Impact of Short-term Exposure to Acid Sulphate Soil Leachate Parameters on the Biochemical Condition of Stressed Pacific Oysters, *Crassostrea gigas*.

School of Biological Sciences

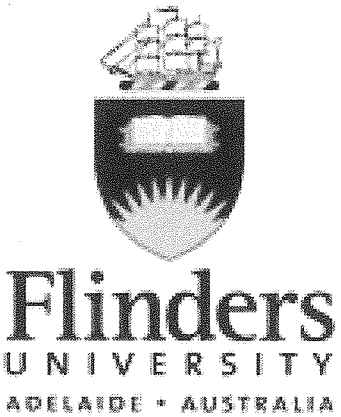
Flinders University of South Australia

Bachelor of Technology (Aquaculture) (Honours)

December, 2009

by Joel Kellam-Stock

Supervisor: Dr James Harris



ISBN: 978-1-925982-45-9

Table of Contents

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person where due reference is not made in the text.

Joel Kellam-Stock

Acknowledgments

Firstly I would like to acknowledge the strong support and funding from the Australian Seafood Cooperative Research Centre, Flinders University and Gary Zippel of Zippel Oysters.

I am extremely grateful to my honours supervisor James Harris for his continuous advice and assistance. I would also like to acknowledge the advice and assistance of Keryn Simons, Andy Ball, Yan Li and Kirsten Benkendorff.

Furthermore I would like thank Graham Mair, Jian Qin and Josie Mair for their help and feedback and Alan McLennan, David Rudd, Leslie Morrison and Sandra Marshall for assistance in acquiring materials and equipment.

Abstract

This study investigated the impact of short-term exposure to acid sulphate soil leachate on the biochemical condition of the Pacific oyster *Crassostrea gigas*. Oysters were exposed to artificially acidified seawater, aluminium and iron treatments for a period of six hours and assessed for changes in mantle glycogen, mantle protein and haemolymph protein between treatments. An additional challenge using *Vibrio harveyi* extracellular products was conducted to reduce oyster condition and make them more vulnerable to impacts of acidified treatments. Despite depressed mantle protein levels no significant differences in biochemical condition were found between control treatments and acidified water treatments indicating that short periods of exposure to acid leachate in the field would be unlikely to have significant impacts on general oyster condition.

1. Introduction

The Pacific oyster, *Crassostrea gigas*, is the most widely cultivated oyster in the world. In contrast to other cultivated oyster species, *C. gigas* suffers from few major infectious diseases leading to significant production growth in the last few decades as it replaces struggling native oyster industries around the world (FAO 2009). However, a growing concern in the industry is the regular occurrence of summer mortality outbreaks with no identified disease agents (Berthelin et al. 2000). These mortalities have been hypothesised to result from a combination of biological and environmental stressors reducing oyster condition and suppressing immune capabilities (Li et al. 2009b). Due to the extensive nature of oyster production, their filter-feeding behaviour and open circulatory system, oysters are continually exposed to a wide array of environmental stressors (Gagnaire et al. 2004; Lacoste et al. 2001; Canesi et al. 2002). An environmental stressor recently attributed to production declines of the oyster *Saccostrea glomerata* on the east coast of Australia is water acidification from acid sulphate soils (Dove and Sammut 2007a).

Acid sulphate soils are potentially present in most low-lying coastal regions of South Australia. These are soil sediments containing iron sulphides usually under oxygen depleted

conditions found in water logged swamps, mangroves and salt marshes (CPB 2003). When exposed to air, through drainage or disturbance, iron sulphides oxidise generating sulphuric acid. The acid generated can dissolve metals present in the soil sediments and this toxic leachate can be transported into and throughout coastal waterways through artificial drains, rainfall and tidal movement (Powell and Martens 2005). Acid leachate can potentially depress estuarine water to a pH of 3 for weeks at a time with a range of deleterious impacts on aquatic biota and habitats (Dove and Sammut 2007b).

Acidification rapidly damages the gills and skin of fish and can initiate disease and result in large mortality events (Dove and Sammut 2007b, Callinan et al. 1993). In bivalves, acidic water conditions (pH ≤7) can suppress feeding activity and growth, cause shell dissolution and (at pH 5.1) lead to deleterious changes in soft tissues (Dove and Sammut 2007a and 2007b). Bamber (1990) found that chronic exposure to pH 6 or less over a 30 day period causes significant mortalities in *C. gigas*. Furthermore aluminium in acid leachate has been shown to increase abnormalities in *S. glomerata* embryonic development (Wilson and Hyne 1997) and has been associated with intensified tissue inflammation in adult oysters (Dove and Sammut 2007b). Dove and Sammut (2007a) examined the impact of acidification on *S. glomerata* aquaculture in an estuary in New South Wales finding that higher mortalities occur in sites frequented by acid leachate exposure linked to shell dissolution and perforation and recommended the movement of production away from regions facing episodic acidification.

The majority of *C. gigas* aquaculture in South Australia is conducted in intertidal bays where potential acid leachate would likely be quickly buffered or flushed by tidal movement and may be negligible as an environmental stressor. However, the synergistic impact of multiple stressors as hypothesised in summer mortality events may make oysters more sensitive to the impacts of short-term acid leachate exposure. Bivalves primarily respond to stressors by diverting energy resources from physiological functions such as growth and reproduction to sustain metabolic and behavioural adaptations that maintain homeostasis (Lacoste et al. 2001; Lacoste et al. 2002). Chronic exposure to significant stressors such as extreme temperatures and salinity reduced feed quality and availability, air exposure, handling and pollutants can reduce immune capabilities and decrease biochemical and physiological

condition in bivalves as energy reserves are utilised (Whyte et al. 1990; Hummel at al. 1989; Lacoste et al. 2001). Low energetic condition in oysters may further increase their sensitivity to additional stressors and may be useful in assessing the potential impacts of otherwise mild stressors. Mechanical disturbance simulating the handling stresses of grading have been found to significantly increase mortalities in oyster challenged with a *Vibrio* bacterium (Lacoste et al. 2001). Similar results have been observed in infected mussels, *Mytilus edulis*, when exposed simultaneously to dissolved copper pollutants (Pipe and Coles 1995).

The primary objective of this study was to establish if short-term exposure to acid leachate should be a major concern to the intertidal culture of Pacific oysters on the coast of South Australia. To achieve this objective, this study aimed to determine the impact of acidified water, aluminium and iron treatments on the biochemical condition of oysters already in poor condition due to additional stressors of *Vibrio harveyi* bacterial toxins and incidental high ambient temperatures.

2 Materials and Methods

2.1 Animals

Adult *C. gigas* were donated by Zippel oysters (Smoky Bay, South Australia) and were received at Flinders University (Bedford Park, South Australia) in two separate batches. On arrival oysters were cleaned of fouling biota and randomly distributed into 90L re-circulating seawater systems. Oysters were acclimatised for at least two weeks at ambient temperature with salinity maintained between 30 to 35‰; pH maintained above 8 ; and dissolved oxygen above 5mg.L^{-1}. Oysters were fed 33.5mg.$oyster^{-1}$ of Nosan M-1 Bivalve Diet (Aquasonic) twice daily and tanks were cleaned with approximately 50% water exchanges weekly.

2.2 Bacterial extracellular products

Extracellular products of *Vibrio harveyi* were produced based on the methods of Labreuche et al. (2006), Li et al. (2009c) and Liu (1957) to be used in a simulated challenge in attempt to weaken the condition of oysters and make them more sensitive to acidic stressors. *V.*

harveyi was used as it common marine bacterium in the coastal regions of southern Australia and would be potentially encountered by oysters in the field. *V. harveyi* has also been shown to produce lethal extracellular toxins which are considered to be crucial in determining the virulence of *Vibrio* species towards bivalves (Labreuche et al. 2006, Li et al. 2009c; Pass et al. 1987). *V. harveyi* stock cultures stored at -80°C with 15% glycerol were streaked onto nutrient agar (Oxoid) plates supplemented with 2% NaCl (Merck) and incubated at 25°C for 24 hours. Bacterial growth was re-streaked on thiosulfate citrate bile salts sucrose (TCBS) agar (Oxoid) plates for single colonies and incubated at 25°C for 36 hours. Single colonies from the TCBS agar plates were inoculated into 10mL tubes containing 5mL nutrient broth (Oxoid) supplemented with 2% NaCl and incubated at 28°C for 24 hours on a shaker table. Cellophane sheets were cut to size and autoclaved in deionised water at 121°C for 15 minutes and overlaid on nutrient agar plates supplemented with 2% NaCl. 2mL of bacterial suspension was spread onto the cellophane plates and incubated at 25°C for 48 hours. Cellophane sheets were removed from the agar and transferred to empty Petri dishes with cells washed off using 4mL of ice-chilled deionised and autoclaved seawater. The resulting solution was centrifuged for 30 minutes at 10,000*g* and 4°C. Supernatant was filtered (0.2µm) and stored in 1mL aliquots at -80°C until used in the bacterial challenge.

2.3 Experimental challenges

2.3.1 Water treatments

Two experiments were conducted using different acidified water treatments designed to resemble potentially harmful pH, aluminium and iron concentrations caused by acid leachate within a range in which oysters would maintain feeding behaviour based on the methods and results of Dove & Sammut (2007a and 2007b). Water parameters for the different treatments measured in experiments one and two are listed in tables 1 and 2 respectively. Oysters were exposed to the different treatments for a period six hours to simulate acid and metal exposure during a tidal cycle. The pH level in treatments 2 to 5 in experiment one and treatments 4 to 7 in experiment two were reduced by the addition of sulphuric acid (Scharlau). Due to the carbonate buffering of seawater the pH was measured and adjusted back to the desired levels every hour, hence the mean values recorded

represent the mean maximum pH during the exposure period. Aluminium chloride (Chem-Supply) and iron chloride (Sigma-Aldrich) were added to an isolated reservoir of seawater and thoroughly mixed to achieve a desired nominal concentration of total aluminium and total iron of 7.5mg.L^{-1} prior to addition to experimental systems for treatments 5 and 7 of experiments one and two respectively. The actual dissolved and suspended aluminium and iron concentration were later determined by Waite Analytical Services (Glen Osmond, South Australia) using inductively coupled plasma atomic emission spectrometry with nitric/perchloric acid digestions.

Treatment	Components	Mean pH ±95% CI	Dissolved Fe (mg.L^{-1})	Dissolved Al (mg.L^{-1})	Suspended Fe (mg.L^{-1})	Suspended Al (mg.L^{-1})
1	Seawater	8.43 ± 0.07	ND	ND	ND	ND
2	Seawater & H_2SO_4	8.00 ± 0.24	ND	ND	ND	ND
3	Seawater & H_2SO_4	7.44 ± 0.42	ND	ND	ND	ND
4	Seawater & H_2SO_4	5.84 ± 1.30	ND	ND	ND	ND
5	Seawater, H_2SO_4, $FeCl_3.6H_2O$ & $AlCl_3.6H_2O$	5.62 ± 0.20	2.3	3.4	5.6	4.1

Table 1: Experiment 1 pH, Al & Fe concentrations during a six hour exposure period. ND = Not Detectable.

Treatment	Components	Mean pH ±95% CI	Dissolved Fe (mg.L^{-1})	Dissolved Al (mg.L^{-1})	Suspended Fe (mg.L^{-1})	Suspended Al (mg.L^{-1})
1	Seawater	8.56 ± 0.04	ND	ND	ND	ND
2	Seawater	8.61 ± 0.03	ND	ND	ND	ND
3	Seawater	8.59 ± 0.03	ND	ND	ND	ND
4	Seawater & H_2SO_4	7.29 ± 0.15	ND	ND	ND	ND
5	Seawater & H_2SO_4	6.04 ± 0.20	ND	ND	ND	ND
6	Seawater & H_2SO_4	4.99 ± 0.21	ND	ND	ND	ND
7	Seawater, H_2SO_4 $FeCl_3.6H_2O$ & $AlCl_3.6H_2O$	5.00 ± 0.08	2.3	3.4	5.6	4.1

Table 2: Experiment 2 pH, Al & Fe concentrations during a six hour exposure period. ND = Not Detectable.

2.3.2 Experimental systems

Exposure treatments were conducted simultaneously in separate 90L re-circulating seawater systems. Each system consisted of 4 replicate 10L treatment tanks which drained into 40L sump containing biological filter media from which water was pumped into a 10L header tank and fed back into the treatment tanks.

2.3.3 Oysters

For experiment one 240 oysters (mean live weight 69.00±2.34g) were randomly and evenly distributed between 5 of the experimental systems for exposure treatments. Prior to experiment two a large mortality event occurred while acclimatising oysters, attributed to extreme ambient air temperatures above 40°C and poor water quality due to initial oyster mortalities. As a result many of the smaller and weaker oysters died impacting on initial oyster size and potentially, biochemical condition in experiment two. For experiment two, 252 surviving oysters (mean live weight 85.97±1.58g) were randomly and evenly distributed between 7 of the experimental systems.

2.3.4 Bacterial Extracellular Products Challenge

Oysters used in experiment two were subjected to a simulated bacteria challenge based on the methods of Li et al. (2009c) using *V. harveyi* extracellular products. The protein concentration of the produced extracellular products was quantified using the Bradford (1976) method of the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) at 595nm with bovine serum albumin (Sigma-Aldrich) as a standard. All oysters used in experiment two were anaesthetised in a 50g.L^{-1} $MgCl_2$ (Merck) bath (2:3, v:v, seawater:freshwater) to facilitate shell opening. Then 200µl of extracellular products (equivalent to 1.8µg protein) was injected into the adductor muscle of oysters for treatments 3 to 7 using a 1mL syringe with a 29 gauge needle. As a control, 200µl of filtered (0.2µm) seawater was injected into the adductor muscle of oysters used in treatment 2 while oysters used in treatment 1 received no injections.

2.3.5 Survival

Following treatment exposure, oysters were returned to seawater and monitored for 7 days for mortalities. Due to extreme ambient water temperatures (31.93±0.81°C) following experiment two oysters were moved to air conditioned facilities after 4 days in an attempt to reduce mortalities and allow for sufficient biochemical analysis.

2.4 Biochemical analysis

2.4.1 Sampling procedure

There were no oyster mortalities during experiment one and hence all 12 oysters per tank could be sampled for biochemical analysis of mantle glycogen, mantle protein and haemolymph protein. Mortalities during experiment 2 limited sampling to six oysters per tank from the original 9 oysters. During sampling oysters were shucked and approximately 300µl of haemolymph was removed from the pericardial cavity using a 1mL syringe with a 29 gauge needle. Oysters were then blotted dry on paper towel in a systematic manner and flesh weight recorded. Mantle tissue was then quickly dissected and frozen in liquid nitrogen. To ensure enough tissue and haemolymph was available for sampling and to minimise inter-animal variability samples were pooled within each tank. Linehan et al. (1999) showed that the adoption of pooling has an acceptable impact on results as the mean value of an assay from the pool is very similar to the weighted average of the same assay conducted on each individual in the pool. Pooled mantle tissue was ground with a mortar and pestle on dry ice and stored at -80°C until analysed for protein and glycogen content. Pooled haemolymph samples were centrifuged at 1500*g* and 4°C for 10 minutes to remove tissue and gamete contamination and the supernatant was stored at -80°C until analysed for protein content.

2.4.2 Glycogen analysis

The glycogen content in the mantle tissue was determined using the colorimetric iodine methods of Li et al. (2007) and Kristman (1962). Glycogen was extracted by adding 1g of ground mantle tissue to 5mL of 0.6M perchloric acid. Samples were then vortexed for 30 seconds, left on ice for ten minutes and then homogenised. Tissue was removed from the

homogenate by centrifugation for 10 minutes at 1,500*g* and 4°C. In a 96-well microplate, triplicate 40µl samples of mantle supernatant were mixed with 260µl of iodine solution consisting of 1.92mL of Lugol's iodne and 500mL of saturated $CaCl_2$. The plate was shaken and incubated for 20 minutes at 25°C before absorbance was measured at 460nm on a BMG Labtech FLUOstar omega microplate reader. Glycogen concentration in $mg.g^{-1}$ of tissue was determined using purified mussel glycogen (Sigma-Aldrich) standards.

2.4.3 Mantle protein analysis

The protein content in the mantle tissue was determined using the methods of Li et al. (2009a). Protein was extracted by homogenising 100mg of ground mantle tissue in 8mL of 20mM Tris-HCl buffer (pH 8) made from tris(hydroxymethyl)aminomethane (Sigma-Aldrich, USA), 1M HCl (BRAND), 2% NaCl and 0.1% $NaNO_3$. Tissue was removed from the homogenate by centrifugation for 30 minutes at 1,500*g* and 4°C. In a 96-well microplate, Triplicate 10µl samples of mantle supernatant were mixed with 200µl of Protein Assay Dye Reagent (Bio-Rad Laboratories, USA). The plate was shaken and incubated for 5 minutes at 25°C before absorbance was measured at 595nm on a BMG Labtech FLUOstar omega microplate reader. Protein concentration in $mg.g^{-1}$ of tissue was determined using bovine serum albumin (Sigma-Aldrich) standards.

2.4.4 Haemolymph protein analysis

Haemolymph protein content was determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, USA). Haemolymph samples were thawed on ice and triplicate 10-fold dilutions prepared using distilled and deionised water. In a 96-well microplate, 10µl of diluted haemolymph samples were mixed with 200µl of Protein Assay Dye Reagent (Bio-Rad Laboratories, USA). The plate was shaken and incubated for 5 minutes at 25°C before absorbance was measured at 595nm on a BMG Labtech FLUOstar omega microplate reader. Protein concentration in $mg.mL^{-1}$ of haemolymph was determined using bovine serum albumin (Sigma-Aldrich, USA) standards.

2.5 Statistical analysis

Statistical analysis was conducted using SPSS for Windows v.17. Levene statistic tests and Shapiro-Wilk tests for homogeneity and normality were conducted to ensure data adhered to ANOVA assumptions. One-way ANOVA comparisons of means were then conducted between treatments for survival and mean mantle glycogen, mantle protein and haemolymph protein from the triplicate absorbance measurements. Pooling samples and using mean measurements reduced sample power, however this statistical measurement was not considered to be a true reflection of total sample size as shown by Linehan et al. (1999). All tests were considered significant at α=0.05.

3. Results

ANOVA assumptions of normality and homogeneity were met for all ANOVA comparisons between treatments. ANOVA results indicated no significant differences exist between the mean survival, mantle glycogen, mantle protein and haemolymph protein of different treatments in both experiments at significant level P<0.05 as listed in table 3.

	Between Treatments Effects	
Parameter	Experiment One	Experiment Two
Survival	-	0.467
Mantle Glycogen	0.825	0.070
Mantle Protein	0.331	0.917
Haemolymph Protein	0.746	0.877

Table 3: Probability of differences from One-Way ANOVA comparison of means between treatments for survival, mantle glycogen, mantle protein and haemolymph protein parameters measured in experiment one and two, significant level set at P<0.05.

3.1 Survival results

No mortalities were recorded during experiment one for any treatment. Survival during experiment two ranged between 66.67 and 100% for the different treatments. Lowest

mean survival of 72.23% and 75.00% was recorded for treatments 1 and 2 respectively which were the non injection and seawater injection control groups. Highest mean survival of 88.89% was recorded in treatments 4 and 5 in which oysters injected with *V. harveyi* extracellular products were exposed to seawater with pH 7.29 and 6.04 respectively.

3.2 Mantle glycogen

Mantle glycogen for the different treatments in experiment one ranged between 4.16 and 11.63mg.g^{-1} of tissue wet weight. The lowest mean glycogen concentration recorded was 6.73mg.g^{-1} in treatment 2 in which oysters were exposed to pH 8.00. The highest mean glycogen concentration recorded was 8.54mg.g^{-1} in treatment 1 which was a normal seawater control treatment with pH 8.43. In experiment two, mantle glycogen ranged between 3.62 and 14.36mg.g^{-1} of tissue wet weight. The lowest mean glycogen of 5.99mg.g^{-1} was recorded in treatment 6 in which oysters were exposed to acidified water at mean pH 4.99 while the highest mean glycogen concentration recorded was 10.43mg.g^{-1} in treatment 4 with oysters exposed to pH 7.29, with oysters from both treatments receiving *V. harveyi* extracellular product injections.

3.3 Mantle protein

Mantle protein content in experiment one ranged between 11.82 and 27.98mg.g^{-1} of tissue wet weight. The lowest mean mantle protein recorded was 16.51mg.g^{-1} in treatment 4 in which oysters were exposed to water acidified to pH 5.84 while the highest mean protein was recorded in treatment 5 at 21.36mg.g^{-1} following exposure to seawater acidified to pH 5.62 and total Al and Fe concentrations of 7.5 and 7.9mg.L^{-1} respectively. In experiment two, mantle glycogen ranged between 6.50 and 23.41mg.g^{-1} of tissue wet weight with the lowest mean protein of 11.18mg.g^{-1} recorded in treatment 3 in which oysters were exposed to a normal seawater control following injection with *V. harveyi* extracellular products. The highest mean protein recorded was 15.66mg.g^{-1} in treatment 5 with oysters exposed to seawater acidified to pH 6.04 following injection with *V. harveyi* extracellular products.

3.4 Haemolymph protein

Haemolymph protein in experiment one varied between 1.05 and 1.41 $mg.mL^{-1}$ of haemolymph. The lowest mean haemolymph protein occurred in treatment 2 at 1.18$mg.mL^{-1}$ in which oysters were exposed to slightly reduced pH of 8.00. The highest mean protein content recorded was 1.28$mg.mL^{-1}$ in the normal seawater control of treatment 1. During experiment two, haemolymph protein content ranged between 1.48 and 2.53$mg.mL^{-1}$ with the lowest mean level of 1.75$mg.mL^{-1}$ measured in treatment 1, the seawater and injection control treatment. The highest mean haemolymph protein was recorded in treatment 6 at 1.99$mg.mL^{-1}$ in which oysters were exposed to water acidified to pH 4.99 following injection with *V. harveyi* extracellular products.

4. Discussion

The objective of this study was to establish the potential impact of short-term exposure to acid leachate on the biochemical condition of oysters. The results observed in the conducted laboratory experiments seem to clearly indicate that short-term exposure to acid leachate during a tidal cycle would be unlikely to have major implications on the metabolic condition of *C. gigas* in the field. In experiment one all 240 oysters survived up to a week following exposure to a range of lowered pH, aluminium and iron water treatments during a six hour period. Comparisons between mean measurements of mantle glycogen, mantle protein and haemolymph protein between control and acidified treatments indicated that no significant differences exist. Furthermore high and low mean concentrations seem to vary randomly between all treatments from normal seawater to severely acidified water with relatively high metal concentrations. This result is somewhat surprising given that Dove and Sammut (2007b) observed noticeable changes in the soft tissues of *S. glomerata* including extensive inflammation and lesions when exposed to similar acidity and metal concentrations over the same time period.

The additional stress imposed by abnormally high temperatures, which led to a mass mortality event preceding experiment two, as well as the addition of challenges with bacterial toxins, was expected to increase the sensitivity of oysters to the acidified treatments. Lacoste et al. (2001) found increased mortality rates in juvenile *C. gigas*

challenged by *Vibrio splendidus* when subjected to 15 minute mechanical disturbance stress. Similar results were also observed in the mussel *M. edulis* challenged by *Vibrio tubiashi* when exposed to treatments of copper. Whyte et al. (1990) also identified an increasing impact on biochemical condition in adult *C. gigas* by increasing the severity of starvation stresses. While there was a marked increase in mortalities during experiment two, there was no significant difference in mean survival between treatments. In addition, the highest mortalities occurred in the seawater and bacterial challenge control groups further supporting the fact that they were not the result of acidified water, metal or bacterial toxins. The most likely case is that the mortalities during experiment two were a result of the poor condition created by the preceding mortality event and continuing high water temperatures during and following treatment exposure. Surprisingly comparisons of mean mantle glycogen, mantle protein and haemolymph protein from experiment two also indicated that no significant differences between treatments resulted from the six hour exposure or from the simulated bacterial challenge adopted in this study.

A major flaw was later identified in the production of the *V. harveyi* extracellular products which reduced capable dosage rates and possibly prevented its effectiveness during this study. Washing bacterial cells off the cellophane overlay with chilled seawater was ineffective in achieving the desired bacterial concentrations and resulting protein content in the injected supernatant. This methodology would be greatly improved by scraping cells from the cellophane sheets into the chilled water to increase concentrations prior to centrifugation. Despite the ineffectiveness of the simulated bacterial challenge impacting on the survival and biochemical condition of experimental oysters, there is reasonable evidence to suggest that the incidentally high ambient temperatures and poor initial condition of oysters was sufficient in creating low biochemical condition to increase the oysters' sensitivity to acid leachate. In addition to observed mortalities in experiment two comparisons can be drawn between the measured biochemical conditions with the seasonal cycle in conditions of a *C. gigas* population in Stansbury, South Australia determined by Li et al. (2009a). Li et al. (2009a) found that within a normal seasonal cycle mantle glycogen varied between 2.93 to 8.15mg.g^{-1} wet tissue weight, mantle protein varied between 25.86 and 50.59mg.g^{-1}wet tissue weight and haemolymph protein approximately varied between

1.5 and 3mg.mL^{-1} of haemolymph. While the mantle glycogen concentration of oysters in both experiments one and two of this study is well within the normal seasonal range, mantle protein was significantly depressed below the normal range. Mantle protein levels can be similarly depressed down to approximately 10mg.g^{-1} wet tissue weight in starved *C. gigas* (Li et al. 2009b). This result suggests that mantle protein had been considerably catabolised by the oysters, possibly to maintain homeostasis during ambient temperature stressors.

In conclusion, this study demonstrates that short-term exposure (6 hours) to artificially acidified seawater (pH≥5), Al (≤7.5mg.L^{-1}) and Fe (≤7.9mg.L^{-1}) have little impact on the survival and biochemical condition of *C. gigas* even when challenged by other extreme stressors. Given these results, it is unlikely that acid leachate exposure during a single tidal cycle, such as during abnormally high tidal movements, would significantly affect oysters in the field. These conclusions could assist in aquaculture management decisions regarding oyster translocation and timing in culture areas where potential acid sulphate soils exist. While the impact of short-term exposure on oyster condition may be minimal it is important to note the potential for the accumulation of metals, particularly iron precipitates, within the oyster tissues. Greater research is needed regarding the clearance of metals from coastal habitats following acute acid leachate exposure and potential food safety impact on oyster aquaculture.

5. References

Bamber R. 1990. The effects of acidic seawater on three species of lamellibranch mollusc, *Journal of Experimental. Marine Biology and Ecology*, vol. 143, pp.181-191.

Berthelin C., Kellner K. and Mathieu M. 2000. Storage metabolism in the Pacific oyster (*Crassostrea gigas*) in relation to summer mortalities and reproductive cycle (West Coast of France), *Comparative Biochemistry and Physiology Part B*, vol. 125, pp. 359-369.

Bradford M. 1976. A rapid and sensitive method for the quantification of microgram quanitites of protein utilizing the principle of protein-dye binding, *Analytical. Biochemistry*, vol.59, pp. 124-132.

Callinan R., Fraser G. and Melville M. 1993. Seasonally recurrent fish mortalities and ulcerative disease outbreaks associated with acid sulphate soils in Australian estuaries. In: Dent D. and van Mensvoort M., editors. Selected papers of the Ho Chi Minh City Symposium on Acid Sulphate Soils, Wageningen, The Netherlands: International Institute for Land Reclamation and Improvement. pp.403-410.

Canesi L., Gallo G., Gavioli M. And Pruzzo C. 2002. Bacteria-hemocyte interactions and phagocytosis in marine bivalves, *Microscopy Research and Technique*, vol. 57, pp. 469-476.

Dove M. and Sammut J. 2007a. Impacts of estuarine acidification on survival and growth of Sydney rock oysters *Saccostrea glomerata* (Gould 1850), *Journal of Shellfish Research*, vol. 26, pp. 519-527.

Dove M. and Sammut J. 2007b. Histological and feeding response of Sydney rock oysters, *Saccostrea glomerata*, to acid sulphate soil outflows, *Journal of Shellfish Research*, vol. 26, pp. 509-518.

Gagnaire B., Thomas-Guyon H. and Renault T. 2004. In vitro effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes, *Fish and Shellfish Immunology*, vol. 16, pp. 501-502.

Hummel H., Wolf L., Zurburg W., Apon L., Bogaards R. and Ruitenburg M. 1989. The glycogen content in stressed marine bivalves: The initial absence of a decrease, *Comparative Biochemistry and Physiology Part B*, vol. 94, pp. 729-733.

Kristman C. 1962. A method for the colorimetric estimation of glycogen with iodine, *Analytical. Biochemistry*, vol.4, pp. 17-23.

Labreuche Y., Soudant P., Goncalves M., Lambert C. and Nicolas J. 2006. Effects of extracellular products from the pathogenic *Vibrio aestuarianus* strain 01/32 on lethality and

cellular immune responses of the oyster *Crassostrea gigas*, *Developmental and Comparative Immunology*, vol. 30, pp. 367-379.

Lacoste A., Malham S., Gelebart F., Cueff A. and Poulet S. 2002. Stress-induced immune changes in the oyster *Crassostrea gigas*, *Developmental and Comparative Immunology*, vol. 26, pp. 1-9.

Lacoste A., Jalabert F., Malham S., Cueff A. and Poulet S. 2001. Stress and stress-induced neuroendocrine changes increase the susceptibility of juvenile oysters (*Crassostrea gigas*) to *Vibrio splendidus*, *Applied and Environmental Microbiology*, vol. 67, pp. 2304-2309.

Li Y., Qin J., Li X. and Benkendorff K. 2009a. Monthly variation of condition index, energy reserves and antibacterial activity in Pacific oysters, *Crassostrea gigas*, in Stansbury (South Australia), *Aquaculture*, vol.286, pp. 64-71.

Li Y., Qin J., Li X. and Benkendorff K. 2009b. Spawning-dependent stress response to food deprivation in Pacific oyster *Crassostrea gigas*, *Aquaculture*, vol.286, pp. 309-317.

Li Y., Qin J., Li X. and Benkendorff K. 2009c. Spawning-dependent stress responses in Pacific oysters *Crassostrea gigas*: A simulated bacterial challenge in oysters, *Aquaculture*, vol.293, pp. 164-171.

Li Y., Qin J., Abbott C., Li X. and Benkendorff K. 2007. Synergistic impacts of heat shock and spawning on the physiology and immune health of *Crassostrea gigas*: an explanation for summer mortality in Pacific oysters, *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, vol. 293, pp. 2353-2362.

Linehan L., O'Connor T. and Burnell G. 1999. Seasonal variation in the chemical composition and fatty acid profile of Pacific oysters (*Crassostrea gigas*), *Food Chemistry*, vol. 64, pp. 211-214.

Pass D., Dybdahl R. and Mannion M. 1987. Investigations into the causes of mortality of the pearl oyster, *Pinctada maxima* (Jamson), in Western Australia, *Aquaculture*, vol. 65, pp. 149-169.

Powell B. and Martens M. 2005. A review of acid sulphate soil impacts, actions and policies that impact on water quality in Great Barrier Reef catchments, including a case study on remediation at East Trinity, *Marine Pollution Bulletin*, vol. 51, pp. 149-164.

Pipe R. and Coles J. 1995. Environmental contaminants influencing immune function in marine bivalve molluscs, *Fish and Shellfish Immunology*, vol.5, pp. 581-595.

Whyte J., Englar J. and Carswell B. 1990. Biochemical composition and energy reserves in *Crassostrea gigas* exposed to different levels of nutrition, *Aquaculture*, vol. 90, pp. 157-172.

Wilson S. and Hyne R. 1997. Toxicity of acid-sulfate soil leachate and aluminum to Embryos of the Sydney rock oyster, *Ecotoxicology and Environmental Safety*, vol. 37, pp.30-36.

Appendices

A.1 Experiment 1 SPSS Output

Descriptives

pH

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	7	8.4271	.07387	.02792	8.3588	8.4955	8.33	8.53
2.00	7	8.0029	.26171	.09892	7.7608	8.2449	7.53	8.24
3.00	7	7.4386	.45867	.17336	7.0144	7.8628	6.69	8.02
4.00	7	5.8414	1.40696	.53178	4.5402	7.1427	4.10	7.61
5.00	7	5.6200	.21825	.08249	5.4182	5.8218	5.38	5.86
Total	35	7.0660	1.31828	.22283	6.6132	7.5188	4.10	8.53

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		

pH	1.00	8	8.5863	.05397	.01908	8.5411	8.6314	8.50	8.65
	2.00	8	8.6175	.06902	.02440	8.5598	8.6752	8.52	8.70
	3.00	8	8.5963	.08733	.03088	8.5232	8.6693	8.48	8.72
	4.00	8	8.5775	.06944	.02455	8.5194	8.6356	8.51	8.73
	5.00	8	8.5150	.06481	.02291	8.4608	8.5692	8.45	8.64
	Total	40	8.5785	.07471	.01181	8.5546	8.6024	8.45	8.73
Temperature	1.00	8	23.7500	4.54878	1.60824	19.9471	27.5529	18.90	29.70
	2.00	8	23.6125	4.54389	1.60651	19.8137	27.4113	18.70	29.60
	3.00	8	23.4250	4.52414	1.59953	19.6427	27.2073	18.50	29.20
	4.00	8	23.6000	4.67822	1.65400	19.6889	27.5111	18.50	29.50
	5.00	8	23.8750	4.80409	1.69850	19.8587	27.8913	18.80	30.30
	Total	40	23.6525	4.38038	.69260	22.2516	25.0534	18.50	30.30
Salinity	1.00	8	34.7625	.43404	.15346	34.3996	35.1254	34.00	35.20
	2.00	8	34.7750	.48624	.17191	34.3685	35.1815	34.20	35.40
	3.00	8	35.5000	.41748	.14760	35.1510	35.8490	34.90	36.20
	4.00	8	35.1375	.63231	.22356	34.6089	35.6661	34.10	35.80

	5.00	8	34.5375	.56300	.19905	34.0668	35.0082	33.70	35.20
	Total	40	34.9425	.59480	.09405	34.7523	35.1327	33.70	36.20

Tests of Normality

	Treatment	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Length	1.00	.206	4	.	.962	4	.791
	2.00	.243	4	.	.966	4	.818
	3.00	.366	4	.	.812	4	.126
	4.00	.251	4	.	.926	4	.570
	5.00	.255	4	.	.960	4	.776
Live.Weight	1.00	.356	4	.	.771	4	.059
	2.00	.266	4	.	.903	4	.444
	3.00	.242	4	.	.908	4	.470
	4.00	.198	4	.	.963	4	.798
	5.00	.286	4	.	.936	4	.630
Flesh.Weight	1.00	.294	4	.	.851	4	.229

	2.00	.199	4	.	.988	4	.950
	3.00	.271	4	.	.859	4	.256
	4.00	.400	4	.	.720	4	.019
	5.00	.247	4	.	.950	4	.716
Flesh.Ratio	1.00	.202	4	.	.957	4	.757
	2.00	.236	4	.	.889	4	.381
	3.00	.172	4	.	.988	4	.945
	4.00	.382	4	.	.719	4	.019
	5.00	.235	4	.	.906	4	.460
Haemolymph.Protein	1.00	.283	4	.	.939	4	.650
	2.00	.227	4	.	.974	4	.864
	3.00	.238	4	.	.895	4	.407
	4.00	.207	4	.	.981	4	.909
	5.00	.247	4	.	.890	4	.384
Mantle.Protein	1.00	.223	4	.	.970	4	.841
	2.00	.148	4	.	.998	4	.995
	3.00	.187	4	.	.964	4	.802

	4.00	.246	4	.	.927	4	.576
	5.00	.282	4	.	.939	4	.648
Mantle.Glycogen	1.00	.270	4	.	.897	4	.416
	2.00	.223	4	.	.931	4	.600
	3.00	.222	4	.	.970	4	.842
	4.00	.368	4	.	.816	4	.135
	5.00	.216	4	.	.960	4	.779

a. Lilliefors Significance Correction

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Length	1.250	4	15	.332
Live.Weight	.466	4	15	.759
Flesh.Weight	6.154	4	15	.004
Flesh.Ratio	7.061	4	15	.002
Haemolymph.Protein	.257	4	15	.901
Mantle.Protein	.192	4	15	.939

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Length	1.250	4	15	.332
Live.Weight	.466	4	15	.759
Flesh.Weight	6.154	4	15	.004
Flesh.Ratio	7.061	4	15	.002
Haemolymph.Protein	.257	4	15	.901
Mantle.Protein	.192	4	15	.939
Mantle.Glycogen	.458	4	15	.765

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Length	1.00	4	80.3325	2.76914	1.38457	75.9262	84.7388	77.17	83.33
	2.00	4	75.4575	2.55209	1.27604	71.3966	79.5184	72.67	78.83
	3.00	4	77.3325	1.70338	.85169	74.6220	80.0430	76.00	79.83
	4.00	4	76.8325	.71751	.35876	75.6908	77.9742	76.17	77.83

	5.00	4	80.8750	3.86071	1.93036	74.7317	87.0183	76.67	86.00
	Total	20	78.1660	3.11932	.69750	76.7061	79.6259	72.67	86.00
Live.Weight	1.00	4	68.9350	3.57281	1.78640	63.2499	74.6201	66.55	74.22
	2.00	4	70.9875	7.20381	3.60190	59.5246	82.4504	60.83	77.28
	3.00	4	67.7275	6.06444	3.03222	58.0776	77.3774	62.53	75.90
	4.00	4	67.6025	3.92862	1.96431	61.3512	73.8538	63.68	72.83
	5.00	4	69.7300	5.63142	2.81571	60.7692	78.6908	63.48	77.17
	Total	20	68.9965	5.01571	1.12155	66.6491	71.3439	60.83	77.28
Flesh.Weight	1.00	4	7.1850	.65292	.32646	6.1461	8.2239	6.45	7.75
	2.00	4	7.3950	1.12417	.56209	5.6062	9.1838	5.98	8.70
	3.00	4	6.8450	.98963	.49481	5.2703	8.4197	5.83	7.80
	4.00	4	10.7400	6.50362	3.25181	.3913	21.0887	6.70	20.45
	5.00	4	7.5050	.67777	.33888	6.4265	8.5835	6.67	8.33
	Total	20	7.9340	3.04923	.68183	6.5069	9.3611	5.83	20.45
Flesh.Ratio	1.00	4	10.2200	.69824	.34912	9.1090	11.3310	9.54	11.14
	2.00	4	10.2125	.77878	.38939	8.9733	11.4517	9.57	11.30
	3.00	4	10.0850	.81937	.40968	8.7812	11.3888	9.17	11.06

	4.00	4	15.9375	10.04601	5.02300	-.0479	31.9229	9.98	30.91
	5.00	4	10.7450	.92378	.46189	9.2751	12.2149	9.95	12.03
	Total	20	11.4400	4.66109	1.04225	9.2585	13.6215	9.17	30.91
Haemolymph.Protein	1.00	4	1.2800	.11972	.05986	1.0895	1.4705	1.12	1.41
	2.00	4	1.1750	.08737	.04368	1.0360	1.3140	1.08	1.29
	3.00	4	1.2175	.08057	.04029	1.0893	1.3457	1.15	1.33
	4.00	4	1.2125	.11701	.05851	1.0263	1.3987	1.06	1.34
	5.00	4	1.2250	.12793	.06397	1.0214	1.4286	1.05	1.33
	Total	20	1.2220	.10217	.02285	1.1742	1.2698	1.05	1.41
Mantle.Protein	1.00	4	16.7925	4.72667	2.36334	9.2713	24.3137	11.82	23.08
	2.00	4	20.3250	3.11859	1.55930	15.3626	25.2874	16.73	24.14
	3.00	4	20.2275	3.66131	1.83065	14.4015	26.0535	16.58	25.06
	4.00	4	16.5050	2.92208	1.46104	11.8553	21.1547	12.77	19.18
	5.00	4	21.3550	5.03819	2.51910	13.3381	29.3719	15.72	27.98
	Total	20	19.0410	4.08962	.91447	17.1270	20.9550	11.82	27.98
Mantle.Glycogen	1.00	4	8.5375	2.45763	1.22882	4.6269	12.4481	6.41	11.63
	2.00	4	6.7250	1.86964	.93482	3.7500	9.7000	4.16	8.44

	3.00	4	7.8550	2.60499	1.30249	3.7099	12.0001	5.12	11.32
	4.00	4	7.6775	1.50267	.75134	5.2864	10.0686	5.48	8.88
	5.00	4	7.6325	1.99620	.99810	4.4561	10.8089	5.56	10.01
	Total	20	7.6855	1.97901	.44252	6.7593	8.6117	4.16	11.63

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Length	Between Groups	87.365	4	21.841	3.360	.037
	Within Groups	97.508	15	6.501		
	Total	184.873	19			
Live.Weight	Between Groups	32.238	4	8.059	.271	.892
	Within Groups	445.752	15	29.717		
	Total	477.990	19			
Flesh.Weight	Between Groups	40.380	4	10.095	1.111	.388
	Within Groups	136.278	15	9.085		
	Total	176.658	19			
Flesh.Ratio	Between Groups	102.167	4	25.542	1.233	.339

	Within Groups	310.623	15	20.708		
	Total	412.790	19			
Haemolymph.Protein	Between Groups	.023	4	.006	.486	.746
	Within Groups	.176	15	.012		
	Total	.198	19			
Mantle.Protein	Between Groups	79.592	4	19.898	1.253	.331
	Within Groups	238.182	15	15.879		
	Total	317.775	19			
Mantle.Glycogen	Between Groups	6.720	4	1.680	.372	.825
	Within Groups	67.693	15	4.513		
	Total	74.413	19			

A.2 Experiment 2 SPSS Output

Descriptives

pH

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	Minimum	Maximum

					Lower Bound	Upper Bound		
1.00	7	8.5614	.04670	.01765	8.5182	8.6046	8.49	8.62
2.00	7	8.6114	.03024	.01143	8.5835	8.6394	8.56	8.65
3.00	7	8.5929	.02812	.01063	8.5669	8.6189	8.56	8.62
4.00	7	7.2914	.14736	.05570	7.1551	7.4277	7.10	7.49
5.00	7	6.0357	.19663	.07432	5.8539	6.2176	5.80	6.30
6.00	7	4.9900	.21000	.07937	4.7958	5.1842	4.68	5.21
7.00	7	5.0029	.07610	.02876	4.9325	5.0732	4.87	5.09
Total	49	7.0122	1.56115	.22302	6.5638	7.4607	4.68	8.65

Descriptives

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
pH	1.00	9	8.6100	.03279	.01093	8.5848	8.6352	8.54	8.65
	2.00	9	8.6156	.03283	.01094	8.5903	8.6408	8.56	8.68
	3.00	9	8.5889	.03408	.01136	8.5627	8.6151	8.52	8.63
	4.00	9	8.6144	.04475	.01492	8.5800	8.6488	8.53	8.70

	5.00	9	8.5889	.04014	.01338	8.5580	8.6197	8.52	8.64
	6.00	9	8.5622	.05215	.01738	8.5221	8.6023	8.49	8.63
	7.00	9	8.5633	.04359	.01453	8.5298	8.5968	8.52	8.63
	Total	63	8.5919	.04406	.00555	8.5808	8.6030	8.49	8.70
Temperature	1.00	9	26.9222	5.43548	1.81183	22.7441	31.1003	20.20	33.30
	2.00	9	26.7667	5.29670	1.76557	22.6953	30.8381	20.20	33.10
	3.00	9	26.4444	5.00752	1.66917	22.5953	30.2936	20.10	32.20
	4.00	9	26.4222	4.92818	1.64273	22.6341	30.2104	20.30	32.20
	5.00	9	26.4333	4.94924	1.64975	22.6290	30.2377	20.20	32.20
	6.00	9	26.6000	5.10637	1.70212	22.6749	30.5251	20.20	32.80
	7.00	9	26.8667	5.35654	1.78551	22.7493	30.9841	20.30	33.20
	Total	63	26.6365	4.90603	.61810	25.4009	27.8721	20.10	33.30
Salinity	1.00	9	35.0444	.87765	.29255	34.3698	35.7191	33.60	36.10
	2.00	9	35.0556	.93956	.31319	34.3333	35.7778	33.40	36.10
	3.00	9	35.2444	.76340	.25447	34.6576	35.8312	34.00	36.10
	4.00	9	34.8778	1.01953	.33984	34.0941	35.6615	33.30	36.10
	5.00	9	34.9556	1.05488	.35163	34.1447	35.7664	33.20	36.10

	6.00	9	35.0667	1.09087	.36362	34.2281	35.9052	33.20	36.10
	7.00	9	35.1333	.94340	.31447	34.4082	35.8585	33.30	36.10
	Total	63	35.0540	.92017	.11593	34.8222	35.2857	33.20	36.10
DO	1.00	9	5.8111	.23154	.07718	5.6331	5.9891	5.50	6.00
	2.00	9	5.9556	.10138	.03379	5.8776	6.0335	5.70	6.00
	3.00	9	5.9222	.08333	.02778	5.8582	5.9863	5.80	6.00
	4.00	9	5.9778	.04410	.01470	5.9439	6.0117	5.90	6.00
	5.00	9	5.9889	.03333	.01111	5.9633	6.0145	5.90	6.00
	6.00	9	5.9222	.08333	.02778	5.8582	5.9863	5.80	6.00
	7.00	9	5.8667	.17321	.05774	5.7335	5.9998	5.60	6.00
	Total	63	5.9206	.13339	.01681	5.8870	5.9542	5.50	6.00

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Treatment	Statistic	df	Sig.	Statistic	df	Sig.
Survival	1.00	.441	4	.	.630	4	.001
	2.00	.283	4	.	.863	4	.272

	3.00	.298	4	.	.849	4	.224
	4.00	.260	4	.	.827	4	.161
	5.00	.250	4	.	.945	4	.683
	6.00	.151	4	.	.993	4	.972
	7.00	.283	4	.	.863	4	.272
Length	1.00	.193	4	.	.990	4	.955
	2.00	.251	4	.	.917	4	.518
	3.00	.231	4	.	.895	4	.405
	4.00	.322	4	.	.817	4	.136
	5.00	.247	4	.	.922	4	.551
	6.00	.223	4	.	.941	4	.660
	7.00	.152	4	.	.997	4	.990
Live.Weight	1.00	.319	4	.	.872	4	.306
	2.00	.248	4	.	.929	4	.591
	3.00	.292	4	.	.925	4	.565
	4.00	.205	4	.	.942	4	.668
	5.00	.237	4	.	.937	4	.638

	6.00	.234	4	.	.942	4	.667
	7.00	.178	4	.	.994	4	.977
Flesh.Weight	1.00	.207	4	.	.958	4	.765
	2.00	.371	4	.	.764	4	.052
	3.00	.243	4	.	.957	4	.763
	4.00	.222	4	.	.978	4	.893
	5.00	.286	4	.	.813	4	.129
	6.00	.265	4	.	.929	4	.588
	7.00	.174	4	.	.987	4	.943
Flesh.Ratio	1.00	.185	4	.	.993	4	.970
	2.00	.331	4	.	.834	4	.177
	3.00	.271	4	.	.897	4	.417
	4.00	.147	4	.	.995	4	.983
	5.00	.211	4	.	.965	4	.813
	6.00	.173	4	.	.988	4	.946
	7.00	.299	4	.	.843	4	.205
Haemolymph.Protein	1.00	.305	4	.	.880	4	.339

	2.00	.184	4	.	.973	4	.859
	3.00	.349	4	.	.826	4	.158
	4.00	.214	4	.	.983	4	.917
	5.00	.284	4	.	.878	4	.329
	6.00	.184	4	.	.993	4	.973
	7.00	.250	4	.	.890	4	.385
Mantle.Protein	1.00	.244	4	.	.919	4	.530
	2.00	.203	4	.	.982	4	.916
	3.00	.173	4	.	.995	4	.981
	4.00	.272	4	.	.948	4	.702
	5.00	.164	4	.	.991	4	.964
	6.00	.383	4	.	.793	4	.090
	7.00	.235	4	.	.959	4	.775
Mantle.Glycogen	1.00	.390	4	.	.775	4	.065
	2.00	.283	4	.	.870	4	.297
	3.00	.224	4	.	.973	4	.860
	4.00	.178	4	.	.991	4	.964

	5.00	.255	4	.	.902	4	.442
	6.00	.209	4	.	.981	4	.908
	7.00	.223	4	.	.955	4	.746

a. Lilliefors Significance Correction

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Survival	.800	6	21	.581
Length	1.938	6	21	.121
Live.Weight	.511	6	21	.793
Flesh.Weight	.851	6	21	.546
Flesh.Ratio	.822	6	21	.565
Haemolymph.Protein	1.419	6	21	.254
Mantle.Protein	.659	6	21	.683
Mantle.Glycogen	1.308	6	21	.297

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Survival	1.00	4	72.2250	11.11000	5.55500	54.5465	89.9035	66.67	88.89
	2.00	4	75.0025	10.63702	5.31851	58.0766	91.9284	66.67	88.89
	3.00	4	80.5575	16.66500	8.33250	54.0398	107.0752	66.67	100.00
	4.00	4	88.8900	15.71191	7.85596	63.8888	113.8912	66.67	100.00
	5.00	4	88.8900	9.07128	4.53564	74.4556	103.3244	77.78	100.00
	6.00	4	83.3350	14.34295	7.17147	60.5122	106.1578	66.67	100.00
	7.00	4	80.5575	10.63702	5.31851	63.6316	97.4834	66.67	88.89
	Total	28	81.3511	12.84780	2.42801	76.3692	86.3329	66.67	100.00
Length	1.00	4	80.1675	1.17710	.58855	78.2945	82.0405	78.67	81.50
	2.00	4	81.3325	1.67476	.83738	78.6676	83.9974	79.83	83.67
	3.00	4	79.0000	3.59341	1.79671	73.2821	84.7179	76.00	84.00
	4.00	4	81.8350	.91409	.45705	80.3805	83.2895	81.17	83.17
	5.00	4	80.4175	2.12929	1.06465	77.0293	83.8057	78.50	83.17
	6.00	4	79.3725	1.76462	.88231	76.5646	82.1804	77.50	81.33

	7.00	4	80.2100	.84289	.42145	78.8688	81.5512	79.17	81.17
	Total	28	80.3336	1.95227	.36894	79.5766	81.0906	76.00	84.00
Live.Weight	1.00	4	89.1075	2.79794	1.39897	84.6553	93.5597	86.70	93.13
	2.00	4	88.8125	3.95355	1.97678	82.5215	95.1035	85.03	93.60
	3.00	4	83.0700	3.86681	1.93340	76.9170	89.2230	77.72	86.93
	4.00	4	86.7725	2.87554	1.43777	82.1969	91.3481	82.90	89.47
	5.00	4	83.7025	2.96275	1.48138	78.9881	88.4169	80.72	87.15
	6.00	4	84.0675	4.45359	2.22680	76.9808	91.1542	79.82	89.70
	7.00	4	86.2750	5.10148	2.55074	78.1574	94.3926	79.87	92.10
	Total	28	85.9725	4.07012	.76918	84.3943	87.5507	77.72	93.60
Flesh.Weight	1.00	4	7.0500	.46007	.23004	6.3179	7.7821	6.60	7.65
	2.00	4	7.3625	1.01631	.50816	5.7453	8.9797	6.68	8.87
	3.00	4	7.1350	.86172	.43086	5.7638	8.5062	5.97	8.02
	4.00	4	7.0400	.86568	.43284	5.6625	8.4175	5.93	8.03
	5.00	4	6.5375	.71107	.35554	5.4060	7.6690	5.85	7.20
	6.00	4	6.7875	.32898	.16449	6.2640	7.3110	6.33	7.10
	7.00	4	6.7050	.65424	.32712	5.6640	7.7460	5.92	7.43

	Total	28	6.9454	.70055	.13239	6.6737	7.2170	5.85	8.87
Flesh.Ratio	1.00	4	7.9800	.61150	.30575	7.0070	8.9530	7.28	8.75
	2.00	4	8.2975	.76960	.38480	7.0729	9.5221	7.70	9.42
	3.00	4	8.6125	.69806	.34903	7.5017	9.7233	7.74	9.22
	4.00	4	8.1100	.84711	.42356	6.7621	9.4579	7.11	9.09
	5.00	4	7.9000	.87977	.43989	6.5001	9.2999	6.83	8.81
	6.00	4	7.9675	.82935	.41468	6.6478	9.2872	6.96	8.88
	7.00	4	7.7825	.31288	.15644	7.2846	8.2804	7.52	8.14
	Total	28	8.0929	.69626	.13158	7.8229	8.3628	6.83	9.42
Haemolymph.Protein	1.00	4	1.7525	.23557	.11778	1.3777	2.1273	1.55	2.09
	2.00	4	1.7650	.07326	.03663	1.6484	1.8816	1.69	1.86
	3.00	4	1.8475	.46679	.23339	1.1047	2.5903	1.48	2.53
	4.00	4	1.9000	.21894	.10947	1.5516	2.2484	1.65	2.18
	5.00	4	1.7800	.34995	.17498	1.2231	2.3369	1.49	2.28
	6.00	4	1.9850	.21174	.10587	1.6481	2.3219	1.74	2.25
	7.00	4	1.7800	.19201	.09600	1.4745	2.0855	1.62	2.05
	Total	28	1.8300	.25607	.04839	1.7307	1.9293	1.48	2.53

Mantle.Protein	1.00	4	14.5625	6.19370	3.09685	4.7069	24.4181	9.00	23.18
	2.00	4	14.8775	5.98175	2.99087	5.3592	24.3958	7.53	22.10
	3.00	4	11.1825	3.85061	1.92531	5.0553	17.3097	6.50	15.80
	4.00	4	14.8125	4.66744	2.33372	7.3856	22.2394	9.60	20.95
	5.00	4	15.6600	6.67951	3.33976	5.0314	26.2886	7.53	23.09
	6.00	4	14.1975	1.28669	.64334	12.1501	16.2449	13.21	16.09
	7.00	4	15.5550	6.39703	3.19852	5.3759	25.7341	7.75	23.41
	Total	28	14.4068	4.90202	.92639	12.5060	16.3076	6.50	23.41
Mantle.Glycogen	1.00	4	6.7000	1.08943	.54472	4.9665	8.4335	5.90	8.31
	2.00	4	6.3225	1.51234	.75617	3.9160	8.7290	5.11	8.27
	3.00	4	6.2875	2.01392	1.00696	3.0829	9.4921	4.13	8.95
	4.00	4	10.4325	3.52916	1.76458	4.8168	16.0482	5.95	14.36
	5.00	4	6.5750	1.80652	.90326	3.7004	9.4496	5.04	8.97
	6.00	4	5.9875	2.16642	1.08321	2.5402	9.4348	3.62	8.81
	7.00	4	6.5200	.81154	.40577	5.2287	7.8113	5.55	7.35
	Total	28	6.9750	2.30043	.43474	6.0830	7.8670	3.62	14.36

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Survival	Between Groups	969.824	6	161.637	.973	.467
	Within Groups	3486.957	21	166.046		
	Total	4456.780	27			
Length	Between Groups	24.016	6	4.003	1.065	.414
	Within Groups	78.890	21	3.757		
	Total	102.907	27			
Live.Weight	Between Groups	143.327	6	23.888	1.650	.183
	Within Groups	303.952	21	14.474		
	Total	447.280	27			
Flesh.Weight	Between Groups	1.916	6	.319	.592	.734
	Within Groups	11.335	21	.540		
	Total	13.251	27			
Flesh.Ratio	Between Groups	1.897	6	.316	.593	.732
	Within Groups	11.193	21	.533		
	Total	13.089	27			

Haemolymph.Protein	Between Groups	.178	6	.030	.391	.877
	Within Groups	1.593	21	.076		
	Total	1.770	27			
Mantle.Protein	Between Groups	54.957	6	9.159	.324	.917
	Within Groups	593.847	21	28.278		
	Total	648.803	27			
Mantle.Glycogen	Between Groups	57.082	6	9.514	2.328	.070
	Within Groups	85.801	21	4.086		
	Total	142.883	27			