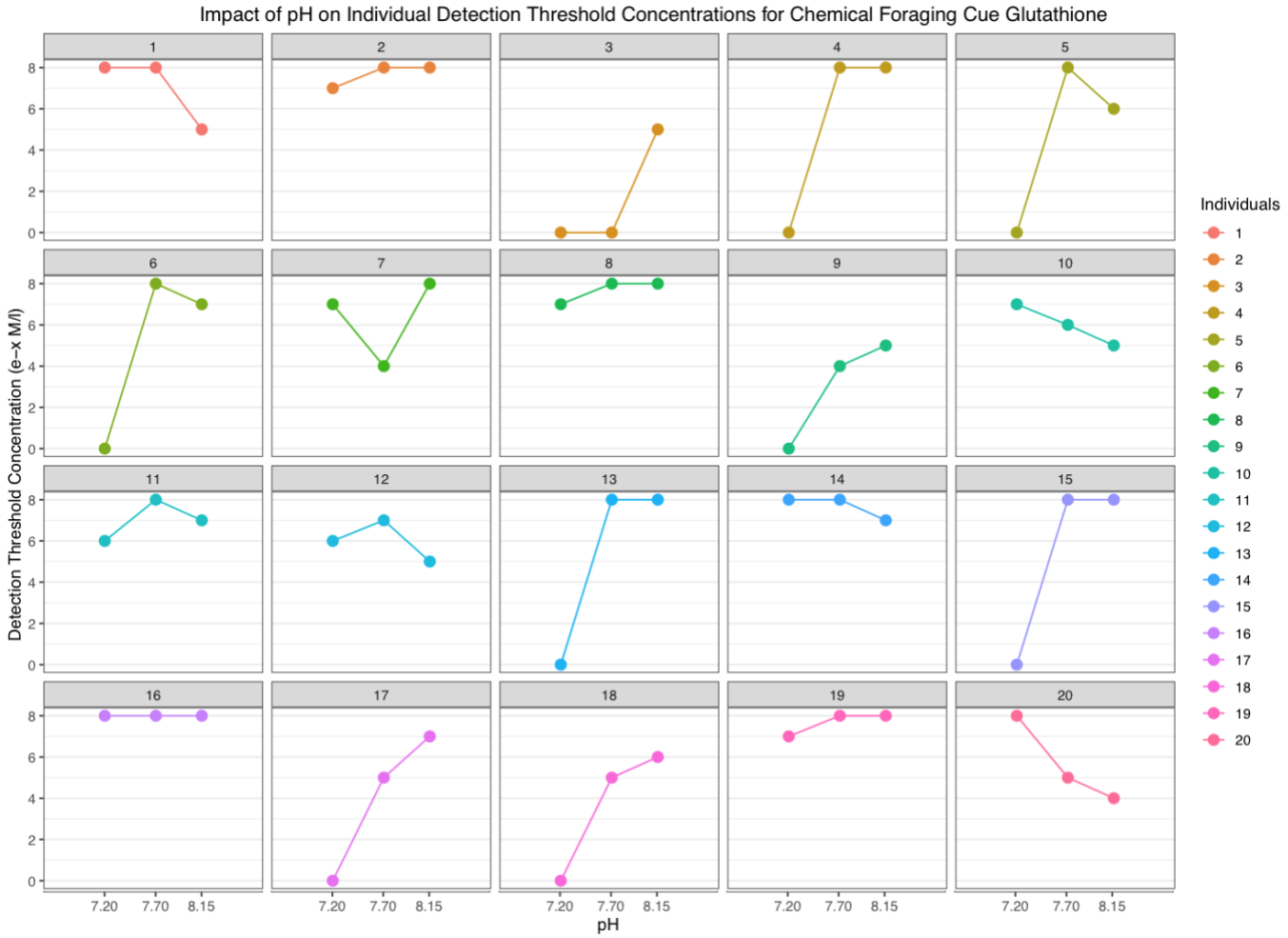

Figure 7: Detection threshold concentration (M/l) for chemical foraging cue glutathione and variation resulting from change in pH, for 20 individuals. Individuals as defined by key. Detection threshold concentration e^{-0} M/l indicates instances were cue was not detected at any concentration in trials.

Determination of pH and Overall Drop in Oxygen Concentration (%)

Figure 8 demonstrates overall drop in oxygen concentration (%) in accordance to pH level and trial addition: A) artificial seawater, B) mussel conditioned artificial seawater and C) chemical foraging cue GSH. Data obtained with the addition of GSH was found to be non-normally distributed ($P=1.43x10^{-6}$). This was also evident after log transformation ($P<0.05$). Results of the Kruskal-Wallis statistical test determined there to be no effect of pH level on overall drop in oxygen concentration with the addition of chemical foraging cue GSH ($X^2_{57,\ 20} = 1.088$, $P = 0.58$).

Similarly, data for drop in overall oxygen concentration (%) with the addition of artificial seawater and mussel conditioned artificial seawater was found to be non-normally distributed ($P = 3.16\ x10^{-6}$ and 0.0028, respectively). After log transformation, such data became normally distributed $P>0.05$. Both data sets also met the assumption of homogeneity of variances, required by One-Way ANOVA

statistical test. Artificial seawater (F = 1.27, P = 0.29) and mussel conditioned artificial seawater (F = 0.072, P = 0.93). One-Way ANOVA confirmed pH level had no effect on mean drop in overall oxygen concentration, with additions: artificial seawater ($F_{2, 57}$ = 1.35, P = 0.27) and mussel conditioned artificial seawater ($F_{2, 57}$ = 0.76, P = 0.47). Leading to the rejection of hypothesis 1b). Reduction in pH level did not result in decreased drop in overall oxygen concentration (%) with trial additions chemical foraging cue GSH and mussel conditioned artificial seawater.

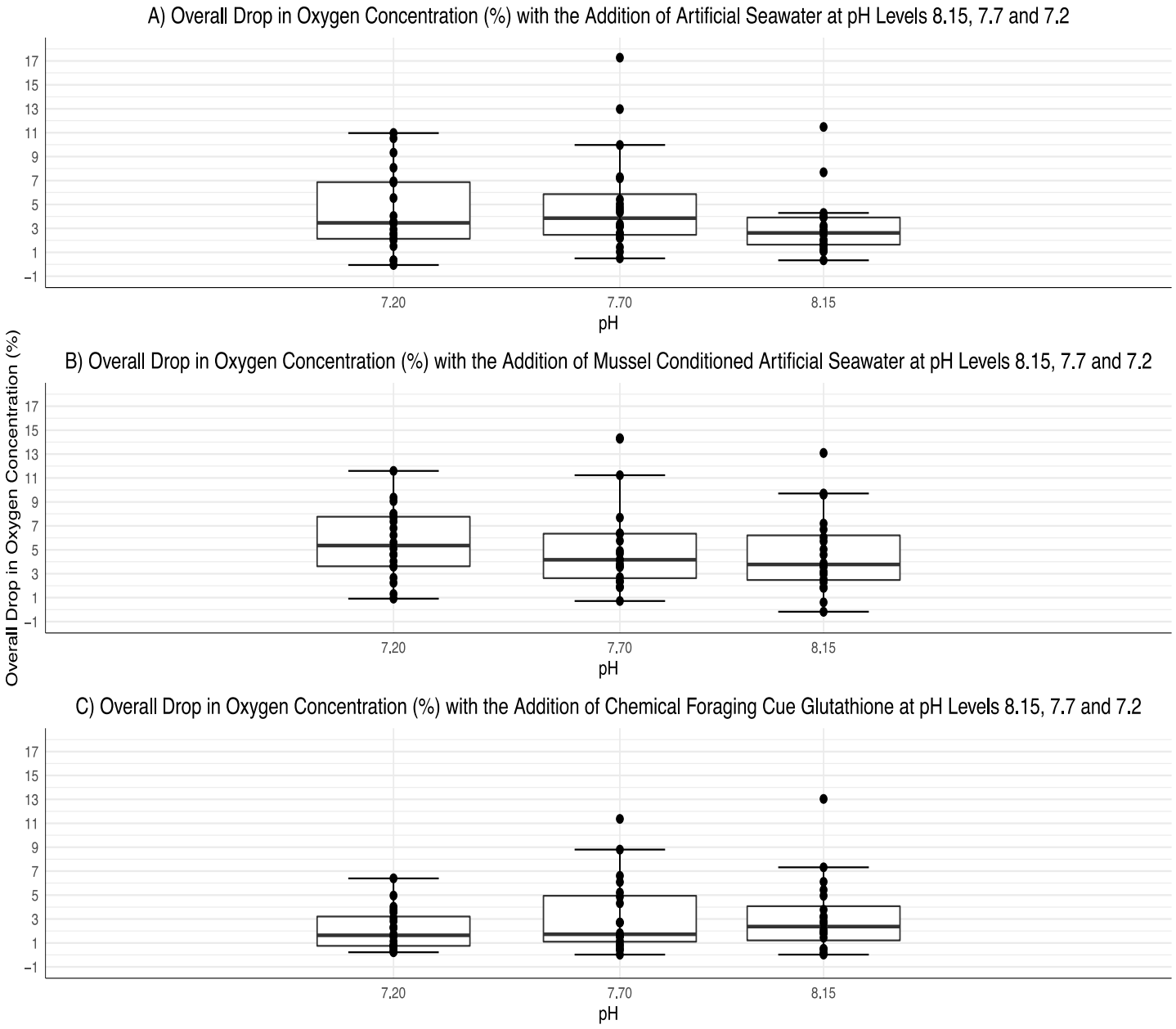

Figure 8: Overall drop in oxygen concentration (%), for the duration of each trial in accordance to pH level: 8.15, 7.7 and 7.2. With variation in trial addition for 20 individuals. Plot A) addition on artificial seawater at all pH levels. Plot B) addition of mussel conditioned artificial seawater. Plot C) addition of chemical foraging cue glutathione. Whiskers signify 95% confidence limits (Cis). Outliers are represented by singular points.

Impact of Olfactory Cues and Control Additions on Overall Drop in Oxygen Concentration (%)

To examine if variance in means for the overall drop in oxygen concentration (%) between different trial environemental additions at the same pH level exist, a series of statistical analysis was incorporated. One-Way ANOVA determined trial addition effected mean overall drop in oxygen concentration (%), at both pH 7.7 ($F_{2, 57}$ = 3.30, P = 0.044, Fig: 9) and 7.2 ($F_{2, 57}$ = 8.81, P = 0.00046, Fig: 9). This leads to the acceptance of the hypothesis 1c); overall drop in oxygen concentration (%) differed with trial environment addition at pH 7.7 and 7.2. Tukey HSD for pH 7.7 data observed differences between trials with environemental additions: mussel conditioned artificial seawater and chemical foraging cue GSH (P = 0.05). Similarly for pH 7.2, differences were observed between: chemical foraging cue GSH and artificial seawater (P = 0.044) in addition to mussel conditioned artificial seawater and chemical foraging cue GSH (P = 0.0003).

No differences exist trial addition at pH 8.15 ($F_{2, 57}$ = 1.85, P = 0.17, Fig: 9). All data met assumptions of homogeneity of variance ($F_{2, 57}$ = 0.74, 0.22 and 2.74, P = 0.48, 0.80 and 0.073, for pH 8.15, 7.7 and 7.2 respectively). However, data was non-normally distributed for each pH level: 8.15 (P = 9.39 $x10^{-6}$), 7.7 (P = 5.05 $x10^{-6}$) and 7.2 (P = 0.0018). Data was therefore log transformed to generate normal distribution for all pH levels P>0.05.

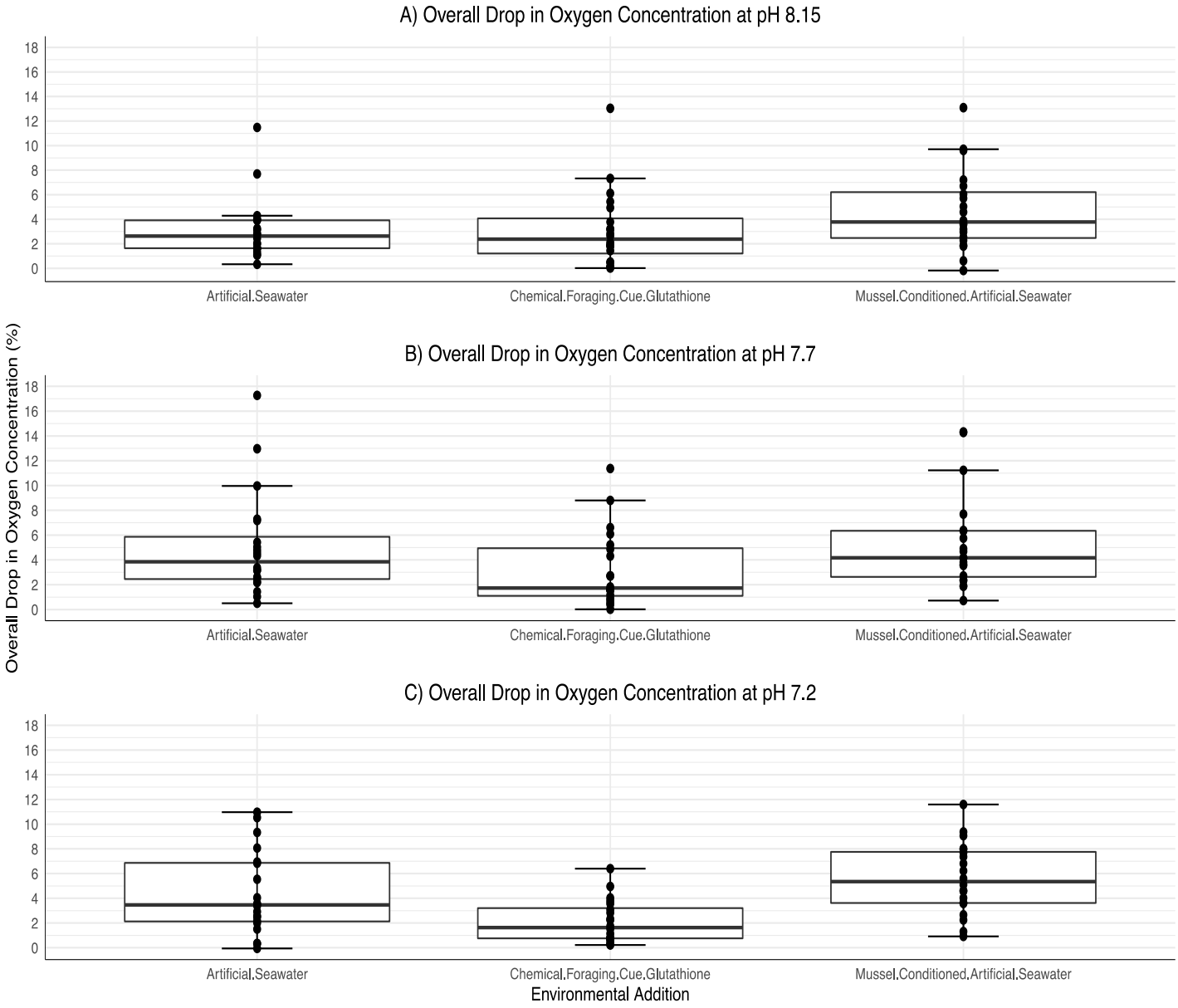

Figure 9: Overall drop in oxygen concentration (%), for the duration of each trial. With varying trail addition: chemical foraging cue glutathione, mussel conditioned artificial seawater and artificial seawater and pH level. Plot A) pH 8.15, plot B) pH 7.7 and plot C) pH 7.2. Whiskers signify 95% confidence limits (Cis). Outliers are represented by singular points.

Pagurus bernhardus Weight

Linear regression analysis determined indivdual *P. bernhardus* weight (g) had no effect on the overall drop in oxygen concentration (%), for pH 8.15 ($F_{1, 12}$ = 3.05, P = 0.11, Fig: 10), with the addition of GSH. Data was of normal distribution (P = 0.38). Similarly, weight had no effect on individual detection thresholds for GSH ($F_{1, 12}$ = 4.65, P = 0.052, Fig: 10). Such data was non-normally distributed (P = 0.0087) and therefore was log-transformed. Leading to the rejection of hypothesis 1d).

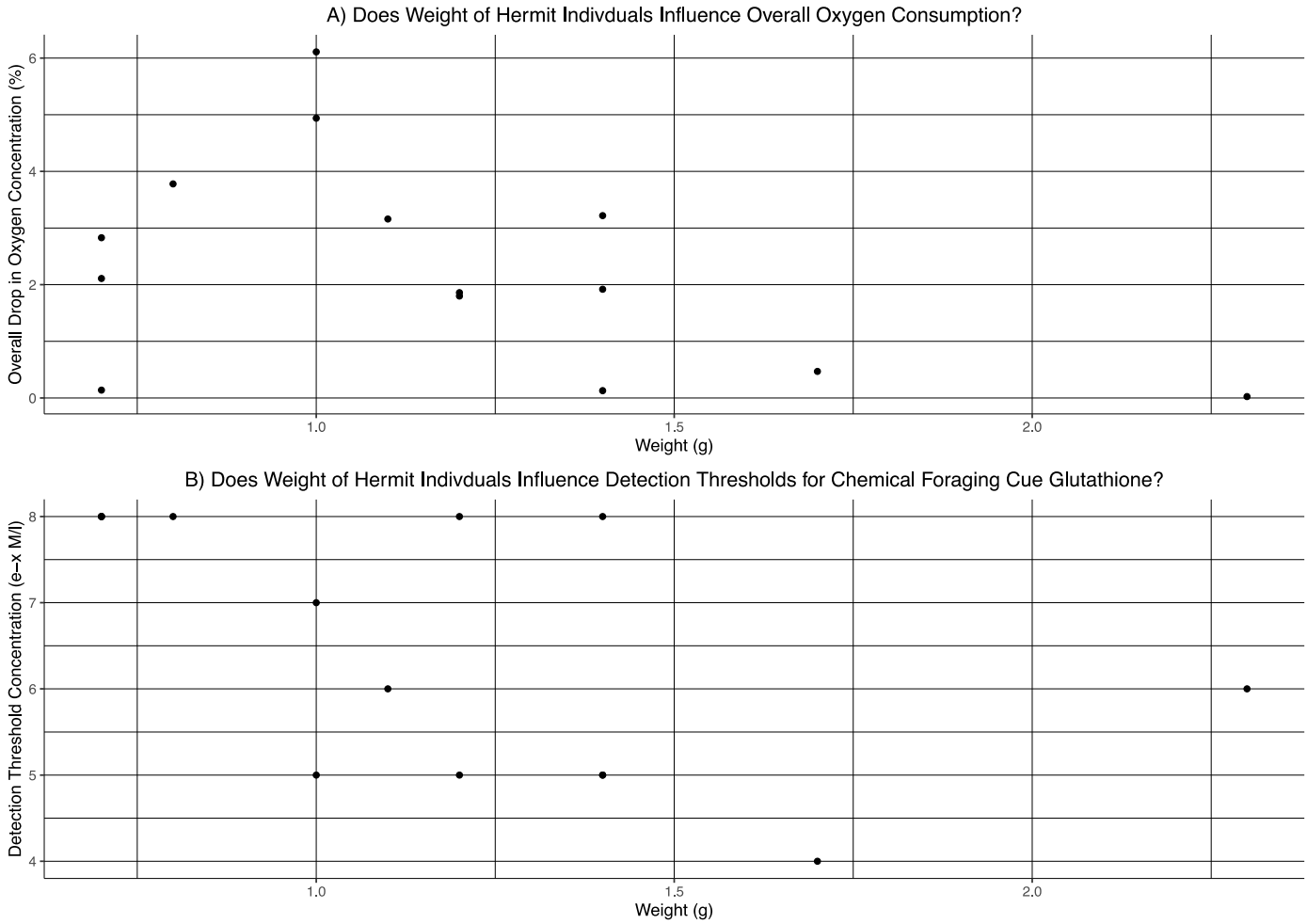

Figure 10: Known weight of 13 *P. bernhardus* individuals and: A) overall drop in oxygen concentration (%); B) detection threshold concentration for chemical foraging cue glutathione (e^{-x} M/l).

3.7 Discussion

Analysis of Chapter Three results gives greater insight into the effect of reduced pH levels on the olfactory ability of hermit crab, *Pagurus bernhardus*, individuals. Such knowledge can prove useful in attempts to distinguish impacts of future OA conditions within coastal environments. Findings suggest that detection threshold concentrations for the chemical foraging cue GSH increased in concentration with reduced pH. Particularly from the drop in pH 7.7 to 7.2, as seen in Figure 6 and in agreement with similar findings of other studies (Velez et al., 2019).

Response to low pH demonstrated by individuals varied greatly amongst the sample population (Figures 3, 4, 5 and 7). One individual, from the sample population, failed to respond with all concentration additions of chemical foraging cue GSH at pH 7.7. Suggesting such individual failed to detect the presence of its addition into the environment. Similarly, a greater quantity of individuals failed to detect the presence of chemical foraging cue GSH of any concentration at pH 7.2. Further

suggesting individuals required greater concentration of chemical foraging cue GSH for successful detection at reduced pH levels. Concentration of GSH required by some individuals for successful detection therefore needed to be greater than 10^{-4} M/l, which was the highest concentration in trials. Such key findings lead to the acceptance of hypothesis 1a). Detection threshold concentrations for chemical foraging cue GSH differ and increase with reduced pH conditions for the population of *P. bernhardus* individuals trialed. However, results also suggest within the population tested, great variation exists both; between and within individual responses.

Upon further exploration hypothesis 1c) can be partially accepted. Overall drop in oxygen concentration (%) of the trial environment for individuals was greatest with the addition of mussel conditioned artificial seawater. However, was true for pH levels 7.7 and 7.2 only. Figure 9 demonstrates the average greatest drop in oxygen concentration at pH 8.15 to be greatest with the addition of mussel conditioned artificial seawater, statistical results did not determine this to be of significance. This occurrence however could have been a result of limitations amongst *P. bernhardus* sample size and issues could have also arisen from not allowing for non-independence of data. As a result, significance of statistical analysis can be biased, from the inflation or deflation in estimated relationships (Grawitch & Munz, 2004).

Hypotheses 1b) and 1d) are both rejected in accordance to statistical results. Which confirm the overall drop in oxygen concentration was not impacted by reduced pH level. Additionally, the weight of individuals did not influence detection threshold concentrations for chemical foraging cue. As a result of such findings, differences amongst results can be attributed to olfactory and chemical signaling processes. Contrastingly, Kim et al. (2016) studied the impact of low pH conditions on the olfactory abilities of the deep sea hermit crab *P. tanneri.* Such study determined the respiration rate of individuals increased in reduced pH waters (Kim et al., 2016). Explanations to this occurrence were linked to the metabolic rate and performance of individuals. Suggesting higher metabolic rates increased as a result of higher CO_2 exposure. Whereby individuals required greater energetic expenditure for processes such as up-regulation of homeostatic mechanisms and acid-base balance (Kim et al., 2016). Similarly, Cooper et al. (2016) also found reduced oxygen consumption for krill species, *Euphausia pacifica*, when subjected to elevated pCO_2. However, such comparisons to the current study should be made with caution, as both *P. tanneri* and *E. pacifica* are exposed to relative stable pH natural environments. Unlike variation amongst conditions of coastal and intertidal environments, which *P. bernhardus* inhabit.

Investigations by Almut and Bamber (2013) unveiled that acute exposure and stepwise pH reduction resulted in immediate and increased locomotion activity of shrimp, *Crangon crangon.* Followed by

individuals resuming their usual resting behaviour. Suggesting effects due to reduced pH and short-term exposure may be partially reversible. The pH level of such experiments was decreased by 0.2 units hourly. Individuals were found to be extremely sensitive to this change and rapidly displayed avoidance behaviour (Almut & Bamber, 2013). Short term exposure of reduced pH to *C. crangon* individuals increased activity levels (Almut & Bamber, 2013). Such results are in contrast to the current study as reduced pH had no significant impact on overall drop in oxygen concentration for *P. bernhardus* individuals. Which suggests no overall effect on activity levels. As briefly stated previously, this further confirms any differences amongst data recordings are the result of the presence and detection of foraging cues.

There are numerous possibilities as to why in the current study at reduced pH levels the respiration rate of *P. bernhardus* individuals did not increase when presented with a foraging cue. One theory proposes as a result of subjection to pH stress individuals experienced physiological changes. The energy availability of such individuals was therefore limited. Further impacting metabolic processes, as reviewed by Pörtner et al. (2004). Secondly it could be hypothesised individuals experienced neurological changes as a result of reduced pH levels, which altered mechanisms involved in the processing of information. Suggesting individuals may have detected the presence of cue but failed to show an appropriate response (Nilsson et al., 2012; Tresguerres & Hamilton, 2017). However, such reasonings are limited in plausibility within the current study. Overall drop in oxygen concentration (%) was not effected by reduced pH, implying there was no change in respiration rate of individuals. Additionally, such physiological changes are unlikely to occur within acute exposure trials.

Individuals displayed appropriate response to the presence of mussel conditioned artificial seawater at reduced pH. As a result of this, it is unlikely reduced foraging with the addition of GSH at reduced pH occurred due to pH induced stress, individual health or changes amongst neurotransmitters. This evidence therefore supports the theory, whereby reduced foraging, at reduced pH, occurred due to chemoreception processes, for the addition of chemical foraging cue GSH to the environment of individuals (Ashur et al., 2017; Briffa et al., 2012; Brown et al., 2002; Roggatz et al., 2016; Tierney & Atema, 1988). It is possible damage occurred to receptor organs, altering receptors functionality and receptor-ligand interactions or reduced pH caused direct changes to the signaling molecules of GSH and interactions with receptors (Velez et al., 2019). Mussel conditioned artificial seawater contains a mixture of chemical molecules and is less likely to be effected by reduction in pH. Therefore, mussel conditioned artificial seawater is still detected by individuals as the quantity added to the environment contains compounds unaffected by reduced pH levels. Similarly, a mixture of

odourants was also considered in a study involving detection of intestinal fluid by seabream, *Sparus aurata,* whilst subjected to reduced pH conditions (Velez et al., 2019).

Amino acids and tripeptides are structurally effected by low pH and therefore OA related conditions. This is due to ionizable functional groups they occupy (Velez et al., 2019). Roggatz et al. (2016) studied the functionality of three tripeptides in behavioural bioassays as cues to mimic egg ventilation in *Carcinus maenas,* subjected to reduced pH levels. Findings determined individuals required a greater concentration of cue to display egg ventilation behavioural response (Roggatz et al., 2016). The results of this study and others suggest that chemical cues and odourants are pH sensitive as; protonation states, charge conformation and distribution of the signaling molecules are significantly altered (Porteus et al., 2018; Roggatz et al., 2016; Roggatz et al., 2019; Velez et al., 2019). in addition to signaling molecules becoming structurally impaired, chemoreceptors (peptides) are also structurally effected (Hardege et al., 2011). Consequently, receptor-ligand interactions become impaired due to pH-induced changes to receptor sites of the receiving organism, which impacts capabilities of ligands docking and initiating signal transduction (Hardege et al., 2011). Conformational changes of pheromone binding proteins have been found to occur among insects under reduced pH conditions, particularly *Bombyx mori* (Damberger et al., 2000; Wojtasek & Leal, 1999). Conformational changes involve the loss and unfolding of rigid tertiary chemoreceptor protein structures. This sensitivity to pH leads to the eventual failed binding of ligands to binding proteins mediating ligand-membrane release and fusion at reduced pH levels (Damberger et al., 2000; Wojtasek & Leal, 1999).

Changes to molecular properties of GSH therefore has the potential to reduce the bioavailability of ‘active’ forms. Such reasoning could be used to explain why individuals failed to detect the presence of GSH at reduced pH levels. Overall resulting in unsuccessful chemoreception, namely perception of chemical cues via olfaction. In circumstance whereby molecules are unable to successfully bind to receptors of receiving organisms, there is a lower binding affinity and lack/reduction of olfactory stimulation. Requiring increased detection threshold concentrations to enable successful detection (Velez et al., 2019). De la Haye et al. (2011) produced very similar findings, indicating reduced pH levels further reduced antennule flicking by *P. bernhardus* individuals. Directly as a result of disruption to chemo-receptiveness of individuals. Roggatz et al (2019) also explored bioavailability of keystone molecules (saxitoxin and tetrodotoxin) of harmful algal blooms and other marine species resulting from reduced pH. Findings predict under future OA related conditions, the bioavailability of molecules will increase. Velez et al. (2019) highlight the scope of impact on differing molecules by reduced pH levels. Confirming, varying response by differing amino acids and subsequent chemical

odours. As a result, acute exposure leads to ranges of olfactory sensitivity amongst marine individuals (Velez et al., 2019).

Impaired chemosensory ability under acidified conditions may result in, hermit crabs relying more heavily on other sensory modalities such as; visual and tactile senses (Ashur et al., 2017; Dodd et a., 2015). However, such senses may not be fully compensatory for reduced capabilities in olfaction. This is due to low visual acuity and the proximity individuals will have to be, in location to sources of food, for tactile sense to be deemed useful in foraging (Ashur et al., 2017). Mesce (1993) proposed two species of hermit crab, *Pagurus samuelis* and *Pagurus hirsutiusculus*, depended on upon chemical cues to locate and uncover partially buried shells to inhabit. Visual and tactile modalities were not relied upon. Failure to successfully detect foraging cues via chemoreception and unsatisfactory compensatory mechanisms may ultimately lead to the deterioration of individual fitness and health. Due to lack in abilities to detect and obtain food. This may further threaten the stability of crab populations with potential cascading ecosystem effects (Jarrold et al., 2017).

P. bernhardus individuals used in the study are coastal/intertidal organisms and experience natural variation. This therefore could explain why, when undergoing experimental trials only very low pH 7.2 impacted the ability of individuals to successfully detect the presence of the chemical foraging cue GSH, compared to pH 8.15 and 7.7. Such variation in pH within their natural environment, may promote resilience to future OA conditions (Maas et al., 2012). A study by Jarrold et al. (2017), investigated the impact of diel pCO_2 cycles on two species of coral reef fish, *Acanthochromis polyacanthus* and *Amphiprion percula.* Findings suggest exposure to fluctuating levels of pCO_2 reduced the occurrence of behavioural abnormalities compared to continued subjection. This may therefore, alleviate the extent of future impacts in the event of OA. However, species whom currently experience such variation in pH, may already be living at their maximum tolerance limits. Such conditions are likely to be amplified by further acidification exposing organisms to conditions past their tolerance limits (Chan et al., 2017; Rose et al., 2020).

A study investigating response to conspecific alarm pheromones by minnows (*Pimephales promelas),* and dace (*Phoxinus neogaeus)* established individuals failed to display antipredator behaviour when subjected to reduced pH levels of 6.0. However, individuals were able to exhibit such behaviour once subjected to pH levels of 8.0 (Brown et al., 2002). Authors stated that this occurred as a result of irreversible covalent changes to the alarm pheromone molecule, as individuals failed to respond to the stimulus when the stimulus sample had been buffered to low pH levels and rebuffered to pH 7.5 (Brown et al., 2002). Velez et al. (2019) stated the protonation state of some chemical odours were reversible when pH was normalised following subjection to reduced

pH levels. Additionally this study also found after four weeks of exposure to reduced pH levels *S. aurata*, failed to regain high olfactory sensitivity (Velez et al., 2019). Laubenstein et al. (2019) discovered that foraging behaviours of juvenile spiny chromis, *Acanthochromis polyacanthus.* Juveniles appeared significantly different between groups reared; under reduced pH levels and current day control conditions. This suggests previous exposure as juveniles to reduced pH, benefited adult individuals. Current day exposure to reduced pH of intertidal environments, therefore may be beneficial to species, considering what is predicted of the future (Maas et al., 2012).

Within the results of Chapter Three it is clear to see that behavioural variation exists as seen through the range of detection thresholds required by individuals. Chapter Four, aims to confirm this via alternate methodology and further highlight level of response that may be displayed by individuals. In addition to chemoreception and sensitivity to the presence of chemical cues, foraging behaviour has the potential to be influenced by a number of aspects. Hunger levels were standardised for all individuals by following a strict feeding routine for the duration of trials. However, it cannot be confirmed hunger levels of all individuals were equal to one another. This could only be assumed. Aspects for instance, and not limited to, moulting, reproductive stage, size and maturation of individuals may have the potential to influence hunger levels (Hayden et al., 2007; Laidre & Elwood, 2008; Lancaster, 1988). The hunger level of an individual can have great influence upon the motivation to attain food (Billock & Dunbar, 2009; Laidre & Elwood, 2008). Those that appear starved are more likely to display greater activity levels and make costly tradeoffs as they have greater motivation. Particularly under stressful environmental conditions (Billock & Dunbar, 2009; Laidre, 2007; Ramsay et al., 1997). Increased activity and motivation would have impacted the concentration of oxygen in the environment of trial individuals within this study and therefore influenced results. However, where possible, precautions were taken to avoid such circumstance

Between trials, individuals were noted to moult. This process involves the shedding of exoskeletons to enable construction of new calcified structures. Often to fit growing individuals (Hazlett, 1981; Lancaster, 1988). Moulting of female hermit crabs has been noted to be significant in the success outcome of reproduction, particularly for *Pagurus* species (Wada et al., 2007). During the moulting period individuals are at their most vulnerable and this may have the potential to impact behaviour (e.g. foraging, fighting, locomotion, etc.). Moulting is also an energetically costly process (Hazlett, 1981; Lancaster, 1988; Wada et al., 2007). During this study exoskeletons were removed from the environment of individuals who did moult, in order to keep their holding container cleaner and to prevent individuals feeding on them. Feeding shed exoskeletons is known to take place in the wild to enable reabsorption of calciferous matter and aid future growth (Lancaster, 1988). It is unknown if individuals fed on shed exoskeleton, before removal. Providing further reasoning towards why

hunger levels of individuals could not be quantified with certainty. Dawirs (1984) found the respiration rate of *P. bernhardus* individuals during megalopa development decreased substantially and remained decreased throughout the duration of their moult cycle. Whether this occurs during adult moulting stages is yet to be determined. Moulting did not occur during biological assay trials, but in-between experimental days whilst held within the universities aquaria. To improve the current study, shedding of exoskeletons should be recorded and correlation with result outcomes by individuals investigated.

P. bernhardus individuals used in trials also were of unknown age/maturation level, as a result of their random selection from the sample collection site of Scarborough, UK. Studies focused upon the impact of OA have suggested that reduced pH conditions can impact individuals of different ages and life-stages varyingly. Organisms of later life-stages may have developed a greater tolerance to environmental change as a result of previous experience (Pörtner, 2008). Larvae and juvenile species are considered more vulnerable to environmental change and OA (Bechmann et al., 2011; Kroeker et al., 2010; Kurihara, 2008). This therefore, may be a contributing factor towards variation observed in behaviour between individuals within trials of this study. Although it can be confirmed that no larvae were amongst the population of *P. bernhardus* investigated and individuals were assumed to be mature adults.

The sex of all *P. bernhardus* individuals used for trials was unknown. Sexing individuals with confidence would require the forced removal of them from shells (Lancaster, 1988). This process was not considered appropriate in the scheme of experimental trials. However, the sex of organisms has been found to influence feeding stimulatory response and activeness of individuals (Hayden et al., 2007). This was determined to occur amongst *Carcinus maenas* individuals. Whereby males displayed a significantly reduced feeding response. Particularly during summer reproductive seasons compared female response when presented with synthetic and natural food cues (Hayden et al., 2007). Females demonstrated strong feeding response throughout the year duration of trials (Hayden et al., 2007). The study further suggested that roles of 20-hydroxyecdysone, the moulting hormone, and release of female sex pheromone during the reproductive season of *C. maenas,* contributed to reduced feeding response of male individuals (Hayden et al., 2007). As reproductive seasons finish, individuals may be more responsive to the presence of foraging cues, as the motivation to detect and respond to reproductive cues diminishes. Such findings could therefore propose an explanation for the existence of variation amongst detection threshold concentrations of GSH, for *P. bernhardus* individuals of this study. As the sex of individuals was largely unknown. However, Turra et al. (2020) found no synergistic impact of pH and sex in the hermit crab, *Pagurus*

criniticornis. Sexing trial organisms would improve significance of findings of this study, if it was to be repeated for future research.

Given the many factors that impact behaviour and physiology, it is not surprising that the level of impact of OA varies greatly between studies. However, caution must be taken in applying findings to other contexts (Clark et al., 2020; Clements & Hunt, 2015; Kroeker et al., 2013). Upon reflection of the significant variation found, small sample sizes may have impacted results. For future development, the use of larger samples would be beneficial and promote reliability of results generated. Synthetic cues both pH sensitive and non-sensitive could also provide further insight into the mechanisms underpinning the observed impacts of reduced pH on behaviour. Betaine has been used as a chemical cue shown to produce foraging responses in existing literature. Betaine is an amino acid, that is pH stable, with stable protonation stages between pH levels 2 and 10. Appropriate for testing impacts of OA (Ferner & Jumars, 1999; Hayden et al., 2007; Ramos-Payán et al., 2018). Mussel conditioned artificial seawater although prepared and used in the same way each time may have the potential to vary in concentration strength and even chemical composition between trials. Solutions were thoroughly mixed/shaken and homogenized before use, limiting the potential of this occurring. Alongside analysing drop in oxygen concentration within trials and respiration rates, additional experiments including the analysis of antennule flicking by individuals would have benefitted the findings of this study. Behavioural responses have been described as reliable methods for quantifying olfactory behaviour in hermit crabs (de la Haye et al., 2011; Kim et al., 2016).

Overall, results show that reduction in pH does have some effect on the ability of *P. bernhardus* individuals to detect and respond to chemical foraging cues via olfaction. Although, significant individual variability exists, highlighted in Figure 7. The average minimum concentration of chemical foraging cue glutathione required to induce a greater drop in oxygen concentration and increased respiration rate was found to be at 10^{-7} M/1 for pH 8.15 and 10^{-8} M/l for pH 7.7. Significantly greater concentrations of 10^{-6}M/l were required at pH 7.2. Many individuals did not demonstrate any response at pH 7.2. Similar to existing literature regarding hermit crab ability to assess quality of shells (de la Haye et al., 2011). Whereby, during exposure to reduced pH individuals failed to exchange and investigate the presence of optimal shells. Leading to individuals occupying less favourable shells, which posed limited amount of protection (de la Haye et al., 2011).

Differences in response to low pH conditions within and between populations of the same species is an important factor to consider when analysing potential impacts of future OA conditions. Experimental design must reflect this in such investigations (Ashur et al., 2017; White & Briffa,

2017). More research is needed to gain specific knowledge of how reduced pH conditions impact chemicals used by organisms as part of their everyday functioning (Roggatz et al., 2019). Laboratory experiments that incorporate; natural variation and fluctuation of environmental conditions and of multiple stressors to better replicate real life environmental context need to be considered (Ashur et al., 2017; Jarrold et al., 2017). Sample individuals collected from varying locations, to determine if they have acclimatised to settings of different environments and how this impacts behavioural response, would also be enlightening (Melzner et al., 2020). Future studies must also incorporate work in the field as there is a lack of this in current literature.

Chapter Four: Biological Assay Exploring Individual Response to Ocean Acidification

4.1 Introduction

As the rate of OA progresses, there is a need to further understand the effect it imposes upon marine organisms. The response to OA by marine organisms is currently at the forefront of research. Response to such conditions has been documented to vary widely between differing species (Clements & Hunt, 2015; Kroeker et al., 2013). Recently research has begun to investigate differences in response between populations of the same species and between individuals of the same population (Gutowska et al., 2010; White & Briffa, 2017). Differences in response could potentially be the result of: abilities to acclimate, act plastically, adapt to novel environments and the existence of personalities (Briffa et al., 2008; Briffa et al., 2013; Donelson et al., 2019; Munday, 2014; Schunter et al., 2018; Thomsen et al., 2017).

Identifying and quantifying differences in response to OA by marine organisms can help in our understanding of the future impacts of such environmental change. This may also lead to the generation of appropriate mitigations of negative implications. Coastal species prove a useful tool to explore possibilities of acclimation, plasticity, adaptation and personality, as they are subject to rapidly changing environments naturally (Carstensen & Duarte, 2019; Garcia et al., 2020). Organisms may therefore have undergone such coping mechanisms already (Jarrold et al., 2017, Jarrold & Munday, 2019; Kwiatkowski & Orr, 2018; Landschützer et al., 2018). There is a limited quantity of data exploring such matters and so the focus of Chapter Four is to acknowledge and make contributions to this research area.

Chapter Four is the second of two data chapters, building upon techniques detailed within general methodology Chapter Two and results of data Chapter Three. Results of Chapter Three highlighted differences which exist between *P. bernhardus* individual responses to subjection of OA related conditions. Chapter Four therefore strives to explore this further, regarding foraging response to the addition of foraging cues within trials and differences displayed between *P. bernhardus* individuals. This will be achieved with use of alternate behavioural assay setting and observation. Again, two foraging cues and a control addition have been selected for investigations. These are the chemical foraging cue GSH (of particular concentration determined by Chapter Three results), mussel conditioned artificial seawater and control addition artificial seawater. Upon observations of behavioural response to trial conditions and additions, the: frequency, time taken by individuals and the differences between them with regards to foraging, will be investigated. As previously stated,

the weight of individuals will also be considered as studies have found size of marine organisms to impact foraging behaviour (Hayden et al., 2007; Laidre & Elwood, 2008; Lancaster, 1988).

4.2 Aims and Objectives

The aim of this data chapter is to investigate the impact of reduced pH conditions on the behavioral response of *P. bernhardus* individuals when presented with a food source in the form of a chemical foraging cue. Particular interest will be taken in the variation of behavioural response (e.g. frequency, time and level). It is important to investigate, not only, the average response of the population but, additionally, the variation between individuals (Laubenstein et al., 2019). Behavioural assays conducted at pH 8.15, 7.7 and 7.2 will attempt to identify such differences, in addition to determining if the detection threshold concentration for chemical foraging cue of individuals, analysed in Chapter Three, influences behavioural assay outcomes within trials incorporating paired individuals. Overall, investigations will aim to more clearly understand the potential impacts of future OA conditions upon foraging response of *P. bernhardus* individuals and the variation of affects experienced across individuals of a population.

4.3 Hypotheses

2a i) Individuals will engage more frequently with the presence of chemical foraging cue GSH and mussel conditioned artificial seawater compared to the addition of artificial seawater. Such level of response, will reduce with reduction in pH.

2a ii) Individuals will display the same level of response unique to them across trials with the same test conditions (pH and environmental addition).

2b) Individuals with lower detection threshold concentrations for GSH will display greater frequency of engagement responses, with the addition of GSH and mussel conditioned artificial seawater to trial environments.

2c i) *P. bernhardus* individuals with the lowest detection threshold concentration for GSH will; reach, make contact with and arrive at the source of the cue, before trial partners with higher detection threshold concentration.

2c ii) Reduction in pH will influence such results.

2c iii) In trial pairings, whereby individuals have the same detection threshold concentration, bolder individuals will make contact with the cue source first.

2d) Heavier and therefore larger *P. bernhardus* individuals will make contact with the cue source first before smaller trial partners.

2e) The time taken by individuals to initially move towards the source of chemical foraging cue GSH and display engagement response, with the cue source will increase with reduced pH.

4.4 Methods

The set-up of the behavioural assay for Chapter Four included a partly partitioned chamber illustrated by Figure 11, constructed with use of beige grey polycarbonate 0.6 cm thick sheets. Purchased from a local plastics fabrication company - Kingston Plastics. Dimensions of such chamber were as follows: 35.0 cm x 22.0 cm x 15.0 cm, with inside partition of 27.0 cm x 10.0 cm. For each trial the chamber was filled with 2000ml of artificial seawater (measuring 35 PSU salinity and a temperature of 19 ± 0.5°C, both determined as stated in Chapter 2 – General Methodology), Artificial seawater was prepared the day of experimental trials. Dependent on trial, pH was adjusted accordingly by bubbling CO_2 through the water using a pressurised canister and recorded with hand-held pH meter (Fisherbrand™ accumet™ AB150 pH Benchtop Meter). pH levels attained and used within trials include 8.15, 7.7 and 7.2. Laboratory lights were dimmed in attempts to avoid visible shadows within the chamber however sufficiently bright to observe trials and simulate daylight. Laboratory temperature was controlled and set to 19°C using thermostat. Trials took place in 3 stages for each pH condition and with 3 rest days in-between. Individuals were starved for two days prior to trials in an attempt to standardise hunger levels.

Due to an unfortunate amount of *P. bernhardus* deaths in the time period between the end of Chapter Three experimental trials and the start of Chapter Four experimental trials, only 8 individuals with known threshold concentrations were available to subjection of Chapter Four behavioural assays. Trialing individuals from the same collection dismisses possibilities of variation in results due to alternative sample collections. For each pH condition (8.15, 7.7 and 7.2) a total of 60 trials were completed each with two randomly allocated *P. bernhardus* individuals. 20 out of 60 trials incorporated the addition of chemical foraging cue GSH to filter paper. A further 20 trials incorporated the addition of mussel conditioned artificial seawater to filter paper. The remaining 20 trials incorporated the addition of artificial seawater to filter paper. This resulted in the subjection of each *P. bernhardus* individual to five trials at each pH condition per filter paper addition. Each trial

of the same test conditions was randomly allocated differing *P. bernhardus* pairings. 8 crabs were available for Chapter Four experiments therefore, individuals were used multiple times within trials. Repeated use of a small sample size may result in limitations amongst statistical results, such as not allowing for non-independence. However, organisms will repeatedly react to the presence of food. Additionally, individuals were rested between trials, and were not subjected to trials consecutively, with rest breaks of 30 minutes. Trial condition order also varied.

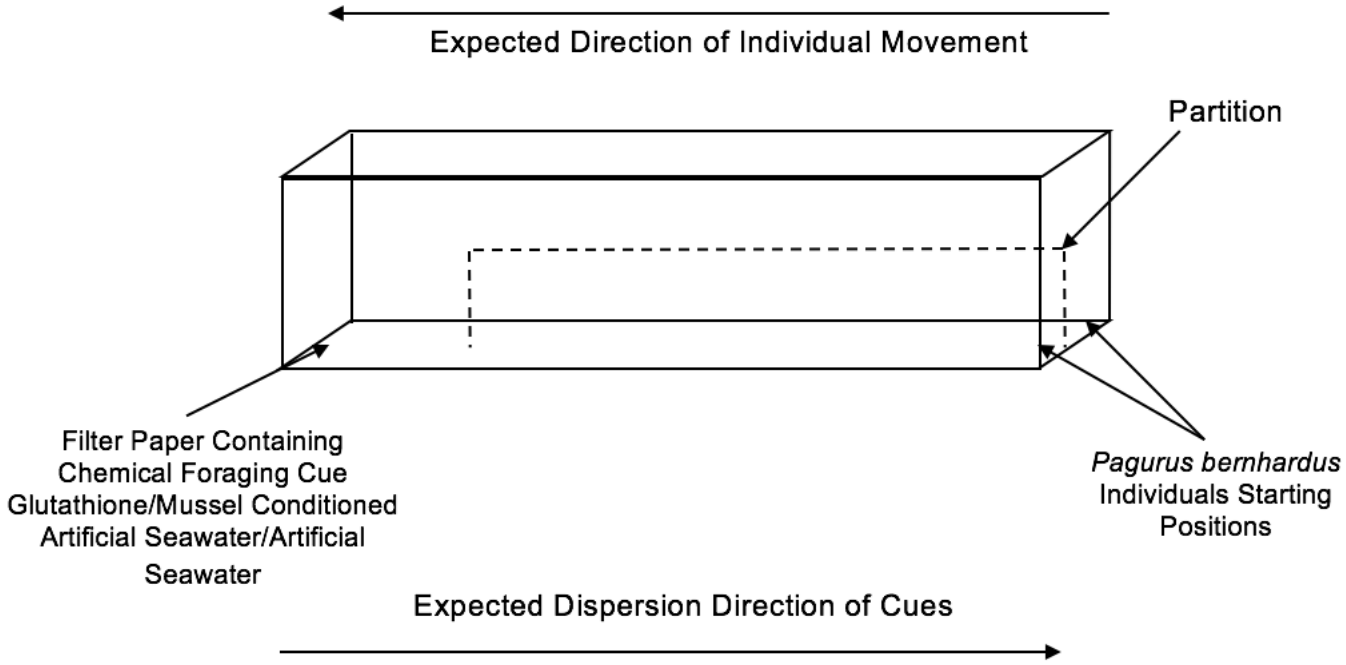

Figure11: Behavioural assay design for Chapter Four observations.

Once 200ml of artificial seawater was added to the chamber, rectangular segments of filter paper (Whatman Filter NO. 3 Qualitative Circles, 70mm) were cut, measuring 2.0 cm x 4.0 cm for each behavioural assay trial. One segment of filter paper was placed at the far end of the chamber centrally, as seen in Figure 11, per trial. Before the addition of filter paper segment to the chamber, 1.0ml of either: artificial seawater, mussel conditioned artificial seawater or GSH was dispensed onto the respective segment for absorption, via 1.0ml syringe. GSH of concentration 10^{-5}M/l, was chosen for the addition amongst trials of Chapter Four in light of previous results determined in Chapter Three. Regarding the minimum threshold concentration required to initiate behavioural response at pH 8.15 for *P. bernhardus* individuals. The cue concentration selected was detected by most individuals. Indicating individuals would respond to its addition within the environment. However such concentration appears lower than the detection threshold concentration required by some individuals and would therefore prove difficult to detect by others.

Filter paper, with appropriate cue addition, was submerged quickly at the start of each trial to avoid untimely dispersion and water disturbance within the trial environment. Once added to the chamber, a period of 60 seconds was granted to allow the formation of an odour signal trail within the environment. Such time period was deemed sufficient to form signal odour trails. Preliminary odour trials involved timely observation of the addition of food colourant from filter paper. Taking note of dispersion within the chamber. This was also substantially longer than the 30 second time period allocated in similar experimental trials by Roggatz et al. (2019). Also, important to note the chamber was a static system, as determined in preliminary trials natural dispersion of cues and corresponding response by individuals sufficed. This system further, replicated the mostly static system of rock pools, which is why such procedure was also executed in experiments by de la Haye et al. (2011).

Simultaneously within this 60 second period two *P. bernhardus* individuals (randomly selected using a random number generator) were placed into separate acclimation baths (container 12 cm x 8 cm) containing 100 ml of artificial seawater, pH relevant to trial conditions. Individuals were placed in such pH baths to allow short term acclimation and reduce the potential of environmentally enhanced shock. After this period, individuals were collected and gently inverted, resulting in the retraction of individuals within shells. Individuals were then placed at the end of the assay chamber, as demonstrated by Figure 11. Individuals were gently placed with their aperture facing downwards, either side of the chamber partition. Originally, methods considered use of shelters placed over individuals in the chamber. Whereby the removal of shelters would release individuals into the experiment. However, preliminary trials determined that this approach caused significant disturbance of the water body.

Once individuals were placed in to the trial environment, the timing of the trial begun. During trials the response displayed by individuals was observed and recorded using SONY Handycam HDR-CX405 Camcorder. Trials ran for a total of three minutes. Preliminary trials established individuals engaged with one another typically longer than three minutes. Reasoning experiments to be terminated after this time period. After each trial, equipment was rinsed thoroughly, in preparation of refill with artificial seawater of relevant pH and appropriate cues. Upon chemoreception of foraging cues, individuals initially displayed ‘searching’ behaviour and oriented towards the source of the cue (Derby et al., 2016). Proceeding this, individuals displayed varied behavioural response. Figure 12 demonstrates the expected outcomes.

The response of individuals, following initiation, was grouped into three levels. The first level was categorised as ‘no interaction’. Which occurred in the event individuals did not purposefully travel towards or make contact with the filter paper placed at the opposite end of the chamber. The second

level of response was categorised as ‘walked over’, which saw individuals travel towards the filter paper and proceed to walk or hover over its location. Such contact was noted if occurrence lasted for a period of time longer than five seconds. The final level was categorised as ‘Interaction’. Whereby individuals made attempts to pick up or consume the filter paper. Both ‘walked over’ and ‘interaction’ level responses are later combined and defined by the inclusive term ‘engagement response’. The individual out of the pairing, to arrive at the cue source was recorded. Additionally, time taken by individuals to display initial searching behaviour and make contact with the filter paper was also recorded.

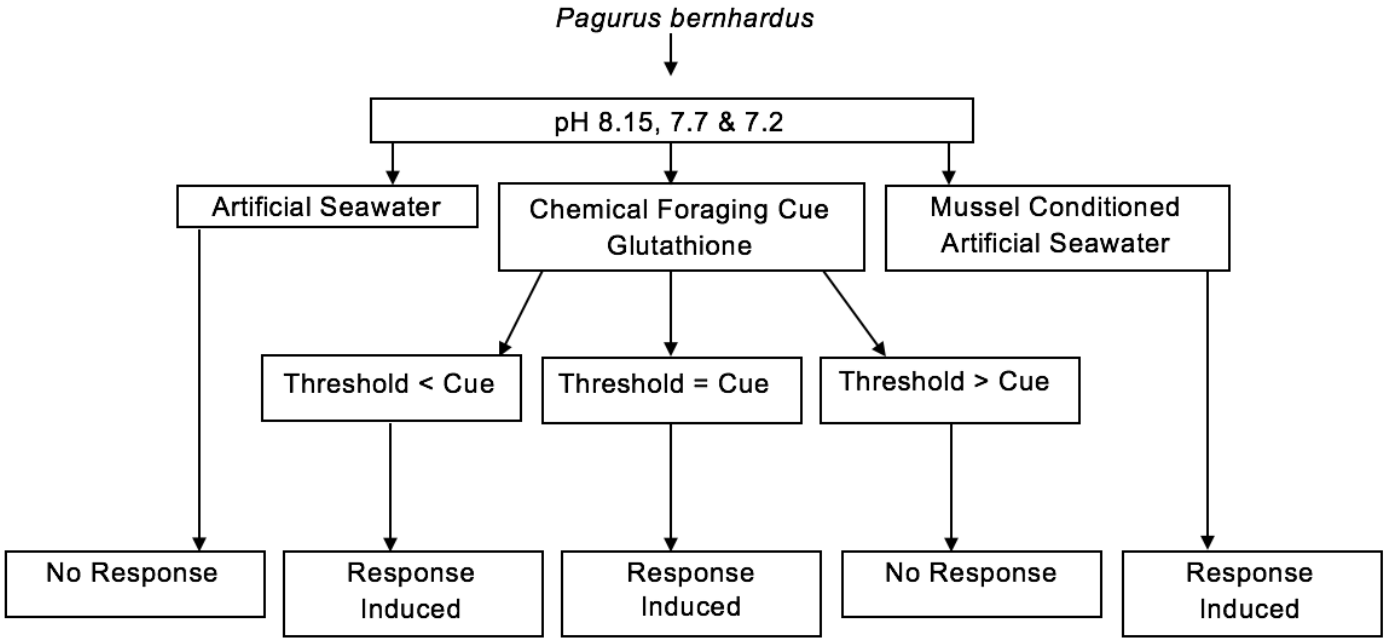

Figure 12: Expected outcomes for Chapter Four behavioural assays.

4.5 Statistical Methods

Impact of Olfactory Cues, pH and Identifying Individual Response

Firstly, to answer hypotheses 2a i) and 2a ii) the frequency of engagement response was calculated. This was defined as the total number of times individuals displayed either: ‘walked over’ or ‘interaction’ responses. Whereby individuals were seen to actively make contact with the presence of filter paper within their environment. One-Way ANOVA statistical test was considered to be the most appropriate statistical test to perform. Such test was used to determine if variation in: environmental cue addition or pH effected mean frequency of engagement response by individuals as a population. Levene’s test of homogeneity and Shapiro-Wilks normality test were further used to determine if assumptions of the One-Way ANOVA were met. However, upon application of the

Shapiro-Wilks normality test, data was non-normally distributed after log transformation. Such findings were confirmed by QQ plots of distribution. The non-parametric statistical equivalent, Kruskal-Wallis was therefore applied to data sets regarding cue addition and pH. Following significant results, further analysis was conducted in the form of Pairwise Wilcoxon test to determine which factors such significance lied between. Statistical analysis incorporated use of programming software R (3.6.0), as seen in appendix.

Detection Threshold Concentration of Olfactory Cues and Response Frequency

To determine if correlation existed between the detection threshold concentration for chemical foraging cue GSH, calculated in Chapter Three at pH 8.15, and the frequency of engagement response, linear regression analysis was conducted. This was applied to Chapter Four behavioural assays incorporating the addition of chemical foraging cue GSH and mussel conditioned artificial seawater. Shapiro-Wilks normality test was used to determine the distribution of data. Further confirmed by QQ plots of distribution. Non-normally distributed data underwent log transformation to create normal distribution. Coding routine as seen in appendix.

Detection Threshold Concentration of Olfactory Cues and Impact of Reduced pH

To answer hypotheses 2c) data was gathered for each pH level (8.15, 7.7 and 7.2) investigated amongst trials, giving a total of three data sets. For each data set, data included the frequency of trials, in which at least one individual made contact with and arrived at the GSH cue. Further, for each trial the individual to arrive first at the source of cue was identified. The detection threshold concentrations for both individuals within that trial was recorded and compared. Amongst trials whereby individuals of the pair had the same detection threshold concentration for chemical foraging cue GSH this was recorded. Whilst also identifying which individual engaged with the source of chemical foraging cue GSH first before their trial partner.

To determine if individuals with the lower detection threshold concentration, for chemical foraging cue GSH, engaged with the cue source before trial partner at each pH, unpaired-t-tests were most appropriate. However, Shapiro-Wilks test and QQ plots of normality, determined data to be non-normally distributed after log transformation. The non-parametric test equivalent Wilcoxon-Mann-Whitney was therefore applied with use of the statistical programme R (version 3.6.0) and additional R packages (coding as seen in appendix). Levene's test of variance was additionally used to determine if assumption of homogeneity in variance were met.

The Influence of Weight upon Behavioural Assay Response Outcomes

For each behavioural assay with the addition of chemical foraging cue GSH the following was recorded and subsequently categorised:

1) Individuals which reached and interacted with the cue source before trial partners;
2) Trials whereby neither individual of the pair displayed engagement response to the cue source.

The weights of 7 from 8 trial individuals was known. To distinguish if the weight of individuals influenced trial outcome the Wilcoxon-Mann-Whitney statistical test was applied to data attained at all pH levels (8.15, 7.7 and 7.2). Sample sizes were small, therefore analysis at each pH level separately was not appropriate. Shapiro-Wilks test and QQ plots of normality were used to determine distribution of data.. Levene's test of variance was again additionally run to determine if data met the assumptions of homogeneity in variance. As before, all statistical analysis was performed utilising the statistical programme R (version 3.6.0) and additional R packages. Coding routines were as seen in appendix.

Impact of Reduced pH on Response Time to the Presence of Olfactory Cues

To best approach hypothesis 2e) data was split into two categories for corresponding analysis. Application of One-Way ANOVA was used to determine the impact of pH on time taken by individuals to both: initially move and make contact with the cue source. Shapiro-Wilks normality test and QQ plots of normality were used to distinguish the distribution of both data-sets. Non-normally distributed data was log transformed to create normal distribution. Further, Levene's test of variance was used to determine if assumptions of One-Way ANOVA were met. Tukey multiple comparisons of means, 95% family-wise confidence level post hoc tests were used for significant results to determine the levels in which such significance existed.

4.6 Results

Impact of Olfactory Cues, pH and Identifying Individual Response

Figures 13, 14 and 15 demonstrate great variance in the level of response between individuals and reduction in pH. For trials with the addition of chemical foraging cue GSH and mussel conditioned artificial seawater, the total frequency of engagement responses displayed by individuals decreased with pH. From 34 occurrences at pH 8.15, to 24 at pH 7.7 and 20 at pH 7.2. Figures also indicate individuals dominantly displayed 'no interaction' level response with the addition of artificial

seawater. Such response was consistent with reduction in pH. Figure 16, represents the average response of the population and how such response varied with pH level (8.15, 7.7 and 7.2) and cue addition. Additionally, Figure 16 shows as a population, individuals engaged less frequently with the addition of artificial seawater and more frequently with the addition of mussel conditioned artificial seawater. Response to mussel conditioned artificial seawater also decreased with reduced pH.

Shapiro-Wilks normality test determined engagement response data to be of non-normal distribution ($P = 7.01 \times 10^{-7}$). Data was non-normally distributed after log transformation also, confirmed via QQ plots of distribution. Levene's test of homogeneity determined data met One-Way ANOVA assumption of homogeneity of variance. $F_2 = 1.46$, $P = 0.24$ for data with variation in cue addition. $F_2 = 1.12$, $P = 0.33$for data with reduction in pH. Kruskal-Wallis statistical test was applied to both data sets. Upon application of Kruskal-Wallis and Pairwise Wilcoxon statistical test, the addition of mussel conditioned artificial seawater was found to result in increased engagement response frequency compared to the addition of artificial seawater ($X^2_{2,8} = 11.82$, $P = 0.0027$ and $P=0.0033$, respectively). pH level had no impact on frequency of engagement response displayed by individuals ($X^2_{2,8} = 5.033$, $P = 0.081$)).

This leads to the partial acceptance of hypotheses 2ai), individuals did display engagement response more frequently, with the addition of mussel conditioned artificial seawater compared to the addition of artificial seawater. However, hypothesis 2aii) must be rejected. Although, during observation of trials individuals did not engage with cues as frequently in pH conditions 7.2 and 7.7 compared to pH 8.15, there was no statistical significance found.

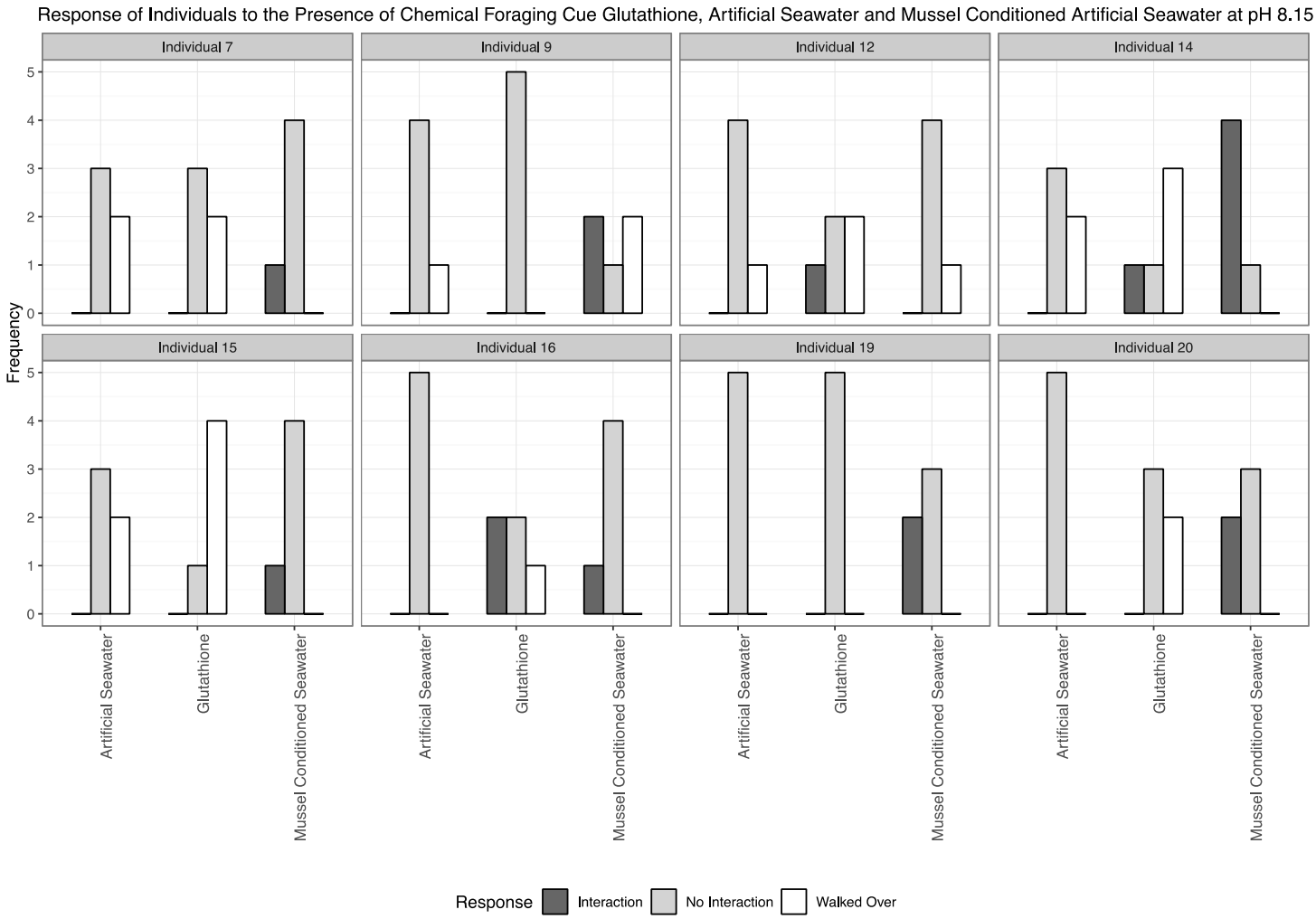

Figure 13: Variation in response by *P. bernhardus* individuals with addition of chemical foraging cue GSH, artificial seawater and mussel conditioned artificial seawater at pH 8.15. Response is categorised as: interaction, no interaction and walked over.

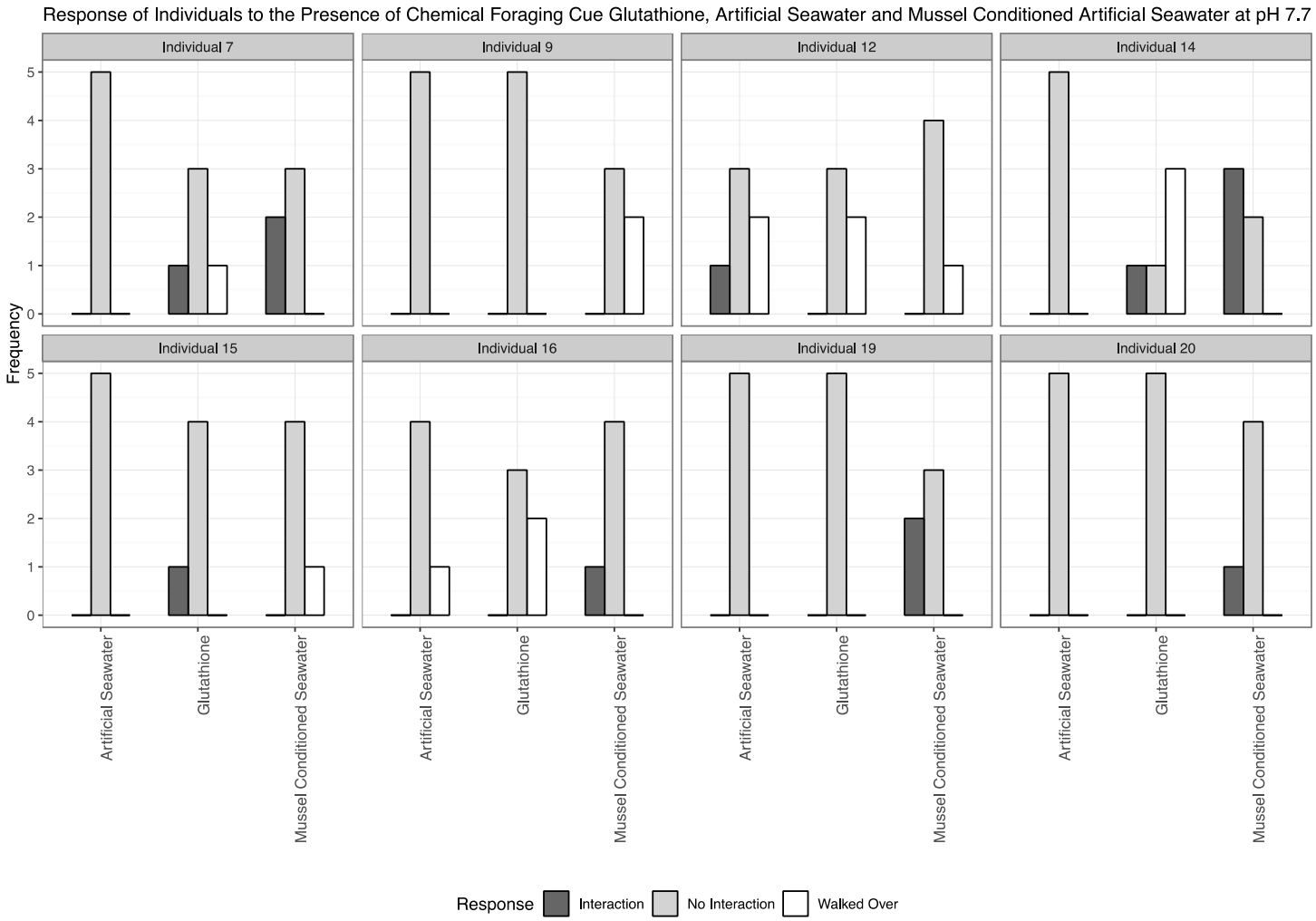

Figure 14: Variation in response by *P. bernhardus* individuals with addition of chemical foraging cue GSH, artificial seawater and mussel conditioned artificial seawater at pH 7.7. Response is categorised as: interaction, no interaction and walked over.

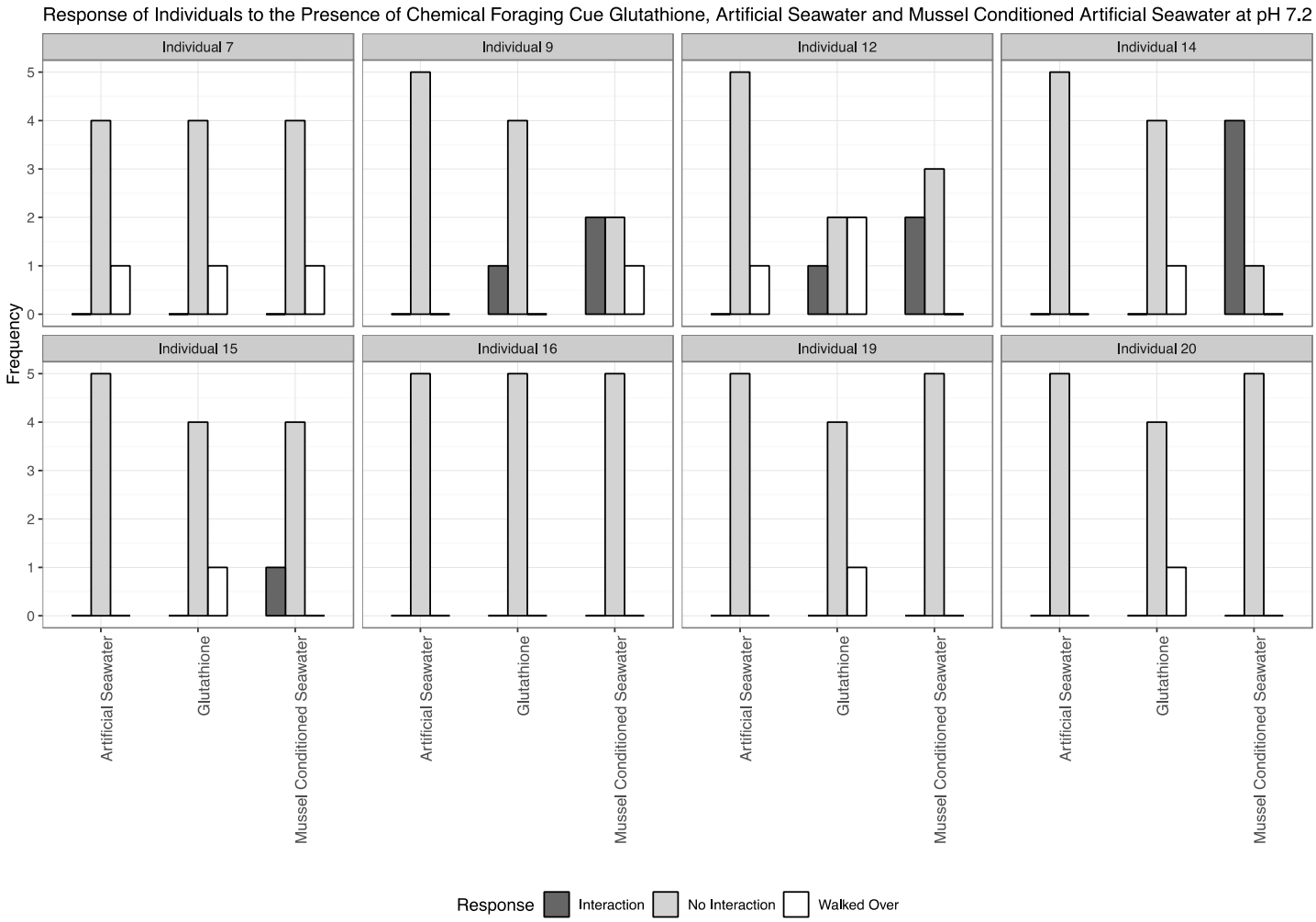

Figure 15: Variation in response by *P. bernhardus* individuals with addition of chemical foraging cue GSH, artificial seawater and mussel conditioned artificial seawater at pH 7.2. Response is categorised as: interaction, no interaction and walked over.

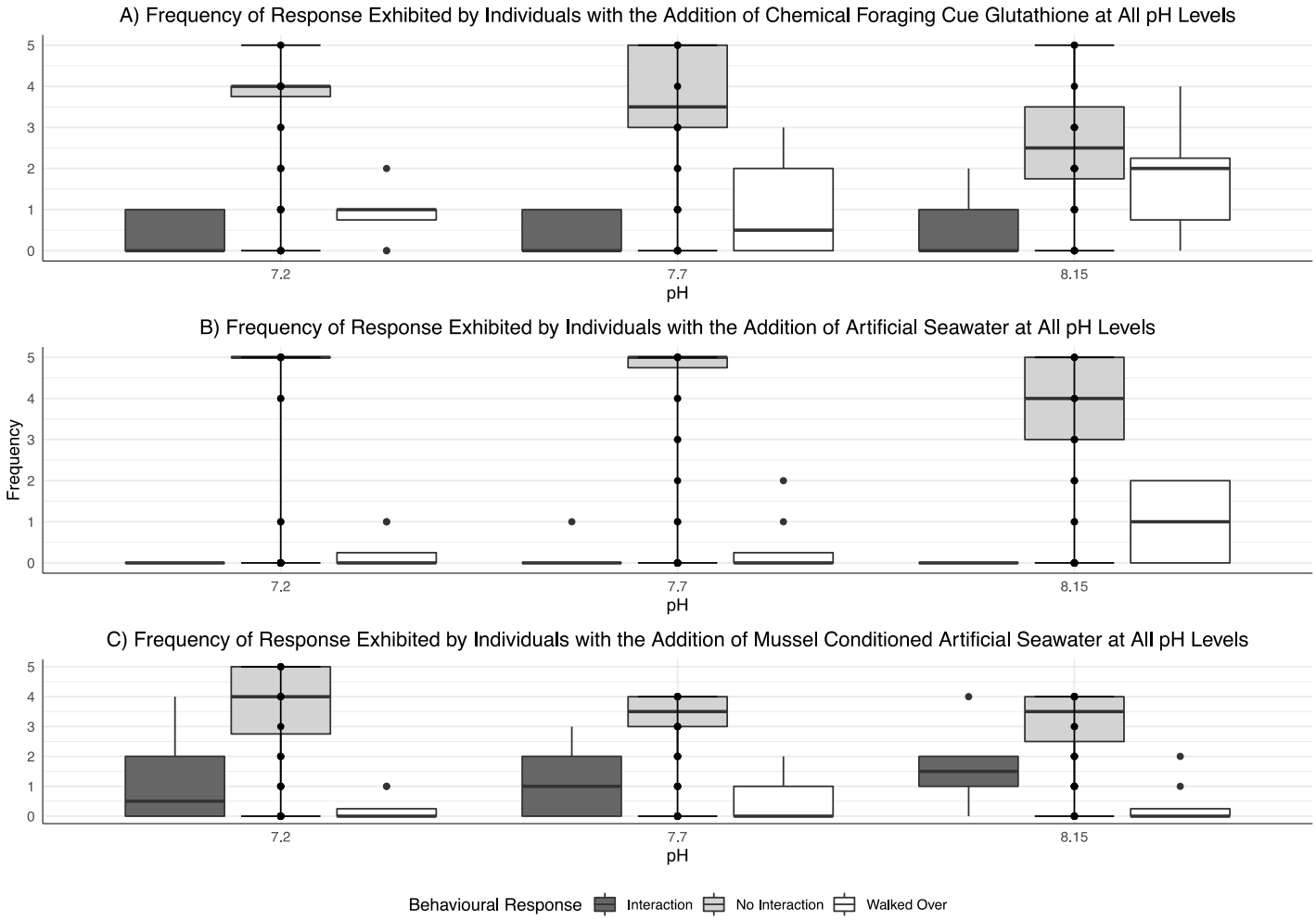

Figure 16: Frequency of response expressed by 8 *P. bernhardus* individuals at pH levels 8.15, 7.7 and 7.2. Response is categorised as: interaction, no interaction and walked over. Plot A) represents trials with addition of chemical foraging cue GSH, B) artificial seawater and C) mussel conditioned artificial seawater. Whiskers signify 95% confidence limits (Cis). Outliers are represented by singular points.

Detection Threshold Concentration of Olfactory Cues and Response Frequency

To determine if detection threshold concentrations impacted engagement response frequency, data was split into two groups. The first included data with the addition of chemical foraging cue GSH at pH 8.15. The second included data with the addition of mussel conditioned artificial seawater at pH 8.15. Shapiro-Wilks normality test determined data with the addition of GSH to be of normal distribution ($P = 0.18$). Further confirmed by QQ plots of distribution. Data regarding mussel conditioned artificial seawater was non-normally distributed and therefore log transformed ($P = 0.0077$). Following this, linear regression analysis determined detection threshold concentration had no correlation to the frequency of engagement response, displayed by individuals with the addition of GSH ($F_6 = 0.25$, $P = 0.633$). Similarly, no correlation was found between detection threshold concentration for chemical foraging cue GSH and frequency of engagement response by individuals with the addition of mussel conditioned artificial seawater ($F_6 = 0.89$, $P = 0.38$).

Figure 17 highlights such results. Combining graphical evidence with results of statistical tests, suggests that individuals with lower detection threshold concentrations for chemical foraging cue GSH did not appear to be, more sensitive to the addition of chemical foraging cue GSH or mussel conditioned artificial seawater. As frequency of engagement response did not correlate with detection threshold concentrations, leading to the rejection of hypothesis 2b).

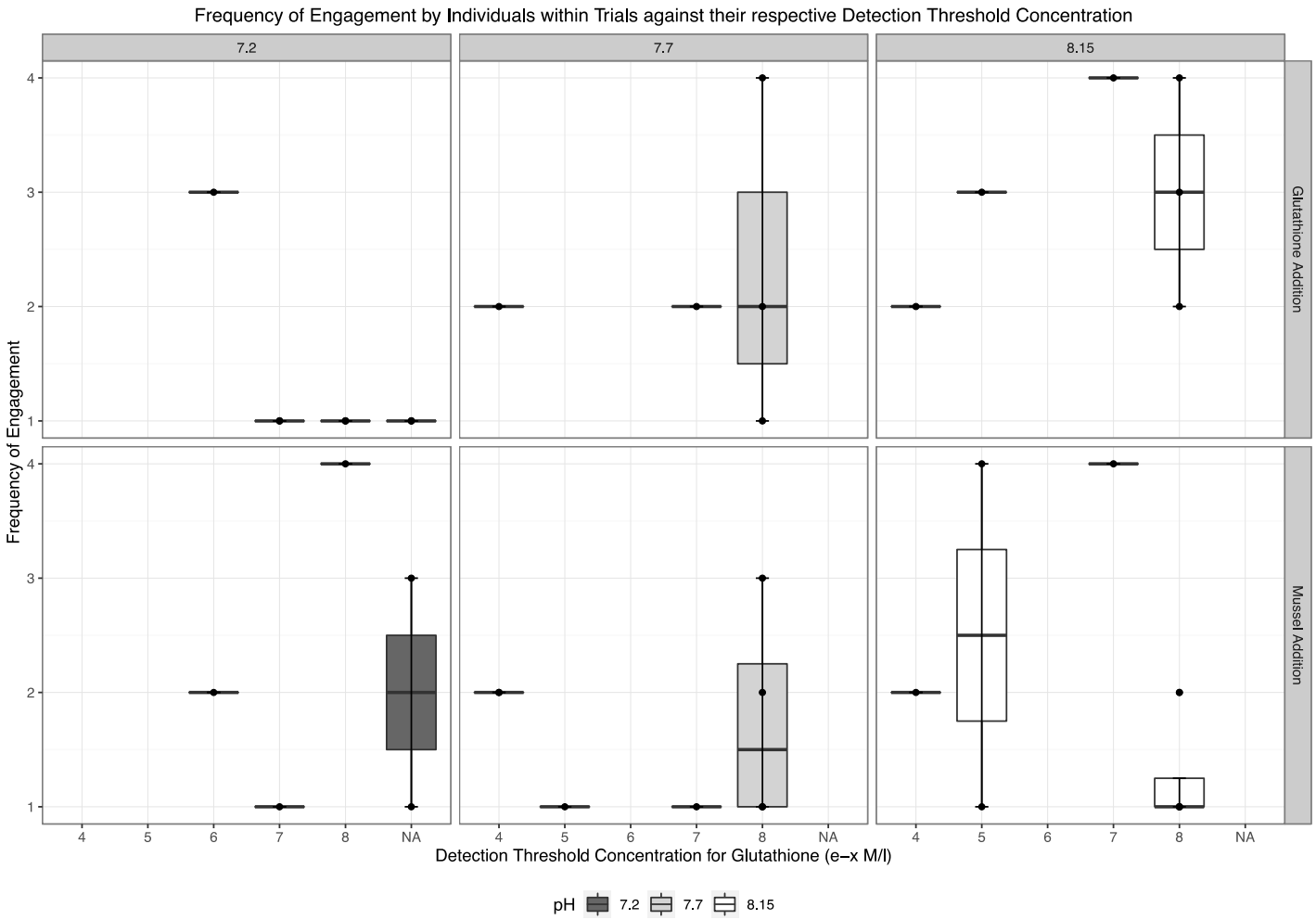

Figure 17: Frequency of engagement response (interaction or walked over) expressed by 8 *P. bernhardus* individuals at pH levels 8.15, 7.7 and 7.2 in accordance to detection threshold concentrations for GSH (i.e. 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}M/l). NA represents circumstance whereby, individuals engaged within Chapter Four experimental trials but detection threshold concentration was undetermined. For additions: GSH (top) and mussel conditioned artificial seawater (bottom). Whiskers signify 95% confidence limits (Cis). Outliers are represented by singular points.

Detection Threshold Concentration of Olfactory Cues and Impact of Reduced pH

Wilcoxon-Mann-Whitney statistical test determined detection threshold concentration of individuals had no influence on which trial individual reached the cue source GSH first, before trial partners at pH 8.15 ($P = 0.9025$). Shapiro-Wilks test and QQ plots of normality determined such data to be of non-normal distribution ($P = 0.00042$). Data met assumptions of homogeneity of variance confirmed by Levene's test of variance ($F_{1, 7} = 0.81$, $P = 0.40$). Similarly, Wilcoxon-Mann-Whitney

statistical test determined no influence found amongst results for pH levels 7.7 (P = 0.26) and 7.2 (P = 0.25). Again data was non-normally distributed for pH levels 7.7 (P = $2.1x10^{-5}$) and 7.2 (P = 0.0014). Levene's test of variance established assumptions of homogeneity of variance. Results of the three data sets can be seen in Figures 18 and 19. Table 2 highlights the outcomes of trials whereby paired individuals had the same detection threshold concentration for chemical foraging cue GSH. Such results can only suggest the individual which engaged with the cue source first before their trial partner was random. Overall this leads to the rejection of hypotheses 2c.

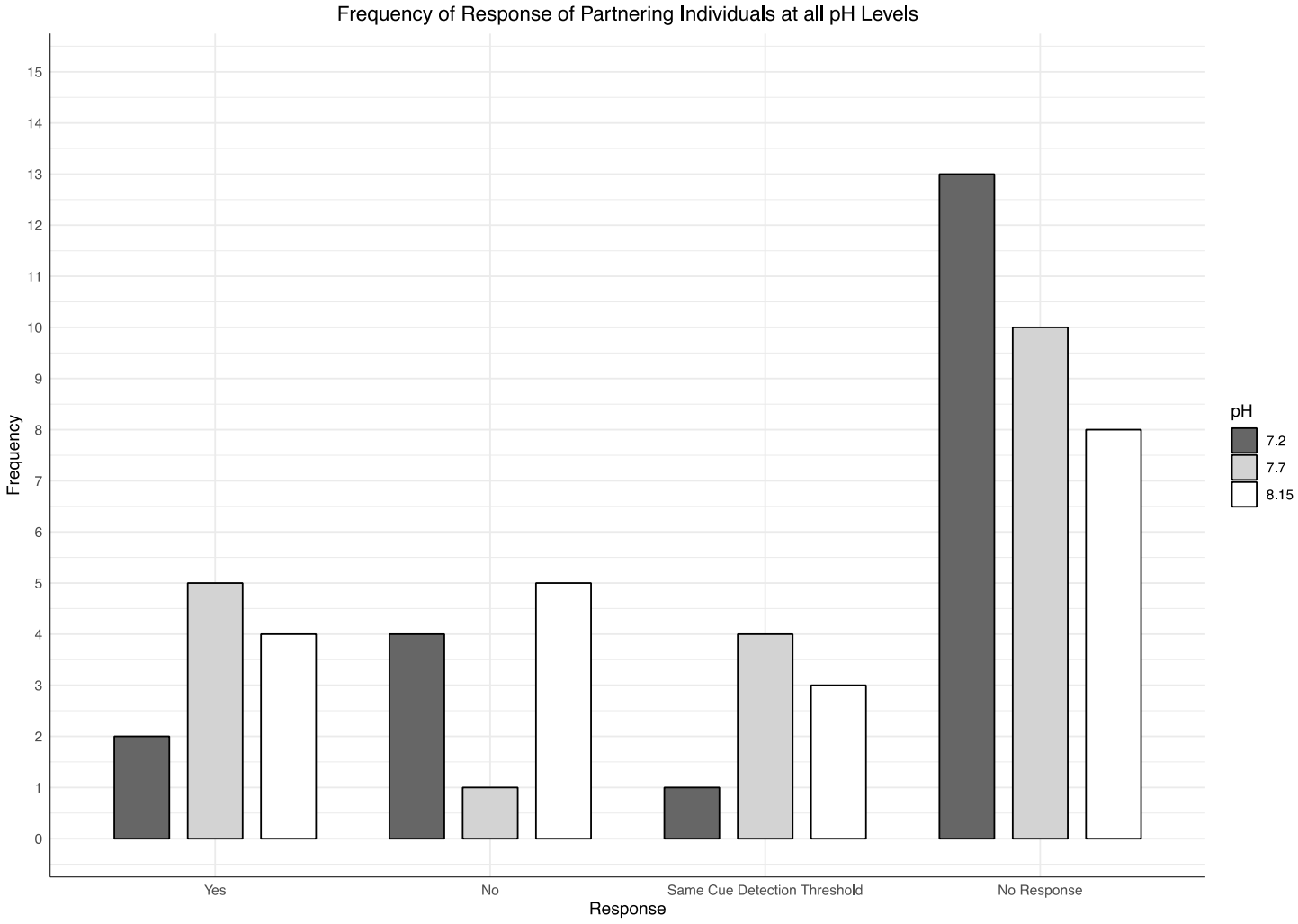

Figure 18: *P. bernhardus* pair response frequency of trials observed with the addition of chemical foraging cue GSH at all pH levels. Responses are categorised by: yes, no, same cue detection threshold and no response. Yes category represents trials whereby the individual with lowest threshold for GSH engaged with the cue source before its trial partner of higher threshold. No category, represents trials whereby individuals with higher thresholds engaged with cue source first.

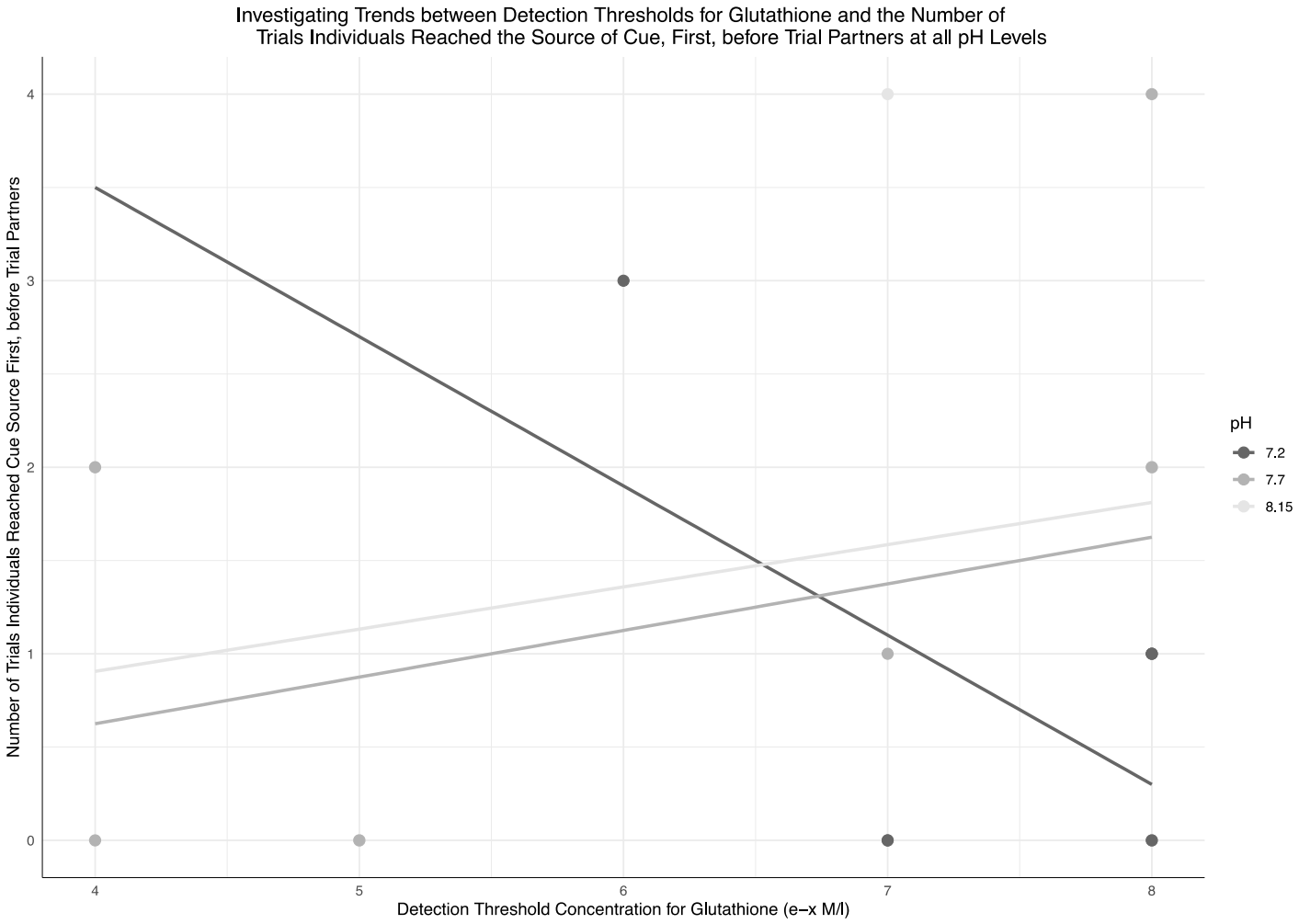

Figure 19: Detection threshold concentration for chemical foraging cue GSH and the frequency of trials individuals displayed engagement response, before their trial partner.

	Competitive Trial Pairings		
pH	**Hermit Individual A**	**Hermit Individual B**	**First Individual to Arrive and Engage with Cue Source**
8.15	7	19	7
8.15	15	7	15
8.15	7	16	16
7.7	14	19	14
7.7	14	16	14
7.7	7	9	7
7.7	15	14	14
7.2	14	16	14

Table 2: Outcome of trials whereby trial pairs had the same detection threshold concentration for chemical foraging cue GSH.

The Influence of Weight upon Behavioural Assay Response Outcomes

Data regarding the weight of individuals and trial outcome was determined to be of non-normal distribution by Shapiro-Wilks test and QQ plots of normality ($P = 2.57 \times 10^{-5}$). Levene's test of found data to meet assumptions of homogeneity in variance ($F_{1, 14} = 0.15$, $P = 0.70$). Application of Wilcoxon-Mann-Whitney non-parametric statistical test for data across all pH levels, determined there to be no influence by weight of individuals on trial outcome ($P = 0.87$). Hypothesis 2d) stating heavier and larger individuals of trial pairings are more likely to reach the source of chemical foraging cue GSH, first before lighter and smaller trial partners, must therefore be rejected. Figure 20 demonstrates such findings and includes the frequency of trials both individuals failed to display engagement response. This clearly increased in frequency with reduced pH level.

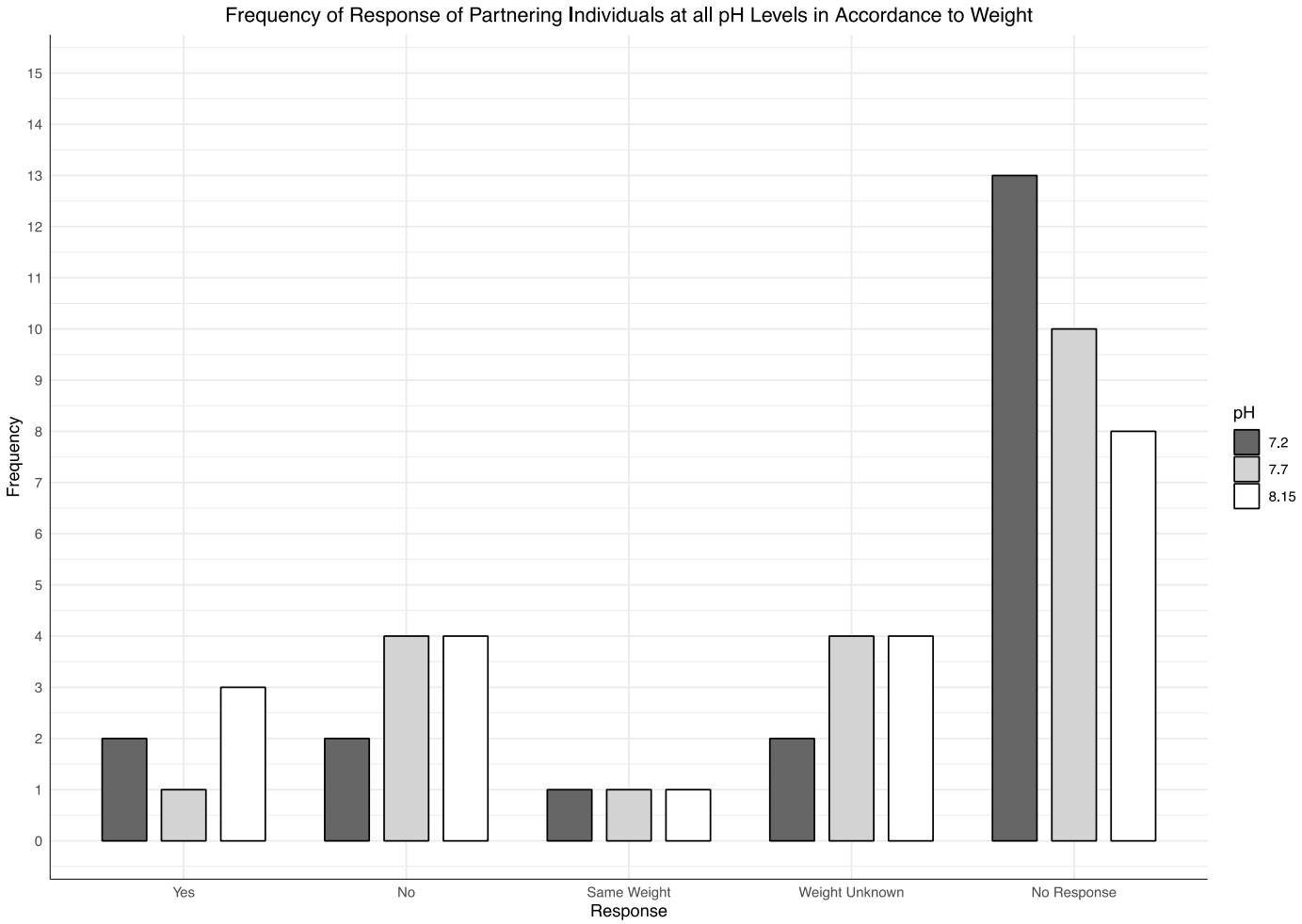

Figure 20: P. bernhardus pair response frequency of trials observed with the addition of chemical foraging cue GSH at all pH levels. Responses are categorised as: yes, no, same weight, weight unknown and no response. Yes category represents trials whereby the heaviest individual engaged with the cue source before its lighter trial partner. No category, represents trials whereby lighter individuals engaged with cue source first.

Impact of Reduced pH on Response Time to the Presence of Olfactory Cues

One-Way ANOVA was carried out to determine if pH impacted the time taken by individuals to initially move towards the direction of cue source. Such results established pH did impact such time ($F_{2, 35}$ = 6.14, P = 0.0052). Data was log transformed as Shapiro-Wilks normality proved original data to be of non-normal distribution (P = 0.025). Log transformed values were of normal distribution (P = 0.072). Levene's test of variance determined the One-Way ANOVA assumption of homogeneity in variance to be met ($F_{2, 35}$ = 1.43, P = 0.25). Further application of Tukey HSD found the time taken to initially respond increased with reduction in pH from 8.15 to 7.7 (P = 0.0059). No impact was found to exist for reduction in pH from 8.15 to 7.2 (P = 0.078) and 7.7 to 7.2 (P = 0.71). This can be seen in Figure 21 A) which demonstrates the average time taken for initial response equated to 18 seconds at pH 8.15, 52 seconds for pH 7.7 and 50 seconds for pH 7.2.

One-Way ANOVA determined reduced pH impacts the time taken by individuals to display engagement response with the cue source ($F_{2, 35}$ = 11.3, P = 0.00016). Again, data was log transformed as data was determined to be of non-normal distribution (P = 0.023). Log transformed data was normally distributed (P = 0.41). One-Way ANOVA of variance was met, confirmed by Levene's test of variance ($F_{2,35}$ = 1.71, P = 0.20). Further application of Tukey HSD post hoc statistical test suggests the reduction of pH from 8.15 to both 7.7 and 7.2 impacted the time taken for individuals to display engagement response with the cue source (P = 0.00027 and 0.0077, respectively). The reduction in pH from 7.7 to 7.2 had no impact (P= 0.68). As demonstrated by Figure 21 B). The average time taken by individuals to make contact with the cue source equated to 33 seconds at pH 8.15 followed by 92 and 78 seconds at pH 7.7 and 7.2, respectively. Results suggest that hypothesis 2e) can be partially accepted, as pH impacted time taken by individuals to initially respond and display engagement response. However, this effect was not evident across the reduction of all pH.

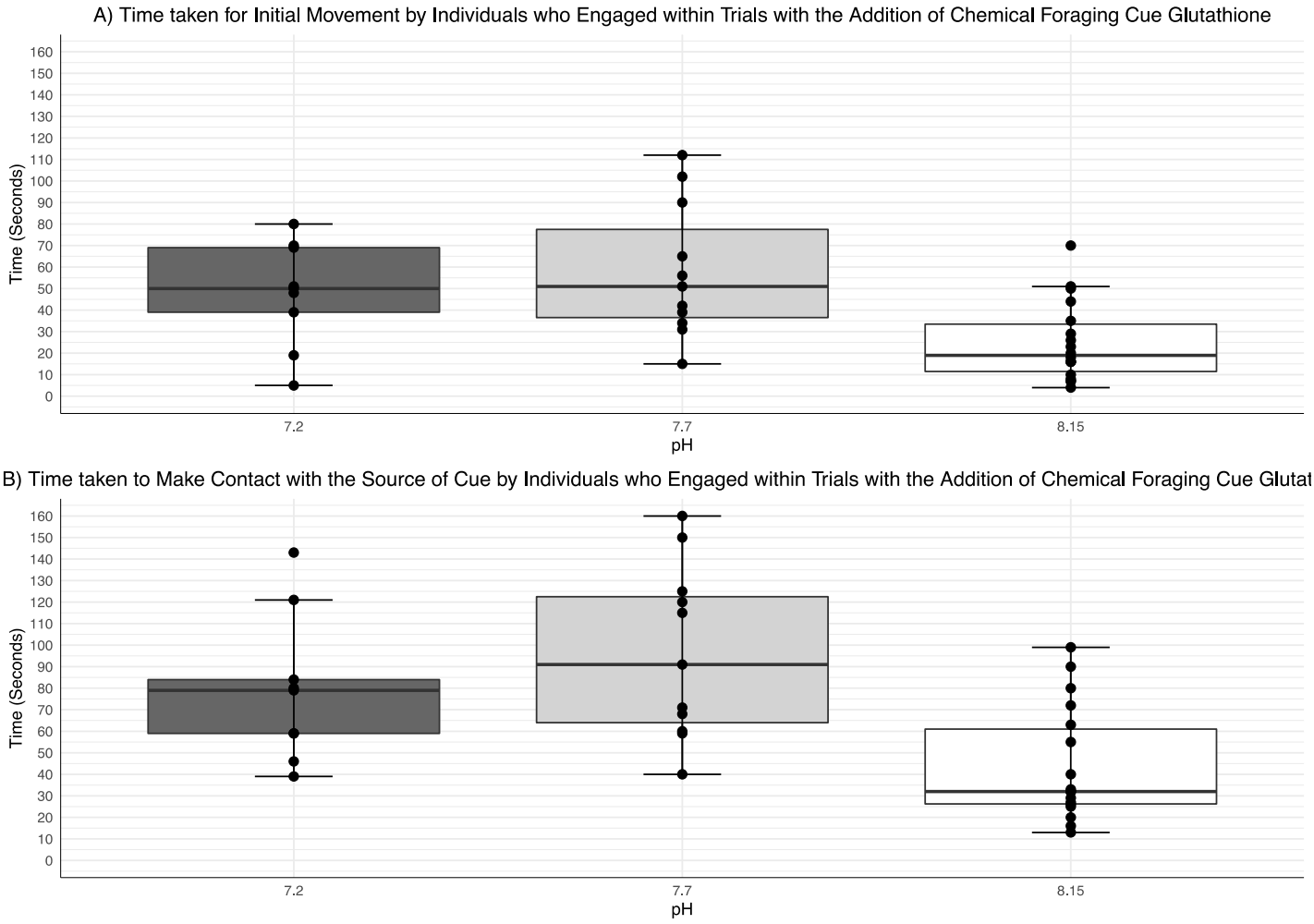

Figure 21: Impact of reduced pH upon A) the time (s) taken for initial response movement towards the source of chemical foraging cue GSH and B) the time taken for individuals to display engagement response with the cue source. Whiskers signify 95% confidence limits (Cis). Outliers are represented by singular points.

4.7 Discussion

Results reiterate findings of Chapter Three, confirming in context of foraging behaviour, there is great variation in response between *P. bernhardus* individuals, environmental addition and pH level. Frequency of engagement response was greater with the addition of mussel conditioned artificial seawater than the addition of chemical foraging cue GSH. As expected and discussed in Chapter Three, mussel conditioned artificial seawater likely contains a mixture of molecular components and so is less likely to be affected by reduction in pH. Unlike GSH molecules, which have the potential to drastically change under such conditions (Roggatz et al., 2016; Velez et al., 2019). Additionally, in accordance to analysis there appeared to be no impact of reduced pH, with the addition of GSH, upon the level of response displayed by individuals. However, during observation reduction in activity and locomotion of individuals appeared to increase with reduced pH. Small sample sizes in this instance, influenced result outcomes.

Chapter Four results additionally demonstrate the effect of reduced pH on the time taken for individuals to both initially respond and engage with the source of chemical foraging cue GSH. Reduction in pH resulted in increased time taken by individuals to display such responses.. Suggesting, individuals required a longer time period to successfully detect the presence of chemical foraging cue GSH. This can be proposed to occur due to the impact of low pH on the structure and binding affinity of chemical foraging cue GSH molecules and chemoreceptor peptides, as previously discussed within discussions of Chapter Three. Under reduced pH conditions seabream, *S. aurata,* required greater concentrations of some chemical odourants to enable their detection (Velez et al., 2019). Time-period to therefore detect the relevant chemosensory information is likely to increase. Results also suggest reduction in pH leads to increased time for individuals to display engagement response however, many factors may have the potential to contribute towards this outcome. For example, and not limited to: health or fitness of individuals, sex, size, hunger levels and motivation for food (Billock & Dunbar, 2009; Hayden et al., 2007; Laidre & Elwood, 2008). Results of Chapter Four analysis however, can rule out the proposed influence of weight or size on the outcome of trials.

During short-term exposure to reduced pH within behavioral assays, limited activity and foraging response displayed by *P. bernhardus* individuals, may have been a strategy to conserve energy. Exposure to stressful environments can result in energy expenditure for maintenance of acid-base regulatory processes (Wang et al., 2018). Metabolism rates have also been noted to be suppressed, as a short-term adaptive approach, by *Dosidicus gigas,* in times of hypoxia and hypercapnia (Fabry et al., 2008). Similar to behavioural assay observations, Dodd et al. (2015) found reduced foraging rates by the mud crab, *Panopeus herbstii*, on oyster prey, *Crassostrea virginica*, when subjected to

OA related conditions. However, consistent with Chapter Four statistical output, discovered no reduced levels of locomotion. This is to be expected if the concentration for chemical foraging cue used, within trials, is equal to or greater than the minimum detection threshold concentration of individuals. Whereby there should be few differences in resulting response level (stereotyped behaviour) or frequency, independent of the pH. Contrastingly, pH induced stress upon hermit crabs by de la Haye et al. (2011), resulted in reduced activity and locomotion of individuals, during trials investigating shell assessments.

Observations also suggest identifiable patterns in response by particular individuals. Proposing the existence of bold and shy personalities amongst the sample population, within a foraging behaviour context. Therefore, explanation of limited statistical implications regarding engagement response and foraging behaviour could be as descried by Sih et al. (2004). Sih et al. (2004) suggest, individuals who typically display greater levels of boldness can alter this, in accordance to environmental conditions. However, individuals will still continue to exhibit greater levels of boldness in comparison to its conspecifics, no matter the subjection conditions and can additionally occur in circumstance whereby such response is not favourable (Sih et al., 2004). This therefore justifies the need to further explore such area of research amongst future investigations.

Similar to aggressiveness, increased foraging activity is often correlated to bolder individuals. Suggesting individuals who portray greater foraging activity compared to conspecifics at pH level 8.15 will show greater foraging activity than conspecifics when subjected to reduced pH levels: 7.7 and 7.2. Amongst a real-world context this could lead to individuals becoming vulnerable, particularly in situations of high risk. For example, circumstance whereby there is greater risk of predation. Bolder individuals will actively forage, increasing their susceptibility of been preyed upon (Sih et al., 2004). Additionally, this may result in bold individuals actively foraging whilst subjected to stressful environmental conditions such as those related to future rates of OA. OA negatively impacts successfulness of chemoreception. In such event, bold individuals will actively forage, not as a result of successful chemoreception of foraging cues, but as a result of their bold personalities. Which are responsible for greater frequency of exploratory behaviour. *Conus marmoreus*, conch individuals have previously been noted to demonstrate increased activity under subjection to OA related conditions. However both; foraging and prey-capture was reduced and largely unsuccessful (Watson et al., 2017).

Evaluation of individual responses in each trial condition leads to the belief that such theory is the cause to results established here. Examples of this include frequency of engagement response displayed by individuals 14 and 12. Which suggests both individuals have bolder personalities

compared to others. As the frequency of engagement response demonstrated by both individuals was greater in comparison to other individuals across all pH levels. Especially in comparison to individuals 19 and 20 whom demonstrated lower engagement frequencies, linked to shy personalities, across all pH levels. Alternatively, the frequency in which individuals demonstrated engagement response to the addition of foraging cues (GSH and mussel conditioned artificial seawater) may have varied as a result of hunger level and other environmental factors discussed in Chapter Three. Whereby hungry individuals may have been more motivated and therefore engaged more frequently with cue sources, independent of pH level (Lancaster, 1988). However, such conclusions are speculative due to small sample sizes and inherent limitations of subsequent statistical results. Future studies must therefore investigate the prospect of personality of much larger sample sizes, to increase significance of theories. Outcomes discussed would hence benefit from the use of larger samples. Particularly as detection thresholds determined in Chapter Three were under-represented in statistical analysis.

Additionally, individuals will display differing capabilities to act plastically, particularly in circumstance involving environmental change (Briffa et al., 2008; Kroeker et al., 2013). Amongst many contexts plasticity is a necessary progression required by organisms to survive in rapidly changing environments, but to act plastically can result in potentially costly trade-offs for individuals such as reduction in foraging as a result of reallocation in energy expenditure (de la Haye et al., 2011; Kim et al., 2016; Thomsen et al., 2017; Wang et al., 2018). Trials of the current study involved the acute exposure of individuals to reduced pH levels. Therefore it is unlikely variation in foraging response occurred as a result of individuals acting plastically and acclimatising to trials specifically. For such occurrence, a longer time-period of subjection would be required. However, as a result of the intertidal environment sample organisms inhabit, they experience fluctuating pH naturally. It is therefore possible individuals were acclimatised and able to perform plastically in reduced pH environments (Bueno-Guerra & Amici, 2018; Carstensen & Duarte, 2019; Pörtner et al., 2004).

This theory could potentially reason Chapter Three. Whereby individuals were more negatively affected by extremely low pH 7.2 than pH 7.7. Amongst Chapter Four results, such theory could explain the variation in engagement frequency between individuals. Further, greater ability of some individuals to act plastically may have also determined the individual to engage with the cue source first, before trial partners. Particularly due to some individuals consistently displaying greater engagement frequency with the cue source, across all pH levels, compared to others. Therefore, suggesting some individuals were better acclimatised and able to perform more actively. This was seen within a study involving deep sea hermit crabs by Kim et al. (2016). Whereby differences in response were potentially a result of individual capabilities to acclimate.

Findings of Chapter Four, also suggest the detection threshold concentrations for GSH, determined in Chapter Three, did not impact the level of foraging behaviour and frequency of engagement with cue source. Therefore, individuals with lower detection threshold concentrations, were neither less or more likely to engage with the addition of chemical foraging cue GSH compared to individuals requiring higher threshold concentration. However, again due to small sample sizes there was under-representation of some threshold concentrations within analysis and therefore not enough evidence to fully support this outcome. Furthermore, in the context of trial pairings, there was no influence by threshold concentrations upon the individual of the pair to engage with the cue source first. The individual to reach the cue source first was therefore not dependent on their specific detection threshold concentrations. Such outcome was observed to be consistent with reduction in pH also.

In summary, outcomes Chapter Four can speculate conclusions and agree with findings observed within Chapter Three. Which suggests future OA predictions are likely to have significant impact on the foraging ability, via olfaction and chemoreception, of *P. bernhardus* individuals. Great variation in response to such conditions will occur between individuals of the same population. Chapter Four does highlight the potential of personality portrayal to persist across different environmental conditions in the form of bold and shy individuals with reduction in pH levels. However small samples sizes limit the significance of findings. Future research should investigate in greater depth the response personality amongst foraging behaviour in marine organisms. Acknowledging potential long-term impacts and recovery of such environmental changes (Pörtner et al., 2004). Studies must also focus upon variation amongst the reaction norms of populations both; intra- and interspecifically (Hattich et al., 2016). Trials incorporating populations collected from differing sample sites would additionally expand insight into the variation of foraging response across *P.* bernhardus populations (Vargas et al., 2017). Further giving the opportunity to determine the existence presence of local adaptation, plasticity and tolerance to OA related pH conditions (Vargas et al., 2017).

Chapter Five: Conclusion

5.1 Conclusion

Marine ecosystems are vulnerable to changes in the environment (Clements & Hunt, 2015). Such changes are becoming more frequent and appear to be accelerated by anthropogenic activity especially for predicted climate change events (Clements & Hunt, 2015). This study aimed to highlight the potential impact of future pH conditions of marine ecosystems, upon *Pagurus bernhardus* individuals whilst; investigating abilities to display foraging behaviour, exploring variation in response between individuals of the same population and considering the existence of response personalities. Chapter Three findings demonstrate that reduction in pH to 7.2 significantly reduced the ability of *P. bernhardus* individuals to successfully detect and respond to the presence of chemical foraging cue GSH. Increased detection threshold concentrations were required to initiate a behavioural response. Some individuals failed to detect any concentration of cue within trials. Direct impairment to chemoreception via changes of the molecular cue, is thought to be the cause of this as exposure to reduced pH was acute (Roggatz et al., 2016).

Chapter Four observations established a reduction in engagement response to foraging cues with reduced pH, however small samples sizes determined this not to be of statistical significance. The time taken by individuals to initially respond and engage with the cue source GSH increased with reduced pH. Such a factor could have detrimental effects of the successfulness of foraging in the field (Billock & Dunbar, 2009; Laidre, 2007; Ramsay et al., 1997). Amongst paired trials, the individual to engage with the cue source first was not significantly dependent upon detection threshold concentrations. Results suggest this could be random or dependent on other factors such as; crab fitness, appetite levels or physiological state. Weight of individuals had no impact on behavioural assay outcomes. However, it is proposed, the boldness of individuals is influences such outcomes. Future research should focus upon response personality potentials and the effect of OA on this area of study.

Subjection of individuals to reduced pH levels of 7.7 had significant impact of foraging response of individuals, within Chapter Four investigations. Reduction in pH to 7.7 resulted in individuals taking a longer period of time to detect and respond to the presence of chemical foraging cue GSH. Further reduction in pH to 7.2 had little more effect in such circumstance. Contrastingly within Chapter Three trials, pH 7.2 had greatest impact upon detection threshold concentrations compared to response observed at pH 7.7. However, such findings are a result of impairment to the olfactory molecule and chemoreceptor, rather than fitness or motivation of the individual, linked to Chapter Four findings.

As previously discussed some results are suggested to occur due to the variation within their natural environment. As an intertidal species, individuals may have demonstrated varied response due to abilities to act plastically and exhibit acclimation (Carstensen & Duarte, 2019; Donelson et al., 2019). individuals also displayed personality traits. Particular individuals consistently displayed higher or lower frequency of engagement, when compared to that of conspecifics, with the addition of chemical foraging cues across all pH levels. The combination of both Chapter Three and Four findings conclusively determine that reduced pH significantly impacts foraging response of *P. bernhardus* individuals. Level of impact was dependent upon the individual, particularly amongst detection threshold concentrations for cue, required by individuals and the time taken by individuals to detect and display a response to foraging cue presence.

Although this study attempted to replicate accurately predicted conditions for intertidal environments of the coastal species *P. bernhardus,* by investigating reduced pH of 7.2, further exploration of existing data has suggested the need for experiments to incorporate the natural variation and fluctuation of their ecosystem (Vargas et al., 2017). This includes application of natural diel and seasonal cycles within experiments and aquaria (Jarrold et al., 2017, Jarrold & Munday, 2019; Kwiatkowski & Orr, 2018). Observing foraging behaviour proves to be sufficient in the assessment of impacts of OA conditions upon behaviours controlled via chemical cues and olfaction. Using behaviour additionally allows the study of other environmental factors, physiology and animal personality or plasticity. As responses can be easily quantifiable, especially amongst *P. bernhardus* individuals. For example, and not limited to, activeness and antennule flicking. This was found by studies for alternate behaviours in existing literature, for example the portrayal of personalities amongst *P. bernhardus* individuals in the context of boldness and shyness within shell selection, startle response and aggressive acts (Bridger et al., 2015; Briffa et al., 2008; de la Haye et al., 2011; Garcia et al., 2020). Additionally, it is of high importance to explore the potential negative impacts upon foraging behaviours, as such interactions within the natural environment, are vital in the functioning, structure and health of ecosystems (Doney et al., 2012; Draper & Weissburg, 2019; Watson et al., 2017). Future research should focus upon transgenerational and multigenerational studies and the aspect of further quantifying tolerance levels possessed by organisms to OA (Clements & Hunt, 2015). Finally, investigations must expand current knowledge regarding; acclimation, plasticity and genetic adaptation in the event of environmental change.

Ethical Statement

No harm or injury was imposed on *Pagurus bernhardus* individuals upon collection from sample site in Scarborough and transportation back to the University of Hull., All individuals comfortably survived the collection and transportation processes. Whilst held within aquaria during the length of experimental trials, deaths did occur, however, unpreventable as high quality care and husbandry was given for the duration. Handling of individuals was as minimal as possible. On completion of experiments, individuals were kept at the university for further use by students to avoid unnecessary collections of more organisms. Additionally, the study was granted ethical approval by the University of Hull's Ethical Review Committee, as per obligation of university policy.

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Appendix: R Script for Statistical Analysis

Chapter Three: Biological Assay Determining Individual Thresholds

Statistical Analysis – R Script

Detection Threshold Concentration of Olfactory Cues

```
##########################################################################################################################################################################
###Chapter Three###

##########################################################################################################################################################################
###Detection Threshold Concentration of Olfactory Cues###

###Figures###

### Generating Boxplots to Demostrate Drop in Oxygen Concentration (%) at pH 8.15 with the Addition of Chemical Foraging Cue Glutathione for All 20 Individuals ###

rm(list=ls()) ## Clears R from any previous workspace sessions/objects

## Inputting data into R and exploring it's dimensions ##

pH8.15PercentageDrop<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##

ls(pH8.15PercentageDrop)                         ## examine objects ##
head(pH8.15PercentageDrop)
str(pH8.15PercentageDrop)
tail(pH8.15PercentageDrop)
summary(pH8.15PercentageDrop)

library(dplyr)      ## Made packages and functions available to use in script ##
library(ggplot2)    ## graphics package ##
library(viridis)    ## colour install for graph/plot made available to use in script ##

pH8.15PercentageDrop$Chemical.foraging.cue.concentration..ml.<-factor(pH8.15PercentageDrop$Chemical.foraging.cue.concentration..ml.)

ggplot(pH8.15PercentageDrop,aes(group=pH8.15PercentageDrop$Individual, x=pH8.15PercentageDrop$Individual, y=pH8.15PercentageDrop$Drop.in.Oxygen.Concentration....))+
  geom_boxplot()+                                  ##generates boxplot##
  stat_boxplot(geom = 'errorbar', width=0.4)+      ##Adds whisker ends to boxplots##
  geom_point(aes(color=pH8.15PercentageDrop$Chemical.foraging.cue.concentration..ml.),size=3)+                    ##formats points##
  theme_minimal() +                                ##Inserts theme##
  theme(legend.position = 'bottom')+               ##alters legend position##
  ggtitle("Drop in Oxygen Concentration (%) per Individual at pH 8.15 with the Addition of Chemical Foraging Cue Glutathione")+  ##adds main title##
  theme(plot.title = element_text(hjust = 0.5))+   ##centers main title##
  xlab("Individual")+                              ##x axis label##
  ylab("Drop in Oxygen Concentration (%)")+        ##y axis label##
  expand_limits(x=c(0,20),y=c(-1,4))+             ##determines the upper and lower limits of scales for both the x and y axis##
  scale_y_continuous(breaks = seq(-1,4,by=0.25))+  ##determines the size of the increments/scale for the y axis##
  scale_x_continuous(breaks = seq(1,20,by=1))+     ##determines the size of the increments/scale for the x axis##
  labs(color="Chemical Foraging Cue Glutathione Concentration (e-x M/l)")+           ##labeling the figure legend##
  theme(axis.line = element_line(color = "black",size = 0.2))   ##adding and formatting axis lines##
```

```
##########################################################################################

### Generating Boxplots to Demostrate Drop in Oxygen Concentration (%) at pH 7.7 with the Addition of Chemical Foraging Cue Glutathione for All 20 Individuals ###

rm(list=ls()) ## Clears R from any previous workspace sessions/objects

## Inputting data into R and exploring it's dimensions ##

pH7.7PercentageDrop<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##

ls(pH7.7PercentageDrop)                         ## examine objects ##
head(pH7.7PercentageDrop)
str(pH7.7PercentageDrop)
tail(pH7.7PercentageDrop)
summary(pH7.7PercentageDrop)

library(dplyr)      ## Made packages and functions available to use in script ##
library(ggplot2)    ## graphics package ##
library(viridis)    ## colour install for graph/plot made available to use in script ##

pH7.7PercentageDrop$Chemical.foraging.cue.concentration..ml.<-factor(pH7.7PercentageDrop$Chemical.foraging.cue.concentration..ml.)

ggplot(pH7.7PercentageDrop,aes(group=pH7.7PercentageDrop$Individual, x=pH7.7PercentageDrop$Individual, y=pH7.7PercentageDrop$Drop.in.Oxygen.Concentration....))+
  geom_boxplot()+                                  ##generates boxplot##
  stat_boxplot(geom = 'errorbar', width=0.4)+      ##Adds whisker ends to boxplots##
  geom_point(aes(color=pH7.7PercentageDrop$Chemical.foraging.cue.concentration..ml.),size=3)+                    ##formats points##
  theme_minimal() +                                ##Inserts theme##
  theme(legend.position = 'bottom')+               ##alters legend position##
  ggtitle("Drop in Oxygen Concentration (%) per Individual at pH 7.7 with the Addition of Chemical Foraging Cue Glutathione")+  ##adds main title##
  theme(plot.title = element_text(hjust = 0.5))+   ##centers main title##
  xlab("Individual")+                              ##x axis label##
  ylab("Drop in Oxygen Concentration (%)")+        ##y axis label##
  expand_limits(x=c(0,20),y=c(-1,4))+             ##determines the upper and lower limits of scales for both the x and y axis##
  scale_y_continuous(breaks = seq(-1,4,by=0.25))+  ##determines the size of the increments/scale for the y axis##
  scale_x_continuous(breaks = seq(1,20,by=1))+     ##determines the size of the increments/scale for the x axis##
  labs(color="Chemical Foraging Cue Glutathione (e-x M/l)")+           ##labeling the figure legend##
  theme(axis.line = element_line(color = "black",size = 0.2))   ##adding and formatting axis lines##

```

```r
################################################################################

### Generating Boxplots to Demostrate Drop in Oxygen Concentration (%) at pH 7.2 with the Addition of Chemical Foraging Cue Glutathione for All 20 Individuals ###

rm(list=ls()) ## Clears R from any previous workspace sessions/objects

## Inputting data into R and exploring it's dimensions ##

pH7.2PercentageDrop<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##

ls(pH7.2PercentageDrop)                         ## examine objects ##
head(pH7.2PercentageDrop)
str(pH7.2PercentageDrop)
tail(pH7.2PercentageDrop)
summary(pH7.2PercentageDrop)

library(dplyr)      ## Made packages and functions available to use in script ##
library(ggplot2)    ## graphics package ##
library(viridis)    ## colour install for graph/plot made available to use in script ##

pH7.2PercentageDrop$Chemical.foraging.cue.concentration..ml.<-factor(pH7.2PercentageDrop$Chemical.foraging.cue.concentration..ml.)

ggplot(pH7.2PercentageDrop,aes(group=pH7.2PercentageDrop$Individual, x=pH7.2PercentageDrop$Individual, y=pH7.2PercentageDrop$Drop.in.Oxygen.Concentration....))+
  geom_boxplot()+                                       ##generates boxplot##
  stat_boxplot(geom = 'errorbar', width=0.4)+           ##Adds whisker ends to boxplots##
  geom_point(aes(color=pH7.2PercentageDrop$Chemical.foraging.cue.concentration..ml.),size=3)+                              ##formats points##
  theme_minimal() +                                     ##Inserts theme##
  theme(legend.position = 'bottom')+                    ##alters legend position##
  ggtitle("Drop in Oxygen Concentration (%) per Individual at pH 7.2 with the Addition of Chemical Foraging Cue Glutathione")+  ##adds main title##
  theme(plot.title = element_text(hjust = 0.5))+        ##centers main title##
  xlab("Individual")+                                   ##x axis label##
  ylab("Drop in Oxygen Concentration (%)")+             ##y axis label##
  expand_limits(x=c(0,20),y=c(-1,4))+                  ##determines the upper and lower limits of scales for both the x and y axis##
  scale_y_continuous(breaks = seq(-1,4,by=0.25))+       ##determines the size of the increments/scale for the y axis##
  scale_x_continuous(breaks = seq(1,20,by=1))+          ##determines the size of the increments/scale for the x axis##
  labs(color="Chemical Foraging Cue Concentration Glutathione (e-x M/l)")+           ##labeling the figure legend##
  theme(axis.line = element_line(color = "black",size = 0.2))   ##adding and formatting axis lines##
```

```r
################################################################################################################################################################################
###Box Plot of Averages per pH on Detection Concentration Thresholds at all pH Levels for Chemical Foraging Cue Glutathione for all 20 Individuals###

rm(list = ls())            ## Clears R from any previous workspace sessions/objects

library(dplyr)           ## Made packages and functions available to use in script ##
library(ggplot2)         ## graphics package ##

Threshold.concentration.pH.All.individuals<-read.csv(file.choose())        ## Read in data as this name from chosen file which have the option to locate ##

ls(Threshold.concentration.pH.All.individuals)              ## examine objects ##
head(Threshold.concentration.pH.All.individuals)
str(Threshold.concentration.pH.All.individuals)
tail(Threshold.concentration.pH.All.individuals)
summary(Threshold.concentration.pH.All.individuals)

Threshold.concentration.pH.All.individuals$pH<-as.factor(Threshold.concentration.pH.All.individuals$pH)

ggplot(Threshold.concentration.pH.All.individuals,aes(group=Threshold.concentration.pH.All.individuals$pH,
                                                      x=Threshold.concentration.pH.All.individuals$pH, y=Threshold.concentration.pH.All.individuals$Detection.Concentration))+
  geom_boxplot()+                                       ##generates boxplot##
  geom_point(size=3)+                                        ##adds data points to boxplots##
  stat_boxplot(geom = 'errorbar', width=0.2)+           ##Adds whisker ends to boxplots##
  theme_minimal() +                                     ##Inserts theme##
  ggtitle("Detection Threshold Concentration for Chemical Foraging Cue Glutathione at each pH level for 20 Individuals")+  ##adds main title##
  theme(plot.title = element_text(hjust = 0.5))+        ##centers main title##
  xlab("pH")+                                           ##x axis label##
  ylab("Detection Threshold Concentration (e-x M/l)")+      ##y axis label##
  expand_limits(y=c(0,10))+                              ##determines the upper and lower limits of scales for the y axis##
  scale_y_continuous(breaks = seq(0,10,by=1))+           ##determines the size of the increments/scale for the y axis##
  theme(axis.line = element_line(color = "black",size = 0.2))   ##adding and formatting axis lines##

################################################################################################################################################################################
###Effect of pH on Individual Detection Thresholds for Chemical Foraging Cue Glutathione Concentrations - Facet-Wrapped Scatter Plots###

rm(list=ls()) ## Clears R from any previous workspace sessions/objects ##

library(dplyr)    ## downloading relevent packages ##
library(ggplot2)

Detection.Threshold.pH<-read.csv(file.choose())   ## reading in data ##
glimpse(Detection.Threshold.pH)                   ## exploring data ##
str((Detection.Threshold.pH))

Detection.Threshold.pH$Individual<-factor(Detection.Threshold.pH$Individual)    ## setting individuals as a factor ##
ggplot(data=Detection.Threshold.pH, mapping = aes(x=Detection.Threshold.pH$pH, y=Detection.Threshold.pH$Detection.Concentration, color=Detection.Threshold.pH$Individual))+
  geom_point(size=3)+
  geom_line()+                                                                  ## ploting scatter graphs ##
  facet_wrap(facets = vars(Detection.Threshold.pH$Individual))+                 ## multiple graphs according to data ##
  labs(title = "Impact of pH on Individual Detection Threshold Concentrations for Chemical Foraging Cue Glutathione",
       x="pH",
       y="Detection Threshold Concentration (e-x M/l)")+                        ## setting title and labels ##
  labs(color="Individuals")+                                                    ## key to represent individuals by colour ##
  theme(plot.title = element_text(hjust = 0.5))+                                ## adjusting title position ##
  theme_bw()+                                                                   ## applying formatting theme ##
scale_x_discrete(breaks=c(7.2,7.7,8.15))                                       ## setting axis limits ##
```

```
##########################################################################################################################################################################
###Statistical Analysis###

#############################################################################
#### Is There a Significant Difference in Individual Detection Threshold Concentrations for Chemical Foraging Cue Glutathione with Differing pH  ####

rm(list=ls()) ## Clears R from any previous workspace sessions/objects

## Inputting data into R and exploring it's dimensions ##

Impact.of.pH.on.Detection.Threshold<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##

ls(Impact.of.pH.on.Detection.Threshold)                         ## examine objects ##
head(Impact.of.pH.on.Detection.Threshold)
str(Impact.of.pH.on.Detection.Threshold)
tail(Impact.of.pH.on.Detection.Threshold)
summary(Impact.of.pH.on.Detection.Threshold)

library(ggplot2)    ## downloading packages to aid analysis ##
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###
library(car)

#################################################################
### shapiro-wilks test for normality ###
shapiro.test(Impact.of.pH.on.Detection.Threshold$Detection.Concentration)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
## data:  Impact.of.pH.on.Detection.Threshold$Detection.Concentration
## W = 0.74361, p-value = 6.788e-09


### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(Impact.of.pH.on.Detection.Threshold$Detection.Concentration,
         main="Q-Q plot to Visualise Distribution of Individual Detection Thresholds with the Addition of Chemical Foraging Cue Data at all pH Levels",
         xlab = "Individual Detection Thresholds")

## CONFIRMS NON-NORMAL DISTRIBUTION

#################################################################
###Testing Assumption 2 - Homogeneity of Variance###

Impact.of.pH.on.Detection.Threshold$pH<-factor(Impact.of.pH.on.Detection.Threshold$pH)
leveneTest(Impact.of.pH.on.Detection.Threshold$Detection.Concentration~Impact.of.pH.on.Detection.Threshold$pH,
           data=Impact.of.pH.on.Detection.Threshold)

## Levene's Test for Homogeneity of Variance (center = median)
##        Df F value Pr(>F)
## group  2  7.4536 0.001332
##       57
##Assumption not met of homegeneity of variance
```

```
#################################################################
### Transforming non-normal data ###
Impact.of.pH.on.Detection.Threshold$log.Detection.Concentration<-
  log(Impact.of.pH.on.Detection.Threshold$Detection.Concentration)      ##log transforming data to create normally distributed data##

shapiro.test(Impact.of.pH.on.Detection.Threshold$log.Detection.Concentration)
ggqqplot(Impact.of.pH.on.Detection.Threshold$log.Detection.Concentration,
         main="Q-Q plot to Visualise Distribution of log Transformed Individual Detection Thresholds Data With the Addition of Chemical Foraging Cue at All pH Levels",
         xlab = "Individual Detection Thresholds")    ###checking distribution of log transformed data###

##Still non-normal distribution - choose non-parametric version of test##

###Applying non-parametric version of one-way ANOVA - Kruskal-Wallis test###
kruskal.test(Impact.of.pH.on.Detection.Threshold$Detection.Concentration~Impact.of.pH.on.Detection.Threshold$pH, data = Impact.of.pH.on.Detection.Threshold)

##Kruskal-Wallis rank sum test
##data:  Impact.of.pH.on.Detection.Threshold$Detection.Concentration by Impact.of.pH.on.Detection.Threshold$pH
##Kruskal-Wallis chi-squared = 7.1973, df = 2, p-value = 0.02736

## As the p-value is less than the significance level 0.05, we can conclude that there are significant differences between the treatment groups.

###Determining between which groups significance exists###
pairwise.wilcox.test(Impact.of.pH.on.Detection.Threshold$Detection.Concentration, Impact.of.pH.on.Detection.Threshold$pH, p.adjust.method = "BH")

## Pairwise comparisons using Wilcoxon rank sum test
## data:  Impact.of.pH.on.Detection.Threshold$Detection.Concentration and Impact.of.pH.on.Detection.Threshold$pH
##      7.2   7.7
## 7.7  0.037 -
## 8.15 0.070 0.561
## P value adjustment method: BH

##The pairwise comparison shows that only Detection thresholds at pH 7.7 and 7.2 are significantly different p<0.05
```

Determination of pH and Overall Drop in Oxygen Concentration (%)

```
##############################################################################################################################################################################################
##############################################################################################################################################################################################
###Determination of pH and Overall Drop in Oxygen Concentration (%)###

###Figures###

##### Overall Drop in Oxygen Concetrations for Controls and Chemical Foraging Cue Glutathione at all pH Levels #####
rm(list = ls())

library(dplyr)      ## Made packages and functions available to use in script ##
library(ggplot2)    ## graphics package ##
library(gridExtra)    #layout of graphics window#

##############################################################################################################################################################################################
###Negative Control###
#Gerenating Boxplots for Artificial Seawater at all pH Levels###

## Inputting data into R and exploring it's dimensions ##
Negative.Control<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##

ls(Negative.Control)                         ## examine objects ##
head(Negative.Control)
str(Negative.Control)
tail(Negative.Control)
summary(Negative.Control)

plot.negative<-ggplot(Negative.Control,aes(group=Negative.Control$pH, x=Negative.Control$pH, y=Negative.Control$Overall.Drop.in.Oxygen.Concentration...))+
  geom_boxplot()+                                          ##generates boxplot##
  geom_point(size=2)+
  stat_boxplot(geom = 'errorbar', width=0.2)+               ##Adds whisker ends to boxplots##
  theme_minimal() +                                         ##Inserts theme##
  ggtitle("A) Overall Drop in Oxygen Concentration (%) with the Addition of Artificial Seawater at pH Levels 8.15, 7.7 and 7.2")+  ##adds main title##
  theme(plot.title = element_text(hjust = 0.5))+            ##centers main title##
  xlab("pH")+                                          ##x axis label##
  ylab("")+                                            ##y axis label##
  expand_limits(x=c(8.15,7.2),y=c(-1,18))+                  ##determines the upper and lower limits of scales for both the x and y axis##
  scale_y_continuous(breaks = seq(-1,18,by=2))+           ##determines the size of the increments/scale for the y axis##
  scale_x_discrete(limits=c(8.15,7.7,7.2))+               ##determines the size of the increments/scale for the x axis##
  theme(axis.line = element_line(color = "black",size = 0.2))   ##adding and formatting axis lines##

##############################################################################################################################################################################################
###Positive Control###
#Gerenating Boxplots for Mussel Conditioned Artificial Seawater and Overall Drop in Oxygen Concentration (%) at all pH Levels###

## Inputting data into R and exploring it's dimensions ##
Positive.Control<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##
ls(Positive.Control)                         ## examine objects ##
head(Positive.Control)
str(Positive.Control)
tail(Positive.Control)
summary(Positive.Control)

plot.positive<-ggplot(Positive.Control,aes(group=Positive.Control$pH, x=Positive.Control$pH, y=Positive.Control$Overall.Drop.in.Oxygen.Concentration...))+
  geom_boxplot()+                                          ##generates boxplot##
  geom_point(size=2)+
  stat_boxplot(geom = 'errorbar', width=0.2)+               ##Adds whisker ends to boxplots##
  theme_minimal() +                                         ##Inserts theme##
  ggtitle("B) Overall Drop in Oxygen Concentration (%) with the Addition of Mussel Conditioned Artificial Seawater at pH Levels 8.15, 7.7 and 7.2")+  ##adds main title##
  theme(plot.title = element_text(hjust = 0.5))+            ##centers main title##
  xlab("pH")+                                          ##x axis label##
  ylab("Overall Drop in Oxygen Concentration (%)")+                    ##y axis label##
  expand_limits(x=c(8.15,7.2),y=c(-1,18))+                  ##determines the upper and lower limits of scales for both the x and y axis##
  scale_y_continuous(breaks = seq(-1,18,by=2))+           ##determines the size of the increments/scale for the y axis##
  scale_x_discrete(limits=c(8.15,7.7,7.2))+               ##determines the size of the increments/scale for the x axis##
  theme(axis.line = element_line(color = "black",size = 0.2))   ##adding and formatting axis lines##

##############################################################################################################################################################################################
###Chemical Foraging Cue Glutathione###
#Gerenating Boxplots for Chemical Foraging Cue Glutathione at all pH Levels###

## Inputting data into R and exploring it's dimensions ##
Chemical.Foraging.Cue.Glutathione<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##
ls(Chemical.Foraging.Cue.Glutathione)                         ## examine objects ##
head(Chemical.Foraging.Cue.Glutathione)
str(Chemical.Foraging.Cue.Glutathione)
tail(Chemical.Foraging.Cue.Glutathione)
summary(Chemical.Foraging.Cue.Glutathione)

plot.cue<-ggplot(Chemical.Foraging.Cue.Glutathione,aes(group=Chemical.Foraging.Cue.Glutathione$pH, x=Chemical.Foraging.Cue.Glutathione$pH, y=Chemical.Foraging.Cue.Glutathione$Overall.Drop.in.Oxygen.Concentration...))+
  geom_boxplot()+                                          ##generates boxplot##
  geom_point(size=2)+
  stat_boxplot(geom = 'errorbar', width=0.2)+               ##Adds whisker ends to boxplots##
  theme_minimal() +                                         ##Inserts theme##
  ggtitle("C) Overall Drop in Oxygen Concentration (%) with the Addition of Chemical Foraging Cue Glutathione at pH Levels 8.15, 7.7 and 7.2")+  ##adds main title##
  theme(plot.title = element_text(hjust = 0.5))+            ##centers main title##
  xlab("pH")+                                          ##x axis label##
  ylab("")+                                            ##y axis label##
  expand_limits(x=c(8.15,7.2),y=c(-1,18))+                  ##determines the upper and lower limits of scales for both the x and y axis##
  scale_y_continuous(breaks = seq(-1,18,by=2))+           ##determines the size of the increments/scale for the y axis##
  scale_x_discrete(limits=c(8.15,7.7,7.2))+               ##determines the size of the increments/scale for the x axis##
  theme(axis.line = element_line(color = "black",size = 0.2))   ##adding and formatting axis lines##

##############################################################################################################################################################################################
####combining plots in same graphics window####
grid.arrange(plot.negative,plot.positive,plot.cue)
```

```
###Statistical Analysis###

### Is There a Difference of Means in Drop in Oxygen Concentration for Chemical Foraging Cue Glutathione with Differing pH ###

rm(list=ls()) ## Clears R from any previous workspace sessions/objects

## Inputting data into R and exploring it's dimensions ##

Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##

ls(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration)                          ## examine objects ##
head(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration)
str(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration)
tail(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration)
summary(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration)

library(ggplot2)    ## downloading packages to aid analysis ##
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###
library(car)

#####################################################################
### shapiro-wilks test for normality ###
shapiro.test(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$Overall.Drop.in.Oxygen.Concentration...)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
## data:  Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$Overall.Drop.in.Oxygen.Concentration...
## W = 0.83862, p-value = 1.429e-06

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$Overall.Drop.in.Oxygen.Concentration...,
         main="Q-Q plot to Visualise Distribution of Overall Drop in Oxygen Concentration with the Addition of Chemical Foraging Cue Data at all pH Levels",
         xlab = "Overall Drop in Oxygen Concentration")

## CONFIRMS NON-NORMAL DISTRIBUTION

#####################################################################
###Testing Assumption 2 - Homogeneity of Variance###

Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$pH<-factor(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$pH)

leveneTest(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$Overall.Drop.in.Oxygen.Concentration...~Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$pH,
           data=Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration)

## Levene's Test for Homogeneity of Variance (center = median)
##        Df F value Pr(>F)
## group  2  0.9069 0.4095
##       57
##Assumption met of homegeneity of variance
```

```
#####################################################################
### Transforming non-normal data and looking at distribution###

Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$log.Overall.Drop.in.Oxygen.Concentration...<-
  log(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$Overall.Drop.in.Oxygen.Concentration...)      ##log transforming data to create normally distributed data##

ggqqplot(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$log.Overall.Drop.in.Oxygen.Concentration...,
         main="Q-Q plot to Visualise Distribution of log Transformed Overall Drop in Oxygen Concentration Data With the Addition of Chemical Foraging Cue at All pH Levels",
         xlab = "Overall Drop in Oxygen Concentration")    ###checking distribution of log transformed data###

### shapiro-wilks test for normality ###
shapiro.test(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$log.Overall.Drop.in.Oxygen.Concentration...)    ### Carrying out a Shapiro-Wilk normality test ###

#Shapiro-Wilk normality test
#data:  Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$log.Overall.Drop.in.Oxygen.Concentration...
#W = 0.91686, p-value = 0.0005746

##Still Non-normal distribution - choose non-parametric version of test##

###Applying non-parametric version of one-way ANOVA - Kruskal-Wallis test###
kruskal.test(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$Overall.Drop.in.Oxygen.Concentration...~Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$pH, data = Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration)

##Kruskal-Wallis rank sum test
##data:  Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$Overall.Drop.in.Oxygen.Concentration... by Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration$pH
##Kruskal-Wallis chi-squared = 1.0877, df = 2, p-value = 0.5805

## As the p-value is not less than the significance level 0.05, we can conclude that there are no significant differences between overall drop in oxygen concentration with varying pH and the addition of
##chemical foraging cue glutathioine##

#####################################################################################################################################################################################################
#### Does Overall Drop in Oxygen Concentration Change with pH with the Addition of Mussel Conditioned Artificial Seawater ####

rm(list=ls()) ## Clears R from any previous workspace sessions/objects

## Inputting data into R and exploring it's dimensions ##

Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##

ls(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive)                          ## examine objects ##
head(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive)
str(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive)
tail(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive)
summary(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive)

library(ggplot2)    ## downloading packages to aid analysis ##
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###
library(car)
```

```
#####################################################################
### shapiro-wilks test for normality ###
shapiro.test(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$Overall.Drop.in.Oxygen.Concentration...)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
## data:  Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$Overall.Drop.in.Oxygen.Concentration...
## W = 0.9334, p-value = 0.002775

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$Overall.Drop.in.Oxygen.Concentration...,
         main="Q-Q plot to Visualise Distribution of Overall Drop in Oxygen Concentration with the Addition of Mussel Conditioned Artificial Seawater Data at all pH Levels",
         xlab = "Overall Drop in Oxygen Concentration")

## CONFIRMS NON-NORMAL DISTRIBUTION

#####################################################################
###Testing Assumption 2 - Homogeneity of Variance###

Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$pH<-factor(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$pH)
leveneTest(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$Overall.Drop.in.Oxygen.Concentration...~Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$pH,
           data=Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive)
## Levene's Test for Homogeneity of Variance (center = median)
##        Df F value Pr(>F)
## group  2  0.0722 0.9305
##       57
##Assumption met of homegeneity of variance

#####################################################################
### Transforming non-normal data and applying test###

Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$log.Overall.Drop.in.Oxygen.Concentration...<-
  log(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$Overall.Drop.in.Oxygen.Concentration...+1)      ##log transforming data to create normally distributed data##

ggqqplot(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$log.Overall.Drop.in.Oxygen.Concentration...,
         main="Q-Q plot to Visualise Distribution of log Transformed Overall Drop in Oxygen Concentration Data With the Addition of Mussel Conditioned Artificial Seawater at All pH Levels",
         xlab = "Overall Drop in Oxygen Concentration")    ###checking distribution of log transformed data###

### shapiro-wilks test for normality ###
shapiro.test(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$log.Overall.Drop.in.Oxygen.Concentration...)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
#data:  Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$log.Overall.Drop.in.Oxygen.Concentration...
#W = 0.97533, p-value = 0.2635

#log-transformed data normally distributed
```

```
lm(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$log.Overall.Drop.in.Oxygen.Concentration...~Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$pH,
   data = Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive)       ##fitting a linear model##

qqnorm(resid(lm(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$log.Overall.Drop.in.Oxygen.Concentration...~Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$pH,
                data = Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive)))                      ##checking fit of model##

anova(lm(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$log.Overall.Drop.in.Oxygen.Concentration...~Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$pH,
         data = Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive))     ##Running a one-way ANOVA##

## Analysis of Variance Table
## Response: Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$log.Overall.Drop.in.Oxygen.Concentration...
##                                                    Df  Sum Sq Mean Sq F value Pr(>F)
## Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.positive$pH  2  0.5139 0.25696  0.7574 0.4735
## Residuals                                         57 19.3373 0.33925

## As the p-value is not less than the significance level 0.05, we can conclude that there are no significant differences between overall drop in oxygen concentration with varying pH and the addition of
##mussel conditioned artificial seawater##

#############################################################################
#### Does overall drop in oxygen concentration change with pH with the addition of artificial seawater ####

rm(list=ls()) ## Clears R from any previous workspace sessions/objects

## Inputting data into R and exploring it's dimensions ##

Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##

ls(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative)                         ## examine objects ##
head(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative)
str(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative)
tail(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative)
summary(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative)

library(ggplot2)    ## downloading packages to aid analysis ##
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###
library(car)

#####################################################################
### shapiro-wilks test for normality ###
shapiro.test(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$Overall.Drop.in.Oxygen.Concentration...)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
##data:  Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$Overall.Drop.in.Oxygen.Concentration...
## W = 0.85056, p-value = 3.163e-06

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$Overall.Drop.in.Oxygen.Concentration...,
         main="Q-Q plot to Visualise Distribution of Overall Drop in Oxygen Concentration with the Addition of Artificial Seawater Data at all pH Levels",
         xlab = "Overall Drop in Oxygen Concentration")

## CONFIRMS NON-NORMAL DISTRIBUTION
```

```r
#####################################################################
###Testing Assumption 2 - Homogeneity of Variance###

Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$pH<-factor(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$pH)
leveneTest(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$Overall.Drop.in.Oxygen.Concentration...~Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$pH,
           data=Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative)

## Levene's Test for Homogeneity of Variance (center = median)
##        Df F value Pr(>F)
## group  2  1.2693 0.2888
##       57
##Assumption met of homegeneity of variance

#####################################################################
### Transforming non-normal data and applying test###

Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$log.Overall.Drop.in.Oxygen.Concentration...<-
  log(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$Overall.Drop.in.Oxygen.Concentration...+1)      ##log transforming data to create normally distributed data##

ggqqplot(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$log.Overall.Drop.in.Oxygen.Concentration...,
         main="Q-Q plot to Visualise Distribution of log Transformed Overall Drop in Oxygen Concentration Data With the Addition of Artificial Seawater at All pH Levels",
         xlab = "Overall Drop in Oxygen Concentration")    ###checking distribution of log transformed data###

#####################################################################
### shapiro-wilks test for normality ###
shapiro.test(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$log.Overall.Drop.in.Oxygen.Concentration...)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
## data:  Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$log.Overall.Drop.in.Oxygen.Concentration...
## W = 0.98865, p-value = 0.8511

#log-transformed data normally distributed

lm(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$log.Overall.Drop.in.Oxygen.Concentration...~Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$pH,
   data = Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative)        ##fitting a linear model##

qqnorm(resid(lm(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$log.Overall.Drop.in.Oxygen.Concentration...~Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$pH,
                data = Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative)))                      ##checking fit of model##

anova(lm(Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$log.Overall.Drop.in.Oxygen.Concentration...~Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$pH,
         data = Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative))                              ##Running a one-way ANOVA##

## Analysis of Variance Table
## Response: Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$log.Overall.Drop.in.Oxygen.Concentration...
##                                                    Df  Sum Sq Mean Sq F value Pr(>F)
## Impact.of.pH.on.Overall.Drop.in.Oxgen.Concentration.negative$pH  2  1.0619 0.53095  1.3543 0.2663
## Residuals                                          57 22.3468 0.39205
# hypothesis not accepted, cant reject the null. No significant difference between any pairs of means###


## As the p-value is not less than the significance level 0.05, we can conclude that there are no significant differences between overall drop in oxygen concentration with varying pH and the addition of
##artificial seawater##
```

Impact of Olfactory Cues and Control Additions on Overall Drop in Oxygen Concentration (%)

```
##############################################################################################################################################################################
###Impact of Olfactory Cues and Control Additions on Overall Drop in Oxygen Concentration (%)###

###Figures###
#############################################################################################
##Boxplot to Visualise how Overall Drop in Oxygen Concentration (%) Varies with Environmental Addition (Artificial Seawater, Chemical Foraging Cue Glutathione and Mussel Conditiones Artificial Seawater) at pH 8.15##
rm(list=ls()) ## Clears R from any previous workspace sessions/objects
library(ggplot2)  ##Makes packages available for use##
library(dplyr)
library(gridExtra)

## Inputting data into R and exploring it's dimensions ##
Overall.Drop.8.15<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##
ls(Overall.Drop.8.15)                         ## examine objects ##
head(Overall.Drop.8.15)
str(Overall.Drop.8.15)
tail(Overall.Drop.8.15)
summary(Overall.Drop.8.15)

plot8.15<-ggplot(Overall.Drop.8.15, aes(group=Overall.Drop.8.15$Addition,x=Overall.Drop.8.15$Addition,y=Overall.Drop.8.15$Overall.Drop.in.Oxygen.Concentration...))+
  geom_boxplot()+                    #Generates boxplot
  geom_point(size=2)+                #formats plot individual data points
  stat_boxplot(geom='errorbar',width=0.2)+    #adds and formats error bars
  theme_minimal()+                  #inserts theme
  ggtitle("A) Overall Drop in Oxygen Concentration at pH 8.15")+      #inserts title
  theme(plot.title = element_text(hjust = 0.5))+            #adjusts title position
  xlab(" ")+          #Blanks x and y axis labels
  ylab(" ")+
  theme(axis.line = element_line(color = "black",size = 0.2))+    #inserts theme
  scale_y_continuous(breaks = seq(0,18,by=2))+      #sets y axis scale
  expand_limits(y=c(0,18))
plot8.15

#############################################################################################
##Boxplot to Visualise how Overall Drop in Oxygen Concentration (%) Varies with Environmental Addition (Artificial Seawater, Chemical Foraging Cue Glutathione and Mussel Conditiones Artificial Seawater) at pH 7.7##
## Inputting data into R and exploring it's dimensions ##
Overall.Drop.7.7<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##
ls(Overall.Drop.7.7)                         ## examine objects ##
head(Overall.Drop.7.7)
str(Overall.Drop.7.7)
tail(Overall.Drop.7.7)
summary(Overall.Drop.7.7)

plot7.7<-ggplot(Overall.Drop.7.7, aes(group=Overall.Drop.7.7$Addition,x=Overall.Drop.7.7$Addition,y=Overall.Drop.7.7$Overall.Drop.in.Oxygen.Concentration...))+
  geom_boxplot()+                    #Generates boxplot
  geom_point(size=2)+                #formats plot individual data points
  stat_boxplot(geom='errorbar',width=0.2)+      #adds and formats error bars
  theme_minimal()+                  #inserts theme
  ggtitle("B) Overall Drop in Oxygen Concentration at pH 7.7")+     #inserts title
  theme(plot.title = element_text(hjust = 0.5))+           #adjusts title position
  xlab(" ")+        #blanks x axis label
  ylab("Overall Drop in Oxygen Concentration (%)")+       #inserts y axis label
  theme(axis.line = element_line(color = "black",size = 0.2))+    #inserts theme
  scale_y_continuous(breaks = seq(0,18,by=2))+     #sets y axis scale
  expand_limits(y=c(0,18))
plot7.7
```

```
#############################################################################################
##Boxplot to Visualise how Overall Drop in Oxygen Concentration (%) Varies with Environmental Addition (Artificial Seawater, Chemical Foraging Cue Glutathione and Mussel Conditiones Artificial Seawater) at pH 7.2##
## Inputting data into R and exploring it's dimensions ##
Overall.Drop.7.2<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##
ls(Overall.Drop.7.2)                         ## examine objects ##
head(Overall.Drop.7.2)
str(Overall.Drop.7.2)
tail(Overall.Drop.7.2)
summary(Overall.Drop.7.2)

plot7.2<-ggplot(Overall.Drop.7.2, aes(group=Overall.Drop.7.2$Addition,x=Overall.Drop.7.2$Addition,y=Overall.Drop.7.2$Overall.Drop.in.Oxygen.Concentration...))+
  geom_boxplot()+                    #Generates boxplot
  geom_point(size=2)+                #formats plot individual data points
  stat_boxplot(geom='errorbar',width=0.2)+        #adds and formats error bars
  theme_minimal()+                  #inserts theme
  ggtitle("C) Overall Drop in Oxygen Concentration at pH 7.2")+     #inserts title
  theme(plot.title = element_text(hjust = 0.5))+           #adjusts title position
  xlab("Environmental Addition")+         #adds x axis label
  ylab(" ")+                    #blanks y axis label
  theme(axis.line = element_line(color = "black",size = 0.2))+    #inserts theme
  scale_y_continuous(breaks = seq(0,18,by=2))+     #sets y axis scale
  expand_limits(y=c(0,18))
plot7.2

##Combining plots into same graphics window##
grid.arrange(plot8.15,plot7.7,plot7.2)
```

```
##################################################################################################
###Statistical Analysis###

##################################################################################################
###Are There Significant Differences Between Treatment (Chemical Foraging Cue Glutathione, Mussel Condiioned Artificial Seawater, Artificial Seawater) and response (overall drop in oxygen concentration) at pH 8.15 ###
###testing assumption 1 - are residuals normally distributed?###

rm(list=ls()) ## Clears R from any previous workspace sessions/objects
library(ggplot2)  ##Makes packages available for use##
library(dplyr)
library(ggpubr)
library(car)

## Inputting data into R and exploring it's dimensions ##
Treatment.Overall.Drop.8.15<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##

ls(Treatment.Overall.Drop.8.15)                         ## examine objects ##
head(Treatment.Overall.Drop.8.15)
str(Treatment.Overall.Drop.8.15)
tail(Treatment.Overall.Drop.8.15)
summary(Treatment.Overall.Drop.8.15)

### shapiro-wilks test for normality ##
shapiro.test(Treatment.Overall.Drop.8.15$Overall.Drop.in.Oxygen.Concentration...)    ### Carrying out a Shapiro-Wilk normality test ###

##Shapiro-Wilk normality test output
##data:  Treatment.Overall.Drop.8.15$Overall.Drop.in.Oxygen.Concentration...
##W = 0.86606, p-value = 9.385e-06

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(Treatment.Overall.Drop.8.15$Overall.Drop.in.Oxygen.Concentration...,
         main="Q-Q plot to Visualise Distribution of Overall Drop in Oxygen Concentration Data at pH 8.15 with All Treatments",
         xlab = "Overall Drop in Oxygen Concentration")

##Confirms non-normal distribution##

###testing assumption2 - homogeneity of variance###
leveneTest(Treatment.Overall.Drop.8.15$Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.8.15$Addition, data=Treatment.Overall.Drop.8.15)

## Levene's Test for Homogeneity of Variance (center = median)
##       Df F value Pr(>F)
## group  2  0.7394 0.4819
##  57
##Assumption met of homegeneity of variance
```

```
##Transforming non-normal data
Treatment.Overall.Drop.8.15$log.Overall.Drop.in.Oxygen.Concentration...<-log(Treatment.Overall.Drop.8.15$Overall.Drop.in.Oxygen.Concentration...+1)
##log transforming data to create normally distributed data##
ggqqplot(Treatment.Overall.Drop.8.15$log.Overall.Drop.in.Oxygen.Concentration...,
         main="Q-Q plot to Visualise Distribution of log Transformed Overall Drop in Oxygen Concentration Data at pH 8.15 with All Treatments",
         xlab = "Overall Drop in Oxygen Concentration")

### shapiro-wilks test for normality ##
shapiro.test(Treatment.Overall.Drop.8.15$log.Overall.Drop.in.Oxygen.Concentration...)    ### Carrying out a Shapiro-Wilk normality test ###

##Shapiro-Wilk normality test output
##data:  Treatment.Overall.Drop.8.15$log.Overall.Drop.in.Oxygen.Concentration...
##W = 0.98416, p-value = 0.6265

##Confirms normal distribution

###perfroming one-way ANOVA (with log transformed data)###
lm(Treatment.Overall.Drop.8.15$log.Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.8.15$Addition,
   data = Treatment.Overall.Drop.8.15)       ##fitting a linear model##
qqnorm(resid(lm(Treatment.Overall.Drop.8.15$log.Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.8.15$Addition,
                data = Treatment.Overall.Drop.8.15)))                          ##checking fit of model##
anova(lm(Treatment.Overall.Drop.8.15$log.Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.8.15$Addition,
         data = Treatment.Overall.Drop.8.15))                                  ##Running a one-way ANOVA##

## Analysis of Variance Table
## Response: Treatment.Overall.Drop.8.15$log.Overall.Drop.in.Oxygen.Concentration...
##                                   Df  Sum Sq Mean Sq F value Pr(>F)
## Treatment.Overall.Drop.8.15$Addition  2  1.4981 0.74903  1.8515 0.1663
## Residuals                            57 23.0593 0.40455

## As the p-value is not less than the significance level 0.05, we can conclude that there are no significant differences between overall drop in oxygen concentration with varying trial environment addition of
##(chemical foraging cue glutathione, artificial seawater and mussel conditioned artificial seawater) at pH 8.15##
```

```r
###############################################################################################
###Are There Significant Differences Between Treatment (Chemical Foraging Cue Glutathione, Mussel Condiioned Artificial Seawater, Artificial Seawater) and response (overall drop in oxygen concentration) at pH 7.7 ###

###testing assumption 1 - are residuals normally distributed?###

rm(list=ls()) ## Clears R from any previous workspace sessions/objects
library(ggplot2)
library(dplyr)
library(ggpubr)
library(car)

## Inputting data into R and exploring it's dimensions ##
Treatment.Overall.Drop.7.7<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##
ls(Treatment.Overall.Drop.7.7)                         ## examine objects ##
head(Treatment.Overall.Drop.7.7)
str(Treatment.Overall.Drop.7.7)
tail(Treatment.Overall.Drop.7.7)
summary(Treatment.Overall.Drop.7.7)

### shapiro-wilks test for normality ##
shapiro.test(Treatment.Overall.Drop.7.7$Overall.Drop.in.Oxygen.Concentration...)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test output
## data:  Treatment.Overall.Drop.7.7$Overall.Drop.in.Oxygen.Concentration...
## W = 0.85734, p-value = 5.049e-06

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(Treatment.Overall.Drop.7.7$Overall.Drop.in.Oxygen.Concentration...,
         main="Q-Q plot to Visualise Distribution of Overall Drop in Oxygen Concentration Data at pH 7.7 with All Treatments",
         xlab = "Overall Drop in Oxygen Concentration")

##Confirms non-normal distribution##

###testing assumption2 - homogeneity of variance###
leveneTest(Treatment.Overall.Drop.7.7$Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.7.7$Addition, data=Treatment.Overall.Drop.7.7)

## Levene's Test for Homogeneity of Variance (center = median)
##        Df F value Pr(>F)
## group  2  0.2197 0.8034
## 57
##Assumption met of homegeneity of variance

##Transforming non-normal data
Treatment.Overall.Drop.7.7$log.Overall.Drop.in.Oxygen.Concentration...<-log(Treatment.Overall.Drop.7.7$Overall.Drop.in.Oxygen.Concentration...+1)

ggqqplot(Treatment.Overall.Drop.7.7$log.Overall.Drop.in.Oxygen.Concentration...,
         main="Q-Q plot to Visualise Distribution of log Transformed Overall Drop in Oxygen Concentration Data at pH 7.7 with All Treatments",
         xlab = "Overall Drop in Oxygen Concentration")

### shapiro-wilks test for normality ##
shapiro.test(Treatment.Overall.Drop.7.7$log.Overall.Drop.in.Oxygen.Concentration...)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test output
## data:  Treatment.Overall.Drop.7.7$log.Overall.Drop.in.Oxygen.Concentration...
## W = 0.9911, p-value = 0.9411

##Confirms normal distribution
```

```r
###perfroming one-way ANOVA###

lm(Treatment.Overall.Drop.7.7$log.Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.7.7$Addition,
   data = Treatment.Overall.Drop.7.7)        ##fitting a linear model##

qqnorm(resid(lm(Treatment.Overall.Drop.7.7$log.Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.7.7$Addition,
                data = Treatment.Overall.Drop.7.7)))                    ##checking fit of model##

aov.7.7<-anova(lm(Treatment.Overall.Drop.7.7$log.Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.7.7$Addition,
                  data = Treatment.Overall.Drop.7.7))                           ##Running a one-way ANOVA##

## Analysis of Variance Table
## Response: Treatment.Overall.Drop.7.7$log.Overall.Drop.in.Oxygen.Concentration...
##                                  Df  Sum Sq Mean Sq F value  Pr(>F)
## Treatment.Overall.Drop.7.7$Addition  2  2.6195 1.30975  3.3027 0.04394 *
##  Residuals                          57 22.6048 0.39657
##  Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

## As the p-value is  less than the significance level 0.05, we can conclude that there are significant differences between overall drop in oxygen concentration with varying trial environment addition of
##(chemical foraging cue glutathione, artificial seawater and mussel conditioned artificial seawater) at pH 7.7##


###post hoc to determine which means are significantly different
TukeyHSD(aov(lm(Treatment.Overall.Drop.7.7$log.Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.7.7$Addition,
                data = aov.7.7)),
         conf.level=0.95)

##Tukey multiple comparisons of means
##95% family-wise confidence level
##Fit: aov(formula = lm(Treatment.Overall.Drop.7.7$log.Overall.Drop.in.Oxygen.Concentration... ~ Treatment.Overall.Drop.7.7$Addition, data = aov.7.7))
##$`Treatment.Overall.Drop.7.7$Addition`
##                                                    diff          lwr        upr       p adj
##Chemical.Foraging.Cue-Artificial.Seawater                  -0.39562361 -0.8748422568 0.08359503   0.1247470
##Mussel.Conditioned.Artificial.Seawater-Artificial.Seawater    0.08339003 -0.3958286111 0.56260868   0.9080330
##Mussel.Conditioned.Artificial.Seawater-Chemical.Foraging.Cue  0.47901365 -0.0002049984 0.95823229   0.0501207

##The Tukey HSD shows that for log transformed data only overall drop in oxygen concentration recorded with the addition of chemical foraging cue glutathione and
##mussel conditioned artificial seawater are significantly different p ≤ 0.05
```

```
###################################################################################################################################################################################################
###Are There Significant Differences Between Treatment (Chemical Foraging Cue Glutathione, Mussel Condtioned Artificial Seawater, Artificial Seawater) and response (overall drop in oxygen concentration) at pH 7.2 ###

###testing assumption 1 - are residuals normally distributed?###
rm(list=ls()) ## Clears R from any previous workspace sessions/objects
library(ggplot2)    #makes packages available to use
library(dplyr)
library(ggpubr)
library(car)

## Inputting data into R and exploring it's dimensions ##
Treatment.Overall.Drop.7.2<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##
ls(Treatment.Overall.Drop.7.2)                         ## examine objects ##
head(Treatment.Overall.Drop.7.2)
str(Treatment.Overall.Drop.7.2)
tail(Treatment.Overall.Drop.7.2)
summary(Treatment.Overall.Drop.7.2)

### shapiro-wilks test for normality ##
shapiro.test(Treatment.Overall.Drop.7.2$Overall.Drop.in.Oxygen.Concentration...)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
## data:  Treatment.Overall.Drop.7.2$Overall.Drop.in.Oxygen.Concentration...
## W = 0.92904, p-value = 0.001808

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(Treatment.Overall.Drop.7.2$Overall.Drop.in.Oxygen.Concentration...,
         main="Q-Q plot to Visualise Distribution of Overall Drop in Oxygen Concentration Data at pH 7.2 with All Treatments",
         xlab = "Overall Drop in Oxygen Concentration")

##Confirms non-normal distribution##

###testing assumption2 - homogeneity of variance###
leveneTest(Treatment.Overall.Drop.7.2$Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.7.2$Addition, data=Treatment.Overall.Drop.7.2)

## Levene's Test for Homogeneity of Variance (center = median)
##        Df F value  Pr(>F)
## group  2  2.7431 0.07287 .
## 57
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##Assumption met of homegeneity of variance

##Transforming non-normal data
Treatment.Overall.Drop.7.2$log.Overall.Drop.in.Oxygen.Concentration...<-log(Treatment.Overall.Drop.7.2$Overall.Drop.in.Oxygen.Concentration...+1)
ggqqplot(Treatment.Overall.Drop.7.2$log.Overall.Drop.in.Oxygen.Concentration...,
         main="Q-Q plot to Visualise Distribution of log Transformed Overall Drop in Oxygen Concentration Data at pH 7.2 with All Treatments",
         xlab = "Overall Drop in Oxygen Concentration")

### shapiro-wilks test for normality ##
shapiro.test(Treatment.Overall.Drop.7.2$log.Overall.Drop.in.Oxygen.Concentration...)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
## data:  Treatment.Overall.Drop.7.2$log.Overall.Drop.in.Oxygen.Concentration...
## W = 0.96671, p-value = 0.1006

##confirms normal distribution of log transformed data
```

```
###perfroming one-way ANOVA###
lm(Treatment.Overall.Drop.7.2$log.Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.7.2$Addition,
   data = Treatment.Overall.Drop.7.2)        ##fitting a linear model##

qqnorm(resid(lm(Treatment.Overall.Drop.7.2$log.Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.7.2$Addition,
                data = Treatment.Overall.Drop.7.2)))                    ##checking fit of model##

aov.7.2<-anova(lm(Treatment.Overall.Drop.7.2$log.Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.7.2$Addition,
         data = Treatment.Overall.Drop.7.2))                            ##Running a one-way ANOVA##

## Analysis of Variance Table
## Response: Treatment.Overall.Drop.7.2$log.Overall.Drop.in.Oxygen.Concentration...
##                                Df  Sum Sq Mean Sq F value    Pr(>F)
## Treatment.Overall.Drop.7.2$Addition  2  6.3115 3.15576  8.8138 0.0004622 ***
##  Residuals                          57 20.4088 0.35805
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

## As the p-value is  less than the significance level 0.05, we can conclude that there are significant differences between overall drop in oxygen concentration with varying trial environment addition of
##(chemical foraging cue glutathione, artificial seawater and mussel conditioned artificial seawater) at pH 7.2##

###post hoc to determine which means are significantly different
TukeyHSD(aov(lm(Treatment.Overall.Drop.7.2$log.Overall.Drop.in.Oxygen.Concentration...~Treatment.Overall.Drop.7.2$Addition,
                data = aov.7.2)),
         conf.level=0.95)

##Tukey multiple comparisons of means
##95% family-wise confidence level
##Fit: aov(formula = lm(Treatment.Overall.Drop.7.2$log.Overall.Drop.in.Oxygen.Concentration... ~ Treatment.Overall.Drop.7.2$Addition, data = aov.7.2))
##$`Treatment.Overall.Drop.7.2$Addition`
##                                                    diff        lwr         upr     p adj
##Chemical.Foraging.Cue-Artificial.Seawater                -0.4659364 -0.9212830 -0.01058973 0.0438056
# Mussel.Conditioned.Artificial.Seawater-Artificial.Seawater    0.3242948 -0.1310518  0.77964143 0.2088636
##Mussel.Conditioned.Artificial.Seawater-Chemical.Foraging.Cue  0.7902312  0.3348845  1.24557780 0.0002989

##The Tukey HSD shows that for log transformed data overall drop in oxygen concentration recorded with the addition of chemical foraging cue glutathione and artificial seawater
## glutathione are significanlty different p ≤ 0.05, alongside chemical foraging cue glutathione and mussel conditioned artificial seawater p ≤ 0.05
```

Pagurus bernhardus Weight

```
###############################################################################################################################################################
###Pagurus bernhardus Weight###

###Figures###

#################################################################################################
##### Is there Correlation Between Weight and Overall Drop in Oxygen Concentration or Detection Threshold Concentration for Chemical Foraging Cue at pH 8.15###

rm(list = ls())       #Clears R from previous workspace session/objects#
library(dplyr)        #Relevent packages
library(ggplot2)
library(gridExtra)    #layout of graphics window#

weight<-read.csv(file.choose())     #locate and read in file#
str(weight)  #looking at data#

###Scatter Plot of weight Against Overall Drop in Oxygen Concentration (%), pH 8.15 Only####

plot1<-ggplot(weight, aes(x=weight$Weight.g., y=weight$Overall.Drop.in.Oxygen.Concetration...))+    #Produces scatter plot
  geom_point()+
  ggtitle("A) Does Weight of Hermit Indivduals Influence Overall Oxygen Consumption?")+   #Adds title#
  theme(plot.title = element_text(hjust = 0.5))+    #Adjust postion of title#
  xlab("Weight (g)")+    #Adds x axis label#
  ylab("Overall Drop in Oxygen Concentration (%)")+     #Adds y axis label#
  theme(axis.line = element_line(color = "black",size = 0.2))+    #Adds axis line#
  theme(panel.grid.major = element_line(colour = "black",size = 0.05),panel.grid.minor = element_line(colour = "black",size = 0.05),
        panel.background = element_blank(), axis.line = element_line(colour = "black"))  #Formats grid background and eliminates grey colour#
plot1

###Scattergraph of Weight Against Detection Threshold Concentration for Chemical Foraging Cue Glutathione, pH 8.15 Only####

plot2<-ggplot(weight, aes(x=weight$Weight.g.,y=weight$cue.concentration))+
  geom_point()+
  ggtitle("B) Does Weight of Hermit Indivduals Influence Detection Thresholds for Chemical Foraging Cue Glutathione?")+   #Adds title#
  theme(plot.title = element_text(hjust = 0.5))+    #Adjust postion of title#
  xlab("Weight (g)")+    #Adds x axis label#
  ylab("Detection Threshold Concentration (e-x M/l)")+     #Adds y axis label#
  theme(axis.line = element_line(color = "black",size = 0.2))+    #Adds axis line#
  theme(panel.grid.major = element_line(colour = "black",size = 0.05),panel.grid.minor = element_line(colour = "black",size = 0.05),
        panel.background = element_blank(), axis.line = element_line(colour = "black"))  #Formats grid background and eliminates grey colour#
plot2

####Formatting plots to lay side-by-side####
grid.arrange(plot1,plot2)
```

```
###Statistical Analysis###

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

###Is There Correlation Between Weight and Overall Drop in Oxygen Concentration (%), with the Addition of Chemical Foraging Cue Glutathione at pH 8.15###

rm(list=ls()) ## Clears R from any previous workspace sessions/objects

## Inputting data into R and exploring it's dimensions ##
weight.overall.oxygen.consumption<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##

ls(weight.overall.oxygen.consumption)                         ## examine objects ##
head(weight.overall.oxygen.consumption)
str(weight.overall.oxygen.consumption)
tail(weight.overall.oxygen.consumption)
summary(weight.overall.oxygen.consumption)

library(ggplot2)  ###load in packages###
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###

### shapiro-wilks test for normality ###
shapiro.test(weight.overall.oxygen.consumption$Overall.Drop.in.Oxygen.Concetration...)    ### Carrying out a Shapiro-Wilk normality test ###

##Shapiro-Wilk normality test output##
## data:  weight.overall.oxygen.consumption$Overall.Drop.in.Oxygen.Concetration...
##W = 0.93709, p-value = 0.3823

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(weight.overall.oxygen.consumption$Overall.Drop.in.Oxygen.Concetration...,
         main="Q-Q plot to Visualise Distribution of Overall Oxygen Drop at pH 8.15",
         xlab = "Overall Drop in Oxygen Concentration")

## CONFIRMS NORMAL DISTRIBUTION
```

```r
###Application of Linear Regression Analysis###

linearmod2<-lm(weight.overall.oxygen.consumption$Overall.Drop.in.Oxygen.Concetration...~weight.overall.oxygen.consumption$Weight.g.,
               data = weight.overall.oxygen.consumption)
linearmod2

## Call:
##   lm(formula = weight.overall.oxygen.consumption$Overall.Drop.in.Oxygen.Concetration... ~
##        weight.overall.oxygen.consumption$Weight.g., data = weight.overall.oxygen.consumption)
## Coefficients:
##   (Intercept)  weight.overall.oxygen.consumption$Weight.g.
## 4.528                                       -1.861

summary(linearmod2)

## Call:
##   lm(formula = weight.overall.oxygen.consumption$Overall.Drop.in.Oxygen.Concetration... ~
##        weight.overall.oxygen.consumption$Weight.g., data = weight.overall.oxygen.consumption)

## Residuals:
##   Min      1Q  Median      3Q     Max
## -3.0852 -0.7939 -0.3085  0.7255  3.4432

## Coefficients:
##                                          Estimate Std. Error t value Pr(>|t|)
## (Intercept)                                 4.528      1.344   3.369  0.00558 **
##   weight.overall.oxygen.consumption$Weight.g.   -1.861      1.066  -1.747  0.10624

##   Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

## Residual standard error: 1.711 on 12 degrees of freedom
## Multiple R-squared:  0.2027, Adjusted R-squared:  0.1362
## F-statistic:  3.05 on 1 and 12 DF,  p-value: 0.1062

## Both p values are greater than 0.05, t=-1.7, R(squared)=0.20 therefore not significant and we cannot reject the null hypothesis
##there is no correlation between weight and overall drop in oxygen concetration (%) and any correlation, if seen, is likely to be because of chance.
```

```r
################################################################################
###Is there Correlation Between Weight and Detection Threshold Concentrations for Chemical Foraging Cue Glutathione at pH 8.15###

rm(list=ls()) ## Clears R from any previous workspace sessions/objects

## Inputting data into R and exploring it's dimensions ##
weight.detection.threshold<-read.csv(file.choose())    ## Read in data as this name from chosen file which have the option to locate ##

ls(weight.detection.threshold)                         ## examine objects ##
head(weight.detection.threshold)
str(weight.detection.threshold)
tail(weight.detection.threshold)
summary(weight.detection.threshold)

library(ggplot2)  ###load in packages##
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###

### shapiro-wilks test for normality ###
shapiro.test(weight.detection.threshold$cue.concentration)   ### Carrying out a Shapiro-Wilk normality test ###

##Shapiro-Wilk normality test output

##Shapiro-Wilk normality test
##data:  weight.detection.threshold$cue.concentration
##W = 0.81926, p-value = 0.008723

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(weight.detection.threshold$cue.concentration,
         main="Q-Q plot to Visualise Distribution of Detection Threshold Concentration data at pH 8.15",
         xlab = "Detection Threshold Concentration")

## CONFIRMS NON-NORMAL DISTRIBUTION

#####LOG TRANSFORMING DATA###

weight.detection.threshold$log.cue.concentration<-log(weight.detection.threshold$cue.concentration)

### shapiro-wilks test for normality ###
shapiro.test(weight.detection.threshold$log.cue.concentration)   ### Carrying out a Shapiro-Wilk normality test ###

##Shapiro-Wilk normality test output

##Shapiro-Wilk normality test
##data:  weight.detection.threshold$log.cue.concentration
##W = 0.82915, p-value = 0.1171

ggqqplot(weight.detection.threshold$log.cue.concentration)

##Confirms normal distribution of log-transformed data
```

```
###linear regression###
linearmod1<-lm(weight.detection.threshold$log.cue.concentration~weight.detection.threshold$Weight.g.,
               data = weight.detection.threshold)

print(linearmod1)

## print(linearmod1)
## Call:
## lm(formula = weight.detection.threshold$log.cue.concentration ~
##     weight.detection.threshold$Weight.g., data = weight.detection.threshold)
## Coefficients:
## (Intercept)  weight.detection.threshold$Weight.g.
## 2.1894                              -0.2904

summary(linearmod1)
## Call:
## lm(formula = weight.detection.threshold$log.cue.concentration ~
## weight.detection.threshold$Weight.g., data = weight.detection.threshold)
## Residuals:
##  Min       1Q   Median       3Q      Max
## -0.30936 -0.17334  0.07017  0.11516  0.29666
## Coefficients:
##                                    Estimate Std. Error t value Pr(>|t|)
## (Intercept)                         2.1894     0.1698  12.895 2.16e-08 ***
## weight.detection.threshold$Weight.g.  -0.2904     0.1346  -2.157    0.052 .
##Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## Residual standard error: 0.2161 on 12 degrees of freedom
## Multiple R-squared:  0.2794, Adjusted R-squared:  0.2193
## F-statistic: 4.652 on 1 and 12 DF,  p-value: 0.052

##### Both p values are greater than 0.05, t=-2.157, R(squared)=0.2794  therefore not significiant and we cannot reject the null hypothesis, there is no correlation
#### between weight and the detection threshold concentration of chemical foraging cue glutathione at pH 8.15 - any correlation, if seen, will be due to chance
```

Chapter Four: Biological Assay Exploring Individual Response to Ocean Acidification

Statistical Analysis – R Script

Impact of Olfactory Cues, pH and Identifying Individual Response

```
#####################################################################################################################################################
#####################################################################################################################################################
###facet wrapped bar plots at pH 8.15 with all additions to show frequency of behavioural responses###
rm(list = ls())       #Clears R from previous workspace session/objects#
library(ggplot2)

pH8.15.chapter.four<-read.csv(file.choose())     #locate and read in file#
str(pH8.15.chapter.four)                    #looking at data#


pH8.15.chapter.four$Individual2<-factor(pH8.15.chapter.four$Individual, levels = c("Individual 7","Individual 9","Individual 12",
                                                                                   "Individual 14","Individual 15","Individual 16","Individual 19","Individual 20"))   #Reorder facet plot
Response<-pH8.15.chapter.four$Response                                                                                                                  #used to change legend title#

ggplot(data = pH8.15.chapter.four, aes(fill=Response, x=pH8.15.chapter.four$Addition, y=pH8.15.chapter.four$Frequency))+    #creates bar plot#
  geom_bar(position="dodge", stat = "identity", colour="black", width = 0.6)+
  scale_fill_manual(values = c("Grey40","LightGrey","White"))+                                                                                  #formats colours of bars - aimed to match format of previous thesis graphs#
  ggtitle("Response of Individuals to the Presence of Chemical Foraging Cue Glutathione, Artificial Seawater and Mussel Conditioned Artificial Seawater at pH 8.15")+   #Adds title#
  theme(plot.title = element_text(hjust = 0.5), axis.text.x = element_text(size = 10, angle = 90, hjust = 1))+    #Adjust postion of title#
  xlab("")+                                                                                                                                     #Adds x axis label (no label wanted)#
  ylab("Frequency")+                                                                                                                            #Adds y axis label#
  theme(axis.line = element_line(color = "black",size = 0.2))+                                                                                  #Adds axis line#
  theme(panel.grid.major = element_line(colour = "grey90",size = 0.2),panel.grid.minor = element_line(colour = "grey98",size = 0.5),
        panel.background = element_rect(fill = "white",colour = NA), axis.line = element_blank(), panel.border = element_rect(fill=NA, colour="grey50"),
        strip.background = element_rect(fill = "grey80",colour = "grey50"), legend.position = "bottom", plot.title = element_text(size = 12))+      #Formats grid background, border, axis lines, legend position and text size#
  facet_wrap(~pH8.15.chapter.four$Individual2, nrow = 2)                                                                                        #facet wraps acording to individual#

#####################################################################################################################################################
#####################################################################################################################################################
###facet wrapped bar plots at pH 7.7 with all additions to show frequency of behavioural responses###
rm(list = ls())       #Clears R from previous workspace session/objects#
library(ggplot2)

pH7.7.chapter.four<-read.csv(file.choose())     #locate and read in file#
str(pH7.7.chapter.four)                    #looking at data#


pH7.7.chapter.four$Individual2<-factor(pH7.7.chapter.four$Individual, levels = c("Individual 7","Individual 9","Individual 12",
                                                                                 "Individual 14","Individual 15","Individual 16","Individual 19","Individual 20"))   #Reorder facet plot
Response<-pH7.7.chapter.four$Response                                                                                                                 #used to change legend title#

ggplot(data = pH7.7.chapter.four, aes(fill=Response, x=pH7.7.chapter.four$Addition, y=pH7.7.chapter.four$Frequency))+    #creates bar plot#
  geom_bar(position="dodge", stat = "identity", colour="black", width = 0.6)+
  scale_fill_manual(values = c("Grey40","LightGrey","White"))+                                                                                #formats colours of bars - aimed to match format of previous thesis graphs#
  ggtitle("Response of Individuals to the Presence of Chemical Foraging Cue Glutathione, Artificial Seawater and Mussel Conditioned Artificial Seawater at pH 7.7")+   #Adds title#
  theme(plot.title = element_text(hjust = 0.5), axis.text.x = element_text(size = 10, angle = 90, hjust = 1))+    #Adjust postion of title#
  xlab("")+                                                                                                                                   #Adds x axis label (no label wanted)#
  ylab("Frequency")+                                                                                                                          #Adds y axis label#
  theme(axis.line = element_line(color = "black",size = 0.2))+                                                                                #Adds axis line#
  theme(panel.grid.major = element_line(colour = "grey90",size = 0.2),panel.grid.minor = element_line(colour = "grey98",size = 0.5),
        panel.background = element_rect(fill = "white",colour = NA), axis.line = element_blank(), panel.border = element_rect(fill=NA, colour="grey50"),
        strip.background = element_rect(fill = "grey80",colour = "grey50"), legend.position = "bottom", plot.title = element_text(size = 12))+      #Formats grid background, border, axis lines, legend position and text size#
  facet_wrap(~pH7.7.chapter.four$Individual2, nrow = 2)                                                                                       #facet wraps acording to individual#
```

```
###facet wrapped bar plots at pH 7.2 with all additions to show frequency of behavioural responses###
rm(list = ls())       #Clears R from previous workspace session/objects#
library(ggplot2)

pH7.2.chapter.four<-read.csv(file.choose())     #locate and read in file#
str(pH7.2.chapter.four)                    #looking at data#


pH7.2.chapter.four$Individual2<-factor(pH7.2.chapter.four$Individual, levels = c("Individual 7","Individual 9","Individual 12",
                                                                                 "Individual 14","Individual 15","Individual 16","Individual 19","Individual 20"))   #Reorder facet plot
Response<-pH7.2.chapter.four$Response                                                                                                                 #used to change legend title#

ggplot(data = pH7.2.chapter.four, aes(fill=Response, x=pH7.2.chapter.four$Addition, y=pH7.2.chapter.four$Frequency))+    #creates bar plot#
  geom_bar(position="dodge", stat = "identity", colour="black", width = 0.6)+
  scale_fill_manual(values = c("Grey40","LightGrey","White"))+                                                                                #formats colours of bars - aimed to match format of previous thesis graphs#
  ggtitle("Response of Individuals to the Presence of Chemical Foraging Cue Glutathione, Artificial Seawater and Mussel Conditioned Artificial Seawater at pH 7.2")+   #Adds title#
  theme(plot.title = element_text(hjust = 0.5), axis.text.x = element_text(size = 10, angle = 90, hjust = 1))+    #Adjust postion of title#
  xlab("")+                                                                                                                                   #Adds x axis label (no label wanted)#
  ylab("Frequency")+                                                                                                                          #Adds y axis label#
  theme(axis.line = element_line(color = "black",size = 0.2))+                                                                                #Adds axis line#
  theme(panel.grid.major = element_line(colour = "grey90",size = 0.2),panel.grid.minor = element_line(colour = "grey98",size = 0.5),
        panel.background = element_rect(fill = "white",colour = NA), axis.line = element_blank(), panel.border = element_rect(fill=NA, colour="grey50"),
        strip.background = element_rect(fill = "grey80",colour = "grey50"), legend.position = "bottom", plot.title = element_text(size = 12))+      #Formats grid background, border, axis lines, legend position and text size#
  facet_wrap(~pH7.2.chapter.four$Individual2, nrow = 2)                                                                                       #facet wraps acording to individual#
```

```
##########################################################################################################################################################
### Boxplots of averages at all pH levels ###

###repsonse boxplot with the addition of chemical foraging cue###
rm(list = ls())   #clear R workspace#
library(ggplot2)  #installs graphical package#
Chapter.Four.All.pH.Cue.Response<-read.csv(file.choose()) #reads in data#

ls(Chapter.Four.All.pH.Cue.Response)    #examines data#
head(Chapter.Four.All.pH.Cue.Response)
str(Chapter.Four.All.pH.Cue.Response)
tail(Chapter.Four.All.pH.Cue.Response)
summary(Chapter.Four.All.pH.Cue.Response)

Response1<-Chapter.Four.All.pH.Cue.Response$Response<-factor(Chapter.Four.All.pH.Cue.Response$Response, labels = c("Interaction","No Interaction","Walked Over"))   #creating factors and respective labels#
Chapter.Four.All.pH.Cue.Response$pH<-factor(Chapter.Four.All.pH.Cue.Response$pH)    #creates factor#

Cue<-ggplot(Chapter.Four.All.pH.Cue.Response, aes(x=Chapter.Four.All.pH.Cue.Response$pH, y=Chapter.Four.All.pH.Cue.Response$Frequency))+
  geom_boxplot(aes(fill=Response1), position = position_dodge(0.8))+    #generates boxplot#
  scale_fill_manual(name="Behavioural Response", values = c("Grey40","lightgrey","white"))+ #labels legend and manually selects graphics colours#
  geom_point()+                                     ##adds data points to boxplots##
  stat_boxplot(geom = 'errorbar', width=0.2)+       ##Adds whisker ends to boxplots##
  theme_minimal() +                                 ##Inserts theme##
  ggtitle("A) Frequency of Response Exhibited by Individuals with the Addition of Chemical Foraging Cue at All pH Levels")+  ##adds main title##
  theme(plot.title = element_text(hjust = 0.5))+    ##centers main title##
  xlab("pH")+                                       ##x axis label##
  ylab("")+        ##y axis label/blank##
  theme(axis.line = element_line(color = "black",size = 0.2), legend.position = "none")   ##adding and formatting axis lines/removing legend##


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

###repsonse boxplot with the addition of Artificial Seawater###
Chapter.Four.All.pH.Seawater.Response<-read.csv(file.choose())     #reads in data#

ls(Chapter.Four.All.pH.Seawater.Response)     #examines data#
head(Chapter.Four.All.pH.Seawater.Response)
str(Chapter.Four.All.pH.Seawater.Response)
tail(Chapter.Four.All.pH.Seawater.Response)
summary(Chapter.Four.All.pH.Seawater.Response)

Response2<-factor(Chapter.Four.All.pH.Seawater.Response$Response, labels = c("Interaction","No Interaction","Walked Over"))       #creating factors and respective labels#
Chapter.Four.All.pH.Seawater.Response$pH<-factor(Chapter.Four.All.pH.Seawater.Response$pH)    #creates factor#

Seawater<-ggplot(Chapter.Four.All.pH.Seawater.Response, aes(x=Chapter.Four.All.pH.Seawater.Response$pH, y=Chapter.Four.All.pH.Seawater.Response$Frequency))+
  geom_boxplot(aes(fill=Response2), position = position_dodge(0.8))+          #generates boxplot#
  scale_fill_manual(name="Behavioural Response", values = c("Grey40","lightgrey","white"))+    #labels legend and manually selects graphics colours#
  geom_point()+                                     ##adds data points to boxplots##
  stat_boxplot(geom = 'errorbar', width=0.2)+       ##Adds whisker ends to boxplots##
  theme_minimal() +                                 ##Inserts theme##
  ggtitle("B) Frequency of Response Exhibited by Individuals with the Addition of Artificial Seawater at All pH Levels")+  ##adds main title##
  theme(plot.title = element_text(hjust = 0.5))+    ##centers main title##
  xlab("pH")+                                       ##x axis label##
  ylab("Frequency")+        ##y axis label##
  theme(axis.line = element_line(color = "black",size = 0.2), legend.position = "none")   ##adding and formatting axis lines/removing legend##

```

```
###repsonse boxplot with the addition of Mussel Conditioned Artificial Seawater###
Chapter.Four.All.pH.Mussel.Seawater.Response<-read.csv(file.choose())       #reads in data#

ls(Chapter.Four.All.pH.Mussel.Seawater.Response)    #examines data#
head(Chapter.Four.All.pH.Mussel.Seawater.Response)
str(Chapter.Four.All.pH.Mussel.Seawater.Response)
tail(Chapter.Four.All.pH.Mussel.Seawater.Response)
summary(Chapter.Four.All.pH.Mussel.Seawater.Response)

Response3<-factor(Chapter.Four.All.pH.Mussel.Seawater.Response$Response, labels = c("Interaction","No Interaction","Walked Over"))        #creating factors and respective labels#
Chapter.Four.All.pH.Mussel.Seawater.Response$pH<-factor(Chapter.Four.All.pH.Mussel.Seawater.Response$pH)      #creates factor#

Mussel<-ggplot(Chapter.Four.All.pH.Mussel.Seawater.Response, aes(x=Chapter.Four.All.pH.Mussel.Seawater.Response$pH, y=Chapter.Four.All.pH.Mussel.Seawater.Response$Frequency))+
  geom_boxplot(aes(fill=Response3), position = position_dodge(0.8))+          #generates boxplot#
  scale_fill_manual(name="Behavioural Response", values = c("Grey40","lightgrey","white"))+   #labels legend and manually selects graphics colours#
  geom_point()+                                     ##adds data points to boxplots##
  stat_boxplot(geom = 'errorbar', width=0.2)+       ##Adds whisker ends to boxplots##
  theme_minimal() +                                 ##Inserts theme##
  ggtitle("C) Frequency of Response Exhibited by Individuals with the Addition of Mussel Conditioned Artificial Seawater at All pH Levels")+  ##adds main title##
  theme(plot.title = element_text(hjust = 0.5))+    ##centers main title##
  xlab("pH")+                                       ##x axis label##
  ylab("")+        ##y axis label/blank##
  theme(axis.line = element_line(color = "black",size = 0.2), legend.position = "bottom")   ##adding and formatting axis lines/repositioning legend##

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

###Combining all three plots together###
library(gridExtra)
grid.arrange(Cue,Seawater,Mussel)
```

```
#####################################################################################################################################################
###One-Way ANOVA for pH and engagment response frequency###
#################################################################
### shapiro-wilks test for normality ###
shapiro.test(frequency.of.response.pH.and.addition$Frequeny.of.Engagement)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
## data:  frequency.of.response.pH.and.addition$Frequeny.of.Engagement
##W = 0.85258, p-value = 7.005e-07


### Plotting a QQ plot to visualise data distribution/normality ###
library(ggplot2)                                        #graphical package#
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###
ggqqplot(frequency.of.response.pH.and.addition$Frequeny.of.Engagement,
         main="Q-Q plot to Visualise Distribution of Engagement Frequency with all Additions Data at all pH Levels",
         xlab = "Individual Detection Thresholds")

## CONFIRMS NON-NORMAL DISTRIBUTION

#################################################################
###Testing Assumption 2 - Homogeneity of Variance###
library(car)
leveneTest(frequency.of.response.pH.and.addition$Frequeny.of.Engagement~frequency.of.response.pH.and.addition$pH,
           data=frequency.of.response.pH.and.addition)

## Levene's Test for Homogeneity of Variance (center = median)
##        Df F value Pr(>F)
## group  2  1.1237  0.331
##       68
##Assumption met of homegeneity of variance

leveneTest(frequency.of.response.pH.and.addition$Frequeny.of.Engagement~frequency.of.response.pH.and.addition$Addition,
           data=frequency.of.response.pH.and.addition)

## Levene's Test for Homogeneity of Variance (center = median)
##        Df F value Pr(>F)
## group  2  1.4615 0.2391
##       68
##Assumption met of homegeneity of variance

#################################################################
### Transforming non-normal data ###
frequency.of.response.pH.and.addition$log.Frequeny.of.Engagement<-
  log(frequency.of.response.pH.and.addition$Frequeny.of.Engagement)      ##log transforming data to create normally distributed data##

shapiro.test(frequency.of.response.pH.and.addition$log.Frequeny.of.Engagement)
ggqqplot(Impact.of.pH.on.Detection.Threshold$log.Detection.Concentration,
         main="Q-Q plot to Visualise Distribution of log Transformed Engagement Frequency with all Additions Data at all pH Levels",
         xlab = "Individual Detection Thresholds")    ###checking distribution of log transformed data###

##Still non-normal distribution - choose non-parametric version of test##
##Kruskal-Wallis##
```

```
kruskal.test(frequency.of.response.pH.and.addition$Frequeny.of.Engagement~frequency.of.response.pH.and.addition$pH,data = frequency.of.response.pH.and.addition)

##Kruskal-Wallis rank sum test
##data:  frequency.of.response.pH.and.addition$Frequeny.of.Engagement by frequency.of.response.pH.and.addition$pH
##Kruskal-Wallis chi-squared = 5.0335, df = 2, p-value = 0.08072


#p-value is greater the 0.05, so can confirm that there is no significant difference in mean frequency of engagement
#response with variation in pH
```

```r
###One-Way ANOVA for addition and engagment response frequency###
#####################################################################
### shapiro-wilks test for normality ###
shapiro.test(frequency.of.response.pH.and.addition$Frequeny.of.Engagement)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
## data:  frequency.of.response.pH.and.addition$Frequeny.of.Engagement
##W = 0.85258, p-value = 7.005e-07


### Plotting a QQ plot to visualise data distribution/normality ###
library(ggplot2)                                    #graphical package#
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###
ggqqplot(frequency.of.response.pH.and.addition$Frequeny.of.Engagement,
         main="Q-Q plot to Visualise Distribution of Engagement Frequency with all Additions Data at all pH Levels",
         xlab = "Individual Detection Thresholds")

## CONFIRMS NON-NORMAL DISTRIBUTION

#####################################################################
###Testing Assumption 2 - Homogeneity of Variance###
library(car)
leveneTest(frequency.of.response.pH.and.addition$Frequeny.of.Engagement~frequency.of.response.pH.and.addition$Addition,
           data=frequency.of.response.pH.and.addition)

## Levene's Test for Homogeneity of Variance (center = median)
##        Df F value Pr(>F)
## group  2  1.4615 0.2391
##       68
##Assumption met of homegeneity of variance

#####################################################################
### Transforming non-normal data ###
frequency.of.response.pH.and.addition$log.Frequeny.of.Engagement<-
  log(frequency.of.response.pH.and.addition$Frequeny.of.Engagement)      ##log transforming data to create normally distributed data##

shapiro.test(frequency.of.response.pH.and.addition$log.Frequeny.of.Engagement)
ggqqplot(Impact.of.pH.on.Detection.Threshold$log.Detection.Concentration,
         main="Q-Q plot to Visualise Distribution of log Transformed Engagement Frequency with all Additions Data at all pH Levels",
         xlab = "Individual Detection Thresholds")    ###checking distribution of log transformed data###

##Still non-normal distribution - choose non-parametric version of test##
##Kruskal-Wallis##

kruskal.test(frequency.of.response.pH.and.addition$Frequeny.of.Engagement~frequency.of.response.pH.and.addition$Addition,data = frequency.of.response.pH.and.addition)

#Kruskal-Wallis rank sum test
#data:  frequency.of.response.pH.and.addition$Frequeny.of.Engagement by frequency.of.response.pH.and.addition$Addition
#Kruskal-Wallis chi-squared = 11.82, df = 2, p-value = 0.002712

#p-value is smaller the 0.05, so can confirm that there is significant difference in mean frequency of engagement
#response with variation in environmental addition (chemical foraging cue glutathione/artificial seawater/mussel conditioned artificial seawater)
```

```r
###pairwise-comparison between groups###
pairwise.wilcox.test(frequency.of.response.pH.and.addition$Frequeny.of.Engagement,frequency.of.response.pH.and.addition$Addition,
                     p.adjust.method = "BH")

#Pairwise comparisons using Wilcoxon rank sum test
#data:  frequency.of.response.pH.and.addition$Frequeny.of.Engagement and frequency.of.response.pH.and.addition$Addition
#                                  Artificial Seawater Glutathione
#Glutathione                         0.0107              -
#Mussel Conditioned Artificial Seawater 0.0033             0.7490

##Pairwise comparisonshows that only mussel conditioned artificial seawater and artificial seawater are significantly different

#####################################################################################################################################################################
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```

```r
##Chapter Four - Detection Threshold Concentration of Olfactory Cues and Response Level##
##Frequency of Engagement and Detection Threshold Concentration##
##generating boxplots of frequency of engagement against threshold concentration at all pH levels##
##chemical foraging cue glutathione and mussel conditioned artificial seawater##

rm(list=ls())         #clears workspace#

threshold.frequency<-read.csv(file.choose())   #reads in data#
str(threshold.frequency)                       #examine data#
head(threshold.frequency)

library(ggplot2)

threshold.frequency$pH<-factor(threshold.frequency$pH, levels = c("7.2","7.7","8.15"), labels =c("7.2","7.7","8.15"))               #sets pH as factor#
threshold.frequency$Detection.Threshold.Concentration<-factor(threshold.frequency$Detection.Threshold.Concentration)    #sets detection threshold concentration as a factor#
threshold.frequency$Addition<-factor(threshold.frequency$Addition, levels = c("cue","mussel"),labels = c("Glutathione Addition","Mussel Addition"))

plot1<-ggplot(threshold.frequency, aes(x=threshold.frequency$Detection.Threshold.Concentration, y=threshold.frequency$Frequency.of.Engagement))+
  geom_boxplot(aes(fill=threshold.frequency$pH), position = position_dodge(0.8))+    #generates boxplot#
  scale_fill_manual(name="pH", values = c("Grey40","lightgrey","white"))+   #sets colours#
  geom_point()+                                  #adds data points to boxplots#
  stat_boxplot(geom = 'errorbar', width=0.2)+    #Adds whisker ends to boxplots#
  ggtitle("Frequency of Engagement by Individuals within Trials against their respective Detection Threshold Concentration")+  ##dds main title#
  theme(plot.title = element_text(hjust = 0.5))+ #centers main title#
  xlab("Detection Threshold Concentration for Glutathione (e-x M/l)")+              #x axis label#
  ylab("Frequency of Engagement")+        #y axis label#
  scale_y_continuous(breaks = seq(0,5,by=1))+   #sets y axis scale#
  facet_grid(threshold.frequency$Addition~threshold.frequency$pH)+      #facet wraps plot according to pH and trial addition#
  theme(axis.line = element_line(color = "black",size = 0.2))+                                  #Adds axis line#
  theme(panel.grid.major = element_line(colour = "grey90",size = 0.2),panel.grid.minor = element_line(colour = "grey98",size = 0.5),
        panel.background = element_rect(fill = "white",colour = NA), axis.line = element_blank(), panel.border = element_rect(fill=NA, colour="grey50"),
        strip.background = element_rect(fill = "grey80",colour = "grey50"), legend.position = "bottom", plot.title = element_text(size = 12))      # sets theme/formats for grid, background and legend#
plot1
```

```r
###performing linear regression to determine if correlation exists between frequency of engagment response of the population and detection threshold concentration ###

rm(list=ls())         #clears workspace#

frequency.of.response.detection.threshold.8.15.glutathione<-read.csv(file.choose())   #reads in data#
str(frequency.of.response.detection.threshold.8.15.glutathione)                       #examine data#
head(frequency.of.response.detection.threshold.8.15.glutathione)

library(ggplot2)    #oading relevant packages#
library(dplyr)
library(ggpubr)

###detection threshld at 8.15 with chemical foraging cue and frequency of engagement response###
### shapiro-wilks test for normality ###
shapiro.test(frequency.of.response.detection.threshold.8.15.glutathione$Frequency.of.Engagement.Response)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
## data:  frequency.of.response.detection.threshold.8.15.glutathione$Frequency.of.Engagement.Response
## W = 0.87676, p-value = 0.1753

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(frequency.of.response.detection.threshold.8.15.glutathione$Frequency.of.Engagement.Response,
         main="Q-Q plot to Visualise Distribution of Engagement Frequency with Glutathione Addition Data at pH 8.15",
         xlab = "Engagement Frequency")

## CONFIRMS NORMAL DISTRIBUTION

linearmodel1<-lm(frequency.of.response.detection.threshold.8.15.glutathione$Frequency.of.Engagement.Response~frequency.of.response.detection.threshold.8.15.glutathione$Detection.Threshold.Concentration,
                 data = frequency.of.response.detection.threshold.8.15.glutathione)
#Call:
#  lm(formula = frequency.of.response.detection.threshold.8.15.glutathione$Frequency.of.Engagement.Response ~
#       frequency.of.response.detection.threshold.8.15.glutathione$Detection.Threshold.Concentration,
#     data = frequency.of.response.detection.threshold.8.15.glutathione)
#Coefficients:
#(Intercept)
#1.0000
#frequency.of.response.detection.threshold.8.15.glutathione$Detection.Threshold.Concentration
#0.1887
```

```
summary(linearmodel1)
#Call:
#  lm(formula = frequency.of.response.detection.threshold.8.15.glutathione$Frequency.of.Engagement.Response ~
#       frequency.of.response.detection.threshold.8.15.glutathione$Detection.Threshold.Concentration,
#     data = frequency.of.response.detection.threshold.8.15.glutathione)
#Residuals:
#  Min      1Q  Median      3Q     Max
#-2.5094 -0.8679  0.3679  1.1651  1.6792
#Coefficients:                                                                                    Estimate
#(Intercept)                                                                                      1.0000
#frequency.of.response.detection.threshold.8.15.glutathione$Detection.Threshold.Concentration     0.1887
#                                                                                                 Std. Error
#(Intercept)                                                                                        2.5555
#frequency.of.response.detection.threshold.8.15.glutathione$Detection.Threshold.Concentration      0.3753
#                                                                                                 t value
#(Intercept)                                                                                      0.391
#frequency.of.response.detection.threshold.8.15.glutathione$Detection.Threshold.Concentration     0.503
#                                                                                                 Pr(>|t|)
#(Intercept)                                                                                       0.709
#frequency.of.response.detection.threshold.8.15.glutathione$Detection.Threshold.Concentration      0.633

#Residual standard error: 1.673 on 6 degrees of freedom
#Multiple R-squared:  0.04043,  Adjusted R-squared:  -0.1195
#F-statistic: 0.2528 on 1 and 6 DF,  p-value: 0.633

###Both p values are greater than 0.05, cannot accept the hyothesis - there is no correlation between detection
#threshold concentration for chemical foraging cue glutathione and frequency of engagement response
#however small sample size could have significantly affected this
```

```
###detection threshld at 8.15 with mussel conditioned artificial seawater and frequency of engagement response###
rm(list=ls())        #clears workspace#

frequency.of.response.detection.threshold.8.15.mussel<-read.csv(file.choose())   #reads in data#
str(frequency.of.response.detection.threshold.8.15.mussel)                       #examine data#
head(frequency.of.response.detection.threshold.8.15.mussel)

library(ggplot2)  #loading relevant packages#
library(dplyr)
library(ggpubr)

### shapiro-wilks test for normality ###
shapiro.test(frequency.of.response.detection.threshold.8.15.mussel$Frequency.of.Engagement.Response)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
## data:  frequency.of.response.detection.threshold.8.15.mussel$Frequency.of.Engagement.Response
## W = 0.74784, p-value = 0.007732

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(frequency.of.response.detection.threshold.8.15.mussel$Frequency.of.Engagement.Response,
         main="Q-Q plot to Visualise Distribution of Engagement Frequency with Mussel Conditioned Artificial Seawater Addition Data at pH 8.15",
         xlab = "Engagement Frequency")

## CONFIRMS NON-NORMAL DISTRIBUTION

### Transforming non-normal data ###
frequency.of.response.detection.threshold.8.15.mussel$log.Frequeny.of.Engagement.Response<-
  log(frequency.of.response.detection.threshold.8.15.mussel$Frequency.of.Engagement.Response)      ##log transforming data to create normally distributed data##

shapiro.test(frequency.of.response.detection.threshold.8.15.mussel$log.Frequeny.of.Engagement.Response)
ggqqplot(frequency.of.response.detection.threshold.8.15.mussel$log.Frequeny.of.Engagement.Response,
         main="Q-Q plot to Visualise Distribution of log Transformed Engagement Frequency with Mussel Conditioned Artificial Seawater Addition Data at pH 8.15",
         xlab = "Individual Detection Thresholds")    ###checking distribution of log transformed data###

#Shapiro-Wilk normality test
#data:  frequency.of.response.detection.threshold.8.15.mussel$log.Frequeny.of.Engagement.Response
#W = 0.78233, p-value = 0.01846

## CONFIRMS NORMAL DISTRIBUTION for log-transformed data
```

```
linearmodel2<-lm(frequency.of.response.detection.threshold.8.15.mussel$log.Frequeny.of.Engagement.Response~frequency.of.response.detection.threshold.8.15.mussel$Detection.Threshold.Concentration,
                 data = frequency.of.response.detection.threshold.8.15.mussel)

#Call:
#  lm(formula = frequency.of.response.detection.threshold.8.15.mussel$log.Frequency.of.Engagement.Response ~
#       frequency.of.response.detection.threshold.8.15.mussel$Detection.Threshold.Concentration,
#     data = frequency.of.response.detection.threshold.8.15.mussel)
#Coefficients:
#(Intercept)  frequency.of.response.detection.threshold.8.15.mussel$Detection.Threshold.Concentration
#1.3863                                                                                      -0.1308

summary(linearmodel2)

#Call:
#  lm(formula = frequency.of.response.detection.threshold.8.15.mussel$log.Frequeny.of.Engagement.Response ~
#       frequency.of.response.detection.threshold.8.15.mussel$Detection.Threshold.Concentration,
#     data = frequency.of.response.detection.threshold.8.15.mussel)
#Residuals:
#  Min       1Q  Median      3Q     Max
#-0.7324 -0.3400 -0.2550  0.4283  0.9155
#Coefficients:
#                                                                                    Estimate Std. Error t value Pr(>|t|)
#(Intercept)                                                                          1.3863     0.9463   1.465    0.193
#frequency.of.response.detection.threshold.8.15.mussel$Detection.Threshold.Concentration  -0.1308     0.1390  -0.941    0.383

#Residual standard error: 0.6195 on 6 degrees of freedom
#Multiple R-squared:  0.1286, Adjusted R-squared:  -0.01658
#F-statistic: 0.8858 on 1 and 6 DF,  p-value: 0.3829


###Both p values are greater than 0.05, cannot accept the hyothesis - there is no correlation between detection
#threshold concentration for log-transformed mussel conditioned artificial seawater and frequency of engagement response
#however small sample size could have significantly affected this
```

```r
##Chapter four - Exploring the importance of Detection Threshold Concentration and Impact of Reduced pH##
##Generating scatterplots to determine trends between detection threshold concentrations of individuals and the number of trials in which they arrived
##at the source of chemical foraging cue, glutathione, in trials first (wins) vs trial partners at all levels of pH##

rm(list=ls())         #clears workspace#

threshold.wins.glutathione.all.pH<-read.csv(file.choose())   #reads in data#
str(threshold.wins.glutathione.all.pH)                       #examine data#
head(threshold.wins.glutathione.all.pH)
library(ggplot2)                                              #graphical package#

ggplot(threshold.wins.glutathione.all.pH, aes(x=threshold.wins.glutathione.all.pH$Detection.Threshold.Concentration,
                                              y=threshold.wins.glutathione.all.pH$Number.of.Wins,
                                          color=as.factor(threshold.wins.glutathione.all.pH$pH)))+    #generating scatter plot#
  geom_point(size=3)+                                         #formats data point size#
  geom_smooth(method = lm,se=FALSE, fullrange=TRUE)+          #inserts trend lines of best fit#
  theme_minimal() +                                           #Inserts theme#
  scale_color_manual(values = c("Grey40","grey70","grey90"))+ #set colours#
  ggtitle("Investigating Trends between Detection Thresholds for Glutathione and the Number of
          Trials Individuals Reached the Source of Cue, First, before Trial Partners at all pH Levels")+  #adds main title#
  theme(plot.title = element_text(hjust = 0.5))+               #centers main title#
  xlab("Detection Threshold Concentration for Glutathione (e-x M/l)")+          #x axis label#
  ylab("Number of Trials Individuals Reached Cue Source First, before Trial Partners")+                                    #y axis label#
  scale_y_continuous(breaks = seq(0,5,by=1))+                 #sets y axis scale#
  theme(axis.line = element_line(color = "black",size = 0.2), legend.position = "right")+   ##adding and formatting axis lines/repositioning legend##
  labs(colour="pH")         #labeling legend title#
```

```r
##Chapter four - Exploring the importance of Detection Threshold Concentration and Impact of Reduced pH##
##Generating bar plots to investigate...
##a) if crabs used had significiantly different detection thresholds for chemical foraging cue glutathione, did the individual with the lower threshold arrive at the cue source first?
##b) if crab used has similar detection thresholds for chemical foraging cue glutathione, were results random?##

rm(list=ls())         #clears workspace#

first.arrival.frequency<-read.csv(file.choose())   #reads in data#
str(first.arrival.frequency)                       #examine data#
head(first.arrival.frequency)
library(ggplot2)                                    #graphical package#

first.arrival.frequency$Response<-factor(first.arrival.frequency$Response, levels = c("Yes","No","Same Cue Detection Threshold","No Response"))   #generating factors#

ggplot(data=first.arrival.frequency, aes(x=first.arrival.frequency$Response, y=first.arrival.frequency$Frequency, fill=factor(first.arrival.frequency$pH))) +
  geom_bar(stat="identity", width = 0.6, position = position_dodge(0.8), color="black")+   #creating bar plot#
  theme_minimal()+                                  #inserting theme#
  ggtitle("Frequency of Response of Partnering Individuals at all pH Levels")+   #Adds title#
  theme(plot.title = element_text(hjust = 0.5))+    #Adjust postion of title#
  xlab("Response")+                                                              #Adds x axis label#
  ylab("Frequency")+                          #Adds y axis label#
  expand_limits(y=c(0,15))+
  scale_y_continuous(breaks = seq(0,15,by=1))+              #sets y axis scale#
  theme(axis.line = element_line(color = "black",size = 0.2), legend.position = "right")+            #Adds axis line#
  scale_fill_manual(values = c("Grey40","lightgrey","white"))+      #colour formatting bars and figure legend#
  labs(fill="pH")                     #title of figure legend#
```

```r
##Chapter four - Exploring the importance of Detection Threshold Concentration and Impact of Reduced pH##
#performing t test to determine if individuals with lower threshods significantly reached the source of glutathione first before trial partners##
################################################################################################################################################################
#pH 8.15#

rm(list=ls())         #clears workspace#

pH8.15.response<-read.csv(file.choose())   #reads in data#
str(pH8.15.response)                       #examine data#
head(pH8.15.response)

library(ggplot2)    ## downloading packages to aid analysis ##
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###
library(car)

#####################################################################
### shapiro-wilks test for normality ###
shapiro.test(pH8.15.response$Response)    ### Carrying out a Shapiro-Wilk normality test ###

##Shapiro-Wilk normality test
##data:  pH8.15.response$Response
##W = 0.65474, p-value = 0.0004194

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(pH8.15.response$Response,
         main="Q-Q plot to Visualise Distribution of Response Data",
         xlab = "Response")

## CONFIRMS NON-NORMAL DISTRIBUTION

###Testing Assumption 2 - Homogeneity of Variance###

leveneTest(pH8.15.response$Trial~pH8.15.response$Response,
           data=pH8.15.response)

#Levene's Test for Homogeneity of Variance (center = median)
#      Df F value Pr(>F)
#group  1  0.8057 0.3992
#       7

##Assumption met of homegeneity of variance

##choose non-parametric version of unpaired t test - Wilcoxon rank sum test##
wilcox.test(pH8.15.response$Trial~pH8.15.response$Response, data = pH8.15.response, exact=FALSE)

#Wilcoxon rank sum test with continuity correction
#data:  pH8.15.response$Trial by pH8.15.response$Response
#W = 11, p-value = 0.9025
#alternative hypothesis: true location shift is not equal to 0

##P value (0.9025) is greater than significance level 0.05, there is no significance in the amount of trials
## whereby individuals with lower detection thresholds reached the source of cue glutathione first before trial partners (with higher thresholds) at pH 8.15#
```

```r
#####################################################################################################################################################
#pH 7.7#

rm(list=ls())         #clears workspace#

pH7.7.response<-read.csv(file.choose())   #reads in data#
str(pH7.7.response)                       #examine data#
head(pH7.7.response)

library(ggplot2)    ## downloading packages to aid analysis ##
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###
library(car)

#################################################################
### shapiro-wilks test for normality ###
shapiro.test(pH7.7.response$Response)    ### Carrying out a Shapiro-Wilk normality test ###

##Shapiro-Wilk normality test
##data:  pH7.7.response$Response
##W = 0.49609, p-value = 2.073e-05

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(pH7.7.response$Response,
         main="Q-Q plot to Visualise Distribution of Response Data",
         xlab = "Response")

## CONFIRMS NON-NORMAL DISTRIBUTION

###Testing Assumption 2 - Homogeneity of Variance###
leveneTest(pH7.7.response$Trial~pH7.7.response$Response,
           data=pH7.7.response)

#Levene's Test for Homogeneity of Variance (center = median)
#      Df F value Pr(>F)
#group  1  1.7143 0.2606
#       4

##Assumption met of homegeneity of variance

##choose non-parametric version of unpaired t test - Wilcoxon rank sum test##
wilcox.test(pH7.7.response$Trial~pH7.7.response$Response, data = pH7.7.response, exact=FALSE)

#Wilcoxon rank sum test with continuity correction
#data:  pH7.7.response$Trial by pH7.7.response$Response
#W = 5, p-value = 0.2416
#alternative hypothesis: true location shift is not equal to 0

##P value (0.2416) is greater than significance level 0.05, there is no significance in the amount of trials
## whereby individuals with lower detection thresholds reached the source of cue glutathione first before trial partners (with higher thresholds) at pH 7.7#
```

```r
#####################################################################################################################################################
#pH 7.2#

rm(list=ls())         #clears workspace#

pH7.2.response<-read.csv(file.choose())   #reads in data#
str(pH7.2.response)                       #examine data#
head(pH7.2.response)

library(ggplot2)    ## downloading packages to aid analysis ##
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###
library(car)

#################################################################
### shapiro-wilks test for normality ###
shapiro.test(pH7.2.response$Response)    ### Carrying out a Shapiro-Wilk normality test ###

##Shapiro-Wilk normality test
##data:  pH7.2.response$Response
##W = 0.63989, p-value = 0.001351

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(pH7.2.response$Response,
         main="Q-Q plot to Visualise Distribution of Response Data",
         xlab = "Response")

## CONFIRMS NON-NORMAL DISTRIBUTION

###Testing Assumption 2 - Homogeneity of Variance###
leveneTest(pH7.2.response$Trial~pH7.2.response$Response,
           data=pH7.2.response)

#Levene's Test for Homogeneity of Variance (center = median)
#      Df F value Pr(>F)
#group  1  0.1212 0.7453
#       4

##Assumption met of homegeneity of variance

##choose non-parametric version of unpaired t test - Wilcoxon rank sum test##
wilcox.test(pH7.2.response$Trial~pH7.2.response$Response, data = pH7.2.response, exact=FALSE)

#Wilcoxon rank sum test with continuity correction
#data:  pH7.2.response$Trial by pH7.2.response$Response
#W = 7, p-value = 0.2472
#alternative hypothesis: true location shift is not equal to 0

##P value (0.2472) is greater than significance level 0.05, there is no significance in the amount of trials
## whereby individuals with lower detection thresholds reached the source of cue glutathione first before trial partners (with higher thresholds) at pH 7.2#
```

```r
##############################################################################################################################################################################
##Chapter four - The influence of weight upon behavioural assay response outcomes##
##Generating bar plots to investigate...
##if crabs used had significiantly different weights, did the heaviest individual arrive at the cue source first?

rm(list=ls())        #clears workspace#

weight.first.arrival.frequency<-read.csv(file.choose())   #reads in data#
str(weight.first.arrival.frequency)                       #examine data#
head(weight.first.arrival.frequency)
library(ggplot2)                                             #graphical package#

weight.first.arrival.frequency$Response<-factor(weight.first.arrival.frequency$Response, levels = c("Yes","No","Same Weight","Weight Unknown","No Response"))   #generating factors#

ggplot(data=weight.first.arrival.frequency, aes(x=weight.first.arrival.frequency$Response, y=weight.first.arrival.frequency$Frequency, fill=factor(weight.first.arrival.frequency$pH))) +
  geom_bar(stat="identity", width = 0.6, position = position_dodge(0.8), color="black")+   #creating bar plot#
  theme_minimal()+                                  #inserting theme#
  ggtitle("Frequency of Response of Partnering Individuals at all pH Levels in Accordance to Weight")+   #Adds title#
  theme(plot.title = element_text(hjust = 0.5))+    #Adjust postion of title#
  xlab("Response")+                                                                  #Adds x axis label#
  ylab("Frequency")+                          #Adds y axis label#
  expand_limits(y=c(0,15))+
  scale_y_continuous(breaks = seq(0,15,by=1))+              #sets y axis scale#
  theme(axis.line = element_line(color = "black",size = 0.2), legend.position = "right")+            #Adds axis line#
  scale_fill_manual(values = c("Grey40","lightgrey","white"))+      #colour formatting bars and figure legend#
  labs(fill="pH")                       #title of figure legend#
```

```r
##############################################################################################################################################################################
##Statisitical Analysis##
#performing t test to determine if heavier individuals significantly reached the source of glutathione first before competitive trial partners##

rm(list=ls())         #clears workspace#

weight.response<-read.csv(file.choose())   #reads in data#
str(weight.response)                       #examine data#
head(weight.response)

library(ggplot2)    ## downloading packages to aid analysis ##
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###
library(car)

#####################################################################
### shapiro-wilks test for normality ###
shapiro.test(weight.response$Response)    ### Carrying out a Shapiro-Wilk normality test ###

##Shapiro-Wilk normality test
##data:  weight.response$Response
##W = 0.62089, p-value = 2.566e-05

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(weight.response$Response,
         main="Q-Q plot to Visualise Distribution of Response and Weight Data",
         xlab = "Response")

## CONFIRMS NON-NORMAL DISTRIBUTION

###Testing Assumption 2 - Homogeneity of Variance###

leveneTest(weight.response$Trial~weight.response$Response,
           data=weight.response)

#Levene's Test for Homogeneity of Variance (center = median)
#      Df F value Pr(>F)
#group  1  0.1544 0.7003
#      14

##Assumption met of homegeneity of variance

##choose non-parametric version of unpaired t test - Wilcoxon rank sum test##
wilcox.test(weight.response$Trial~weight.response$Response, data = weight.response, exact=FALSE)

#Wilcoxon rank sum test with continuity correction
#data:  weight.response$Trial by weight.response$Response
#W = 32, p-value = 0.8708
#alternative hypothesis: true location shift is not equal to 0

##P value (0.8708) is greater than significance level 0.05, there is no significance in the amount of trials
## whereby heavier/larger individuals reached the source of cue glutathione first before competitive trial partners (weighing less (g)) at all pH levels#
```

```
##Chapter four - Impact of Reduced pH on both Time for Initial Movement and Time to Make Contact with the Source of Chemical Foraging Cue Glutathione##
##Generating boxplots##

#########################################################################################################################################
##generating a boxplot to determine if reduced pH impacts the time taken for individuals to make initial movement##

rm(list = ls())
intial.time.and.pH<-read.csv(file.choose())   #reads in data#
str(intial.time.and.pH)                       #examine data#
head(intial.time.and.pH)
library(ggplot2)                            #running necessary graphics package#

intial.time.and.pH$pH<-factor(intial.time.and.pH$pH)               #sets pH as factor#

initial.movement<-ggplot(intial.time.and.pH, aes(x=intial.time.and.pH$pH, y=intial.time.and.pH$Time..s.))+
  geom_boxplot(aes(fill=intial.time.and.pH$pH), position = position_dodge(0.8))+    #generates boxplot#
  scale_fill_manual(name="pH", values = c("Grey40","lightgrey","white"))+   #sets colours#
  geom_point(size=2.5)+                                                  #adds data points to boxplots#
  stat_boxplot(geom = 'errorbar', width=0.2)+                  #Adds whisker ends to boxplots#
  theme_minimal() +                                            #Inserts theme#
  ggtitle("A) Time taken for Initial Movement by Individuals who Engaged within Trials with the Addition of Chemical Foraging Cue Glutathione")+  ##dds main title#
  theme(plot.title = element_text(hjust = 0.5))+               #centers main title#
  xlab("pH")+               #x axis label#
  ylab("Time (Seconds)")+        #y axis label#
  theme(axis.line = element_line(color = "black",size = 0.2))+     #adding and formatting axis lines#
  expand_limits(y=c(0,160))+                          #sets y axis scale#
  scale_y_continuous(breaks = seq(0,160,by=10))+   #sets y axis scale#
  theme(legend.position = "none")                 #no figure legend#

initial.movement
```

```
#########################################################################################################################################
##generating a boxplot to determine if reduced pH impacts the time taken for individuals to make contact with the source of chemical foraging cue glutathione##

contact.time.and.pH<-read.csv(file.choose())   #reads in data#
str(contact.time.and.pH)                       #examine data#
head(contact.time.and.pH)
library(ggplot2)                            #running necessary graphics package#

contact.time.and.pH$pH<-factor(contact.time.and.pH$pH)               #sets pH as factor#

contact.time<-ggplot(contact.time.and.pH, aes(x=contact.time.and.pH$pH, y=contact.time.and.pH$Time..s.))+
  geom_boxplot(aes(fill=contact.time.and.pH$pH), position = position_dodge(0.8))+    #generates boxplot#
  scale_fill_manual(name="pH", values = c("Grey40","lightgrey","white"))+   #sets colours#
  geom_point(size=2.5)+                                                  #adds data points to boxplots#
  stat_boxplot(geom = 'errorbar', width=0.2)+                  #Adds whisker ends to boxplots#
  theme_minimal() +                                            #Inserts theme#
  ggtitle("B) Time taken to Make Contact with the Source of Cue by Individuals who Engaged within Trials with the Addition of Chemical Foraging Cue Glutathione")+  ##dds main title#
  theme(plot.title = element_text(hjust = 0.5))+               #centers main title#
  xlab("pH")+               #x axis label#
  ylab("Time (Seconds)")+        #y axis label#
  theme(axis.line = element_line(color = "black",size = 0.2))+     #adding and formatting axis lines#
  expand_limits(y=c(0,160))+                          #sets y axis scale#
  scale_y_continuous(breaks = seq(0,160,by=10))+   #sets y axis scale#
  theme(legend.position = "none")                 #no figure legend#

contact.time

#Combining plots together in same graphics window#
library(gridExtra)
grid.arrange(initial.movement,contact.time)
```

```r
#####################################################################################################################################################################
#statistical analysis to determine if reduced pH impacts the time taken for individuals to make initial movement towards the source of chemical foraging cue glutathione#
#One-Way ANOVA to be carried out#

rm(list = ls())
initial.time.and.pH<-read.csv(file.choose())   #reads in data#
str(initial.time.and.pH)                       #examine data#
head(initial.time.and.pH)

library(ggplot2)                          #running necessary graphics and statistical packages#
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###
library(car)

#####################################################################
### shapiro-wilks test for normality ###
shapiro.test(initial.time.and.pH$Time..s.)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
## data:  initial.time.and.pH$Time..s.
## W = 0.93328, p-value = 0.02549

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(initial.time.and.pH$Time..s.,
         main="Q-Q plot to Visualise Distribution of Initial Time for Movement Data with the Addition of Chemical Foraging Cue Data at all pH Levels",
         xlab = "Time")

## CONFIRMS NON-NORMAL DISTRIBUTION

#####################################################################
###Testing Assumption 2 - Homogeneity of Variance###

initial.time.and.pH$pH<-factor(initial.time.and.pH$pH)

leveneTest(initial.time.and.pH$Time..s.~initial.time.and.pH$pH,
           data=initial.time.and.pH)

## Levene's Test for Homogeneity of Variance (center = median)
##        Df F value Pr(>F)
## group  2   1.431 0.2527
## 35

##Assumption met of homegeneity of variance

#####################################################################
### Transforming non-normal data and looking at distribution###

initial.time.and.pH$log.Time..s.<-
  log(initial.time.and.pH$Time..s.)      ##log transforming data to create normally distributed data##

ggqqplot(initial.time.and.pH$log.Time..s.,
         main="Q-Q plot to Visualise Distribution of log Transformed Initial Time for Movement Data with the Addition of Chemical Foraging Cue Data at all pH Levels",
         xlab = "Log - Time")    ###checking distribution of log transformed data###

### shapiro-wilks test for normality ###
shapiro.test(initial.time.and.pH$log.Time..s.)    ### Carrying out a Shapiro-Wilk normality test ###

#Shapiro-Wilk normality test
#data:  initial.time.and.pH$log.Time..s.
#W = 0.94728, p-value = 0.07247

##log-transformed data normally distributed##

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

lm(initial.time.and.pH$log.Time..s~initial.time.and.pH$pH,
   data = initial.time.and.pH)        ##fitting a linear model##

qqnorm(resid(lm(initial.time.and.pH$log.Time..s~initial.time.and.pH$pH,
                data = initial.time.and.pH)))                    ##checking fit of model##

anova(lm(initial.time.and.pH$log.Time..s~initial.time.and.pH$pH,
         data = initial.time.and.pH))     ##Running a one-way

#Analysis of Variance Table
#Response: initial.time.and.pH$log.Time..s
#                      Df  Sum Sq Mean Sq F value   Pr(>F)
#initial.time.and.pH$pH  2  7.1143  3.5572  6.1394 0.005182 **
#Residuals              35 20.2789  0.5794
#Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

## As the p-value (0.005182) is less than the significance level 0.05,
##we can conclude that there is significant differences between time taken for individuals to initially move with reduced pH with the presence of glutathione##

#####################################################################
###post hoc to determine which means are significantly different
TukeyHSD(aov(lm(initial.time.and.pH$log.Time..s~initial.time.and.pH$pH,
                data = initial.time.and.pH)),
         conf.level=0.95)

#Tukey multiple comparisons of means 95% family-wise confidence level
#Fit: aov(formula = lm(initial.time.and.pH$log.Time..s ~ initial.time.and.pH$pH, data = initial.time.and.pH))
#$`initial.time.and.pH$pH`
#               diff       lwr         upr     p adj
# 7.7-7.2   0.2690100 -0.568266  1.10628610 0.7138134
# 8.15-7.2 -0.6965252 -1.457019  0.06396855 0.0781159
# 8.15-7.7 -0.9655352 -1.678450 -0.25262036 0.0059271

##The Tukey HSD shows that for log transformed data only initial time for movement recorded between pH 8.15 and 7.7 is significantly different p ≤ 0.05,
##indicating that with reduced pH from 8.15 to 7.7 time taken to initially move towards the source of chemical foraging cue glutathione significantly increased.
```

```r
#####################################################################################################################################################################
#statistical analysis to determine if reduced pH impacts the time taken for individuals to make contact with the source of chemical foraging cue glutathione#
#One-Way ANOVA to be carried out#

rm(list = ls())
contact.time.and.pH<-read.csv(file.choose())   #reads in data#
str(contact.time.and.pH)                       #examine data#
head(contact.time.and.pH)

library(ggplot2)                           #running necessary graphics and statistical packages#
library(dplyr)
library(ggpubr)   ### for ggplot-based data visualisation ###
library(car)

#####################################################################
### shapiro-wilks test for normality ###
shapiro.test(contact.time.and.pH$Time..s.)    ### Carrying out a Shapiro-Wilk normality test ###

## Shapiro-Wilk normality test
## data:  contact.time.and.pH$Time..s.
## W = 0.93164, p-value = 0.02261

### Plotting a QQ plot to visualise data distribution/normality ###
ggqqplot(contact.time.and.pH$Time..s.,
         main="Q-Q plot to Visualise Distribution of Contact Time Data with the Addition of Chemical Foraging Cue Data at all pH Levels",
         xlab = "Time")

## CONFIRMS NON-NORMAL DISTRIBUTION

#####################################################################
###Testing Assumption 2 - Homogeneity of Variance###

contact.time.and.pH$pH<-factor(contact.time.and.pH$pH)

leveneTest(contact.time.and.pH$Time..s.~contact.time.and.pH$pH,
           data=contact.time.and.pH)

## Levene's Test for Homogeneity of Variance (center = median)
##        Df F value Pr(>F)
## group  2  1.7103 0.1956
## 35

##Assumption met of homegeneity of variance
```

```r
#####################################################################
### Transforming non-normal data and looking at distribution###

contact.time.and.pH$log.Time..s.<-log(contact.time.and.pH$Time..s.)      ##log transforming data to create normally distributed data##

ggqqplot(contact.time.and.pH$log.Time..s.,
         main="Q-Q plot to Visualise Distribution of log Transformed Contact Time Data with the Addition of Chemical Foraging Cue Data at all pH Levels",
         xlab = "Log - Time")    ###checking distribution of log transformed data###

### shapiro-wilks test for normality ###
shapiro.test(contact.time.and.pH$log.Time..s.)    ### Carrying out a Shapiro-Wilk normality test ###

#Shapiro-Wilk normality test
#data:  contact.time.and.pH$log.Time..s.
#W = 0.97059, p-value = 0.4078

##log-transformed data normally distributed##

#####################################################################
lm(contact.time.and.pH$log.Time..s.~contact.time.and.pH$pH,
   data = contact.time.and.pH)        ##fitting a linear model##

qqnorm(resid(lm(contact.time.and.pH$log.Time..s~contact.time.and.pH$pH,
                data = contact.time.and.pH)))                    ##checking fit of model##

anova(lm(contact.time.and.pH$log.Time..s~contact.time.and.pH$pH,
         data = contact.time.and.pH))     ##Running a one-way

#Analysis of Variance Table
#Response: contact.time.and.pH$log.Time..s
#                        Df Sum Sq Mean Sq F value    Pr(>F)
# contact.time.and.pH$pH  2 6.0327 3.01636  11.298 0.0001638 ***
# Residuals              35 9.3445 0.26699
# Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

## As the p-value (0.0001638) is less than the significance level 0.05,
##we can conclude that there is significant differences between time taken for individuals to make contact with the source of glutathione with reduced pH##

#####################################################################
###post hoc to determine which means are significantly different
TukeyHSD(aov(lm(contact.time.and.pH$log.Time..s~contact.time.and.pH$pH,
                data = contact.time.and.pH)),
         conf.level=0.95)

#Tukey multiple comparisons of means 95% family-wise confidence level
#Fit: aov(formula = lm(contact.time.and.pH$log.Time..s ~ contact.time.and.pH$pH, data = contact.time.and.pH))
#$`contact.time.and.pH$pH`
#               diff        lwr        upr     p adj
# 7.7-7.2   0.1942722 -0.3740903  0.7626347 0.6831617
# 8.15-7.2 -0.6786860 -1.1949269 -0.1624451 0.0076553
# 8.15-7.7 -0.8729582 -1.3569015 -0.3890150 0.0002675

##The Tukey HSD shows that for log transformed data only time taken to make contact wiith the source of chemical foraging cue glutathione recorded between pH 8.15 and 7.7 and pH 8.15 and 7.2
##is significantly different p ≤ 0.05, indicating that with reduced pH from 8.15 to 7.7 and from 8.15 to 7.2, time taken to make contact with the
##source of chemical foraging cue glutathione significantly increased.
```