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# Striking a balance between melanosis and weight recovery in western rock lobster (*Panulirus Cygnus*)

Hannah Williams, Glen Davidson and John Mamo

Food Science
and Technology Program

Curtin
University of Technology

ROCK LOBSTER POST-HARVEST SUBPROGRAM
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**OBJECTIVES:**

1. To establish the impact of temperature on the activity of *P. cygnus* hemolymph PPO *in vitro*
2. To establish the impact of post-harvest transportation and holding on PPO activity, weight recovery and melanosis formation
3. To establish the impacts of current commercial practises on weight recovery and melanosis formation in *P. cygnus*
4. To validate the use of experimentally determined thermal kinetic parameters for prevention of melanosis in *P. cygnus*
5. To establish the impact of antibrowning agents on the activity of *P. cygnus* hemolymph PPO *in vitro*.
6. To determine the effects of anti-browning agents on weight recovery and melanosis formation when applied during processing

**NON TECHNICAL SUMMARY:**

**OUTCOMES ACHIEVED TO DATE**

The cause of melanosis was identified as the enzyme: polyphenoloxidase. The enzyme's kinetic parameters were calculated and applied to current processing methods. The thermal kinetics values for western rock lobster were determined and the theoretical lethality ($F_{90}$) was derived. The theoretical time required to deactivate 99.9% of the enzyme at a reference temperature (90°C) ($F_{90}$) is 36.12 minutes. The actual process lethality achieved in current processing was calculated using

$$F_{process} = \sum 10^{\frac{T_i - T_r}{z}} \Delta t$$

Comparison of the actual process lethality to the desired theoretical value was used to predict how effective the processing system was in preventing melanosis. It was clearly demonstrated that current processing methods do not deactivate the enzyme. To deactivate the enzyme much longer cooking times at higher temperatures would be needed. Long cook times, however, increase weight loss. Current best practice transportation and post-harvest holding methods do not impact on melanosis or weight recovery. The theoretical values were validated for use in western rock lobster processing which enables the evaluation of a processing system's effectiveness in the prevention of melanosis formation.

Each year 4-5,000 tonnes of western rock lobster are cooked and exported to countries such as Japan and Taiwan. When existing cooking methods are used a proportion of cooked western rock lobster go black, this results in a product that is unacceptable to the customer

The blackening of the flesh is known as melanosis and is caused by an enzyme called polyphenoloxidase (PPO). Melanosis is a major problem for the western rock lobster industry and it has been estimated that it costs the industry over $1 million per year. Finding a solution to the problem of melanosis by stopping PPO activity is therefore necessary. Currently, cooking is the most common method used in food processing to stop enzyme activity. However, as the PPO enzyme is evidently still

active after export lobsters have been cooked it was important to first understand what was happening to the enzyme when it was heated.

This study showed that when lobsters were heated to an internal temperature between 60 and 80°C there was a large increase in enzyme activity. It was not until after temperatures reached 90°C, or more, the enzyme activity stopped. Overall it was found that for 50% of the cooking time, temperatures only reached between 60-80°C. Therefore they did not get high enough to kill the enzyme; rather, current cooking methods actually increase the enzyme 's activity.

It was thought that some factors that occur before cooking, such as transportation and holding conditions could also have an impact on enzyme activity. To test this a series of experiments were undertaken to simulate the existing transportation and holding conditions of lobsters before they are cooked. The results of these experiments showed that when current practises in post-harvest handling of live lobsters were followed there was no observable impact on the activity of the enzyme.

What was still unknown was how long the temperature needed to remain at 90°C in order to completely stop the enzyme activity causing melanosis. Therefore a series of experiments was undertaken using a range of time-temperature combinations to produce a range from severely undercooked to severely overcooked lobsters. From this study it was found that lobsters must be cooked for at least 36.12 minutes at 90°C in order to prevent melanosis.

However, increased cooking times results in increased weight loss in lobsters. For the producer this means lost money. A solution was needed as to how to reduce the length of cooking time required, in order to minimise both weight loss and melanosis. One possibility lies in the use of chemical compounds called "antibrowning agents". These chemicals work in a similar way to putting lemon juice on cut apples to stop them going brown.

Four different antibrowning agents (ascorbic acid (vitamin C), citric acid, 4-hexylresorcinol (the active ingredient in "Everfresh") and carbon dioxide were selected on the basis of their "naturalness". A comparison of their impact on enzyme activity was undertaken using laboratory-based tests. Ascorbic acid was not effective. Citric acid, 4-hexylresorcinol, and carbon dioxide all prevented enzyme activity even when applied at low concentrations. The next stage was to trial the

effectiveness of these antibrowning agents in prevention of melanosis during commercial processing of western rock lobster.

Different strengths of the three effective antibrowning agents were applied to lobsters during the preparation for cooking. Melanosis development was then measured after cooking. Significant reduction in melanosis was achieved with all of the agents, however, lobsters treated with 4-hexylresorcinol showed the greatest reduction in melanosis. Stopping the melanosis completely was not achieved by any of the agents. It was therefore established that antibrowning agents may be of assistance but further work needs to be undertaken to establish the best way to use them.

This study showed that prolonged cooking at higher temperatures can prevent melanosis but that this results in weight losses. Therefore a further project is proposed to look at different methods of cooking, using higher cooking temperatures over shorter time periods to establish their impact on preventing weight loss and melanosis formation.

**KEYWORDS: Rock lobster, processing, cooking, melanosis, polyphenoloxidase**

**ACKNOWLEDGEMENTS**

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## BACKGROUND

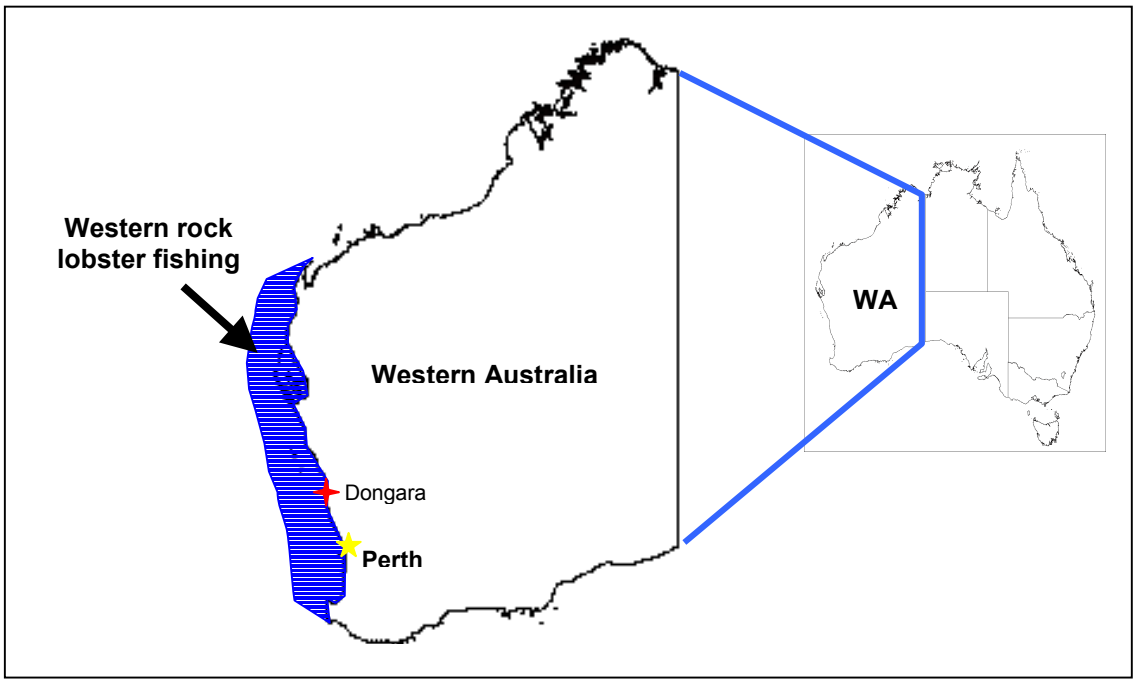


*Figure 1: Location of Western Rock Lobster Fishery*

The Western Rock Lobster Fishery extends along the west coast of Western Australia from the North West Cape in the north to Cape Leeuwin in the south (Figure 1) and harvests only the western rock lobster (*Panulirus cygnus*). The fishery is the third largest commercial lobster fishery in the world and the most valuable single species fishery in Australia with an average catch of 11.3 million kg and a commercial value of AU$200 to $400 million p.a. (Fisheries WA 2002). The value of the fishery equates to 20% of the total value of all Australian fisheries (Fisheries WA 2002).

The Western Rock Lobster Fishery supplies nine major processors and several smaller ones. The processors on-sell rock lobster in a variety of forms with the major types being: live, whole raw frozen, whole cooked frozen, and tails chilled or frozen to the major export markets in Japan, Singapore, United States, China, Hong Kong and Chinese Taipei (Figures 2 and 3).

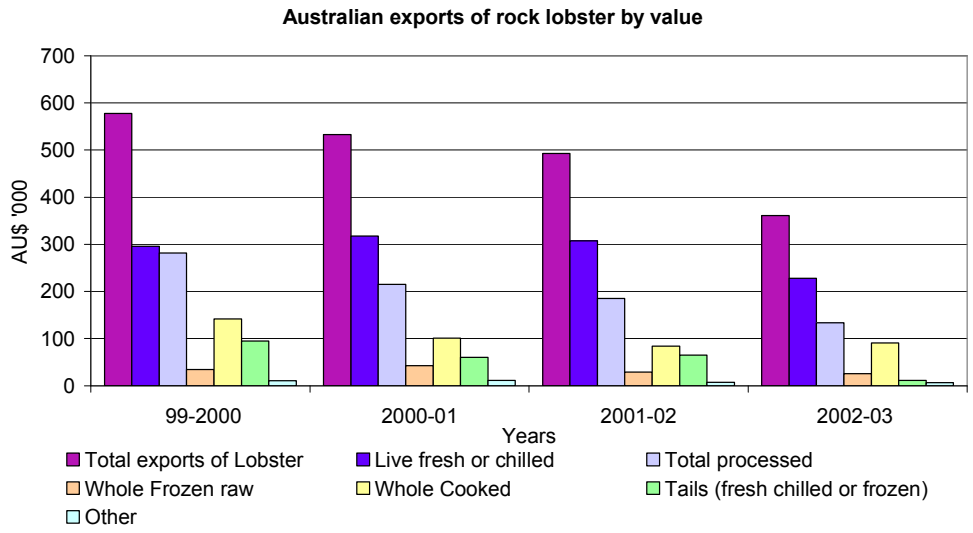


*Figure 2: Breakdown of Australian rock lobster exports by value (ABARE 2004)*

*Table 1: Four-year summary for Australian rock lobster exports (ABARE 2004)*

| Year | % Live | | % Processed | | % of processed sold as whole cooked | |
|---|---|---|---|---|---|---|
| | % by weight | $'000 | % by weight | $'000 | % by weight | $'000 |
| 99-2000 | 52.17 | 51.2 | 47.83 | 48.8 | 57.77 | 50.28 |
| 2000-01 | 57.47 | 59.65 | 42.53 | 40.35 | 53.52 | 46.96 |
| 2001-02 | 60.77 | 62.39 | 39.23 | 37.61 | 52.67 | 45.4 |
| 2002-03 | 50.91 | 63.09 | 49.09 | 36.91 | 49.82 | 67.87 |
| Average | 55.3 | 59.1 | 44.7 | 40.9 | 53.4 | 52.6 |

Live lobster continues to be the highest earner from the export market (Figure 2) with the majority of the live product being derived from the southern fisheries, however over the last four years 35.5 to 46.4% of the value of lobster exports has come from within the processed categories (Table 1). The majority of processed product is produced in the Western Rock Lobster Fishery and of this, 42.3 to 63% of the value of exports has been derived from whole, cooked lobster (Table 1, Figure 2), which means this is the single most important commodity in the processed sector of the market. However, different markets prefer different types of product as shown in Figure 3.

The majority of the whole cooked lobster is exported to Japan (37.7%) closely followed by China Taipei (28.7%) (Figure 3). In these markets, most of the product is bought by catering establishments and restaurants. Since lobster is seen as a luxury food to be eaten on special occasions, appearance is of paramount importance in determining acceptability.

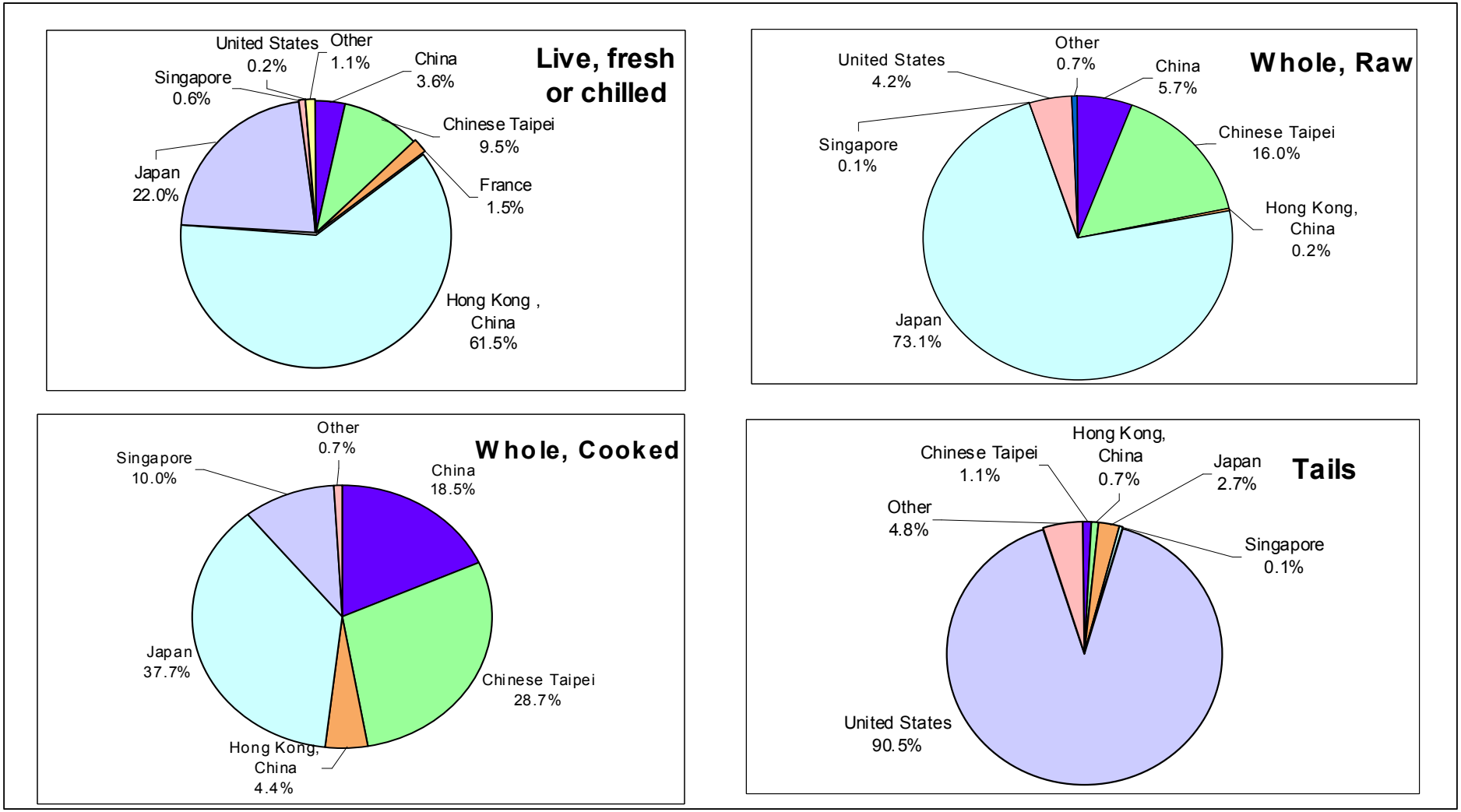


*Figure 3: Market preference for product type, 2002-03 (ABARE 2004)*

Discussions with western rock lobster processors in 2000 highlighted an important quality defect occurring in cooked western rock lobster. Japanese restaurateurs and caterers were complaining of blackening, or **melanosis**, of the tissues during preparation of western rock lobster for banquets. The cooked lobsters were purchased frozen and split with an electric band saw then placed in the refrigerator overnight to thaw. When removed from the refrigerator the following day between 5 and 100% of the lobsters had developed black regions in the head and at the anterior end of the tail meat. (Mr Ross McGregor, Mr Stephen Hood, pers.comm., June 2003). An example of melanosis in western rock lobster is shown in Figure 4.

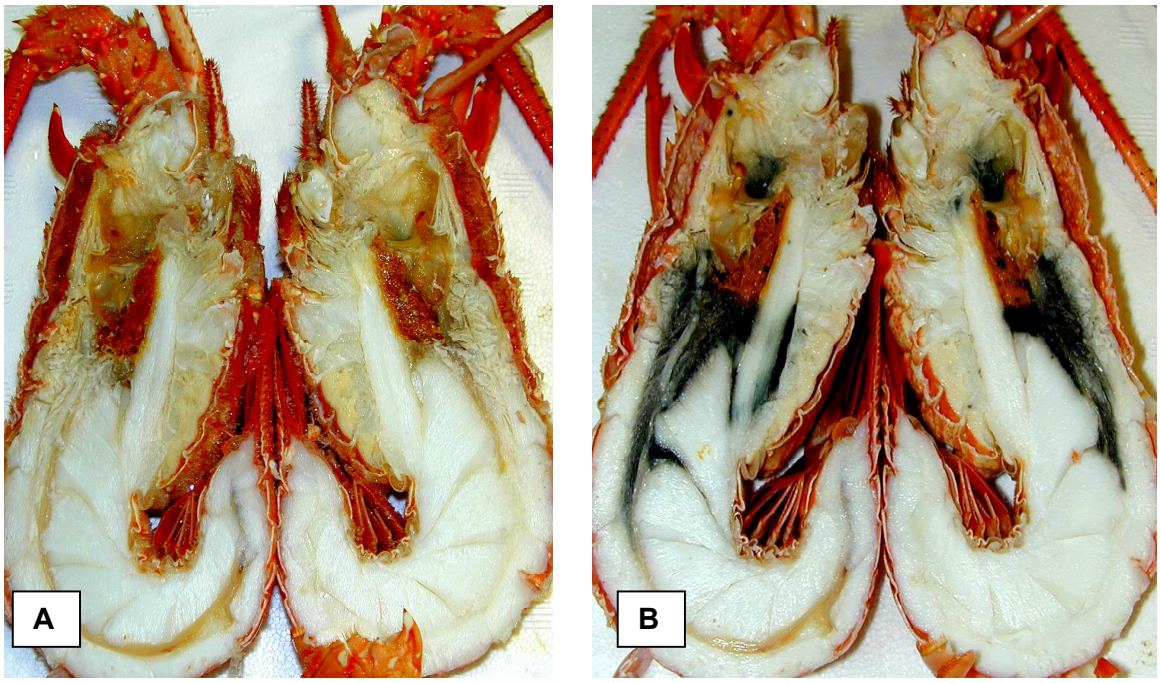


*Figure 4: Melanosis in processed western rock lobster*
*(A) Freshly cut processed lobster, (B) Same lobster after 3 hours*

Since western rock lobster is deemed a luxury food in many markets, and is reserved to be eaten on special occasions, appearance is of paramount importance in determining acceptability. While the occurrence of melanosis during preparation is not harmful to human health, it violates the perceived appearance norm for lobsters. Violation of expectations takes on heightened meaning when associated with socially important occasions, such as banquets and weddings. The elevated level of emotional and financial input raises the standard such that variations from the culturally accepted norm that would be tolerated within the home are not accepted at this level of social interaction.

It is also important to realise the symbolism associated with colour and food in the cultures of Asian nations. Colours have a significant meaning for the Chinese and Japanese in particular (Jacobs et al. 1991). In Japan, red is an auspicious colour, as are other bright colours. In the Chinese culture, blue and black are considered 'sad' colours while gold, red and green are perceived as positive colours (Jacobs et al. 1991). Black is frowned upon by the older generation, especially during festive occasions. Colour thus provides a code for "safe" as well as appropriate foods. In these markets, western rock lobster is highly prized for weddings and other important celebrations due to the bright red of the shell in both the raw and cooked state. However, the blackening of the flesh violates the culturally accepted norm and may be seen as inauspicious thus having an impact beyond concerns of health and safety. It is therefore of vital interest to the western rock lobster industry to establish what causes the blackening and identify possible means of prevention in order to maximise sales of processed whole western rock lobster into the lucrative Asian markets.

Research conducted by Johnson & Evans (1991) identified the location and onset point of melanosis in western rock lobster. It was noted that melanosis was isolated in the heart, arteries and ventral sinus of the lobster, i.e. those organs and regions high in hemolymph content, leading to the conclusion that a constituent of the hemolymph is responsible for melanosis formation. No further work has been carried out to determine the causative agents or factors that may impact on the melanosis formation in western rock lobster.

Processors believe that increasing the cooking time can reduce melanosis. However, this also results in reduced cooked weight recoveries and substantial financial losses to the processor. Examination of the literature does not confirm or refute this belief, as it does not appear to have been evaluated by a scientific study. At present, cooked weight recoveries average between 93-95% of the landed beach weight. Assuming that approximately 50% of the annual catch is processed as whole cooked lobster, Mr. Steven Hood, Compliance and Projects Manager, of M.G. Kailis International Pty Ltd estimated that a 5% loss in beach weight during cooking would cost the industry approximately $9.5 million per annum based on a beach price of $26 per kg (pers.comm., 4 July 2000). Thus, there appears to be significant scope for improvement. In addition, Mr. Ross McGregor, Marketing Manager, of Lobster Australia Ltd. estimated that the occurrence of melanosis after

processing costs the industry approximately $1 million per annum in destroyed stock without taking into account lost repeat sales (pers.comm., June 27th 2000).

Considerable work has been done on cuticular melanosis (black spot) in prawns by other investigators and the use of anti-browning agents, such as sodium metabisulphite, has been shown to be effective for reducing the incidence of blackspot (Ogawa 1984; Ogawa 1987; Otwell, Iyengar & McEvily 1992; Slattery, Williams & Cusack 1995; Chinivasagam, Bremner & Reeves 1998; Adachi et al. 2001) Application of anti-browning agents may also reduce melanosis in rock lobsters, but very little information on their use is available. A study is required to establish the most effective anti-browning agent (or agents), and their effective dose rates, for processing.

To date a comprehensive study to establish the impacts of post-harvest handling and processing on cooked weight recovery and melanosis formation in western rock lobster has not been undertaken. For the western rock lobster industry to make informed decisions resulting in rationalization and/or formulation of handling and processing protocols, fundamental studies aimed at obtaining a better understanding of the mechanisms of melanosis in western rock lobster and the impacts of processing on melanosis formation are needed.

**Causes of discolouration in crustacean species**

Johnson & Evans (1991) carried out a study to identify the location and onset point of melanosis in western rock lobsters. They discovered that it was isolated in the heart, arteries and ventral sinus of the lobster, i.e. those organs and regions high in hemolymph content, leading to the conclusion that a constituent of the hemolymph is responsible for melanosis formation.

Boon (1975) reviewed a series of studies into discolouration of heat-processed crabmeat and noted that the condition known as ‘blueing’ in crabmeat was related to the hemolymph content of the meat. Differences in the degree of blueing were noted between species of crab and those species that underwent blueing were found to have similar hemolymph characteristics. These included high copper content in the hemolymph, appreciable amounts of phenolic substances, for example tyrosine, and the presence of polyphenoloxidase enzymes such as tyrosinase (Boon 1975). Boon (1975) also noted that other decapods with similar hemolymph characteristics show similar discolouration.

'Black spot' is the name given to post-mortem melanosis of the cuticle in prawns and lobsters. The discolouration is the result of oxidative enzyme reactions followed by non-enzymatic oxidation and polymerisation of reaction products to form melanin pigments (Ogawa 1987). Blackspot has been shown to be influenced by the degree of stress the crustacean suffers; physical damage to the cuticle; the moult stage of the crustacean and gender (Ogawa 1984; Ogawa 1987). Uninjured lobsters that were drowned in iced water immediately post harvest showed no melanosis formation, while organisms that were injured, and/or stressed, invariably underwent melanosis (Ogawa 1987). Females and those animals in the pre-moult stage of ecdysis also showed higher rates of black spot. It is thought that the levels of enzymes are increased during the pre-moult stage making these animals more susceptible to melanosis. Ogawa (1987) also noted that freezing did not prevent melanosis, it merely delayed the onset until thawing. Research into the physiological role of the oxidative enzymes that initiate the formation of melanin has shown that they play a part in the mechanisms of the host defence system (Söderhäll 1982).

**Role of melanin in crustacean immunity**

Söderhäll and others (Ashida & Söderhäll 1984; Ferrer et al. 1989a; Ferrer et al. 1989b; Chen et al. 1991; Söderhäll & Cerenius 1998) have carried out extensive research into the immune reactions and wound repair systems of crustaceans. Like most creatures, the host defence system of arthropods is largely based in the blood cells or **hemocytes**. These cells can remove foreign particles by phagocytosis or encapsulation activities. They also participate in the rapid sealing of wounds to prevent the loss of hemolymph and the ingress of microorganisms.

Research has shown that bacterial invasion or wounding sets off a complex cascade of reactions in the hemolymph and/or cuticle of arthropods, the final product of which is melanin. The components of the cascade act to stimulate the cellular defence system of arthropods resulting in phagocytosis, clotting and encapsulation of foreign matter. Since the cascade acts to activate **prophenoloxidase (proPO)** to **polyphenoloxidase (PPO)** it is known as the **prophenoloxidase activating system (proPOA)** and is illustrated in Figure 5.

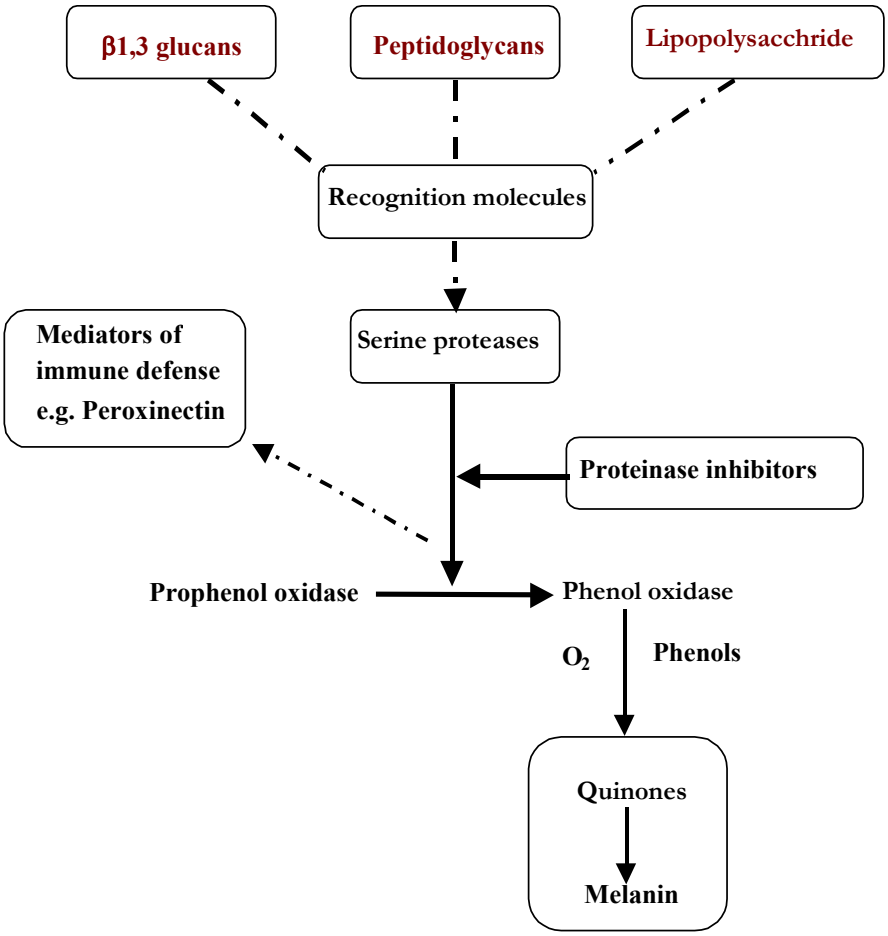


*Figure 5: The prophenoloxidase activation system (Johansson & Söderhäll 1989; Söderhäll & Cerenius 1992)*

**Polyphenoloxidase (PPO)**

Polyphenoloxidase (PPO; monophenol, L-dopa: oxygen oxidoreductase EC 1.14.18.1) is a copper containing protein that shows strong similarities to the hemocyanin found in the hemolymph of arthropods (Terwilliger 1999). Arthropod hemocyanins are typically produced in the hepatopancreas while proPO is produced and stored in the hemocytes (Terwilliger 1999). The hepatopancreas does not exhibit polyphenoloxidase activity although the hemocyanins may oxidise phenols when partially denatured (Terwilliger 1999).

Tests carried out to establish the substrate specificity of crayfish *(Pacifastacus leniusculus)* proPO established that it readily oxidises monophenols, such as tyrosine and tyramine, and *o*-diphenols, such as dopamine and l-dihydroxyphenylalanine (L-dopa) (Aspan et al. 1995). However, it will not oxidise *p*-diphenols such as hydroquinone (Aspan et al. 1995). It is, therefore, similar in reactivity to other tyrosinases. It has also been shown that the L-dopa-oxidising

activity of crayfish *(Pacifastacus leniusculus)* proPO is inhibited by tyrosinase inhibitors such as phenylthiourea (Aspan et al. 1995).

The activity of PPO can be affected by temperature and pH (Kim & Marshall 2000). While thermodurablity of an enzyme is known to vary with species and the enzyme source, most PPOs identified are heat labile above 70°C (Chen et al. 1991). In a study carried out by Chen et al (1991) to compare the activity of PPO extracted from the cuticle of western rock lobster and Florida spiny lobster it was found that activity of the enzyme was raised by increasing temperature but stability was reduced (Chen et al. 1991). The temperature/time profile required to ensure denaturation of PPO was not determined in this study as enzyme activity was present after 30 minutes at 60°C and higher temperatures were not used (Chen et al. 1991). Investigation into the effects of pH showed that a similar pattern of sensitivity to pH changes existed in the two species, with the optimal pH for activity lying between pH 6 and pH 8. Maximum stability was obtained at pH 7 and low pH resulted in inhibition of the enzymes (Chen et al. 1991).

**Reactions of PPO**

Tyrosine is the classic substrate for enzymatic oxidation leading to the formation of melanin (Mathew & Parpia 1971). PPO carries out two enzymatic activities utilizing molecular oxygen as shown in Figure 6. They are:

1. The hydroxylation of monophenols (Tyrosine) to *o*-diphenols. This is known as monophenolase or cresolase activity;

2. The dehydrogenation of *o*-diphenols to *o*-quinones. This is known as diphenolase or catecholase activity (Zawistowski, Biliaderis & Eskin 1991).

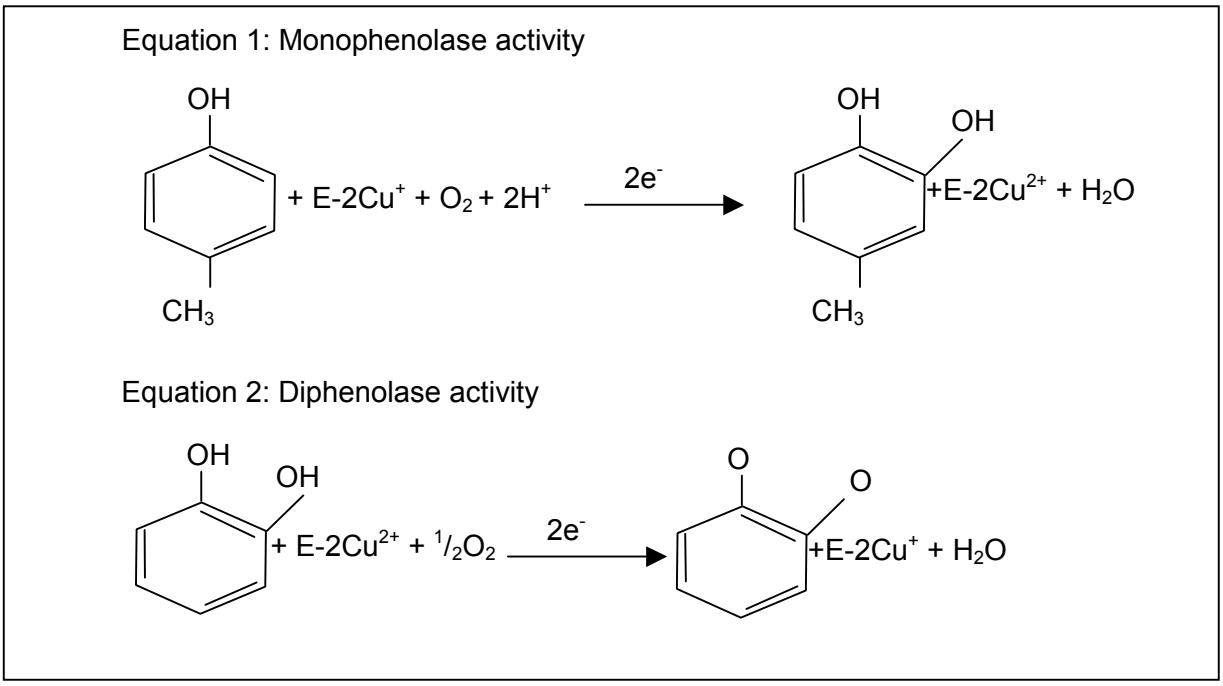


*Figure 6: Reactions catalysed by PPO*
*(Zawistowski, Biliaderis & Eskin 1991)*

The *o*-quinones formed vary from colourless to reddish brown pigments depending on the original phenol oxidised (Chinivasagam, Bremner & Reeves 1998). They undergo secondary reactions that influence the degree of pigmentation. These reactions include:

- Coupled oxidation with other substrates, for example ascorbic acid, effectively results in inhibition of quinone formation so that browning will not occur until the excess proton donor is used up

  *o*–quinone +RH ⟶ *o*-diphenol + R

- Complexing with amino acids and proteins which results in intensely coloured red to purple compounds; and

- Polymerisation via oxidative condensation resulting in melanin formation (Mathew & Parpia 1971). This is illustrated in Figure 7.

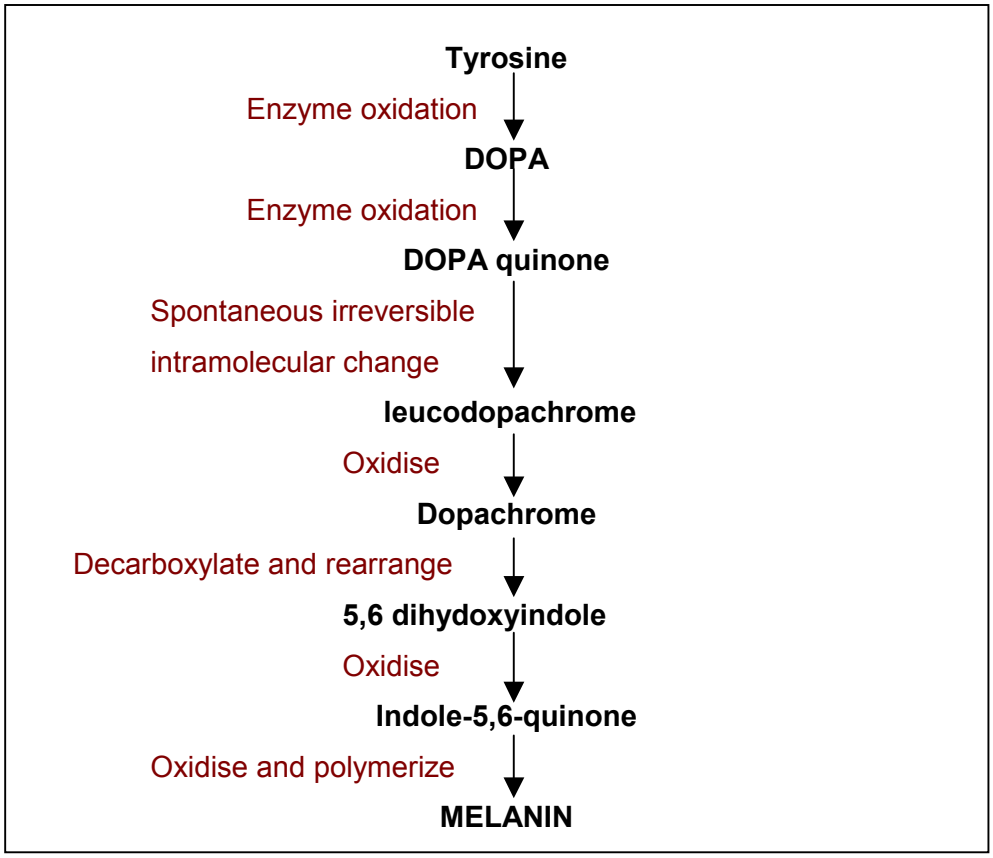


*Figure 7: Production of melanin*

*(Mathew & Parpia 1971)*

Deviations to the pathway depicted in Figure 7 are known to occur but it has been determined that the bulk of melanin formation occurs as shown (Zawistowski, Biliaderis & Eskin 1991).

**Alternative methods of activation of polyphenoloxidase**

It has been shown that the release of proPO may occur spontaneously in response to homeostatic changes, such as a sudden drop in calcium ion content of the hemolymph, resulting in recognition molecules in the hemolymph stimulating the proPOA cascade (Johansson & Söderhäll 1989). This has been associated with the wound response reaction and control of homeostasis (Johansson & Söderhäll 1989). Native inhibitors of proPO activating enzymes have been identified in the plasma of crayfish. They act as competitive inhibitors of the protease enzymes and so effectively block the activation of the proPOA system. Their action will continue until a high enough concentration of activators is present to override inhibitor activity (Söderhäll & Smith 1986).

Non-physiological inducers of polyphenoloxidase activity have also been determined such as heat or the presence of sodium dodecyl sulphate (SDS), or trypsin

(Söderhäll & Smith 1986). Ashida & Söderhäll (1984) showed that heating an extract of crayfish (*Astacus astacus*) hemolymph to more than 45°C resulted in activation of polyphenoloxidase. The activity increased rapidly to an optimum at 58°C. At temperatures greater than 65°C no heat-induced activity was observed (Ashida & Söderhäll 1984). It appeared that non-physiological activators impact directly on the prophenoloxidase, not the rest of the proPOA cascade. It was postulated that they produce a conformational change of prophenoloxidase to bring about activation (Söderhäll & Smith 1986). It was also proposed that these non-physiological activators may impact on secondary factors that can exhibit PPO-type activity under certain conditions, such as hemocyanin (Adachi et al. 2003b; Lee, Lee & Söderhäll 2004) .

Hemocyanin plays a major role as an oxygen transporter within the hemolymph of crustaceans and also functions as a storage protein, osmolyte, and precursor for anti-microbial peptides (Adachi et al. 2003a). However the ability of hemocyanin to act as a polyphenoloxidase-type enzyme has only been established recently (Zlateva et al. 1996; Adachi et al. 2001; Adachi et al. 2003b; Lee, Lee & Söderhäll 2004). It has been shown that hemocyanin can act as a PPO enzyme, however the extent of the contribution that hemocyanin makes to melanin formation has yet to be determined. The ability of hemocyanin to exhibit PPO-type activity after heating may have an important role to play in the formation of melanosis in processed western rock lobsters.

## Prevention of enzymatic browning

Various techniques have been applied to a wide variety of foodstuffs to prevent PPO activity throughout the post-harvest period. The techniques utilised aim to eliminate one or more of components essential for activity to occur. The essential components are; oxygen, copper, the enzyme, or the substrate (Kim & Marshall 2000).

### Heat

Thermal processing is the most commonly used method for stablilization of foods due to its ability to destroy micro-organisms and deactivate enzymes (Kim & Marshall 2000). Bigelow and Esty established the basis of modern thermal kinetics calculations in 1920 when they demonstrated that the destruction of micro-organisms through the application of heat obeys first order kinetics in that the destruction is a semi-logarithmic function of time at any given temperature

(Ramaswamy, Van de Voort & Ghazala 1989). Numerous studies have since followed and established that when heated at a constant temperature the rate of deactivation of an enzyme is also a first order reaction (Martens et al. 2001). In a first order reaction, the time required to deactivate a fixed percentage of the enzyme is independent of the initial concentration (Whitaker 1994). Consequently, when the log of enzyme activity is plotted against time, at a specified temperature, the resultant graph is a straight line (Toledo 1991).

As the deactivation occurs logarithmically, a 90% reduction in activity will be achieved within a constant time interval for any given temperature. The time interval is called the D value or decimal reduction value (Scott & Weddig 1998) and is equal to the negative reciprocal of the slope of the regression line specific to the given temperature (Toledo 1991). The D value is expressed as the time in minutes at a constant temperature necessary to destroy 90% of the enzyme activity Scott, (1998. The smaller the D value, the faster the rate of deactivation (Sharma, Mulvaney & Rizvi 2000). Once the D value for a specific temperature is known it is possible to calculate the time required to achieve any given level of deactivation for the enzyme at that temperature using Equation 1

*Equation 1:*

$$F_t = S \times D_t$$

where S is the number of log cycles to be achieved and $D_t$ is the decimal reduction value at the specified temperature and $F_t$ is the theoretical process lethality at that temperature (Toledo 1991). However, to be able to conduct comparisons between different temperatures, it is necessary to calculate the rate reaction constant, k, for the deactivation of the enzyme at each temperature (Sharma, Mulvaney & Rizvi 2000) using Equation 2:

*Equation 2:*

$$\mathrm{k} = \frac{\ln(10)}{\mathrm{D_t}}$$

Once k values have been calculated for a range of temperatures it is possible to determine $E_a$ and z values for the enzyme, where;

- $E_a$ equals the activation energy required; and
- z equals the temperature change required to change the D value by a factor of 10 (Toledo 1991)

Plotting ln(k) against the reciprocal of absolute temperature results in a thermal resistance graph (Sharma, Mulvaney & Rizvi 2000). The thermal resistance graph has a slope equal to $-E_a/R$, therefore $E_a$ can be calculated using Equation 3 and the z value can be determined using Equation 4:

*Equation 3:*
$$E_a = slope \times R$$

*Equation 4:*
$$z = \frac{\ln(10)(T_1 T_2)}{(E_a/R)}$$

Where;

- $E_a$ is the activation energy;
- R is the gas constant (8.314 J/K/mol or 1.98717 cal/K/mol);
- $T_1$ is the lowest temperature used; and
- $T_2$ is the highest temperature used to create the thermal resistance graph (Toledo 1991)

McCord & Kilara (McCord & Kilara 1983) noted that the higher the $E_a$ value the more sensitive the enzyme is to increases in temperature. Once the z value is known it can be used to calculate the lethal rate (L) for the enzyme of interest using Equations 5 and 6.

*Equation 5:*
$$L = 10^{A}$$

And

*Equation 6*:
$$A = \frac{T_i - T_r}{z}$$

Where

- $T_r$ is the reference temperature;
- T is the product temperature; and

- z is the z value for the enzyme of interest (Toledo 1991; Fellows 1997; Scott & Weddig 1998)

The lethal rate converts the time spent at a constant temperature to the equivalent number of minutes at the reference temperature (90°C) necessary to achieve the same degree of inactivation (Toledo 1991; Fellows 1997; Scott & Weddig 1998). However, the lethal rate only allows comparison between processes that occur at constant temperatures and times. Such a system does not often occur in food processing due to the complex nature of foods. Therefore, calculation of the process lethality ($F_r$) is required.

The process lethality ($F_{process}$) is a reflection of the **total** lethal effect of the heat applied. $F_{process}$ is calculated by multiplying the lethal rate by the time at the given temperature ($T_t$) when temperature is constant and is expressed as the equivalent minutes at the reference temperature for a specific z value (Toledo 1991; Fellows 1997; Scott & Weddig 1998). $F_{process}$ is calculated using Equation 7:

*Equation 7:*
$$F_{process} = L \times T_T$$

During cooking, temperatures are not constant. Therefore, to calculate the **total accumulated process lethality** that occurs in a food product during processing the measurement of the temperature-time profile is required. The integration of the lethal rate for every time interval throughout the process can then be used to calculate the total accumulated process lethality (Toledo 1991; Fellows 1997; Scott & Weddig 1998). Equation 8 is used to achieve this when $\Delta t$ is the measurement period in minutes:

*Equation 8:*
$$F_{process} = \sum L_T \Delta t$$

The calculated total accumulated process lethality can then be compared to the theoretical $F_t$ value (Equation 1) in order to predict the effectiveness of the process in deactivating the enzyme.

The time-temperature profile required to achieve an irreversible deactivation varies between species since thermal stability of the enzyme is dependent on the relative amounts of free and bound water; pH of the solution; and the presence of salts (Kim & Marshall 2000). It is important to establish the thermodurablity of the PPO in

western rock lobster in order to make informed decisions regarding the severity of heat treatment required to ensure denaturation of the enzyme and thus prevention of melanosis.

**Refrigeration or freezing**

Refrigeration and freezing result in a reduction of the enzyme's catalytic rate during storage. However, lowering the temperature only delays the onset of melanosis, it does not prevent it (Ogawa 1985). In freezing the increase in solute concentration combined with decreased water activity, and resultant changes in pH, appear to exert an inhibitory effect. Upon thawing disruption of the cells by ice crystals results in enhanced enzyme activity due to increased contact between the enzyme and substrates (Ogawa 1985). The increased activity results in higher levels of enzymatic browning after freezing and thawing when compared to unfrozen product (Ogawa 1985; Kim & Marshall 2000)

**Irradiation**

Irradiation has been postulated as a means of prevention of blackspot in prawns (Savagon & Sreenivasan 1978). However, trials have shown that while irradiation of fresh prawns appears to have an inhibitory effect, if melanosis has already begun the irradiation will accelerate blackspot formation. Irradiation also increases free radical production, which results in other detrimental changes such as increased rancidity of lipids (Savagon & Sreenivasan 1978).

**Carbon dioxide**

Carbon dioxide ($CO_2$) has been used during processing of fruit and vegetables to modify the atmosphere and so prevent enzymatic browning. Wang & Brown (1983) carried out a study to determine the impact of $CO_2$ modified atmospheric storage on fresh water crayfish *(Pacifastacus leniusculus)* and showed that chemical and microbial changes were retarded and organoleptic properties were maintained in $CO_2$-treated crayfish. Chen et al. (1993) also showed that purified spiny lobster (*Panulirus argus*) PPO was more sensitive to increases in temperature when in the presence of $CO_2$. Samples of purified PPO heated at 33 °C and 43°C showed very slight loss of activity after heating for 30 minutes. Samples heated to 33°C in the presence of $CO_2$ showed a 98.5% loss of activity after 30 minutes, while samples heated to 43°C in the presence of $CO_2$ showed no activity after 20 minutes (Chen et al. 1993). Samples heated without $CO_2$ showed an increase in activity during

storage while samples heated with $CO_2$ showed no restoration of activity (Chen et al. 1993). It was postulated that $CO_2$ bound irreversibly with the enzyme to deactivate it (Chen et al. 1992). Carbon dioxide has the advantage of being inexpensive, nontoxic, nonflammable and readily available.

**Pressure**

Pressure influences biochemical reactions by increasing the inter-chain interactions (Kim & Marshall 2000). High pressure (>1Mpa) can produce irreversible changes while maintaining the organoleptic quality of a food. It is however dependant on the immersion media, pH, temperature and the duration of treatment (Kim & Marshall 2000). When used in combination with carbon dioxide pressure has been shown to have a synergistic effect in the prevention of enzymatic browning (Chen et al. 1992). Chen et al. (1992) showed that after two minutes, at 43°C in the presence of high pressure (5.88Mpa) $CO_2$ Florida spiny lobster (*Panulirus argus*) PPO showed no activity. High pressure increased the susceptibility of PPO to the effects of heat and $CO_2$, which meant lower temperatures could be used during processing to deactivate the enzyme (Chen et al. 1992).

**Anti-browning agents**

The alternatives to processing treatments are the use of anti-browning agents. Antibrowning agents interact with the enzyme or its substrates and/or products in such a way as to inhibit the formation of the pigments. Their use is constrained by concerns about: performance, cost, regulatory concerns based on toxicity and the possible negative impacts on the organoleptic characteristics of the food (Kim & Marshall 2000). Anti-browning agents may be classified according to their primary mode of action as shown in Table 2.

*Table 2: Inhibitors of enzymatic browning (McEvily & Iyengar 1992)*

| | | | |
|---|---|---|---|
| **Reducing agents** | Sulphites<br>Ascorbic acid and analogs<br>Sulphydryl compounds e.g. cysteine. | **Enzyme treatments** | Oxygenases<br>o-Methyl transferases<br>Proteases |
| **Enzymatic inhibitors** | Substitute resorcinols<br>Aromatic carboxylic acids<br>Anions<br>Peptides<br>Kojic acid | **Chelating agents** | Phosphates<br>EDTA |
| **Acidulates** | Citric acid<br>Phosphoric acid | **Complexing agents** | Cyclodextrins |

***Reducing agents***

Reducing agents or antioxidants react with the *o*-quinones produced to form stable colourless compounds or to reduce them back to the less reactive colourless diphenols and so prevent pigment formation (McEvily & Iyengar 1992). However, reducing agents are only effective for the time period determined by their rate of consumption as they are irreversibly oxidised during the reaction with the pigment intermediaries (McEvily & Iyengar 1992). They are also non-specific in nature and so react with other compounds that may result in production of undesirable flavours and colours (McEvily & Iyengar 1992).

*Sulphiting agents*

Sulphiting agents are compounds that release sulphur dioxide ($SO_2$) under the conditions of use. They exist as a mixture of ionic species in aqueous solution, bisulphite ($HSO_3^-$) and sulphite ($SO_3^{2-}$). The equilibrium between species is dependent on the pH of the solution. Bisulphite is the most effective species as it reduces the quinone products of the enzyme and acts as a competitive inhibitor by binding to the sulphydryl group found at the enzyme's active site (Kim & Marshall 2000).

Until recently, sulphites were the most widely used inhibitors of enzymatic browning. However, they also exhibit several undesirable characteristics. High levels of sulphites result in the bleaching of naturally occurring pigmentation, as well as having a negative impact on flavour (McEvily & Iyengar 1992) Health risks also exist for sensitive individuals. McEvily & Iyengar (1992) report that several deaths have occurred among steroid dependent asthmatics due to the consumption of sulphite treated foods. As a result of these deaths there is an increasing level of regulatory

control over the use of sulphites with maximum residue limits being set for those products where their use is accepted (Food Standards Australia New Zealand 2004). The adverse health effects, increased regulatory scrutiny and resultant heightened consumer awareness of associated risks has created a need for functional, inexpensive and safe alternatives to sulphites (McEvily & Iyengar 1992).

*Ascorbic acid and analogs*

The main role of ascorbic acid and its isomer, erythorbic acid, in the prevention of enzymatic browning is to reduce the *o*-quinones to diphenols and to act as free radical or oxygen scavengers. There also appears to be some degree of interaction with the enzyme resulting in its deactivation (McEvily & Iyengar 1992). Spectrophotometric assays show that there is an initial lag phase followed by a slow increase in reaction rate (McEvily & Iyengar 1992). The greater the reductant concentration, the longer the lag phase. Ascorbic acid is irreversibly oxidized to dehydroascorbic acid during reaction with the *o*-quinones. Once the ascorbic acid becomes fully oxidised enzyme activity will recommence and browning will occur (Kim & Marshall 2000).

While the mode of action of ascorbic and erythorbic acids are similar, ascorbic acid appears to be more efficient in most food systems (McEvily & Iyengar 1992). In particular, erythorbic acid is more susceptible to the copper-catalysed oxidation carried out by PPO and is therefore used up faster than an equivalent concentration of ascorbic acid (McEvily & Iyengar 1992). These reducing agents are however relatively reactive and may react with other components of the food system, such as endogenous enzymes resulting in deleterious effects (McEvily & Iyengar 1992). Ascorbic acid has been shown to have a pro-oxidant impact when oxidised or used at elevated concentrations. In tests on shrimps samples treated with ascorbic acid developed a distinct yellow colour (McEvily & Iyengar 1992).

Ascorbyl phosphate esters have been utilised as more stable sources of ascorbic acid. They release ascorbic acid when hydrolysed by endogenous acid phosphatases and exhibit a longer lag phase than the equivalent concentrations of ascorbic acid (McEvily & Iyengar 1992). However, they are not as effective in acid systems possibly due to the low activity of the endogenous acid phosphatases (McEvily & Iyengar 1992). Therefore, the suitability of ascorbyl phosphate esters as browning inhibitors is dependent on the ability of the food system to absorb the

compound, the pH of the system and the activity of the endogenous acid phosphatases (McEvily & Iyengar 1992).

Ascorbic acid and/or it's derivatives are commonly used in conjunction with other anti-browning agents such as citric acid. This results in a synergistic effect allowing for the use of lower concentrations (Kim & Marshall 2000).

*Sulphydryl compounds*

Sulphydryl compounds react with *o*-quinones to produce stable colourless compounds or reduce the carbonyl groups and double bonds of the pigment so that a colourless compound results (Kim & Marshall 2000). Many sulphydryl containing compounds are not approved for food use by the regulatory authorities and others are too expensive to be practicable alternatives, for example glutathione. Practical alternatives are the sulphur containing amino acids such as cystine, methionone and cysteine. Of these, cysteine has been shown to be as effective as sodium bisulphite in preventing browning in some food systems (Kim & Marshall 2000). Unfortunately the concentrations required also produce negative impacts on the taste of the treated foods (McEvily & Iyengar 1992).

***Enzymatic inhibitors***

*Substituted resorcinol*

Extracts of figs were shown to have an inhibitory effect on PPO activity (Taoukis et al. 1990). Isolation of the inhibitory fractions showed that the inhibitors present were substituted resorcinols. These compounds are structurally related to the phenolic substrates of PPO in that they are *m*-diphenols (Kim & Marshall 2000). Resorcinol can be substituted at the 1,3-,2-,4- and 5-positions. Those substituted at the 1,3- and 2- positions were the least effective as antibrowning agents (McEvily & Iyengar 1992). 4- and 5-position substitutions showed the same high level of inhibition however the 5-substituted resorcinols also posses toxic and irritant properties that make them unsuitable for use in the food industry (McEvily, Iyengar & Otwell 1991).

Of the 4-substituted resorcinols, hydrophobic substitutions such as hexyl- showed the highest levels of inhibition (Kim & Marshall 2000). 4-hexylresorcinol has the greatest potential for use in the food industry. 4-hexylresorcinol has a long, safe history of human use in non-food applications, for example as a topical antiseptic (McEvily, Iyengar & Otwell 1991). It is non-toxic, non-carcinogenic and non-

mutagenic and is therefore considered to be a GRAS (generally recognized as safe) compound (McEvily, Iyengar & Otwell 1991). Trials have shown it to be more effective than bisulphite in the prevention of blackspot in prawns and it has no associated negative impacts (McEvily, Iyengar & Otwell 1991; Otwell, Iyengar & McEvily 1992; Guandalini et al. 1998). There are several advantages in the use of 4-hexylresorcinol that include:

1. they are specific and potent inhibitors of PPO;
2. non-bleaching of native pigments;
3. no impact on organoleptic characteristics;
4. they can be used at low concentrations and so are cost competitive; and
5. they are chemically stable under proposed conditions of use (McEvily, Iyengar & Otwell 1991).

4-hexylresorcinol is sold blended with a carrier as 'Everfresh'™ and is widely used in the food industry. It is currently approved for use with prawns in Australia and this approval maybe extended to rock lobster if sufficient justification for its use can be shown (Food Standards Australia New Zealand 2003).

*Aromatic carboxylic acids*

These act to inhibit PPO due to their structural similarities to the phenolic substrates (Kim & Marshall 2000). Their effectiveness varies from system to system dependent on the substrate specificity of the PPO present and therefore the type of inhibition that occurs. Inhibition can be competitive, non-competitive or mixed. Unfortunately these compounds may be converted to PPO substrates by other enzymes present in the system and therefore act to induce browning rather than inhibit it (Kim & Marshall 2000).

*Anions*

Inorganic halides act as inhibitors of PPO by reacting with the positively charged imidazole group at the active site of PPO (McEvily & Iyengar 1992). Their effectiveness is pH dependent and decreases as pH increases. Ideally the pH of the system should lie between 3.5 and 5 (McEvily & Iyengar 1992). Other anions such as nitrite or sulphates are ineffective due to steric effects (McEvily & Iyengar 1992).

*Proteins, peptides and amino acids*

Proteins, peptides or amino acids can affect enzymatic browning by direct inhibition of the enzyme and by reaction with the quinone products. Amino acids containing thiol groups are more effective than others due to their high affinity for the $Cu^{2+}$ at the enzyme's catalytic site (McEvily & Iyengar 1992). L-cysteine is the most effective of these compounds (see also 'Sulphydryl compounds') (Dudley & Hotchkiss 1989). The quinone intermediate may be preferentially trapped as a sulphur adduct so blocking polymer formation and preventing browning (Dudley & Hotchkiss 1989). Unfortunately, if proteins, peptides or amino acids are able to bind with the final pigments, enhancement of the colouration may result (Kim & Marshall 2000).

*Kojic acid*

Kojic acid is a metabolite of several species of *Aspergillus* and *Penicillium* and so is found as a natural constituent in many Japanese fermented foods. It is a natural antibacterial and anti-fungal agent, a reducing agent and an antioxidant. Kojic acid has been shown to prevent melanosis in shrimp when used as a 1% dipping solution and is thus comparative in activity to sulphiting agents (Kim & Marshall 2000). It is effective through the direct inhibition of the enzyme by chelation of the copper ions at the active site, and it is thought to reduce the pigment or its precursors to colourless compounds (Kim & Marshall 2000). A blend of kojic acid and ascorbic acid has been patented as an anti-browning agent for foods in Japan (Chen, Wei & Marshall 1991). However, it has been shown that when kojic acid is added to tissues possessing peroxidase activity, and low levels of hydrogen peroxide, a yellow pigmentation results even though enzymatic browning is prevented (Kahn, Lindner & Zakin 1995). When consideration is given to this, and kojic acid's potential as a mutagenic agent and concerns over toxicity, it is doubtful if kojic acid will be permitted as a food additive (Kim & Marshall 2000).

***Acidulates***

Any change in pH will have an impact on the ionic side chains of the enzyme. The ionic groups must be maintained in the most desirable ionic form to maintain the conformation of the active site so that binding of the substrate and catalysis can occur (Kim & Marshall 2000). Changes in pH may also have an impact on the substrate such that it can no longer bind to the enzyme. The pH optimum of PPO varies with enzyme source and substrate but is generally within the range of pH 6-7. Irreversible denaturation of the enzyme will only occur at extremes of pH, for

example lowering the pH below 3 will effectively inhibit enzyme activity. The resistance of the enzyme to changes in pH is dependent on the buffering capacity of the food system, the type of substrate it utilizes and the resistance of co-factors and activators to pH changes (Kim & Marshall 2000). The presence of enzyme isoforms may mitigate the impact of pH changes to some degree (Kim & Marshall 2000).

*Citric acid*

Citric acid is the most widely used acidulant in the food industry. Organic acids like citric have a dual inhibitory effect on PPO. They reduce the pH and chelate the copper at the enzyme active site thus being more effective than inorganic acids (McEvily & Iyengar 1992). Other acidulates, such as malic acid and phosphoric acid, exhibit disadvantages such as poor availability, high price, and negative impacts on food flavours (McEvily & Iyengar 1992).

***Enzyme treatments***

Modification of the enzyme or its substrate through the use of appropriate enzymes, such as oxygenases, *o*-methyl transferases and protesases, has been proposed as a method of prevention of enzymatic browning (McEvily & Iyengar 1992). However, the use of these methods is restricted due to the high cost of the enzymes and the impacts they may have on the food system (McEvily & Iyengar 1992).

***Chelating agents***

Chelating agents either bind with the copper at the enzyme's active site or reduce the level of copper available for incorporation with the holoenzyme (McEvily & Iyengar 1992). Examples include EDTA and sodium acid pyrophosphate. As anti-browning agents these compounds are generally used in conjunction with other agents, which results in either an additive or a synergistic enhancement of activity relative to the impact of any single agent (Kim & Marshall 2000).

***Complexing agents***

Cyclodextrins inhibit enzymatic browning by the entrapment or formation of inclusion complexes with the substrates or products of PPO activity (McEvily & Iyengar 1992). A similar activity is exhibited by chitosan, a naturally occurring polymer (McEvily & Iyengar 1992). However due to the large molecular size of these compounds their use is restricted to liquid systems (McEvily & Iyengar 1992).

**Application of antibrowning agents to the rock lobster industry**

Overall, selection of an antibrowning agent for a particular purpose must consider several constraints. The agent must be:

- non-toxic, and permissible for food use;
- ideally be perceived as “natural”;
- have no negative impacts on organoleptic characteristics of the food;
- relatively easy to apply;
- cheap; and
- above all effective.

Food Standards Australia New Zealand (FSANZ) (Food Standards Australia New Zealand 2004) lists several antibrowning agents that are permissible for use in seafood including sulphites, citric acid, ascorbic acid and 4-hexylresorcinol. While sulphites may be used in the lobster industry, there is consumer resistance to their use due to the negative impacts on asthmatics and the elderly. However, the effectiveness of other antibrowning agents has never been scientifically evaluated for application to western rock lobster.

While some work has been done to evaluate the uptake of antibrowning agents in live fish (Thed & Erickson 1992) a corresponding study in lobsters is yet to be undertaken. In the prawn industry antibrowning agents such as sulphites are applied by dipping the live or dead prawns in dilute solutions of the agents (Ogawa 1987). A study conducted by Slattery, Williams & Cusack (1995) showed that live prawns dipped in 4-hexylresorcinol solutions had higher residue levels and a lower incidence of blackspot than those dipped when dead. The western rock lobster industry uses freshwater drowning to humanely kill lobsters prior to cooking (Western Rock Lobster Development Association Inc. 1999). It is therefore possible that antibrowning agents maybe applied at this step in during normal processing. It is essential that the effectiveness of individual antibrowning agents in prevention of melanosis is evaluated and any negative impacts from their use identified before adoption for use by the industry.

**Instrumental colour analysis in food research**

In the manufacturing world of today, the ability to produce foods with consistent colour attributes has become increasingly important. In many applications, reliance on human assessors is sufficient to determine consistency of colour attributes, given that the human sense of vision is amazingly acute. However, while the human eye can accurately assess the colour of an object, it is unable to accurately measure the area and distribution of specific colours on an object (Lawless & Heymann 1998). In order to determine colour distributions and areas other means have been sought.

Instrumental methods of colour measurement, such as colorimetry and spectrophotometry, have limited application to the food industry, as they are only applicable to flat, opaque and evenly coloured materials (Hutchings, Luo & Ji 2002). Most solid foods are distinguished by the non-uniformity of their colour so that these criteria could not be met without destruction of the food, for example grinding the food to a homogeneous paste. Since this would in turn change the colour relationships, such techniques are not applicable for evaluation of complex whole foods such as lobster.

Recent advances in digital imaging and computer-based digital analysis have created opportunities for rapid measurement of food colour and appearance characteristics in whole foods utilising methodology developed in the health sciences and geographical information systems (GIS) technology. The technology is based on the use of digital cameras to capture the images of food followed by computer based image analysis.

In recent publications, several such systems have been evaluated for use in food research (Papadakis et al. 2000; Ramos et al. 2004; Yam & Papadakis 2004). These systems are useful research tools in that they are simple, versatile and relatively cheap while capable of providing a lot of information about the food (Yam & Papadakis 2004). The systems utilise a light source; a digital camera to collect the image; a computer to display the image; and image analysis software to analyse the colour distributions.

Digital cameras use an electric light sensor to record the diffused light as millions of tiny points. Each point or *pixel* is assigned a location and a specific colour value based on the camera's built-in colour reference framework. The greater the number of pixels a camera is capable of recording the better the resolution of images will be.

Thus, the use of a high-resolution camera (1600 x 1200 pixels, 32Mb memory card) will ensure maximisation of image clarity.

Image analysis software is used to measure and classify a wide range of colour distribution parameters such as area, modal and average values, size distribution, height, width, roundness, perimeter, minimum and maximum distances, perpendicular distances, line length etc. The information sought determines the specific parameters utilised in any analysis. The values are reported based on colour model utilised by the software.

**Application of image analysis to post harvest processing of western rock lobster**

A discussion with processors and restaurateurs has shown anecdotally that development of melanosis in western rock lobster is initiated upon splitting of the cooked lobster and develops over time. It is possible that PPO and/or the products of its activity will not be destroyed during processing thus leading to the formation of melanosis after processing. Comparison between the impacts of different handling and processing techniques, such as methods of transportation or application of antibrowning agents, requires evaluation of the rate, extent and intensity of melanosis development over time in the processed lobster.

Chemical analysis of enzyme activity requires destruction of the food structure, which would preclude analysis of the rate, extent and intensity of melanosis development in the whole lobster. The use of digital imaging over time followed by digital analysis of the images would allow such a determination. The use of a digital system would also allow storage of the image for future evaluation of issues such as which anatomical features are most associated with the development of melanosis in western rock lobster.

Earlier studies of melanosis development have utilised photography over extended periods as a means of recording melanosis in lobsters however, digital image analysis was not used to measure degree of change over time (Johnson & Evans 1991). The increasing sophistication of the systems now available has opened up opportunities for research that have never existed before.

## Conclusions

Söderhäll and others (Ashida & Söderhäll 1984; Ferrer et al. 1989a; Ferrer et al. 1989b; Chen et al. 1991; Söderhäll & Cerenius 1998) have carried out extensive research into the role and activation of the polyphenoloxidase with respect to crustacean immunity. However, little has been done on the impact of these inherent systems on lobster processing and quality.

From the foregoing discussion, the following conclusions may be drawn:

- Melanosis is caused by the action of polyphenoloxidase enzymes in the hemolymph;
- Phenolic compounds are oxidised by these enzymes to *o*-quinones that then undergo polymerisation via non-enzymatic oxidative condensation to form melanin pigments (Söderhäll 1982);
- Heat may activate the enzyme during processing and may therefore increase the formation of coloured pigments (Mathew & Parpia 1971);
- Determination of the effects of heat on western rock lobster PPO activity is required; and
- Anti-browning agents may have a role to play in melanosis prevention.

Not all of the possible methods for prevention of enzymatic browning are suitable for use with whole rock lobster. Several restrictions on different anti-browning agents use can be readily identified. They include:

- While sulphites are the most effective, they have negative connotations in the minds of consumers and their use would be detrimental to the industry;
- Those anti-browning agents of high molecular weight, such as the cyclodextrins, are unlikely to be absorbed by lobsters in sufficient quantity to be effective;
- Enzyme treatments, such as proteases, may have a negative impact on meat quality as such enzymes have been shown to cause mushiness in crabmeat (Queensland Department of Primary Industries 1987);
- Regulatory approval is required for use and is not available in all cases, for example Kojic acid; and

- High concentrations of anti-browning agents may have deleterious effects on organoleptic quality, as has been shown with ascorbic acid and L-cysteine in shrimp (Kim & Marshall 2000).

Human evaluation of melanosis is capable of detecting its presence or absence. However, the human eye is unable to accurately determine area and intensity. It is possible however to use digital image analysis as a useful and objective tool for the assessment of the rate, extent and intensity of melanosis development in western rock lobster.

**Directions for research**

It is possible that physiological stress induced by post harvest handling and transportation may impact on PPO activity and subsequent melanosis formation in processed lobster. Therefore a study to evaluate the impact of these factors on PPO activity, melanosis formation and weight recovery is required.

The possibility that the enzyme may be activated by heat and maintain activity at high temperatures is most important when considering the impacts of heat processing on processed lobster quality. If the enzyme activity increases greatly during processing this may result in a build up of quinones, which can then undergo non-enzymatic polymerisation after processing and cause melanosis. Similarly, if the enzyme is not deactivated during processing then post-processing activity will result in quinone production and subsequent melanosis formation.

The determination of the temperature/time profiles required to activate the pro-enzyme and to deactivate the enzyme is central to understanding the possible impacts of the enzyme on melanosis development post-processing. Fundamental studies aimed at obtaining a better understanding of the impacts of heat on PPO activity in western rock lobster are needed. *In vitro* studies are required to establish the relationship between temperature and duration of heating. These studies can be then be used to predict the activity during processing of lobsters based on thermal kinetic calculations. Validation of the obtained values through assessment of the impacts on melanosis of different temperature-time profiles will be needed.

The modification of PPO activity using selected antibrowning agents must also be investigated. *In vitro* studies can be utilised to screen selected agents for effectiveness in reduction of PPO activity. Evaluation of the effectiveness of the selected agents when applied during processing will then be required.

**NEED**

Currently, melanosis is prevented by increasing cooking time, which results in reduced weight recoveries. At present, cooked weight recoveries average between 92-95% of landed beach weight. An average increaser of 1% over the entire industry would result in an increased return of $1 – 2 million per season. Thus there is significant scope for improvement. In addition, prevention of melanosis would result in further savings of approximately $1 million per season.

For the industry to consistently achieve maximum cooked weight recoveries, whilst reliably controlling melanosis, a systematic investigation of the impact of processing on these factors is essential. The information generated by this study will be used to define best processing practises in order to increase overall quality of the product and profitability of the industry.

**OBJECTIVES**

1. To establish the impact of temperature on the activity of *P. cygnus* hemolymph PPO *in vitro*
2. To establish the impact of post-harvest transportation and holding on PPO activity, weight recovery and melanosis formation
3. To establish the impacts of current commercial practises on weight recovery and melanosis formation in *P. cygnus*
4. To validate the use of experimentally determined thermal kinetic parameters for prevention of melanosis in *P. cygnus*
5. To establish the impact of antibrowning agents on the activity of *P. cygnus* hemolymph PPO *in vitro*.
6. To determine the effects of anti-browning agents on weight recovery and melanosis formation when applied during processing

## METHODS

### Materials

All laboratory chemicals were purchased from Sigma-Aldrich unless otherwise stated.

### Lobsters

In undertaking this project, consideration was given to the variation that may exist between lobsters taken from different geographical locations, or at different times of the season. If not accounted for, these factors may confound experimental treatment effects. Therefore, as far as was practicable, all comparisons within experiments were made between lobsters from the same geographical region and catch history. All lobsters used were evaluated as healthy according to the vigour index proposed by Spanoghe & Bourne (1999). Every lobster utilised in the study had a unique numbered plastic tag attached to an antennae with a cable tie. The tags remained in place throughout the study to enable identification of every lobster at all times.

For the laboratory-based experiments 30 live western rock lobsters in good condition were purchased from a local commercial supplier and were maintained in tanks continuously supplied with aerated flow-through ambient temperature seawater at the University of Western Australia (UWA) Marine Research Laboratories, Perth, Western Australia. The lobsters were fed twice weekly on a maintenance diet of local blue mussels (*Mytilus edulis*) for the duration of the study. Several studies have shown superior growth and survival results in lobsters feed a diet of blue mussel rather than processed foods (James & Tong 1997; James 1998; Crear et al. 2000). These lobsters were utilised as the source of hemolymph for all in-vitro experiments.

A 100-litre seawater aquarium with bio-filtration and aeration was established in the laboratories at Curtin University. Water pH and ammonia concentration were monitored using an aquarium kit. One third of the water was replaced with fresh seawater every two weeks. Four lobsters were transported in chiller boxes to Curtin and held in the tank for no more then 4 days. During that time, each lobster was bled only once. At the end of four days, they were returned to the tanks at the UWA research laboratory. Care was taken to ensure that each lobster was not bled more than once within a 6-week period. The lobsters were kept for a total of 18 months after which time they were disposed of.

For the factory-based experiments live lobsters were taken from a single catch at the landing dock and held overnight in the MG Kailis Pty Ltd Live Holding Facility, Dongara, Western Australia to ensure their condition was stabilised. They were then processed within 24 hours, according to the requirements of the experiment being carried out.

**Anticoagulants:**

***Cacodylate anticoagulant buffer***

Cacodylate buffer, (0.01 M sodium cacodylate, 0.45 M sodium chloride, 10 mM calcium chloride hexahydrate, 26 mM magnesium chloride dihydrate) (Smith & Söderhäll 1991) was used for preparation of all solutions. The buffer was adjusted to pH 7.0 as this has been shown to be optimal for lobster PPO activity (Chen et al. 1991). The ratio of hemolymph to anticoagulant was 1:1.

**Phenol oxidase activity**

***L-Dopa***

300 mg of L-dihydroxyphenylalanine was dissolved in 100 ml of buffer to give a solution concentration of 3 mg/ml. As L-DOPA oxidises on standing this solution was freshly made up immediately prior to use.

***Trypsin***

A solution of 0.1% trypsin (porcine pancreas, Type II-S Lyophilised powder 1,000-2,000 BAEE units/mg solid, Sigma T7409) was freshly prepared in buffer immediately prior to use.

**Standard Methods**

**Collection of hemolymph**

Hemolymph was drawn from the pericardial sinus surrounding the heart. The lobster was held with the abdomen firmly bent beneath the cephalothorax, exposing the unsclerotized membrane between the cephalothorax and the first segment of the abdomen. The required volume of hemolymph was withdrawn using a pre-chilled hypodermic needle (0.65x32 mm) and plastic syringe. The hemolymph was immediately mixed with an equal volume of cacodylate buffer and held on ice to

prevent coagulation. All experiments used whole blood preparations from individual lobsters.

**Protein assay**

Due to the difficulty involved in conducting protein assays in the field, a rapid non-destructive method using refractometry was utilised. The refractive index (RI) of hemolymph was measured on a handheld refractometer. The refractometer was calibrated against distilled water at ambient temperature. The RI was then converted to blood protein values using the equation developed by Paterson, Davidson & Spanoghe (1999) for western rock lobster hemolymph.

$$\text{Total protein (mg/ml)} = 5402.398 \times (\text{RI} - 7214.877)$$

**Phenol oxidase activity**

Baseline and total PPO activity in whole blood was estimated spectrophotometrically at 490 nm according to the method published by Smith & Söderhäll (1991). L-dihydroxyphenylalanine (L-DOPA) is oxidised by PPO resulting in the formation of highly coloured orange-red DOPA-chrome (Ali et al. 1994). **Baseline activity** is the level of active polyphenoloxidase innately present in the plasma of the hemolymph. **Total activity** is defined as the maximal enzyme activity following trypsin-mediated activation (Smith & Söderhäll 1991). The two measures provide a profile of all the changes in enzyme availability and activity that occurred under the experimental conditions.

It is normal laboratory procedure to report enzyme activity in relation to mg protein in a solution. However, large differences in protein content exist between individual lobsters (Paterson, Davidson & Spanoghe 1999; Oliver & MacDiarmid 2001) and PPO represents a minor fraction of total hemolymph protein. Therefore to normalize the response, all enzyme activities were calculated as change of absorbance per minute per ml hemolymph as defined by Smith & Söderhäll (1991).

***Baseline PO activity***

200 µl of hemolymph in anticoagulant was diluted with 600 µl of cacodylate buffer and incubated at 25°C for 30 minutes. Then 200 µl of L-DOPA (3 mg/ml) was added. The absorbance was determined over time at 490 nm. Absorbance was measured against a blank containing L-DOPA and anticoagulant to control for spontaneous oxidation of the substrate (Smith & Söderhäll 1991).

***Total PO activity***

200 µl of hemolymph in anticoagulant was diluted with 400 µl of anticoagulant buffer. Then 200 µl of 0.1% trypsin was added and the solution incubated at 25°C for 30 minutes. After incubation, 200 µl of L-DOPA (3 mg/ml) was added and the absorbance was determined over time at 490 nm. Absorbance was measured against a blank containing L-DOPA, trypsin and anticoagulant to control for spontaneous oxidation of the substrate (Smith & Söderhäll 1991).

**Method for standardized commercial processing**

Discussions with processors revealed that considerable differences in the time-temperature profiles used to process whole lobsters exist throughout the season and between processors. Therefore, for the purposes of this study a “representative” processing protocol was devised based on the information that was provided by members of the industry.

The standardised method consisted of three stages:

1. **Drowning:** Drowning is utilised prior to cooking since it has been determined as the most rapid and humane way to ensure death of large quantities of lobster whilst maintaining flesh quality (Western Rock Lobster Development Association Inc. 1999). Live healthy lobsters were placed in ambient (21 ± 4.5°C) potable fresh water. They were held for 20 minutes to ensure they were drowned. The drowned lobsters were then packed into stainless steel cooking baskets or ‘kibbles’, which are 57cm long by 34cm wide and 41cm high. The lobsters are packed in four layers to each basket. Each layer consisted of 16 lobsters arranged in two rows of eight. The lobsters were all placed with their abdomens flexed under the cephalothorax and the heads toward the mid line of the basket.

2. **Cooking:** A double burner gas-fired commercial cooker was used for all treatments in this phase of the study. It held four baskets in each cook with 5 cm clearance at the sides and 15 cms at either end. The water depth was maintained so that the top layer of lobsters was completely covered through out the cooking process. No additives were included in the water for this phase of the study. Four baskets were placed into the cooker with a maximum of two minutes between the first and the last basket going into the

cooker. The cook time was timed for 20 minutes from the entry of the first basket into the cooker

3. **Cooling**: After cooking, the baskets were removed in the same order as they were placed into the cooker with a maximum of two minutes between the first and last basket being removed. They were immediately plunged into a tank filled with fresh water ice slurry and left for a further 20 minutes to cool. The tank was large enough to ensure the baskets were completely submerged and surrounded with ice slurry. No agitation was used. After cooling, the lobsters were removed from the tank, rinsed with fresh water, drained for 10 to 15 minutes, wrapped in cellophane, packed into standard cardboard freezer boxes and blast frozen to -30°C within 2 hours. They were then maintained at this temperature until required for analysis.

This "standard" protocol was used in all processing experiments.

**Digital image analysis**

***Sample preparation***

All lobsters were processed at the M.G Kailis Pty Ltd lobster processing facility in Dongara, Western Australia. Immediately after processing, they were washed, drained and wrapped in cellophane. The wrapped lobsters were then packed into boxes and blast frozen to below -28°C. They were held at that temperature until required for analysis.

Prior to undertaking digital imaging, the frozen lobsters were placed on metal trays in a refrigerator for 16 hours at 4±2°C to thaw. After thawing, the lobsters were removed from the refrigerator and the identification tag number and final weight were recorded. The legs and antennae were then removed and the lobsters split along the midsagittal axis from the supraorbital spines to the telson. The two halves were then placed, cut surface up and back-to-back, on a polystyrene tile covered in clean white paper. The paper acts as a neutral background for photography. Toothpicks were used to secure the pieces as shown in Figure 8 in order to prevent the pieces from moving during transfer to and from the refrigerator.

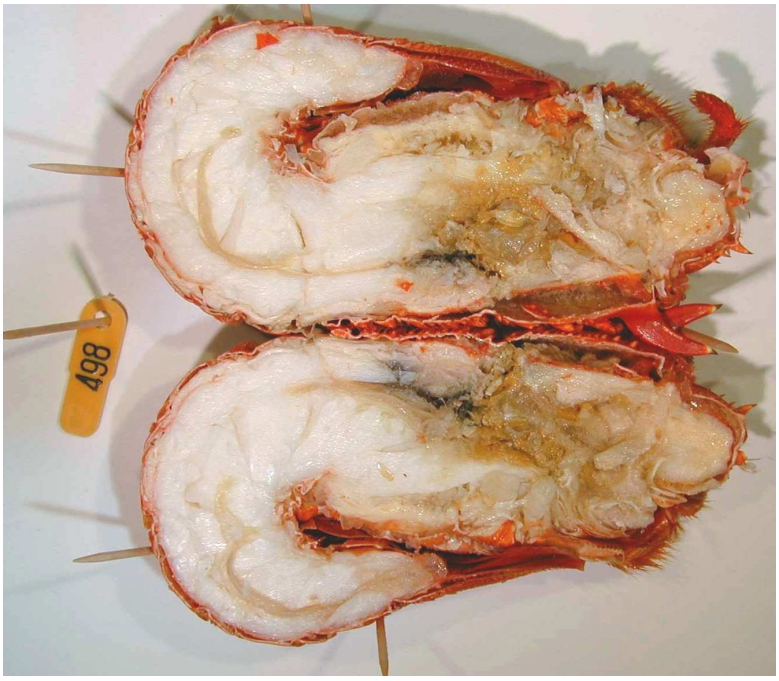


*Figure 8: Sample prepared for digital image analysis*

***Photographic set-up***

The tray with the pinned lobster was placed inside a black box illuminated by four halogen lamps (Thorn 38 degrees 12 Volt 50 W). The use of a standard light source that delivers consistent illumination precludes variation between photographs. It is important to ensure that the light source is set at a 45° angle to the camera lens as this corresponds to the angle of diffuse reflection, which is responsible for colour perception (Yam & Papadakis 2004). The black box was utilised to prevent introduction of variation from outside light sources.

The digital camera (Nikon Coolpix950) was attached to a tripod directly above the lobsters at a distance of 38.5cms as shown in Figure 9. The camera aperture was set at 3.0 (f-number) and the shutter speed was 1/250$^{th}$ of a second. All images taken were saved in TIFF format. Of the common types of image format, Tagged Image File Format (TIFF) is preferred as it is an uncompressed image file format giving a true image without introducing variations as will occur with compressed formats like JPG.

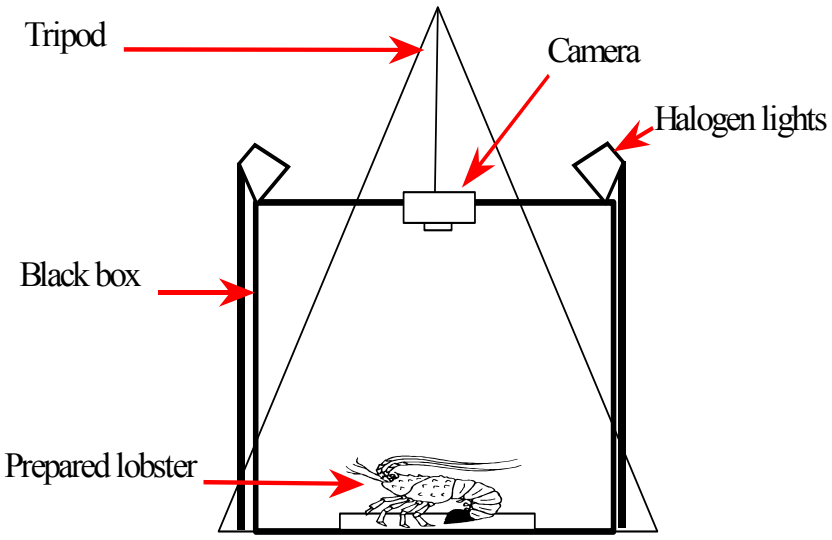


*Figure 9: Photographic set-up*

Photographs of each lobster were taken at time zero, at 40 minutes and then at 20-minute intervals for a period of three hours. If it was clear that no melanosis was developing then the number of photographs taken was reduced. However, a minimum of four were taken between time zero and the three hour time point. The lobsters were then held for 24 hours from time zero and a final photograph was taken. All images were downloaded to the computer.

Subsequent photographs were compared to the time zero photograph using PhotoBase 3 Version 3.0.0.106 for Canon© image viewer (ArcSoft Inc 2002) to identify those showing changes and development of melanosis. Development of melanosis was defined as areas showing a mean pixel density in excess of 100 where 0 equals white and 255 equals black. Photographs meeting this criterion were then selected for further data analysis.

***Image analysis procedure***

Image analysis was undertaken using Scion Image for Windows Version 4.0.2 (Rasband 2000). Scion Image (SI) utilises RGB imaging and greyscale imaging for digital analysis. When an image is open in SI opens it appears in two versions; a grey image and a RGB indexed colour image as shown in Figure 10. The greyscale image was used for digital analysis of melanosis in lobsters.

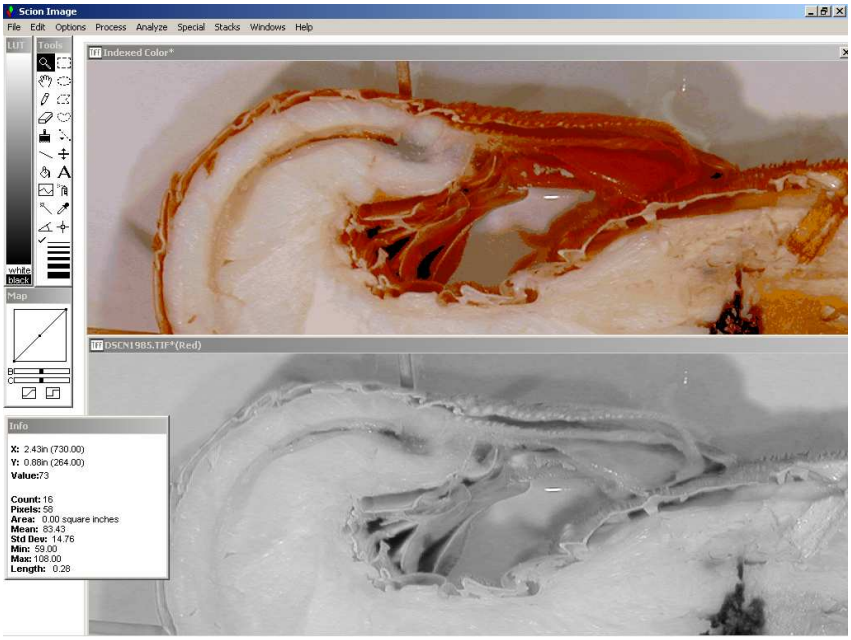


*Figure 10: Screen view of Scion Image*

As the TIFF images were 5.50 MB in size they were too large to be fully displayed and analysed on the screen. Therefore, the image was resized by changing the horizontal and vertical scales. By using the EDIT >SCALE AND ROTATE function both scales were reduced from 1.0 to 0.70, resulting in the image being fitted to the screen for ease of viewing and analysis (Figure 11). Bilinear interpolation was used to ensure a smoother image reduction. The reduced image automatically opened in a new window ready for spatial calibration, which allowed results from length and area measurements to be presented in calibrated units, such as centimetres.

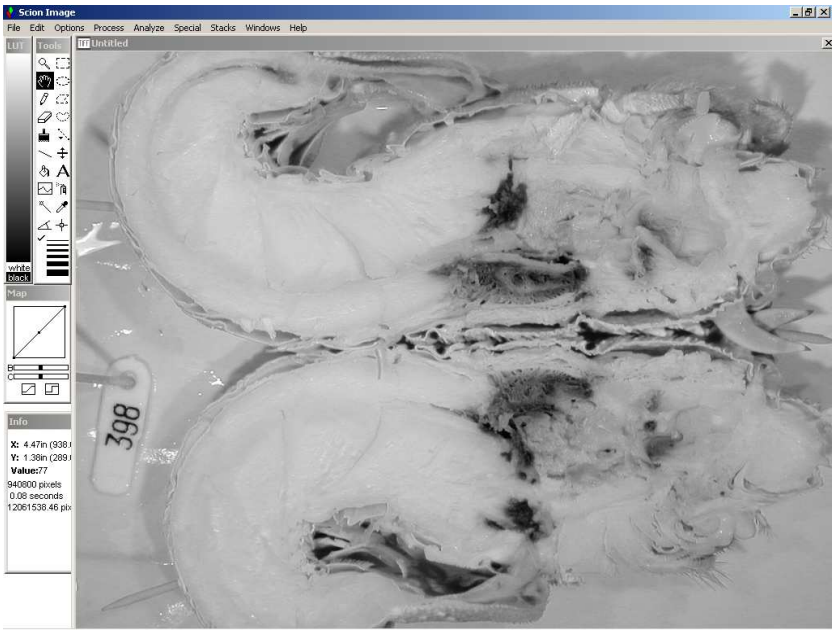


*Figure 11: Scaled image in Scion image*

Before setting the scale, a known distance was measured as the reference point. The identification tags used for each lobster were identical in size and were therefore used as the reference point for all spatial calibration. The tags were measured using the LINE SELECTION tool in order to obtain the tags measured distance in pixels. Combining the known distance in centimetres and the measured distance in pixels provides the scale expressed as pixels per cm. Scion Image used the scale value generated to compute all area measurements.

The next step in the process was to select the variables of measurement required. ANALYSE>OPTIONS brings up the window shown in Figure 12 so that selection of the types of measurements to be recorded can be selected.

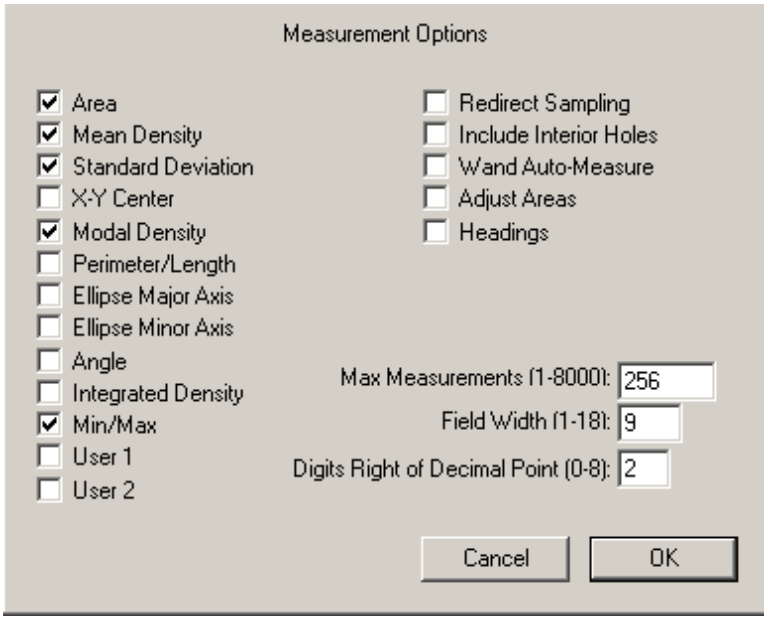


*Figure 12: Measurement options window*

The following variables were recorded for every measurement undertaken:

- **Area** – selected area in calibrated units
- **Mean Density** – Average grey value within the selection. This is the sum of the grey values of all the pixels in the selection divided by the number of pixels
- **Standard Deviation** - Standard Deviation of the grey values used to generate the mean grey value
- **Modal Value** – Most frequently occurring grey value within the selection
- **Min/Max** – Minimum and maximum grey values within the selection

Manual digitisation was then used to select the areas to be measured. For the time zero image, the total surface area of the split lobster was measured and the area in $cm^2$ was recorded. The mean pixel density was taken as the baseline colour intensity value for that lobster.

Each area was outlined using the POLYGON SELECTION tool. The tool is used to create polygon-shaped regions for use by various commands in the EDIT, PROCESS and ANALYZE menus. The outline or border of the area concerned was traced around with the mouse. Irregular shapes could be selected as clicking the mouse anchors the selection line at that point so allowing curves to be drawn. Multiple areas could be selected enabling measurement of broken areas of melanosis. Then all areas selected were measured and all measurements taken were shown in the RESULTS window as shown in Figure 13.

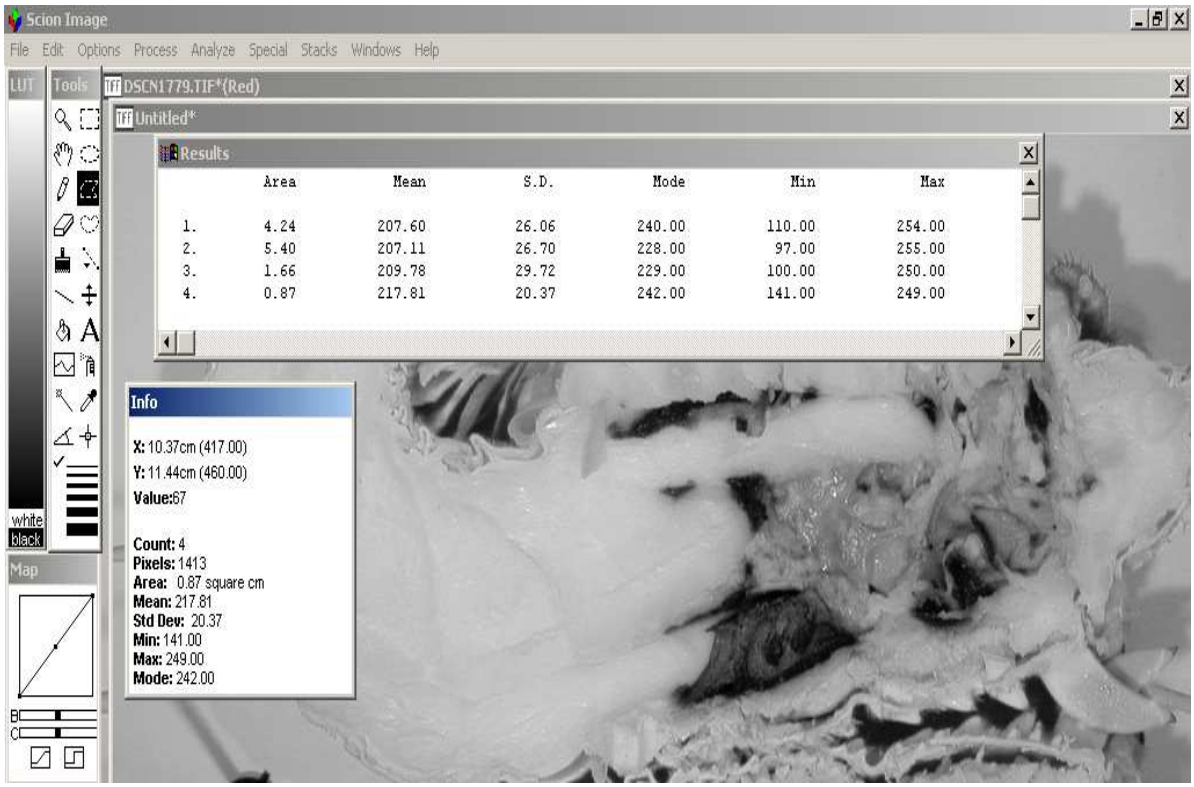


*Figure 13: Results of melanosis measurement*

The results were copied and pasted into Excel or a similar program for recording and further analysis. The recorded values were then used to further calculate the development and spread of melanosis. Final percentage area of melanosis was used as a measure of the extent of melanosis and the final average pixel density was used to evaluate the intensity of melanosis. The increase in percentage area of melanosis over time was used to determine the rate of melanosis development.

**Statistical analysis**

The statistical package SPSS for Windows version 11 (SPSS 2000) was used for analysis of all experimental results. After ensuring the assumption of normality was not violated, Analysis of Variance (ANOVA) and Tukey's honestly significant difference post hoc comparisons were used to establish significant variations. For those parameters where assumption of normality was not met the Kruskal-Wallis (K-W ANOVA) test of variance was used.

Student's T tests were used to determine the variability for values with two classes such as gender and grade. Correlation and regression were used to establish any relationships between physiological factors and melanosis development. Significance was defined as $p \leq 0.05$. A full summary of the statistics is available in Appendix 2.

## Specialised Methods

### Objective 1: The impact of temperature on the activity of *P. cygnus* hemolymph PPO *in vitro*

#### *Identification of the impact of temperature on PPO activity*

Thermal stability of PPO was determined for both steady state and unsteady state heating. Steady state temperatures were used to enable evaluation of the impact of constant temperatures over time on all forms of PPO activity. The impact of a single temperature in terms of activation and deactivation effects was then established. The information generated enabled calculation of thermal kinetic constants for the enzyme. Unsteady state temperatures were used to allow investigation of the impact of changing temperature on all forms of PPO. The changing temperature established the additive effect of temperature over time as an indication of effects occurring during heat processing.

In steady state heating, 1 ml of hemolymph was mixed with 3 ml of pre-heated cacodylate buffer in each of six test tubes and placed in the water bath at a set temperature. After 5 minutes a tube was removed from the bath and plunged into a bed of ice and 2 ml of iced buffer was added. Baseline PPO activity and total PPO activity were then determined using the standard method (Smith & Söderhäll 1991). The procedure was repeated at 5-minute intervals to a maximum time of 30 minutes. Initial baseline and total PPO activity were determined using an unheated sample.

The tests were conducted at 40, 50, 60, 70, 80, 90 and 100ºC. Each temperature series was repeated four times.

For unsteady state heating, 1 ml of hemolymph was mixed with 3 ml of buffer at room temperature. Eight test tubes in all were set up and then seven were placed in the water bath. The initial temperature was recorded and the water bath switched on. When the temperature in the tubes reached a predetermined set value (40, 50, 60, 70, 80, 90 or 100ºC) one tube was removed from the bath and plunged into a bed of ice. Two ml of iced buffer was added and the contents of the tube mixed thoroughly. Baseline PPO activity and total PPO activity were then determined using the standard method (Smith & Söderhäll 1991). Initial baseline and total PPO activity were determined using the remaining unheated sample. A total of eight lobsters were used for the test series.

***Calculation of thermal kinetic constants***

The data from the *in vitro* experiments was used to calculate the thermal kinetic constants and theoretical thermal lethality input required to produce deactivation of the enzyme during cooking as outlined by Ramaswamy, Van de Voort & Ghazala (1989)

The thermal kinetic constants determined were;

- the decimal reduction value (D);
- the rate reaction constant (k);
- the activation energy ($E_a$); and
- the z values for the enzyme.

These values were then used to calculate the theoretical lethal rate (L) and the theoretical process lethality value ($F_r$) required to produce 99.9% deactivation of the enzyme (Toledo 1991; Whitaker 1994; Scott & Weddig 1998). The equations and calculations utilised are summarised on pages 14 to 17 of this report.

**Objective 2: The impact of post-harvest transportation and holding on PO activity, weight recovery and melanosis formation**

***Simulation of selected transportation methods***

The planned programme for this objective called for three field trips spanning the season to identify any seasonal effects, however modification of the experimental plan was required to accommodate the delay experienced in establishing the pilot plant at Dongara. Therefore, four repeats of the transportation simulations were conducted over a 4-day period in January 2003.

Four common methods of transportation of live lobster were selected for simulation:

- Live tank on boat - Lobsters were submerged in fresh ambient temperature (26±0.5ºC) seawater flowing through the tank, with adequate aeration
- Spray truck - Lobsters were placed in a closed system tank with chilled (15 ±2 ºC) seawater recirculating via a spray head delivering 11 L/min
- Carrier boat - Lobsters were placed in a tank with ambient temperature (26±0.5ºC) seawater flowing into the tank via a spray head delivering 11 L/min and freely draining
- Dry chiller truck - Lobsters were placed in a chilled still air truck cabinet and maintained at 15 ±2 ºC

Large tanks capable of holding two prawn baskets were set up to simulate the three transportation methods using seawater (Figure 14).

*Figure 14: Tanks used in transportation simulations*

*Evaluation of lobster physiology*

Two hundred lobsters from the previous day's catch were randomly subdivided into subgroups of 50. Blood samples were taken from 20 lobsters in each group. The sampled lobsters were then tagged and randomly distributed throughout each basket of lobsters and the lid clamped on. As soon as each basket was ready it was placed in one of the four treatments for five hours. On completion of the treatment the tagged lobsters from each treatment were bled and then all the lobsters were processed using the standardised processing protocol and frozen at –40ºC. The blood samples were analysed for total and basal PO activity immediately after bleeding and sub-samples frozen in liquid nitrogen and held at –80ºC for later ammonia determination. Weight on receipt, after treatment and after processing (cook, cool and drain) was recorded for each lobster. The trials were repeated over 4 days using size 'A' lobsters.

*Determination of transportation environmental factors*

Water quality (ammonia concentration, and oxygen saturation) and temperature was monitored over the respective treatments. Temperature was monitored using thermocouples inserted into the water lines at the inlet and outlet of each tank. Oxygen saturation of Treatment A (submerged flow through simulation) was monitored using a dissolved oxygen meter and the airflow regulated so that aeration was maintained at $PO_2$ equal to 86±5% saturation at 26±2ºC. Treatment D (chiller truck simulation) was maintained at 60 ± 20% relative humidity and 15±2ºC over the treatment time. Ammonia concentration at the point of exit from the tank was monitored in all seawater treatments using the alternative spectrophotometric method detailed by Parsons, Maita & Lalli (1984).

**Evaluation of the impacts of post harvest holding on PPO activity substrate levels and subsequent melanosis development**

The aim of this experiment was to determine if post harvest holding might increase the risk of melanosis formation by inducing changes in PPO activity and substrate concentration in lobsters prior to cooking. 100 lobsters were selected directly from the landing ramp at the live holding facility. Hemolymph was immediately collected from 20 lobsters and analysed for total and baseline PPO, protein content and total phenols content. The entire group was then placed in the live holding tanks for 48 hours. After that period they were processed using the standard method. Hemolymph was taken from groups of 20 lobsters not previously bled before and

after drowning. All samples were analysed spectrophotometrically for PPO activity and total substrate concentrations (total phenols). A further twenty lobsters were collected following cooking and digital image analysis was used to determine melanosis development. Sex, moult stage, physical damage, weight on receipt, post-drown, post-cook and post-cool were recorded for every lobster used. The experiment was repeated at 3 times throughout the season to control for any seasonal variation occurring.

***Post processing evaluation***

Digital image analysis was conducted on all lobsters utilised in these studies according to the standard method. Evaluation of the extent, intensity and rate of melanosis development assists in establishing the impact of handling and transportation on physiological factors on melanosis development

**Objective 3: The impacts of current commercial practises on weight recovery and melanosis formation in *P. cygnus***

***Validation of thermal profile measurement***

A series of tests were conducted to establish the best position for the measurement of the core temperature of lobsters during cooking. The aim was to insert a thermocouple in such a way as to minimise disruption of the normal internal state and restrict water ingress, whilst measuring the core temperature at the point of slowest heating.

In the first test, four ‘A’ grade lobsters were drowned in fresh water. Each lobster then had thermocouples inserted through 4 different points, namely; the anus and up the hindgut, through the foregut, and though the autotomised stump of two different legs. Each probe was inserted far enough to ensure the end was within the tail meat situated at the base of the cephalothorax. Different leg positions were evaluated on different lobsters, ensuring that a number of possible positions were compared. The temperature was recorded during cooking and cooling, and then the lobsters were split and the position of the end of each thermocouple was determined. From this trial, it was found that insertion through the third leg on the left and/or the right resulted in the most consistent and slowest rate of heating.

In a second trial, ten grade ‘A’ lobsters were drowned in fresh water. Then thermocouples were inserted through the stump of the third leg on the left and the

third leg on the right. A thermocouple was also strapped to the exterior surface of each lobster to measure water temperature. The change in temperature for each thermocouple was logged over a 25 minute cooking and cooling period. After cooking and cooling, the lobsters were split and the position of the end of each thermocouple was determined as shown in Figure 15.

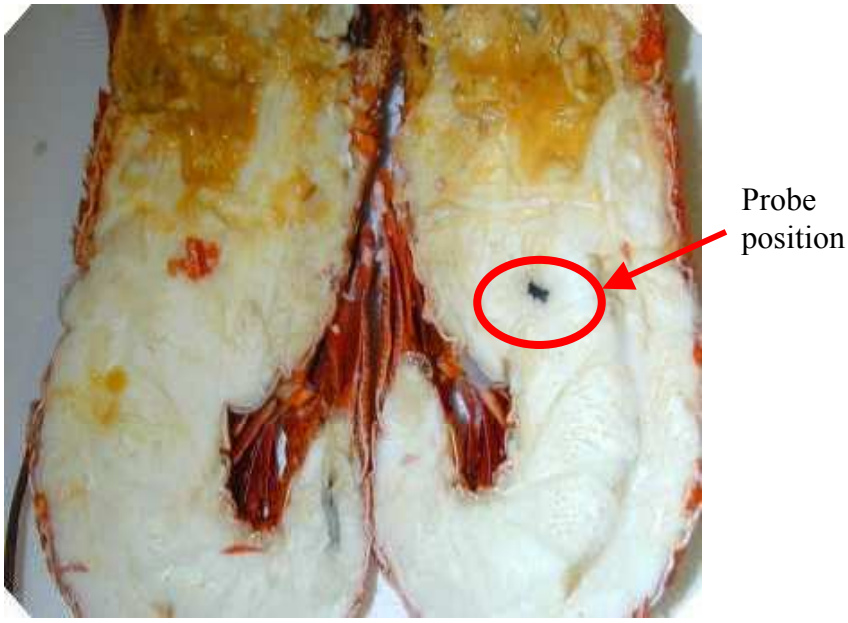


*Figure 15: Location of probe used for thermal profile analysis of western rock lobster processing*

The rate of temperature change for each thermocouple was compared to the water temperature and damage to tissues evaluated. From these trials it was noted that the insertion of the thermocouple through the base of the third leg on the right caused the least disruption to tissues and resulted in the probe being consistently positioned in the muscle mass at the base of the tail as shown in Figure 15. The region also demonstrated the slowest rate of heating.

***Determination of temperature time profile for standardised commercial cooking***

Three field trips were run over the 2001-2002 season. The work was carried out at the M.G. Kailis factory in Dongara, Western Australia. A commercial system was used comprising a drown tank, cooker and chill tank as illustrated in Figure 16.

After drowning, fourteen rock lobsters were selected and thermocouples attached to them for recording internal and external temperatures. On each lobster, the third leg on the right hand side was removed at the joint closest to the body and a nickel

cadmium thermocouple probe inserted (type TK; Teflon coated, Positive = Ni, 10% Cr, negative = Ni, 2%Mn, 2%Al). The probe was positioned in the anterior muscle mass within the cephalothorax. Another thermocouple was placed on the dorsal carapace between the supraorbital spines in order to describe the immediate thermal environment of each lobster during the cook. The thermocouples were secured with plastic cable ties as shown in Figure 17.

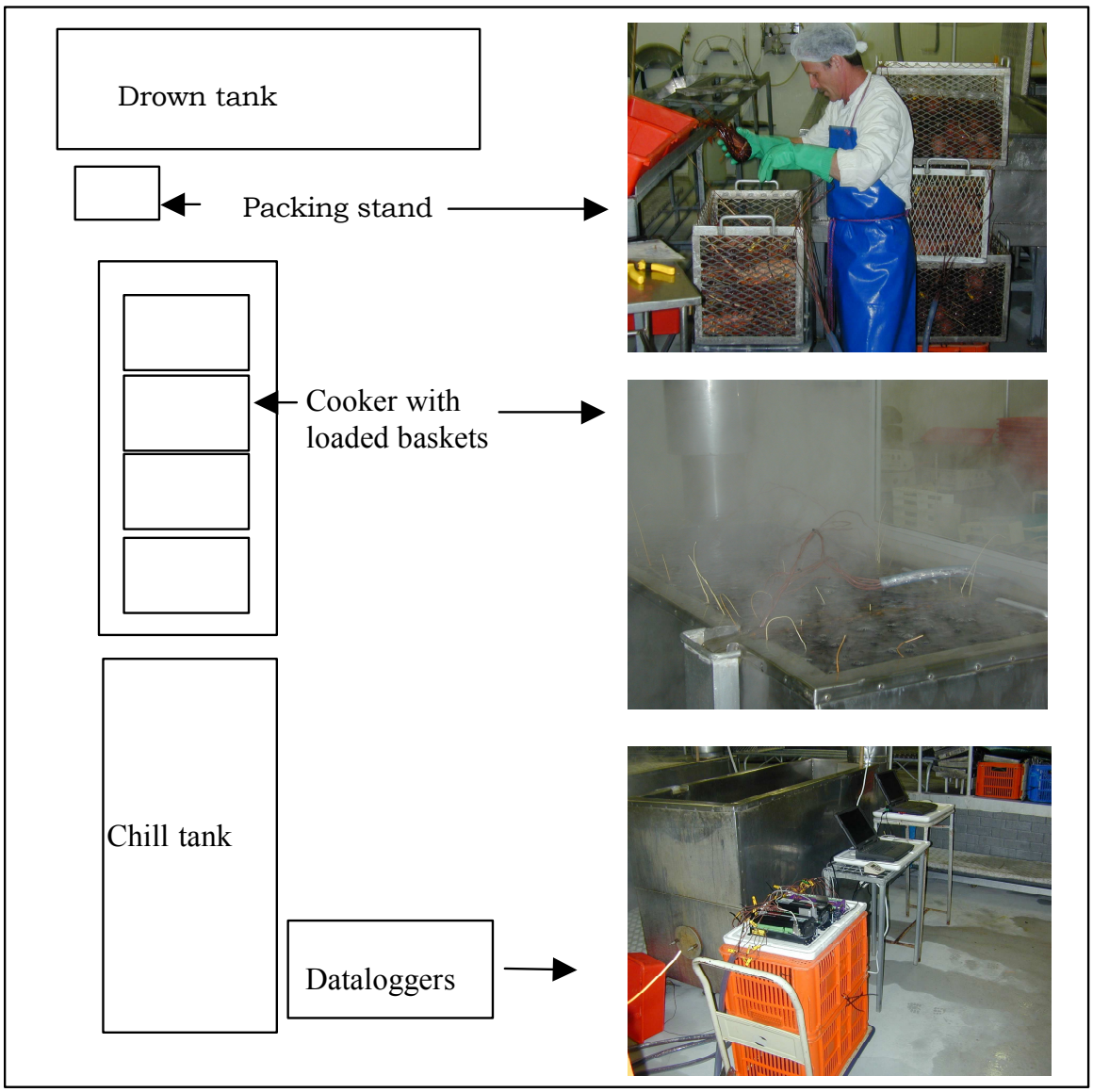


*Figure 16: Layout of western rock lobster processing*

*Figure 17: Lobsters used for measurement of thermal profile during processing*

Every basket in each cook held 64 lobsters arranged in four layers. Instrumented lobsters were placed into each basket at selected strategic positions to give a 3D array throughout each cook of 100kg as shown in Figures 18 and 19. The water inlet and burners were situated at the back of the cooker.

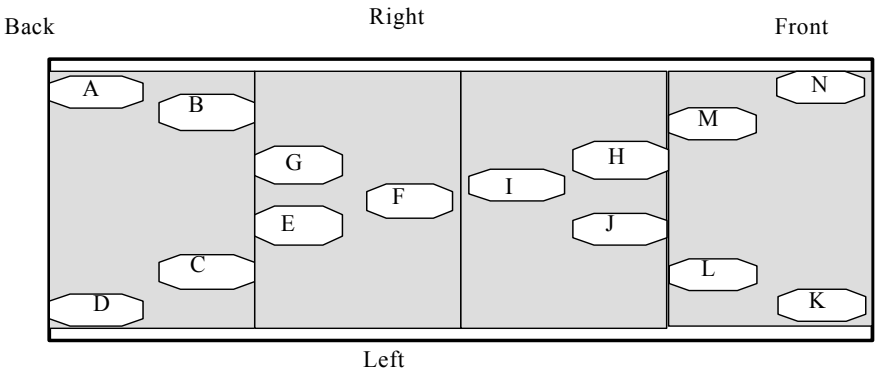


*Figure 18: Top view of the 3-D array of instrumented lobsters within cooker*

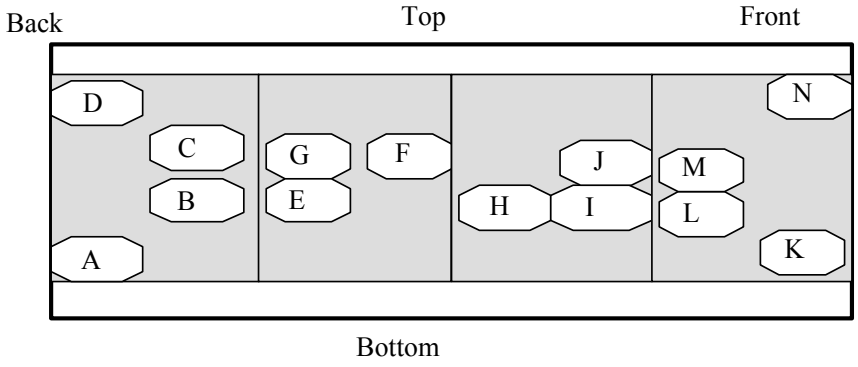


*Figure 19: Side view of 3D array of instrumented lobsters within cooker*

Letters between A and N were used to identify each position. The arrangement ensured measurement of spatial variations in cooking conditions within the cooker. Temperature variations throughout the cook will result in variations in the degree of cooking experienced by lobsters within a single cook-load. The thermocouples remained *in situ* whilst the lobsters were cooled for 20 minutes in ice slurries, and were removed immediately prior to freezing.

Datataker DT500 dataloggers connected to a laptop PC were used for real time recording of the thermal profile for each lobster. The trial was repeated to give 14 cooks of Grade A lobsters (average weight = 433 $\pm$ 20.7g) spread over three one-week field trips spanning the lobster season (November 15$^{th}$ 2001 to June 30$^{th}$ 2002).

Since lobster size may affect heat penetration and therefore, the rate at which lethal internal temperature is reached, single cooks were run over 3 days at 3 times throughout the season using Grade 'B' lobsters (average weight = 497 $\pm$ 29g). The results for the larger Grade B lobsters were then compared to the Grade A lobsters. Ideally, a wider size range should have been used, however this was not possible.

The information generated from this phase of the study can be used to determine the minimum lethality point within the cooker in order to identify the preferred position for the determination of the end point of cooking should a significant variation exist. It is acknowledged that any identified minimum lethality point will vary from cooker to cooker due to variations in cooker design.

***Evaluation of lobster physiology***

Sex, moult stage, physical damage as indicated by appendage loss, and weight on receipt were recorded for every lobster used. All instrumented lobsters were bled prior to drowning and after drowning according to the standard method. Total and baseline PPO activity was determined according to the standard method to enable evaluation of the impact of drowning on PPO activity. Protein content was also determined using the method developed by Paterson, Davidson & Spanoghe (1999). A separate group of 20 lobsters were weighed before and after drowning to establish levels of water uptake by lobsters during drowning.

### *Post processing evaluation*

Digital image analysis was conducted on all lobsters utilised in these studies according to the standard method. Evaluation of the extent, intensity and rate of melanosis development may assist in establishing the impact of physiological factors and heat input during cooking on melanosis development.

**Objective 4: Validation of the theoretical thermal kinetic parameters**

Three field trips were conducted over the course of the 2003-2004 season. On each trip a series of cooking trials was run every day for 3 days to give 80 cooks using 800 test lobsters and 160 instrumented lobsters.

### *Processing*

On each day 120 Grade A lobsters were taken from the previous day's catch and held in a flow through seawater system at ambient temperature until required. Twenty lobsters were bled for determination of protein and initial phenol oxidase enzyme activity. These lobsters were then used for temperature recording during the cooking process. Two instrumented lobsters plus another 10 non-instrumented lobsters were used in each cook. The weights of the non-instrumented lobsters in each cook were recorded before and after drowning, cooking, and cooling. The physical damage, gender, and moult stage data was recorded for every lobster utilised.

Ten lobsters and two bled lobsters (for temperature measurement) were drowned in fresh water at ambient temperature for 20 minutes. After drowning, thermocouples were inserted into the two bled lobsters in each batch prior to cooking. Thermocouples were also attached to the outsides of each bled lobster in order to describe the immediate thermal environment during the cook. The lobsters were then placed in a single layer in the bottom of a cook basket to ensure an even temperature distribution around them during cooking. The lobsters with thermocouples attached were placed in the central position of the baskets every time to ensure consistent determination of the average lethality of the cooking process. The thermocouples were connected to a Datataker DT500 and laptop PC to enable real-time monitoring of lethality. The baskets were placed into a cooker filled with boiling water and cooked to a predetermined lethality value. After removal from the cooker, the lobsters were immediately placed into ice slurry and held there for 20 minutes prior to washing, draining, packing and freezing. The thermocouples

remained *in situ* whilst the lobsters were cooled in ice slurries, but were removed immediately prior to packing and freezing. Cook time was determined by the lethality reached and the lethality values were varied for each basket of lobsters so that a range of lethality values spanning the calculated required process lethality was achieved. A gradation from severely undercooked to severely overcooked lobsters resulted.

***Digital image analysis***

After processing, all the lobsters were blast frozen and held until digital image analysis could be done. The digital imaging was carried out using the standard method and the extent, intensity and rate of melanosis development were determined for each of the 10 un-instrumented lobsters in every cook. Care was taken to ensure that all lobsters were analysed in a blind randomised manner to prevent bias of the digital results. At no time during digital image analysis was it possible to determine the cook time or lethality of the cook for any lobster. All data was recorded on a separate work sheet and not collated with cook values until the digital image analysis had been completed.

**Objective 5: The impact of antibrowning agents on PPO activity in western rock lobster hemolymph**

***Antibrowning agent solutions***

A range of concentrations was selected for each agent as shown in Table 3, based on previous research (Otwell & Marshall 1986; Wagner & Gunnar 1986; Dudley & Hotchkiss 1989; Slattery, Williams & Cusack 1995; Guandalini et al. 1998; Montero, Avalos & Perez-mateos 2001). All solutions were made up with analytical grade chemicals to prevent interference from contaminants. Evaluation of each concentration was carried out four times utilising hemolymph from a different lobster each time (four lobsters x 15 antibrowning solutions = 60 lobster in total). Within each test series, all tests were done in triplicate.

*Table 3: Concentrations of selected antibrowning agents*

| Agent | Solution concentration |
|---|---|
| Ascorbic acid | 100 & 400µg/ml |
| Citric acid | 3.3, 1.5, 1.0, 0.7, 0.5, 0.2 mg/ml |
| 4-Hexylresorcinol: | 87, 49, 20.5,10.25, 7 µg/ml |
| Carbon dioxide | Saturated solution |

***Evaluation of antibrowning agent impact on PPO activity***

For each test series, hemolymph was collected from a lobster into a chilled tube, and held on ice. A sub-sample was taken to determine initial PO activity levels. One ml of hemolymph was mixed with three mls of a set concentration of anti-browning agent (in cacodylate buffer) in each of four test tubes. A control series in cacodylate buffer (without antibrowning agent) was run in parallel utilising hemolymph from the same lobster. The tubes were mixed thoroughly using a vortex mixer and placed in a preheated water bath at 70°C for 30 minutes. A thermocouple was inserted into the fourth tube prior to heating and remained in situ throughout the test series. The temperature within the tubes reached 70°C within two seconds of being placed in the heated water bath. After heating for 30 minutes, the tubes were removed and plunged into a bed of ice. Two mls of iced buffer was added and the contents of the tube mixed thoroughly. The temperature in the tubes dropped to 20°C immediately upon addition of the iced buffer. Baseline PO activity and total PO activity were then determined spectrophotometrically according to the standard method.

Solutions were heated at 70°C as previous work (Objective 1) had shown that at this temperature heat activation of the enzyme would occur in the first five minutes. It was also apparent in the previous study that heating for 30 minutes at 70°C is not enough to ensure significant deactivation of the enzyme. Therefore, any deactivation effect noticed will be due to the impact of the antibrowning agent on the enzyme.

A modification of the method above was used in the evaluation of $CO_2$. Firstly, $CO_2$ was bubbled through the buffer solution for 30 minutes at a rate of 50ml/minute. The solution was held on ice for this period and pH was measured at the beginning and end of testing. Hemolymph was added and the solution sub-sampled. A control series was run in parallel without $CO_2$ addition. Baseline PO activity and total PO activity were determined spectrophotometrically according to the standard method. The trials were repeated with the hemolymph being added to the buffer and then $CO_2$ bubbled through for 30 minutes while the solution was held on ice. A control series was run in parallel without $CO_2$ addition. Baseline PO activity and total PO activity were determined spectrophotometrically before and after the $CO_2$ addition. Each solution was then divided between four tubes and heated at 70°C for thirty minutes. One tube was removed for each time interval and baseline PO activity and total PO activity were determined.

## Objective 6: Application of antibrowning agents in western rock lobster processing

### *Application of antibrowning agents*

Based on the work conducted in Objective 5 the agents and concentrations selected were citric acid (2.5mg/ml and 1.5mg/ml), carbon dioxide (saturated gaseous) and 4-hexylresorcinol (10, 50 and 87 µg/ml 4-H). Ascorbic acid was not utilised, as by itself it did not appear to be effective against total PPO activity in western rock lobster.

The trials were run over a four-day period as shown in Table 4. On each day, receipt weight, physical damage and gender were determined for all lobsters used prior to treatment. Lobsters were divided into groups of 50 and 20 lobsters were bled to allow determination of moult stage, initial baseline PPO activity, total PPO activity and hemolymph protein content according to the standard methods. Each group was then assigned to a treatment. The lobsters were drowned in 50 litres of fresh water (controls) or 50 litres of the appropriate antibrowning agent solution made up with fresh water (treatments).

*Table 4: Timetable for antibrowning agent trials*

| Day | Test | Numbers of Lobsters |
|---|---|---|
| Day 1 | Control | 50 |
| | $CO_2$ x2 | 100 |
| Day 2 | Control | 50 |
| | Citric acid 1.5mg/ml x2 | 100 |
| | Citric acid 2.0 mg/ml x2 | 100 |
| Day 3 | Control | 50 |
| | 4-H 10µg/ml x2 | 100 |
| | 4-H 20µg/ml x2 | 100 |
| Day 4 | Control | 50 |
| | 4-H 87µg/ml x2 | 100 |

All treated and control lobsters were evaluated for physical damage and weighed again after drowning. All lobsters were cooked and chilled using the same standardised processing protocol as in previous experiments. After freezing, the extent, intensity and rate of melanosis development was observed for all treated and control lobsters. Evaluation of melanosis factors was carried out using the standard digital imaging analysis process.

## RESULTS AND DISCUSSION

### Objective 1: The impact of temperature on the activity of *P. cygnus* hemolymph PPO *in vitro*

#### Introduction

Several studies have shown that there is considerable variaration in the thermal stablity of PPO extracted from different organisms or foods (McCord & Kilara 1983; Whitaker 1994; Kim & Marshall 2000). Typically, PPO enzymes are heat labile so that exposure to temperatures greater than 60°C results in partial or total denaturation (Kim & Marshall 2000). Paradoxically, Ashida & Söderhäll (1984) showed that increasing temperature to 65°C actually increased PPO activity in crayfish.

A study looking at the activity of PPO extracted from the cuticle of western rock lobster also showed that western rock lobster PPO activity increased with increasing temperature while stability decreased, yet enzyme activity was still present after 30 minutes at 60°C (Chen et al. 1991). However, the study did not cover the range of temperatures (40 to 100°C) that may exist within a lobster during heat processing and the study utilized purified extracts of proPO, which precludes the inclusion of activity from secondary sources such as hemocyanin.

Since other factors present in the hemolymph, such as magnesium and calcium, also act as modifiers of PPO activity (Ashida & Söderhäll 1984; Sung et al. 1998; Lee & Shiau 2002) and hemocyanin may contribute to the production of melanin precursors during processing of western rock lobster it is important to evaluate the impacts of heat on whole hemolymph to ensure all factors are taken into consideration.

The results of any study looking at the impacts of heat should cover those temperatures that are evidenced during heat processing. If complete deactivation of the enzyme does not occur during the cooking process, melanosis will result. In addition, if the internal temperature reached by lobsters during cooking is sufficient to result in significant activation of the enzyme, increased levels of melanosis will result.

Therefore, the objective of this phase of the study was to examine the impact of temperature on the PPO activity in western rock lobster hemolymph and calculate

the thermal lethality values that should be reached in commercial cooking. From this information, it will be possible to ascertain if current commercial practice is sufficient to significantly decrease PPO activity and subsequently reduce the incidence of melanosis in cooked western rock lobster.

**Determination of the impact of heat on PPO activity *invitro***

When western rock lobster hemolymph was exposed to 40ºC under steady state conditions, baseline PPO activity did not change significantly (P>0.05) (Figure 20)(Williams, Mamo & Davidson 2003). Ashida & Söderhäll (1984) showed that heating purified proPO, derived from freshwater crayfish hemolymph, to temperatures greater than 45°C resulted in a rapid increase in PPO activity. However, in western rock lobster hemolymph, no baseline PPO activity was detected after heating for five minutes at 50 and 60ºC (Figure 20) (Williams, Mamo & Davidson 2003). From the data, it can be concluded that at 50 to 60ºC, heat deactivation of the active PPO forms exceeds any heat-induced activation of the pro-enzyme and/or other compounds expressing PPO activity such as hemocyanin. No significant recovery of PPO activity was observed over the 30-minute heating period at 50°C or 60ºC (P>0.05) (Figure 20) (Williams, Mamo & Davidson 2003).

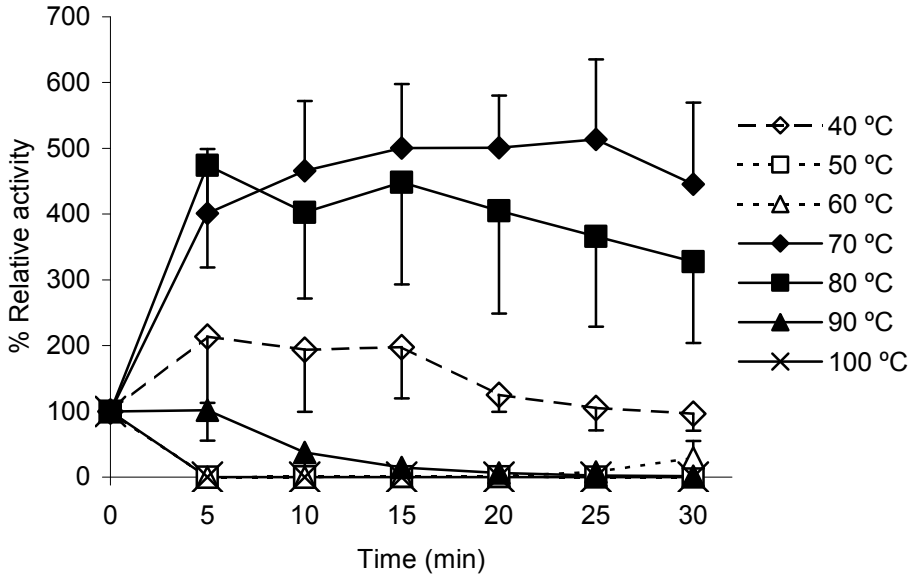


*Figure 20: Baseline relative PPO activity in western rock lobster hemolymph over time under steady state heating conditions (mean±SEM, n=4)*

The activation effect was much more marked at 70 and 80ºC with significant increases (P<0.05) in baseline PPO activity occurring within five minutes (Figure 20)

(Williams, Mamo & Davidson 2003). It would appear then that at 70 to 80°C heat-induced activation of the pro-enzyme and/or other forms of PPO activity exceeds heat deactivation of the active forms. Once the temperature exceeded 80°C baseline PPO activity followed a downward trend throughout the rest of the heating period but was still significantly higher ($P<0.05$) than the time zero value. There was no increase in baseline PPO activity at 90ºC and levels decreased steadily until there was no detectable baseline PPO activity after 20 minutes, which leads to the supposition that heat deactivation of the active enzyme and other forms of PPO activity exceeds any heat-induced activation at 90°C. No baseline PPO activity was detected in samples exposed to 100ºC for 5 minutes or more (Figure 20) (Williams, Mamo & Davidson 2003).

Under steady state conditions, total PPO activity appeared to increase slightly over time at 50 and 60ºC (Figure 21) (Williams, Mamo & Davidson 2003). The increase to greater than 100% initial total PPO activity is not significant ($P>0.05$) but it may be related to the activation of the secondary factors such as hemocyanin by heat. Terwilliger (1999) noted that when the hemocyanin of crustacean hemolymph is exposed to conditions that result in partial disruption of the tertiary structure, such as mild heating, it demonstrates PPO-type activity.

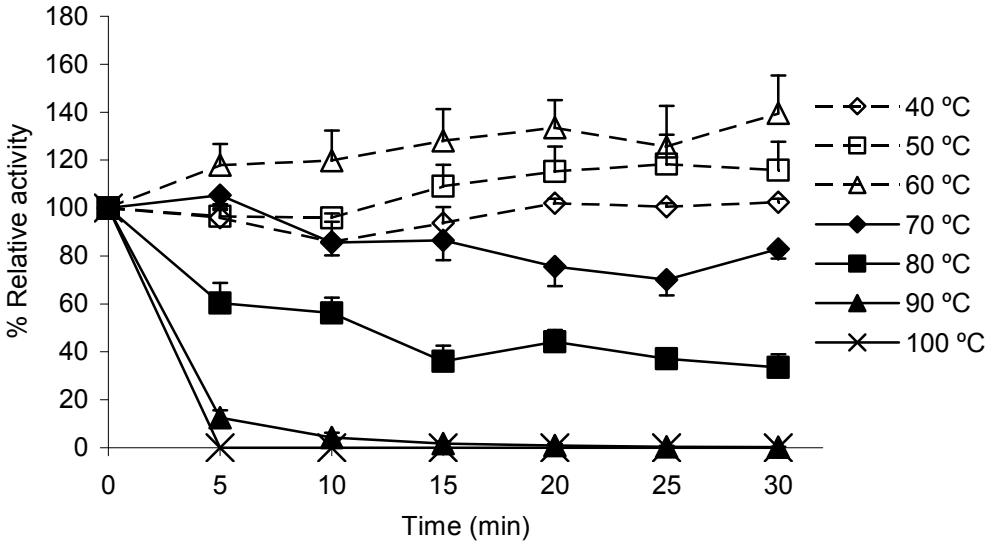


*Figure 21: Total relative PPO activity in western rock lobster hemolymph over time under steady state heating conditions (mean±SEM, n=4)*

For the temperatures greater than 80°C, total PPO activity decreased over time ($P<0.05$) (Figure 21) (Williams, Mamo & Davidson 2003). After 20 minutes at 90ºC,

total PPO activity was approaching zero. When heated at 100ºC no PPO activity was detected after 5 minutes, indicating that all compounds demonstrating PPO-type activity had been deactivated (Figure 21) (Williams, Mamo & Davidson 2003). These results would indicate that the internal temperature of lobsters must exceed 90°C for approximately 20 minutes in order to achieve significant deactivation of PPO.

In the unsteady state trials baseline PPO activity followed a similar pattern to that shown in the steady state trials (Figure 22) (Williams, Mamo & Davidson 2003). There was no significant impact on baseline PPO activity as the temperature approached 40ºC, but a sharp decrease occurred as temperature increased to 50ºC ($P<0.05$). No baseline PPO activity was detected at 60ºC. However, significant increases ($P<0.05$) in baseline PPO activity occurred as the temperature moved towards 70ºC (Figure 22) (Williams, Mamo & Davidson 2003). The increase may be attributed to heat activation of the pro-enzyme or other forms of PPO activity and/or disruption of the hemocytes. As the temperature approached 90ºC, baseline PPO activity decreased and no baseline PPO activity was detected in the samples at 100ºC (Figure 22) (Williams, Mamo & Davidson 2003).

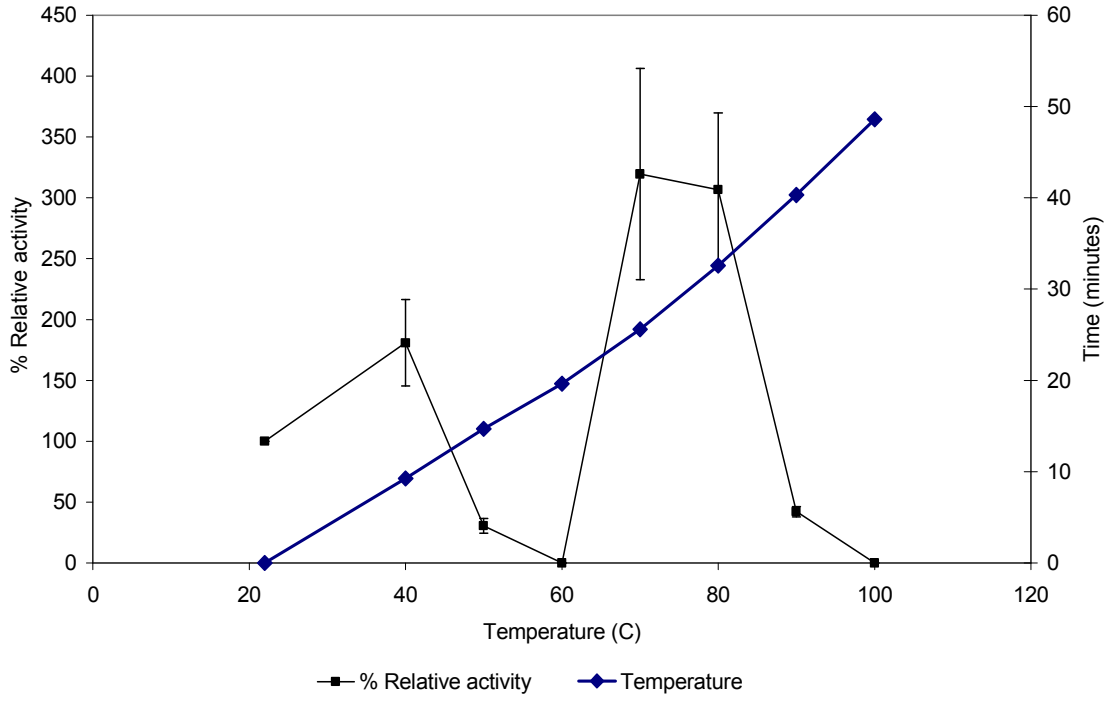


*Figure 22: Baseline relative PPO activity in western rock lobster hemolymph under unsteady state heating conditions (mean±SEM, n=8)*

While there was a shorter period of exposure to each temperature, the level of heat activation of the pro-enzyme and other compounds expressing PPO-type activity approached the maximal values achieved in steady state heating (Williams, Mamo & Davidson 2003).

Total PPO activity did not change significantly (P>0.05) during unsteady state heating until the temperature exceeded 70ºC (Figure 23) (Williams, Mamo & Davidson 2003). The apparent increase in total activity noted during steady state heating at the lower temperatures (Figure 21) is not significant during unsteady state heating (P>0.05) (Figure 23) (Williams, Mamo & Davidson 2003). Given that at least 15 minutes at 50°C under steady state conditions was required for the higher levels of activity to become noticeable (Figure 23) it is possible that the rate of change in temperature utilized in unsteady state trials was too rapid to allow for the heat-induced changes to occur in the other factors of the hemolymph that exhibit PPO-type activity (Williams, Mamo & Davidson 2003). Decreasing total PPO activity was observed as the temperature reached 80ºC, and at 90ºC total PPO activity was approaching zero (Figure 23) (Williams, Mamo & Davidson 2003). When the samples reached 100ºC no PPO activity was detected indicating all measured forms of PPO activity had been deactivated (Figure 23) (Williams, Mamo & Davidson 2003).

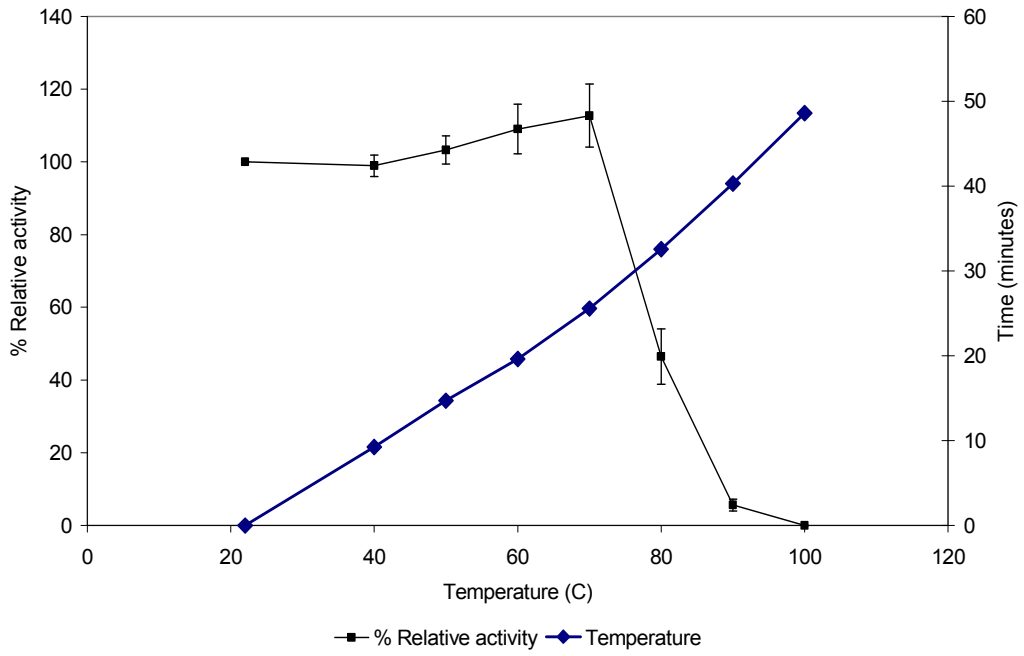


*Figure 23: Total relative PPO activity in western rock lobster hemolymph under unsteady state heating conditions (mean±SEM, n=8)*

When the baseline PPO values are compared to the total PPO activity values at the same temperature it appears that at 70ºC, 50% of the total available enzyme existed in the active PPO form, and at temperatures of 80ºC or more, 100% of the enzyme was in the active PPO form. However, even at the point of maximal activation of the pro-enzyme, the level reached did not exceed 50% of the initial level of total enzyme activity. It can therefore be concluded that for any given temperature the availability of compounds expressing PPO activity will be determined by a balance between temperature-induced activation and temperature deactivation of all measured forms (Williams, Mamo & Davidson 2003).

***Calculation of thermal constants***

The rate of deactivation of an enzyme is generally a first order reaction (Whitaker 1994). In a first order reaction the time required to deactivate a fixed percentage of the enzyme is independent of the initial concentration (Whitaker 1994). When the logarithm of percentage (%) relative activity at specified temperatures is plotted against time, a straight line graph results (Toledo 1991) as shown in Figure 24 for western rock lobster PPO. There is no value given for 100°C because the enzyme was completely deactivated within the first 5-minute time interval.

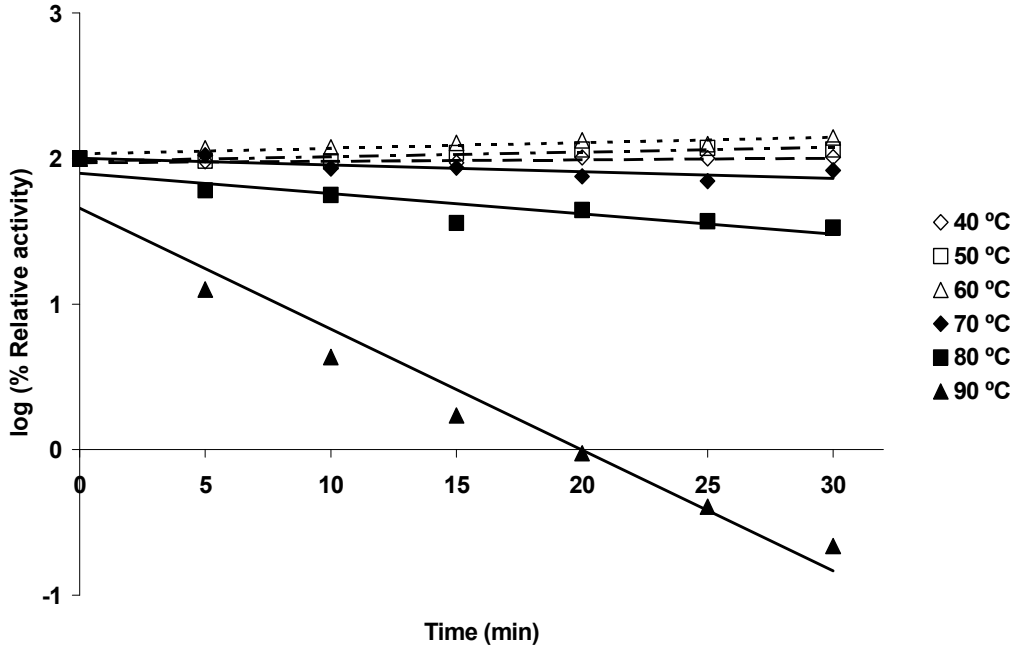


*Figure 24: Log % relative activity versus time for western rock lobster PPO*

Within a constant time interval at any given temperature, a 90% reduction in activity will be achieved when the deactivation occurs logarithmically (Scott & Weddig

1998). The constant time interval is called the *D value* or decimal reduction value (Scott & Weddig 1998) and is equal to the negative reciprocal of the slope of the regression line specific to the given temperature (Toledo 1991). The smaller the D value, the faster the rate of deactivation (Sharma, Mulvaney & Rizvi 2000). The D values derived from Figure 24 for western rock lobster PPO are shown in Table 5.

*Table 5: D value calculation for Western rock lobster PPO*

| Temperature | Slope of regression line | P [a] | D value | k |
|---|---|---|---|---|
| 40°C | 0.001037 | 0.36 ([b]NS) | - | - |
| 50°C | 0.003193 | 0.01 | -313.19 | 0.00 |
| 60°C | 0.003826 | 0.01 | -261.37 | 0.00 |
| 70°C | -0.004672 | 0.03 | 214.04 | 0.01 |
| 80°C | -0.013953 | 0.01 | 71.67 | 0.03 |
| 90°C | -0.083072 | 0.00 | 12.04 | 0.19 |

[a] ANOVA significance of variation from zero

[b] NS = not significant

From the data in Table 5 it can be seen that the slope of the line for 50 and 60°C is positive i.e. the D values are negative. A negative D value is indicative of no deactivation i.e. there is an increase in activity at these temperatures. Once the D value is known it is possible to calculate the rate reaction constant, k, for the deactivation of the enzyme at each temperature using Equation 2 (Sharma, Mulvaney & Rizvi 2000).

*Equation 2:*

$$k = \frac{\ln(10)}{D_t}$$

The values of k for western rock lobster PPO are also shown in Table 5. The highest rate of deactivation calculated was at 90°C therefore this temperature is used as the reference temperature in all following calculations.

When the natural log of k is plotted against the reciprocal of absolute temperature a thermal resistance graph or **Arrhenius plot** results as shown in Figure 25.

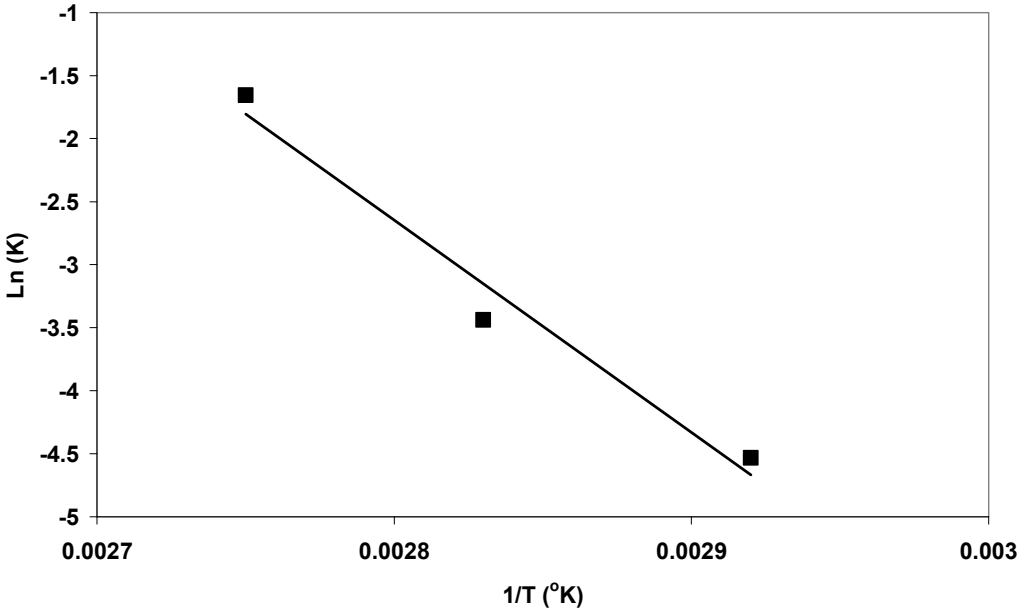


*Figure 25: Arrhenius plot for inactivation of western rock lobster PPO*

The Arrhenius plot has a slope equal to $-E_a/R$ where $E_a$ is the activation energy and R is the gas constant (8.314 J/K/mol or 1.98717 cal/K/mol (Toledo 1991). It is therefore possible to determine the activation energy, $E_a$ (Sharma, Mulvaney & Rizvi 2000) using Equation 3.

*Equation 3:*
$$E_a = slope \times R$$

McCord & Kilara (1983) noted that the higher the $E_a$ value the more sensitive the enzyme is to increases in temperature.

The z value can also be calculated from the slope of the Arrhenius plot using Equation 4:

*Equation 4*:
$$z = \frac{\ln(10)(T\ T_r)}{(E_a/R)}$$

where $T_r$ is the reference temperature (90°C), and T is the product temperature (Toledo 1991) The z value is the temperature change required to change the D value by a factor of 10.

From Figure 25, the Arrhenius plot for western rock lobster PPO has a slope of -17872, when $T_1$ = 70°C and $T_r$ = 90°C. Therefore $E_a$ = 148.59 KJ/mol (or 35.51 kcal/mol), and z = 16.0°C for western rock lobster PPO.

The $E_a$ value falls within the range of published values for PPO inactivation energy of various fruits (Robert et al. 1995; Weemaes et al. 1998; Gomez-Lopez 2002) and vegetables (McCord & Kilara 1983). However, values for D, z and $E_a$ for Florida spiny lobster PPO at 33 to 43°C were reported by Kim & Marshall (2000) and were significantly different (D = 320 to 229 minutes, z = 69.1°C and Ea = 26.6 kJ/mol) to that reported in the present study. However, several researchers have reported that the heat inactivation of PPO is a biphasic process (Robert et al. 1995; Weemaes et al. 1998). For temperatures below a specific break point temperature, characteristic to the PPO source, a log-linear relationship exists that is different to the log-linear relationship above the break point temperature. A higher $E_a$ value for temperatures above the break point, i.e. an increased sensitivity to heat, will result. Examination of Figures 20 and 21 suggests that the break point for western rock lobster PPO is between 60 and 70°C. Therefore it would be expected that the $E_a$ value for temperatures beyond 70°C would be much higher than that determined at temperatures below 60°C.

Using the $D_{90}$ value, it is possible to calculate the time required to achieve 99.9% deactivation of the enzyme at 90°C, or theoretical process lethality ($F_{90}$) using Equation 1:

*Equation 1:* $$F_t = S \times D_t$$

where S is the number of log cycles to be achieved and $D_t$ is the decimal reduction value at the reference temperature i.e. 90°C (Toledo 1991; Fellows 1997; Scott & Weddig 1998).

Given $D_{90}$= 12.04 (from Table 5) and 99.9% is equal to 3 log cycles then using Equation 1:

$$\begin{aligned} F_{90} &= 3 \text{ x } 12.04 \\ &= 36.12 \text{ minutes} \end{aligned}$$

Therefore, for a process to achieve a minimum deactivation level of 99.9% of western rock lobster PPO it must attain a process lethality value equal to or greater than 36.12 minutes at 90°C.

**Conclusions**

Hemolymph from live healthy western rock lobsters was exposed to a range of temperatures (40-100°C) for 30 minutes using steady state and unsteady state heating. Under the conditions used, at temperatures between 60 and 80°C heat-induced activation of the pro-enzyme and/or other forms of PPO activity exceeded heat deactivation of the active forms. Prolonged exposure to temperatures above 80°C was required to produce significant deactivation of the enzyme. These results lead to the conclusion that rapid heating of western rock lobster during processing is required in order to reduce the exposure time at moderate temperatures and thus limit the heat-induced activation of PPO (Williams, Mamo & Davidson 2003).

Calculation of the $F_{90}$ value showed that to produce 99.9% deactivation of the enzyme, the internal temperature of lobsters during cooking must exceed 90ºC for more than 36 minutes. Evaluation of the actual temperature time profile of standard commercial cooking is therefore required to establish if current processing regimes approach this level of deactivation and are sufficient to prevent development of melanosis in western rock lobster.

If the calculated level of heat treatment was applied during cooking of western rock lobster, it is possible that it could negatively impact on lobster quality through overcooking. Overcooking is associated with weight loss and decrease in eating quality through toughening of the meat. Therefore, further research into the use of alternative processing technologies such as steaming or microwave cooking to ensure rapid heating of western rock lobster during processing is required. The use of processing aids, such as anti-browning agents, may also enable a reduction in the severity of the temperature–time profile required to deactivate the enzyme. Research into the impact of anti-browning agents on melanosis formation is therefore indicated. Comparison of the impact of current and alternative methods on PPO activity and subsequent melanosis formation would assist the industry to determine the most effective thermal processing method for prevention of melanosis in western rock lobster.

## Objective 2: The impact of post-harvest transportation and holding on PPO activity, weight recovery and melanosis formation

### Introduction

Several different methods are used to transport western rock lobsters from the fishing boats to the processing factory. A study conducted by Tod & Spanoghe (1996) indicated that lobster health could be maintained during transportation. However, it is possible that the production of PPO in lobsters may occur as the result of stressing factors experienced by lobsters during prolonged transportation (Söderhäll & Cerenius 1998) such aerial exposure, temperature fluctuations and variable water quality in circulating systems.

It was therefore necessary to determine the effects of common transportation methods on PPO activity during and after transportation. These values could then be correlated with the level of melanosis development in the lobsters after processing, to establish if transportation method was a contributing factor to melanosis development in western rock lobster.

### Evaluation of the effects of transportation

Four common methods of transportation of live lobster were selected for simulation:

(A.)Live tank on boat - Lobsters were submerged in fresh ambient temperature (26±0.5ºC) seawater flowing through the tank, with adequate aeration

(B.)Spray truck - Lobsters were placed in a closed system tank with chilled (15 ±2 ºC) seawater recirculating via a spray head delivering 11 L/min

(C.) Carrier boat - Lobsters were placed in a tank with ambient temperature (26±0.5ºC) seawater flowing into the tank via a spray head delivering 11 L/min and freely draining

(D.) Dry chiller truck - Lobsters were placed in a chilled still air truck cabinet at 15 ±2 ºC

Temperature for Treatment A (ambient, submerged flow through simulation) and Treatment C (ambient spray, flow through) was constant at 26± 0.5ºC and Treatment B (recirculating spray, chilled) was constant at 15 ± 2ºC over the 4 day period. Oxygen saturation of Treatment A (submerged flow through simulation) was maintained at $PO_2$ equal to 86±5% saturation at 26±2ºC. Ammonia

concentration at the point of exit from the tank was monitored in seawater treatments and showed significant increases in Treatment B (recirculating spray, chilled) reaching levels of 100 µmol Nitrogen per litre compared to 10 µmol N/L in the other treatments. Treatment D (chiller truck simulation) was maintained at 60 ± 20% relative humidity and 15±2ºC over the treatment time. The day a trial was run on had no significant impact on the measured values. Evaluation of lobster vigour according to Spanoghe's classification system (Spanoghe & Bourne 1999) after treatments showed Treatments A and B were similar with the most vigorous lobsters (vigour indices = 3 or higher) while lobsters from C and D were significantly less vigorous (vigour indices = 3 or less).

Weight on receipt, after treatment and after processing (cook, cool and drain) was recorded for each lobster. There was a significant difference in weights after treatment with Treatment D showing a 4% weight loss ($P<0.05$) compared to a 2% weight gain for Treatment A ($P<0.05$). There was no significant change in weight with Treatment B or C ($P>0.05$). The day the treatment was run on had no impact on the result. Significant differences existed in weight after treatment with treatment D (chiller truck) showing an average weight loss of 4% (±1%SEM, $P<0.05$). There was no significant difference between other treatments ($p>0.05$). There was also no significant difference in weight recovery after processing for any treatment (98±2%SEM, $p>0.05$). There was no significant difference ($p>0.05$) in leg loss between treatments. Leg loss equated to 0.17±0.03 legs per lobster over the 4 treatments.

Evaluation of hemolymph chemistry in the lobsters after treatment showed that Treatment D had significantly higher baseline PPO activity than any other treatment ($P<0.05$, +3.7 EU ±1.57SEM). Baseline PPO activity also increased significantly in Treatment B ($P<0.05$, +1.7 EU ± 0.32 SEM). There was no significant change in baseline PPO activity for Treatments A and C ($P>0.05$). Total PPO activity increased significantly in Treatment D ($P<0.05$, +34.91 EU ± 3.41 SEM) but showed no significant change in the other three treatments ($P>0.05$). There was no correlation between initial baseline or total PPO activity and measurements of melanosis post processing.

Initial hemolymph ammonia content varied according to day before treatment with lobsters used on Day 2 showing significantly lower initial ammonia levels than lobsters used on the other three days ($P< 0.05$, 0.065 ± 0.003 SEM µmol N /ml). However ammonia levels after treatment varied according to day ($P<0.05$) with

Day 1 and 4, and Day 2 and 3 having similar final ammonia concentrations (see table 6). Comparison of hemolymph ammonia concentration after treatment showed that Treatment A had significantly higher levels of ammonia in the hemolymph compared to other treatments (P<0.05, 0.08 µmol N/ml ±0.02 SEM).

| Day | Mean | ± SEM |
|---|---|---|
| 1.00 | 0.036 | 0.0058 |
| 4.00 | 0.061 | 0.0079 |
| 2.00 | 0.146 | 0.0120 |
| 3.00 | 0.117 | 0.0110 |
| Total | 0.097 | 0.0061 |

*Table 6: Ammonia concentration (µmol N /ml) post t treatment*

**Evaluation of the effects of post harvest handling**

Comparison of receipt values by gender for PPO activity, total phenols and protein showed no significant differences for any parameter apart from an increased baseline PPO activity in females (+2U, P<0.05). During the holding period the females' baseline activity decreased to show no significant differences to that experienced by males. Overall baseline PO activity showed a significant decrease on holding (-2U, P<0.001) and total PO activity increased (+5U, P<0.05). There was no impact on total phenols or protein content during the holding period.

Drowning resulted in significant decreases in protein content (-10mg/ml, P<0.01), and total PO activity (-2U, P<0.01). It also resulted in significant increases in baseline PO activity (+3, P<0.001) and total phenol content (+12mg/ml, P<0.05). The decreased values may be related to dilution effects of the hemolymph serum while the increases in baseline activity and total phenol content may be caused by cellular disruption resulting release of these components into the hemocoelom.

Variations according to fieldtrip were noted. Total PO activity was significantly higher at the beginning of the season (+8U, P<0.01) and total phenol content showed significant increases over the season (40<56<94mg/ml, P<0.001). Baseline activity showed no significant differences throughout the season (P>0.05).

Correlation of the physiological parameters before and after holding, and drowning with the melanosis development factors (extent, intensity and rate) showed that current post harvest holding conditions had no negative impact melanosis development when lobsters were held for 48 hours. Evaluation of longer holding

periods maybe of value should the industry consider pursuing aquaculture on long term holding options in the future.

**Conclusions**

The aim of these experiments was to determine the impact of simulated transportation methods on weight recovery and those factors that may impact on melanosis formation after processing. From these results it can be concluded that:

- The simulated transportation and holding methods had no impact on weight recovery after processing.
- The simulated transportation and holding methods had no impact on melanosis formation after processing
- Flow through seawater systems showed the least impact on PPO activity and chilled air showed the greatest impact on PPO activity.

## Objective 3: The impacts of current commercial practises on weight recovery and melanosis formation in *P. cygnus*

**Introduction**

The calculated theoretical $F_{90}$ value previously determined in objective 1 gives a valuable insight into the possible parameters required for processing of western rock lobster, however it is not the whole answer. The calculated lethality was determined using whole hemolymph in a test tube, which does not take into account factors that may modify the impact of the heat on the enzyme within a whole lobster during processing.

Researchers have shown that variations in crustacean physiology, such as gender, moult stage and physiological stress can impact on the level of PPO activity that exists in crustaceans (Ogawa 1984; Ogawa 1987; Bartolo & Brik 1998). Ogawa found in investigations of raw shrimp (*Penaeus subtilis*) that females showed higher levels of discolouration compared to males (Ogawa 1984; Ogawa 1987). He also noted that moult stage played a role in increased levels of melanosis formation, with individuals in moult stage C (intermoult) and D (premoult) showing higher levels of melanosis (Ogawa 1984; Ogawa 1987). Moult stage was also investigated in the Norway lobster (*Nephrops norvegicus*) and it was noted that moulting was associated with increased PPO activity (Bartolo & Brik 1998).

It has been shown that a lobster undergoing rough or repetitive handling will become physiologically stressed (Paterson & Spanoghe 1997; Norton, Levy & Field 1999; Spanoghe & Bourne 1999; Bergmann, Taylor & Moore 2001). Bartolo and Brik (1998) investigated the impacts of post-harvest handling on PPO activity in Norway lobster (*Nephrops norvegicus*) and noted that rough handling, as indicated by increased claw loss, contributed to increased PPO activity. Different methods of transportation may also contribute to levels of physiological stress in lobsters (Tod & Spanoghe 1996). Evaluation of gender, moult stage and physiological stress as modifiers of melanosis formation in western rock lobster should therefore be undertaken.

In the western rock lobster industry, lobsters are graded according to weight. Investigation of the differences in heating rate is necessary to evaluate variations in heat transfer characteristics that may exist between different grades of lobster and the possible impacts on melanosis. Initial lobster temperature; maximum temperature reached; and the rate of heating & cooling may also modify the heat input experienced by a lobster during processing. It is therefore necessary to determine the actual lethal rate (L) and the total accumulated process lethality values ($F_r$) for standardised commercial cooking. Application of this method is widely used in evaluation of heat processing of foods throughout the food industry.

The lethal rate is reported in terms of minutes at the reference temperature that will give an equivalent lethal effect to the actual minutes of exposure at a constant temperature. The lethal rate is calculated for any temperature at any point in time as previously discussed. However, in order to determine the total lethal effect of a process it is necessary to calculate the total accumulated process lethality. The integration of the lethal rate over time is required and can be achieved using Equation 8 (Toledo 1991; Fellows 1997; Scott & Weddig 1998).

*Equation 8:*

$$F_r = \sum L_T \Delta t$$

Comparison of the calculated total process lethality to the theoretical $F_{90}$ value, previously determined in objective 1, will allow prediction of the effectiveness of the process in deactivating the enzyme.

The objectives of this phase of the study were therefore:

- To determine the initial total and baseline PPO activity, total phenols, protein content, gender, moult stage, and physical damage for every lobster used and evaluate the impact of these physiological factors on melanosis formation;

- To determine the temperature-time profile and the total accumulated process lethality of the standardised cooking process for western rock lobster; and

- To evaluate the effectiveness of the determined lethality in the prevention of melanosis formation in western rock lobster after processing.

**Characteristics of the lobsters utilised**

All the lobsters were at stage C (intermoult) in the moult cycle therefore the evaluation of the impact of moult stage on melanosis was not carried out in this study. When data was broken down into field trips and compared for all values, there were no significant differences between field trips for any of the physiological parameters measured (One way ANOVA, $p>0.05$).

There was an even distribution of gender across each grade with Grade A having 99 females and 97 males, and Grade B utilising 62 females and 64 males. Comparison of initial baseline and total PPO activity showed that females had significantly higher levels of baseline PPO activity upon receipt compared to males (Independent T test, $p<0.05$) (see Table 7). There was no significant difference by gender in weight, protein content, or initial total PPO activity levels at the time of receipt (Independent T test, $p>0.05$).

*Table 7: Comparison of initial physiological parameters by gender (n=161 for both genders)*

| Physiological parameters | Female | | Male | |
|---|---|---|---|---|
| | Mean | SEM | Mean | SEM |
| Protein initial (mg/ml) | 87.37 | 5.09 | 83.89 | 3.51 |
| *Total PPO initial | 19.52 | 1.77 | 17.03 | 1.04 |
| ♣*Baseline PPO initial | 6.29 | 0.44 | 4.90 | 0.33 |

*PPO activity measured as ∆ Absorbance/min/ml

♣ Independent T test, significant difference between genders $p<0.05$

Evaluation of the data showed that the grade of lobster had no impact on the initial total and baseline PPO activity nor did grade impact on the protein level in lobsters (One way ANOVA, $p>0.05$).

Blood measures have been widely evaluated for use as indicators of lobster stress during post harvest handling (Paterson & Spanoghe 1997; Jussila et al. 1999; Spanoghe & Bourne 1999; Bergmann, Taylor & Moore 2001; Jussila et al. 2001) Protein level is one of the easiest parameters to measure in the field requiring only a handheld refractometer (Paterson, Davidson & Spanoghe 1999). Protein level did show a significant correlation to initial total PPO activity with a Pearson's correlation coefficient of 0.493 ($P<0.001$). Examination of the linear regression showed that 24.3% of the variation on total PPO activity was related to the calculated protein level for western rock lobster (Figure 26). However, there was no correlation between protein level and initial baseline PPO activity or, more importantly, to melanosis development. Therefore, to use measurement of protein in the hemolymph, as a predictor of melanosis development, does not appear to be justified as more factors may be involved than total PPO activity alone

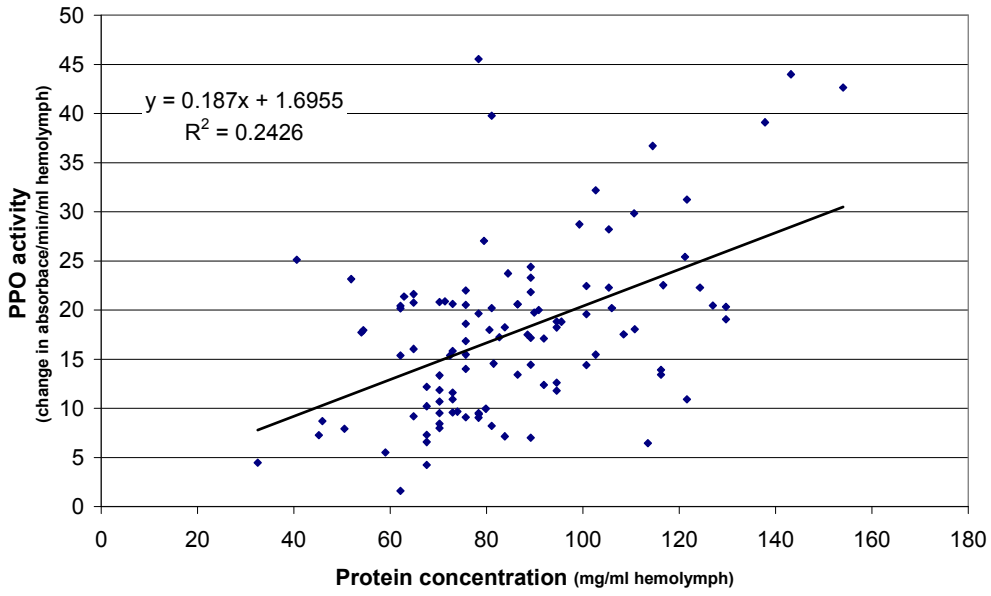


*Figure 26: Correlation of protein to initial total PPO activity in western rock lobster hemolymph*

Evaluation of the weight data showed that significant changes in weight occurred during processing (Paired T tests, $p<0.01$). Since lobsters are osmotic conformers, they cannot control the movement of water in and out of their tissues (Ruppert & Barnes 1994). Therefore, when placed in freshwater there is an influx of water to the tissues. During drowning both grades of lobsters took up 6.6 $\pm$ 0.2% of their own body weight or approximately 30 grams of water as shown in Figure 27. During cooking, the weight of lobsters decreased but was still higher than weight upon receipt into the factory. After cooling the Grade A lobsters showed an overall weight

gain of 0.54 ± 0.5% and Grade B lobster showed an overall weight gain of 0.7 ± 0.65% (approx 3g). The difference in weight gain between grades and between receipt weight & final weight was not significant (One-way ANOVA and Paired T test respectively, p>0.05).

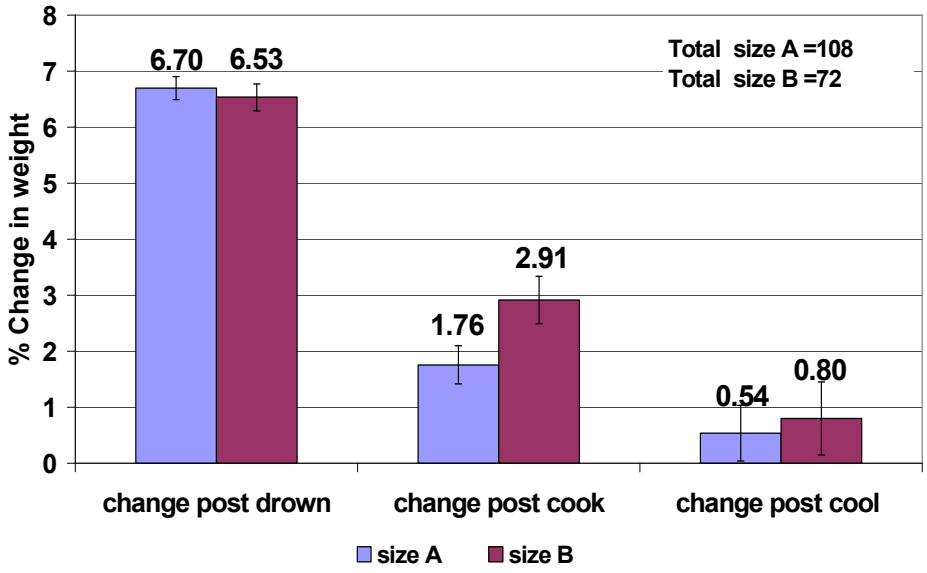


*Figure 27: Change in weight of lobsters during processing compared to receipt weight*

It is interesting to note from Table 8 that drowning resulted in a significant decrease in protein concentration (-10mg/ml, Paired T test, P<0.01), while baseline PPO activity increased (+3U, Paired T test P<0.001). It is probable that the influx of fresh water results in increased cellular disruption thus releasing proPO into the hemocoelom where it is activated by the endogenous activating enzymes. The decrease in protein occurred due to the dilution effect of the water influx.

*Table 8: Overall measures of physiological parameters before and after drowning*

| Physiological Parameters | Mean | SEM |
|---|---|---|
| Protein initial (mg/ml) | 85.47 | 2.80 |
| ♣Protein post drown (mg/ml) | 74.53 | 2.43 |
| *Total PO initial | 17.71 | 0.92 |
| Total PO post drown | 16.60 | 0.90 |
| *Baseline PO initial | 5.35 | 0.28 |
| ♣Baseline PO post drown | 8.47 | 0.71 |

*PPO activity measured as Δ Absorbance/min/ml hemolymph

♣Paired T test significance of difference P<0.01

The increases in the baseline activity of PPO upon drowning may be of importance in the development of melanosis. If lobsters are drowned and then left to sit for a period before processing this will increase the time where PPO activity can take place. Since there is no indication that the products of PPO activity are destroyed by heating, an increase in activity before processing may correlate with an increased risk of melanosis development after processing. Application of antibrowning agents during drowning may overcome this effect.

There is also the possibility that the use of anti-browning agents may act to reduce the temperature–time profile required to deactivate the enzyme which would have the added advantage of increasing returns to the industry due to decreased weight loss during commercial cooking. Research into the impact of anti-browning agents acceptable to the industry on melanosis formation is therefore needed.

**Evaluation of the thermal profile**

For each cook the change in temperature over time during cooking and cooling was plotted for the internal and external probes of each lobster. A thermal curve similar to that shown in Figure 28 was recorded for each cook.

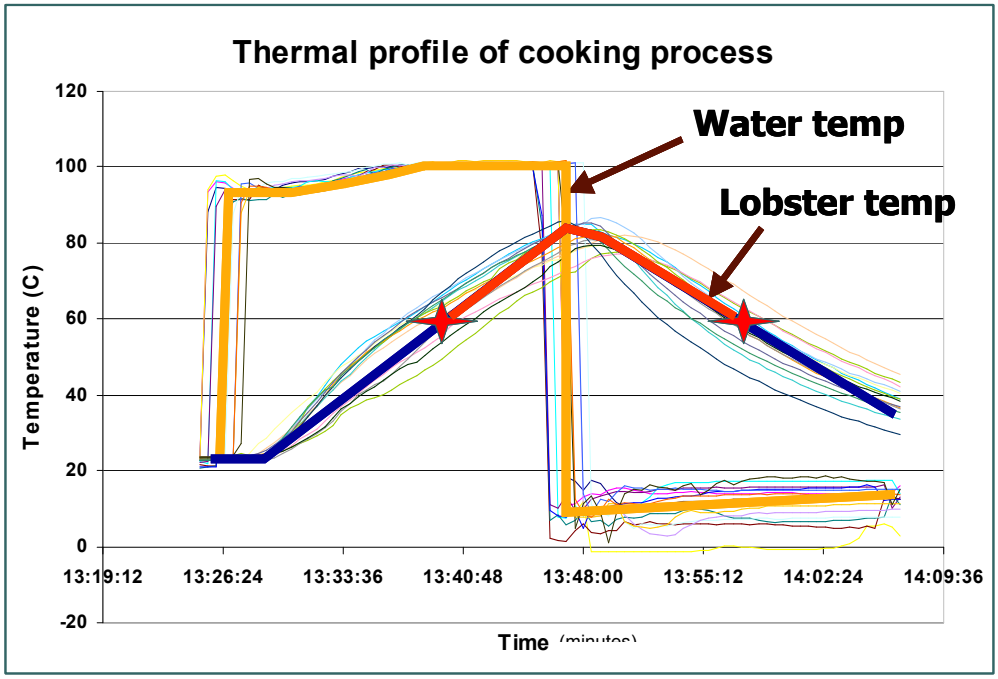


*Figure 28: Typical thermal profile of western rock lobster processing*

At the beginning of the cooking period, the water temperature in the cooker was 100ºC. When the lobsters were put in the cooker, the temperature dropped between 3 and 5°C. There was a gradual increase until the external temperature for all

positions in the cooker reached 100ºC again. A sharp drop in external temperature occurred when the baskets were removed from the cooker and plunged into the ice slurry. The internal temperature of the individual lobsters showed a gradual increase in temperature throughout the cooking period and an equally gradual decrease in temperature during cooling.

Analysis of the thermal transfer rate showed there was no significant difference between cooks utilising the same grade of lobsters (K-W ANOVA, $p>0.05$). However, there were significant differences in thermal transfer rate between the cooks of different grades with the larger Grade B lobsters showing a significantly slower rate of heating and cooling (K-W ANOVA, $p<0.05$) as shown in Table 9. Table 9 also shows the average maximum and minimum temperature reached during heating and cooling.

*Table 9: Comparison of thermal transfer rate during cooking (grade A n=14, grade B n=9)*

| Probe Position | Heating rate: (°C per minute) | | Maximum temperatures (°C) | | Cooling rate: (°C per minute) | | Minimum temperatures (°C) | |
|---|---|---|---|---|---|---|---|---|
| | **Grade A** | **Grade B** | **Grade A** | **Grade B** | **Grade A** | **Grade B** | **Grade A** | **Grade B** |
| A | 3.07 | 2.95 | 84.24* | 80.03* | -2.71* | -2.41 | 32.29* | 38.69 |
| B | 3.14 | 2.97 | 82.95 | 82.97 | -2.42 | -2.20 | 36.78 | 38.21 |
| C | 3.13 | 3.04 | 83.37 | 83.18 | -2.35 | -2.37 | 34.52 | 37.42 |
| D | 3.08 | 2.88 | 84.06 | 81.11 | -2.47* | -2.20 | 37.84* | 42.74 |
| E | 3.06 | 2.85 | 83.08 | 80.87 | -2.46 | -2.20 | 37.64 | 41.39 |
| F | 2.89 | 2.80 | 82.58* | 79.41* | -2.40 | -2.18 | 38.75 | 40.47 |
| G | 2.90 | 2.87 | 81.83 | 81.98 | -2.35 | -2.39 | 37.93 | 39.24 |
| H | 3.00 | 2.68 | 81.92 | 79.72 | -2.49* | -2.27 | 37.51 | 41.44 |
| I | 2.96 | 2.64 | 83.00 | 81.62 | -2.56 | -2.42 | 37.66 | 40.40 |
| J | 3.10 | 2.68 | 83.78* | 81.19* | -2.59 | -2.27 | 36.24 | 39.67 |
| K | 3.06 | 2.80 | 83.96 | 82.36 | -2.73 | -2.55 | 34.27 | 38.93 |
| L | 3.21 | 2.81 | 83.76 | 81.27 | -2.48* | -2.40 | 33.85* | 40.80 |
| M | 2.94 | 2.88 | 84.19 | 83.23 | -2.53 | -2.29 | 39.15 | 41.58 |
| N | 2.82 | 2.89 | 83.26 | 82.97 | -2.41 | -2.52 | 37.92 | 41.41 |
| Overall averages | Mean = 3.0, SEM = 0.03 | Mean = 2.8 SEM= 0.03 | Mean =83.2 SEM= 0.24 | Mean =81.9 SEM= 0.34 | Mean =-2.5 SEM= 0.03 | Mean = -2.3 SEM = 0.03 | Mean = 36.6 SEM = 0.55 | Mean = 40.2 SEM = 0.41 |

***** One way ANOVA significant difference, P<0.05

The greatest differences between grades in maximum temperature reached occurred at positions A, F and J with Grade A reaching much higher temperatures than Grade B in all cases (One way ANOVA, $p<0.05$) (Positions shown in Figures 18 and 19). There were no significant differences between maximum temperatures reached by different grades in at any other position (One way ANOVA, $p>0.05$) as shown in Figure 29.

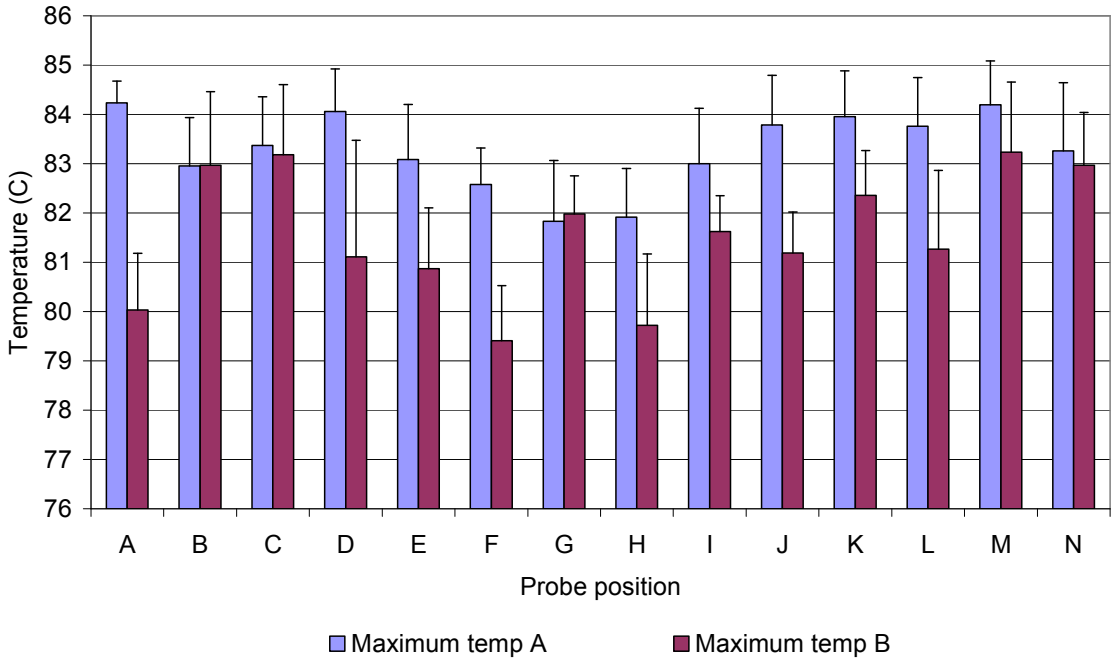


*Figure 29: Average maximum temperature reached during cooking of western rock lobster (mean±SEM)*

The average minimum temperature for each position during cooling is shown in Figure 30. There were significant differences between grades in minimum temperature at positions A, D, and L (One way ANOVA, $p<0.05$) (Positions shown in Figures 18 and 19). There was no significant difference in minimum temperature reached by different grades at any other positions (One way ANOVA, $p>0.05$).

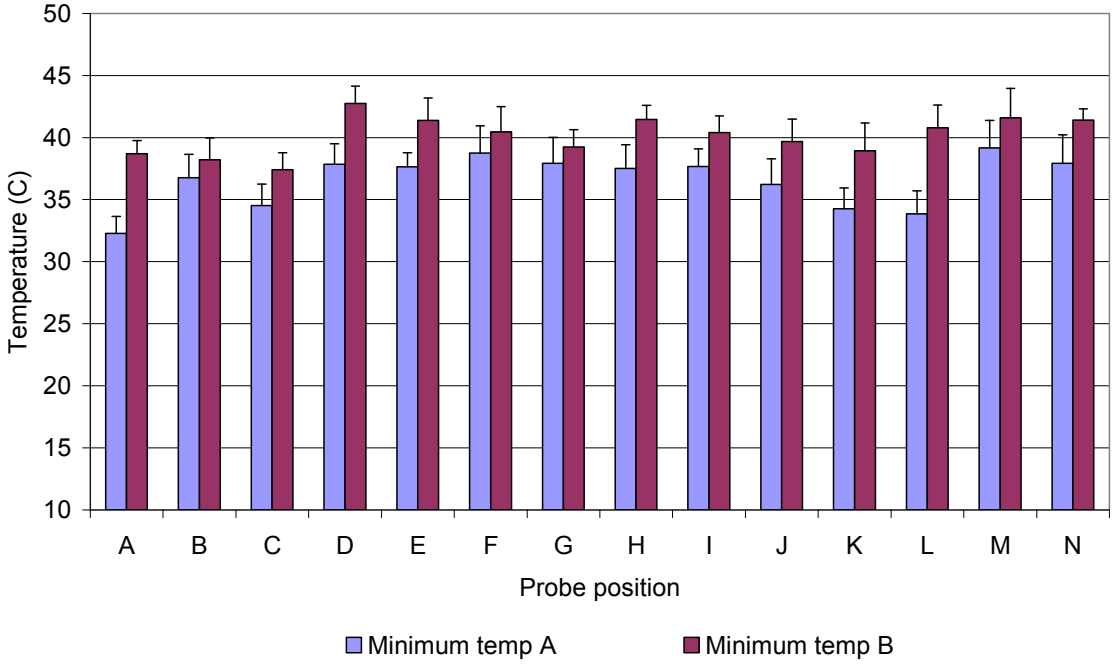


*Figure 30: Average minimum temperature reached during cooling of western rock lobster after cooking (mean±SEM)*

These results clearly indicate that size is an impacting factor on the rates of heating and cooling during processing. Larger lobsters will take longer to heat up and cool down thus having a higher final temperature compared to smaller lobsters. It can also be seen that heat transfer gradients exist within the cooker and the cooling tank. Lobsters towards the middle of the cook reached lower maximum temperatures during heating, but also maintained a higher final temperature during cooling. The differences in final temperatures at different positions maybe attributable to different packing densities with the larger grade B lobsters being more tightly packed in the baskets and so resulting in restriction of water movement around the lobsters during cooking. The weight to water volume ratio may also be an influencing factor on these trials.

The total accumulated process lethality was calculated using equations 5, 6 and 8 (Appendix 3) as demonstrated in Table 10. Comparison to the theoretical $F_{90}$ value previously determined allows prediction of the effectiveness of the process in deactivating the enzyme.

*Table 10: Example of a process lethality calculation table*

| Time (Minutes) | Temperature °C | A ($T_i$-90)/ z | L $L=10^A$ | Cumulative lethality ($F_r= \Sigma L\Delta t$) |
|---|---|---|---|---|
| 0 | 25.4 | -4.04 | 4.57E-05 | 4.59E-05 |
| 10 | 64.6 | -1.59 | 0.013 | 0.0519 |
| 15 | 78.3 | -0.73 | 0.093 | 0.547 |
| 20 | 86.4 | -0.22 | 0.299 | 2.46 |
| 25 | 64.8 | -1.58 | 0.0133 | 3.50 |
| 30 | 49 | -2.56 | 0.00137 | 3.54 |
| 35 | 37.7 | -3.29 | 0.000269 | 3.55 |
| 40 | 29.8 | -3.76 | 8.64E-05 | 3.55 |

The values in Table 10 were taken from a processing run. In each run temperature was recorded at 30-second intervals however only the 5 minute results are shown here. For the lethality calculations, $\Delta t$ was 0.5 minutes (Equation 8, appendix 3). Figures 31 and 32 demonstrate the changes in lethality during processing of western rock lobster. The first 20 minutes of the scale constitutes the cooking period and the second 20 minutes constitutes the cooling period. Lethality was calculated across the entire 40 minute time span to take in to account the lethal effect occurring in the initial part of the cooling curve where internal temperature of the lobsters was above 60°C

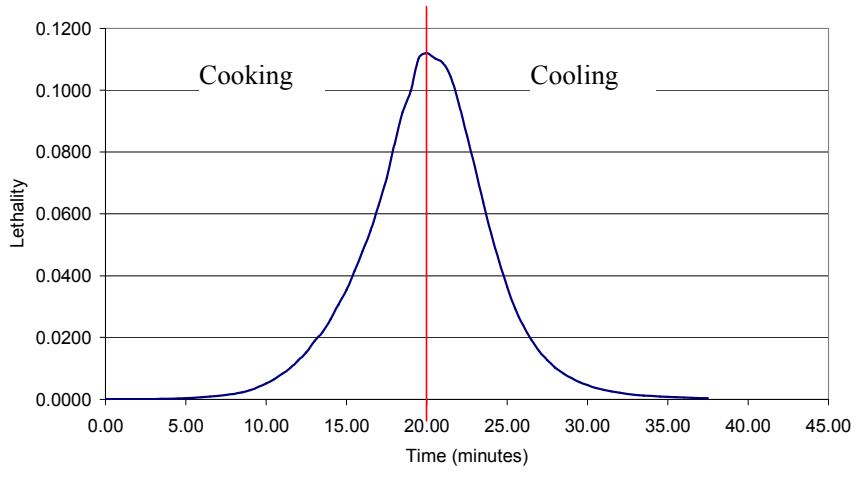


*Figure 31: Typical lethal input curve for heat processing of western rock lobster*

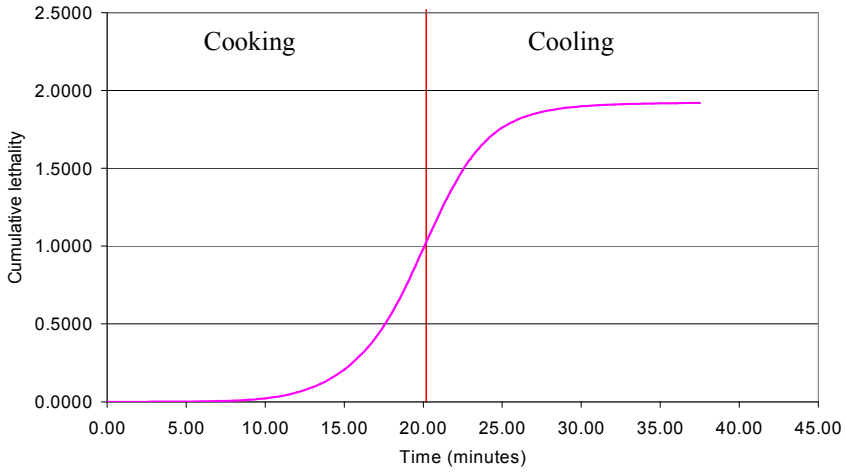


*Figure 32: Typical cumulative lethality curve for heat processing of western rock lobster*

The total accumulated process lethality values for standardised cooking of western rock lobster were calculated, using the thermal profiles from the fourteen cooks of Grade A and nine cooks of Grade B lobster during the 2001-2002 season. Calculations were carried out for every position in the cook. Comparison of process lethality at each position showed no significant differences by gender or between cooks within grades (one way ANOVA, $P>0.05$). There was significant difference with respect to position between grades (one way ANOVA, $P<0.05$). The results are shown in Table 11 and show that lobsters towards the outside of cooks typical experience higher lethalities than those towards the middle. Therefore, measurements of lethality during cooking should be taken from those lobsters positioned centrally during processing. By this means, the processor can be sure of measuring the point of lowest lethality and thus preventing under processing.

*Table 11: Process lethality values, as equivalent minutes at 90°C, for commercial cooking of western rock lobster (2001-2002 season)*

| Probe position | Grade A | Grade B |
|---|---|---|
| Probe A | 3.04 | 2.16 |
| Probe B | 2.74 | 2.55 |
| Probe C | 2.72 | 2.76 |
| Probe D | 3.36 | 3.04 |
| Probe E | 2.92 | 2.53 |
| Probe F | 2.85 | 1.97 |
| Probe G | 2.50 | 2.45 |
| Probe H | 2.69 | 2.03 |
| Probe I | 2.91 | 2.30 |
| Probe J | 3.15 | 2.16 |
| Probe K | 3.16 | 2.69 |
| Probe L | 2.87 | 2.64 |
| Probe M | 3.52 | 3.50 |
| Probe N | 3.37 | 3.20 |
| Mean ± standard deviation | 2.99± 0.81 | 2.57 ±0.84 |
| Maximum value | 8.26 | 9.82 |
| Minimum value | 1.05 | 0.74 |

These results also indicate that Grade B lobsters experienced significantly less lethal impact when compared to the smaller grade A lobsters. If larger lobsters were processed it would be expected that the lethality differences would be greater. Therefore, it is recommended that lobster processors ensure that cooks are made up of similar sized lobsters and larger lobsters are cooked for longer to ensure sufficient lethality is reached to deactivate the enzyme. When these results are compared to the theoretical process value of $F_{90}$ equivalent to 36.12 minutes at 90°C then it is clear that the system cannot produce 99.9% deactivation of the enzyme.

**Digital image analysis**

Every lobster cooked using the standardised protocol was evaluated for melanosis development after freezing, storage and thawing. Every lobster processed exhibited melanosis development. The area of blackening, intensity and rate of development

was evaluated over a 24-hour period and a summary of the results is shown in Table 12.

*Table 12: Summary of melanosis development post processing*

| Factor | Grade | Minimum | Maximum | Mean | Std. Error |
|---|---|---|---|---|---|
| % Area | A grade | 0 | 21.32 | 6.11 | 0.56 |
| | B grade | 0 | 20.57 | 6.61 | 0.76 |
| | | | Total | 6.31 | 0.45 |
| Intensity (average pixel density) | A grade | 93.13 | 204.61 | 174.24 | 2.58 |
| | B grade | 77.6 | 223.84 | 178.50 | 3.70 |
| | | | Total | 176.00 | 2.15 |
| Rate (change in area over time) | A grade | 0 | 0.11 | 0.018 | 0.0024 |
| | B grade | 0 | 0.06 | 0.016 | 0.0022 |
| | | | Total | 0.017 | 0.0017 |

Analysis of the results showed that significant differences existed between field trips with respect to extent, intensity and rate of melanosis development (Kruskal-Wallis $P<0.05$). Overall, Field trip 3 showed the lowest levels of all melanosis parameters while Field trip 1 exhibited the highest values for extent and rate of melanosis development. It is possible that these values maybe be explained by moult effects. The 'white' run of recently moulted lobsters occurs in November to late December, which corresponds to Field trip 1 (December 16$^{th}$ to 21$^{st}$, 2001). It would be expected that these lobsters would be more stressed due to their recent moult and be more susceptible to the impacts of change (Chen & S. 1993; Andersen, M.G. & Roepstroff 1996; Le Moullac et al. 1997; Mugnier & Justou 2004) thus increasing the risk of melanosis. The low levels of melanosis experienced by lobsters from Field trip 3 (March 1$^{st}$ to 8$^{th}$, 2002) can be explained given that this trip was held in the "reds" post moult stage of lobster development. Red lobsters have moulted sometime previously and have regained condition so should not be physiologically stressed. However, there is no certainty to these inferences as few of the lobsters used were not at moult stage C (intermoult).

Neither gender nor grade of lobsters had a significant impact on the extent, intensity and rate of melanosis development (One way ANOVA, $p>0.05$). It was also shown that position in the cooker did not impact significantly on the parameters measured for melanosis development (K-W ANOVA, $p>0.05$). There were significant

differences in melanosis factors between cooks, as shown in Table 13 and 143, yet when lethality of a cook was correlated with its melanosis factors, no significant relationships existed (Pearson correlation $P>0.05$).

It may be concluded that the variation in lethality within the cooker and between cooks is not significant at the levels experienced during these trials. However, it should be noted that with larger lobsters these variations would be more pronounced and may be significant in determining post-processing quality. A wider range of lethality may be required to elucidate the impact of lethality on melanosis factors.

*Table 13: Melanosis values by cook for Grade A lobsters in order of increasing value*

| Grade A | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % Area of melanosis | | | | Intensity (Average Pixel density) | | | | Rate (change in %area over time) | | | | Average lethality | |
| | Mean | | | | Mean | | | | Mean | | | | |
| COOK | Rank | Mean | SEM | COOK | Rank | Mean | SEM | COOK | Rank | Mean | SEM | COOK | Lethality |
| Cook 20 | 26.40 | 2.59 | 0.71 | Cook 1 | 28.20 | 165.78 | 3.54 | Cook 18 | 25.80 | 0.006 | 0.002 | Cook 28 | 1.92 |
| Cook 27 | 27.80 | 2.64 | 1.12 | Cook 17 | 37.40 | 165.64 | 8.98 | Cook 4 | 32.90 | 0.020 | 0.002 | Cook 5 | 2.37 |
| Cook 4 | 36.20 | 3.67 | 0.71 | Cook 20 | 41.40 | 169.84 | 9.32 | Cook 20 | 39.30 | 0.009 | 0.004 | Cook 4 | 2.52 |
| Cook 18 | 41.80 | 4.49 | 1.42 | Cook 27 | 44.40 | 161.13 | 17.83 | Cook 27 | 39.60 | 0.009 | 0.004 | Cook 3 | 2.58 |
| Cook 26 | 46.80 | 4.58 | 1.61 | Cook 4 | 47.20 | 169.93 | 6.36 | Cook 26 | 41.70 | 0.010 | 0.004 | Cook 26 | 2.68 |
| Cook 19 | 51.00 | 4.80 | 0.63 | Cook 26 | 51.20 | 164.05 | 17.65 | Cook 19 | 42.40 | 0.009 | 0.003 | Cook 25 | 2.86 |
| Cook 17 | 54.60 | 5.29 | 1.80 | Cook 2 | 52.40 | 179.14 | 4.90 | Cook 17 | 57.00 | 0.018 | 0.008 | Cook 29 | 2.89 |
| Cook 5 | 60.80 | 6.60 | 2.18 | Cook 18 | 55.00 | 173.40 | 6.97 | Cook 1 | 63.60 | 0.020 | 0.008 | Cook 27 | 2.93 |
| Cook 3 | 62.60 | 5.85 | 0.39 | Cook 5 | 57.20 | 117.64 | 6.21 | Cook 29 | 67.00 | 0.016 | 0.002 | Cook 1 | 2.98 |
| Cook 29 | 67.60 | 6.35 | 0.76 | Cook 19 | 58.40 | 178.95 | 5.49 | Cook 5 | 72.40 | 0.020 | 0.005 | Cook 18 | 3.13 |
| Cook 1 | 67.80 | 7.23 | 1.81 | Cook 3 | 64.10 | 180.24 | 5.05 | Cook 3 | 76.70 | 0.020 | 0.002 | Cook 19 | 3.19 |
| Cook 25 | 81.00 | 8.97 | 1.96 | Cook 25 | 65.20 | 181.27 | 7.28 | Cook 2 | 83.40 | 0.048 | 0.023 | Cook 2 | 3.59 |
| Cook 2 | 83.80 | 12.47 | 1.01 | Cook 28 | 73.20 | 185.22 | 7.35 | Cook 25 | 87.10 | 0.032 | 0.006 | Cook 17 | 4.01 |
| Cook 28 | 89.20 | 11.69 | 2.78 | Cook 29 | 83.80 | 190.05 | 7.49 | Cook 28 | 95.90 | 0.041 | 0.010 | Cook 20 | 4.23 |
| Overall average | | 5.81 | 1.24 | Overall average | | 168.63 | 8.22 | Overall average | | 0.02 | 0.005 | Average | 3.00 |
| Overall SEM | | 0.81 | 0.19 | Overall SEM | | 4.65 | 1.16 | Overall SEM | | 0.003 | 0.001 | SEM | 0.17 |

*Table 14: Melanosis values by cook for Grade B lobsters in order of increasing value*

| Grade B | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % Area of melanosis | | | | | Intensity (Average Pixel density) | | | | Rate (change in %area over time) | | | | Average lethality | |
| **COOK** | **Mean** | **Rank** | **Mean** | **SEM** | **COOK** | **Mean** | **Rank** | **Mean** | **SEM** | **COOK** | **Mean** | **Rank** | **Mean** | **SEM** | COOK | **Lethality** |
| Cook 22 | 29.60 | 2.86 | 0.71 | | Cook 8 | 28.70 | 159.96 | 8.39 | | Cook 22 | 31.10 | 0.005 | 0.002 | | Cook 31 | 2.12 |
| Cook 23 | 43.60 | 4.18 | 1.66 | | Cook 22 | 29.20 | 158.72 | 8.44 | | Cook 21 | 46.30 | 0.012 | 0.006 | | Cook 30 | 2.13 |
| Cook 32 | 46.80 | 4.42 | 1.38 | | Cook 23 | 36.00 | 153.73 | 19.30 | | Cook 23 | 47.20 | 0.011 | 0.004 | | Cook 32 | 2.20 |
| Cook 30 | 50.60 | 4.83 | 1.01 | | Cook 30 | 66.80 | 183.20 | 6.72 | | Cook 30 | 48.00 | 0.011 | 0.003 | | Cook 7 | 2.21 |
| Cook 8 | 62.60 | 9.19 | 3.96 | | Cook 7 | 75.80 | 186.23 | 3.62 | | Cook 32 | 50.40 | 0.013 | 0.005 | | Cook 21 | 2.51 |
| Cook 21 | 68.20 | 6.11 | 1.50 | | Cook 6 | 76.40 | 186.52 | 3.42 | | Cook 6 | 61.70 | 0.018 | 0.007 | | Cook 8 | 2.80 |
| Cook 6 | 69.00 | 1.54 | 1.54 | | Cook 21 | 77.20 | 185.49 | 14.12 | | Cook 8 | 64.50 | 0.023 | 0.011 | | Cook 22 | 2.87 |
| Cook 31 | 79.00 | 8.30 | 1.91 | | Cook 31 | 89.80 | 192.76 | 4.06 | | Cook 31 | 71.60 | 0.023 | 0.008 | | Cook 6 | 3.13 |
| Cook 7 | 87.20 | 3.14 | 3.32 | | Cook 32 | 95.00 | 201.13 | 8.23 | | Cook 7 | 88.40 | 0.032 | 0.008 | | Cook 23 | 3.16 |
| | **Overall average** | 4.69 | 1.54 | | | **Overall average** | 156.81 | 7.69 | | | **Overall average** | 0.02 | 0.01 | | **Overall average** | 2.34 |
| | **Overall SEM** | 0.86 | 0.36 | | | **Overall SEM** | 18.11 | 1.73 | | | **Overall SEM** | 0.003 | 0.0009 | | **Overall SEM** | 0.27 |

Physical damage i.e. appendage loss resulted in significant differences in extent and intensity of melanosis development (One way ANOVA, $p<0.05$) but there was no significant difference in rate of melanosis development (One way ANOVA, $p>0.05$). Lobsters which lost two appendages showed higher levels of melanosis formation compared to those which lost none. Numbers of lobsters with more than two legs lost were low so that significance of increased appendage loss could not be calculated. It was not determined if the appendage loss arose from old injuries or had occurred during post harvest handling. It is interesting to note that the level of appendage loss showed no correlation to initial total or baseline PPO activity. It may be that damaged lobsters are more susceptible to the impacts of other factors that influence melanosis development, however, no clear conclusions as to what those factors are can be drawn from this study.

Correlation and regression analysis were used to evaluate the relationships between melanosis and physiological factors. There was no relationship between the initial baseline PPO activity and the extent, intensity and rate of melanosis development. However, a significant relationship existed between initial total PPO activity and the extent (Pearson correlation coefficient=0.217, $r^2 = 0.047$, $p=0.024$) & rate (Pearson correlation coefficient=0.222, $r^2 = 0.049$, $p=0.020$) of melanosis development. While these values are deemed significant by One Way ANOVA ($p<0.05$), the correlation value is too low to be useful in prediction of melanosis from total PPO activity. Protein content showed no correlation to any factor of melanosis development (One way ANOVA, $P>0.05$).

Comparison of the extent, intensity and rate of melanosis development values shows that significant relationships exist, with all three factors showing significant correlation to each other ($p<0.01$)(Table 14). The most significant correlation was the extent of melanosis to the rate of development with 76.2% of the variability in area being explained by the rate of development as shown in Table 14. Since the correlation is positive, it can be concluded that a higher rate of development will result in a larger area being affected by melanosis.

*Table 15: Correlation of melanosis factors*

| Pearson Correlation | Area | Intense | Rate |
|---|---|---|---|
| Area | 1 | .334** | .873** |
| Intense | | 1 | .263** |
| Rate | | | 1 |
| | $R^2$ | | |
| Area | 1 | 0.111 | 0.762 |

** Correlation is significant at the 0.01 level (2-tailed).

**Conclusions**

Several factors have been implicated by researchers in the formation of melanosis. These include moult stage; gender; stress and PPO activity. Moult stage was not evaluated in this study, as there was no variation in moult stage between the lobsters used. Unlike blackspot studies carried out in shrimp (Ogawa 1984), gender did not appear to play a role in development of melanosis in western rock lobster. There was no significant difference between genders for any parameter measured with the exception of initial baseline PPO activity. However, the higher baseline activity evidenced in females did not translate into greater rates of melanosis in processed lobster. Comparison of initial PPO activity levels to melanosis development indicated that lobsters with higher levels of initial total activity are more likely to undergo melanosis after processing. However, since processing factors have a greater impact on the development of melanosis it does not appear that this can be taken as a single objective measure for determining those lobsters most likely to develop melanosis.

It has also been suggested that protein level maybe used as an indicator of lobster stress during post harvest handling and several researchers have linked stress to melanin formation in crustacean species (Ogawa 1987; Söderhäll & Cerenius 1992; Spanoghe & Bourne 1999; Nagai & Kawabata 2000). However, while there was a significant correlation between protein level and initial total PPO activity, protein level did not correlate with initial baseline PPO activity or to melanosis development. Therefore, to use measurement of protein in the hemolymph of western rock lobsters, as a predictor of melanosis development, does not appear to be justified.

Since lobsters are osmotic conformers, they cannot control the movement of water in and out of their tissues, which results in an influx of water to the tissues during drowning. It is probable that the influx of fresh water results in increased cellular

disruption thus releasing proPO into the hemocoelom where it is activated by the endogenous activating enzymes. The increases in the baseline activity of PPO upon drowning may be of importance in the development of melanosis. Since there is no indication that the products of PPO activity are destroyed by heating, an increase in activity before processing may correlate with an increased risk of melanosis development after processing. Application of antibrowning agents during drowning may overcome this effect by inhibition of the enzyme and or removal of the reaction products. Research is therefore required to determine the most effective and acceptable antibrowning agents for use in the western rock lobster industry.

Significant differences existed between the heating profiles of the different grades of lobsters with Grade B lobsters having slower rates of heating and cooling and lower maximum temperatures when compared to Grade A lobsters. A slower rate of thermal transfer means that while the product may not heat up as fast, it retains heat for much longer so has a higher end temperature after cooling. Whether Grade influences temperature exchange rates due to the weight-surface area ratio or due to the difference in packing density is unclear from this study.

It must also be noted that the differences in thermal profile between grades did not translate to differences in melanosis development. It may be that the impact of the low process lethality values achieved disguised the impact of lobster size and subsequent heat transfer rates on melanosis development. It is also possible that the difference between a Grade A and a Grade B lobster is not large enough to have a significant impact. Further work is required with a larger size difference to explain this fully.

Comparison of the actual process lethality values to the calculated theoretical $F_{90}$ value clearly shows that the thermal profile of the standardised commercial process is inadequate to achieve significant deactivation of the enzyme. Further examination of the cooking thermal profiles shows that the maximum temperature reached did not exceed 84.0 ±1.22ºC for Grade A lobsters and 81.6± 1.3ºC for Grade B lobsters over all positions in the three dimensional array. Comparison of these values to the *in vitro* heating trials (objective 1) shows that they would be insufficient to achieve significant deactivation of the enzyme. Each cook also spent 15.7 ± 0.77 minutes between 60 and 80°C during cooking and cooling. Examination of the *in vitro* heating data shows that this is the zone were maximum activation of the enzyme occurs. It would therefore be expected that melanosis would occur upon thawing of the lobsters, which was borne out by the digital image analysis results.

In order to reduce the exposure time at moderate temperatures it would appear that rapid heating of western rock lobster to temperatures greater than 80°C is required. From these trials, it is obvious that current practice is not capable of achieving the desired rates and levels of heating. It would also be of value to the industry to investigate the use of other methods of melanosis prevention such as the use of antibrowning agents. The use of antibrowning agents may render the enzyme more sensitive to the impacts of heat and thus modify the thermal profile required to deactivate the enzyme. Reducing the level of heating required then enables the use of current technology for processing. The use of antibrowning agents has the added advantage that reduced thermal inputs will result in increased weight recovery and reduced energy costs. Evaluation of alternative cooking methods such as steaming or microwave cooking to establish a process that achieves a faster rate of heating and/or a higher end-point would also be of value to the industry. Evaluation of the impact of these methods on PPO activity and subsequent melanosis formation would assist the industry to determine the most effective alternative processing method for prevention of melanosis in western rock lobster.

### Objective 4: Validation of the theoretical thermal kinetic parameters

#### Introduction

For western rock lobster, the identification of the kinetics of polyphenoloxidase enzyme behaviour under steady state heating allows the prediction of enzyme behaviour at different temperature-time combinations. The thermal kinetic values for western rock lobster PPO deactivation were determined in objective 1 of this study. For any given concentration of enzyme, the time at constant temperature required to produce a 90% reduction in activity over a constant time interval is called the **decimal reduction value** or D value (Toledo 1991). It was determined in objective 1 that the D value for western rock lobster PPO at 90°C ($D_{90}$) was 12.04 minutes. Using the $D_{90}$ value it is possible to calculate the time required to achieve 99.9% deactivation of the enzyme at 90°C, or **theoretical process lethality** ($F_{90}$). It was calculated in objective 1 that, theoretically, for a process to achieve a minimum deactivation level of 99.9% of western rock lobster PPO it must attain a process lethality value equal to or greater than 36.12 minutes at 90°C. While this calculated theoretical $F_{90}$ value gives a valuable insight into the possible parameters required for processing of lobster it is not the whole answer.

Since processing of lobsters occurs under conditions of constantly changing temperature, it is necessary to calculate the actual total accumulated process lethality applied to lobsters during processing. Actual process lethality is calculated by integration of the lethal rate over time (Toledo 1991; Fellows 1997; Scott & Weddig 1998) using Equation 6.

*Equation 6:*

$$F_r = \sum L_T \Delta T$$

Comparison of the actual total accumulated process lethality to the theoretical $F_{90}$ value previously determined enables prediction of the effectiveness of the process in deactivating the enzyme.

Evaluation of the standardised commercial process utilised in this study was reported in objective 3. The total accumulated process lethality values for standardised cooking of western rock lobster were calculated, using the thermal profiles from fourteen cooks of Grade A and nine cooks of Grade B lobster during the 2001-2002 season. An average process lethality value of 2.99 $\pm$ 0.81 (Grade A) and 2.57 $\pm$ 0.84 (Grade B) was achieved. Comparison of these results to the calculated theoretical $F_{90}$ value of 36.12 minutes clearly showed that the thermal profile of the standardised commercial process is inadequate to achieve significant deactivation of PPO.

It, therefore, became necessary to determine the cooking time required to reach the theoretical process lethality value in whole lobster. At the same time it was important to validate the effectiveness of the theoretical value in predicting the required process lethality required to prevent melanosis in lobsters. After all, it had not been established if 99.9% deactivation of the enzyme is required to prevent melanosis. If the actual heat input required is less than previously thought, there may be significant advantages in terms of weight recoveries and energy input savings for the industry

Therefore, the aim of this phase of the study was to determine if cooking alone could prevent melanosis and if so, to determine the level of processing lethality required, based on the theoretically determined process lethality previously calculated. The information generated could then be used to determine the optimum cooking time required to ensure minimal melanosis whilst maximising weight recoveries in western rock lobster.

**Characteristics of the lobsters utilised**

Of the 800 lobsters utilised across the season only three were not at stage C of the moult cycle. Therefore, moult stage did not confound the results. There was also no significant difference (t-test, $p>0.05$) in the numbers of males to females used for each of the three field trips (average ratio 1.2:1).

Gender did not impact on initial baseline PPO activity, however total PPO activity varied between genders across field trips with females having consistently higher levels of total PPO activity (K-W ANOVA, $p<0.05$). In previous work, females had higher baseline levels and there was no significant difference in the total PPO activity. Therefore, it can be concluded that the variations in the level of both forms of PPO activity are not solely due to gender but are influenced by other factors, which cannot be identified from this data.

It is apparent that there was no significant difference between lobsters in Field trip 8 (December 2003) and 9 (February 2004) with respect to protein and total PPO activity (Kruskal Wallis ANOVA, $p>0.05$) (Table 16). Lobsters in Field trip 10 (April 2004) showed an average protein content significantly higher than in Field trip 8 and 9, however total PPO activity was significantly lower (Kruskal Wallis ANOVA, $p<0.05$) (Table 16). Baseline PPO activity was significantly different across the season with Field trip 8 showing higher levels than Field trip 9 (Kruskal Wallis ANOVA, $p<0.05$). Field trip 9 in turn had significantly higher baseline PPO activity than Field trip 10 (Kruskal Wallis ANOVA, $p<0.05$) (Table 16).

*Table 16: Physiological parameters by field trip (2003-2004 season)*

| Parameter | Field trip | Mean Rank |
|---|---|---|
| Protein | 8 | 86.767 |
| | 9 | 82.583 |
| | 10 | 125.185* |
| Total PPO | 8 | 118.592 |
| | 9 | 123.583 |
| | 10 | 71.241* |
| Baseline PPO | 8 | 147.525* |
| | 9 | 102.708* |
| | 10 | 63.575* |

*Significantly different at p=0.05

When evaluating the physiological parameters, the life cycle of the animal must be considered. Each year in late November and again in February western rock lobsters moult (Donohue 1998). Therefore, during Field trips 8 and 9, the animals were in the immediate post-moult stage. Based on work done in other arthropods and decapod crustaceans, the demands for active PPO to sclerotise the cuticle will be high resulting in elevated activity (Andersen, M.G. & Roepstroff 1996; Le Moullac et al. 1997). The higher total and baseline activities for Field trip 8 and 9 demonstrated in Table 16 reflect this expectation. PPO activity would then decrease until it reaches the lowest level immediately pre-moult (Andersen, M.G. & Roepstroff 1996; Le Moullac et al. 1997). It has also been demonstrated in other decapod crustacean that hemolymph protein would be low immediately post moult and then increase as the animal begins to build a new shell for further growth (Chen & S. 1993; Le Moullac et al. 1997; Mugnier & Justou 2004). Therefore the low PPO activity and high protein concentration evidenced by lobsters used in Field trip 10 indicates that they are in the late intermoult to pre-moult stage of development.

Overall, comparison of the melanosis factors shows significant correlations ($p<0.05$) existed between the melanosis factors and the physiological parameters (Table 17). However, the regression values indicate that the degree of variability attributable to other factors exceeds that directly attributable to any single physiological factor. The data gathered here reflects the trends indicated in objective 3. Therefore drawing conclusions based on physiological parameters measured is not applicable in predicting melanosis rates.

*Table 17: Correlation of physiological parameters and melanosis factors*

| | **Protein** | | **Total PPO** | | **Baseline PPO** | |
|---|---|---|---|---|---|---|
| | Coeff. | $R^2$ | Coeff. | $R^2$ | Coeff. | $R^2$ |
| % Area | 0.28** | 0.077 | -0.079* | 0.006 | 0.078** | -0.279 |
| Intensity | 0.36** | 0.129 | -0.211** | 0.045 | 0.179** | -0.424 |
| Rate | 0.2** | 0.04 | -0.082* | 0.007 | 0.046** | -0.215 |

*correlation is significant at $p<0.05$

**correlation is significant at $p<0.01$

Correlation of the field trip averages for factors of melanosis with the physiological parameters measured before processing is not valid because processing conditions were not consistent between field trips. Field trip 10 had significantly lower rates of lethality across all treatments when compared to Field trip 8 and 9 (Table 18).

*Table 18: Averages of lethality by field trip*

| Field trip | Mean | N | Std. Dev |
|---|---|---|---|
| 8 | 36.79 | 230 | 21.82 |
| 9 | 25.10 | 230 | 17.20 |
| 10 | 14.35 | 370 | 17.64 |
| Total | 23.55 | 830 | 20.95 |

The difference in lethality was due to errors in calculation of the lethality in the initial trials of Field trip 8. A lower z value than that required was used to determine the lethality of the cooks during processing resulting in lower calculated lethality values than actually existed. In routine sampling and checking of the data the error was discovered but resulted in more undercooks having to be undertaken in Field trip 10 to bolster the data set of undercooks. The error resulted in Field trip 8 having a predominance of over cooks, and Field trip 10 a predominance of undercooks. Therefore, all melanosis data generated must be compared based on the lethality of the cook rather than the time of the season or field trip.

**Evaluation of the calculated lethality**

Over the course of the field trips, 80 cooks were run with 10 lobsters plus two instrumented lobsters in each. The thermal profiles of the instrumented lobsters in each cook were used to calculate the process lethality for each treatment. The process lethality was calculated by integration of the change in temperature over changing time using Equation 8 as detailed previously.

*Equation 8:*

$$F_r = \sum L_T \Delta t$$

The range of calculated process lethality values was from 0.313 to 83.45 and cook times were between 12 and 49.5 minutes. With longer cooking times, higher final internal temperatures resulted. When lethality values are plotted against cook time, the exponential relationship between the lethal input and duration of cooking time can be clearly seen (see Figure 33).

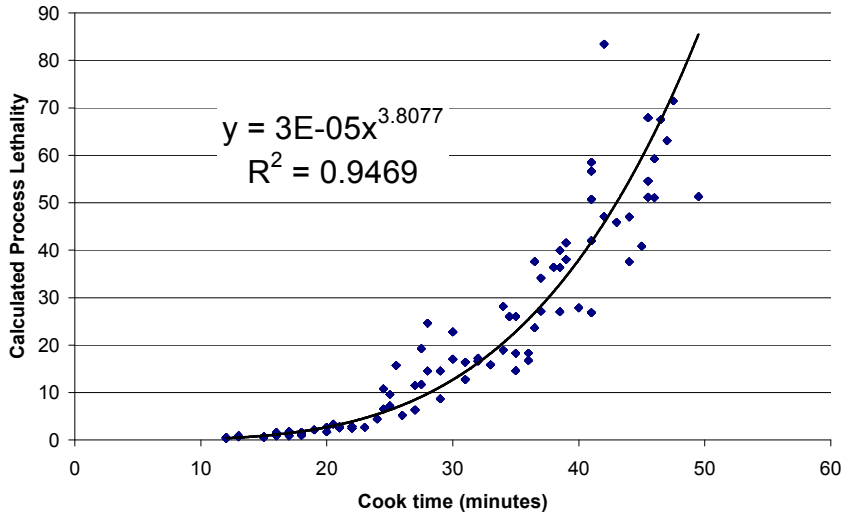


*Figure 33: Lethality vs. cook time for cooks of western rock lobster in 2003- 2004 season*

When the calculated process lethality for each cook is plotted against the extent of melanosis development for each lobster processed then Figure 34 results. The graph shows that as lethality approached the theoretical lethality value determined previously, the resultant area of melanosis decreased. When the theoretical lethality value was exceeded then no melanosis was apparent in any lobster processed in that cook.

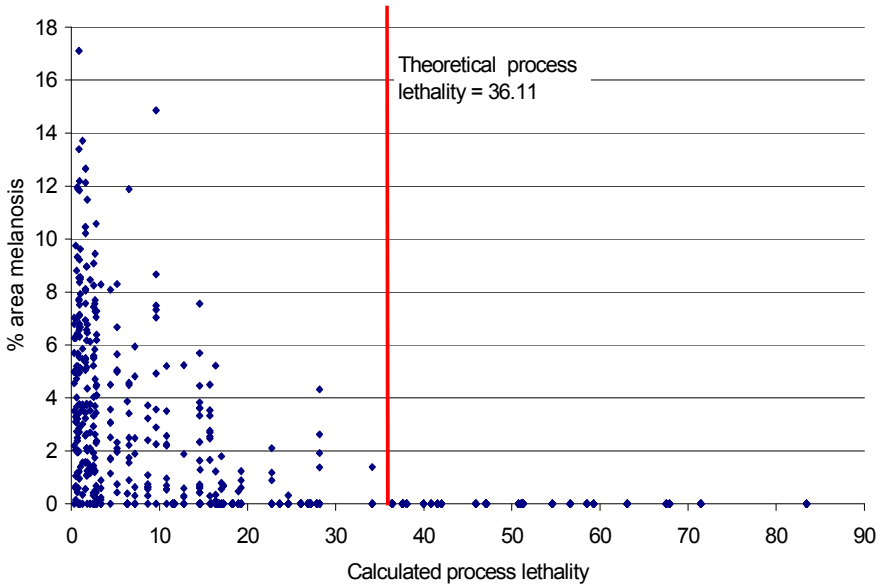


*Figure 34: Extent (% Area) of melanosis in western rock lobster versus lethality of cooking*

Determination of the percentage of lobsters within each cook that showed melanosis was then plotted against lethality to give Figure 35.

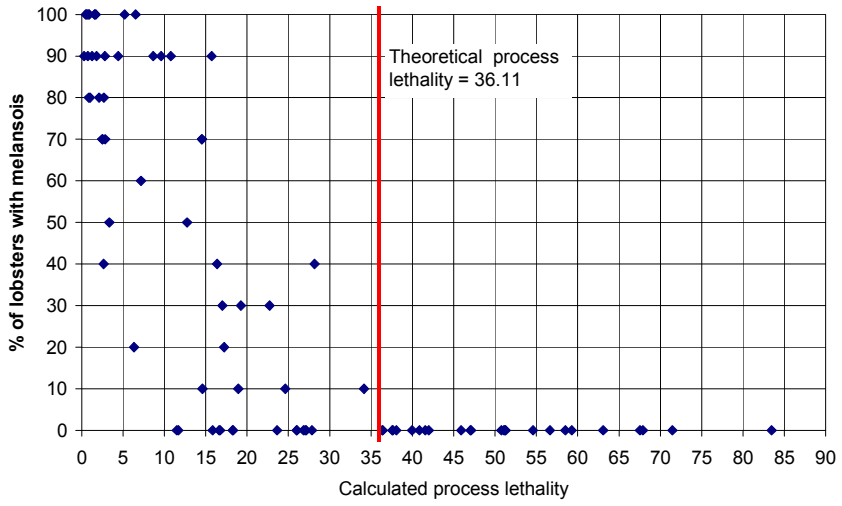


*Figure 35: Percentage of western rock lobster with melanosis versus lethality of processing*

The graph shows that as the lethality increased the percentage of lobsters with melanosis decreased. It was calculated in objective 1 that the equivalent of 12.04 minutes at 90°C ($D_{90}$) was required to produce a 90% reduction in PPO activity. It is interesting to note from Figures 34 and 35 that as lethality increased the number of lobsters with melanosis and the extent of melanosis decreased. From these two graphs, it is clear that the determined theoretical value of lethality equivalent to 36.12 minutes at 90°C must be reached to ensure that complete deactivation of PPO and hence prevention of melanosis. The prevention of melanosis by the use of heat alone also suggests that no heat stable products of enzyme activity are produced prior to processing.

Intensity of the regions of melanosis was defined according to the average pixel density for the region based on the RGB greyscale where 0 = white and 255 = black. Visual observation of the lobsters utilised in objective 3 had determined that the human eye assesses the onset of melanosis as commencing at pixel density grey values greater than 120 on the RGB greyscale. Therefore, only those lobsters with visible melanosis (areas with pixel density greater than 120) are shown in Figure 36 where the intensity of melanosis regions is plotted against the calculated process lethality.

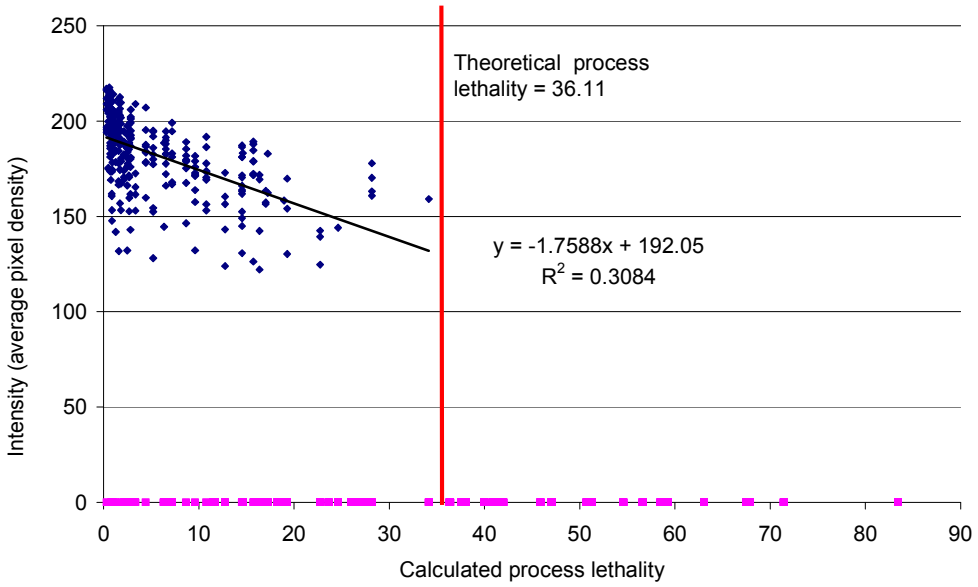


*Figure 36: Intensity of melanosis in western rock lobster versus lethality of processing*

Figure 36 shows that regions of melanosis had average pixel densities of 120 to 218. As commonly experienced in processing, at every lethality point some lobsters did not exhibit melanosis. However, it can also be seen that a negative correlation between lethality and intensity existed such that as lethality increased, the intensity of the regions of melanosis decreased until the point where the determined theoretical value of process lethality was exceeded. After that point, no regions identified as melanosis existed.

Examination of the rate of melanosis development was also undertaken. The rate of melanosis development was defined as the change in area of melanosis over the first 3 hours of the photography period. Melanosis developed most rapidly within that period and then appeared to level off over the subsequent hours to the final 24-hour point. It was believed that the development of melanosis within the first 3 hours after thawing would be the critical time frame for the consumer or end user of lobster based on standard restaurant and catering practise. If a lobster develops identifiable melanosis within 3 hours of thawing and splitting then the expectations of the consumer have been violated and the product will not be deemed acceptable. Comparison of the rate of blackening to the lethality of the cook experienced by each lobster is shown in Figure 37.

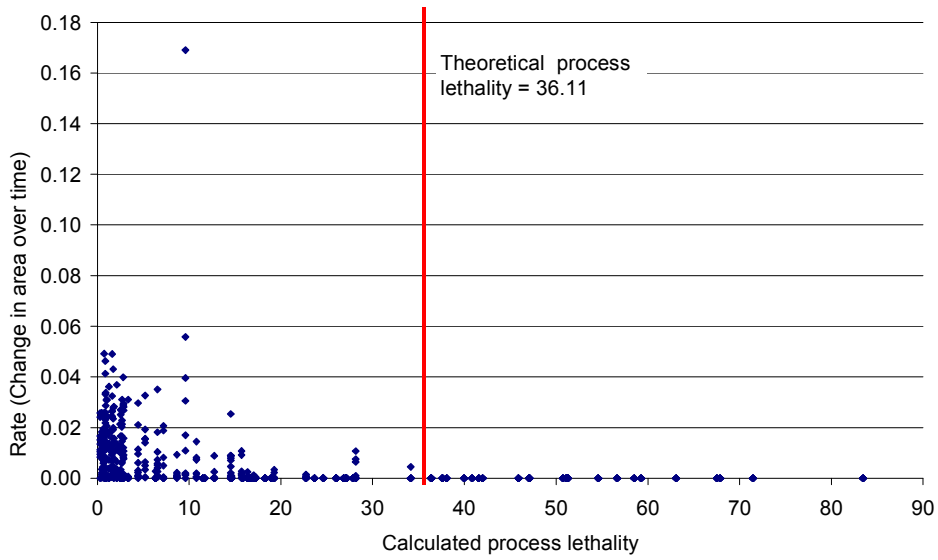


*Figure 37: Rate of melanosis development in western rock lobster versus lethality of processing*

There was considerable variability between the rates of melanosis formation for individual lobsters. The average rate was 0.01135 and the highest rate exhibited was 0.169 at a lethality of 9.06. From Figure 37 it can also be seen that as lethality approached the 2 x $D_{90}$ value, there was a large decrease in the rate of melanosis development. However, complete cessation of melanosis development did not occur until the lethality value exceeded the calculated process lethality value of 36.12 minutes.

For all lobsters, a lethality equal to or exceeding the calculated process lethality, $F_{90}$ equal to 36.12, was required to achieve prevention of melanosis. Translation of the lethal value into cook times can be carried out using Equation 9, which is derived from the trendline of cook time versus lethality (Figure 33).

*Equation 9*

Where:

$$y = 0.00003x^{3.8077}$$

- y is the calculated lethality; and
- x is the associated cook time.

The resultant conversion is shown graphically in Figure 38, where the theoretical process lethality of 36.12 equates to a cook time of 39.4 minutes under the conditions pertaining to the system used.

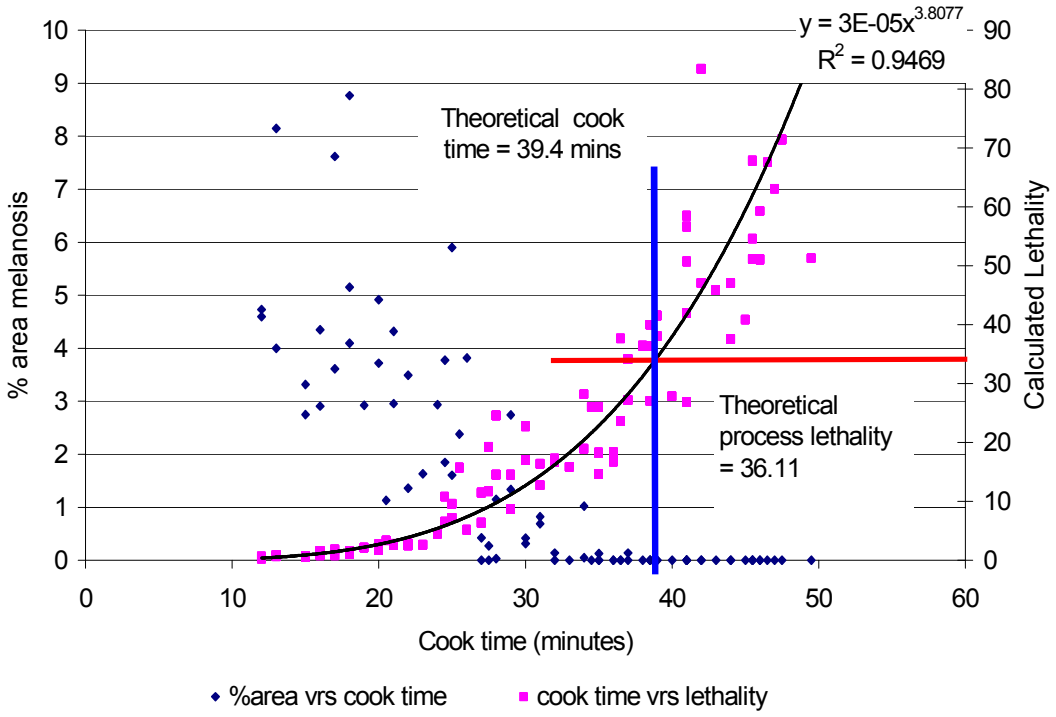


*Figure 38: % area of melanosis and lethality vs. cook time utilised in processing of western rock lobster*

From Figure 38 it can be seen no melanosis was present in lobsters that were cooked for slightly shorter periods than the determined cook time. For example, lobsters cooked for 38 minutes did not go black even though they were cooked for less than 39.4 minutes. However, they achieved a lethality equivalent to 36.42 minutes at 90 °C. Using Equation 9 the expected lethality value was 35.66. The variation from this expected value can be explained by variations in the heating profile of the cooker. Variations in the pressure of the gas supply resulted in variations in the stability of the heat provided to the cooker. In turn, the heat profile experienced by the lobsters during cooking was affected. Heating that is more rapid resulted in a higher lethality being achieved in a shorter time. Therefore, it is recommended that processors use lethality as the determinant of the end-point of processing and not cook time.

Weight recovery is an important processing parameter for western rock lobster processors. Since lobsters are sold by weight anything that impacts on their weight impacts on the final value. Weight recovery is defined as:

$$\%\ \text{weight recovery} = 100 + \left[\left(\frac{\text{processed weight - receipt weight}}{\text{receipt weight}}\right) \times 100\right]$$

Comparison of average overall weight recoveries for each cook showed significant differences existed between treatments (Kruskal Wallis ANOVA, $p<0.05$). The overall trend was for increasing cook time and lethality to correlate with decreased weight recoveries as shown in Figure 39 and 40.

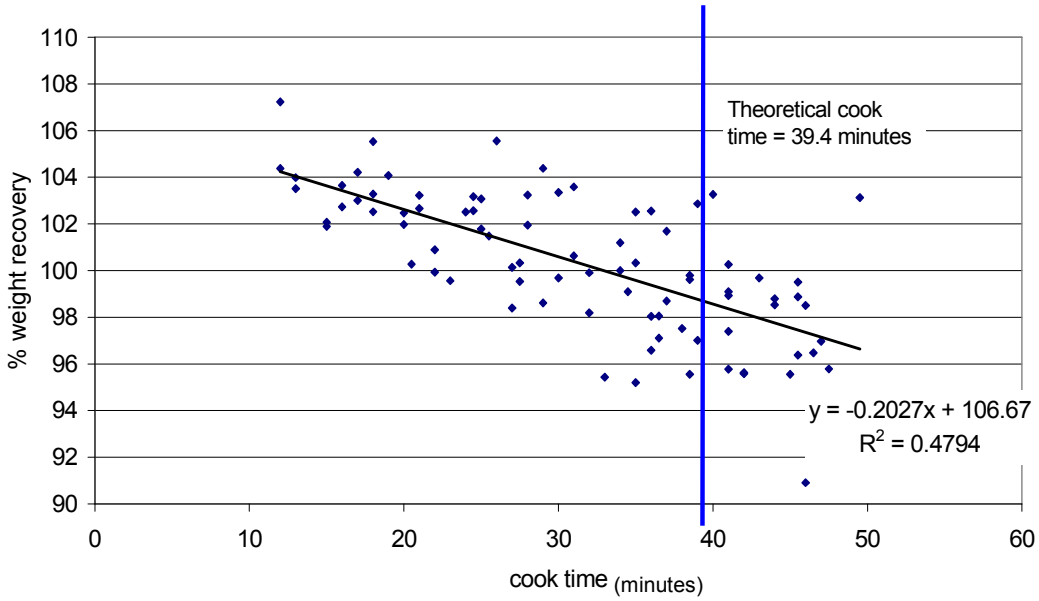


*Figure 39: Correlation of weight recovery to cook time in processed western rock lobster*

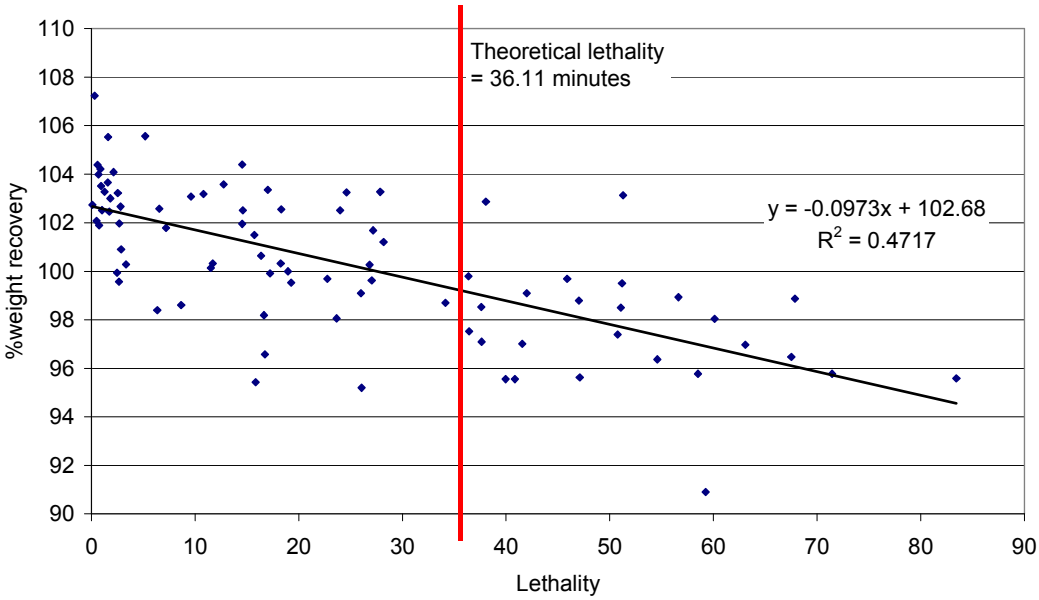


*Figure 40: Correlation of weight recovery to lethality in processed western rock lobster*

From Figures 39 and 40 it can be calculated that cooking for 39.4 minutes to the process lethality value of 36.12 gave a weight recovery of 98 to 99% under the processing conditions used in this project. It is clear, however, that while temperature contributes to weight loss ($r^2 = 0.4717$), it should not be considered as the sole factor in determining the final weight of lobsters after processing. These

figures are higher then the norm of 92-93% achieved in industry (Mr S. Hood, pers.comm., 4 July 2000) and it must be remembered that they were not achieved using standard commercial practises. Whilst the cooker used was a standard commercial cooker, conditions were not identical to that occurring in a commercial process. For instance, each cook consisted of 12 lobsters, each lobster was handled individually and with extra care, thereby reducing damage through leg loss and possible subsequent weight loss. By way of comparison, these lobsters had lost 0.46 legs per lobster compared with 0.85 legs lost per lobster used in the evaluation of current processing (Objective 3).

**Conclusions**

Lobsters were processed to specific lethality values and digital analysis of the melanosis results was conducted. Significant reduction occurred in melanosis levels as lethality increased. Lobsters cooked to lethality values less than the calculated process lethality ($F_{90}$) of 36.12 minutes at 90°C developed melanosis. Lobsters cooked to lethality values greater than the calculated $F_{90}$ value did not develop melanosis. Therefore, if heat is to be used as the sole measure of controlling melanosis development in western rock lobsters, the lobsters must reach lethality values equivalent to or greater than 36.12 minutes at 90°C.

The slope of the lethality vs. cook time curve is specific to the cooker utilised, as it is a reflection of the energy input over time. Energy input is directly related to the efficiency and design of the cooker so that different cookers will produce different curves. Determination of the process lethality inputs for a series of predetermined cook times in a cooker will enable recalibration of the process lethality curve for each cooker used. Once the process lethality curve has been determined for each cooker, it can be used to determine the endpoint of cooking based on achievable lethal input for that cooker.

Comparison of the impact of cook time and lethality showed that lethality was a better predictor of the endpoint of cooking than cook time. Calculation of the theoretical time to achieve the desired lethality was carried out based on the lethality versus cook time curve. A value of 39.4 minutes was determined. All samples cooked to the specified lethality value, regardless of the time taken to reach it, did not go black while samples cooked to the time associated with the theoretical lethality were possibly overcooked. Overcooking is undesirable due to loss of sensory quality, the increase in processing cost and added weight loss.

Cooking lobsters for 39 minutes in boiling water exceeds current practise in the industry where cook times vary from 12 to 30 minutes (T. Gibson, Western Rock Lobster Development Association, pers. comm. 2004). Evaluation of the level of weight loss versus lethality of cooks showed that increasing cook time/lethality resulted in an overall increase in weight loss. Weight loss equates to lost money for the processor so the industry will be strongly resistant to increasing their cooking times. Increased cook times also correlates in increased processing costs due to the increased man-hours and energy cost involved.

Further research into alternatives to heat for prevention of melanosis such as antibrowning agents is therefore required. Antibrowning agents are chemicals that act to prevent melanosis by inhibition of enzyme activity, removal of substrates (oxygen) or interaction with compounds formed by enzymes to prevent the formation of pigments. Their application may also modify the temperature-time profile of the enzyme by increasing its sensitivity to heat. The application of antibrowning agents in the western rock lobster industry must be carefully investigated to ensure that no negative impacts on the sensory characteristic of lobster will result from their use.

Alternative cooking methods, such as steam or retort cooking, that produce a faster rate of heat input may also significantly improve the post harvest quality of lobsters by decreasing melanosis rates while utilising shorter cook times. It is postulated that shorter cook times would result in increased weight recovery and, possibly, better textural quality. If at the same time melanosis was decreased or prevented, the processes would be of great value to the industry. Systems that utilise steam or autoclave (steam and pressure) cooking maybe effective. Several researchers have looked at the effects of alternative technologies on PPO enzymes, such as hydrostatic pressure, ultra high pressure and microwave cooking (Leblanc & Leblanc 1990; Ohshima, Ushio & Koizumi 1993; Gomes & Ledward 1996; Devece et al. 1999; Palou et al. 1999; Rodriguez-lopez et al. 1999; MacDonald & Schaschke 2000). Of these innovative technologies, microwave cooking may be applicable to processing of western rock lobster. Several researchers have suggested that microwave cooking has an impact on PPO activity beyond the heat input (Datta & Anantheswaran 2001). The application of these systems to western rock lobster processing requires investigation.

It has been clearly demonstrated in this study that the determined thermal kinetic values hold true under current processing conditions and are directly related to the incidence of melanosis after processing. Therefore, the ability of any processing

system to prevent melanosis and improve post harvest quality can be assessed based on the thermal kinetic values validated in this study.

## Objective 5: The impact of antibrowning agents on PPO activity in western rock lobster hemolymph

### Introduction

Comparison of the theoretical thermal kinetics parameters for western rock lobster PPO to the actual process lethality clearly demonstrated that the standardised process is inadequate to deactivate the enzyme and prevent subsequent melanosis formation. Evaluation of the lethal input required to ensure prevention of melanosis under standard processing conditions also showed that the theoretical value of 36.12 minutes at 90°C must be exceeded to ensure melanosis prevention. However, while such severe heating regimes may result in prevention of melanosis, the associated increase in costs due to increased energy input, longer processing time and weight loss, makes increased heating an unattractive option to the western rock lobster industry.

Alternative methods of preventing enzymatic browning have been widely researched in numerous sections of the food industry, such as fruit and vegetable post-harvest processing; vegetable oil production; wine and fruit juice production; and seafood processing (McCord & Kilara 1983; McEvily & Iyengar 1992; Lee & Whitaker 1995; Martinez & Whitaker 1995; Kim & Marshall 2000; Laurila, Kervinen & Ahvenainen 2000; Gomez-Lopez 2002; Perez-mateos, Lopez-Caballero & Montero 2002; Soliva-Fortuny et al. 2002). From the range of possible preventative measures, the application of various antibrowning agents has been the most heavily investigated.

Antibrowning agents are compounds that interact with the enzyme, its substrates and/or the products of reaction to prevent the formation of the pigments responsible for melanosis (McEvily & Iyengar 1992). When selecting an antibrowning agent for use several constraints must be considered. The agent must:

1. Be non-toxic and permissible for food use - Any legal constraints such as allowable residue limits and the recommended dosage rates from similar studies should be considered;

2. Be perceived as “Natural” - There is a growing resistance among consumers to the perceived use of chemicals as food additives. If the

chemical used is perceived as a natural compound then this resistance is considerably weakened. For example, ascorbic acid is acceptable when added to foods and labelled as Vitamin C;

3. Have no negative impacts on organoleptic characteristics of the food – The western rock lobster industry is founded on the perceived organoleptic superiority of the western rock lobster. The use of a compound that would impact negatively on any of sensory factor would be detrimental to the industry. Consumers have high expectations of luxury foods like lobster and should their expectations be violated in any way the chance of repeat purchase is considerably diminished;

4. Be cheap - The cost of any additive must be considerably less than the cost of increasing the heat treatment for it to be acceptable to the industry;

5. Be easy to apply – Lobster processors will not accept an agent that requires considerable modification of existing practise;

6. Be effective – The application of antibrowning agents must have a demonstrated benefit for acceptance by the industry and the consumer. However, it cannot be assumed that because an agent is successful in one application, for example prevention of browning in apples, that it will be equally successful in preventing melanosis in western rock lobster.

Four anti-browning agents were selected for evaluation based on their mode of action:

- Ascorbic acid –reducing agent and oxygen scavenger
- Citric acid – chelating agent, acidulant
- 4-Hexylresorcinol – enzyme inhibitor
- Carbon dioxide ($CO_2$) – inactivator

Each of these agents meets the first five criteria listed. However, they must be evaluated for application in the western rock lobster industry to ensure that the final and most important criterion is also met, namely, that they are effective in preventing melanosis in western rock lobster.

### PPO activity in control samples

Figure 41 shows the average percentage relative baseline and total activity for all control samples run in this phase of the study. Control solutions were made up from the same lobster hemolymph as used in the test series.

Overall, baseline activity showed a heat activation over the first 10 minutes followed by a gradual reduction in activity as time increased. There was a steady decline in total activity over heating time with appreciable activity remaining after 30 minutes at 70°C. These results match expectations based on the work detailed in Objective 1.

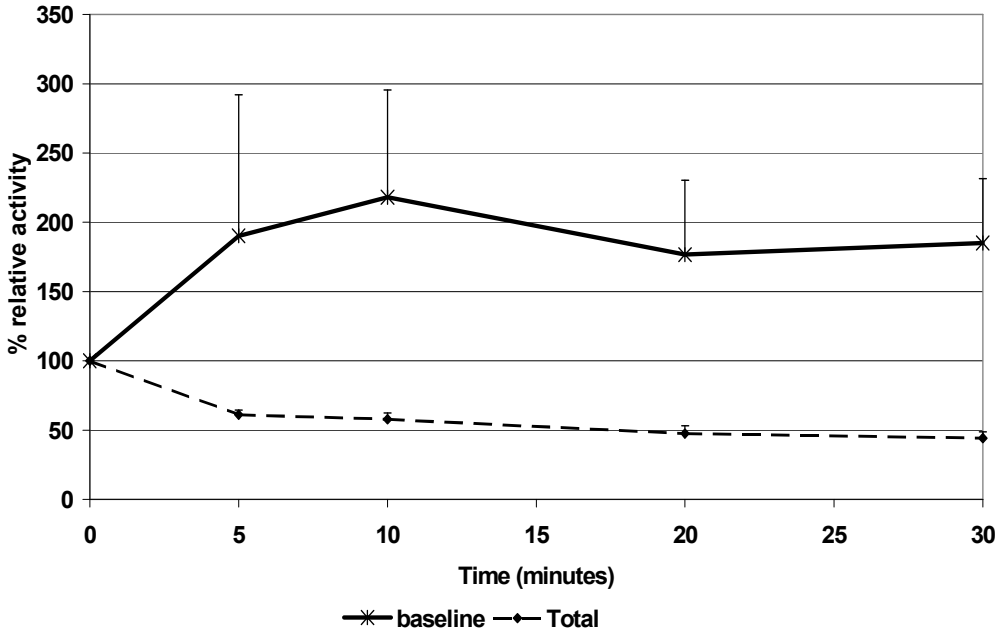


*Figure 41: PPO activity in western rock lobster hemolymph over time in control samples used in antibrowning evaluation (n=60, ±SEM)*

### Ascorbic acid

Ascorbic acid and its derivatives are leading GRAS (Generally Recognised As Safe) antioxidants for use in food products. However, the Food Standards Australia New Zealand (FZANZ) Food Standards Code currently specifies limits of no more than 400µg/ml for frozen fish products and does not provide for the use of ascorbic acid in cooked crustaceans (Food Standards Australia New Zealand 2004). If a substantial benefit can be shown, with no risk to the consumer, the use of ascorbic may be approved.

Several other issues must also be considered. Ascorbic acid may be destroyed by increasing temperatures during processing since it is quite heat sensitive. It has also been reported that low concentrations of ascorbic acid may activate the PPO enzyme (McEvily & Iyengar 1992); while it has been shown that high concentrations leads to yellowing of the flesh in prawns (Kim & Marshall 2000).

However, these factors must be balanced against the fact that ascorbic acid has wide consumer appeal due to its vitamin functionality (Vitamin C). It is also relatively cheap and readily obtainable.

Ascorbic acid concentrations of 100µg/ml and 400µg/ml were tested. The selection of these concentrations was based on the FSANZ restriction of 400µg/ml as a maximum residue limit for ascorbic acid in frozen fish products (Food Standards Australia New Zealand 2003). The lower concentration was tested on the assumption that treatment of a whole lobster would preclude absorption of the entire amount of available ascorbic acid.

The control samples for this series of showed an increase in baseline activity to 185% (SEM 46%) of the initial activity, while samples exposed to 100µg/ml of ascorbic acid showed a significant decrease in baseline activity (Paired samples t test, $p<0.05$). At 400µg/ml, ascorbic acid inhibited the baseline activity of the enzyme completely (Figure 42).

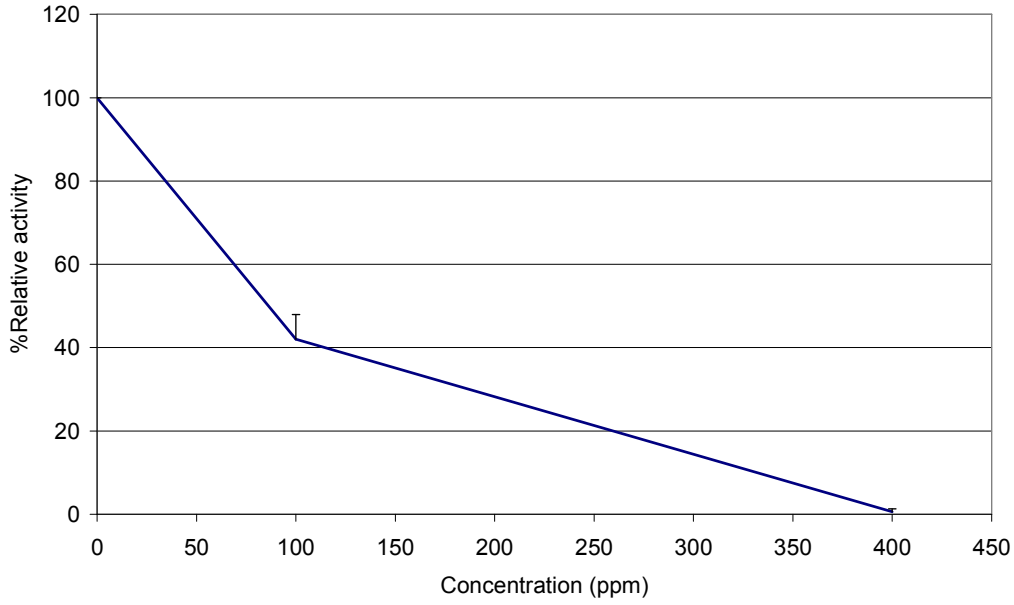


*Figure 42: Average % relative baseline PPO activity at 70°C in samples of western rock lobster hemolymph treated with ascorbic acid (mean± SEM, n=4)*

After heating for 30 minutes at 70°C, total activity of control samples dropped to 48% (SEM 4.5%) of the initial activity. Comparison of total activity for the 100µg/ml and 400µg/ml solutions with the controls clearly shows that the addition of ascorbic acid does not have a significant impact on total PPO activity. Treated samples showed a slightly lower final activity than the controls (0µg/ml) however, the Paired t-test showed no significant difference between total PPO activity results for the two concentrations and the control samples (P>0.05)(Figure 43).

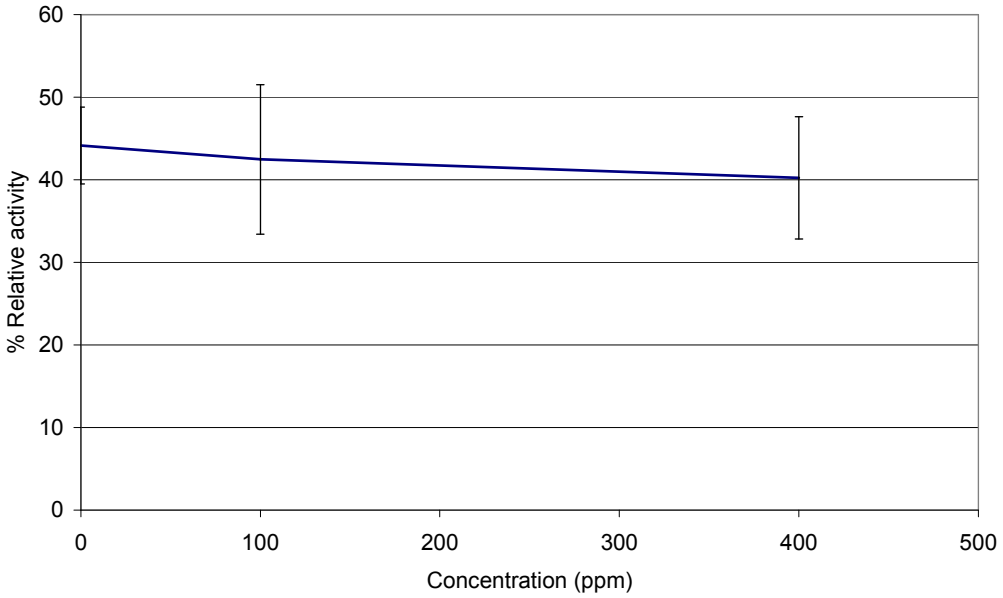


*Figure 43: Average Total % relative PPO Activity in samples of western rock lobster hemolymph treated with ascorbic acid at 70°C (mean± SEM, n=4)*

Since neither concentration of ascorbic acid modified total activity during heating, enzyme activity may occur after processing and result in melanosis. Therefore, it is concluded that ascorbic acid treatment by itself is not a suitable antibrowning agent for use by the western rock lobster industry.

**Citric acid**

Citric acid possesses dual activity against PPO as an acidulant and a chelator. As a chelator it binds with the copper ions at the active site of the enzyme rendering it inactive. The acidulant effect requires the use of a concentration high enough to drop the pH below 3 (Kim & Marshall 2000) which may effect the texture of meat due to denaturation of proteins. Citric acid has high consumer appeal due to its perceived natural origin (lemon juice), is very cheap and readily available. Typical dosage rates in antibrowning treatments range from 2mg/ml to 10mg/ml so a wide range of concentrations should be evaluated.

Since citric acid may have an effect based on low pH, this parameter was measured across all concentrations. (Kim & Marshall 2000) noted that pH had to drop to below 3 for the effect to be apparent. None of the solutions reached this level, as the lowest pH reached was 3.4 when citric acid concentration was 3.34mg/ml. Therefore, any effect noted should be attributed to the chelation of the copper ions by citric acid.

Figure 44 shows the impact of heating for 30 minutes at 70°C in the presence of citric acid. When citric acid concentration equals zero, the baseline and total activity value equates to the activity exhibited by the control.

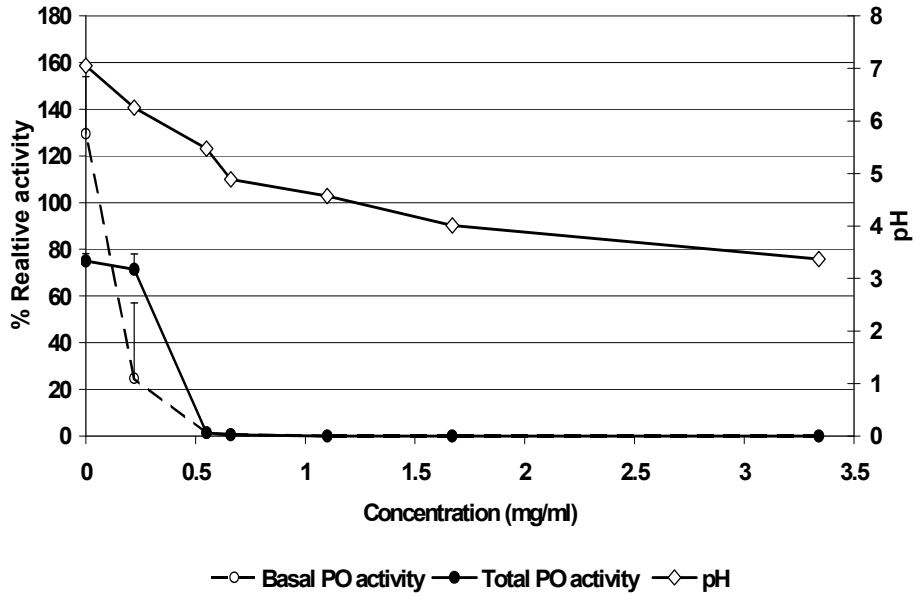


*Figure 44: Impact of citric acid concentration on % relative PPO activity and pH in western rock lobster hemolymph after heating at 70°C for 30 minutes (mean± SEM, n=4)*

After heating for 30 minutes at 70°C, total and baseline PPO activity were detectable in solutions with concentrations less than 1mg/ml (Figure 44). However, solutions of higher concentration showed no activity. At 0.2 mg/ml there was no significant difference between the control and the citric acid solution for total PO activity (Kruskal-Wallis ANOVA, $p>0.05$), however baseline activity was significantly reduced (Kruskal-Wallis ANOVA, $p<0.05$).

Therefore, it can be concluded that citric acid concentrations greater than 1mg/ml are effective against PPO in western rock lobster hemolymph. Their use may be

effective in prevention of melanosis in western rock lobster provided sufficient concentrations can be reached within the lobster.

**4-Hexylresorcinol**

4-hexylresorcinol was originally derived from figs. It has many uses in the cosmetics industry and is a GRAS substance. It has been shown to be a very effective PPO inhibitor at low concentrations. It is allowed by the Food Standards Code for use in crustaceans (Food Standards Australia New Zealand 2004) and is widely used in some branches of the seafood industry as a component of an antibrowning solution called “Everfresh” which contains approximately 5% 4-hexylresorcinol. At the recommended dose, this equates to a dose rate of 87µg/ml of 4-hexylresorcinol. While 4-hexylresorcinol is relatively expensive, the low dosage rates and potentially low residual rates favour its use.

Several concentrations were screened to determine the lowest concentration that effectively inhibited PPO activity. The highest concentration of 87µg/ml corresponds to the dosage rate recommended for Everfresh. All concentrations were effective against the baseline PPO activity with no baseline activity apparent after heating. However, for 10.25 and 7µg/ml samples residual total PO activity was detected (Figure 45). The level of activity was at the limit of detection (5% relative activity) and therefore was not significant (Paired T-test, p>0.05).

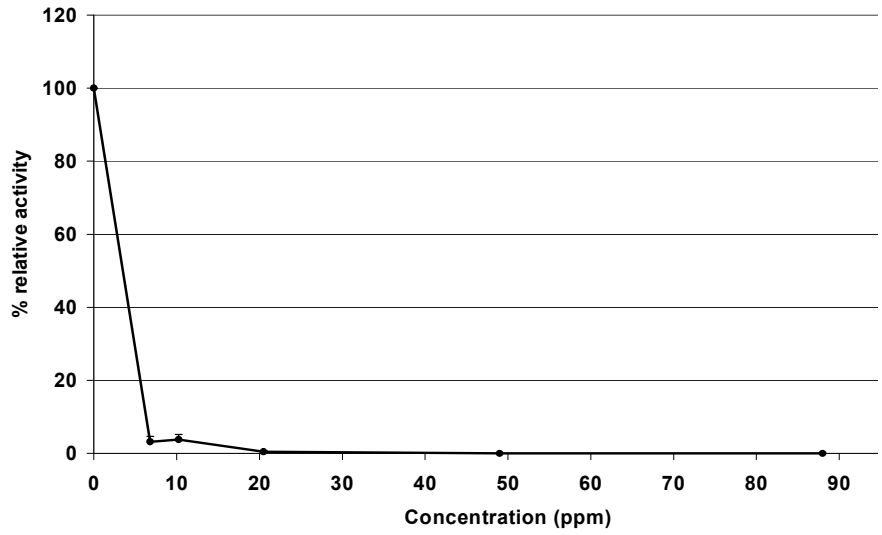


*Figure 45: The impact of 4-hexylresorcinol concentration on % relative total PPO activity in western rock lobster hemolymph (mean± SEM, n=4)*

From these results, it maybe concluded that 4-hexylresorcinol should be effective against PPO activity during western rock lobster processing when applied at concentrations in excess of 20µg/ml.

**Carbon dioxide ($CO_2$)**

Carbon dioxide ($CO_2$) is a colourless, odourless gas. It is cheap to produce, readily available and leaves no residue in the tissues of treated foods. The use of $CO_2$ has a high degree of acceptability, as it would require no special labelling on packaging and poses no health threat. It has been suggested that elevated levels of $CO_2$ increase the susceptibility of PPO to the impact of heat thus enabling reduced cook times (Chen et al. 1993). The extension of this idea to lobster processing has yet to be tested.

As the selected antibrowning agents are currently not used in the western rock lobster industry, it is essential to first screen them to determine:

- firstly, if they are effective against PPO activity in western rock lobster; and
- secondly, which concentrations are most effective against total and baseline PPO activity in western rock lobster.

It is important to determine if the agent is capable of overcoming the heat activation effects experienced by PPO between 60 and 80°C (Objective 1). It is therefore the aim of this study to investigate the impact of the selected antibrowning agents on PPO activity in western rock lobster hemolymph in the presence of heat.

Carbon dioxide was applied in two ways. Firstly, the buffer solution was saturated with $CO_2$ gas and then the hemolymph added before heating. In the second method, the hemolymph was added and exposed to $CO_2$ for thirty minutes and then heated. Both methods of exposure to $CO_2$ resulted in complete inhibition of all forms of PPO activity. The inhibition effect occurred immediately upon exposure of the hemolymph to carbon dioxide and before heating commenced. During and after heating there was no recovery of PPO activity in any form.

Measurement of pH before and after carbonation showed that in all samples pH dropped to 5.5 $\pm$ 0.3. Previous work has shown that this drop is insufficient for pH to impact on PPO activity (Kim & Marshall 2000) therefore the reduction in PPO activity

was due to the inhibitory effects of $CO_2$. No pH drop occurred in control samples (pH6.8 $\pm$ 0.2).

From the results obtained, it can be concluded that carbon dioxide possesses potential for use as an antibrowning agent in the western rock lobster processing industry.

**Overall comparison of agents**

Examination of the values of activity in control samples shows that the lobsters utilised in the ascorbic acid trials were more sensitive to heating than those used in the other trials. However, the treated samples did not vary significantly from the matched controls indicating that ascorbic acid had little effect on total PPO activity. When the remaining total activity is compared across all treatments, several groupings can be seen (Table 19).

*Table 19: Kruskal-Wallis ranking of total PPO activity after 30 minutes at 70°C*

| Agent | Concentration | Mean Rank | Average Total PPO activity | SEM |
|---|---|---|---|---|
| Ascorbic acid | 0µg/ml | 14.00 | 44.15 | 4.65 |
| | 100µg/ml | 13.00 | 42.48 | 9.06 |
| | 400µg/ml | 12.00 | 40.24 | 7.42 |
| 4-Hexylresorcinol | 0µg/ml | 16.00 | 75.00 | 4.54 |
| | 6.8µg/ml | 10.00 | 3.23 | 1.46 |
| | 10.25µg/ml | 11.00 | 3.79 | 1.40 |
| | 20.5µg/ml | 7.00 | 0.46 | 0.25 |
| | 49µg/ml | 3.50 | 0 | 0 |
| | 87.9µg/ml | 3.50 | 0 | 0 |
| Citric acid | 0mg/ml | 16.00 | 75.00 | 4.54 |
| | 3.34mg/ml | 3.50 | 0 | 0 |
| | 1.67mg/ml | 3.50 | 0 | 0 |
| | 1.1mg/ml | 3.50 | 0 | 0 |
| | 0.66mg/ml | 8.00 | 0.54 | 0.12 |
| | 0.55mg/ml | 9.00 | 1.40 | 0.21 |
| | 0.22mg/ml | 15.00 | 71.45 | 6.54 |
| $CO_2$ | 0mg/ml | 16.00 | 75.00 | 4.54 |
| | Saturated | 7 | 0 | 0 |

The samples that were not significantly different from their matched control were 0.22mg/ml citric acid and the ascorbic acid treatments. Samples with total PPO activity still present at reduced rates were the 6.8 to 20.5µg/ml 4-hexylresorcinol, 0.66 and 0.55mg/ml citric acid. Samples with no remaining activity were 49µg/ml and 87.9µg/ml 4-hexylresorcinol, 1.1 to 3.34mg/ml citric acid and both $CO_2$ treatments. Evaluation of the prevention of melanosis in lobsters should utilise those antibrowning agents and concentrations that show no total PPO activity remaining.

**Conclusions**

The aim of these experiments was to determine the minimum concentrations of the various agents required to achieve inhibition of the PPO enzyme. From this phase of the study several conclusions can be reached:

1. Ascorbic acid by itself is not suitable for use in the western rock lobster industry. Its inability to impact on total PPO activity at acceptable residual concentrations means that PPO activity could occur after processing and result in melanosis. Further research is required to determine if its use in combination with other antibrowning agents may be more effective. Ascorbic acid is often used in conjunction with citric acid and appears to have a synergistic effect producing a higher level of inhibition at lower concentrations of both agents (Kim & Marshall 2000; Laurila, Kervinen & Ahvenainen 2000).

2. Citric acid is effective against baseline and total PPO activity at concentrations greater than 1mg/ml. Since pH did not drop below three it was not a factor in the inhibition of western rock lobster PPO.

3. 4-Hexylresorcinol is effective against baseline and total PPO activity at concentrations greater than 20 µg/ml. At these dosage rates, the use of 4-hexylresorcinol becomes economically feasible.

4. Both methods of application of carbon dioxide were effective in inhibition of western rock lobster PPO. The use of carbon dioxide in processing of western rock lobster should therefore be investigated.

It must be noted however that these experiments were conducted under controlled conditions in a test tube, which in no way reflects the complex systems that exist within a lobster. In order to truly evaluate the effectiveness of these compounds in

prevention of melanosis in western rock lobster they must be applied and evaluated during commercial processing. Evaluation of melanosis development can then be undertaken after processing and freezing to determine effectiveness of the agents in prevention of melanosis at the given concentrations.

## Objective 6: Application of antibrowning agents in western rock lobster processing

### Introduction

The experiments conducted in objective 5 identified three antibrowning agents with potential for use in the western rock lobster industry. However, the experiments were conducted under controlled conditions in a test tube, which in no way reflects the complex systems that exist during processing of lobster. In order to evaluate the effectiveness of these compounds in prevention of melanosis in western rock lobster they must be applied to whole lobster and evaluated under the conditions of commercial processing. Evaluation of a range of antibrowning agents with different modes of action will assist in identification of those action modes most effective in prevention of melanosis in lobster. These can then be expanded in further research to identify specific agents within that group.

Evaluation of the flow of product during processing identifies several points where application of antibrowning agents may be practicable. These points have been identified with stars in Figure 46 and are;

- during drowning;
- during cooking; and
- during cooling.

Of the three processing stages indicated, application during drowning seems to offer the most advantages. A study conducted by Slattery, Williams & Cusack (1995) showed that live prawns dipped in 4-hexylresorcinol solutions had higher residue levels and a lower incidence of blackspot than those dipped when dead. It has been shown in Objective 3 that lobsters absorb significant quantities of water during drowning due to the hypo-osmotic gradient. It is therefore possible that lobsters drowned in solutions of antibrowning agents may demonstrate effects similar to prawns. It was also shown that drowning resulted in a significant increase in baseline PPO activity that may act to increase melanosis after processing.

Application of antibrowning agents at this point may therefore act to counter this effect

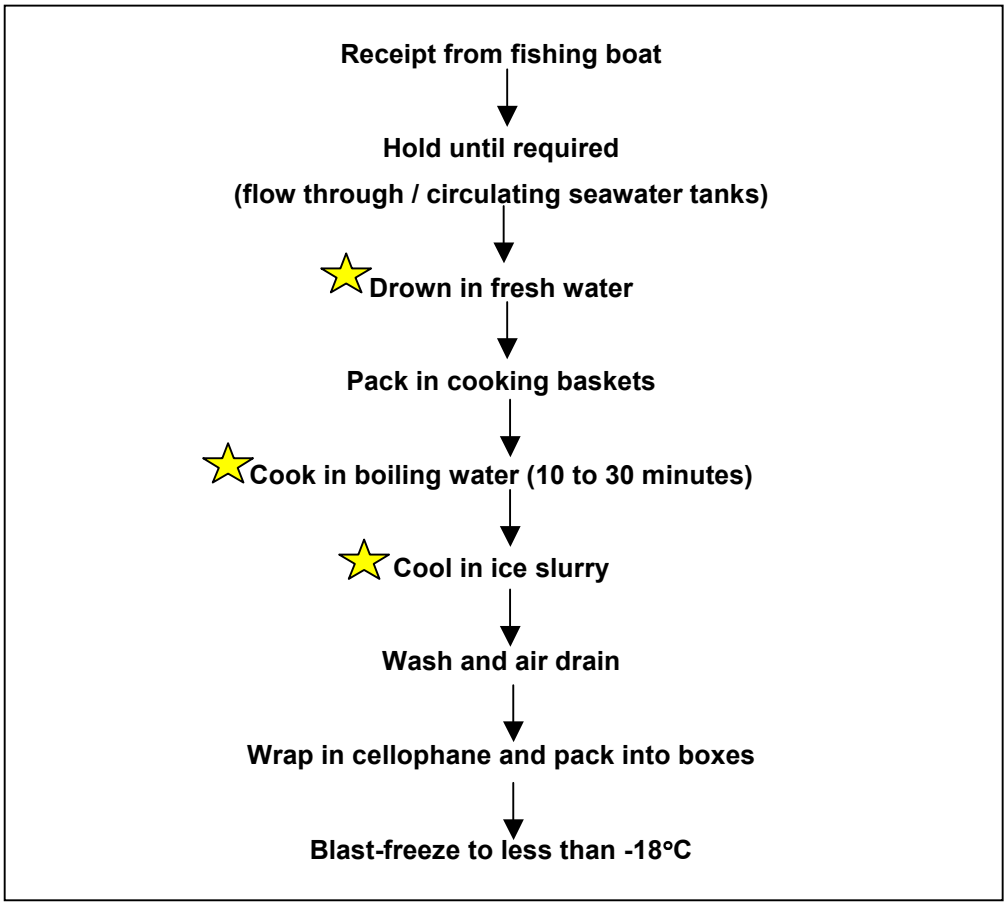


*Figure 46: Flow diagram of western rock lobster processing indicating possible points of antibrowning agent addition*

Application during cooking by dissolving the agent in the cooking water does not appear to be applicable as the agent may be destroyed by the heating process and/or not be taken up by the lobster tissues. It is postulated that the effects of cooking may be mitigated if the agent is applied during drowning as it is taken within the tissues and is somewhat protected from leaching and heat destruction.

The study in Objective 3 also showed that lobsters continued to lose weight during the cooling period immediately post-cooking. It can therefore be concluded that they will not take water in from the cooling slurry in sufficient quantities to enable an effective dose of antibrowning agent to be absorbed in the time allowed.

These points lead to the conclusion that application of agents during drowning merits first investigation as an effective point of delivery. In determination of concentrations to be used, consideration must be given to the dilution effect that

may occur during application. It is postulated that the lobster will not absorb large enough volumes of the antibrowning agent for its tissues to reach the same concentration as the solution being applied. The common practise of drowning several batches of lobsters in the same tank of water may also lead to a decrease in concentration of the solution over time. Therefore, a range of concentrations should be evaluated for each agent to determine if uptake varies with concentration of agent and the impact of these varying concentrations on melanosis.

The aim of this phase of the study was therefore to apply selected concentrations of the identified antibrowning agents to lobster during drowning and evaluate their effectiveness in the prevention of melanosis formation after processing. Post processing evaluation was undertaken using digital image analysis. The program required to meet this objective called for one week of testing carried out during the last week of April 2003. The trials constituted a pilot project to clarify directions that may require further research.

Complete inhibition of melanosis was not achieved by any treatment within the exposure times, solution concentrations and processing conditions used. However, significant reductions in the extent, intensity and rate of melanosis development were achieved.

**Evaluation of initial physiological parameters**

Examination of the physiological parameters of initial protein level, total and baseline PPO activity were undertaken at the beginning of the study as they may have a significant impact on the development of melanosis after processing. It was noted that significant differences existed between groups of lobsters (Tables 20, 21 and 22).

Comparison of controls to their matched treatment groups showed that baseline PPO was significantly higher (+2.4 U $p<0.05$, K-W ANOVA) in the Also total PPO was higher for the $CO_2$ treatment group (+11 U) and, the 2.5mg/ml citric acid group showed significantly lower total PPO activity (-8.44U) when compared to the matched control groups ($p<0.05$, K-W ANOVA). There were no significant variations between controls and matched treatment groups for protein content ($p>0.05$, K-W ANOVA) (Table 22).

Comparison of treatment groups showed several significant differences. The lobsters to be treated with $CO_2$ had significantly lower initial baseline PPO activity

and the 50µg/ml 4-hexylresorcinol treatment group had significantly higher initial baseline PPO activity compared to other treatment groups (P<0.05, K-W ANOVA) (Table 20). The lobsters in the $CO_2$ treatment group had a higher initial total PPO activity (P<0.05, K-W ANOVA) while the lobsters in the 87µg/ml 4-hexylresorcinol group had a significantly lower initial total PPO activity compared to other treatments (P<0.05, K-W ANOVA) (Table 21).

*Table 20: Initial Baseline PPO activity*

| Treatment | Mean | Std err |
|---|---|---|
| 50 µg/ml 4_H | 4.82* | 0.08 |
| Citric 2.5mg/mL | 3.26 | 0.08 |
| 87 µg/ml 4_H | 3.01 | 0.28 |
| Citric1.5mg/mL | 2.85 | 0.26 |
| Control citric | 2.42 | 0.28 |
| 10µg/ml 4_H | 2.35 | 0.21 |
| Control 87µg/ml 4_H | 1.78 | 0.19 |
| Control 10µg/ml&50µg/ml 4_H | 1.24 | 0.46 |
| $CO_2$ | 0.57 | 0.26 |
| Control $CO_2$ | 0.41 | 0.45 |

*is significantly different to the matched control, P<0.05

*Table 21: Initial Total PPO activity*

| Treatment | Mean | Std err |
|---|---|---|
| $CO_2$* | 32.28 | 2.06 |
| Control citric | 25.06 | 2.93 |
| 50 µg/ml 4_H | 22.61 | 2.00 |
| Control 10µg/ml&50µg/ml 4_H | 22.31 | 1.12 |
| Control $CO_2$ | 21.14 | 1.37 |
| Citric 1.5mg/mL | 20.24 | 2.04 |
| Control 87µg/ml 4_H | 19.76 | 1.06 |
| 10µg/ml 4_H | 18.46 | 1.40 |
| Citric 2.5mg/mL* | 16.62 | 1.25 |
| 87 µg/ml 4_H | 15.46 | 1.00 |

*is significantly different to the matched control, P<0.05

*Table 22: Initial Protein content*

| Treatment | Mean | Std err |
|---|---|---|
| Control citric | 107.29 | 7.48 |
| Control $CO_2$ | 103.59 | 4.34 |
| Citric1.5 | 101.52 | 6.69 |
| Control 10µg/ml&50µg/ml 4_H | 100.51 | 4.51 |
| $CO_2$ | 100.35 | 4.41 |
| Citric 2.5 | 98.89 | 7.64 |
| 50 µg/ml 4_H | 98.15 | 2.99 |
| 10µg/ml 4_H | 94.64 | 3.89 |
| 87 µg/ml 4_H | 84.84 | 4.34 |
| Control 87µg/ml 4_H | 82.63 | 3.38 |

In considering the variation of the controls from their matched treatments it must be noted that correlation of physiological factors with the final area, intensity and rate of melanosis development showed no significant correlations existed for individual treatments or controls (one way ANOVA, $p>0.05$). That is, variations in physiological parameters had no impact on melanosis formation factors after processing. Therefore, reliable inferences about the outcomes of melanosis after treatment with antibrowning agents cannot be drawn based on the initial values of physiological parameters in these trials.

**Extent of melanosis formation**

On examination of the extent of melanosis formation in the lobsters, the controls for all treatments showed much larger areas of blackening than the treated lobsters (Table 23). The control samples for the $CO_2$ showed greatest area of blackening and were significantly different to other controls ($P<0.05$, K-W ANOVA).

*Table 23: Average area of melanosis after 24 hours refrigeration*

| Treatment | Mean | Std dev |
|---|---|---|
| Control $CO_2$ | 12.33 | 6.66 |
| Control 87µg/ml 4_H | 9.02 | 5.53 |
| Control 10µg/ml&50µg/ml 4_H | 9.00 | 4.12 |
| Control citric | 7.70 | 2.48 |
| $CO_2$ | 5.30 | 4.68 |
| Citric 2.5mg/mL | 4.23 | 3.74 |
| Citric 1.5mg/mL | 3.98 | 3.00 |
| 50 µg/ml 4_H | 1.46 | 1.86 |
| 10µg/ml 4_H | 1.46 | 1.99 |
| 87 µg/ml 4_H | 1.34 | 1.41 |

All treatment samples were significantly lower than their corresponding controls (P<0.05, K-W ANOVA) with the 4-hexylresorcinol treatments showing the greatest reduction in final melanosis area (85%) (Table 24). Citric acid treatments and $CO_2$ showed the least reduction in final area of melanosis with $CO_2$ giving a 57% reduction and the citric acid treatments approximately a 46.5% reduction in final melanosis area.

*Table 24: Change in average area of melanosis between samples and controls*

| Treatment | Mean Area | % Reduction |
|---|---|---|
| Control 87µg/ml 4_H | 9.02 | |
| 87 µg/ml 4_H | 1.34 | 85.1% |
| Control 10µg/ml&50µg/ml 4_H | 9.00 | |
| 50 µg/ml 4_H | 1.46 | 83.8% |
| 10µg/ml 4_H | 1.46 | 83.8% |
| Control $CO_2$ | 12.33 | |
| $CO_2$ | 5.30 | 57% |
| Control citric | 7.70 | |
| Citric 2.5mg/mL | 4.23 | 45.1% |
| Citric 1.5mg/mL | 3.98 | 48.3% |

Significant differences between the treatments were identified. Samples in the $CO_2$ treatment showed significantly larger areas of blackening when compared to the 4-hexylresorcinol treatments (P<0.05, K-W ANOVA). There was no significant difference in the final area of melanosis between the three individual 4-hexylresorcinol treatments (P>0.05, K-W ANOVA). There was also no significant difference in the final area of melanosis between the two citric acid treatments (P>0.05, K-W ANOVA) or between the citric acid treatments and the $CO_2$ treatment (Tables 23 and 24).

**Evaluation of the average intensity of melanosis**

In this study, the measure of average pixel density was utilised as a measure of how black the areas of melanosis were. Average pixel density is therefore referred to as the intensity value in all tables and figures. All measures are related to the greyscale

values as defined in the RGB colour model where 0 is equal to white and 255 is equal to black.

Table 25 shows that the citric acid control was significantly darker than the other controls and there was no significant difference in intensity of melanosis between the $CO_2$ control and 4-hexylresorcinol controls (P>0.05, K-W ANOVA). All controls were significantly darker than their matched treatments (P<0.05, K-W ANOVA).

*Table 25: Average intensity after 24 hours (Pixel values, 0=white, 255= black)*

| Treatment | Mean | Std dev |
|---|---|---|
| Control citric | 185.44 | 14.92 |
| Control 10µg/ml&50µg/ml 4_H | 177.53 | 16.64 |
| Control $CO_2$ | 174.30 | 18.13 |
| Citric 2.5mg/mL | 174.32 | 15.10 |
| Citric1.5mg/mL | 173.98 | 21.24 |
| $CO_2$ | 162.16 | 21.99 |
| Control 87µg/ml 4_H | 156.75 | 21.18 |
| 10µg/ml 4_H | 138.48 | 22.37 |
| 50 µg/ml 4_H | 130.97 | 24.06 |
| 87 µg/ml 4_H | 136.93 | 26.65 |

It was shown during the evaluation of current processing that while it was not possible to predict the level of melanosis from the total PPO level, those lobsters with higher levels of total PPO activity were more likely to undergo a greater degree of blackening. It is interesting to note that the lobsters from the citric acid control had a high initial total PPO and were significantly darker (P<0.05, K-W ANOVA) compared to other controls and treatments. The $CO_2$ treatment group also had a high initial total PPO activity, however the antibrowning agent action of $CO_2$ resulted in a significant reduction in intensity and extent of melanosis after processing.

Comparison between treatments showed that all 4-hexylresorcinol treatments had significantly lower values of melanosis intensity compared to other treatments (P< 0.05, K-W ANOVA) but were not significantly different to each other (P>0.05, K-W ANOVA). There was no significant difference between the citric acid and $CO_2$ treatments with respect to intensity of melanosis (P>0.05, K-W ANOVA).

**Evaluation of the rate of melanosis development**

Evaluation of the rate of melanosis development showed that there was no significant difference between the individual controls (P>0.05, K-W ANOVA) (Table 26).

*Table 26: Rate of melanosis development (change in area over time)*

| Treatment | Mean | Std dev |
|---|---|---|
| Control $CO_2$ | 0.029 | 0.018 |
| Control 87µg/ml 4_H | 0.028 | 0.022 |
| Control citric | 0.025 | 0.011 |
| Control 10µg/ml&50µg/ml 4_H | 0.021 | 0.013 |
| $CO_2$ | 0.012 | 0.012 |
| Citric 2.5mg/mL | 0.0098 | 0.0097 |
| Citric 1.5mg/mL | 0.0087 | 0.0099 |
| 50 µg/ml 4_H | 0.0055 | 0.0089 |
| 10µg/ml 4_H | 0.0048 | 0.0064 |
| 87 µg/ml 4_H | 0.0028 | 0.0042 |

There were significant differences in the rate of melanosis development between the controls and their matched treatments (P< 0.05, K-W ANOVA). Overall, the 87µg/ml 4-hexylresorcinol treatment showed the greatest reduction in the rate of melanosis development (90%) (Table 27). It is interesting to note that while the samples treated with 10µg/ml & 50 µg/ml 4-hexylresorcinol achieved a 75% reduction in the rate of melanosis formation, this was not considered significantly different from the 87µg/ml treatment (P>0.05, K-W ANOVA). All 4-hexylresorcinol samples were significantly different from the citric acid and $CO_2$ treatments. However, there was no significant difference between the two citric acid treatments and the $CO_2$ treatment (P>0.05, K-W ANOVA) as they all showed a 60 - 65% reduction in rate of melanosis formation (Table 27).

*Table 27: Reduction in the rate of melanosis development*

| Treatment | Mean | % Reduction in rate |
|---|---|---|
| Control 87µg/ml 4_H | 0.028 | |
| 87 µg/ml 4_H | 0.0028 | 90% |
| Control 10µg/ml&50µg/ml 4_H | 0.021 | |
| 50 µg/ml 4_H | 0.0055 | 73.8% |
| 10µg/ml 4_H | 0.0048 | 77.1% |
| Control citric | 0.025 | |
| Citric 2.5mg/mL | 0.0098 | 60.8% |
| Citric 1.5mg/mL | 0.0087 | 65.2% |
| Control $CO_2$ | 0.029 | |
| $CO_2$ | 0.012 | 58.6% |

**Evaluation of melanosis development factors**

Comparison of the correlation coefficients between melanosis factors showed significant relationships existed between factors (Table 28). For all treatments, an increased rate of development leads to a larger area becoming melanized. For the control samples of the 4-hexylresorcinol treatments and the two treatment groups, 10µg/ml 4-hexylresorcinol and $CO_2$, darker samples occurred with increasing area and rate of development.

*Table 28: Correlation coefficients of melanosis factors*

| Treatment | Area vs. Rate | Area vs. intensity | Rate vs. intensity |
|---|---|---|---|
| Control 87µg/ml 4_H | 0.853[a] | 0.609[a] | 0.513[b] |
| 87 µg/ml 4_H | 0.724[a] | ns | ns |
| Control 10µg/ml&50µg/ml 4_H | 0.665[a] | 0. 559[b] | 0.506[b] |
| 50 µg/ml 4_H | 0.958[a] | ns | ns |
| 10µg/ml 4_H | 0.881[a] | 0.465[b] | 0.434[b] |
| Control citric | 0.933[a] | ns | ns |
| Citric 2.5mg/mL | 0.825[a] | ns | ns |
| Citric 1.5mg/mL | 0.628[a] | ns | ns |
| Control $CO_2$ | 0.887[a] | ns | ns |
| $CO_2$ | 0.908[a] | 0.533[a] | 0.49[b] |

[a] Correlation is significant at the 0.01 level (2-tailed).

[b] Correlation is significant at the 0.05 level (2-tailed), and ns = not significant

When the percentage area of melanosis is plotted against time, the area under the curve corresponds to the interaction between area and rate of melanosis development. Those samples that experienced delays in initiation of melanosis show a much smaller area under the curve than those that rapidly blackened. Evaluation of the areas showed that the controls were not significantly different to each other but were significantly different to all treatments (K-W ANOVA< $p<0.05$). In addition, significant differences existed between treatments as shown in Table 29 (K-W ANOVA< $p<0.05$). Comparison of the areas under the rate curve with the ratio of samples within each treatment that did not experience melanosis allows some conclusions to be drawn about the effectiveness of the agents

*Table 29: Area under regression curve*

| **Treatment** | **Mean area under rate curve** | **Proportion without melanosis** |
|---|---|---|
| 87 µg/ml 4_H [a, c] | 44.15 | 0.24 |
| 10µg/ml 4_H [a, c] | 81.66 | 0.36 |
| 50 µg/ml 4_H [a, c] | 104.34 | 0.20 |
| Citric1.5 [a, d] | 136.31 | 0.175 |
| $CO_2$ [a, d] | 178.54 | 0.125 |
| Citric 2.5 [a, d] | 196.85 | 0.075 |
| Control 10µg/ml&50µg/ml [b] | 325.62 | 0.05 |
| Control citric [b] | 470.33 | 0 |
| Control $CO_2$ [b] | 494.26 | 0 |
| Control 87µg/ml [b] | 524.48 | 0.05 |

[a] is significantly different to [b], $p<0.05$

[c] is significantly different to [d], $p<0.05$

From Table 29 it can be clearly seen that the 4-hexylresorcinol treatments had more individuals that did not go black, and greater delay in the onset than any other treatment. It is also apparent that overall, the 4-hexylresorcinol samples were significantly better than other treatments at reducing melanosis yet all treatments had lower levels, and slower onset of melanosis than their matched controls.

**Evaluation of the impact of antibrowning agents on weight recovery**

Analysis of the weight data recorded for all control and treated lobsters showed that no significant differences in weight recovery existed for the treatments used (One-way ANOVA p>0.05) as shown in Table 30.

*Table 30: Percentage weight recovery for antibrowning trials by treatment*

| Treatment | Mean | Std. Error |
|---|---|---|
| Control CO2 | 103.86 | 0.79 |
| CO2 | 96.69 | 3.21 |
| Control citric | 102.55 | 1.36 |
| Citric1.5 | 102.01 | 1.18 |
| Citric 2.5 | 101.00 | 0.92 |
| Control 10µg/ml&50µg/ml 4_H | 102.37 | 0.97 |
| 10µg/ml 4_H | 104.94 | 1.27 |
| 50 µg/ml 4_H | 97.69 | 4.38 |
| Control 87µg/ml | 103.49 | 0.75 |
| 87 µg/ml 4_H | 99.54 | 0.93 |

**Conclusions**

The results of these experiments clearly reinforce the previous conclusions that initial physiological parameter measures of western rock lobster cannot be used to accurately predict the melanosis outcomes. They also show that antibrowning agents are effective in reducing the incidence and severity of melanosis occurrence in western rock lobster.

The aim of these experiments was to determine the impact of selected antibrowning agents on melanosis formation after processing. There were significant differences between all treatments and their matched control groups. Lobsters treated with 87µg/ml 4-hexylresorcinol showed the greatest reduction in melanosis with respect to rate, intensity and extent. There was also considerable promise exhibited in the use of carbon dioxide and citric acid for the reduction in melanosis formation. However, no treatment achieved complete prevention of melanosis, which may be attributed to a variety of factors. These included:

a) *The ratio of the weight of lobsters to the volume of the drowning solution.* The current trial utilized the lowest volume of solution (50L) possible to drown the lobsters in. It is possible that this will have limited the uptake of the antibrowning agents by restriction of respiratory exchange and localised depletion of the agents. Further trials using a range of lobster to solution ratios should be conducted to determine the optimum drowning volume for most effective uptake of chosen antibrowning solutions.

b) *The temperature of the drowning solution*. All lobsters were drowned at ambient temperature (approx 25ºC) fresh water, however some companies use ice water in drowning which may have an impact on the uptake of the solutions and should be investigated.

c) *Length of drown time*. All lobsters were drowned to a standard time of 20 minutes. It is possible that a longer period in the drown tank could result in higher uptake levels for the antibrowning solutions and so improve effectiveness in reduction of melanosis. Considerable variation in drown time between different companies has been observed and is dependent on the demands of processing. Evaluation of the impacts of these different drowning regimes on antibrowning agent uptake, PPO activity and melanosis in processed lobster needs to be undertaken.

d) *The possible use of combined agents to improve reduction in melanosis formation due to synergistic effects*. Often citric acid is used in conjunction with ascorbic acid to prevent browning in fruit and vegetables. The two agents used together act synergistically to produce a far greater effect than either acting alone (Whitaker 1994; Kim & Marshall 2000). Therefore, it maybe advantageous to combine citric acid with carbon dioxide and/or 4-hexylresorcinol and produce a more economical and more effective antibrowning agent than any one agent on its own. The most effective combination and ratio of agents must be clarified.

Before any agent is used in the western rock lobster industry, the above issues must be addressed along with clarification of the impacts of antibrowning agents on the sensory quality of the processed product. Since lobster is a luxury food and must meet the consumers expectations, in terms of colour, flavour and texture, it is important to ensure that any change to current processing practices does not have a negative impact on the perceived quality of the product.

## BENEFITS AND ADOPTION

It was of vital interest to the western rock lobster industry to establish what causes melanosis in western rock lobster and identify possible means of prevention in order to maximise sales of processed whole western rock lobster into the lucrative Asian markets.

Several benefits have accrued to the industry from this study.

1. The cause of melanosis has been identified as the enzymic reactions of polyphenoloxidase. Therefore the industry now knows that it is not due to contamination or bacteria.

2. The thermal kinetic values of the enzyme have been established which can be used by the industry to evaluate the effectiveness of any method of heat processing to prevent melanosis in western rock lobster. Industry now knows that rapid heating to temperatures in excess of 80°C is required to reduce heat activation of the enzyme. **Heating to a lethality greater than or equivalent to 36.11 minutes at 90°C is required to prevent melanosis totally**.

3. Evaluation of current transportation, post harvest handling and cooking has shown that the impacts of cooking outweigh any impact on melanosis formation arising from the current methods of post-harvest handling.

4. The impacts on heating on weight recovery have been established but the study also showed that temperature was not the major cause of weight loss in processed lobsters.

The findings of the study show that processing must achieve a balance between weight recovery and melanosis prevention. Members of the industry have used them to evaluate and modify their current processing practise to reduce melanosis formation while maximising weight recovery.

## FURTHER DEVELOPMENT

While more severe heating regimes may result in prevention of melanosis, the associated increase in costs due to increased energy input, longer processing time and possible weight loss, makes increased heating an unattractive option to the western rock lobster industry. Alternative cooking methods that produce a faster rate of heat input may also significantly improve the post harvest quality of lobsters. It is postulated that shorter cook times with higher final temperatures would result in increased weight recovery and better textural quality. If at the same time, they decreased or prevented melanosis they would be of great value to the industry. Systems that utilise steam or autoclave (steam and pressure) cooking maybe effective. The ability of any processing system to prevent melanosis and improve post harvest quality can be assessed based on the thermal kinetic values validated in this study. Evaluation of the impact of these alternative processing methods on weight recovery and melanosis formation would assist the industry to determine the most effective processing method for western rock lobster.

Antibrowning agents are effective in reducing the incidence and severity of melanosis occurrence in western rock lobster. Several issues were identified in this study that required elucidation such as the possibility of synergistic effects of selected antibrowning agents. Evaluation of the impact of any selected antibrowning agent on sensory characteristics of lobster is also required. Any change in processing methods that may result in changes in colour, texture or flavour of lobster must be evaluated to ensure acceptability by the end consumer.

The work developed in this study can also be extended to other crustacean industries that experience difficulties with post harvest blackening. Determination of the impacts of processing technologies on PPO activity and the thermal kinetics values of the enzyme for each species will enable those industries to evaluate the effectiveness of future and current technologies for processing the species of interest.

## PLANNED OUTCOMES

The recommendations and guidelines for the commercial processing of western rock lobster arising from this project will allow processors to consistently maximise their cooked weight recoveries whilst reliably ensuring melanosis is prevented, ensuring more profitable and efficient use of the resource. All WA processors will reap the potential benefits of this research through

1) Increased financial returns, resulting from maximised cooked weight recoveries;

2) Minimisation of financial and reputation losses through reduction of the incidence of melanosis; and

3) Improved product quality consistency, resulting in increased competitive advantage in export markets.

All western rock lobster fishers will benefit from maximised cooked recoveries and quality through increased bonuses and beach prices. Overall, participants in the commercial fishery (i.e. both fishers and processors) should benefit through the realisation of increase profitability.

The outcomes of the study have been communicated to industry members though newsletters (The Lobster News) and presentations at workshops and conferences (7th International Workshop on lobster management and Biology, Annual RLEAS & RLPH subprogram workshops).

This report will also be used to further disseminate the information to the industry members.

## CONCLUSIONS

Melanosis was identified as a problem for the western rock lobster (WRL) industry. Understanding of the mechanisms of melanosis and the impacts of processing on melanosis formation were needed. Polyphenoloxidase (PPO) was identified as the major causative factor in melanosis development. Therefore, evaluation of the impacts of heating and antibrowning agents on PPO activity, invitro and invivo, was undertaken.

Heat-induced activation of PPO occurs between 60 and 80°C. Significant deactivation of PPO did not occur until 80°C was exceeded. It can be concluded therefore, that rapid heating of WRL to temperatures greater than 80°C is required to limit the heat activation effects. The decimal reduction value at 90°C ($D_{90}$) was 12.04 minutes; the z value was 16.0°C and the theoretical process lethality value ($F_{90}$) was 36.12 minutes for WRL PPO.

Comparison of the theoretical process lethality value to the commercial cooking lethality values (Grade A lobsters, 2.99 $\pm$ 0.81, Grade B 2.57 $\pm$ 0.84) showed that current commercial cooking methods were unable to achieve deactivation of PPO. Determination of the level of heating required to prevent melanosis development in whole cooked lobster was then undertaken. A series of cooks in the 2003-2004 season used a range of lethality values such that a gradation from severely undercooked to severely overcooked lobsters resulted. Significant reduction in melanosis levels occurred as lethality increased. Lobsters heated to lethality values equivalent to or greater than 36.12 minutes at 90°C did not exhibit melanosis.

While increasing cook time to meet the required lethality level will prevent melanosis it may also have a negative effect on weight recovery. Evaluation of weight recoveries through out this study showed that increasing cook time/lethality correlated with decreased weight recovery. Increased cook times also correlates in increased processing costs and weight loss equates to lost money for the processor so the industry will be strongly resistant to increasing their cooking times even if it will prevent melanosis formation.

Evaluation of the physiological parameters (protein content, total phenols content, baseline and total PPO activity) showed that the impact of cooking on melanosis development is such that it outweighs any pre-processing factor when current best practise is followed. These findings were further reinforced by evaluation of

simulated transportation and holding conditions, which showed that current post harvest handling processes had no negative impact on melanosis development.

Ascorbic acid, citric acid, 4-hexylresorcinol and carbon dioxide were evaluated for their impact on melanosis. Ascorbic acid had no effect on total PPO activity, while citric acid, 4-hexylresorcinol, and carbon dioxide inhibited both forms of PPO activity. The effectiveness of these compounds in prevention of melanosis during commercial processing of western rock lobster was then evaluated. Selected concentrations of the effective antibrowning agents were applied to lobster during drowning and post-processing evaluation of melanosis development was undertaken using digital image analysis. Lobsters treated with 87µg/ml 4-hexylresorcinol showed the greatest reduction in melanosis with respect to rate, intensity and extent yet complete inhibition of melanosis was not achieved by any treatment within the exposure times, solution concentrations and processing conditions used. However, significant reductions in the extent, intensity and rate of melanosis development were achieved with all treatments. Several factors were identified that require further investigation.

Antibrowning agents are effective in reducing the incidence and severity of melanosis occurrence in WRL, yet further investigation of their use in lobster processing is required. The application of alternative cooking methods that produce a faster rate of heat input also requires investigation.

It has been clearly demonstrated in this study that the determined thermal kinetic values hold true under current processing conditions and are directly related to the incidence of melanosis after processing. The results also show that the initial physiological parameter measures of western rock lobster cannot be used to accurately predict the melanosis outcomes.

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## APPENDIX 1: INTELLECTUAL PROPERTY

This study embraces both fundamental and practical questions that impact on the quality of western rock lobster, a vital export commodity for Western Australia. It takes a systematic and stepwise approach to the major issues that are addressed coordinating the fundamental chemical and physical sciences with real-world practice. In the process it assesses the risks and benefits of strategies that may impact on the development of melanosis in western rock lobster. The results have important implications for industry and it was desirable that they be released as soon as possible to enable rapid industry uptake. Some concerns arose during the study regarding the commercial potential and/or sensitivity of the information generated however it was decided that the industry would derive maximum benefit from full disclosure as soon as possible.

The first objective of the project involved the determination of heat stability of the enzyme in vitro. From the data it was possible to develop equations that allow prediction of the process lethality required to ensure deactivation of the enzyme. Process lethality is defined as the lethal effect of all heat applied to a system expressed as equivalent minutes at a specified reference temperature. Determination of the theoretical value enables evaluation of the effectiveness of a heating system in destroying the enzyme during processing. The determined thermal kinetics values for western rock lobster were:

- $D_{90}$= 12.04 minutes (time required at 90°C to deactivate 90% of the enzyme)
- *Z*= 16 °C (increase in temperature required to achieve a 10 fold decrease in the enzyme concentration)
- $F_{90}$ = 36.12 minutes (process lethality required to deactivate 99.9% of the enzyme expressed in minutes at 90°C)

These values are unique to polyphenoloxidase of western rock lobster.

The third objective required the measurement of the thermal profiles achieved during standardized processing of rock lobsters. The actual process lethality achieved in current processing was calculated using the equation derived from the values obtained in Objective 1:

$$F_{process} = \sum 10^{\frac{T_i - T_r}{z}} \Delta t$$

It was clear that the profile used in the standardized process was inadequate to achieve the process lethality required to reach significant deactivation of the enzyme. However, the results of Objective 3 also demonstrated that the equation derived from Objective 1 had to be validated within a commercial system before it could be used commercially. This result was foreseen and was requirement of the project (Objective 4). Improper disclosure without full and accurate information could have resulted in misuse of the information and a resultant backlash from the industry.

Therefore, the theoretical equations and the derived enzyme kinetic values were not released to industry until the validation stage of the project was completed. Objective 4 (Validation) was successfully completed in 2004. Since the intellectual property no longer required protection, it was made available to the industry through the RLPHS.

The information was reported publicly at the Annual RLEAS/RLPHS Workshop in Port Lincoln, SA., on September 15th 2004, and will be further disseminated through this report, scientific papers and workshop proceedings.

## APPENDIX 2: STAFF

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**APPENDIX 3:**

N.B Please note that

- PPO activity is reported as Change in absorbance/min/ml hemolymph for all processing data shown in the appendices.
- PPO activity is reported as % relative activity (activity at time sampled compared to time zero activity) for all in vitro data shown in the appendices

**Thermal kinetics equations**

*Equation 1*:..... $$F_t = S \times D_t$$

*Equation 2*: ..... $$\mathrm{k} = \frac{\ln(10)}{\mathrm{D_t}}$$

*Equation 3*:..... $$E_a = slope \times R$$

*Equation 4*:..... $$\mathrm{z} = \frac{\ln(10)(\mathrm{T_1 T_2})}{(\mathrm{E_a}/\mathrm{R})}$$

*Equation 5*: ..... $$\mathrm{L} = 10^{A}$$

*Equation 6*: ..... $$\mathrm{A} = \frac{T_i - T_r}{z}$$

*Equation 7*:..... $$F_{process} = L \times T_T$$

*Equation 8:* ..... $$F_{process} = \sum L_T \Delta t$$

Equation 8 can also be written as:

$$F_{process} = \sum 10^{\frac{T_i - T_r}{z}} \Delta t$$

## Calculation of the thermal kinetics values

### Calculation of the D value

When the log of % relative activity is plotted against time, at a specified temperature, the resultant graph is a straight line (Toledo 1991). The D value or decimal reduction value (Scott & Weddig 1998) is equal to the negative reciprocal of the slope of the regression line specific to the given temperature (Toledo 1991) and is expressed as the time in minutes at a constant temperature necessary to destroy 90% of the enzyme activity (Scott & Weddig 1998).

### Calculation of the k value

Once the D value is known it is possible to calculate the rate reaction constant, k, for the deactivation of the enzyme at each temperature (Sharma, Mulvaney & Rizvi 2000) since:

$$k = \frac{\ln(10)}{Dt}$$

### Calculation of the $E_a$ value

Plotting ln(k) against the reciprocal of absolute temperature gives a thermal resistance graph (Sharma, Mulvaney & Rizvi 2000). The thermal resistance graph has a slope equal to $-E_a/R$., so:

$$E_a = slope \times R$$

where $E_a$ is the activation energy and R is the gas constant (8.314 J/K/mol or 1.98717 cal/K/mol)(Toledo 1991)

**Calculation of the z value**

The z value is the temperature change required to change the D value by a factor of 10 (Toledo 1991) and is calculated as

$$z = \frac{\ln(10)(T_1 T_r)}{(Ea/R)}$$

**Calculation of the theoretical process lethality ($F_{90}$) value**

Using the $D_{90}$ value it is possible to calculate the time required to achieve 99.9% deactivation of the enzyme at 90°C since

$$F_{90} = S \times D_{90}$$

where S is the number of log cycles to be achieved and $D_{90}$ is the decimal reduction value at 90°C (Toledo 1991).

**Calculation of the lethal rate (L)**

The lethal rate converts the minutes at the product temperature to the equivalent number of minutes at the reference temperature necessary to achieve the same degree of inactivation (Scott & Weddig 1998). The formula used is:

$$L = 10^{\left(\frac{T - T_r}{Z}\right)}$$

where $T_r$ is the reference temperature (90°C), T is the product temperature and z is the z value for the enzyme of interest.

**Calculation of the actual process lethality**

To calculate the total accumulated process lethality requires the integration of the lethal rate over time (Toledo 1991).

$$F_r = \sum L_T \Delta T$$

or

$$F_{process} = \sum 10^{\frac{T_i - T_r}{z}} \Delta t$$

**Calculation of values for western rock lobster**

The semilogarithmic graph for western rock lobster is shown in Figure 71. And the resultant D and Z values in Table 36.

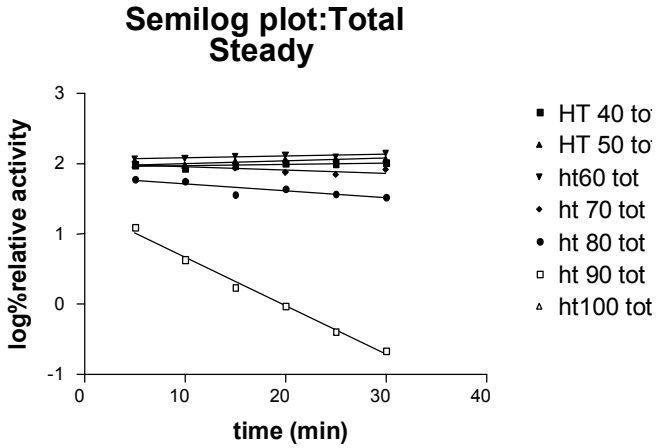


*Figure 47: Log (% relative activity) versus time for western rock lobster PPO*

*Table 31: D and K value calculation values*

| Temperature °C | Temperature Kelvin | 1/T | D value | k | ln (k) |
|---|---|---|---|---|---|
| 50°C | 323.00 | 0.0031 | -313.18 | 0.000 | - |
| 60°C | 333.00 | 0.0030 | -261.37 | 0.000 | - |
| 70°C | 343.00 | 0.0029 | 214.04 | 0.0108 | -4.5321 |
| 80°C | 353.00 | 0.0028 | 71.67 | 0.032 | -3.4380 |
| 90°C | 363.00 | 0.0027 | 12.034 | 0.191 | -1.6540 |

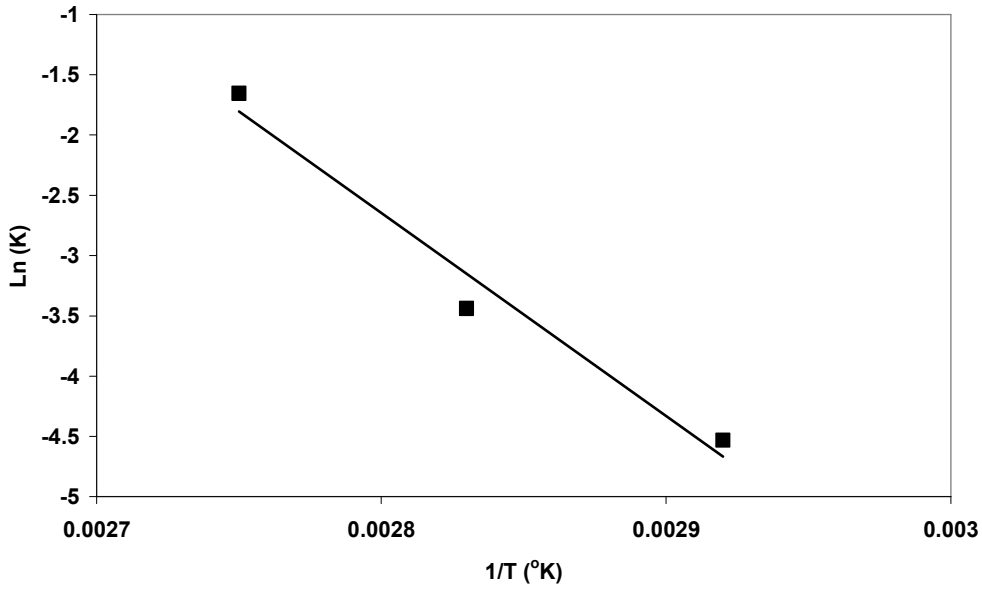


*Figure 48: Ln(K) versus 1/T (Kelvin) for western rock lobster*

From Figure 72, the thermal resistance curve for Western rock lobster PO has a slope of -17872 -°K., when $T_1$ = 70°C and $T_r$ = 90°C. Therefore $E_a$ = 148.59 KJ/mol (or 35.51 kcal/mol), and z = 16.0°C for Western Rock Lobster PPO.

**Calculation of actual process lethality**

The Excel spreadsheet used to calculate the graphs in Figure 73 is shown on the following page

*Figure 49: Lethality graphs calculated for a processing run*

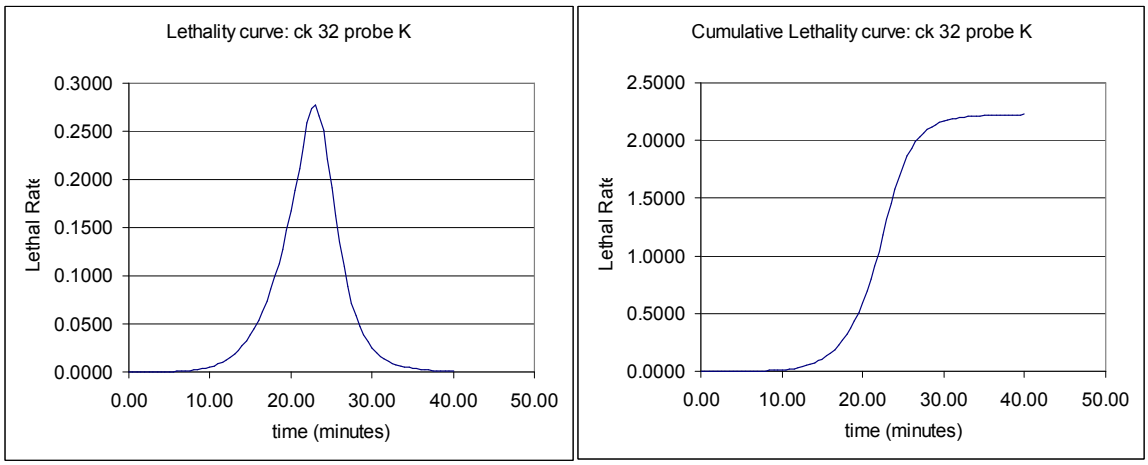

*Figure 50: Excel spreadsheet for calculation of lethality curves*

| | | | | | |
|---|---|---|---|---|---|
| **Lethality calculation from cook profile** | | | | | |
| (for formulas: ? = current row) | | | | | |
| | | | | | |
| **time (mins)** | **temperature** | **A** | **L/min** | **L/min*0.5** | **Cumulative L** |
| | | **(B?-90)/16** | **POWER(10,C?)** | **D?*0.5** | **Fprev+E?** |
| 0.00 | 22.4 | -4.225 | 0.0001 | 0.0000 | 0.0000 |
| 0.50 | 22.1 | -4.24375 | 0.0001 | 0.0000 | 0.0001 |
| 1.00 | 23 | -4.1875 | 0.0001 | 0.0000 | 0.0001 |
| 1.50 | 24.2 | -4.1125 | 0.0001 | 0.0000 | 0.0001 |
| 2.00 | 24.8 | -4.075 | 0.0001 | 0.0000 | 0.0002 |
| 2.50 | 26.7 | -3.95625 | 0.0001 | 0.0001 | 0.0002 |
| 3.00 | 28.3 | -3.85625 | 0.0001 | 0.0001 | 0.0003 |
| 3.50 | 29.8 | -3.7625 | 0.0002 | 0.0001 | 0.0004 |
| 4.00 | 31.5 | -3.65625 | 0.0002 | 0.0001 | 0.0005 |
| 4.50 | 33.5 | -3.53125 | 0.0003 | 0.0001 | 0.0006 |
| 5.00 | 35.3 | -3.41875 | 0.0004 | 0.0002 | 0.0008 |
| 5.50 | 37.2 | -3.3 | 0.0005 | 0.0003 | 0.0011 |
| 6.00 | 39.1 | -3.18125 | 0.0007 | 0.0003 | 0.0014 |
| 6.50 | 41 | -3.0625 | 0.0009 | 0.0004 | 0.0018 |
| 7.00 | 42.8 | -2.95 | 0.0011 | 0.0006 | 0.0024 |
| 7.50 | 44.6 | -2.8375 | 0.0015 | 0.0007 | 0.0031 |
| 8.00 | 46.6 | -2.7125 | 0.0019 | 0.0010 | 0.0041 |
| 8.50 | 48.5 | -2.59375 | 0.0025 | 0.0013 | 0.0054 |
| 9.00 | 50.2 | -2.4875 | 0.0033 | 0.0016 | 0.0070 |
| 9.50 | 51.8 | -2.3875 | 0.0041 | 0.0020 | 0.0091 |
| 10.00 | 53.5 | -2.28125 | 0.0052 | 0.0026 | 0.0117 |
| 10.50 | 55.1 | -2.18125 | 0.0066 | 0.0033 | 0.0150 |
| 11.00 | 56.6 | -2.0875 | 0.0082 | 0.0041 | 0.0190 |
| 11.50 | 58.1 | -1.99375 | 0.0101 | 0.0051 | 0.0241 |
| 12.00 | 59.6 | -1.9 | 0.0126 | 0.0063 | 0.0304 |
| 12.50 | 61 | -1.8125 | 0.0154 | 0.0077 | 0.0381 |
| 13.00 | 62.4 | -1.725 | 0.0188 | 0.0094 | 0.0475 |
| 13.50 | 63.7 | -1.64375 | 0.0227 | 0.0114 | 0.0589 |
| 14.00 | 64.9 | -1.56875 | 0.0270 | 0.0135 | 0.0724 |
| 14.50 | 66.1 | -1.49375 | 0.0321 | 0.0160 | 0.0884 |
| 15.00 | 67.3 | -1.41875 | 0.0381 | 0.0191 | 0.1075 |
| 15.50 | 68.5 | -1.34375 | 0.0453 | 0.0227 | 0.1301 |
| 16.00 | 69.7 | -1.26875 | 0.0539 | 0.0269 | 0.1571 |
| 16.50 | 70.8 | -1.2 | 0.0631 | 0.0315 | 0.1886 |
| 17.00 | 71.9 | -1.13125 | 0.0739 | 0.0370 | 0.2256 |
| 17.50 | 73 | -1.0625 | 0.0866 | 0.0433 | 0.2689 |
| 18.00 | 73.9 | -1.00625 | 0.0986 | 0.0493 | 0.3182 |
| 18.50 | 74.8 | -0.95 | 0.1122 | 0.0561 | 0.3743 |
| 19.00 | 75.7 | -0.89375 | 0.1277 | 0.0639 | 0.4381 |
| 19.50 | 76.7 | -0.83125 | 0.1475 | 0.0737 | 0.5119 |
| 20.00 | 77.6 | -0.775 | 0.1679 | 0.0839 | 0.5958 |
| 20.50 | 78.4 | -0.725 | 0.1884 | 0.0942 | 0.6900 |
| 21.00 | 79.2 | -0.675 | 0.2113 | 0.1057 | 0.7957 |
| 21.50 | 79.9 | -0.63125 | 0.2337 | 0.1169 | 0.9125 |
| 22.00 | 80.6 | -0.5875 | 0.2585 | 0.1293 | 1.0418 |
| 22.50 | 81 | -0.5625 | 0.2738 | 0.1369 | 1.1787 |
| 23.00 | 81.1 | -0.55625 | 0.2778 | 0.1389 | 1.3176 |
| 23.50 | 80.8 | -0.575 | 0.2661 | 0.1330 | 1.4507 |
| 24.00 | 80.4 | -0.6 | 0.2512 | 0.1256 | 1.5763 |
| 24.50 | 79.5 | -0.65625 | 0.2207 | 0.1103 | 1.6866 |
| 25.00 | 78.5 | -0.71875 | 0.1911 | 0.0955 | 1.7821 |
| 25.50 | 77.4 | -0.7875 | 0.1631 | 0.0816 | 1.8637 |
| 26.00 | 76.1 | -0.86875 | 0.1353 | 0.0676 | 1.9313 |
| 26.50 | 74.7 | -0.95625 | 0.1106 | 0.0553 | 1.9866 |
| 27.00 | 73.2 | -1.05 | 0.0891 | 0.0446 | 2.0312 |
| 27.50 | 71.7 | -1.14375 | 0.0718 | 0.0359 | 2.0671 |
| 28.00 | 70.3 | -1.23125 | 0.0587 | 0.0294 | 2.0965 |
| 28.50 | 68.9 | -1.31875 | 0.0480 | 0.0240 | 2.1205 |
| 29.00 | 67.3 | -1.41875 | 0.0381 | 0.0191 | 2.1395 |
| 29.50 | 65.8 | -1.5125 | 0.0307 | 0.0154 | 2.1549 |
| 30.00 | 64.4 | -1.6 | 0.0251 | 0.0126 | 2.1675 |
| 30.50 | 63 | -1.6875 | 0.0205 | 0.0103 | 2.1777 |
| 31.00 | 61.5 | -1.78125 | 0.0165 | 0.0083 | 2.1860 |
| 31.50 | 60 | -1.875 | 0.0133 | 0.0067 | 2.1927 |
| 32.00 | 58.7 | -1.95625 | 0.0111 | 0.0055 | 2.1982 |
| 32.50 | 57.4 | -2.0375 | 0.0092 | 0.0046 | 2.2028 |
| 33.00 | 56.1 | -2.11875 | 0.0076 | 0.0038 | 2.2066 |
| 33.50 | 54.9 | -2.19375 | 0.0064 | 0.0032 | 2.2098 |
| 34.00 | 53.6 | -2.275 | 0.0053 | 0.0027 | 2.2124 |
| 34.50 | 52.3 | -2.35625 | 0.0044 | 0.0022 | 2.2147 |
| 35.00 | 51.2 | -2.425 | 0.0038 | 0.0019 | 2.2165 |
| 35.50 | 50 | -2.5 | 0.0032 | 0.0016 | 2.2181 |
| 36.00 | 48.9 | -2.56875 | 0.0027 | 0.0013 | 2.2195 |
| 36.50 | 47.7 | -2.64375 | 0.0023 | 0.0011 | 2.2206 |
| 37.00 | 46.7 | -2.70625 | 0.0020 | 0.0010 | 2.2216 |
| 37.50 | 45.6 | -2.775 | 0.0017 | 0.0008 | 2.2224 |
| 38.00 | 44.6 | -2.8375 | 0.0015 | 0.0007 | 2.2231 |
| 38.50 | 43.5 | -2.90625 | 0.0012 | 0.0006 | 2.2238 |
| 39.00 | 42.6 | -2.9625 | 0.0011 | 0.0005 | 2.2243 |
| 39.50 | 41.6 | -3.025 | 0.0009 | 0.0005 | 2.2248 |
| 40.00 | 40.7 | -3.08125 | 0.0008 | 0.0004 | **2.2252** |

**Statistics for invitro heat trials**

**Statistics for PPO activity in western rock lobster hemolymph under steady sate heating**

*Table 32: Descriptive statistics for total PPO activity in WRL hemolymph under steady state heating*

| | Temp. | N | Mean | Std. Dev. | Std. Err. | 95% CI for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| TIME0 | 40.00 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 50.00 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 60.00 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 70.00 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 80.00 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 90.00 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 100.00 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | Total | 28.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| TIME5 | 40.00 | 4.00 | 96.02 | 7.77 | 3.89 | 83.65 | 108.39 | 85.74 | 103.38 |
| | 50.00 | 4.00 | 96.50 | 10.11 | 5.06 | 80.41 | 112.60 | 84.89 | 108.33 |
| | 60.00 | 4.00 | 117.97 | 17.39 | 8.69 | 90.30 | 145.64 | 105.02 | 142.21 |
| | 70.00 | 4.00 | 105.40 | 12.36 | 6.18 | 85.73 | 125.07 | 89.89 | 120.12 |
| | 80.00 | 4.00 | 105.40 | 12.36 | 6.18 | 85.73 | 125.07 | 89.89 | 120.12 |
| | 90.00 | 4.00 | 12.59 | 5.97 | 2.98 | 3.10 | 22.08 | 7.70 | 20.53 |
| | 100.00 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Total | 28.00 | 76.27 | 46.69 | 8.82 | 58.16 | 94.37 | 0.00 | 142.21 |
| TIME10 | 40.00 | 4.00 | 85.91 | 16.64 | 8.32 | 59.42 | 112.39 | 62.09 | 100.77 |
| | 50.00 | 4.00 | 95.82 | 3.79 | 1.89 | 89.79 | 101.84 | 92.72 | 100.77 |
| | 60.00 | 4.00 | 119.82 | 24.99 | 12.49 | 80.06 | 159.58 | 94.18 | 153.78 |
| | 70.00 | 4.00 | 85.62 | 10.82 | 5.41 | 68.40 | 102.83 | 78.41 | 101.37 |
| | 80.00 | 4.00 | 85.62 | 10.82 | 5.41 | 68.40 | 102.83 | 78.41 | 101.37 |
| | 90.00 | 4.00 | 4.33 | 3.89 | 1.94 | -1.86 | 10.51 | 0.55 | 9.76 |
| | 100.00 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Total | 28.00 | 68.16 | 45.46 | 8.59 | 50.53 | 85.79 | 0.00 | 153.78 |
| TIME15 | 40.00 | 4.00 | 93.71 | 13.51 | 6.75 | 72.22 | 115.20 | 75.17 | 105.48 |
| | 50.00 | 4.00 | 109.08 | 18.04 | 9.02 | 80.38 | 137.78 | 93.58 | 135.00 |
| | 60.00 | 4.00 | 128.05 | 26.56 | 13.28 | 85.79 | 170.31 | 107.89 | 166.61 |
| | 70.00 | 4.00 | 86.56 | 16.50 | 8.25 | 60.29 | 112.82 | 63.81 | 99.37 |

| | Temp. | N | Mean | Std. Dev. | Std. Err. | 95% CI for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| | 80.00 | 4.00 | 86.56 | 16.50 | 8.25 | 60.29 | 112.82 | 63.81 | 99.37 |
| | 90.00 | 3.00 | 1.72 | 1.66 | 0.96 | -2.40 | 5.84 | 0.00 | 3.31 |
| | 100.00 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Total | 27.00 | 74.85 | 48.94 | 9.42 | 55.49 | 94.21 | 0.00 | 166.61 |
| TIME20 | 40.00 | 4.00 | 101.98 | 3.70 | 1.85 | 96.09 | 107.88 | 97.52 | 105.56 |
| | 50.00 | 4.00 | 115.17 | 20.87 | 10.43 | 81.97 | 148.38 | 97.14 | 144.91 |
| | 60.00 | 4.00 | 133.54 | 23.07 | 11.53 | 96.83 | 170.24 | 110.78 | 165.70 |
| | 70.00 | 4.00 | 75.52 | 16.22 | 8.11 | 49.72 | 101.32 | 58.78 | 97.57 |
| | 80.00 | 4.00 | 75.52 | 16.22 | 8.11 | 49.72 | 101.32 | 58.78 | 97.57 |
| | 90.00 | 4.00 | 0.95 | 1.27 | 0.63 | -1.07 | 2.96 | 0.00 | 2.77 |
| | 100.00 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Total | 28.00 | 71.81 | 51.55 | 9.74 | 51.82 | 91.80 | 0.00 | 165.70 |
| TIME25 | 40.00 | 4.00 | 100.60 | 2.79 | 1.40 | 96.15 | 105.04 | 97.60 | 104.01 |
| | 50.00 | 4.00 | 118.29 | 24.61 | 12.31 | 79.12 | 157.45 | 95.71 | 151.29 |
| | 60.00 | 4.00 | 125.55 | 34.03 | 17.01 | 71.40 | 179.69 | 90.72 | 170.39 |
| | 70.00 | 4.00 | 70.04 | 12.99 | 6.50 | 49.36 | 90.72 | 53.99 | 85.78 |
| | 80.00 | 4.00 | 70.04 | 12.99 | 6.50 | 49.36 | 90.72 | 53.99 | 85.78 |
| | 90.00 | 4.00 | 0.41 | 0.81 | 0.41 | -0.88 | 1.69 | 0.00 | 1.62 |
| | 100.00 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Total | 28.00 | 69.27 | 51.17 | 9.67 | 49.43 | 89.12 | 0.00 | 170.39 |
| TIME30 | 40.00 | 4.00 | 102.35 | 3.19 | 1.60 | 97.27 | 107.44 | 99.51 | 105.93 |
| | 50.00 | 4.00 | 115.73 | 23.94 | 11.97 | 77.63 | 153.82 | 91.84 | 142.82 |
| | 60.00 | 4.00 | 139.58 | 31.31 | 15.66 | 89.76 | 189.40 | 116.64 | 185.86 |
| | 70.00 | 4.00 | 82.88 | 7.97 | 3.99 | 70.19 | 95.56 | 74.11 | 92.59 |
| | 80.00 | 4.00 | 82.88 | 7.97 | 3.99 | 70.19 | 95.56 | 74.11 | 92.59 |
| | 90.00 | 4.00 | 0.22 | 0.44 | 0.22 | -0.47 | 0.91 | 0.00 | 0.87 |
| | 100.00 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Total | 28.00 | 74.80 | 53.32 | 10.08 | 54.13 | 95.48 | 0.00 | 185.86 |

*Table 33: ANOVA for total PPO activity in WRL hemolymph under steady state heating*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| TIME0 | Between Groups | 0.00 | 6.00 | 0.00 | . | . |
| | Within Groups | 0.00 | 21.00 | 0.00 | | |
| | Total | 0.00 | 27.00 | | | |
| TIME5 | Between Groups | 56430.81 | 6.00 | 9405.14 | 81.66 | 0.00 |
| | Within Groups | 2418.60 | 21.00 | 115.17 | | |
| | Total | 58849.41 | 27.00 | | | |
| TIME10 | Between Groups | 52313.72 | 6.00 | 8718.95 | 52.39 | 0.00 |
| | Within Groups | 3494.97 | 21.00 | 166.43 | | |
| | Total | 55808.69 | 27.00 | | | |
| TIME15 | Between Groups | 56982.81 | 6.00 | 9497.14 | 35.98 | 0.00 |
| | Within Groups | 5279.57 | 20.00 | 263.98 | | |
| | Total | 62262.39 | 26.00 | | | |
| TIME20 | Between Groups | 67226.71 | 6.00 | 11204.45 | 51.98 | 0.00 |
| | Within Groups | 4526.54 | 21.00 | 215.55 | | |
| | Total | 71753.25 | 27.00 | | | |
| TIME25 | Between Groups | 64372.14 | 6.00 | 10728.69 | 35.60 | 0.00 |
| | Within Groups | 6329.65 | 21.00 | 301.41 | | |
| | Total | 70701.79 | 27.00 | | | |
| TIME30 | Between Groups | 71674.92 | 6.00 | 11945.82 | 49.45 | 0.00 |
| | Within Groups | 5073.52 | 21.00 | 241.60 | | |
| | Total | 76748.44 | 27.00 | | | |

*Table 34: TUKEY HSD comparisons of total PPO activity in WRL hemolymph under steady state heating*

| TIME5 | N | Subset for alpha = .05 | |
|---|---|---|---|
| TEMP | | 1.00 | 2.00 |
| 100.00 | 4.00 | 0.00 | |
| 90.00 | 4.00 | 12.59 | |
| 40.00 | 4.00 | | 96.02 |
| 50.00 | 4.00 | | 96.50 |
| 70.00 | 4.00 | | 105.40 |
| 80.00 | 4.00 | | 105.40 |
| 60.00 | 4.00 | | 117.97 |
| Sig. | | 0.65 | 0.10 |

| TIME10 | N | Subset for alpha = .05 | | |
|---|---|---|---|---|
| TEMP | | 1.00 | 2.00 | 3.00 |
| 100.00 | 4.00 | 0.00 | | |
| 90.00 | 4.00 | 4.33 | | |
| 70.00 | 4.00 | | 85.62 | |
| 80.00 | 4.00 | | 85.62 | |
| 40.00 | 4.00 | | 85.91 | |
| 50.00 | 4.00 | | 95.82 | 95.82 |
| 60.00 | 4.00 | | | 119.82 |
| Sig. | | 1.00 | 0.92 | 0.17 |

| TIME15 | N | Subset for alpha = .05 | | |
|---|---|---|---|---|
| TEMP | | 1.00 | 2.00 | 3.00 |
| 100.00 | 4.00 | 0.00 | | |
| 90.00 | 3.00 | 1.72 | | |
| 70.00 | 4.00 | | 86.56 | |
| 80.00 | 4.00 | | 86.56 | |
| 40.00 | 4.00 | | 93.71 | 93.71 |
| 50.00 | 4.00 | | 109.08 | 109.08 |
| 60.00 | 4.00 | | | 128.05 |
| Sig. | | 1.00 | 0.49 | 0.10 |

| TIME20 | N | Subset for alpha = .05 | | |
|---|---|---|---|---|
| TEMP | | 1.00 | 2.00 | 3.00 |
| 100.00 | 4.00 | 0.00 | | |
| 90.00 | 4.00 | 0.95 | | |
| 70.00 | 4.00 | | 75.52 | |
| 80.00 | 4.00 | | 75.52 | |
| 40.00 | 4.00 | | 101.98 | 101.98 |
| 50.00 | 4.00 | | | 115.17 |
| 60.00 | 4.00 | | | 133.54 |
| Sig. | | 1.00 | 0.19 | 0.08 |

| TIME25 | N | Subset for alpha = .05 | | |
|---|---|---|---|---|
| TEMP | | 1 | 2 | 3 |
| 100 | 4 | 0 | | |
| 90 | 4 | 0.405 | | |
| 70 | 4 | | 70.04 | |
| 80 | 4 | | 70.04 | |
| 40 | 4 | | 100.59 | 100.59 |
| 50 | 4 | | | 118.29 |
| 60 | 4 | | | 125.55 |
| Sig. | | 1 | 0.213 | 0.425 |

| TIME30 | N | Subset for alpha = .05 | | |
|---|---|---|---|---|
| TEMP | | 1 | 2 | 3 |
| 100 | 4 | 0 | | |
| 90 | 4 | 0.2175 | | |
| 70 | 4 | | 82.88 | |
| 80 | 4 | | 82.88 | |
| 40 | 4 | | 102.35 | |
| 50 | 4 | | 115.73 | 115.73 |
| 60 | 4 | | | 139.58 |
| Sig. | | 1 | 0.085 | 0.351 |

Means for groups in homogeneous subsets are displayed

a Uses Harmonic Mean Sample Size = 4.000.

*Table 35: Descriptive statistics for baseline PPO activity in WRL hemolymph under steady state heating*

| | | | | | | 95% CI for Mean | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | Mean | Std. Dev. | Std. Err. | | | Min. | Max. |
| | | | | | | Lower Bound | Upper Bound | | |
| TIME0 | 40 | 6.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 50 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 60 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 70 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 80 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 90 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 100 | 4.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| | Total | 30.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100.00 | 100.00 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| TIME5 | 40 | 6.00 | 259.11 | 228.82 | 93.42 | 18.97 | 499.24 | 89.38 | 590.45 |
| | 50 | 4.00 | 0.11 | 0.21 | 0.11 | -0.23 | 0.44 | 0.00 | 0.42 |
| | 60 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 70 | 4.00 | 400.94 | 195.79 | 97.89 | 89.41 | 712.48 | 203.48 | 578.78 |
| | 80 | 4.00 | 474.21 | 310.53 | 155.27 | -19.91 | 968.34 | 142.78 | 872.76 |
| | 90 | 4.00 | 101.55 | 91.79 | 45.89 | -44.51 | 247.61 | 37.57 | 237.46 |
| | 100 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Total | 30.00 | 182.06 | 240.94 | 43.99 | 92.09 | 272.03 | 0.00 | 872.76 |
| TIME10 | 40 | 6.00 | 222.53 | 175.65 | 71.71 | 38.19 | 406.86 | 76.29 | 475.89 |
| | 50 | 4.00 | 0.60 | 1.20 | 0.60 | -1.31 | 2.52 | 0.00 | 2.41 |
| | 60 | 4.00 | 0.18 | 0.36 | 0.18 | -0.40 | 0.76 | 0.00 | 0.73 |
| | 70 | 4.00 | 465.80 | 211.93 | 105.97 | 128.56 | 803.03 | 257.02 | 742.60 |
| | 80 | 4.00 | 402.77 | 262.65 | 131.32 | -15.16 | 820.70 | 113.51 | 743.40 |
| | 90 | 4.00 | 37.58 | 52.18 | 26.09 | -45.45 | 120.62 | 0.00 | 114.81 |
| | 100 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Total | 30.00 | 165.43 | 227.63 | 41.56 | 80.43 | 250.43 | 0.00 | 743.40 |
| TIME15 | 40 | 6.00 | 208.36 | 146.94 | 59.99 | 54.16 | 362.57 | 99.55 | 430.10 |
| | 50 | 4.00 | 0.37 | 0.74 | 0.37 | -0.81 | 1.55 | 0.00 | 1.49 |
| | 60 | 4.00 | 1.33 | 1.50 | 0.75 | -1.06 | 3.71 | 0.00 | 3.10 |
| | 70 | 4.00 | 500.57 | 194.22 | 97.11 | 191.52 | 809.62 | 318.84 | 668.99 |
| | 80 | 4.00 | 448.24 | 310.23 | 155.11 | -45.40 | 941.88 | 143.09 | 874.52 |
| | 90 | 3.00 | 14.38 | 16.40 | 9.47 | -26.35 | 55.11 | 0.00 | 32.23 |
| | 100 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Total | 29.00 | 175.70 | 245.11 | 45.52 | 82.47 | 268.94 | 0.00 | 874.52 |
| TIME20 | 40 | 6.00 | 164.01 | 102.27 | 41.75 | 56.68 | 271.34 | 77.02 | 356.25 |
| | 50 | 4.00 | 0.35 | 0.71 | 0.35 | -0.77 | 1.48 | 0.00 | 1.42 |
| | 60 | 4.00 | 1.60 | 1.60 | 0.80 | -0.95 | 4.15 | 0.26 | 3.87 |
| | 70 | 4.00 | 501.01 | 158.93 | 79.46 | 248.13 | 753.90 | 291.65 | 675.29 |
| | 80 | 4.00 | 404.82 | 312.30 | 156.15 | -92.13 | 901.76 | 168.33 | 862.16 |
| | 90 | 4.00 | 6.41 | 10.05 | 5.03 | -9.58 | 22.41 | 0.00 | 21.15 |
| | 100 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Total | 30.00 | 154.69 | 229.26 | 41.86 | 69.09 | 240.30 | 0.00 | 862.16 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| TIME25 | 40 | 5.00 | 143.80 | 104.44 | 46.71 | 14.12 | 273.48 | 51.66 | 297.74 |
| | 50 | 4.00 | 0.14 | 0.28 | 0.14 | -0.31 | 0.59 | 0.00 | 0.57 |
| | 60 | 4.00 | 7.62 | 10.96 | 5.48 | -9.83 | 25.06 | 0.34 | 23.53 |
| | 70 | 4.00 | 513.52 | 243.66 | 121.83 | 125.80 | 901.24 | 232.22 | 722.47 |
| | 80 | 4.00 | 366.02 | 274.00 | 137.00 | -69.97 | 802.01 | 132.54 | 762.53 |
| | 90 | 4.00 | 2.29 | 4.58 | 2.29 | -4.99 | 9.57 | 0.00 | 9.15 |
| | 100 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Total | 29.00 | 147.49 | 232.29 | 43.14 | 59.14 | 235.85 | 0.00 | 762.53 |
| TIME30 | 40 | 6.00 | 123.41 | 79.56 | 32.48 | 39.92 | 206.90 | 39.59 | 263.72 |
| | 50 | 4.00 | 0.23 | 0.46 | 0.23 | -0.50 | 0.96 | 0.00 | 0.92 |
| | 60 | 4.00 | 29.14 | 51.42 | 25.71 | -52.69 | 110.96 | 0.51 | 106.07 |
| | 70 | 4.00 | 445.24 | 248.43 | 124.22 | 49.93 | 840.55 | 234.19 | 739.95 |
| | 80 | 4.00 | 327.74 | 247.19 | 123.60 | -65.60 | 721.08 | 128.42 | 649.16 |
| | 90 | 4.00 | 1.64 | 3.29 | 1.64 | -3.59 | 6.88 | 0.00 | 6.58 |
| | 100 | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Total | 30.00 | 131.88 | 203.73 | 37.20 | 55.81 | 207.96 | 0.00 | 739.95 |

*Table 36: ANOVA of baseline PPO activity in western rock lobster hemolymph under steady state heating*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| TIME0 | Between Groups | 0.00 | 6 | 0.00 | . | . |
| | Within Groups | 0.00 | 23 | 0.00 | | |
| | Total | 0.00 | 29 | | | |
| TIME5 | Between Groups | 992192.24 | 6 | 165365.37 | 5.50 | 0.00 |
| | Within Groups | 691354.30 | 23 | 30058.88 | | |
| | Total | 1683546.54 | 29 | | | |
| TIME10 | Between Groups | 998507.17 | 6 | 166417.86 | 7.59 | 0.00 |
| | Within Groups | 504142.12 | 23 | 21919.22 | | |
| | Total | 1502649.30 | 29 | | | |
| TIME15 | Between Groups | 1171806.60 | 6 | 195301.10 | 8.42 | 0.00 |
| | Within Groups | 510390.06 | 22 | 23199.55 | | |
| | Total | 1682196.66 | 28 | | | |
| TIME20 | Between Groups | 1103221.84 | 6 | 183870.31 | 10.05 | 0.00 |
| | Within Groups | 420982.38 | 23 | 18303.58 | | |
| | Total | 1524204.23 | 29 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| TIME25 | Between Groups | 1063458.78 | 6 | 177243.13 | 8.72 | 0.00 |
| | Within Groups | 447392.29 | 22 | 20336.01 | | |
| | Total | 1510851.07 | 28 | | | |
| TIME30 | Between Groups | 795615.99 | 6 | 132602.67 | 7.47 | 0.00 |
| | Within Groups | 408077.21 | 23 | 17742.49 | | |
| | Total | 1203693.21 | 29 | | | |

*Table 37: Tukey HSD post hoc analysis of baseline PPO activity in WRL hemolymph under steady state heating*

| **TIME5** | **N** | **Subset for alpha = .05** | |
|---|---|---|---|
| TEMP | | 1.00 | 2.00 |
| 60.00 | 4 | 0.00 | |
| 100.00 | 4 | 0.00 | |
| 50.00 | 4 | 0.11 | |
| 90.00 | 4 | 101.55 | 101.55 |
| 40.00 | 6 | 259.11 | 259.11 |
| 70.00 | 4 | | 400.94 |
| 80.00 | 4 | | 474.21 |
| Sig. | | 0.35 | 0.06 |

| **TIME10** | **N** | **Subset for alpha = .05** | |
|---|---|---|---|
| TEMP | | 1.00 | 2.00 |
| 100.00 | 4 | 0.00 | |
| 60.00 | 4 | 0.18 | |
| 50.00 | 4 | 0.60 | |
| 90.00 | 4 | 37.58 | |
| 40.00 | 6 | 222.53 | 222.53 |
| 80.00 | 4 | | 402.77 |
| 70.00 | 4 | | 465.80 |
| Sig. | | 0.34 | 0.25 |

| TIME15 | N | Subset for alpha = .05 | | |
|---|---|---|---|---|
| TEMP | | 1.00 | 2.00 | |
| 100.00 | 4 | 0.00 | | |
| 50.00 | 4 | 0.37 | | |
| 60.00 | 4 | 1.33 | | |
| 90.00 | 3 | 14.38 | | |
| 40.00 | 6 | 208.36 | 208.36 | |
| 80.00 | 4 | | 448.24 | |
| 70.00 | 4 | | 500.57 | |
| Sig. | | 0.48 | 0.14 | |

| TIME20 | N | Subset for alpha = .05 | | |
|---|---|---|---|---|
| TEMP | | 1.00 | 2.00 | 3.00 |
| 100.00 | 4 | 0.00 | | |
| 50.00 | 4 | 0.35 | | |
| 60.00 | 4 | 1.60 | | |
| 90.00 | 4 | 6.41 | | |
| 40.00 | 6 | 164.01 | 164.01 | |
| 80.00 | 4 | | 404.82 | 404.82 |
| 70.00 | 4 | | | 501.01 |
| Sig. | | 0.59 | 0.18 | 0.94 |

| TIME25 | N | Subset for alpha = .05 | | |
|---|---|---|---|---|
| TEMP | | 1.00 | 2.00 | 3.00 |
| 100.00 | 4 | 0.00 | | |
| 50.00 | 4 | 0.14 | | |
| 90.00 | 4 | 2.29 | | |
| 60.00 | 4 | 7.62 | | |
| 40.00 | 5 | 143.80 | 143.80 | |
| 80.00 | 4 | | 366.02 | 366.02 |
| 70.00 | 4 | | | 513.52 |
| Sig. | | 0.77 | 0.32 | 0.75 |

| TIME30 | N | Subset for alpha = .05 | | |
|---|---|---|---|---|
| TEMP | | 1.00 | 2.00 | 3.00 |
| 100.00 | 4 | 0.00 | | |
| 50.00 | 4 | 0.23 | | |
| 90.00 | 4 | 1.64 | | |
| 60.00 | 4 | 29.14 | | |
| 40.00 | 6 | 123.41 | 123.41 | |
| 80.00 | 4 | | 327.74 | 327.74 |
| 70.00 | 4 | | | 445.24 |
| Sig. | | 0.83 | 0.32 | 0.86 |

Means for groups in homogeneous subsets are displayed

a Uses Harmonic Mean Sample Size = 4.200.

b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed

**Statistics for PPO activity in western rock lobster hemolymph under unsteady sate heating**

*Table 38: Descriptive statistics for total PPO activity in western rock lobster hemolymph under unsteady state conditions*

| | N | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|
| T0 | 8.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| T1 | 8.00 | 91.17 | 114.06 | 102.44 | 6.80 |
| T2 | 8.00 | 95.23 | 118.30 | 105.77 | 7.43 |
| T3 | 8.00 | 94.91 | 127.80 | 110.72 | 10.64 |
| T4 | 8.00 | 65.16 | 132.97 | 103.53 | 21.35 |
| T5 | 8.00 | 24.15 | 60.07 | 44.15 | 13.12 |
| T6 | 8.00 | 0.00 | 16.26 | 7.22 | 5.91 |
| T7 | 8.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| T8 | 8.00 | 0.00 | 529.80 | 177.04 | 211.36 |
| Valid N (listwise) | 8.00 | | | | |

*Table 39: Repeated measures ANOVA statistics for total PPO activity in western rock lobster hemolymph under unsteady state heating*

| Source | TEMP | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| TEMP | Linear | 9212.11 | 1.00 | 9212.11 | 0.77 | 0.41 |
| | Quadratic | 23721.35 | 1.00 | 23721.35 | 1.94 | 0.21 |
| | Cubic | 109199.38 | 1.00 | 109199.38 | 12.10 | 0.01 |
| | Order 4 | 55944.86 | 1.00 | 55944.86 | 11.69 | 0.01 |
| | Order 5 | 3333.79 | 1.00 | 3333.79 | 2.30 | 0.17 |
| | Order 6 | 16.16 | 1.00 | 16.16 | 0.04 | 0.84 |
| | Order 7 | 555.27 | 1.00 | 555.27 | 6.10 | 0.04 |
| | Order 8 | 888.75 | 1.00 | 888.75 | 5.81 | 0.05 |
| Error(TEMP) | Linear | 83297.46 | 7.00 | 11899.64 | | |
| | Quadratic | 85574.10 | 7.00 | 12224.87 | | |
| | Cubic | 63150.02 | 7.00 | 9021.43 | | |
| | Order 4 | 33487.72 | 7.00 | 4783.96 | | |
| | Order 5 | 10138.64 | 7.00 | 1448.38 | | |
| | Order 6 | 2584.80 | 7.00 | 369.26 | | |
| | Order 7 | 636.80 | 7.00 | 90.97 | | |
| | Order 8 | 1070.65 | 7.00 | 152.95 | | |

**Tests of Between-Subjects Effects**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig |
|---|---|---|---|---|---|
| Intercept | 501164.00 | 1 | 501164.00 | 90.15 | .000 |
| Error | 38914.62 | 7 | 5559.23 | | |

*Table 40: Descriptive statistics for baseline PPO activity in western rock lobster hemolymph under unsteady state heating*

| | N | Minimum | Maximum | Mean | Std. Dev |
|---|---|---|---|---|---|
| T0 | 8.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| T1 | 8.00 | 59.30 | 281.54 | 159.24 | 82.19 |
| T2 | 8.00 | 0.00 | 43.47 | 19.17 | 15.38 |
| T3 | 8.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| T4 | 8.00 | 127.66 | 529.80 | 291.78 | 158.54 |
| T5 | 8.00 | 127.97 | 549.43 | 303.24 | 147.22 |
| T6 | 8.00 | 0.00 | 208.98 | 67.51 | 63.61 |
| T7 | 8.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| T8 | 8.00 | 0.00 | 529.80 | 177.04 | 211.36 |
| Valid N (listwise) | 8.00 | | | | |

*Table 41: Repeated measures ANOVA statistics for baseline PPO activity in western rock lobster hemolymph under unsteady state heating*

| Source | TEMP | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| TEMP | Linear | 7074.57 | 1.00 | 7074.57 | 0.57 | 0.48 |
| | Quadratic | 22823.79 | 1.00 | 22823.79 | 0.76 | 0.41 |
| | Cubic | 10953.70 | 1.00 | 10953.70 | 0.77 | 0.41 |
| | Order 4 | 228522.96 | 1.00 | 228522.96 | 36.62 | 0.00 |
| | Order 5 | 424334.15 | 1.00 | 424334.15 | 47.37 | 0.00 |
| | Order 6 | 110289.79 | 1.00 | 110289.79 | 10.22 | 0.02 |
| | Order 7 | 59966.87 | 1.00 | 59966.87 | 18.40 | 0.00 |
| | Order 8 | 14763.76 | 1.00 | 14763.76 | 1.70 | 0.23 |
| Error (TEMP) | Linear | 87436.40 | 7.00 | 12490.91 | | |
| | Quadratic | 210575.53 | 7.00 | 30082.22 | | |
| | Cubic | 100212.75 | 7.00 | 14316.11 | | |
| | Order 4 | 43687.37 | 7.00 | 6241.05 | | |
| | Order 5 | 62711.78 | 7.00 | 8958.83 | | |
| | Order 6 | 75553.14 | 7.00 | 10793.31 | | |
| | Order 7 | 22,808.44 | 7.00 | 3,258.35 | | |
| | Order 8 | 60,970.95 | 7.00 | 8,710.14 | | |

**Tests of Between-Subjects Effects**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Intercept | 1,110,993 | 1.00 | 1,110,992.69 | 144.91 | 0.00 |
| Error | 53,667 | 7.00 | 7,666.78 | | |

**Statistics for evaluation of transportation and holding**

*Table 42: Descriptives of holding protein values by field trip*

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Initial Protein | Field trip 1 | 20 | 86.9805 | 14.8514 | 3.3209 | 80.0298 | 93.9312 | 45.50 | 104.12 |
| | Field trip 3 | 20 | 79.9820 | 7.9551 | 1.7788 | 76.2589 | 83.7051 | 70.26 | 99.97 |
| | Fieldtrip 4 | 20 | 89.4355 | 33.6340 | 7.5208 | 73.6943 | 105.1767 | 54.05 | 164.80 |
| | Total | 60 | 85.4660 | 21.7261 | 2.8048 | 79.8536 | 91.0784 | 45.50 | 164.80 |
| Protein post hold | Field trip 1 | 20 | 87.8905 | 16.1463 | 3.6104 | 80.3338 | 95.4472 | 40.08 | 115.64 |
| | Field trip 3 | 20 | 77.9550 | 15.1855 | 3.3956 | 70.8479 | 85.0621 | 56.75 | 110.77 |
| | Fieldtrip 4 | 20 | 100.3760 | 25.5545 | 5.7142 | 88.4161 | 112.3359 | 62.15 | 153.99 |
| | Total | 60 | 88.7405 | 21.3093 | 2.7510 | 83.2357 | 94.2453 | 40.08 | 153.99 |
| Protein post drown | Field trip 1 | 20 | 77.1355 | 14.1964 | 3.1744 | 70.4914 | 83.7796 | 36.60 | 100.32 |
| | Field trip 3 | 20 | 71.0665 | 15.0550 | 3.3664 | 64.0206 | 78.1124 | 40.54 | 110.77 |
| | Fieldtrip 4 | 20 | 75.3900 | 25.5343 | 5.7096 | 63.4396 | 87.3404 | 43.24 | 135.09 |
| | Total | 60 | 74.5307 | 18.8275 | 2.4306 | 69.6670 | 79.3943 | 36.60 | 135.09 |

*Table 43: Oneway anova of holding protein values by field trip*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Initial Protein | Between Groups | 962.498 | 2 | 481.249 | 1.020 | .367 |
| | Within Groups | 26886.822 | 57 | 471.699 | | |
| | Total | 27849.319 | 59 | | | |
| Protein post hold | Between Groups | 5048.687 | 2 | 2524.344 | 6.618 | .003 |
| | Within Groups | 21742.396 | 57 | 381.446 | | |
| | Total | 26791.083 | 59 | | | |
| Protein post drown | Between Groups | 390.481 | 2 | 195.241 | .542 | .584 |
| | Within Groups | 20523.593 | 57 | 360.063 | | |
| | Total | 20914.074 | 59 | | | |

*Table 44: Descriptives of total PPO activity values by field trip*

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Total PO initial | Field trip 1 | 20 | 21.4605 | 6.3884 | 1.4285 | 18.4706 | 24.4504 | 9.84 | 32.21 |
| | Field trip 3 | 20 | 18.3975 | 5.7925 | 1.2952 | 15.6865 | 21.1085 | 10.92 | 30.64 |
| | Fieldtrip 4 | 19 | 13.6489 | 6.7955 | 1.5590 | 10.3736 | 16.9243 | 5.12 | 35.45 |
| | Total | 59 | 17.9066 | 7.0049 | .9120 | 16.0811 | 19.7321 | 5.12 | 35.45 |
| Total PO post hold | Field trip 1 | 20 | 33.2785 | 9.5106 | 2.1266 | 28.8274 | 37.7296 | 20.60 | 54.53 |
| | Field trip 3 | 20 | 16.5170 | 5.1273 | 1.1465 | 14.1173 | 18.9167 | 6.67 | 24.08 |
| | Fieldtrip 4 | 20 | 17.2450 | 10.6176 | 2.3742 | 12.2758 | 22.2142 | 6.37 | 42.66 |
| | Total | 60 | 22.3468 | 11.6083 | 1.4986 | 19.3481 | 25.3456 | 6.37 | 54.53 |
| Total PO post drown | Field trip 1 | 20 | 17.9825 | 5.9035 | 1.3201 | 15.2196 | 20.7454 | 8.31 | 29.79 |
| | Field trip 3 | 18 | 17.9372 | 6.9761 | 1.6443 | 14.4681 | 21.4064 | 7.02 | 39.36 |
| | Fieldtrip 4 | 20 | 14.0620 | 6.8196 | 1.5249 | 10.8703 | 17.2537 | 3.41 | 32.76 |
| | Total | 58 | 16.6166 | 6.7178 | .8821 | 14.8502 | 18.3829 | 3.41 | 39.36 |

*Table 45: Oneway ANOVA of total PPO activity by field trip*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Total PO initial | Between Groups | 601.848 | 2 | 300.924 | 7.509 | .001 |
| | Within Groups | 2244.147 | 56 | 40.074 | | |
| | Total | 2845.996 | 58 | | | |
| Total PO post hold | Between Groups | 3590.340 | 2 | 1795.170 | 23.469 | .000 |
| | Within Groups | 4360.006 | 57 | 76.491 | | |
| | Total | 7950.346 | 59 | | | |
| Total PO post drown | Between Groups | 199.226 | 2 | 99.613 | 2.309 | .109 |
| | Within Groups | 2373.137 | 55 | 43.148 | | |
| | Total | 2572.363 | 57 | | | |

*Table 46: Correlations of total PPO activity to melanosis factors*

| | | area melanosis | intensity melanosis | rate melanosis | Total PO initial | Total PO post hold | Total PO post drown |
|---|---|---|---|---|---|---|---|
| **area melanosis** | Pearson Correlation | 1 | .427 | .922 | .264 | .205 | .009 |
| | Sig. (2-tailed) | . | .001 | .000 | .044 | .116 | .949 |
| | N | 62 | 62 | 62 | 59 | 60 | 58 |
| **intensity melanosis** | Pearson Correlation | .427 | 1 | .394 | -.029 | .107 | -.072 |
| | Sig. (2-tailed) | .001 | . | .002 | .828 | .416 | .594 |
| | N | 62 | 62 | 62 | 59 | 60 | 58 |
| **rate melanosis** | Pearson Correlation | .922 | .394 | 1 | .339 | .293 | .024 |
| | Sig. (2-tailed) | .000 | .002 | . | .009 | .023 | .857 |
| | N | 62 | 62 | 62 | 59 | 60 | 58 |
| **Total PO initial** | Pearson Correlation | .264 | -.029 | .339 | 1 | .153 | .077 |
| | Sig. (2-tailed) | .044 | .828 | .009 | . | .247 | .571 |
| | N | 59 | 59 | 59 | 59 | 59 | 57 |
| **Total PO post hold** | Pearson Correlation | .205 | .107 | .293 | .153 | 1 | .061 |
| | Sig. (2-tailed) | .116 | .416 | .023 | .247 | . | .648 |
| | N | 60 | 60 | 60 | 59 | 60 | 58 |
| **Total PO post drown** | Pearson Correlation | .009 | -.072 | .024 | .077 | .061 | 1 |
| | Sig. (2-tailed) | .949 | .594 | .857 | .571 | .648 | . |
| | N | 58 | 58 | 58 | 57 | 58 | 58 |

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

*Table 47: Descriptives of Baseline PPO activity by field trip*

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Baseline PO initial | Field trip 1 | 20 | 4.8475 | 2.3183 | .5184 | 3.7625 | 5.9325 | 1.31 | 10.07 |
| | Field trip 3 | 20 | 6.1620 | 2.1390 | .4783 | 5.1609 | 7.1631 | 2.65 | 10.74 |
| | Fieldtrip 4 | 19 | 5.2363 | 1.6250 | .3728 | 4.4531 | 6.0196 | 1.83 | 7.55 |
| | Total | 59 | 5.4183 | 2.0959 | .2729 | 4.8721 | 5.9645 | 1.31 | 10.74 |
| Baseline PO post hold | Field trip 1 | 20 | 1.1370 | .8718 | .1949 | .7290 | 1.5450 | .13 | 3.18 |
| | Field trip 3 | 20 | 4.9035 | 2.5599 | .5724 | 3.7054 | 6.1016 | .64 | 9.47 |
| | Fieldtrip 4 | 20 | 4.7225 | 1.9518 | .4364 | 3.8090 | 5.6360 | 1.04 | 7.29 |
| | Total | 60 | 3.5877 | 2.5771 | .3327 | 2.9219 | 4.2534 | .13 | 9.47 |
| Baseline PO post drown | Field trip 1 | 20 | 5.3490 | 3.6883 | .8247 | 3.6228 | 7.0752 | 1.22 | 11.91 |
| | Field trip 3 | 18 | 13.0111 | 6.2474 | 1.4725 | 9.9043 | 16.1179 | 2.43 | 23.99 |
| | Fieldtrip 4 | 20 | 7.3955 | 2.3527 | .5261 | 6.2944 | 8.4966 | 3.04 | 10.87 |
| | Total | 58 | 8.4326 | 5.3248 | .6992 | 7.0325 | 9.8327 | 1.22 | 23.99 |

*Table 48: Oneway ANOVA of baseline PPO activity by field trip*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Baseline PO initial | Between Groups | 18.207 | 2 | 9.104 | 2.155 | .125 |
| | Within Groups | 236.578 | 56 | 4.225 | | |
| | Total | 254.785 | 58 | | | |
| Baseline PO post hold | Between Groups | 180.501 | 2 | 90.250 | 24.342 | .000 |
| | Within Groups | 211.330 | 57 | 3.708 | | |
| | Total | 391.831 | 59 | | | |
| Baseline PO post drown | Between Groups | 589.013 | 2 | 294.507 | 15.770 | .000 |
| | Within Groups | 1027.160 | 55 | 18.676 | | |
| | Total | 1616.173 | 57 | | | |

*Table 49: Correlations of baseline PPO activity to melanosis factors*

| | | area melanosis | intensity melanosis | rate melanosis | Baseline PO initial | Baseline PO post hold | Baseline PO post drown |
|---|---|---|---|---|---|---|---|
| **area melanosis** | Pearson Correlation | 1 | .427 | .922 | -.019 | -.248 | -.146 |
| | Sig. (2-tailed) | . | .001 | .000 | .889 | .056 | .274 |
| | N | 62 | 62 | 62 | 59 | 60 | 58 |
| **intensity melanosis** | Pearson Correlation | .427 | 1 | .394 | -.039 | -.266 | .121 |
| | Sig. (2-tailed) | .001 | . | .002 | .768 | .040 | .365 |
| | N | 62 | 62 | 62 | 59 | 60 | 58 |
| **rate melanosis** | Pearson Correlation | .922 | .394 | 1 | -.031 | -.289 | -.171 |
| | Sig. (2-tailed) | .000 | .002 | . | .817 | .025 | .200 |
| | N | 62 | 62 | 62 | 59 | 60 | 58 |
| **Baseline PO initial** | Pearson Correlation | -.019 | -.039 | -.031 | 1 | .077 | .194 |
| | Sig. (2-tailed) | .889 | .768 | .817 | . | .561 | .149 |
| | N | 59 | 59 | 59 | 59 | 59 | 57 |
| **Baseline PO post hold** | Pearson Correlation | -.248 | -.266 | -.289 | .077 | 1 | .150 |
| | Sig. (2-tailed) | .056 | .040 | .025 | .561 | . | .262 |
| | N | 60 | 60 | 60 | 59 | 60 | 58 |
| **Baseline PO post drown** | Pearson Correlation | -.146 | .121 | -.171 | .194 | .150 | 1 |
| | Sig. (2-tailed) | .274 | .365 | .200 | .149 | .262 | . |
| | N | 58 | 58 | 58 | 57 | 58 | 58 |

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

*Table 50: Descriptives of total phenol content by field trip*

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Todal Phenols Initial | Field trip 1 | 20 | 39.8530 | 10.4342 | 2.3332 | 34.9696 | 44.7364 | 16.18 | 61.76 |
| | Field trip 3 | 20 | 55.6580 | 19.7355 | 4.4130 | 46.4215 | 64.8945 | 21.73 | 84.73 |
| | Fieldtrip 4 | 20 | 93.3305 | 41.3122 | 9.2377 | 73.9958 | 112.6652 | 45.09 | 216.76 |
| | Total | 60 | 62.9472 | 34.9548 | 4.5126 | 53.9174 | 71.9770 | 16.18 | 216.76 |
| Total Phenols post hold | Field trip 1 | 20 | 68.5925 | 14.6824 | 3.2831 | 61.7209 | 75.4641 | 54.25 | 108.49 |
| | Field trip 3 | 20 | 62.0120 | 31.1781 | 6.9716 | 47.4202 | 76.6038 | 9.03 | 117.03 |
| | Fieldtrip 4 | 20 | 69.6275 | 54.1810 | 12.1152 | 44.2700 | 94.9850 | 34.24 | 289.01 |
| | Total | 60 | 66.7440 | 36.5976 | 4.7247 | 57.2898 | 76.1982 | 9.03 | 289.01 |
| Total phenols post drown | Field trip 1 | 20 | 64.4670 | 18.8729 | 4.2201 | 55.6342 | 73.2998 | .00 | 91.98 |
| | Field trip 3 | 20 | 71.1440 | 23.9902 | 5.3644 | 59.9163 | 82.3717 | 39.98 | 134.10 |
| | Fieldtrip 4 | 20 | 89.4960 | 37.4534 | 8.3748 | 71.9673 | 107.0247 | 39.81 | 161.36 |
| | Total | 60 | 75.0357 | 29.4220 | 3.7984 | 67.4351 | 82.6362 | .00 | 161.36 |

*Table 51: Oneway ANOVA of total phenols content by filed trip*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Total Phenols Initial | Between Groups | 30192.389 | 2 | 15096.194 | 20.539 | .000 |
| | Within Groups | 41896.084 | 57 | 735.019 | | |
| | Total | 72088.473 | 59 | | | |
| Total Phenols post hold | Between Groups | 682.467 | 2 | 341.233 | .248 | .781 |
| | Within Groups | 78341.327 | 57 | 1374.409 | | |
| | Total | 79023.794 | 59 | | | |
| Total phenols post drown | Between Groups | 6718.860 | 2 | 3359.430 | 4.317 | .018 |
| | Within Groups | 44354.892 | 57 | 778.156 | | |
| | Total | 51073.753 | 59 | | | |

*Table 52: Correlations of total phenols content to melanosis factors*

| | | area melanosis | intensity melanosis | rate melanosis | Todal Phenols Initial | Total Phenols post hold | Total phenols post drown |
|---|---|---|---|---|---|---|---|
| area melanosis | Pearson Correlation | 1 | .427 | .922 | -.188 | .034 | -.045 |
| | Sig. (2-tailed) | . | .001 | .000 | .150 | .798 | .732 |
| | N | 62 | 62 | 62 | 60 | 60 | 60 |
| intensity melanosis | Pearson Correlation | .427 | 1 | .394 | -.035 | .056 | -.063 |
| | Sig. (2-tailed) | .001 | . | .002 | .789 | .670 | .630 |
| | N | 62 | 62 | 62 | 60 | 60 | 60 |
| rate melanosis | Pearson Correlation | .922 | .394 | 1 | -.194 | .070 | -.104 |
| | Sig. (2-tailed) | .000 | .002 | . | .138 | .596 | .431 |
| | N | 62 | 62 | 62 | 60 | 60 | 60 |
| Todal Phenols Initial | Pearson Correlation | -.188 | -.035 | -.194 | 1 | .114 | .134 |
| | Sig. (2-tailed) | .150 | .789 | .138 | . | .387 | .306 |
| | N | 60 | 60 | 60 | 60 | 60 | 60 |
| Total Phenols post hold | Pearson Correlation | .034 | .056 | .070 | .114 | 1 | -.130 |
| | Sig. (2-tailed) | .798 | .670 | .596 | .387 | . | .321 |
| | N | 60 | 60 | 60 | 60 | 60 | 60 |
| Total phenols post drown | Pearson Correlation | -.045 | -.063 | -.104 | .134 | -.130 | 1 |
| | Sig. (2-tailed) | .732 | .630 | .431 | .306 | .321 | . |
| | N | 60 | 60 | 60 | 60 | 60 | 60 |

** Correlation is significant at the 0.01 level (2-tailed).

*Table 53: Descriptives of change in weight after transportation*

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| A: Spray, recirc, chill | 80 | -.2093 | 3.01485 | .33707 | -.8802 | .4616 | -14.36 | 19.09 |
| B: Submerge, flow thru, ambient | 80 | -2.9674 | 25.06788 | 2.80267 | -8.5460 | 2.6112 | -218.45 | 7.59 |
| C: Spray, flow thru, ambient | 80 | .7939 | 10.24382 | 1.14529 | -1.4857 | 3.0736 | -5.46 | 89.87 |
| D: Air,still,chilled | 80 | 4.1197 | 8.88502 | .99338 | 2.1424 | 6.0969 | .59 | 77.56 |
| Total | 320 | .4342 | 14.48638 | .80981 | -1.1590 | 2.0275 | -218.45 | 89.87 |

*Table 54: Oneway ANOVA of change in weight after transportation*

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 2055.777 | 3 | 685.259 | 3.337 | .020 |
| Within Groups | 64888.015 | 316 | 205.342 | | |
| Total | 66943.792 | 319 | | | |

*Table 55: Tukey's homogenous subsets for change in weight by transportation method*

| | N | Subset for alpha = .05 | |
|---|---|---|---|
| **TREAT** | | **1** | **2** |
| B: Submerge, flow thru, ambient | 80 | -2.9674 | |
| A: Spray, recirc, chill | 80 | -.2093 | -.2093 |
| C: Spray, flow thru, ambient | 80 | .7939 | .7939 |
| D: Air,still,chilled | 80 | | 4.1197 |
| Sig. | | .347 | .226 |

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 80.000.

*Table 56: Descriptives of impact of transportation method on final weight post processing*

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| A: Spray, recirc, chill | 72 | 448.9637 | 44.21565 | 5.21086 | 438.5736 | 459.3539 | 302.79 | 534.88 |
| B: Submerge, flow thru, ambient | 67 | 449.5793 | 42.17191 | 5.15212 | 439.2927 | 459.8658 | 361.26 | 557.85 |
| C: Spray, flow thru, ambient | 72 | 443.1372 | 50.01447 | 5.89426 | 431.3844 | 454.8900 | 335.82 | 576.31 |
| D: Air,still,chilled | 73 | 440.2632 | 49.09293 | 5.74589 | 428.8089 | 451.7174 | 300.57 | 554.87 |
| Total | 284 | 445.3954 | 46.49162 | 2.75877 | 439.9651 | 450.8257 | 300.57 | 576.31 |

*Table 57: Oneway ANOVA of final weight by transportation method*

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 4379.568 | 3 | 1459.856 | .673 | .569 |
| Within Groups | 607316.767 | 280 | 2168.988 | | |
| Total | 611696.335 | 283 | | | |

**Statistics for evaluation of current processing**

*Table 58: Descriptive Statistics for initial physiological parameters*

| Gender | | Total loss leg females | Total loss legs males | receipt weight | post drown weight | post cooling weight | initial total PO | initial baseline PO | Protein |
|---|---|---|---|---|---|---|---|---|---|
| F | M | | | | | | | | |
| 161 | 160 | 133 | 133 | | | | | | |
| | max | 5.0 | 6.0 | 572 | 620.0 | 320.00 | 47.61 | 15.84 | 170.20 |
| | min | 0 | 0 | 380 | 405.0 | 455.71 | 1.60 | 0.20 | 21.91 |
| | average | 0.83 | 0.83 | 459.38 | 488.44 | 2.87 | 18.29 | 4.43 | 86.67 |
| | Std.err | 0.084 | 0.094 | 2.35 | 2.48 | 320.00 | 0.49 | 0.15 | 1.35 |

*Table 59: ANOVA comparison by field trip of initial physiological parameters*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| PROTEIN | Between Groups | 5433.76 | 2 | 2716.88 | 5.770 | .004 |
| | Within Groups | 48967.29 | 104 | 470.84 | | |
| | Total | 54401.05 | 106 | | | |
| INTTOT | Between Groups | 16.15 | 2 | 8.074 | .108 | .897 |
| | Within Groups | 7972.85 | 107 | 74.51 | | |
| | Total | 7989.0 | 109 | | | |
| INTBASE | Between Groups | 10.04 | 2 | 5.02 | .920 | .401 |
| | Within Groups | 583.76 | 107 | 5.46 | | |
| | Total | 593.80 | 109 | | | |

*Table 60: Independent Samples Test of initial physiological parameters by gender*

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | 95% CI of the Difference | |
|---|---|---|---|---|---|---|---|---|---|
| | | F | Sig | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| Initial Protein | Equal variances assumed | 0.18 | 0.67 | 0.49 | 58.00 | 0.62 | 2.93 | 5.92 | -8.92 | 14.77 |
| | Equal variances not assumed | | | 0.48 | 37.58 | 0.64 | 2.93 | 6.11 | -9.44 | 15.29 |
| Total PO initial | Equal variances assumed | 2.81 | 0.10 | 1.33 | 57.00 | 0.19 | 2.51 | 1.89 | -1.28 | 6.30 |
| | Equal variances not assumed | | | 1.23 | 33.30 | 0.23 | 2.51 | 2.04 | -1.64 | 6.66 |
| Baseline PO initial | Equal variances assumed | 0.00 | 0.95 | 2.50 | 57.00 | 0.02 | 1.36 | 0.55 | 0.27 | 2.46 |
| | Equal variances not assumed | | | 2.50 | 41.59 | 0.02 | 1.36 | 0.54 | 0.26 | 2.46 |

*Table 61: Descriptive statistics of melanosis for commercial processing*

| | N | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|
| AREA | 115 | .00 | 21.32 | 6.30 | 4.70 |
| INTENSE | 115 | 77.60 | 223.84 | 176.15 | 22.03 |
| RATE | 115 | .00 | .11 | .017 | .017 |
| Valid N (listwise) | 115 | | | | |

*Table 62: ANOVA statistics for initial physiological parameters and melanosis by grade in commercial processing*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| PROTEIN | Between Groups | 41.60 | 1.00 | 41.60 | 0.08 | 0.78 |
| | Within Groups | 54359.45 | 105.00 | 517.71 | | |
| | Total | 54401.05 | 106.00 | | | |
| INTTOT | Between Groups | 142.48 | 1.00 | 142.48 | 1.97 | 0.16 |
| | Within Groups | 7,723.16 | 107.00 | 72.18 | | |
| | Total | 7,865.65 | 108.00 | | | |
| INTBASE | Between Groups | 1.23 | 1.00 | 1.23 | 0.23 | 0.64 |
| | Within Groups | 585.26 | 107.00 | 5.47 | | |
| | Total | 586.49 | 108.00 | | | |
| AREA | Between Groups | 6.62 | 1.00 | 6.62 | 0.30 | 0.59 |
| | Within Groups | 2,400.48 | 107.00 | 22.43 | | |
| | Total | 2,407.10 | 108.00 | | | |
| INTENSE | Between Groups | 478.64 | 1.00 | 478.64 | 0.95 | 0.33 |
| | Within Groups | 53,984.09 | 107.00 | 504.52 | | |
| | Total | 54,462.74 | 108.00 | | | |
| RATE | Between Groups | 0.00 | 1.00 | 0.00 | 0.23 | 0.63 |
| | Within Groups | 0.03 | 107.00 | 0.00 | | |
| | Total | 0.03 | 108.00 | | | |

*Table 63: Correlations between physiological parameters and melanosis in standardised commercial processing*

| | | PROTEIN | INTTOT | INTBASE | AREA | INTENSE | RATE |
|---|---|---|---|---|---|---|---|
| PROTEIN | Pearson Correlation | 1.00 | 0.49 | 0.06 | 0.31 | 0.42 | 0.35 |
| | Sig. (2-tailed) | . | 0.00 | 0.57 | 0.00 | 0.00 | 0.00 |
| | Sum of Squares and Cross-products | 54401.05 | 10172.88 | 309.81 | 3496.07 | 22,727.99 | 14.83 |
| | Covariance | 513.22 | 95.97 | 2.92 | 32.98 | 214.42 | 0.14 |
| | N | 107.00 | 107.00 | 107.00 | 107.00 | 107.00 | 107.00 |
| INTTOT | Pearson Correlation | 0.49 | 1.00 | 0.11 | 0.22 | 0.18 | 0.22 |
| | Sig. (2-tailed) | 0.00 | . | 0.26 | 0.02 | 0.06 | 0.02 |
| | Sum of Squares and Cross-products | 10172.88 | 7865.65 | 234.26 | 943.61 | 3,696.24 | 3.61 |
| | Covariance | 95.97 | 72.83 | 2.17 | 8.74 | 34.22 | 0.03 |
| | N | 107.00 | 109.00 | 109.00 | 109.00 | 109.00 | 109.00 |
| INTBASE | Pearson Correlation | 0.06 | 0.11 | 1.00 | 0.01 | 0.00 | 0.01 |
| | Sig. (2-tailed) | 0.57 | 0.26 | . | 0.92 | 0.99 | 0.93 |
| | Sum of Squares and Cross-products | 309.81 | 234.26 | 586.49 | 11.16 | 10.26 | 0.04 |
| | Covariance | 2.92 | 2.17 | 5.43 | 0.10 | 0.10 | 0.00 |
| | N | 107.00 | 109.00 | 109.00 | 109.00 | 109.00 | 109.00 |
| | | **PROTEIN** | **INTTOT** | **INTBASE** | **AREA** | **INTENSE** | **RATE** |
| AREA | Pearson Correlation | 0.31 | 0.22 | 0.01 | 1.00 | 0.33 | 0.87 |
| | Sig. (2-tailed) | 0.00 | 0.02 | 0.92 | . | 0.00 | 0.00 |
| | Sum of Squares and Cross-products | 3,496.07 | 943.61 | 11.16 | 2,407.10 | 3,820.16 | 7.86 |
| | Covariance | 32.98 | 8.74 | 0.10 | 22.29 | 35.37 | 0.07 |
| | N | 107.00 | 109.00 | 109.00 | 109.00 | 109.00 | 109.00 |
| INTENSE | Pearson Correlation | 0.42 | 0.18 | 0.00 | 0.33 | 1.00 | 0.26 |
| | Sig. (2-tailed) | 0.00 | 0.06 | 0.99 | 0.00 | . | 0.01 |
| | Sum of Squares and Cross-products | 22,727.99 | 3,696.24 | 10.26 | 3,820.16 | 54,462.74 | 11.25 |
| | Covariance | 214.42 | 34.22 | 0.10 | 35.37 | 504.29 | 0.10 |
| | N | 107.00 | 109.00 | 109.00 | 109.00 | 109.00 | 109.00 |
| RATE | Pearson Correlation | 0.35 | 0.22 | 0.01 | 0.87 | 0.26 | 1.00 |
| | Sig. (2-tailed) | 0.00 | 0.02 | 0.93 | 0.00 | 0.01 | . |
| | Sum of Squares and Cross-products | 14.83 | 3.61 | 0.04 | 7.86 | 11.25 | 0.03 |
| | Covariance | 0.14 | 0.03 | 0.00 | 0.07 | 0.10 | 0.00 |
| | N | 107.00 | 109.00 | 109.00 | 109.00 | 109.00 | 109.00 |

** Correlation is significant at the 0.01 level (2-tailed).

- Correlation is significant at the 0.05 level (2-tailed).

*Table 64: Regression analysis of initial total PPO vs. area of melanosis*

| Model Summary: INTTOT vs. AREA | | | | |
|---|---|---|---|---|
| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate |
| 1.00 | 0.22 | 0.05 | 0.04 | 4.63 |

| ANOVA | | | | | | |
|---|---|---|---|---|---|---|
| **Model** | | **Sum of Squares** | **df** | **Mean Square** | **F** | **Sig.** |
| 1.00 | Regression | 113.20 | 1.00 | 113.20 | 5.28 | 0.02 |
| | Residual | 2293.90 | 107.00 | 21.44 | | |
| | Total | 2407.10 | 108.00 | | | |

*Table 65: Regression analysis of initial total PPO vs. rate of melanosis development*

| Model Summary INTOT vs. RATE | | | | |
|---|---|---|---|---|
| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate |
| 1.00 | 0.22 | 0.05 | 0.04 | 0.02 |

| ANOVA | | | | | | |
|---|---|---|---|---|---|---|
| **Model** | | **Sum of Squares** | **df** | **Mean Square** | **F** | **Sig.** |
| 1 | Regression | 0.00 | 1.00 | 0.00 | 5.56 | 0.02 |
| | Residual | 0.03 | 107.00 | 0.00 | | |
| | Total | 0.03 | 108.00 | | | |

*Table 66: ANOVA analysis of changes in weight during processing by grade*

| | | **Sum of Squares** | **df** | **Mean Square** | **F** | **Sig.** |
|---|---|---|---|---|---|---|
| Receipt weight | Between Groups | 104626.76 | 1.00 | 104626.76 | 270.26 | 0.00 |
| | Within Groups | 68908.99 | 178.00 | 387.13 | | |
| | Total | 173535.75 | 179.00 | | | |
| weight post drown | Between Groups | 122901.33 | 1.00 | 122901.33 | 317.86 | 0.00 |
| | Within Groups | 68,823.62 | 178.00 | 386.65 | | |
| | Total | 191,724.95 | 179.00 | | | |
| weight post cook | Between Groups | 133,579.56 | 1.00 | 133,579.56 | 221.89 | 0.00 |
| | Within Groups | 106,554.06 | 177.00 | 602.00 | | |
| | Total | 240,134 | 178.00 | | | |
| weight post cool | Between Groups | 117,208 | 1.00 | 117,208.33 | 120.19 | 0.00 |
| | Within Groups | 173,586 | 178.00 | 975.20 | | |
| | Total | 290,793.98 | 179.00 | | | |

*Table 67: Paired T-Test: Comparison of weights before and after drowning, cooking and cooling*

| Paired Differences | | Mean | Std. Dev | Std. Error Mean | 95% CI of the Difference | | t | df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|---|
| | | | . | | Lower | Upper | | | |
| Pair 1 | Receipt weight - weight post drown | -30.57 | 8.87 | 0.66 | -31.87 | -29.26 | -46.23 | 179 | 0.00 |
| Pair 2 | Receipt weight - weight post cook | -10.10 | 16.42 | 1.23 | -12.52 | -7.67 | -8.23 | 178 | 0.00 |
| Pair 3 | Receipt weight - weight post cool | -2.87 | 24.35 | 1.81 | -6.45 | 0.71 | -1.58 | 179 | 0.12 |
| Pair 4 | weight post drown - weight post cook | 20.42 | 15.61 | 1.17 | 18.12 | 22.73 | 17.50 | 178 | 0.00 |
| Pair 5 | weight post drown - weight post cool | 27.69 | 24.20 | 1.80 | 24.14 | 31.25 | 15.36 | 179 | 0.00 |
| Pair 6 | weight post cook - weight post cool | 7.37 | 14.57 | 1.09 | 5.22 | 9.52 | 6.77 | 178 | 0.00 |

*Table 68: Evaluation of physiological parameters in western rock lobster hemolymph post drown*

| | | Mean | N | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Pair 1 | Initial Protein | 85.47 | 60.00 | 21.73 | 2.80 |
| | Protein post drown | 74.53 | 60.00 | 18.83 | 2.43 |
| Pair 2 | Total PO initial | 17.71 | 57.00 | 6.96 | 0.92 |
| | Total PO post drown | 16.60 | 57.00 | 6.78 | 0.90 |
| Pair 3 | Baseline PO initial | 5.35 | 57.00 | 2.09 | 0.28 |
| | Baseline PO post drown | 8.47 | 57.00 | 5.36 | 0.71 |

| Paired Differences | | Mean | Std. Dev. | Std. Err. Mean | 95% CI of the Difference | | t | df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Lower | Upper | | | |
| Pair 1 | Initial Protein - Protein post drown | 10.94 | 30.36 | 3.92 | 3.09 | 18.78 | 2.79 | 59.00 | 0.01 |
| Pair 2 | Total PO initial - Total PO post drown | 1.11 | 9.33 | 1.24 | -1.37 | 3.59 | 0.90 | 56.00 | 0.37 |
| Pair 3 | Baseline PO initial - Baseline PO post drown | -3.12 | 5.37 | 0.71 | -4.55 | -1.70 | -4.40 | 56.00 | 0.00 |

*Table 69: Kruskal Wallis comparison of cooks in grade A*

| | CK1 | CK2 | CK3 | CK4 | CK5 | CK17 | CK18 | CK19 | CK20 | CK25 | CK26 | CK27 | CK28 | CK29 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chi-Square | 12.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 |
| df | 12.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 |
| Asymp. Sig. | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 |

*Table 70: Kruskal Wallis comparison of cooks in grade B*

| | CK6 | CK7 | CK8 | CK21 | CK22 | CK23 | CK30 | CK31 | CK32 |
|---|---|---|---|---|---|---|---|---|---|
| Chi-Square | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 |
| df | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 |
| Asymp. Sig. | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 |

a Kruskal Wallis Test
b Grouping Variable: PROBE

*Table 71:Kruskal-Wallis Test for probe grouped by cook Grade A*

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chi-Square | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 12.00 |
| df | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 12.00 |
| Asymp. Sig. | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 |

a Kruskal Wallis Test
b Grouping Variable: cook

*Table 72:Kruskal-Wallis Test Probe grouped by cook Grade B*

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chi-Square | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 |
| df | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 |
| Asymp. Sig. | 0.43 | 0.43 | 0.43 | 0.43 | 0.43 | 0.43 | 0.43 | 0.43 | 0.43 | 0.43 | 0.43 | 0.43 | 0.43 | 0.43 |

a Kruskal Wallis Test

b Grouping Variable: cook

*Table 73: ANOVA probe by field trip*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| A | Between Groups | 0.96 | 2.00 | 0.48 | 1.10 | 0.44 |
| | Within Groups | 1.32 | 3.00 | 0.44 | | |
| | Total | 2.28 | 5.00 | | | |
| B | Between Groups | 0.85 | 2.00 | 0.42 | 2.81 | 0.21 |
| | Within Groups | 0.45 | 3.00 | 0.15 | | |
| | Total | 1.30 | 5.00 | | | |
| C | Between Groups | 0.30 | 2.00 | 0.15 | 0.22 | 0.81 |
| | Within Groups | 2.06 | 3.00 | 0.69 | | |
| | Total | 2.37 | 5.00 | | | |
| D | Between Groups | 10.54 | 2.00 | 5.27 | 13.04 | 0.03 |
| | Within Groups | 1.21 | 3.00 | 0.40 | | |
| | Total | 11.75 | 5.00 | | | |
| E | Between Groups | 0.58 | 2.00 | 0.29 | 0.62 | 0.60 |
| | Within Groups | 1.40 | 3.00 | 0.47 | | |
| | Total | 1.98 | 5.00 | | | |
| F | Between Groups | 2.37 | 2.00 | 1.18 | 2.97 | 0.20 |
| | Within Groups | 1.20 | 3.00 | 0.40 | | |
| | Total | 3.56 | 5.00 | | | |
| G | Between Groups | 0.67 | 2.00 | 0.34 | 2.05 | 0.28 |
| | Within Groups | 0.49 | 3.00 | 0.16 | | |
| | Total | 1.16 | 5.00 | | | |
| H | Between Groups | 0.87 | 2.00 | 0.44 | 0.66 | 0.58 |
| | Within Groups | 1.98 | 3.00 | 0.66 | | |
| | Total | 2.85 | 5.00 | | | |
| I | Between Groups | 0.49 | 2.00 | 0.24 | 0.71 | 0.56 |
| | Within Groups | 1.02 | 3.00 | 0.34 | | |
| | Total | 1.51 | 5.00 | | | |
| J | Between Groups | 0.73 | 2.00 | 0.36 | 0.45 | 0.67 |
| | Within Groups | 2.40 | 3.00 | 0.80 | | |
| | Total | 3.13 | 5.00 | | | |
| K | Between Groups | 0.82 | 2.00 | 0.41 | 0.83 | 0.52 |
| | Within Groups | 1.47 | 3.00 | 0.49 | | |
| | Total | 2.29 | 5.00 | | | |
| L | Between Groups | 0.54 | 2.00 | 0.27 | 0.51 | 0.64 |
| | Within Groups | 1.59 | 3.00 | 0.53 | | |
| | Total | 2.13 | 5.00 | | | |
| M | Between Groups | 1.45 | 2.00 | 0.72 | 2.11 | 0.27 |
| | Within Groups | 1.03 | 3.00 | 0.34 | | |
| | Total | 2.47 | 5.00 | | | |
| N | Between Groups | 3.63 | 2.00 | 1.82 | 1.87 | 0.30 |
| | Within Groups | 2.92 | 3.00 | 0.97 | | |
| | Total | 6.55 | 5.00 | | | |

*Table 74: Descriptive statistics for differences in maximum temperature at probe position by grade*

| | | N | Mean | Std. Dev | Std. Err | 95% CI for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| A | 1.00 | 14.00 | 84.24 | 1.65 | 0.44 | 83.28 | 85.19 | 81.50 | 86.50 |
| | 2.00 | 9.00 | 80.03 | 3.44 | 1.15 | 77.39 | 82.68 | 75.60 | 85.70 |
| | Total | 23.00 | 82.59 | 3.21 | 0.67 | 81.20 | 83.98 | 75.60 | 86.50 |
| B | 1.00 | 13.00 | 82.95 | 3.54 | 0.98 | 80.82 | 85.09 | 77.50 | 87.90 |
| | 2.00 | 9.00 | 82.97 | 4.49 | 1.50 | 79.52 | 86.41 | 78.50 | 90.30 |
| | Total | 22.00 | 82.96 | 3.85 | 0.82 | 81.25 | 84.67 | 77.50 | 90.30 |
| C | 1.00 | 13.00 | 83.37 | 3.57 | 0.99 | 81.21 | 85.52 | 77.20 | 87.70 |
| | 2.00 | 9.00 | 83.18 | 4.27 | 1.42 | 79.89 | 86.46 | 77.50 | 90.10 |
| | Total | 22.00 | 83.29 | 3.77 | 0.80 | 81.62 | 84.96 | 77.20 | 90.10 |
| D | 1.00 | 14.00 | 84.06 | 3.24 | 0.87 | 82.19 | 85.93 | 77.50 | 89.00 |
| | 2.00 | 9.00 | 81.11 | 7.09 | 2.36 | 75.66 | 86.56 | 74.10 | 96.90 |
| | Total | 23.00 | 82.90 | 5.16 | 1.08 | 80.67 | 85.14 | 74.10 | 96.90 |
| E | 1.00 | 13.00 | 83.08 | 4.03 | 1.12 | 80.65 | 85.52 | 76.90 | 90.20 |
| | 2.00 | 9.00 | 80.87 | 3.71 | 1.24 | 78.02 | 83.72 | 77.50 | 87.30 |
| | Total | 22.00 | 82.18 | 3.97 | 0.85 | 80.42 | 83.94 | 76.90 | 90.20 |
| F | 1.00 | 13.00 | 82.58 | 2.66 | 0.74 | 80.97 | 84.19 | 79.30 | 89.10 |
| | 2.00 | 9.00 | 79.41 | 3.34 | 1.11 | 76.84 | 81.98 | 73.80 | 83.40 |
| | Total | 22.00 | 81.28 | 3.29 | 0.70 | 79.82 | 82.74 | 73.80 | 89.10 |
| G | 1.00 | 14.00 | 81.83 | 4.62 | 1.23 | 79.16 | 84.49 | 76.70 | 91.40 |
| | 2.00 | 9.00 | 81.98 | 2.33 | 0.78 | 80.19 | 83.77 | 78.90 | 85.10 |
| | Total | 23.00 | 81.89 | 3.82 | 0.80 | 80.24 | 83.54 | 76.70 | 91.40 |
| H | 1.00 | 13.00 | 81.92 | 3.56 | 0.99 | 79.76 | 84.07 | 75.30 | 88.10 |
| | 2.00 | 9.00 | 79.72 | 4.33 | 1.44 | 76.40 | 83.05 | 73.50 | 84.60 |
| | Total | 22.00 | 81.02 | 3.95 | 0.84 | 79.27 | 82.77 | 73.50 | 88.10 |
| I | 1.00 | 12.00 | 83.00 | 3.90 | 1.13 | 80.52 | 85.48 | 75.30 | 89.80 |
| | 2.00 | 9.00 | 81.62 | 2.18 | 0.73 | 79.95 | 83.30 | 78.70 | 85.70 |
| | Total | 21.00 | 82.41 | 3.28 | 0.72 | 80.92 | 83.90 | 75.30 | 89.80 |
| J | 1.00 | 13.00 | 83.78 | 3.64 | 1.01 | 81.58 | 85.99 | 76.70 | 89.20 |
| | 2.00 | 9.00 | 81.19 | 2.49 | 0.83 | 79.28 | 83.10 | 76.80 | 83.70 |
| | Total | 22.00 | 82.72 | 3.41 | 0.73 | 81.21 | 84.24 | 76.70 | 89.20 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| K | 1.00 | 14.00 | 83.96 | 3.47 | 0.93 | 81.95 | 85.96 | 78.60 | 90.10 |
| | 2.00 | 9.00 | 82.36 | 2.73 | 0.91 | 80.26 | 84.45 | 78.60 | 87.90 |
| | Total | 23.00 | 83.33 | 3.24 | 0.67 | 81.93 | 84.73 | 78.60 | 90.10 |
| L | 1.00 | 13.00 | 83.76 | 3.54 | 0.98 | 81.62 | 85.90 | 79.10 | 89.30 |
| | 2.00 | 9.00 | 81.27 | 4.78 | 1.59 | 77.59 | 84.94 | 73.40 | 89.20 |
| | Total | 22.00 | 82.74 | 4.18 | 0.89 | 80.89 | 84.59 | 73.40 | 89.30 |
| M | 1.00 | 13.00 | 84.19 | 3.22 | 0.89 | 82.25 | 86.14 | 79.30 | 88.60 |
| | 2.00 | 9.00 | 83.23 | 4.27 | 1.42 | 79.95 | 86.52 | 77.50 | 92.60 |
| | Total | 22.00 | 83.80 | 3.62 | 0.77 | 82.19 | 85.41 | 77.50 | 92.60 |
| N | 1.00 | 12.00 | 83.26 | 4.79 | 1.38 | 80.22 | 86.30 | 76.90 | 92.60 |
| | 2.00 | 9.00 | 82.97 | 3.23 | 1.08 | 80.48 | 85.45 | 79.20 | 86.40 |
| | Total | 21.00 | 83.13 | 4.10 | 0.89 | 81.27 | 85.00 | 76.90 | 92.60 |

*Table 75: ANOVA for differences in maximum temperature at probe position by grade*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| A | Between Groups | 96.75 | 1.00 | 96.75 | 15.65 | 0.00 |
| | Within Groups | 129.85 | 21.00 | 6.18 | | |
| | Total | 226.60 | 22.00 | | | |
| B | Between Groups | 0.00 | 1.00 | 0.00 | 0.00 | 0.99 |
| | Within Groups | 311.25 | 20.00 | 15.56 | | |
| | Total | 311.25 | 21.00 | | | |
| C | Between Groups | 0.20 | 1.00 | 0.20 | 0.01 | 0.91 |
| | Within Groups | 298.86 | 20.00 | 14.94 | | |
| | Total | 299.06 | 21.00 | | | |
| D | Between Groups | 47.55 | 1.00 | 47.55 | 1.86 | 0.19 |
| | Within Groups | 538.06 | 21.00 | 25.62 | | |
| | Total | 585.61 | 22.00 | | | |
| E | Between Groups | 26.16 | 1.00 | 26.16 | 1.72 | 0.21 |
| | Within Groups | 304.40 | 20.00 | 15.22 | | |
| | Total | 330.56 | 21.00 | | | |
| F | Between Groups | 53.30 | 1.00 | 53.30 | 6.11 | 0.02 |
| | Within Groups | 174.57 | 20.00 | 8.73 | | |
| | Total | 227.87 | 21.00 | | | |
| G | Between Groups | 0.12 | 1.00 | 0.12 | 0.01 | 0.93 |
| | Within Groups | 320.42 | 21.00 | 15.26 | | |
| | Total | 320.55 | 22.00 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| H | Between Groups | 25.58 | 1.00 | 25.58 | 1.69 | 0.21 |
| | Within Groups | 302.35 | 20.00 | 15.12 | | |
| | Total | 327.93 | 21.00 | | | |
| I | Between Groups | 9.76 | 1.00 | 9.76 | 0.90 | 0.35 |
| | Within Groups | 205.42 | 19.00 | 10.81 | | |
| | Total | 215.18 | 20.00 | | | |
| J | Between Groups | 35.83 | 1.00 | 35.83 | 3.44 | 0.08 |
| | Within Groups | 208.55 | 20.00 | 10.43 | | |
| | Total | 244.38 | 21.00 | | | |
| K | Between Groups | 14.05 | 1.00 | 14.05 | 1.36 | 0.26 |
| | Within Groups | 216.32 | 21.00 | 10.30 | | |
| | Total | 230.37 | 22.00 | | | |
| L | Between Groups | 33.10 | 1.00 | 33.10 | 1.98 | 0.17 |
| | Within Groups | 333.61 | 20.00 | 16.68 | | |
| | Total | 366.71 | 21.00 | | | |
| M | Between Groups | 4.89 | 1.00 | 4.89 | 0.36 | 0.55 |
| | Within Groups | 270.35 | 20.00 | 13.52 | | |
| | Total | 275.24 | 21.00 | | | |
| N | Between Groups | 0.44 | 1.00 | 0.44 | 0.03 | 0.88 |
| | Within Groups | 335.63 | 19.00 | 17.67 | | |
| | Total | 336.07 | 20.00 | | | |

*Table 76: Descriptive statistics for differences in minimum temperature at probe position by grade*

| | | | | | | **95% CI for Mean** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **N** | | **Mean** | **Std. Dev.** | **Std. Error** | | | **Min.** | **Max.** |
| | | | | | | Lower Bound | Upper Bound | | |
| A | 1.00 | 14.00 | 32.29 | 5.08 | 1.36 | 29.35 | 35.22 | 23.70 | 40.70 |
| | 2.00 | 9.00 | 38.69 | 3.18 | 1.06 | 36.25 | 41.13 | 33.80 | 42.40 |
| | Total | 23.00 | 34.79 | 5.40 | 1.13 | 32.46 | 37.13 | 23.70 | 42.40 |
| B | 1.00 | 13.00 | 36.78 | 6.76 | 1.87 | 32.69 | 40.86 | 22.60 | 45.70 |
| | 2.00 | 9.00 | 38.21 | 5.24 | 1.75 | 34.18 | 42.24 | 27.70 | 45.10 |
| | Total | 22.00 | 37.36 | 6.09 | 1.30 | 34.66 | 40.06 | 22.60 | 45.70 |
| C | 1.00 | 13.00 | 34.52 | 6.24 | 1.73 | 30.75 | 38.29 | 22.40 | 43.80 |
| | 2.00 | 9.00 | 37.42 | 4.04 | 1.35 | 34.31 | 40.53 | 31.40 | 44.10 |
| | Total | 22.00 | 35.71 | 5.53 | 1.18 | 33.26 | 38.16 | 22.40 | 44.10 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| D | 1.00 | 14.00 | 37.84 | 6.23 | 1.66 | 34.24 | 41.43 | 23.60 | 45.60 |
| | 2.00 | 9.00 | 42.74 | 4.20 | 1.40 | 39.52 | 45.97 | 33.00 | 47.30 |
| | Total | 23.00 | 39.76 | 5.94 | 1.24 | 37.19 | 42.33 | 23.60 | 47.30 |
| E | 1.00 | 13.00 | 37.64 | 4.06 | 1.13 | 35.19 | 40.09 | 28.30 | 43.70 |
| | 2.00 | 9.00 | 41.39 | 5.39 | 1.80 | 37.24 | 45.53 | 32.70 | 50.20 |
| | Total | 22.00 | 39.17 | 4.90 | 1.05 | 37.00 | 41.35 | 28.30 | 50.20 |
| F | 1.00 | 13.00 | 38.75 | 7.84 | 2.18 | 34.01 | 43.49 | 22.60 | 46.00 |
| | 2.00 | 9.00 | 40.47 | 6.02 | 2.01 | 35.84 | 45.09 | 25.30 | 45.30 |
| | Total | 22.00 | 39.45 | 7.05 | 1.50 | 36.33 | 42.58 | 22.60 | 46.00 |
| G | 1.00 | 14.00 | 37.93 | 7.79 | 2.08 | 33.43 | 42.43 | 22.50 | 46.40 |
| | 2.00 | 9.00 | 39.24 | 4.14 | 1.38 | 36.06 | 42.43 | 30.90 | 43.70 |
| | Total | 23.00 | 38.44 | 6.52 | 1.36 | 35.62 | 41.26 | 22.50 | 46.40 |
| H | 1.00 | 13.00 | 37.51 | 6.96 | 1.93 | 33.30 | 41.71 | 23.70 | 46.60 |
| | 2.00 | 9.00 | 41.44 | 3.47 | 1.16 | 38.78 | 44.11 | 36.60 | 48.00 |
| | Total | 22.00 | 39.12 | 6.01 | 1.28 | 36.45 | 41.78 | 23.70 | 48.00 |
| I | 1.00 | 12.00 | 37.66 | 4.98 | 1.44 | 34.50 | 40.82 | 29.50 | 45.60 |
| | 2.00 | 9.00 | 40.40 | 4.06 | 1.35 | 37.28 | 43.52 | 30.70 | 44.70 |
| | Total | 21.00 | 38.83 | 4.71 | 1.03 | 36.69 | 40.98 | 29.50 | 45.60 |
| J | 1.00 | 13.00 | 36.24 | 7.35 | 2.04 | 31.79 | 40.68 | 23.70 | 46.10 |
| | 2.00 | 9.00 | 39.67 | 5.48 | 1.83 | 35.46 | 43.88 | 32.70 | 47.40 |
| | Total | 22.00 | 37.64 | 6.73 | 1.44 | 34.66 | 40.63 | 23.70 | 47.40 |
| K | 1.00 | 14.00 | 34.27 | 6.26 | 1.67 | 30.66 | 37.89 | 23.00 | 44.00 |
| | 2.00 | 9.00 | 38.93 | 6.70 | 2.23 | 33.78 | 44.09 | 30.00 | 48.50 |
| | Total | 23.00 | 36.10 | 6.70 | 1.40 | 33.20 | 38.99 | 23.00 | 48.50 |
| L | 1.00 | 13.00 | 33.85 | 6.72 | 1.86 | 29.79 | 37.91 | 22.80 | 42.90 |
| | 2.00 | 9.00 | 40.80 | 5.49 | 1.83 | 36.58 | 45.02 | 28.10 | 46.20 |
| | Total | 22.00 | 36.69 | 7.04 | 1.50 | 33.57 | 39.81 | 22.80 | 46.20 |
| M | 1.00 | 13.00 | 39.15 | 7.98 | 2.21 | 34.33 | 43.97 | 23.40 | 51.00 |
| | 2.00 | 9.00 | 41.58 | 7.12 | 2.37 | 36.10 | 47.05 | 28.20 | 48.00 |
| | Total | 22.00 | 40.15 | 7.56 | 1.61 | 36.79 | 43.50 | 23.40 | 51.00 |
| N | 1.00 | 13.00 | 37.92 | 8.30 | 2.30 | 32.90 | 42.93 | 23.90 | 45.70 |
| | 2.00 | 9.00 | 41.41 | 2.71 | 0.90 | 39.32 | 43.50 | 37.10 | 45.30 |
| | Total | 22.00 | 39.35 | 6.73 | 1.43 | 36.36 | 42.33 | 23.90 | 45.70 |

*Table 77: ANOVA for differences in minimum temperature at probe position by grade*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| A | Between Groups | 224.61 | 1.00 | 224.61 | 11.34 | 0.00 |
| | Within Groups | 416.13 | 21.00 | 19.82 | | |
| | Total | 640.74 | 22.00 | | | |
| B | Between Groups | 10.94 | 1.00 | 10.94 | 0.29 | 0.60 |
| | Within Groups | 767.67 | 20.00 | 38.38 | | |
| | Total | 778.61 | 21.00 | | | |
| C | Between Groups | 44.70 | 1.00 | 44.70 | 1.50 | 0.24 |
| | Within Groups | 598.18 | 20.00 | 29.91 | | |
| | Total | 642.88 | 21.00 | | | |
| D | Between Groups | 132.00 | 1.00 | 132.00 | 4.30 | 0.05 |
| | Within Groups | 645.27 | 21.00 | 30.73 | | |
| | Total | 777.28 | 22.00 | | | |
| E | Between Groups | 74.80 | 1.00 | 74.80 | 3.48 | 0.08 |
| | Within Groups | 430.10 | 20.00 | 21.51 | | |
| | Total | 504.90 | 21.00 | | | |
| F | Between Groups | 15.60 | 1.00 | 15.60 | 0.30 | 0.59 |
| | Within Groups | 1027.85 | 20.00 | 51.39 | | |
| | Total | 1043.46 | 21.00 | | | |
| G | Between Groups | 9.49 | 1.00 | 9.49 | 0.22 | 0.65 |
| | Within Groups | 926.13 | 21.00 | 44.10 | | |
| | Total | 935.62 | 22.00 | | | |
| H | Between Groups | 82.42 | 1.00 | 82.42 | 2.43 | 0.13 |
| | Within Groups | 677.21 | 20.00 | 33.86 | | |
| | Total | 759.63 | 21.00 | | | |
| I | Between Groups | 38.66 | 1.00 | 38.66 | 1.82 | 0.19 |
| | Within Groups | 404.29 | 19.00 | 21.28 | | |
| | Total | 442.95 | 20.00 | | | |
| J | Between Groups | 62.50 | 1.00 | 62.50 | 1.41 | 0.25 |
| | Within Groups | 888.87 | 20.00 | 44.44 | | |
| | Total | 951.37 | 21.00 | | | |
| K | Between Groups | 119.06 | 1.00 | 119.06 | 2.88 | 0.11 |
| | Within Groups | 869.47 | 21.00 | 41.40 | | |
| | Total | 988.53 | 22.00 | | | |
| L | Between Groups | 257.17 | 1.00 | 257.17 | 6.57 | 0.02 |
| | Within Groups | 783.11 | 20.00 | 39.16 | | |
| | Total | 1040.28 | 21.00 | | | |
| M | Between Groups | 31.25 | 1.00 | 31.25 | 0.53 | 0.47 |
| | Within Groups | 1169.43 | 20.00 | 58.47 | | |
| | Total | 1200.68 | 21.00 | | | |
| N | Between Groups | 64.99 | 1.00 | 64.99 | 1.47 | 0.24 |
| | Within Groups | 886.01 | 20.00 | 44.30 | | |
| | Total | 951.00 | 21.00 | | | |

*Table 78: Descriptive statistics for heating and cooling gradient grouped by grade*

| | | N | Mean | Std. Dev. | Std. Err. | 95% CI for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| HEATRATE | gradea | 14.00 | 3.03 | 0.11 | 0.03 | 2.96 | 3.09 | 2.82 | 3.21 |
| | gradeb | 14.00 | 2.84 | 0.11 | 0.03 | 2.77 | 2.90 | 2.64 | 3.04 |
| | Total | 28.00 | 2.93 | 0.15 | 0.03 | 2.88 | 2.99 | 2.64 | 3.21 |
| COOLRATE | gradea | 14.00 | -2.50 | 0.12 | 0.03 | -2.56 | -2.43 | -2.73 | -2.35 |
| | gradeb | 14.00 | -2.33 | 0.12 | 0.03 | -2.40 | -2.26 | -2.55 | -2.18 |
| | Total | 28.00 | -2.42 | 0.14 | 0.03 | -2.47 | -2.36 | -2.73 | -2.18 |

*Table 79: ANOVA analysis of heating and cooling rate grouped by grade*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| HEATRATE | Between Groups | 0.25 | 1.00 | 0.25 | 19.29 | 0.00 |
| | Within Groups | 0.33 | 26.00 | 0.01 | | |
| | Total | 0.58 | 27.00 | | | |
| COOLRATE | Between Groups | 0.19 | 1.00 | 0.19 | 12.95 | 0.00 |
| | Within Groups | 0.37 | 26.00 | 0.01 | | |
| | Total | 0.56 | 27.00 | | | |

*Table 80: Descriptive statistics for maximum and minimum temperatures by grade*

| | | N | Mean | Std. Dev. | Std. Err. | 95% CI for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| MAXTEMP | gradea | 14.00 | 84.00 | 2.92 | 0.78 | 82.31 | 85.68 | 81.83 | 93.78 |
| | gradeb | 14.00 | 81.57 | 1.28 | 0.34 | 80.82 | 82.31 | 79.41 | 83.23 |
| | Total | 28.00 | 82.78 | 2.54 | 0.48 | 81.80 | 83.77 | 79.41 | 93.78 |
| MINTEMP | gradea | 14.00 | 36.60 | 2.06 | 0.55 | 35.41 | 37.79 | 32.29 | 39.15 |
| | gradeb | 14.00 | 40.17 | 1.52 | 0.41 | 39.29 | 41.05 | 37.42 | 42.74 |
| | Total | 28.00 | 38.38 | 2.54 | 0.48 | 37.40 | 39.37 | 32.29 | 42.74 |

*Table 81: ANOVA analysis of maximum and minimum temperatures by grade*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| MAXTEMP | Between Groups | 41.46 | 1.00 | 41.46 | 8.15 | 0.01 |
| | Within Groups | 132.21 | 26.00 | 5.09 | | |
| | Total | 173.66 | 27.00 | | | |
| MINTEMP | Between Groups | 89.43 | 1.00 | 89.43 | 27.26 | 0.00 |
| | Within Groups | 85.28 | 26.00 | 3.28 | | |
| | Total | 174.71 | 27.00 | | | |

*Table 82: Descriptive statistics of lethality grouped by probe*

| | | | | | | 95% CI for Mean | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | Mean | Std. Dev. | Std. Err. | | | Min. | Max. |
| | | | | | | Lower Bound | Upper Bound | | |
| PROBA | grade a | 3.00 | 3.04 | 0.57 | 0.33 | 1.64 | 4.45 | 2.41 | 3.50 |
| | grade b | 3.00 | 2.16 | 0.49 | 0.28 | 0.95 | 3.37 | 1.85 | 2.72 |
| | Total | 6.00 | 2.60 | 0.67 | 0.28 | 1.89 | 3.31 | 1.85 | 3.50 |
| PROBB | grade a | 3.00 | 2.74 | 0.15 | 0.09 | 2.35 | 3.12 | 2.58 | 2.88 |
| | grade b | 3.00 | 2.55 | 0.78 | 0.45 | 0.62 | 4.48 | 1.90 | 3.41 |
| | Total | 6.00 | 2.64 | 0.51 | 0.21 | 2.11 | 3.18 | 1.90 | 3.41 |
| PROBC | grade a | 3.00 | 2.72 | 0.59 | 0.34 | 1.25 | 4.20 | 2.04 | 3.12 |
| | grade b | 3.00 | 2.77 | 0.91 | 0.52 | 0.51 | 5.02 | 1.85 | 3.67 |
| | Total | 6.00 | 2.74 | 0.69 | 0.28 | 2.02 | 3.46 | 1.85 | 3.67 |
| PROBD | grade a | 3.00 | 3.36 | 1.12 | 0.65 | 0.57 | 6.14 | 2.48 | 4.62 |
| | grade b | 3.00 | 3.04 | 2.13 | 1.23 | -2.25 | 8.33 | 1.15 | 5.35 |
| | Total | 6.00 | 3.20 | 1.53 | 0.63 | 1.59 | 4.81 | 1.15 | 5.35 |
| PROBE | grade a | 3.00 | 2.92 | 0.85 | 0.49 | 0.81 | 5.02 | 1.98 | 3.62 |
| | grade b | 3.00 | 2.52 | 0.41 | 0.23 | 1.52 | 3.53 | 2.07 | 2.84 |
| | Total | 6.00 | 2.72 | 0.63 | 0.26 | 2.06 | 3.38 | 1.98 | 3.62 |
| PROBF | grade a | 3.00 | 2.84 | 0.86 | 0.50 | 0.71 | 4.98 | 2.12 | 3.79 |
| | grade b | 3.00 | 1.97 | 0.69 | 0.40 | 0.25 | 3.68 | 1.29 | 2.67 |
| | Total | 6.00 | 2.41 | 0.85 | 0.35 | 1.52 | 3.29 | 1.29 | 3.79 |
| PROBG | grade a | 3.00 | 2.50 | 0.63 | 0.36 | 0.94 | 4.06 | 1.80 | 3.02 |
| | grade b | 3.00 | 2.45 | 0.43 | 0.25 | 1.38 | 3.51 | 2.00 | 2.85 |
| | Total | 6.00 | 2.47 | 0.48 | 0.20 | 1.97 | 2.98 | 1.80 | 3.02 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| PROBH | grade a | 3.00 | 2.69 | 1.03 | 0.59 | 0.14 | 5.24 | 1.95 | 3.86 |
| | grade b | 3.00 | 2.03 | 0.22 | 0.12 | 1.49 | 2.56 | 1.87 | 2.27 |
| | Total | 6.00 | 2.36 | 0.76 | 0.31 | 1.56 | 3.15 | 1.87 | 3.86 |
| PROBI | grade a | 3.00 | 2.91 | 0.65 | 0.37 | 1.31 | 4.52 | 2.29 | 3.58 |
| | grade b | 3.00 | 2.30 | 0.23 | 0.13 | 1.72 | 2.88 | 2.14 | 2.57 |
| | Total | 6.00 | 2.61 | 0.55 | 0.22 | 2.03 | 3.18 | 2.14 | 3.58 |
| PROBJ | grade a | 3.00 | 3.15 | 0.67 | 0.39 | 1.49 | 4.82 | 2.64 | 3.91 |
| | grade b | 3.00 | 2.16 | 0.61 | 0.35 | 0.64 | 3.68 | 1.52 | 2.74 |
| | Total | 6.00 | 2.66 | 0.79 | 0.32 | 1.83 | 3.49 | 1.52 | 3.91 |
| PROBK | grade a | 3.00 | 3.16 | 0.35 | 0.20 | 2.29 | 4.03 | 2.94 | 3.56 |
| | grade b | 3.00 | 2.69 | 0.93 | 0.53 | 0.39 | 4.99 | 1.89 | 3.71 |
| | Total | 6.00 | 2.93 | 0.68 | 0.28 | 2.22 | 3.64 | 1.89 | 3.71 |
| PROBL | grade a | 3.00 | 2.87 | 0.91 | 0.53 | 0.60 | 5.14 | 1.88 | 3.69 |
| | grade b | 3.00 | 2.64 | 0.43 | 0.25 | 1.58 | 3.71 | 2.16 | 2.98 |
| | Total | 6.00 | 2.76 | 0.65 | 0.27 | 2.08 | 3.44 | 1.88 | 3.69 |
| PROBM | grade a | 3.00 | 3.52 | 0.55 | 0.32 | 2.15 | 4.88 | 3.04 | 4.11 |
| | grade b | 3.00 | 3.51 | 0.97 | 0.56 | 1.10 | 5.92 | 2.62 | 4.54 |
| | Total | 6.00 | 3.51 | 0.71 | 0.29 | 2.77 | 4.25 | 2.62 | 4.54 |
| PROBN | grade a | 3.00 | 3.37 | 1.77 | 1.02 | -1.03 | 7.77 | 1.77 | 5.27 |
| | grade b | 3.00 | 3.20 | 0.36 | 0.21 | 2.31 | 4.09 | 2.86 | 3.57 |
| | Total | 6.00 | 3.29 | 1.15 | 0.47 | 2.08 | 4.49 | 1.77 | 5.27 |

*Table 83: ANOVA analysis of Lethality: Probes grouped by Grade Lobster*

| | | **Sum of Squares** | **df** | **Mean Square** | **F** | **Sig.** |
|---|---|---|---|---|---|---|
| PROBA | Between Groups | 1.16 | 1.00 | 1.16 | 4.15 | 0.11 |
| | Within Groups | 1.12 | 4.00 | 0.28 | | |
| | Total | 2.28 | 5.00 | | | |
| PROBB | Between Groups | 0.05 | 1.00 | 0.05 | 0.16 | 0.71 |
| | Within Groups | 1.25 | 4.00 | 0.31 | | |
| | Total | 1.30 | 5.00 | | | |
| PROBC | Between Groups | 0.00 | 1.00 | 0.00 | 0.00 | 0.95 |
| | Within Groups | 2.35 | 4.00 | 0.59 | | |
| | Total | 2.36 | 5.00 | | | |
| PROBD | Between Groups | 0.15 | 1.00 | 0.15 | 0.05 | 0.83 |
| | Within Groups | 11.60 | 4.00 | 2.90 | | |
| | Total | 11.75 | 5.00 | | | |
| PROBE | Between Groups | 0.23 | 1.00 | 0.23 | 0.53 | 0.51 |
| | Within Groups | 1.77 | 4.00 | 0.44 | | |
| | Total | 2.00 | 5.00 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| PROBF | Between Groups | 1.15 | 1.00 | 1.15 | 1.89 | 0.24 |
| | Within Groups | 2.43 | 4.00 | 0.61 | | |
| | Total | 3.58 | 5.00 | | | |
| PROBG | Between Groups | 0.00 | 1.00 | 0.00 | 0.02 | 0.91 |
| | Within Groups | 1.16 | 4.00 | 0.29 | | |
| | Total | 1.16 | 5.00 | | | |
| PROBH | Between Groups | 0.66 | 1.00 | 0.66 | 1.20 | 0.34 |
| | Within Groups | 2.21 | 4.00 | 0.55 | | |
| | Total | 2.87 | 5.00 | | | |
| PROBI | Between Groups | 0.57 | 1.00 | 0.57 | 2.40 | 0.20 |
| | Within Groups | 0.94 | 4.00 | 0.24 | | |
| | Total | 1.51 | 5.00 | | | |
| PROBJ | Between Groups | 1.48 | 1.00 | 1.48 | 3.58 | 0.13 |
| | Within Groups | 1.65 | 4.00 | 0.41 | | |
| | Total | 3.12 | 5.00 | | | |
| PROBK | Between Groups | 0.33 | 1.00 | 0.33 | 0.67 | 0.46 |
| | Within Groups | 1.96 | 4.00 | 0.49 | | |
| | Total | 2.29 | 5.00 | | | |
| PROBL | Between Groups | 0.08 | 1.00 | 0.08 | 0.15 | 0.72 |
| | Within Groups | 2.03 | 4.00 | 0.51 | | |
| | Total | 2.11 | 5.00 | | | |
| PROBM | Between Groups | 0.00 | 1.00 | 0.00 | 0.00 | 0.99 |
| | Within Groups | 2.49 | 4.00 | 0.62 | | |
| | Total | 2.49 | 5.00 | | | |
| PROBN | Between Groups | 0.04 | 1.00 | 0.04 | 0.03 | 0.88 |
| | Within Groups | 6.54 | 4.00 | 1.63 | | |
| | Total | 6.58 | 5.00 | | | |

*Table 84: Descriptive statistics for lethality by grade*

| | N | Mean | Std. Dev. | Std. Err. | 95% CI for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1.00 | 42.00 | 2.99 | 0.76 | 0.12 | 2.75 | 3.22 | 1.77 | 5.27 |
| 2.00 | 42.00 | 2.57 | 0.81 | 0.13 | 2.32 | 2.82 | 1.15 | 5.35 |
| Total | 84.00 | 2.78 | 0.81 | 0.09 | 2.60 | 2.95 | 1.15 | 5.35 |

*Table 85: ANOVA analysis of lethality grouped by grade*

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 3.62 | 1.00 | 3.62 | 5.84 | 0.02 |
| Within Groups | 50.77 | 82.00 | 0.62 | | |
| Total | 54.39 | 83.00 | | | |

*Table 86: Descriptive statistics for melanosis factors*

| | N | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|
| AREA | 115.00 | 0.00 | 21.32 | 6.30 | 4.70 |
| INTENSE | 115.00 | 77.60 | 223.84 | 176.15 | 22.03 |
| RATE | 115.00 | 0.00 | 0.11 | 0.02 | 0.02 |
| Valid N (listwise) | 115.00 | | | | |

*Table 87: Kruskal Wallis analysis of melanosis factors grouped by field trip*

| | FLDTRIP | N | Mean Rank |
|---|---|---|---|
| AREA | 1.00 | 40.00 | 66.25 |
| | 3.00 | 35.00 | 45.03 |
| | 4.00 | 40.00 | 61.10 |
| | Total | 115.00 | |
| INTENSE | 1.00 | 40.00 | 53.75 |
| | 3.00 | 35.00 | 47.80 |
| | 4.00 | 40.00 | 71.18 |
| | Total | 115.00 | |
| RATE | 1.00 | 40.00 | 67.95 |
| | 3.00 | 35.00 | 41.30 |
| | 4.00 | 40.00 | 62.66 |
| | Total | 115.00 | |

| | AREA | INTENSE | RATE |
|---|---|---|---|
| Chi-Square | 8.09 | 10.17 | 13.13 |
| df | 2.00 | 2.00 | 2.00 |
| Asymp. Sig. | 0.02 | 0.01 | 0.00 |

a Kruskal Wallis Test

b Grouping Variable: FLDTRIP

*Table 88: Descriptive statistics for melanosis factors by gender*

| | | N | Mean | Std. Dev. | Std. Err. | 95% CI for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| AREA | FEMALE | 58.00 | 6.25 | 4.69 | 0.62 | 5.02 | 7.49 | 0.00 | 21.32 |
| | MALE | 56.00 | 6.43 | 4.75 | 0.64 | 5.15 | 7.70 | 0.00 | 20.57 |
| | Total | 114.00 | 6.34 | 4.70 | 0.44 | 5.47 | 7.21 | 0.00 | 21.32 |
| INTENSE | FEMALE | 58.00 | 178.34 | 22.95 | 3.01 | 172.30 | 184.37 | 77.60 | 213.33 |
| | MALE | 56.00 | 173.71 | 21.14 | 2.83 | 168.05 | 179.37 | 93.13 | 223.84 |
| | Total | 114.00 | 176.06 | 22.11 | 2.07 | 171.96 | 180.17 | 77.60 | 223.84 |
| RATE | FEMALE | 58.00 | 0.02 | 0.02 | 0.00 | 0.01 | 0.02 | 0.00 | 0.11 |
| | MALE | 56.00 | 0.02 | 0.02 | 0.00 | 0.01 | 0.02 | 0.00 | 0.07 |
| | Total | 114.00 | 0.02 | 0.02 | 0.00 | 0.01 | 0.02 | 0.00 | 0.11 |

*Table 89:ANOVA analysis of impact of gender on melanosis factors*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| AREA | Between Groups | 0.85 | 1.00 | 0.85 | 0.04 | 0.85 |
| | Within Groups | 2494.68 | 112.00 | 22.27 | | |
| | Total | 2495.53 | 113.00 | | | |
| INTENSE | Between Groups | 610.08 | 1.00 | 610.08 | 1.25 | 0.27 |
| | Within Groups | 54608.31 | 112.00 | 487.57 | | |
| | Total | 55218.39 | 113.00 | | | |
| RATE | Between Groups | 0.00 | 1.00 | 0.00 | 0.28 | 0.60 |
| | Within Groups | 0.03 | 112.00 | 0.00 | | |
| | Total | 0.03 | 113.00 | | | |

*Table 90: Descriptive statistics of melanosis factors grouped by grade*

| | | N | Mean | Std. Dev. | Std. Err. | 95% CI for Mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| AREA | A grade | 70.00 | 6.10 | 4.43 | 0.53 | 5.05 | 7.16 | 0.00 | 21.32 |
| | B grade | 45.00 | 6.61 | 5.12 | 0.76 | 5.07 | 8.15 | 0.00 | 20.57 |
| | Total | 115.00 | 6.30 | 4.70 | 0.44 | 5.43 | 7.17 | 0.00 | 21.32 |
| INTENSE | A grade | 70.00 | 174.64 | 20.08 | 2.40 | 169.85 | 179.43 | 93.13 | 204.61 |
| | B grade | 45.00 | 178.51 | 24.81 | 3.70 | 171.05 | 185.96 | 77.60 | 223.84 |
| | Total | 115.00 | 176.15 | 22.03 | 2.05 | 172.08 | 180.22 | 77.60 | 223.84 |
| RATE | A grade | 70.00 | 0.02 | 0.02 | 0.00 | 0.01 | 0.02 | 0.00 | 0.11 |
| | B grade | 45.00 | 0.02 | 0.02 | 0.00 | 0.01 | 0.02 | 0.00 | 0.06 |
| | Total | 115.00 | 0.02 | 0.02 | 0.00 | 0.01 | 0.02 | 0.00 | 0.11 |

*Table 91: ANOVA analysis of melanosis factors grouped by grade*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| AREA | Between Groups | 6.93 | 1.00 | 6.93 | 0.31 | 0.58 |
| | Within Groups | 2506.13 | 113.00 | 22.18 | | |
| | Total | 2513.06 | 114.00 | | | |
| INTENSE | Between Groups | 409.53 | 1.00 | 409.53 | 0.84 | 0.36 |
| | Within Groups | 54908.90 | 113.00 | 485.92 | | |
| | Total | 55318.43 | 114.00 | | | |
| RATE | Between Groups | 0.00 | 1.00 | 0.00 | 0.24 | 0.62 |
| | Within Groups | 0.03 | 113.00 | 0.00 | | |
| | Total | 0.03 | 114.00 | | | |

*Table 92: ANOVA analysis of melanosis factors by position*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| AREA | Between Groups | 59.08 | 4.00 | 14.77 | 0.66 | 0.62 |
| | Within Groups | 2453.97 | 110.00 | 22.31 | | |
| | Total | 2513.06 | 114.00 | | | |
| INTENSE | Between Groups | 3475.12 | 4.00 | 868.78 | 1.84 | 0.13 |
| | Within Groups | 51843.31 | 110.00 | 471.30 | | |
| | Total | 55318.43 | 114.00 | | | |
| RATE | Between Groups | 0.00 | 4.00 | 0.00 | 0.56 | 0.69 |
| | Within Groups | 0.03 | 110.00 | 0.00 | | |
| | Total | 0.03 | 114.00 | | | |

*Table 93: Kruskal Wallis analysis of melanosis factors grouped by cook*

| | AREA | INTENSE | RATE |
|---|---|---|---|
| Chi-Square | 35.77 | 38.75 | 39.42 |
| df | 22.00 | 22.00 | 22.00 |
| Asymp. Sig. | 0.03 | 0.02 | 0.01 |

a Kruskal Wallis Test

b Grouping Variable: COOK

*Table 94: ANOVA analysis of the impact of leg loss on melanosis factors*

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| AREA | Between Groups | 229.85 | 4.00 | 57.46 | 2.77 | 0.03 |
| | Within Groups | 2283.20 | 110.00 | 20.76 | | |
| | Total | 2513.06 | 114.00 | | | |
| INTENSE | Between Groups | 6015.88 | 4.00 | 1503.97 | 3.36 | 0.01 |
| | Within Groups | 49302.55 | 110.00 | 448.21 | | |
| | Total | 55318.43 | 114.00 | | | |
| RATE | Between Groups | 0.00 | 4.00 | 0.00 | 1.27 | 0.29 |
| | Within Groups | 0.03 | 110.00 | 0.00 | | |
| | Total | 0.03 | 114.00 | | | |

**Statistics for validation of lethality**

*Table 95: Descriptive Statistics for parameters of lethality validation trials*

| | N | Minimum | Maximum | Mean | Std. Dev. |
|---|---|---|---|---|---|
| cook time | 830.00 | 12.00 | 49.50 | 31.17 | 10.30 |
| lethality | 830.00 | 0.09 | 83.45 | 23.55 | 20.95 |
| area melanosis | 828.00 | 0.00 | 17.11 | 1.48 | 2.76 |
| intensity | 738.00 | 65.50 | 217.70 | 121.16 | 52.67 |
| rate | 828.00 | 0.00 | 0.17 | 0.00 | 0.01 |
| protein | 830.00 | 76.87 | 110.29 | 94.71 | 10.63 |
| total PPO | 830.00 | 9.33 | 37.36 | 17.33 | 8.35 |
| baseline PPO | 830.00 | 0.87 | 5.38 | 2.33 | 1.49 |
| Valid N (listwise) | 738.00 | | | | |

*Table 96: Kruskal Wallis analysis of the impact of gender on physiological parameters*

| Ranks | Gender | N | Mean Rank |
|---|---|---|---|
| protein | female | 372.00 | 455.11 |
| | male | 458.00 | 383.33 |
| | Total | 830.00 | |
| total PPO | female | 372.00 | 430.70 |
| | male | 458.00 | 403.15 |
| | Total | 830.00 | |
| baseline PPO | female | 372.00 | 403.42 |
| | male | 458.00 | 425.31 |
| | Total | 830.00 | |

**Test Statistics**

| | protein | total PPO | baseline PPO |
|---|---|---|---|
| Chi-Square | 18.61 | 2.74 | 1.73 |
| df | 1.00 | 1.00 | 1.00 |
| Asymp. Sig. | 0.00 | 0.10 | 0.19 |

a Kruskal Wallis Test

b Grouping Variable: gender

*Table 97: Kruskal Wallis analysis of the impact of filed trip on physiological parameters*

| Ranks | FT | N | Mean Rank |
|---|---|---|---|
| PROTEIN | 8.00 | 60.00 | 86.77 |
| | 9.00 | 60.00 | 82.58 |
| | 10.00 | 81.00 | 125.19 |
| | Total | 201.00 | |
| TOTAL PPO | 8.00 | 60.00 | 118.59 |
| | 9.00 | 60.00 | 123.58 |
| | 10.00 | 81.00 | 71.24 |
| | Total | 201.00 | |
| BASELINE PPO | 8.00 | 60.00 | 147.52 |
| | 9.00 | 60.00 | 102.71 |
| | 10.00 | 80.00 | 63.58 |
| | Total | 200.00 | |

**Test Statistics**

| | PROTEIN | TOTAL PPO | BASELINE PPO |
|---|---|---|---|
| Chi-Square | 23.66 | 35.73 | 72.26 |
| df | 2.00 | 2.00 | 2.00 |
| Asymp. Sig. | 0.00 | 0.00 | 0.00 |

a Kruskal Wallis Test

b Grouping Variable: FT

*Table 98: Correlation of physiological parameters and melanosis factors*

| Correlations | | area melanosis | intensity | rate | protein | total PPO | baseline PPO |
|---|---|---|---|---|---|---|---|
| area melanosis | Pearson Correlation | 1.00 | 0.75 | 0.86 | 0.28 | -0.08 | -0.28 |
| | Sig. (2-tailed) | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 |
| | N | | 738.00 | 828.00 | 828.00 | 828.00 | 828.00 |
| intensity | Pearson Correlation | 1.00 | 0.56 | 0.36 | -0.21 | -0.42 | |
| | Sig. (2-tailed) | | | 0.00 | 0.00 | 0.00 | 0.00 |
| | N | | | 738.00 | 738.00 | 738.00 | 738.00 |
| rate | Pearson Correlation | | 1.00 | 0.20 | -0.08 | -0.22 | |
| | Sig. (2-tailed) | | | | 0.00 | 0.02 | 0.00 |
| | N | | | | 828.00 | 828.00 | 828.00 |
| protein | Pearson Correlation | | | 1.00 | 0.33 | -0.47 | |
| | Sig. (2-tailed) | | | | | 0.00 | 0.00 |
| | N | | | | | 830.00 | 830.00 |
| total PPO | Pearson Correlation | | | | 1.00 | 0.34 | |
| | Sig. (2-tailed) | | | | | | 0.00 |
| | N | | | | | | 830.00 |

*Table 99: Regression analysis of baseline PPO activity vs. melanosis factors*

**Baseline vr area**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate |
|---|---|---|---|---|
| 1.00 | 0.28 | 0.08 | 0.08 | 2.65 |

**ANOVA**

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 1.00 | Regression | 491.17 | 1.00 | 491.17 | 69.82 | 0.00 |
| | Residual | 5810.55 | 826.00 | 7.04 | | |
| | Total | 6301.71 | 827.00 | | | |

**Baseline vrs intensity**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate |
|---|---|---|---|---|
| 1.00 | 0.42 | 0.18 | 0.18 | 47.74 |

**ANOVA**

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 1.00 | Regression | 366962.24 | 1.00 | 366962.24 | 160.98 | 0.00 |
| | Residual | 1677742.27 | 736.00 | 2279.54 | | |
| | Total | 2044704.51 | 737.00 | | | |

**Baseline vs. rate**

Model Summary

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate |
|---|---|---|---|---|
| 1.00 | 0.22 | 0.05 | 0.05 | 0.01 |

**ANOVA**

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 1.00 | Regression | 0.00 | 1.00 | 0.00 | 40.13 | 0.00 |
| | Residual | 0.08 | 826.00 | 0.00 | | |
| | Total | 0.09 | 827.00 | | | |

*Table 100: Regression analysis of total PPO activity vs. melanosis factors*

**Total vs. area**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate | | |
|---|---|---|---|---|---|---|
| 1.00 | 0.08 | 0.01 | 0.01 | 2.75 | | |

**ANOVA**

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 1.00 | Regression | 39.08 | 1.00 | 39.08 | 5.16 | 0.02 |
| | Residual | 6262.63 | 826.00 | 7.58 | | |
| | Total | 6301.71 | 827.00 | | | |

**Total vs. intensity**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate | | |
|---|---|---|---|---|---|---|
| 1.00 | 0.21 | 0.05 | 0.04 | 51.52 | | |

**ANOVA**

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 1.00 | Regression | 91099.96 | 1.00 | 91099.96 | 34.32 | 0.00 |
| | Residual | 1953604.55 | 736.00 | 2654.35 | | |
| | Total | 2044704.51 | 737.00 | | | |

**Total vs. rate**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate | | |
|---|---|---|---|---|---|---|
| 1.00 | 0.08 | 0.01 | 0.01 | 0.01 | | |

**ANOVA**

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 1.00 | Regression | 0.00 | 1.00 | 0.00 | 5.63 | 0.02 |
| | Residual | 0.09 | 826.00 | 0.00 | | |
| | Total | 0.09 | 827.00 | | | |

*Table 101: Regression analysis of protein values vs. melanosis factors*

**Protein vs area melanosis**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate |
|---|---|---|---|---|
| 1.00 | 0.28 | 0.08 | 0.08 | 2.65 |

**ANOVA**

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 1.00 | Regression | 486.64 | 1.00 | 486.64 | 69.12 | 0.00 |
| | Residual | 5815.08 | 826.00 | 7.04 | | |
| | Total | 6301.71 | 827.00 | | | |

**Protein vs intensity**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate |
|---|---|---|---|---|
| 1.00 | 0.36 | 0.13 | 0.13 | 49.19 |

**ANOVA**

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 1.00 | Regression | 263810.63 | 1.00 | 263810.63 | 109.03 | 0.00 |
| | Residual | 1780893.88 | 736.00 | 2419.69 | | |
| | Total | 2044704.51 | 737.00 | | | |

**Protein vs rate**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate |
|---|---|---|---|---|
| 1.00 | 0.20 | 0.04 | 0.04 | 0.01 |

**ANOVA**

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 1.00 | Regression | 0.00 | 1.00 | 0.00 | 34.37 | 0.00 |
| | Residual | 0.08 | 826.00 | 0.00 | | |
| | Total | 0.09 | 827.00 | | | |

*Table 102: Kruskal Wallis analysis of weight recovery by cook*

| cook number | N | Mean Rank | cook number | N | Mean Rank |
|---|---|---|---|---|---|
| ck49 | 10.00 | 487.10 | ck95 | 10.00 | 47.25 |
| ck50 | 10.00 | 303.15 | ck97 | 10.00 | 305.25 |
| ck51 | 10.00 | 346.15 | ck98 | 10.00 | 206.35 |
| ck52 | 10.00 | 528.05 | ck99 | 10.00 | 574.60 |
| ck53 | 10.00 | 558.85 | ck101 | 10.00 | 323.25 |
| ck54 | 10.00 | 149.80 | ck102 | 10.00 | 297.15 |
| ck55 | 10.00 | 375.20 | ck103 | 10.00 | 365.20 |
| ck56 | 10.00 | 339.35 | ck104 | 10.00 | 296.15 |
| ck57 | 10.00 | 178.70 | ck105 | 10.00 | 430.60 |
| ck58 | 10.00 | 194.50 | ck106 | 10.00 | 410.10 |
| ck60 | 10.00 | 253.60 | ck107 | 10.00 | 486.75 |
| ck61 | 10.00 | 332.25 | ck108 | 10.00 | 549.00 |
| ck62 | 10.00 | 201.15 | ck109 | 10.00 | 474.30 |
| ck63 | 10.00 | 184.40 | ck110 | 10.00 | 658.15 |
| ck64 | 10.00 | 248.20 | ck111 | 10.00 | 397.65 |
| ck65 | 10.00 | 202.65 | ck112 | 10.00 | 544.35 |
| ck67 | 10.00 | 382.75 | ck113 | 10.00 | 490.25 |
| ck68 | 10.00 | 397.75 | ck114 | 10.00 | 537.55 |
| ck69 | 10.00 | 218.80 | ck115 | 10.00 | 364.00 |
| ck70 | 10.00 | 245.80 | ck116 | 10.00 | 491.70 |
| ck72 | 10.00 | 194.50 | ck117 | 10.00 | 533.70 |
| ck73 | 10.00 | 176.85 | ck118 | 10.00 | 562.50 |
| ck74 | 10.00 | 202.80 | ck119 | 10.00 | 519.85 |
| ck75 | 10.00 | 574.15 | ck120 | 10.00 | 772.10 |
| ck76 | 10.00 | 570.20 | ck121 | 10.00 | 290.40 |
| ck77 | 10.00 | 554.35 | ck122 | 10.00 | 430.05 |
| ck78 | 10.00 | 576.70 | ck123 | 10.00 | 587.10 |
| ck79 | 10.00 | 656.50 | ck124 | 10.00 | 343.45 |
| ck80 | 10.00 | 737.25 | ck125 | 10.00 | 498.85 |
| ck81 | 10.00 | 524.55 | ck126 | 10.00 | 546.80 |
| ck82 | 10.00 | 724.60 | ck127 | 10.00 | 637.30 |
| ck83 | 10.00 | 602.35 | ck128 | 10.00 | 585.90 |
| ck84 | 10.00 | 522.75 | ck129 | 10.00 | 534.60 |
| ck86 | 10.00 | 321.15 | ck130 | 10.00 | 620.60 |
| ck87 | 10.00 | 126.65 | ck131 | 10.00 | 323.60 |
| ck88 | 10.00 | 150.15 | ck132 | 10.00 | 467.15 |
| ck89 | 10.00 | 230.50 | ck133 | 10.00 | 425.10 |
| ck90 | 10.00 | 333.95 | ck134 | 10.00 | 574.45 |
| ck91 | 10.00 | 285.60 | ck135 | 10.00 | 625.45 |
| ck92 | 10.00 | 347.60 | ck136 | 10.00 | 600.55 |
| ck93 | 10.00 | 280.70 | ck137 | 10.00 | 566.05 |
| ck94 | 10.00 | 369.30 | Total | 830.00 | |

| **WTRECOV** | |
|---|---|
| Chi-Square | 387.52 |
| df | 82.00 |
| Asymp. Sig. | 0.00 |

**Statistics for evaluation of the impact of antibrowning agents *in vitro***

*Table 103: Paired t test comparison of baseline PPO activity after treatment with ascorbic acid*

| Paired Samples Statistics | | Mean | N | Std. Dev. | Std. Error Mean |
|---|---|---|---|---|---|
| Pair 1 | PPM100 | 58.93 | 5.00 | 23.79 | 10.64 |
| | CONTROL | 174.00 | 5.00 | 44.20 | 19.77 |
| Pair 2 | PPM400 | 20.60 | 5.00 | 44.39 | 19.85 |
| | CONTROL | 174.00 | 5.00 | 44.20 | 19.77 |

| Paired Differences | | Mean | Std. Dev. | Std. Error Mean | 95% CI of the Difference | | t | df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Lower | Upper | | | |
| Pair 1 | PPM100 - CONTROL | -115.07 | 65.41 | 29.25 | -196.29 | -33.85 | -3.93 | 4.00 | 0.02 |
| Pair 2 | PPM400 - CONTROL | -153.40 | 87.15 | 38.97 | -261.60 | -45.19 | -3.94 | 4.00 | 0.02 |

*Table 104: Paired t test comparison of total PPO activity after treatment with ascorbic acid*

| Paired Samples Statistics | | Mean | N | Std. Dev. | Std. Error Mean |
|---|---|---|---|---|---|
| Pair 1 | PPM100 | 66.29 | 5.00 | 22.22 | 9.94 |
| | CONTROL | 62.11 | 5.00 | 22.31 | 9.98 |
| Pair 2 | PPM400 | 62.91 | 5.00 | 22.88 | 10.23 |
| | CONTROL | 62.11 | 5.00 | 22.31 | 9.98 |

| Paired Differences | | Mean | Std. Dev. | Std. Error Mean | 95% Confidence Interval of the Difference | | t | df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Lower | Upper | | | |
| Pair 1 | PPM100 - CONTROL | -4.18 | 6.41 | 2.87 | -3.78 | 12.14 | 1.46 | 4.00 | 0.22 |
| Pair 2 | PPM400 - CONTROL | -0.80 | 3.63 | 1.62 | -3.71 | 5.31 | 0.49 | 4.00 | 0.65 |

*Table 105: Paired t test comparison of baseline PPO activity after treatment with citric acid*

| Paired Samples Statistics | | Mean | N | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Pair 1 | CONTROL | 87.50 | 8.00 | 35.36 | 12.50 |
| | - Citbase | 23.26 | 8.00 | 44.63 | 15.78 |

| Paired Differences | | Mean | Std. Deviation | Std. Error Mean | 95% CI of the Difference | | t | df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Lower | Upper | | | |
| Pair 1 | CONTROL - Citbase | 64.24 | 58.60 | 20.72 | 15.25 | 113.23 | 3.10 | 7.00 | 0.02 |

*Table 106: Paired t test comparison of total PPO activity after treatment with citric acid*

| Paired Samples Statistics | | Mean | N | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Pair 1 | CONTROL | 100.00 | 7.00 | 0.00 | 0.00 |
| | - cittot | 21.20 | 7.00 | 35.56 | 13.44 |

| Paired Differences | | Mean | Std. Deviation | Std. Error Mean | 95% CI of the Difference | | t | df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Lower | Upper | | | |
| Pair 1 | CONTROL - cittot | 78.80 | 35.56 | 13.44 | 45.92 | 111.69 | 5.86 | 6.00 | 0.00 |

*Table 107: Paired t test comparison of total PPO activity after treatment with 4-hexylresorcinol*

| Paired Samples Statistics | | Mean | N | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Pair 1 | CONTROL | 1.50 | 5.00 | 1.86 | 0.83 |
| | 4HTOT | 100.00 | 5.00 | 0.00 | 0.00 |

| Paired Differences | | Mean | Std. Deviation | Std. Error Mean | 95% CI of the Difference | | t | df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Lower | Upper | | | |
| Pair 1 | CONTROL – 4HTOT | -98.50 | 1.86 | 0.83 | -100.81 | -96.19 | -118.41 | 4.00 | 0.00 |

**Statistics for evaluation of the impact of antibrowning agents in commercial processing**

*Table 108: Descriptive statistics for by initial Total PPO of antibrowning agent trials*

| Total PPO | Mean | Std. Error | Minimum | Maximum |
|---|---|---|---|---|
| Control CO2 | 21.14 | 2.06 | 5.64 | 39.57 |
| CO2 | 32.28 | 2.93 | 6.41 | 81.91 |
| Control citric | 25.06 | 2.00 | 10.31 | 43.82 |
| Citric1.5 | 20.24 | 1.12 | 7.81 | 36.72 |
| Citric 2.5 | 16.62 | 1.37 | 3.39 | 35.45 |
| Control 10ppm&50ppm | 22.31 | 2.04 | 9.02 | 41.48 |
| 10ppm 4_H | 18.48 | 1.06 | 6.44 | 37.88 |
| 50 ppm 4_H | 22.61 | 1.40 | 1.15 | 48.25 |
| Control 87ppm | 19.77 | 1.25 | 9.66 | 32.69 |
| 87 ppm 4_H | 15.46 | 1.00 | 6.49 | 33.27 |
| Total | 20.99 | 0.57 | 1.15 | 81.91 |

*Table 109: Descriptive statistics for by initial baseline PPO of antibrowning agent trials*

| Base PPO | Mean | Std. Error | Minimum | Maximum |
|---|---|---|---|---|
| Control CO2 | 0.41 | 0.08 | 0.09 | 1.54 |
| CO2 | 0.57 | 0.08 | 0.03 | 2.12 |
| Control citric | 2.42 | 0.28 | 0.30 | 5.12 |
| Citric1.5 | 2.85 | 0.26 | 0.23 | 6.23 |
| Citric 2.5 | 3.26 | 0.28 | 1.25 | 7.56 |
| Control 10ppm&50ppm | 1.24 | 0.21 | 0.15 | 3.15 |
| 10ppm 4_H | 2.35 | 0.19 | 0.34 | 5.04 |
| 50 ppm 4_H | 4.82 | 0.46 | 2.29 | 9.53 |
| Control 87ppm | 1.78 | 0.26 | 0.50 | 4.50 |
| 87 ppm 4_H | 3.01 | 0.45 | 0.22 | 17.05 |
| Total | 2.30 | 0.11 | 0.03 | 17.05 |

*Table 110: Descriptive statistics for by initial protein of antibrowning agent trials*

| Protein | Mean | Std. Error | Minimum | Maximum |
|---|---|---|---|---|
| Control CO2 | 103.59 | 7.48 | 50.81 | 159.40 |
| CO2 | 100.35 | 4.34 | 54.05 | 167.50 |
| Control citric | 107.29 | 6.69 | 64.85 | 151.29 |
| Citric1.5 | 101.52 | 4.51 | 62.15 | 156.70 |
| Citric 2.5 | 98.89 | 4.41 | 51.35 | 167.50 |
| Control 10ppm&50ppm | 100.51 | 7.64 | 43.24 | 213.42 |
| 10ppm 4_H | 94.64 | 2.99 | 67.56 | 143.19 |
| 50 ppm 4_H | 98.15 | 3.89 | 62.15 | 170.20 |
| Control 87ppm | 82.63 | 4.34 | 58.37 | 135.09 |
| 87 ppm 4_H | 84.84 | 3.38 | 51.35 | 143.19 |
| Total | 96.92 | 1.50 | 43.24 | 213.42 |

- Rate of development is change in %melanosis area/minute

- Melanosis intensity is defined as the average pixel density of the area of melanosis where 0 = white and 255 = black.

*Table 111: Descriptives for antibrowning agent application by melanosis area*

| Area | Mean | Std. Error | Minimum | Maximum |
|---|---|---|---|---|
| Control CO2 | 12.33 | 1.78 | 1.94 | 26.95 |
| CO2 | 5.30 | 0.76 | 0.00 | 18.89 |
| Control citric | 7.70 | 0.60 | 4.27 | 13.57 |
| Citric1.5 | 3.98 | 0.51 | 0.00 | 12.75 |
| Citric 2.5 | 4.23 | 0.61 | 0.00 | 16.04 |
| Control 10ppm&50ppm | 9.00 | 0.97 | 2.19 | 18.47 |
| 10ppm 4_H | 1.46 | 0.36 | 0.00 | 7.76 |
| 50 ppm 4_H | 1.46 | 0.31 | 0.00 | 10.30 |
| Control 87ppm | 9.02 | 1.30 | 1.18 | 18.85 |
| 87 ppm 4_H | 1.34 | 0.24 | 0.00 | 4.85 |
| Total | 4.55 | 0.28 | 0.00 | 26.95 |

*Table 112: Descriptives for antibrowning agent application by melanosis intensity*

| Intensity | Mean | Std. Error | Minimum | Maximum |
|---|---|---|---|---|
| Control CO2 | 174.30 | 4.85 | 135.06 | 198.36 |
| CO2 | 162.16 | 3.67 | 97.54 | 193.44 |
| Control citric | 185.44 | 3.62 | 153.06 | 202.65 |
| Citric1.5 | 173.98 | 3.75 | 105.21 | 209.97 |
| Citric 2.5 | 174.32 | 2.55 | 123.51 | 205.95 |
| Control 10ppm&50ppm | 177.53 | 3.92 | 135.80 | 198.28 |
| 10ppm 4_H | 138.48 | 4.57 | 98.95 | 185.91 |
| 50 ppm 4_H | 130.97 | 4.13 | 84.05 | 180.96 |
| Control 87ppm | 156.75 | 4.98 | 102.63 | 189.33 |
| 87 ppm 4_H | 136.93 | 4.79 | 84.92 | 201.20 |
| Total | 158.83 | 1.73 | 84.05 | 209.97 |

*Table 113: Descriptives for antibrowning agent application by melanosis rate of development*

| Rate | Mean | Std. Error | Minimum | Maximum |
|---|---|---|---|---|
| Control CO2 | 0.0290 | 0.0049 | 0.0030 | 0.0754 |
| CO2 | 0.0108 | 0.0019 | 0.0000 | 0.0471 |
| Control citric | 0.0253 | 0.0026 | 0.0095 | 0.0544 |
| Citric1.5 | 0.0087 | 0.0017 | 0.0000 | 0.0330 |
| Citric 2.5 | 0.0098 | 0.0016 | 0.0000 | 0.0367 |
| Control 10ppm&50ppm | 0.0208 | 0.0031 | 0.0000 | 0.0480 |
| 10ppm 4_H | 0.0048 | 0.0012 | 0.0000 | 0.0238 |
| 50 ppm 4_H | 0.0056 | 0.0014 | 0.0000 | 0.0490 |
| Control 87ppm | 0.0277 | 0.0053 | 0.0000 | 0.0629 |
| 87 ppm 4_H | 0.0029 | 0.0007 | 0.0000 | 0.0176 |
| Total | 0.0116 | 0.0008 | 0.0000 | 0.0754 |

*Table 114: Tukey HSD groups for % area of melanosis*

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **87 ppm 4_H** | **1.34** | | | | |
| 10ppm 4_H | 1.46 | | | | |
| 50 ppm 4_H | 1.46 | | | | |
| Citric1.5 | 3.98 | 3.98 | | | |
| Citric 2.5 | 4.23 | 4.23 | | | |
| $CO_2$ | | 5.30 | 5.30 | | |
| Control citric | | | 7.70 | 7.70 | |
| Control 10ppm&50ppm | | | | 8.99 | |
| Control 87ppm | | | | 9.02 | |
| Control $CO_2$ | | | | | 12.33 |

*Table 115: Tukey HSD groups for average intensity*

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 50 ppm 4_H | 130.97 | | | | |
| 87 ppm 4_H | 136.93 | | | | |
| 10ppm 4_H | 138.48 | 138.48 | | | |
| Control 87ppm | | 156.75 | 156.75 | | |
| CO2 | | | 162.16 | 162.16 | |
| Citric1.5 | | | 173.98 | 173.98 | 173.98 |
| Control CO2 | | | 174.30 | 174.30 | 174.30 |
| Citric 2.5 | | | 174.32 | 174.32 | 174.32 |
| Control 10ppm&50ppm | | | | 177.53 | 177.53 |
| Control citric | | | | | 185.43 |

*Table 116: Tukey HSD groups for total PPO activity*

| | 1 | 2 | 3 |
|---|---|---|---|
| 87 ppm 4_H | 15.46 | | |
| Citric 2.5 | 16.62 | | |
| 10ppm 4_H | 18.48 | 18.48 | |
| Control 87ppm | 19.77 | 19.77 | |
| Citric1.5 | 20.24 | 20.24 | |
| Control CO2 | 21.14 | 21.14 | |
| Control 10ppm&50ppm | 22.31 | 22.31 | |
| 50 ppm 4_H | 22.61 | 22.61 | |
| Control citric | | 25.06 | 25.06 |
| CO2 | | | 32.28 |

*Table 117: Tukey HSD groups for baseline PPO activity*

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Control CO2 | 0.41 | | | | |
| CO2 | 0.57 | | | | |
| Control 10ppm&50ppm | 1.24 | 1.24 | | | |
| Control 87ppm | 1.78 | 1.78 | 1.78 | | |
| 10ppm 4_H | | 2.35 | 2.35 | 2.35 | |
| Control citric | | 2.42 | 2.42 | 2.42 | |
| Citric1.5 | | | 2.86 | 2.86 | |
| 87 ppm 4_H | | | 3.01 | 3.01 | |
| Citric 2.5 | | | | 3.26 | |
| 50 ppm 4_H | | | | | 4.82 |

*Table 118: Pearson Correlation coefficients for melanosis factors and PPO activity*

| | Protein | Total PPO | Baseline PPO |
|---|---|---|---|
| % Area | .263** | .230** | -.253** |
| Intensity | .317** | .170** | -.175** |
| Rate | .294** | .235** | -.200** |

** Correlation is significant at the 0.01 level (2-tailed).

*Table 119: Significant regression values for melanosis factors and physiological parameters by treatment*

| | R | $R^2$ | Std. Error of the Estimate | ANOVA Sig. | Y intercept | Slope |
|---|---|---|---|---|---|---|
| **Intensity vs. rate** | | | | | | |
| CO2 | 0.49 | 0.24 | 0.0104 | 0.002 | -3.11E-02 | 2.62E-04 |
| Control 10ppm&50ppm | 0.506 | 0.256 | 0.01153 | 0.032 | -4.91E-02 | 3.94E-04 |
| 10ppm 4_H | 0.434 | 0.188 | 0.00616 | 0.034 | -1.19E-02 | 1.30E-04 |
| Control 87ppm | 0.513 | 0.264 | 0.01984 | 0.029 | -5.77E-02 | 5.45E-04 |
| **Area vs. rate** | | | | | | |
| 87 ppm 4_H | 0.724 | 0.524 | 0.00293 | 0 | -3.29E-05 | 2.15E-03 |
| Control 87ppm | 0.853 | 0.727 | 0.01208 | 0 | -3.46E-03 | 3.46E-03 |
| 50 ppm 4_H | 0.958 | 0.917 | 0.00264 | 0 | -1.31E-03 | 4.66E-03 |
| 10ppm 4_H | 0.881 | 0.777 | 0.0031 | 0 | 6.69E-04 | 2.85E-03 |
| Control 10ppm&50ppm | 0.665 | 0.443 | 0.00998 | 0.003 | 1.96E-03 | 2.09E-03 |
| Citric 2.5 | 0.825 | 0.68 | 0.00557 | 0 | 6.97E-04 | 2.14E-03 |
| Citric1.5 | 0.628 | 0.394 | 0.00786 | 0 | 4.60E-04 | 2.08E-03 |
| Control citric | 0.933 | 0.87 | 0.00406 | 0 | -6.25E-03 | 4.09E-03 |
| CO2 | 0.908 | 0.825 | 0.00497 | 0 | -1.29E-03 | 2.28E-03 |
| Control CO2 | 0.887 | 0.787 | 0.0088 | 0 | -1.11E-03 | 2.44E-03 |
| **Area vs. intensity** | | | | | | |
| CO2 | 0.533 | 0.285 | 18.87257 | 0.001 | 147.98 | 2.535 |
| Control 10ppm&50ppm | 0.559 | 0.313 | 14.21942 | 0.016 | 157.194 | 2.26 |
| 10ppm 4_H | 0.465 | 0.216 | 20.25837 | 0.022 | 129.315 | 5.013 |
| Control 87ppm | 0.609 | 0.371 | 17.26988 | 0.007 | 135.745 | 2.328 |
| **Baseline PPO vs. intensity** | | | | | | |
| 10ppm 4_H | 0.496 | 0.246 | 1.10501 | 0.014 | 6.391 | -2.76E-02 |