The effectiveness of new anti-melanotic products from Xyrex Ltd. for treating crabs and langoustines

A Scientific Report

by

Dr Amaya Albalat

Professor Douglas Neil

October 2009

University of Glasgow | Faculty of Biomedical & Life Sciences

Introduction

Xyrex Ltd is a Scottish company that develops, produces and markets a range of innovative products for the seafood industry to deliver maximum quality, higher yields and extended shelf life. All the Xyrex products are fully tested and approved as food processing aids, ensuring that they can be used safely and effectively without requirements for declarations on labeling. The Xyrex range of products is now used extensively throughout the world, and the company is a market leader in the production of antimelanotic treatments for shellfish, which prevent melanosis ('blackspot'). The most extensively used of these products is Prawn-Fresh™, which has been approved by the EU (Directive 2006/52/EC article 19), provides sulphite-free melanosis control of langoustines as well as shelf life extension.

The company wishes to adapt its Prawn-Fresh™ product for use on other commercially important shellfish species, particularly crabs, which are extensively marketed in Europe, North America and Asia. In order to do this it requires scientific knowledge in two areas, namely the biochemical processes of melanosis development in the shells and soft tissues of crabs, and procedures for assessing the effectiveness of newly-developed antimelanotic treatments on the target crab species and also langoustines.

The new formulations tested in this project contain a reduction of green tea (one of the current ingredients in Prawn-Fresh™) since this natural anti-oxidant is very expensive and its efficacy has not been proven in Prawn-Fresh™, which has 4-hexylresorcinol as its.active component.

The aim of this project was to establish indices that can be used for scoring the natural rate of melanisation in untreated crabs stored in various ways, including iced, frozen and thawed. The effectiveness of various new formulations of antimelanotic treatments created by Xyrex were then to be evaluated in a series of trials on the target crab species and langoustines, to identify the most effective ones. Optimisation trials were also to be conducted to establish the treatment concentrations and times for these products to be

recommended to users. A biochemical assay was also to be developed for predicting the aggressiveness of melanisation in crab shell from the chemical activity of a key enzyme in the melanin synthetic pathway, namely polyphenol oxidase (PPO). This will provide a significant improvement in detection sensitivity, and will also represent a predictive tool for assessing the susceptibility of different crab species to melanisation.

Expected Deliverables

1. A scoring index for the objective measurement of the extent of melanisation in crabs
2. An evaluation of the effects of various storage methods, including icing and freeze-thawing, on the rate and extent of melanisation in crabs
3. Identification of the optimal formulation for new Xyrex products for suppressing melanisation in crabs
4. Establishment of the most effective treatment conditions for using these new Xyrex products for melanin suppression in crabs
5. A biochemical assay based on the reactivity of the key enzyme, PPO, involved in production of the melanin in crabs

Experimental trials

The expected deliverables were delivered through the following experimental trials:

- Trial 1: Development of melanosis in fresh and frozen/thawed harbour crabs (*Liocarcinus depurator*).
- Trial 2: Effectiveness of Prawn-Fresh™ in delaying melanosis development in frozen/thawed harbour crabs.
- Trial 3: Efficacy of Prawn-Fresh™ new modified formulations in delaying melanosis development in frozen/thawed harbour crabs
- Trial 4: Development of melanosis in fresh and frozen/thawed langoustines (*Nephrops norvegicus*).
- Trial 5: Optimisation of the concentration and time treatment of Prawn-Fresh™ in frozen/thawed langoustines.

- Trial 6: Effectiveness of Prawn-Fresh™ new modified formulations in delaying melanosis development in frozen/thawed langoustines
- Trial 7: Effectiveness of Prawn-Fresh™ new modified formulations in the activity of the enzyme PPO extracted from langoustines heads
- Trial 8: Effectiveness of Prawn-Fresh™ new modified formulations in delaying melanosis development in fresh langoustines

EXPERIMENTAL TRIAL 1: Development of melanosis in fresh and frozen/thawed harbour crabs (*Liocarcinus depurator*)

Methodology: Harbour crabs were collected by otter trawl in the Clyde Sea by the research vessel 'Aplysia' from the University Marine Biological Station Millport (UMBSM). Once on board animals were carefully washed and transported on ice to the facilities at the University of Glasgow. Once in the laboratory, half of the animals were frozen at –20 °C and the other half were directly stored at 4-5 °C for up to 11 days (called 'fresh animals').

Frozen crabs were kept in the freezer for 4 days before being thawed and stored at 4-5 °C for up to 11 days (called 'frozen animals'). Melanosis development was assessed on days 1, 4, 7 and 11 on 'fresh animals' and on days 1, 3, 5 and 7 of storage on 'frozen animals'. Blackening was assessed using digital images and quantified using the Melanosis Index Score shown in Table 1 in the different parts of the crab body (cephalothorax dorsal, first clawed legs, cephalothorax ventral, pereipods and inner ventral part).

Table 1. Scoring system used to assess melanosis development in crabs

Scoring	Attributes
1.0	Total absence of black spots or blackening
2.0	Few black spots or blackening (less than 30 %)
3.0	Considerable blackening (between 30-70 %)
4.0	Substantial blackening (more than 70 %)

Results: As shown in Figure 1 harbour crabs frozen/thawed develop melanosis at a faster rate than crabs that are fresh (not previously frozen). This was the case for all the part of the body studied.

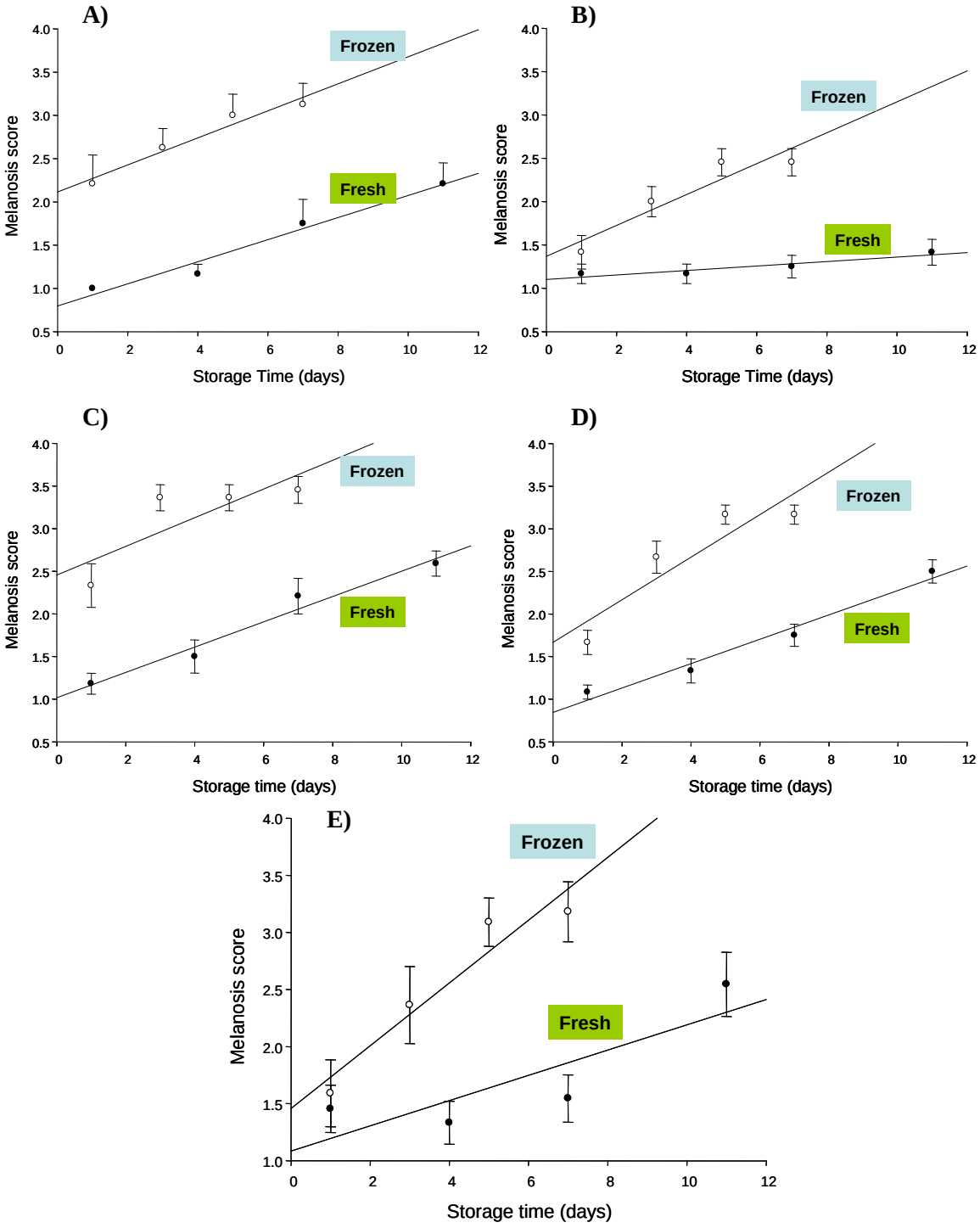


Figure 1. Melanosis score in fresh and frozen/thawed harbour crabs that were stored at 4-5 ºC for up to 11 days. A) Cephalothorax dorsal; B) First clawed legs; C) Cephalothorax ventral; D) Pereipods; E) Inner part containing the pleopods. Values are the mean ± S.E.M. for ten different animals.

EXPERIMENTAL TRIAL 2: Effectiveness of Prawn-Fresh™ in delaying melanosis development in frozen/thawed harbour crabs.

Methodology: Harbour crabs were collected by otter trawl in the Clyde Sea by the research vessel 'Aplysia' from the University Marine Biological Station Millport (UMBSM). Once on board animals were washed carefully and groups of 10 animals each were separated and dipped according to the following treatments:

- Un-dipped (control group)
- Dipped on Prawn-Fresh™ diluted 1/1000 for 15 min (as recommended by manufacturer)
- Dipped on Melacide-SC20 at a concentration of 2 % for 15 min (as recommended by manufacturer)
- Dipped on Sodium metabisulphite (NaMet) at a concentration of 2.5 % for 3 min (as recommended by manufacturer)

Afterwards animals were placed in plastic bags and transported on ice to the facilities at the University of Glasgow. Once in the laboratory, animals were frozen at –20 °C for 4 days and were thawed and stored at 4-5 °C for up to 7 days. Melanosis development was assessed on days 1, 3, 5 and 7 of storage. Blackening was assessed using digital images and quantified using the Melanosis Index Score.

Results: Melanosis development in frozen/thawed crabs was not suppressed or delayed by using Prawn-Fresh™ as shown in Figures 2 and 3. Commercially available sulphite-based treatments (Melacide-SC20 and sodium metabisulphite) performed better than Prawn-Fresh™ although they were not as efficient as in langoustines (data not shown) and on day 7 melanosis was clear in all treatment groups.

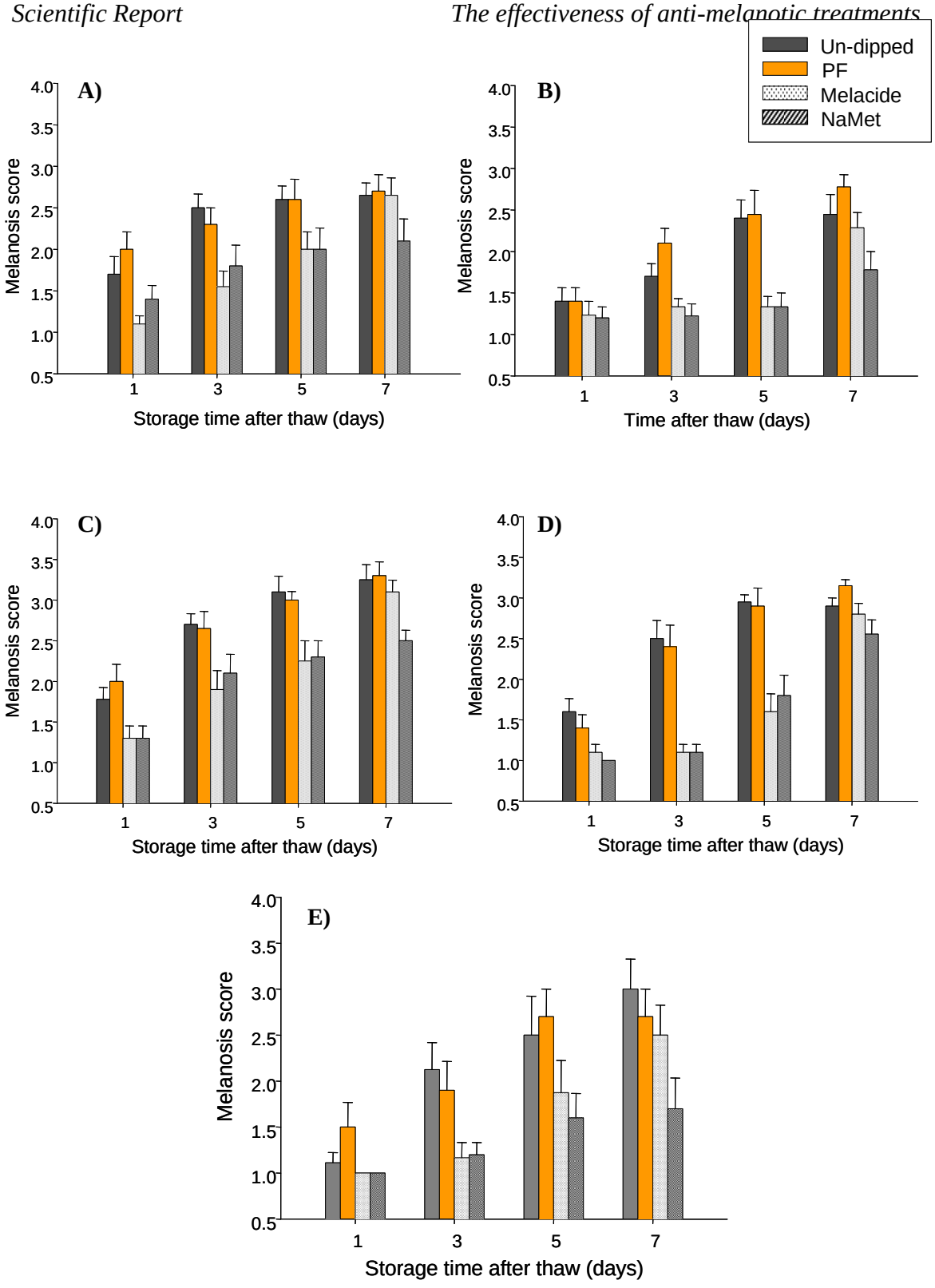


Figure 2. Melanosis score in frozen/thawed harbour crabs un-dipped or dipped using different anti-melanotic treatments that were stored at 4-5 ºC for up to 7 days. A) Cephalothorax dorsal; B) First clawed legs; C) Cephalothorax ventral; D) Pereipods; E) Inner part containing the pleopods. Values are the mean ± S.E.M. for ten different animals.

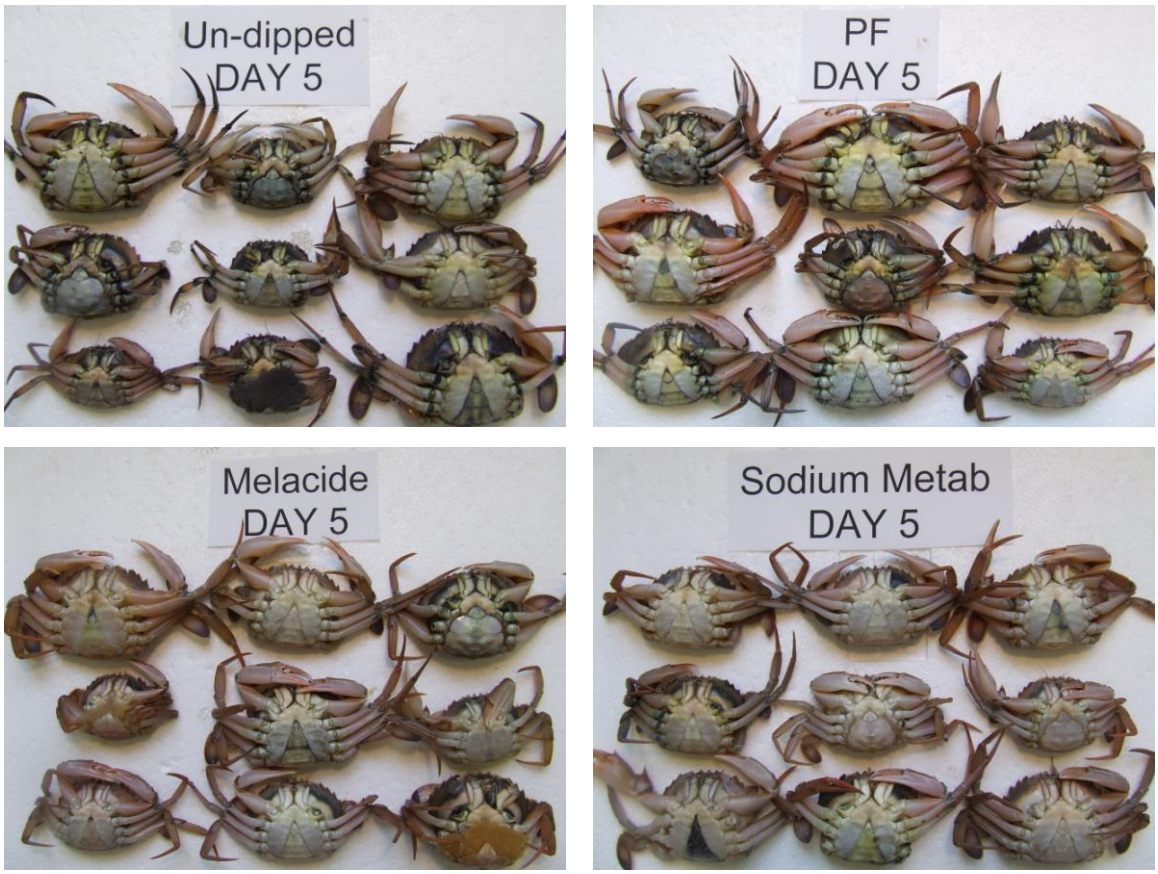


Figure 3. Digital image showing the melanosis development on crabs (ventral side) un-dipped or dipped using different anti-melanotic treatments on day 5 of storage at 4-5 °C.

EXPERIMENTAL TRIAL 3: Efficacy of new modified Prawn-Fresh™ formulations in delaying melanosis development in fresh harbour crabs

Methodology: Harbour crabs were collected by otter trawl in the Clyde Sea by the research vessel 'Aplysia' from the University Marine Biological Station Millport (UMBSM). Once on board animals were washed carefully and groups of 10 animals each were separated and dipped according to the following treatments:

- Un-dipped (control group)
- Dipped on new Prawn-Fresh formulation (PF-A): Same formulation as Prawn-Fresh™ but with 0 % green tea. Treatment concentration and time was 1/1000 for 15 min.
- Dipped on new Prawn-Fresh formulation (PF-B): Same formulation as Prawn-Fresh™ but with 50 % of the active component 4-hexylresorcinol and 0 % of green tea. Treatment concentration and time was 1/1000 for 15 min.
- Dipped on Prawn-Fresh™ diluted 1/1000 for 15 min (as recommended by manufacturer)

Afterwards animals were placed in plastic bags and transported on ice to the facilities at the University of Glasgow. Once in the laboratory, animals were frozen at –20 °C for 4 days and were thawed and stored at 4-5 °C for up to 7 days. Melanosis development was assessed on days 1, 3, 5 and 7 of storage. Blackening was assessed using digital images and quantified using the Melanosis Index Score.

Results: Results from this experiment confirmed the non-suitability of Prawn-Fresh™ to suppress melanosis development in crabs that have been frozen at some point during post-harvest (Figure 4). In this sense, no clear differences were found between crabs that were un-dipped and crabs that had been dipped in Prawn-Fresh™. The use of new formulations did not affect the efficacy of Prawn-Fresh™.

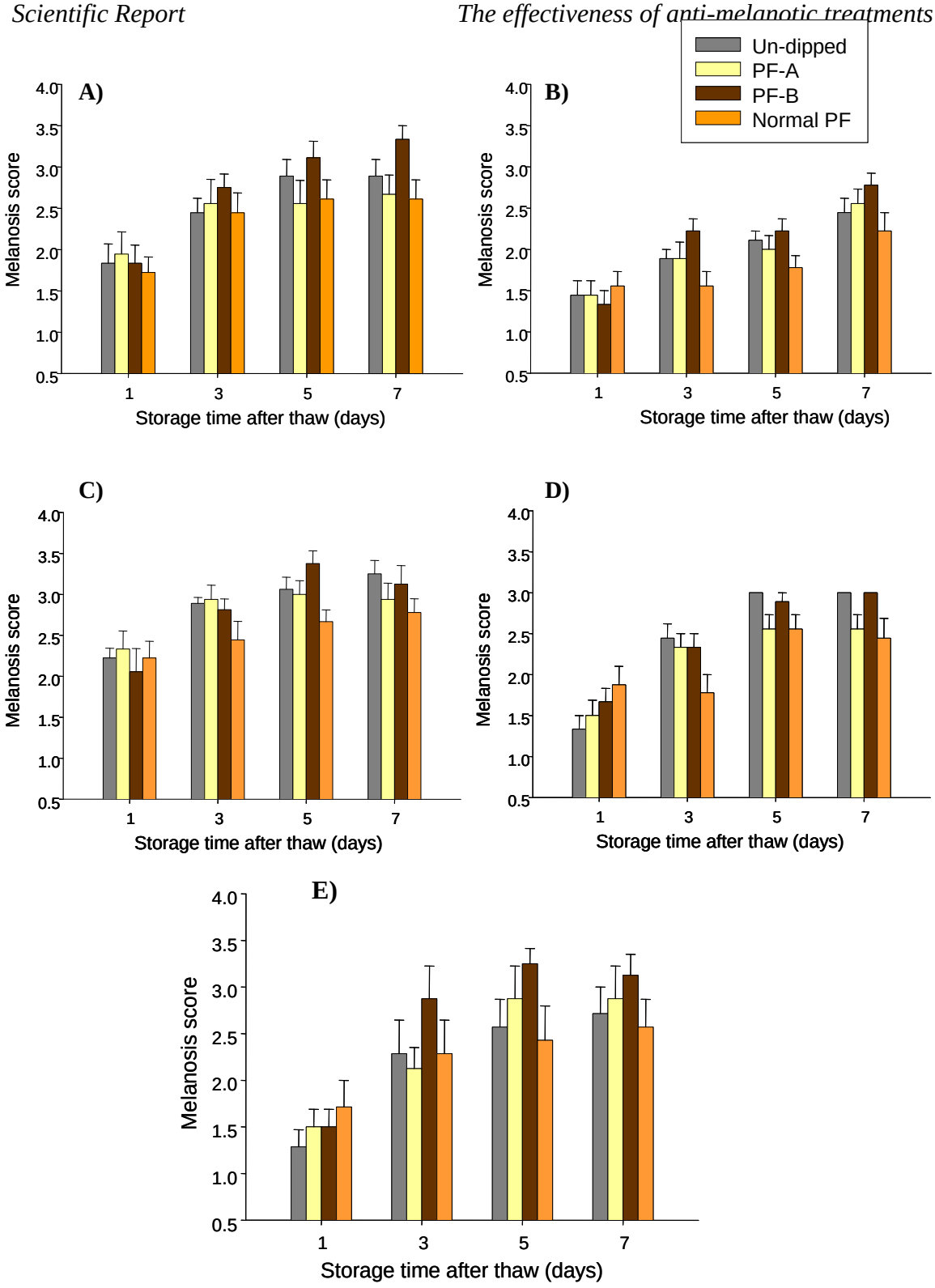


Figure 4. Melanosis score in frozen/thawed harbour crabs un-dipped or dipped using Prawn-Fresh™ and different modified formulations that were stored at 4-5 ºC for up to 7 days. A) Cephalothorax dorsal; B) First clawed legs; C) Cephalothorax ventral; D) Pereipods; E) Inner part containing the pleopods. Values are the mean ± S.E.M. for ten different animals.

EXPERIMENTAL TRIAL 4: Development of melanosis in fresh and frozen/thawed langoustines (*Nephrops norvegicus*) and the effectiveness of Prawn-Fresh™

Methodology: Langoustines were caught by otter trawl in the Clyde Sea by the research vessel 'Aora' from the University Marine Biological Station Millport (UMBSM). Animals were washed carefully and groups of 20 animals each were separated and dipped according to the following treatments:

1 – Un-dipped animals

2 – Dipped animals with Prawn-Fresh™ at a concentration of 1/1000 in fresh seawater for 15 min (as recommended by manufacturer)

Afterwards, animals were transported on ice to the facilities at the University of Glasgow. At this point, each group was separated into 2 further groups. One group was stored at 5-6ºC and the other group was frozen for 2 days and then thawed and stored at 5-6ºC. Therefore the groups in this experiment were as follows:

1 N – Un-dipped animals stored at 5-6ºC

1 F – Un-dipped animals frozen and then thawed and stored at 5-6ºC

2 N – Animals dipped with Prawn-Fresh™ and stored at 5-6ºC

2 F – Animals dipped with Prawn-Fresh™, frozen and then thawed and stored at 5-6ºC.

As in the previous experiment melanosis development was recorded as digital images and also by using the Melanosis Score Index for the different parts of the body of the langoustines.

Results: The first observation from this experiment was that frozen langoustines develop melanosis very quickly when they are thawed (Figure 4). This more rapid blackening in frozen/thawed animals compared to animals that are fresh and never frozen was observed in all the different parts of the body. Therefore, from day 1 un-dipped langoustines that had been frozen/thawed showed clear signs of melanosis in contrast to un-dipped animals that had not previously been frozen. A possible explanation of these results is that the freezing process in some way increases the activity of the enzyme responsible of the blackening, known as polyphenol oxidase enzyme (PPO).

However, Prawn-Fresh™ was able to inhibit melanosis in langoustines that had been subjected to a freeze/thaw cycle then stored at 5-6ºC. This was shown to be the case for all the parts of the body and no differences in blackening were evident between fresh and frozen/thawed langoustines that had been dipped in Prawn-Fresh™. This contrasts with the situation in un-dipped animals, and therefore, it would appear that although melanosis is activated due to the freezing process, Prawn-Fresh™ is able to inhibit this activation if applied before freezing the langoustines.

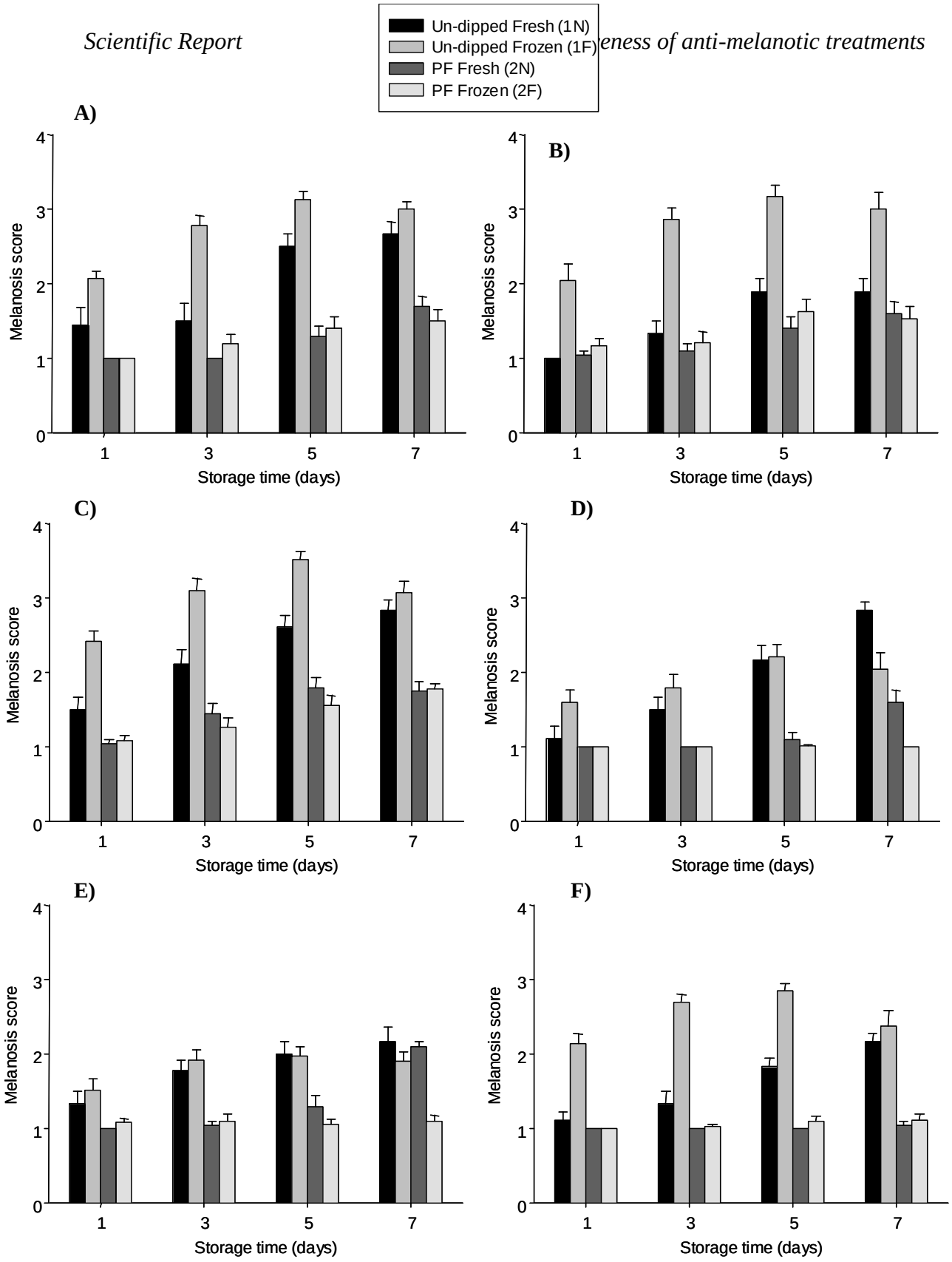


Figure 4. Melanosis score in fresh and frozen/thawed langoustines that were un-dipped or dipped with Prawn-Fresh™ and stored at 5-6 ºC for up to 7 days. A) Cephalothorax dorsal; B) First clawed legs; C) Cephalothorax ventral; D) Pleopods; E) Tails; F) Tail fan. Values are the Mean ± S.E.M. of ten different tails.

EXPERIMENTAL TRIAL 5: Optimisation of the concentration and time treatment of Prawn-Fresh™ in frozen/thawed langoustines

Methodology: Langoustines were caught by otter trawl in the Clyde Sea by the research vessel 'Aplysia' from the University Marine Biological Station Millport (UMBSM). Animals were washed carefully and groups of 20 animals each were separated and dipped according to the following treatments:

- Un-dipped animals
- Animals dipped in a solution of Prawn-Fresh™ 1/500 for:
    - 10 min
    - 5 min
    - 2 min
- Animals dipped in a solution of Prawn-Fresh™ 1/1000 for:
    - 15 min
    - 10 min
    - 5 min
    - 2 min
- Animals dipped in a solution of Prawn-Fresh™ 1/2000 for:
    - 15 min
    - 10 min
    - 5 min
    - 2 min
- Animals dipped in a solution of Prawn-Fresh™ 1/4000 for:
    - 15 min
    - 10 min
    - 5 min
    - 2 min

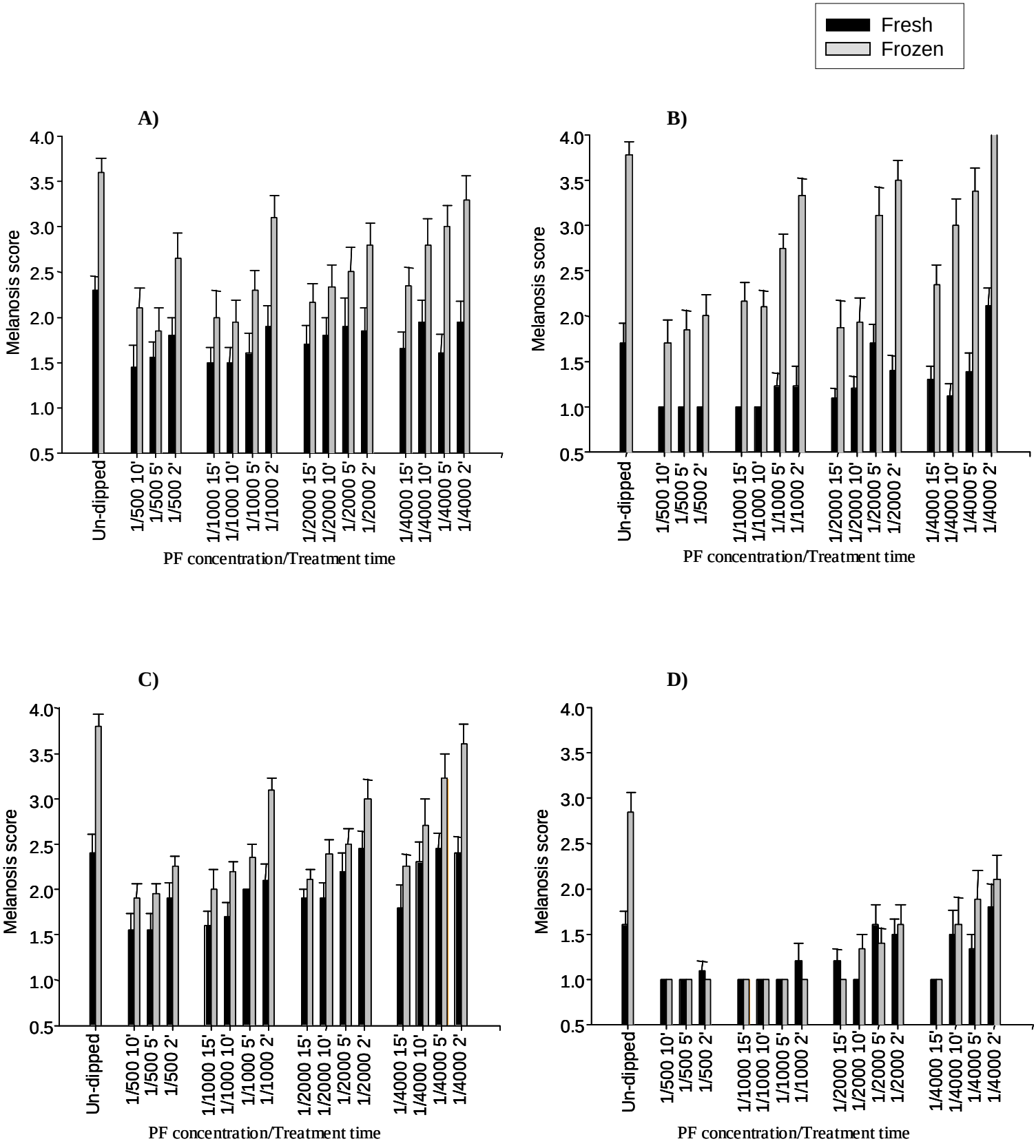
Afterwards, animals were transported on ice to the facilities at the University of Glasgow. Once in the laboratory, half of the animals for each treatment (10 animals/treatment) were frozen at –20 °C and the other half were directly stored at 5-6 °C for up to 7 days (called

'fresh animals'). Frozen packs were kept in the freezer for 2 weeks before being thawed and stored at 5-6 °C for up to 7 days (called 'frozen animals').

Melanosis development was assessed on days 2, 5 and 7 on 'fresh animals' and on days 1, 3, 5 and 7 of storage on 'frozen animals'. Blackening was assessed using digital images and quantified using the Melanosis Index Score in the different parts of the langoustine body.

Results: Figure 5 shows the melanosis score for fresh and frozen/thawed animals stored at 5-6 ºC for 5 days. Animals frozen for 2 weeks and then thawed developed melanosis very quickly in all the different parts of the body, an effect already observed and described previously (Report I). This fact is important since some processors do freeze their animals at some point. Therefore, the concentration/time of Prawn-Fresh™ recommended by Xyrex Ltd. has to be effective not only of fresh animals but also on animals that have been frozen at some point.

When looking at frozen/thawed animals it was observed that the anti-melanotic effect of Prawn-Fresh™ is concentration dependent and concentrations lower than 1/1000 were not as effective. However, not only concentration but also treatment time seemed to be a very important factor for the effectiveness of Prawn-Fresh™.


Fresh
Frozen
A)
B)
C)
D)
Melanosis score
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Un-dipped
1/500 10'
1/500 5'
1/500 2'
1/1000 15'
1/1000 10'
1/1000 5'
1/1000 2'
1/2000 15'
1/2000 10'
1/2000 5'
1/2000 2'
1/4000 15'
1/4000 10'
1/4000 5'
1/4000 2'
PF concentration/Treatment time

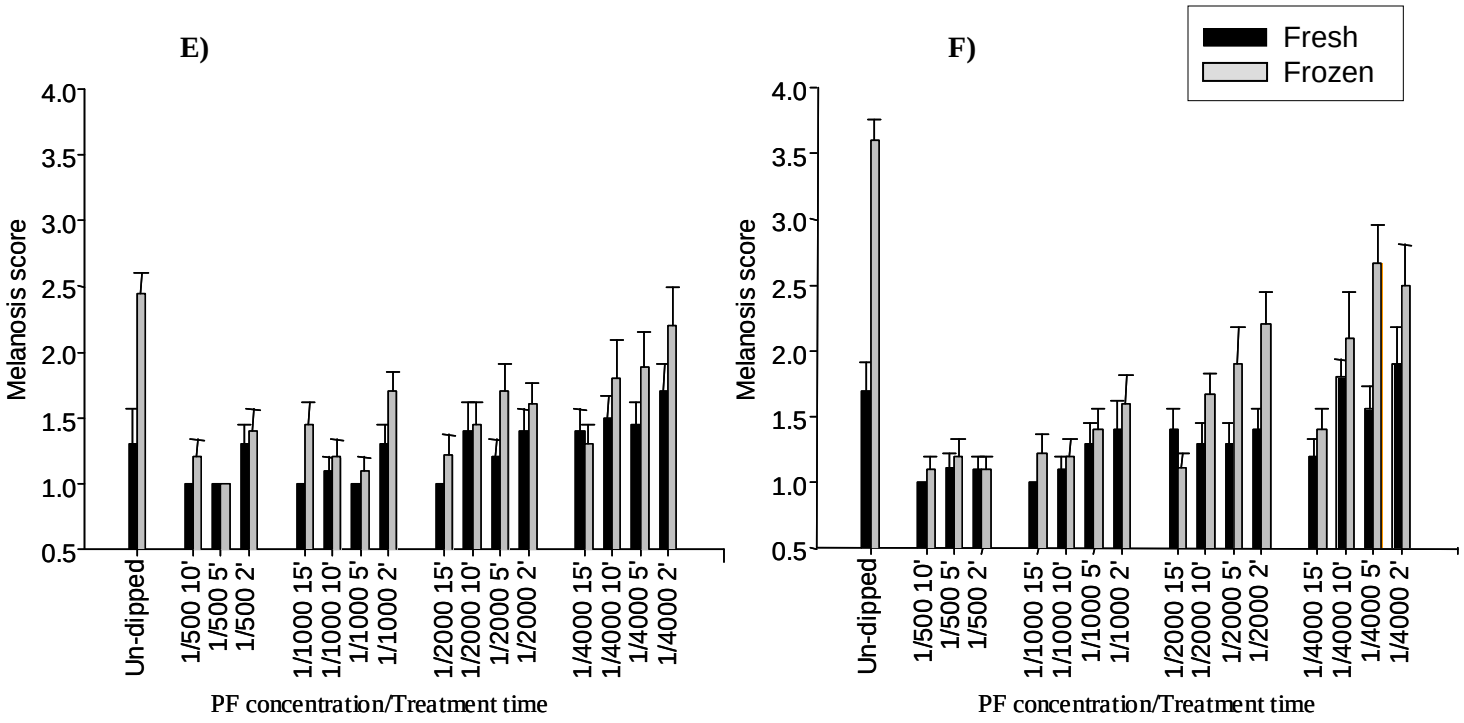


Figure 5. Melanosis score in fresh and frozen/thawed langoustines that were un-dipped or dipped with Prawn-Fresh™ at different concentrations and times and stored at 5-6 ºC for 5 days. A) Cephalothorax dorsal; B) First clawed legs; C) Cephalothorax ventral; D) Pleopods; E) Tails; F) Tail fan.Values are the mean ± S.E.M. for ten different animals.

At the dilution of 1/1000 no differences were observed between the treatment times of 15 and 10 min. However, shorter treatments times (5 and 2 min) gave a more rapid development of blackening, especially in the dorsal cephalothorax, first claw legs, ventral cephalothorax and pereiopods (Figures 5 and 6).

A) Un-dipped

B) PF 1/1000 10 min

C) PF 1/1000 2 min

Figure 6. Images showing blackening in animals un-dipped or dipped with Prawn-Fresh™ at the dilution 1/1000 for 10 or 2 min in frozen/thawed animals after being stored at 6-6 ºC for 5 days.

A more concentrated solution of Prawn-Fresh™ (dilution 1/500) was effective from 5 min onwards but at a shorter treatment time (2 min) blackening in cephalothorax dorsal was more pronounced than with the dilution 1/1000 for 15 or 10 min. The development of blackening at these two concentrations throughout the storage time studied are presented in Figure 7. No clear improvement of anti-melanotic effect is obtained at the higher Prawn-Fresh™ concentration.

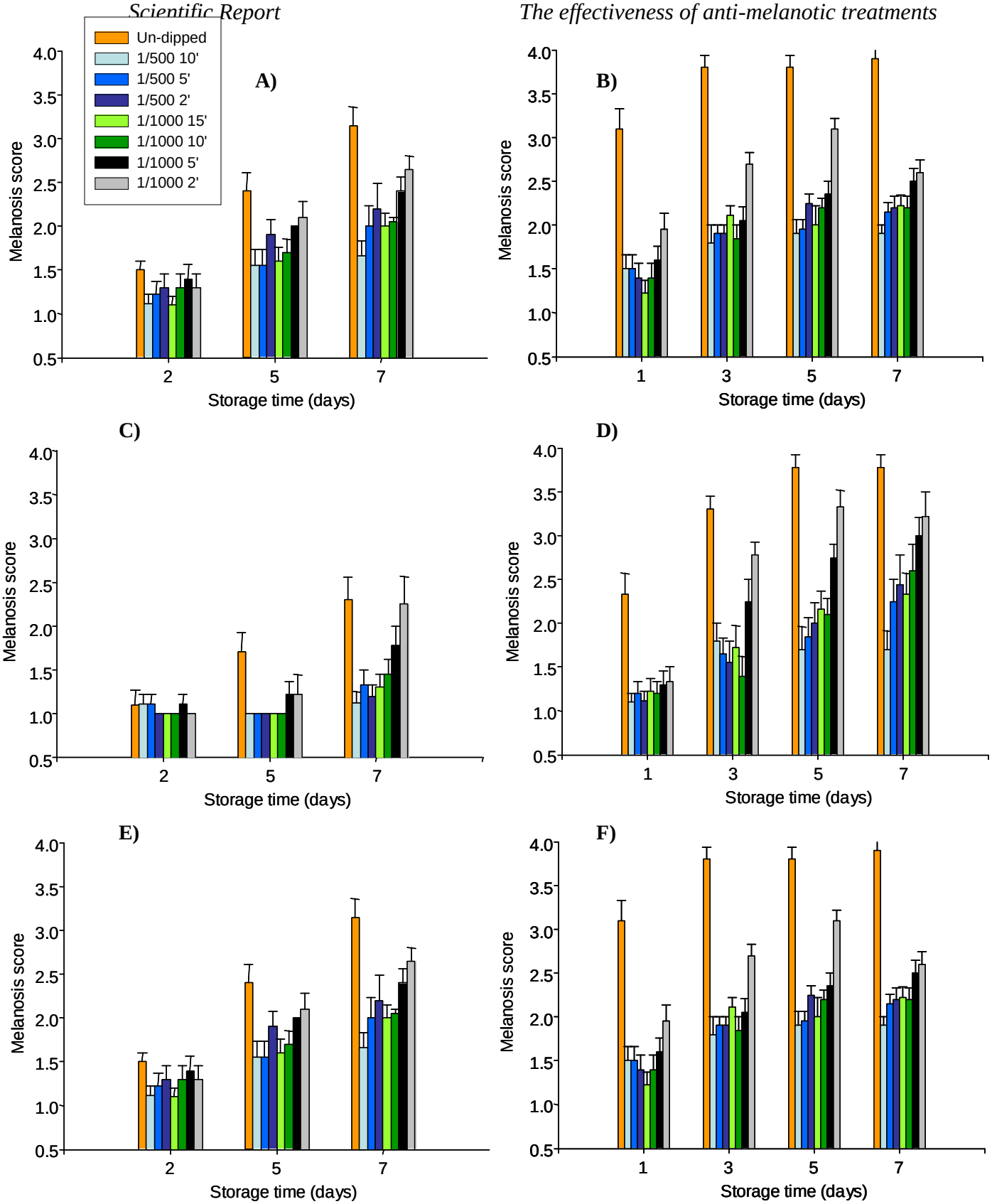


Figure 7. Melanosis score in frozen/thawed langoustines that were un-dipped or dipped with Prawn-Fresh™. A) Cephalothorax dorsal; B) First clawed legs; C) Cephalothorax ventral; D) Pleopods; E) Tails; F) Tail fan. Values are the mean ± S.E.M. for ten different animals.

EXPERIMENTAL TRIAL 6: Effectiveness of new modified Prawn-Fresh™ formulations in delaying melanosis development in frozen/thawed langoustines

Methodology: Langoustines were caught by otter trawl in the Clyde Sea by the research vessel 'Aplysia' from the University Marine Biological Station Millport (UMBSM). Animals were washed carefully and groups of 10-12 animals each were separated and dipped according to the following treatments:

- Un-dipped (control group)
- Dipped on new Prawn-Fresh formulation (PF-75 %): Same formulation as Prawn-Fresh™ but with 75 % green tea.
- Dipped on new Prawn-Fresh formulation (PF-50 %): Same formulation as Prawn-Fresh™ but with 50 % of green tea.
- Dipped on new Prawn-Fresh formulation (PF-25 %): Same formulation as Prawn-Fresh™ but with 25 % green tea.
- Dipped on new Prawn-Fresh formulation (PF-0 %): Same formulation as Prawn-Fresh™ but with 0 % of green tea.
- Dipped on Prawn-Fresh™ diluted 1/1000 for 15 min (as recommended by manufacturer)

Afterwards animals were placed in plastic bags and transported on ice to the facilities at the University of Glasgow. Once in the laboratory, animals were frozen at –20 °C for 4 days and were thawed and stored at 4-5 °C for up to 7 days. Melanosis development was assessed on days 1, 3, 5 and 7 of storage. Blackening was assessed using digital images and quantified using the Melanosis Index Score.

Results: As shown in Figures 8 and 9 the reduction of green tea in the formulation did not produce any significant effect in a concentration-dependent manner. Animals look slightly duller on the dorsal cephalothorax than animals treated with Prawn-Fresh™ with 100 % green tea, but no clear changes in melanosis inhibition were observed.

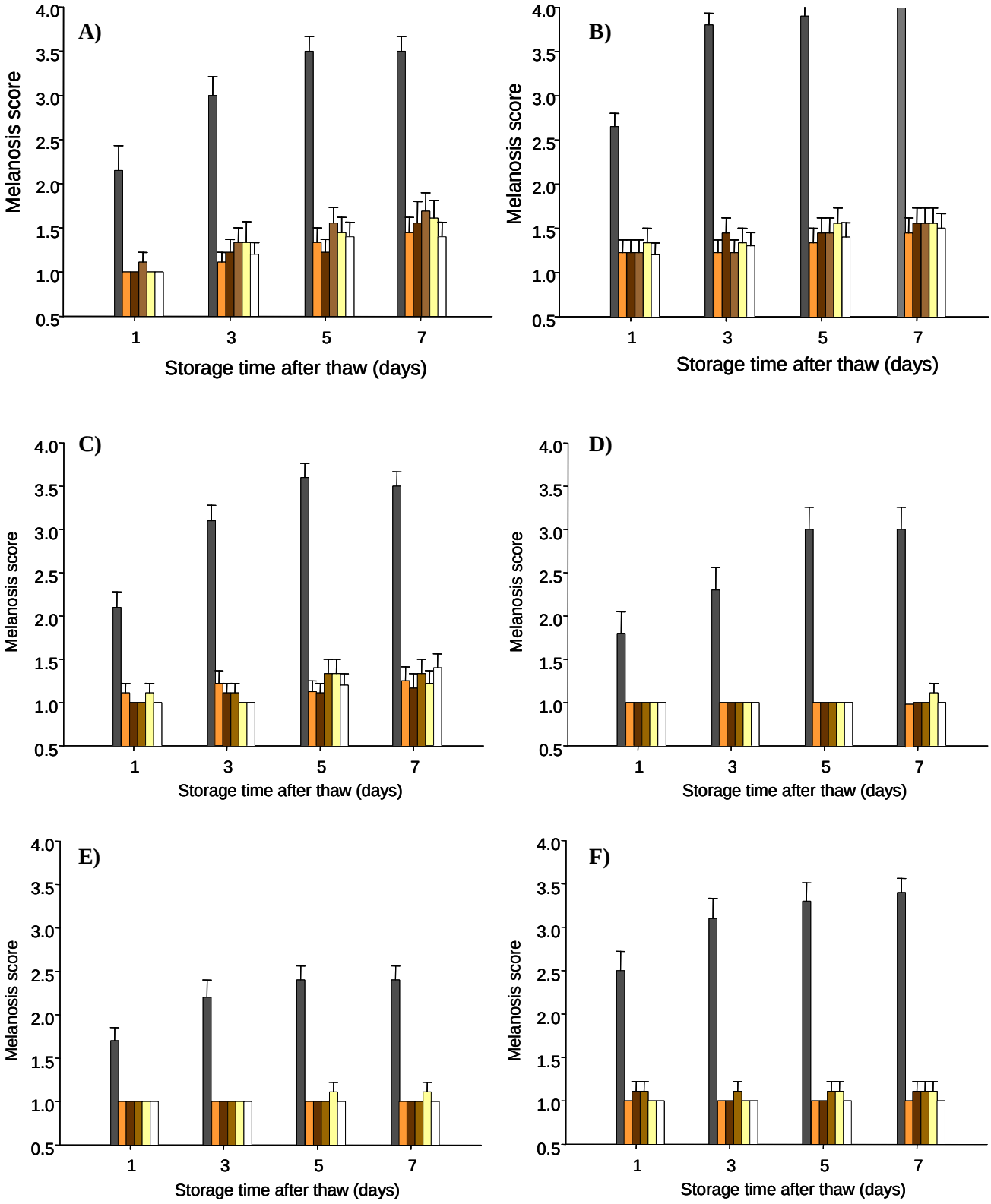


Figure 8. Melanosis score in frozen/thawed langoustines that were un-dipped or dipped with Prawn-Fresh™ and its new formulations. A) Cephalothorax dorsal; B) First clawed legs; C) Cephalothorax ventral; D) Pleopods; E) Tails; F) Tail fan. Values are the mean ± S.E.M. for ten different animals.

However, in order to gain a better insight into the effects of the different products on melanosis inhibition further experiments were conducted on the effect of these different formulations on the activity of the enzyme polyphenol oxidase or PPO.

Figure 9. Digital images of frozen/thawed langoustines that were un-dipped or dipped with Prawn-Fresh™ and its new formulations on day 5 of storage at 4-5 °C.

EXPERIMENTAL TRIAL 7: Effectiveness of new modified Prawn-Fresh™ formulations on the activity of the enzyme PPO extracted from langoustines heads

Methodology: Langoustines were caught by otter trawl in the Clyde Sea by the research vessel 'Aplysia' from the University Marine Biological Station Millport (UMBSM). Animals were carefully washed, placed in plastic bags and transported on ice to the facilities at the University of Glasgow. Once in the laboratory, half of the animals (10-15 animals) were frozen at –20 °C for 4 days; thawed at 5-6 °C and stored at -80 °C until enzyme extracts were prepared (called 'frozen/thawed' samples). The other half of the animals were directly stored at -80 °C until enzyme preparations were done (called 'fresh' samples).

PPO activity was measured in the cephalothorax of whole langoustines as this is the body part were melanosis is more pronounced once the animals are removed from the water. Crude enzyme extracts from the cephalothorax were prepared according to Wang et al. (1992). Between 30-40 g of cephalothorax was added to 2 x of 0.1 M sodium phosphate buffer pH 6.4 and homogenised in an Ultra-Turrax homogenised for 2 min on ice. Homogenates were then centrifuged at 50,000 x g for 30 min at 4 °C. Supernatants were used as the crude polyphenoloxidase preparations and were immediately frozen at -80 °C to minimise alterations.

The enzyme activity was measured using the proline-catechol spectrophotometric assay at saturating conditions as described in Martinez-Alvarez et al. (2005). The reaction mixture contained 480 µl of 30 mM cathechol, 480 µl of 30 mM L-proline and 40 µl of crude enzyme preparation or buffer in blank condition. Cathecol and L-proline were prepared in 0.1 M sodium phosphate buffer. Changes in absorbance at 530 nm were monitored at 25 °C for 11-12 min in a spectrophotometer with a thermostat controller.

In order to study the effect of Prawn-Fresh™ on the PPO activity, crude enzyme preparations from cephalothoraxes from fresh and frozen/thawed animals were pre-incubated for 15 min (or otherwise stated) on ice before the enzyme activity was

measured. In control samples, enzyme extracts were pre-incubated with phosphate buffer in the same conditions.

Results: PPO activity was higher in extracts from cephalothoraxes that had been frozen/thawed compared to cephalothoraxes that were fresh (Figure 10). These results confirm the activation in melanosis obtained in frozen/thawed langoustines in previous trials.

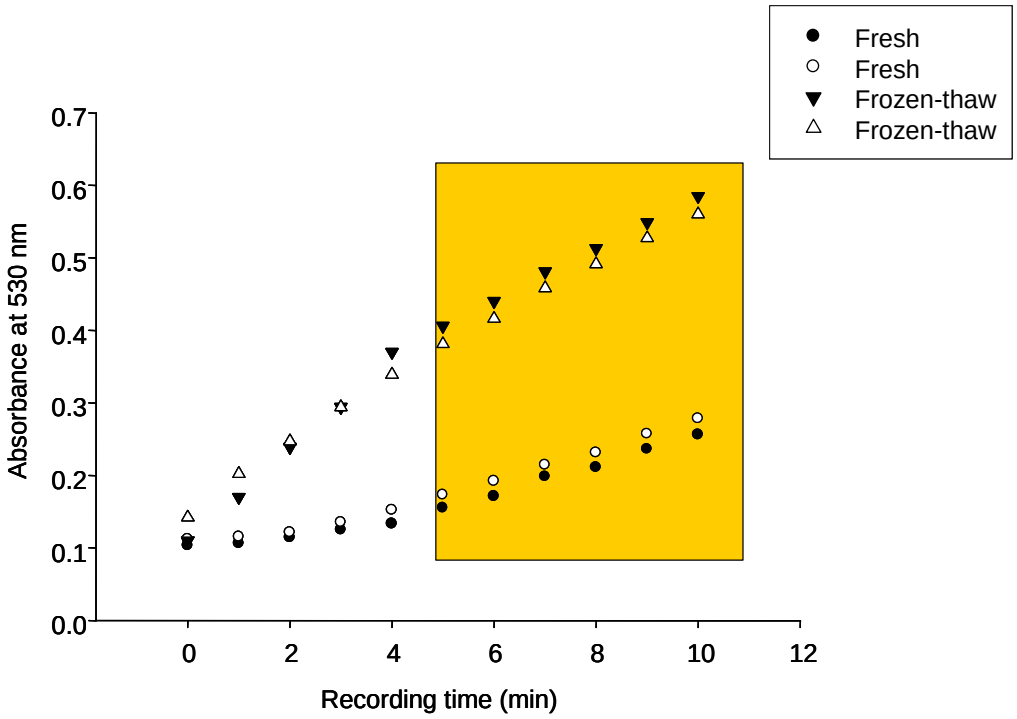


Figure 10. PPO activity in cephalothoraxes from fresh and frozen/thawed animals.

Before testing the effect of the different modified formulations of Prawn-Fresh™ it was necessary to determine the optimal conditions for the experiments. To this end, it was tested for how long it was best to pre-incubate the Prawn-Fresh™ with the enzyme extracts to obtained maximum inhibition levels. As shown in Figure 11, when Prawn-Fresh™ was pre-incubated for 15 min the inhibition of PPO was greater than when it was only pre-incubated for 5 min. For this reason, the modified formulations were pre-incubate for 15 min in order to get more definitive results. Furthermore, higher concentrations of Prawn-Fresh™ elicited a greater inhibition of the PPO, activity although the inhibition never reached to 100 %.

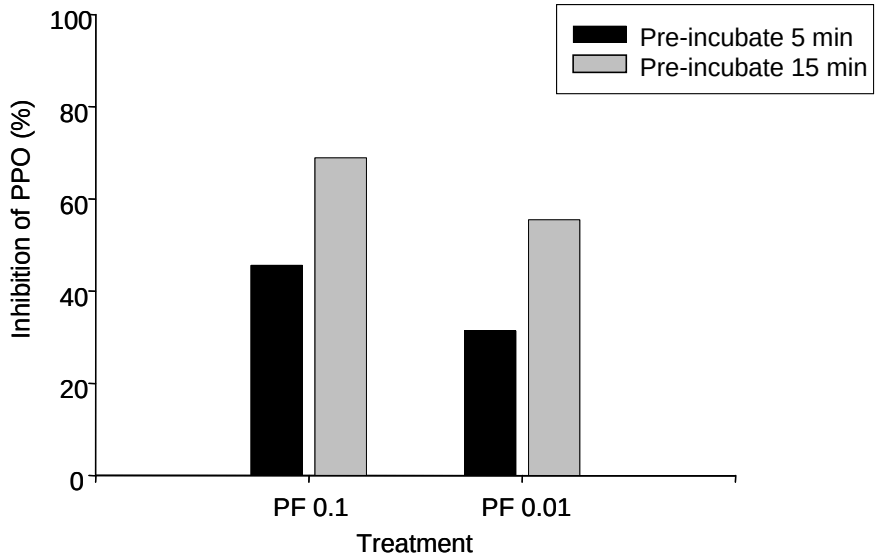


Figure 11. PPO activity in crude extract from cephalothoraxes from fresh animals pre-incubated with Prawn-Fresh™ for 15 or 5 min on ice before the activity was measured.

Experiments also showed that the inhibition of the PPO enzyme by Prawn-Fresh™ varied depending on the nature of the PPO extract. Therefore, as shown in Figure 12 Prawn-Fresh™ was not as effective in inhibiting PPO activity when extracts were obtained from frozen/thawed animals compared to extracts from fresh animals. For this reason, the effects of modified Prawn-Fresh™ formulations was assessed in enzyme extracts from fresh and also from frozen/thawed animals.

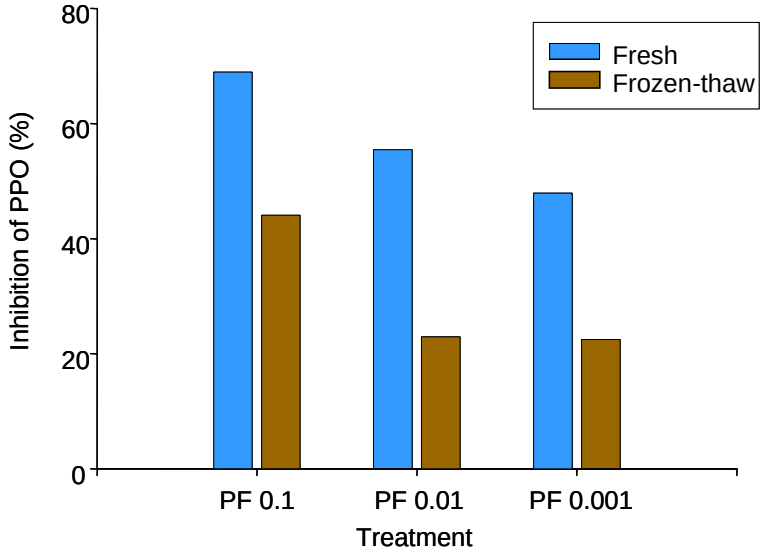


Figure 12. PPO activity in crude extract from cephalothoraxes from fresh and frozen/thawed animals pre-incubated for 15 min on ice with Prawn-Fresh™ before the activity was measured.

Finally, the different new formulations of Prawn-Fresh™ were used to analyse their impact on the PPO activity. As shown in Figure 13 very similar extents of inhibition of PPO activity were reached by Prawn-Fresh™ containing 75 % and 50 % of green tea compared to normal Prawn-Fresh™ in enzyme extracts from fresh animals. Concentrations of green tea in Prawn-Fresh™ lower than 50 % led to significantly lower levels of PPO activity inhibition, so that formulation with no green tea had a 26 % lower strength of inhibition than normal Prawn-Fresh™.

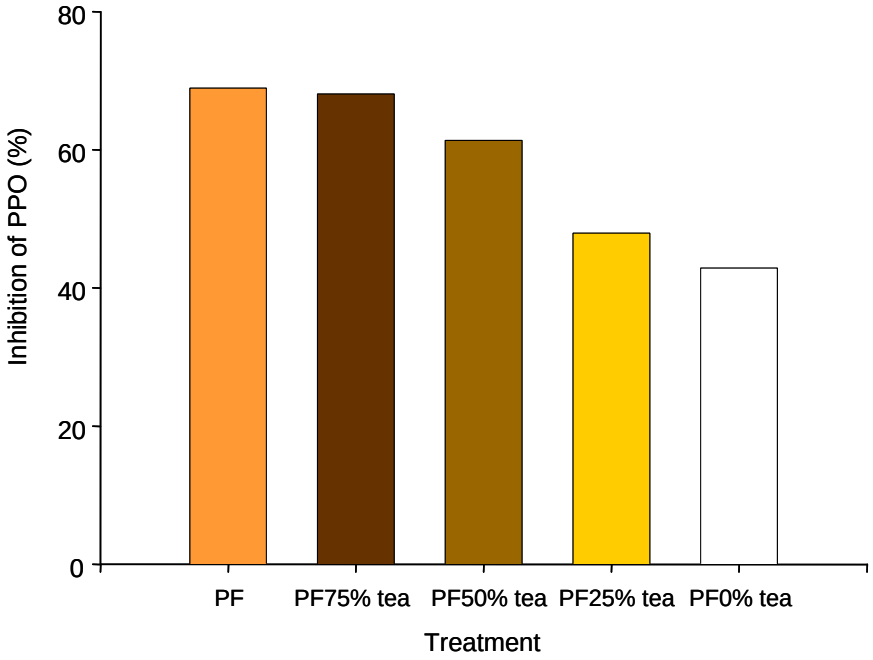


Figure 13. PPO inhibition in crude extract from cephalothoraxes from fresh animals pre-incubated for 15 min on ice with Prawn-Fresh™ and its new formulations before the activity was measured. The concentration used in this trial was 0.1 compared to the commercial use (1/1000)

It was also observed that the effect of all formulations was dependent on the concentration used (Figure 14).

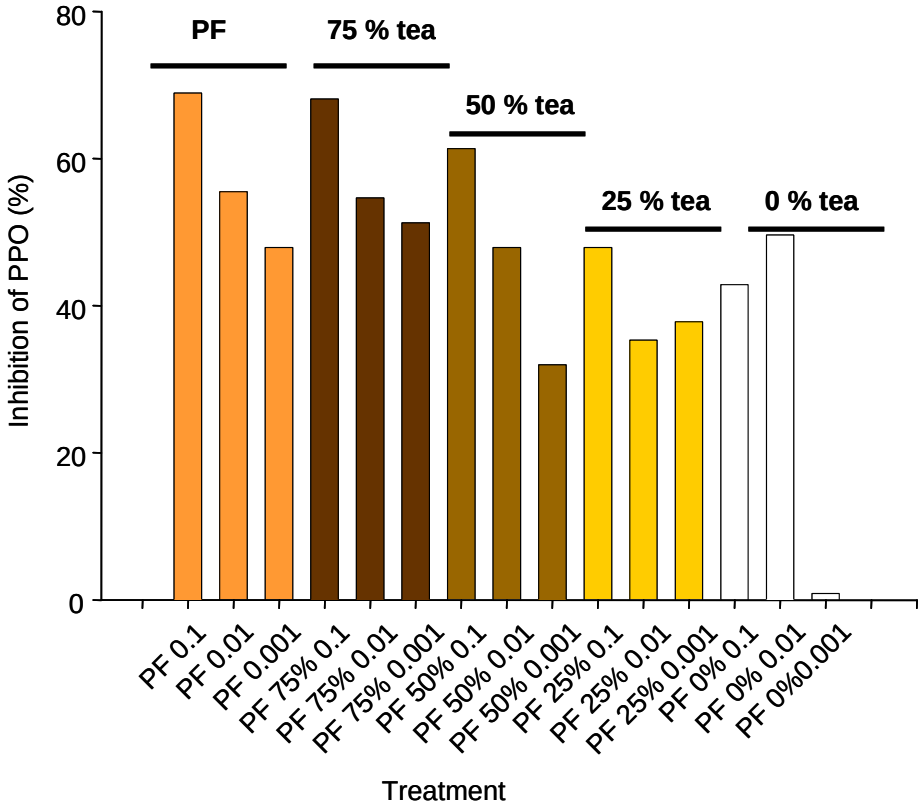


Figure 14. PPO inhibition in crude extract from cephalothoraxes from fresh animals pre-incubated for 15 min on ice with Prawn-Fresh™ and its new formulations before the activity was measured. The concentrations used in this trial were 0.1; 0.01 and 0.001 compared to the commercial use (1/1000)

The same experiments were carried out using enzyme extracts from frozen/thawed animals. Results using these frozen/thawed animals were similar to those obtained using fresh animals (Figure 15). Formulations containing 75 or 50 % of green tea gave similar levels of inhibition compared to normal Prawn-Fresh™ (containing 100 % of green tea). In this case, the formulation with no tea had 30 % less inhibition strength than the normal Prawn-Fresh™.

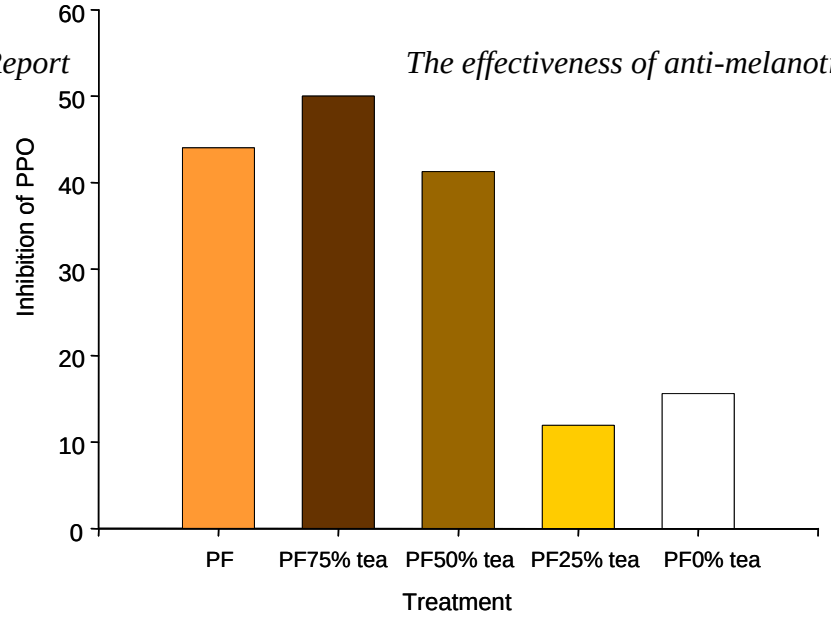


Figure 15. PPO inhibition in crude extract from cephalothoraxes from frozen/thawed animals pre-incubated for 15 min on ice with Prawn-Fresh™ and its new formulations before the activity was measured. The concentration used in this trial was 0.1 compared to the commercial use (1/1000)

Furthermore, a clear concentration-dependent response was observed in the PPO inhibition, indicating the importance of using the appropriate concentration of Prawn-Fresh™ in order to obtain optimal results (Figure 16).

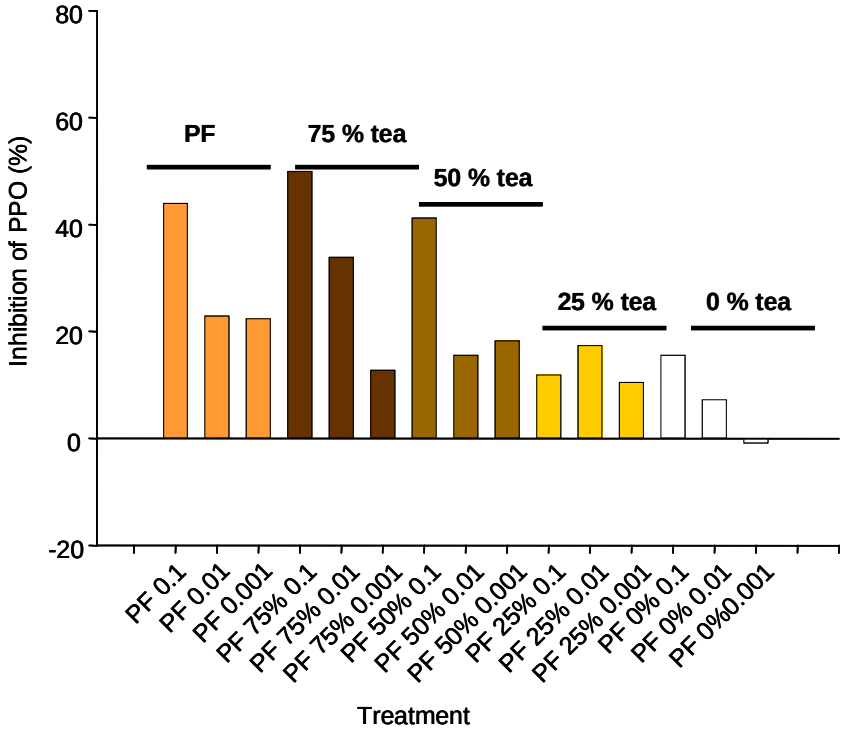


Figure 16. PPO inhibition in crude extract from cephalothoraxes from frozen/thawed animals pre-incubated for 15 min on ice with Prawn-Fresh™ and its new formulations before the activity was measured. The concentrations used in this trial were 0.1; 0.01 and 0.001 compared to the commercial use (1/1000)

EXPERIMENTAL TRIAL 8: Effectiveness of new modified Prawn-Fresh™ formulations in delaying melanosis development in fresh langoustines

Methodology: Langoustines were caught by otter trawl in the Clyde Sea by the research vessel 'Aplysia' from the University Marine Biological Station Millport (UMBSM). Animals were washed carefully and groups of 10-12 animals each were separated and dipped according to the following treatments:

- Un-dipped (control group)
- Dipped on new Prawn-Fresh formulation (PF-50 %): Same formulation as Prawn-Fresh™ but with 50 % of green tea.
- Dipped on new Prawn-Fresh formulation (PF-0 %): Same formulation as Prawn-Fresh™ but with 0 % of green tea.
- Dipped on Prawn-Fresh™ diluted 1/1000 for 15 min (as recommended by manufacturer)

Afterwards animals were placed in plastic bags and transported on ice to the facilities at the University of Glasgow. Once in the laboratory, animals were frozen at –20 °C for 4 days and were thawed and stored at 3-4 °C for up to 11 days. Melanosis development was assessed on days 3, 5, 7 and 11 of storage. Blackening was assessed using digital images and quantified using the Melanosis Index Score.

Results: As revealed in Figure 17 and 18 the reduction of green tea in the formulation of Prawn-Fresh™ did not produce a significant clear effect in a concentration-dependent manner, and no clear changes were observed in fresh langoustines stored at 3-4 °C. From day 7 onwards animals had a strong smell of ammonia and therefore although results are plotted up to day 11 animals were unfit for sale before this time.

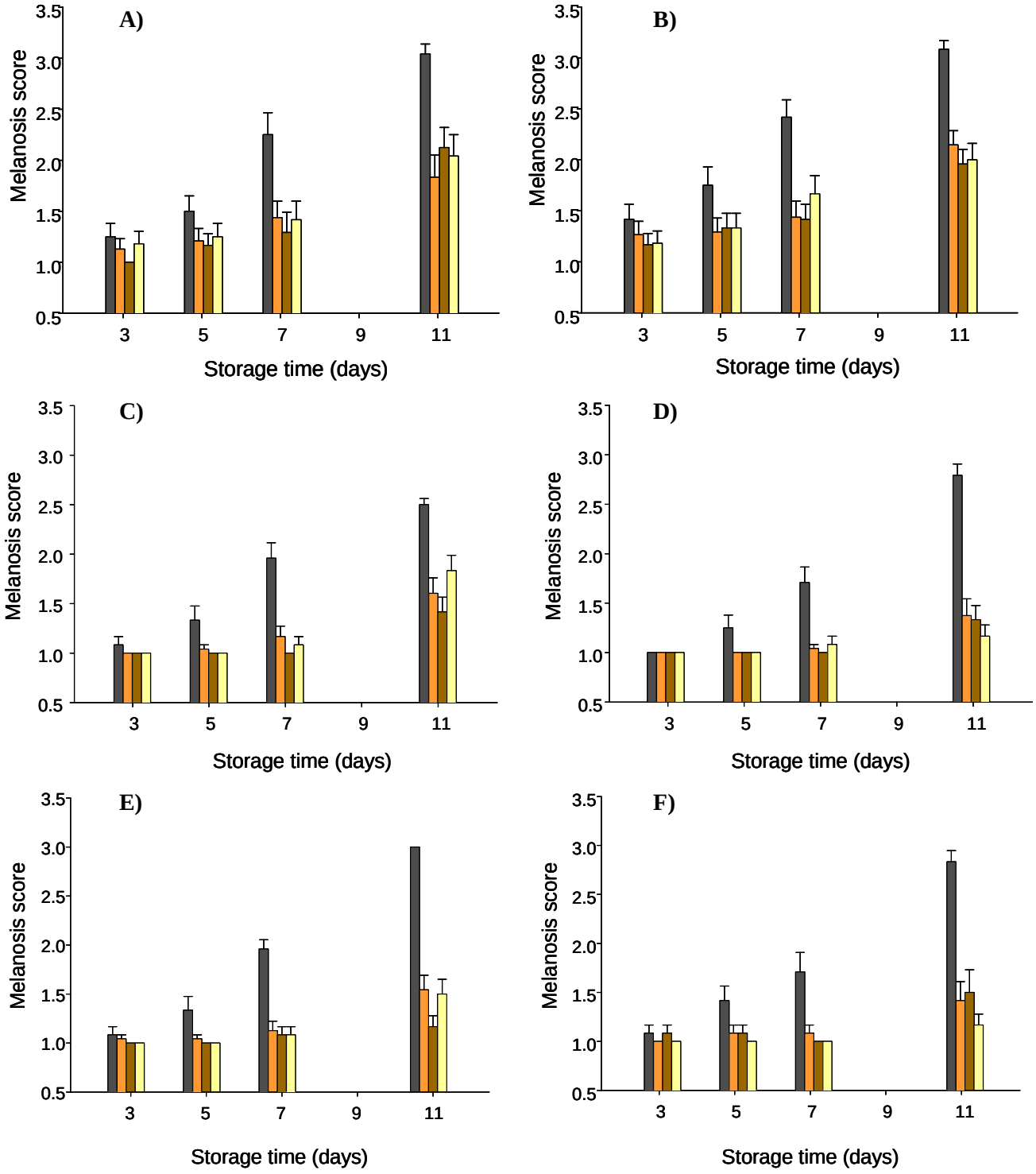


Figure 17. Melanosis score in fresh langoustines that were un-dipped or dipped with Prawn-Fresh™ and its new formulations. A) Cephalothorax dorsal; B) First clawed legs; C) Cephalothorax ventral; D) Pleopods; E) Tails; F) Tail fan. Values are the mean ± S.E.M. for ten different animals.

Figure 18. Digital images of fresh langoustines that were un-dipped or dipped with Prawn-Fresh™ and its new formulations on day 7 of storage at 3-4 °C.

CONCLUSIONS

- Frozen/thawed harbour crabs develop melanosis at a more rapid rate than do fresh ones.
- Prawn-Fresh™ is not able to suppress effectively melanosis development in frozen/thawed harbour crabs. Sulphite-base treatments perform better than Prawn-Fresh™, although they are not as efficient as in langoustines.
- The Prawn-Fresh™ modified formulations tested in this study are not able to suppress melanosis development effectively in frozen/thawed harbour crabs.
- Frozen/thawed langoustines also develop melanosis at a more rapid rate than do fresh ones.
- However, in this case Prawn-Fresh™ is able to suppress melanosis development effectively in frozen/thawed langoustines.
- The lowest effective concentration of Prawn-Fresh™ that can be used in fresh and frozen/thawed langoustines if they are then stored at 5-6 ºC is a dilution of 1/1000 for 10 min. No differences are obtained between treating the animals with Prawn-Fresh™ for 10 or 15 min.
- A shorter treatment time (5 min) could be used if Prawn-Fresh™ is prepared at a higher concentration (1/500). However, the processors could find this combination not cost-effective, as they would need to double the amount of product they use just to shorten the process by 5 min.
- The reduction of green tea in the Prawn-Fresh™ formulation does not produce any significant effect on the inhibition of melanosis in fresh and also in frozen/thawed langoustines.
- Frozen/thawed cephalothoraxes have a higher PPO activity compared to fresh cephalothoraxes
- In an *in-vitro* system no clear differences are found in the inhibition of PPO activity between normal Prawn-Fresh™ and the formulations that contain 75 or 50 % of green tea.
- Prawn-Fresh™ with no green tea is less efficient in inhibiting the PPO enzyme. However, given the fact that no clear visual differences (melanosis score) are

found between any of the formulations tested it is questionable whether green tea provides any real benefit in the Prawn-Fresh™ formulation.

RECOMMENDATIONS

- If Xyrex Ltd. wants to commercialise Prawn-Fresh™ as an anti-melanotic treatment for crabs then a new formulation has to be tested, since the current formulation and also the modified formulations tested in this project did not suppress melanosis efficiently in harbour crabs.

- Prawn-Fresh™ containing less green tea could be patented for the purpose of suppressing melanosis in langoustines since the formulations tested, including the one with no green tea, performed very similarly to normal Prawn-Fresh™ in fresh and frozen/thawed langoustines stored at low temperature.

Acknowledgements

The authors of this report are grateful to the crew members of the research vessels 'Aplysia' and 'Aora' from the University Marine Biological Station Millport for the supply of the animals. This work was in part funded by a First Steps Award from the University of Glasgow.

References

Martinez-Alvarez O, Lopez-Caballero M, Montero P, Gomez-Guillen MC (2005). Spraying of 4-hexylresorcinol based formulation to prevent melanosis and microbiol growth in chilled tiger prawns (*Marsupenaeus japonicus*) from aquaculture. J Food Sci 70, 9: 415-422.

Wang Z, Taylor A, Yan X (1992). Studies on the protease activities in Norway lobster (*Nephrops norvegicus*) and their role in the phenolase activation process. Food Chem 45: 111-116.