Development of techniques for quantification of stress induced catecholamine changes in the haemolymph of the Pacific oyster *Crassostrea gigas*

Xiaoxu Li & Meegan Vandepeer

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Development of techniques for quantification of stress-induced changes in the haemolymph of the Pacific oyster *Crassostrea gigas*
Fisheries Research and Development Corporation project final report

By: Xiaoxu Li & Meegan Vandepeer

South Australian Research and Development Institute
SARDI Aquatic Sciences
2 Hamra Avenue
West Beach SA 5024

Telephone: (08) 8200 2400
Facsimile: (08) 8200 2406
http://www.sardi.sa.gov.au

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Author:	Xiaoxu Li & Meegan Vandepeer
Reviewers:	John Carragher and Stephanie Seddon
Approved by:	John Carragher
Signed:	
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NON TECHNICAL SUMMARY

2002/414 Development of techniques for quantification of stress-induced changes in the haemolymph of the Pacific oyster *Crassostrea gigas*

Principal Investigator: Dr Xiaoxu Li
Address: SARDI Aquatic Sciences Centre
PO Box 120
Henley Beach SA 5022
Telephone: 08 8200 2464 Fax: 08 8200 2481

Objectives:

1. Evaluate, modify and optimise published methodologies to quantify oyster haemolymph catecholamine levels in the South Australian environment using equipment available at the South Australian Research and Development Institute (SARDI).
2. Establish a standard protocol for oyster blood sample collection, preservation and transportation, which will minimise any time effect on the accuracy of the proposed analysis, so that the technologies are useful across the remote oyster farming areas in South Australia.

OUTCOMES ACHIEVED

As a result of the development of a protocol for oyster blood sample collection, preservation and transportation and subsequent catecholamine analysis during the course of this project, a test now exists which can be used to measure stress in oysters. This test will be used in the FRDC project 2003/208 'Reduction in Pacific oyster mortality by improving farming and processing technologies in South Australia' to measure the stress induced by different oyster farming practices. Based on the results from these investigations recommendations will be made to industry regarding low stress practices/technologies that may help minimise death of oysters on farms due to stress related aetiology. In addition, the stress measurement tool developed in this project could be used to improve the efficiency of selective breeding programs.

Non Technical Summary:

Stress is a state of decreased fitness in an animal that is induced by stressors such as adverse physical or environmental events (mechanical injury, unfavourable

temperature or pH, hypoxia etc.), pathogens (bacteria & viruses), predators and competitors (Colombo *et al*., 1990). Stress can be counteracted by eliciting a stress reaction. The stress reaction involves a physiological change induced by hormones that enhance the supply of oxygen and energy to skeletal muscles and the nervous system to enable an animal to flee or fight against a stressor. This means that energy is diverted from other activities such as growth and reproduction, digestive and absorptive processes and alternative routes of defence metabolism like immune response and detoxification, resulting in lowered disease resistance (Colombo *et al*., 1990). This theory is supported by published studies on molluscs that indicate that stress may be associated with high bacterial loads and disease outbreaks (Bricelj *et al*. 1992; Lee *et al*., 1996). For example, experiments conducted to examine the effects of stress on juvenile Pacific oysters resistance to the pathogen *Vibrio splendidus* demonstrated that when oysters were challenged with a low dose of *V. splendidus*, and subjected to a mechanical stress 3 days later, both mortality and *V. splendidus* loads increased in stressed oysters whereas they remained low in unstressed oysters (Lacoste *et al*., 2001a). The ability to detect a stress response in oysters is therefore of interest to aquaculturists because it would allow an assessment of the impact of different farm management techniques. For the Australian oyster aquaculture industry handling, sorting, grading and transportation are the main farming practices likely to cause stress to oysters. In addition to its use as a tool for assessing different farming practices, a stress test could also be of benefit in selective breeding as it could be used to measure stress levels in oysters and identify those more resistant to stress.

Recent studies have shown that stress causes oysters to release the hormones noradrenaline (NA) and dopamine (DA) into their blood (Lacoste *et al*., 2001a,c). These hormones, referred to as catecholamines (CA), were found to increase in Pacific oysters subjected to mechanical, temperature and salinity stressors (Lacoste *et al*., 2001c). Thus measurement of catecholamines could prove to be a useful tool for measuring the degree of stress caused by different oyster farming practices.

Changes in catecholamines can be measured by liquid chromatography. The aim of the current project was to evaluate, and if necessary modify and optimise the published methodologies on oyster haemolymph catecholamine quantification so that a test is available to use on Pacific oysters grown in South Australian waters. Once developed this test will enable stress caused by current farming technologies to be assessed and subsequently recommendations made to farmers as to the best farming practices to use to minimise stress and thus optimise the health of their oysters.

Noradrenaline concentrations from 1.14 to 54.26ng mL^{-1} and dopamine concentrations from 0.10 to 4.58ng mL^{-1} were detected from individual oyster cell-free blood samples with the methods developed in this project. Detection limits for the catecholamines were in the order of 0.01ng mL^{-1}, which is more than adequate to detect normal values of approximately 1ng mL^{-1} in oyster blood.

Catecholamine concentrations in oyster blood stored on ice with the preservative developed in this project remained constant for up to 42 hours. This is substantially greater than that stated by Lacoste *et al*., (2001b) who reported a half-life of 25~30 min in cell-free blood. This could allow the monitoring of catecholamine response to stressors (eg. grading & exposure to air) on oyster farms far from the facilities required for catecholamine extraction and analysis. Although the catecholamine

concentrations in oyster blood remained unchanged for 42 hours on ice with the preservative developed in this project, it is recommended that the blood samples should not be stored for more 30 hours, because in our preliminary experiments the dopamine concentration in one sample increased slightly after 30 hours storage. A protocol has been developed outlining the steps for blood sample collection, preservation and transportation (Appendix 4).

Results from an experiment investigating the effects of water temperature on catecholamines showed that when the temperature was raised from 15^{o}C to 30^{o}C, the average DA concentrations remained at low levels (0.28~0.52ng mL^{-1}) for the first 6 hours and then increased to 2.07±0.17ng mL^{-1} at 10 hours post treatment. In comparison, the average NA concentrations remained at about 4ng mL^{-1} for the first 4 hours and then increased to more than 6ng mL^{-1} at 6 and 8 hours and decreased to about 4ng mL^{-1} at 10 hours post treatment.

The next stage in the development of this test is to use it to measure the stress response induced in oysters by various farming practices used on farms around South Australia. The practices inducing the most and least stress can then be identified. This information can be used by the oyster industry to modify their practices so they minimise the stress placed on their oysters thereby reducing the impact of farming practices on oyster health. This work is to be conducted as part of the FRDC project 2003/208 “Reduction in Pacific oyster mortality by improving farming and processing technologies in South Australia”.

Before this test can be used to measure the stress created by various farming practices on farms in South Australia, preliminary experiments will be required to determine the optimal time when the blood should be sampled. This is because the published data and the data from this study showed that the response times of catecholamines to different stressors were different, with the fastest response time of approximately 15 min being obtained for shaking stress (Lacoste *et al.*, 2001c) in comparison to about 8 hours for high temperature stress (this study and that of Lacoste *et al*., 2001c). The corresponding times for the catecholamines to return to normal levels were approximately 1 hour (shaking stress) and 36 hours (high temperature stress) (Lacoste *et al*., 2001c). In addition, the response from NA was normally quicker than from DA. Therefore, the timing of haemolymph collection will be very critical to the accuracy of any proposed tests on oysters in the future.

It is probable that various environmental and biological variables will also affect catecholamine levels in oysters including salinity, temperature, exposure to air, oyster size, oyster sex and condition. These factors may potentially confound the results obtained from the assessment of various farming practices. For this reason the effects of these variables on catecholamine levels in oysters will also need to be investigated and the results factored in when analysing the responses to different farming practices.

KEYWORDS: Pacific oyster, *Crassostrea gigas*, farming practices, stress, catecholamines, noradrenaline, dopamine.

ACKNOWLEDGMENTS

First of all we would like to thank Seafood Services Australia and the South Australian Oyster Research Council for funding this project and Zippel Enterprises Pty Ltd for providing the oysters. We appreciate and thank the managerial and academic staff at the Aquatic Sciences Centre of SARDI for their support and valuable discussions on the report. We are also grateful to Dr Stephanie Seddon and Dr John Carragher of SARDI for editing this final report.

BACKGROUND

Oysters growing in the sea are permanently exposed to various microbes, and their defence system is continuously activated to prevent accumulation of invading and pathogenic organisms. Impairment of an oyster's defence system is likely to result in poor growth and in severe cases may result in mortality. It is also possible that stressed oysters could carry increased pathogen loads that may be detrimental for human consumption. Published studies indicate that stress decreases the efficiency of the defence system in molluscs and therefore, may be associated with high bacterial loads and disease outbreaks. For example, experiments have shown that the physiological changes imposed by stress, or stress hormones, influenced host-pathogen interactions in oysters and increased juvenile *C. gigas* vulnerability to *Vibrio* sp. (Lacoste *et al.*, 2001c).

Being able to measure stress, both its severity and its duration, is of interest to aquaculturists because it would enable the impact of various farm management techniques that may reduce fitness of oysters to be assessed. For the Australian oyster aquaculture industry, handling, sorting, grading, transportation and different growing conditions (eg varying rack height and therefore exposure) are the main stressful farming practices that could compromise the ability of oysters to perform essential life functions and/or affect their shelf life. Attempts to improve performance and thus survival of the oysters subjected to such stressors, have been difficult because techniques for quantifying the physiological changes in the oysters have not been available.

Recent studies in France have shown that stresses induce neuroendocrine responses in oysters, involving the release of catecholamines such as noradrenaline and dopamine in the haemolymph (Lacoste *et al.*, 2001c). Neuropeptides such as adrenocorticotropic hormone control this catecholaminergic response. The catecholamine changes can be determined by liquid chromatography with electrochemical detection.

The aim of the current project was to evaluate, and if necessary modify and optimise, the published methodologies on oyster haemolymph catecholamine quantification for use on Pacific oysters in South Australian waters. Equipment available at SARDI was used to establish method(s) to preserve the blood samples from stressed oysters so that the stress chemicals remain detectable, representative and comparable; a potential issue in South Australia where oyster farms are in remote locations and a considerable time away from laboratories where liquid chromatography equipment is available. The techniques once developed and validated will then be applied in consultation with the SA oyster farming sector (SAOGA) to refine or compare harvest and post-harvest activities (handling, sorting, grading, transportation, etc) used by different farms and to improve the harvest to market technology. They can also be used as a selection tool in selective breeding programs to manipulate the genetic component of the stress response in a similar fashion as that used for manipulating characteristics such as growth rates.

NEED

One of the major problems facing the Pacific oyster industry throughout southern Australia is minimising the impacts to oysters resulting from general farming activities such as grading and transportation as well as processing before they are sold at the market. A variety of factors have been suggested to be associated with the impacts; however, distinguishing which of these factors is responsible for loss of stocks can not be determined because methods to measure physiological changes in stressed oysters have not been available.

If the method developed by Lacoste *et al*. (2001c) for determining haemolymph catecholamine levels in oysters can be confirmed as suitable for use in the South Australian environment using the facilities at SARDI, the factors suspected as contributing to stock losses in Australia; temperature, grading, transportation, disease infection and the combinations of these factors, can then be identified. The performance of the oysters can then be improved by adopting the best techniques and equipment currently used by the farms and processors or by developing new farming technologies which take into account the physiological requirements of the animals. The methods and equipment currently used by oyster farms and processors have been developed mainly because of their convenience to the farmers, cost effectiveness and the ecological requirement of the animals, not because they optimise the quality of the product.

Another impediment to the development of the oyster industry at present is a lack of stock which have been genetically selected to produce stress-resistant characteristics. This is because suitable selection criteria cannot be determined. Therefore, this project could also provide opportunities for such breeding objectives to be included in the current selective breeding program.

OBJECTIVES

1. Evaluate, modify and optimise the published methodologies to quantify oyster haemolymph catecholamine levels in the South Australian environment using the equipment available at SARDI.
2. Establish a standard protocol for oyster blood sample collection, preservation and transportation, which will minimise any time effect on the accuracy of the proposed analysis, so that the technologies are useful across the remote oyster farming areas in South Australia.

MATERIALS AND METHODS

In all experiments one syringe and one needle per oyster were used for sampling haemolymph.

Experiment 1: Establishment of the standard noradrenaline (NA) and dopamine (DA) elution peaks by using liquid chromatography with electrochemical detection

Extraction of catecholamines from oyster haemolymph

The extraction methods used in this experiment were based on those published by Lacoste *et al*., (2001c) and Goldstein *et al*., (1981).

All chemicals used were purchased from Sigma-Aldrich Co. and were the best available grade. A stock internal standard solution containing 1mg mL^{-1} dihydroxybenzylamine (DHBA) and a stock standard solution containing 1mg mL^{-1} each of noradrenaline (NA), adrenaline (A) and dopamine (DA) were prepared in distilled water and stored at -20^{o}C. Working solutions at a concentration of 10ng mL^{-1} were prepared as required from these stocks.

The oyster haemolymph was sampled from the pericardial cavity using syringes with 22 gauge needles. The samples from individual oysters (30 in total) were centrifuged at 600g for 10 minutes at 4^{o}C and the haemolymph pipetted off the cell pellet. The haemolymph was immediately processed for catecholamine extraction. Standards were subjected to the same extraction procedure.

To 0.5mL of haemolymph were added; 0.5mL TRIS buffer (1.5M, pH8.6), containing 0.07M EDTA; 100µL internal standard; 50µL 5nM sodium metabisulfite and 10mg acid-washed alumina. All solutions were kept on ice prior to use. The sample was mixed for 15 minutes and then centrifuged at 100g for 2 minutes. The supernatant was discarded and the alumina washed for 10 minutes with 1mL of distilled water and then centrifuged for 2 minutes at 1000g. The supernatant was discarded and 100µL 0.2M acetic acid added. The sample was mixed for 10 minutes and the centrifuged for 2 minutes at 1000g. The supernatant (50µL) was injected onto the HPLC column.

Chromatography

Samples were separated on a C18 column (Waters Associates, Symmetry 150x3.9mm, 5µ). The mobile phase consisted of 50mM sodium dihydrogenphosphate, 50mM citric acid, 0.05mM EDTA, 1.0mM octanesulfonic acid and 7% methanol, pH 3.0 at a flow rate of 1mL per minute. A Waters Associates 2465 electrochemical detector was used at a potential of 0.5V against a hydrogen reference with sensitivity set at 5nAFS. The column temperature was 35^{o}C.

Experiment 2: Effect of temperature variations on circulating noradrenaline and dopamine concentrations in oysters

Pacific oysters (*Crassostrea gigas*) (80~100g) were purchased from a seafood wholesaler in Adelaide where they had been delivered in a refrigerated container (about 0^oC) from the South Australian oyster farms and then kept in a cool room at 4^oC. After purchase the oysters were placed in a 600L tank at SARDI where they were maintained for 15 days prior to experiments. During this period the maintenance tank was aerated and supplied with continuously flowing natural seawater at 15 ± 0.5^oC. The oysters were fed with a mixture of *Isochrysis* sp and *Pavlova* sp algae, which were harvested automatically from a bag algal culture system at SARDI.

After 15 days of acclimation the oysters were subjected to high temperature by being immersed in 30^oC seawater. At 0, 4, 6, 8 and 10 hours post treatment the oysters were taken out individually from the treatment tank and then a blood sample collected immediately. The 0 hour samples were taken from the oysters in the maintenance tank. Each oyster was shucked by levering open the hinge and running the knife along the right valve to sever the adductor muscle. The right valve was then removed. The blood sample was collected from the pericardial cavity using 2mL syringes with 22 gauge needles. If more than 0.5mL blood was collected from an individual in less than 1.5 minutes the sample was adjusted to 0.5mL in the syringe and then squeezed into a labelled 1.5mL Eppendorf tube. The tube was immediately put back on ice. If less than 0.5mL blood was collected from one animal, or the time taken to collect blood was over 1.5 minutes, the sample was discarded. Three blood samples were collected respectively from 3 oysters at each time-point.

When the required number of samples were collected, catecholamine extraction commenced immediately using the methods described in Experiment 1. The extracts were analysed that night using an auto-injector on the HPLC cooled to 4^oC.

Experiment 3: Optimisation of the methods for oyster haemolymph sample collection

Five separate trials were conducted in this experiment. The Pacific oysters (80~90g) used for the first and second trials were purchased from a seafood wholesaler at the same time. They were maintained for 12 and 20 days respectively by using the methods described in the Experiment 2. In these trials 0.5mL blood was sampled from individual oysters using 2mL syringes with 22 gauge needles.

In the first trial the period of time from when the oyster knife was placed against the hinge to when the pericardial cavity was exposed, and the period of time from when the needle was inserted into the pericardial cavity to when the needle was removed, were recorded as opening and bleeding times respectively. In total, 30 blood samples were collected from 30 oysters.

In the second trial the total time for sample collection (the period of time from when the oyster knife was placed against the hinge to when the needle was removed from the pericardial cavity) was less than 60 seconds. Two needle sizes, 29 and 22 gauge, were used. Five blood samples were collected from 5 oysters with 29 gauge needles and 7 blood samples were collected from 7 oysters with 22 gauge needles.

The oysters (80~90g) used for the third trial were also purchased from an Adelaide seafood wholesaler and maintained for 9 days by using the same methods as described in Experiment 2 prior to blood sample collection. Three needle sizes, 29, 22 and 18 gauge were used. For the 29 gauge needles the total time for blood collection from individual oysters was managed within 60 seconds. For the 22 and 18 gauge needles the total time for blood collection was managed within 46 and 31 seconds respectively. The bleeding times were also recorded for all collections. For each needle size, 3 replicate samples were collected.

The oysters (90~100g) used for the fourth trial were provided by Zippel Enterprises Pty Ltd and delivered to SARDI overnight by bus in a styrofoam box 47 cm in length, 38 cm in width and 26.5 cm in height. The animals were then maintained for 21 days by using the same methods as described in Experiment 2 before blood collection. Two needle sizes, 29 and 18 gauge, were used. According to the needle size and the bleeding time, the blood samples in this experiment were divided into four groups (2 groups for each needle size). For the 29 gauge needles the two groups were the 10-20 seconds and the 21-30 seconds bleeding time groups, while for the 18 gauge needles the two groups included the 4-6 seconds and the 7-11 seconds bleeding time groups. Three replicates were collected for each group.

In the third and fourth trials all oysters used for blood sample collection were opened in less than 15 seconds and 0.2mL of haemolymph was sampled from each individual oyster. The 0.6mL pooled sample was made up by mixing blood collected from three oysters.

In the fifth trial the effect of needle shear force on catecholamine levels was examined. This was due to concerns that squeezing the haemolymph through the very fine needle on the 29 gauge syringe may rupture the haemocytes affecting the catecholamine levels. To determine if this were true a pooled 8mL haemolymph sample was collected from 16 oysters (0.5mL from each individual) in a 10mL tube using an 18 gauge syringe. The haemolymph from each oyster was collected in < 30 seconds. Before squeezing each 0.5mL sample into the 10mL tube the needle was taken off the syringe. The pooled 8mL sample was mixed thoroughly using a Ratek vortex mixer before being subsampled 12 times as follows:

a) 3 samples x 0.6mL using an 18 gauge syringe (needle removed before squeezing haemolymph into Eppendorf tubes).

b) 3 samples x 0.6mL using a 29 gauge syringe (syringe end cut off before squeezing haemolymph into Eppendorf tubes).

c) 3 samples x 0.6 mL using an 18 gauge syringe (haemolymph squeezed in and out of needle into Eppendorf 4 times).

d) 3 samples x 0.6 ml using a 29 gauge syringe (haemolymph squeezed in and out of needle into Eppendorf 4 times).

The oysters used in this experiment had been obtained from Zippel Enterprises Pty Ltd and kept for 20 days at 15±0.5^{o}C in a flow-through system before being used in the experiment. During this time they were fed with a mixture of *Isochrysis* sp and

Pavlova sp algae, which were harvested automatically from the bag algal culture system at SARDI.

For all trials in Experiment 3 haemolymph samples (either individual samples or pooled samples) were processed and analysed using the same methods as described in Experiment 1.

Experiment 4: Development of a method for stabilisation of catecholamines in oyster haemolymph samples

The oysters used in this experiment were unused oysters left over from Experiments 2 and 3. They had been left undisturbed for about 100 days. The blood was sampled individually using a 1mL syringe and an 18 gauge needle. The amount of blood collected from individual oysters was recorded and then squeezed into a 10mL centrifuge tube containing the required amount of preservative. The preservative was provided by the SARDI Biochemistry Dept. and used at a ratio of 50µL per 1mL of blood sample. The preservative can be kept in a freezer at -18^{o}C for a maximum of 3 months (or on ice in an esky for 3 days). Prior to the commencement of the experiments the solution was taken from the freezer and thawed in running tap water (approximately 20^{o}C). When thawed the solution was stored on ice in an esky and used within 6 hours. The required amount of preservative was transferred into the blood collection tube (10mL centrifuge tube) with a pipette. Blood from 9 to 10 oysters (0.2 to 1.0mL per animal) was pooled to make up the 6mL sample size required in this experiment. In total three 6mL samples were collected. After collection the pooled blood samples were mixed using a vortex mixer (Ratek Instrument Pty. Ltd) to ensure homogeneity of the samples. A 0.5mL subsample was then taken from each of the three samples and immediately processed for catecholamine extraction. The three original samples were then each divided into two subsamples (each containing about 2.25mL of blood). One of each pair from the three samples was then stored on ice in a styrofoam box, the other was kept at room temperature (18~19^{o}C). Subsamples of 0.5mL were taken from all samples stored on ice and at room temperature at 6, 18, 30 and 42 hours post blood sample collection respectively and immediately processed for catecholamine extraction. The processed extracts were kept in a freezer at -18^{o}C until all subsamples had been processed for catecholamine extraction. The frozen extracts were then taken to SARDI's Biochemistry department for HPLC analysis after the last samples had been collected (42 hours).

Statistical Analysis

Comparison of catecholamine levels between treatments was conducted by one way analysis of variance (ANOVA) (Appendix 3). A two way ANOVA was used to examine the effects of shear force and needle size in the fifth trial in Experiment 3 (Appendix 3). Analyses were conducted using GenStat (5th edition). Before analysis, data were checked for normality and homogeneity of variances using residual plots.

RESULTS

Experiment 1: Establishment of the standard noradrenaline (NA) and dopamine (DA) elution peaks by using liquid chromatography with electrochemical detection

The HPLC-ED chromatograms presented in the Figures 1, 2 and 3 show that noradrenaline and dopamine can be detected and quantified with the equipment at SARDI.

Detection limits for the catecholamines were in the order of 0.01ng mL^{-1}, which is more than adequate to detect normal values of approximately 1ng mL^{-1} in oyster haemolymph.

The recovery of standards added to haemolymph ranged from 75% to 80%.

It can be seen in the results section for Experiment 3 that noradrenaline concentrations from 1.14 to 54.26ng mL^{-1} and dopamine concentrations from 0.10 to 4.58ng mL^{-1} were able to be detected from individual oyster cell-free haemolymph samples with the methods developed in this project.

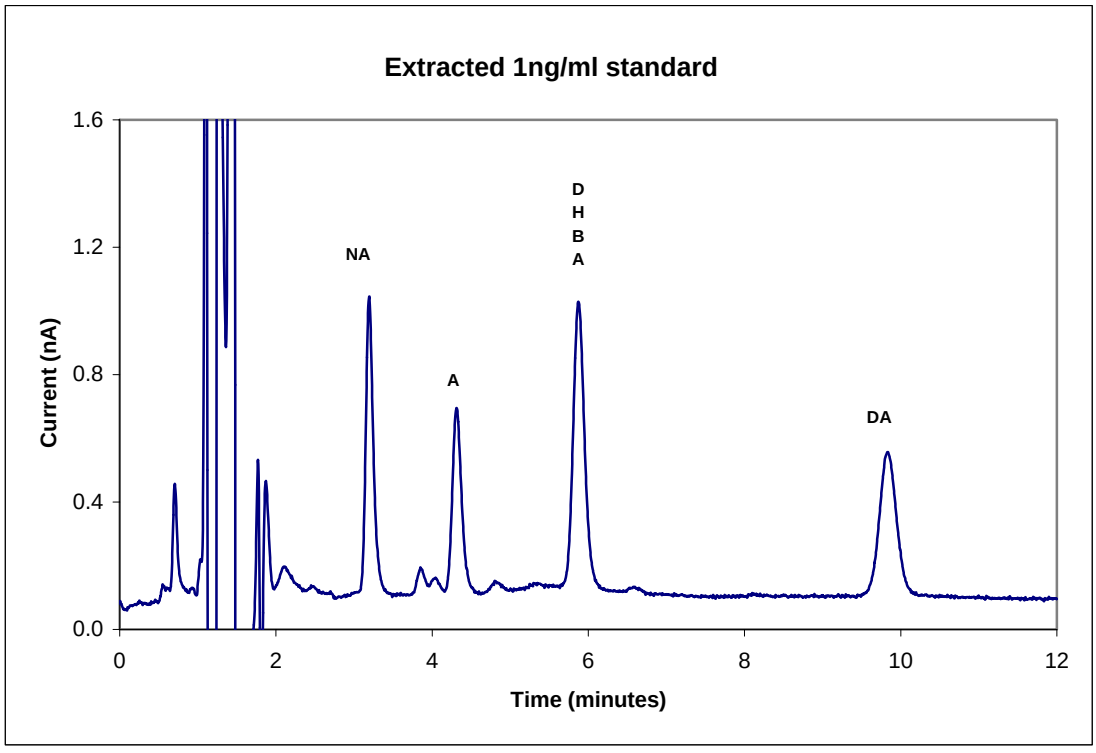


Figure 1. 1 ng mL^{-1} standard catecholamine solution made from Sigma-Aldrich Co. chemicals and extracted using the same method as with oyster haemolymph.

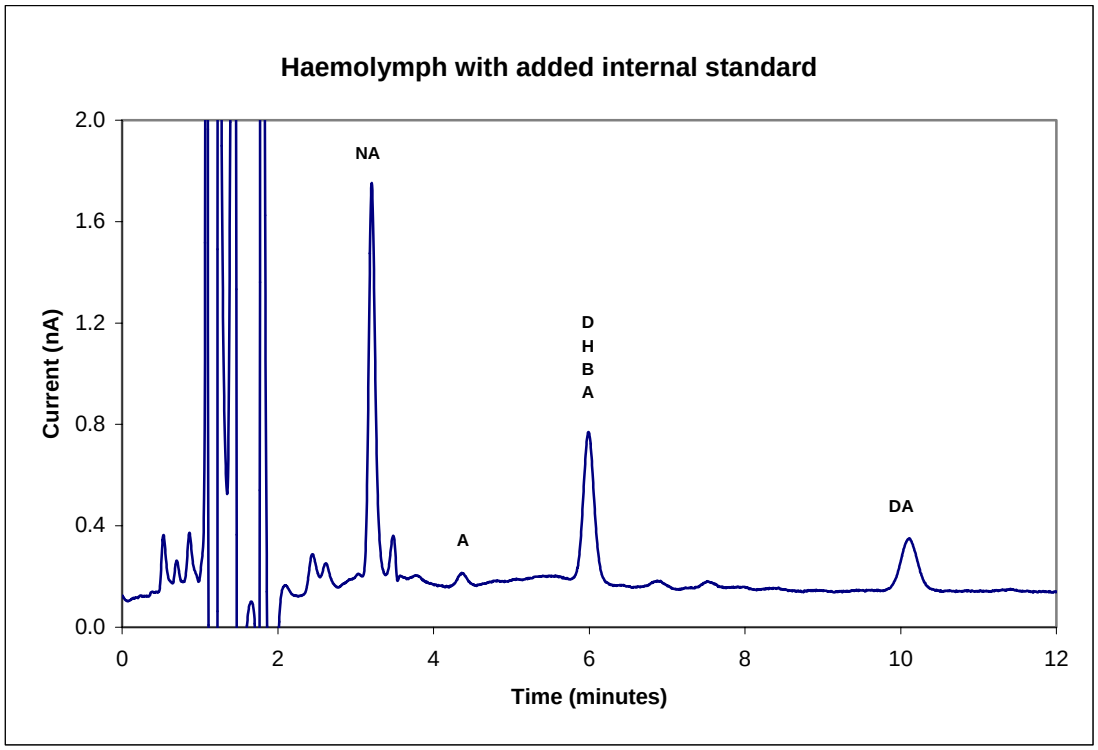


Figure 2. Oyster haemolymph with added internal standard (DHBA).

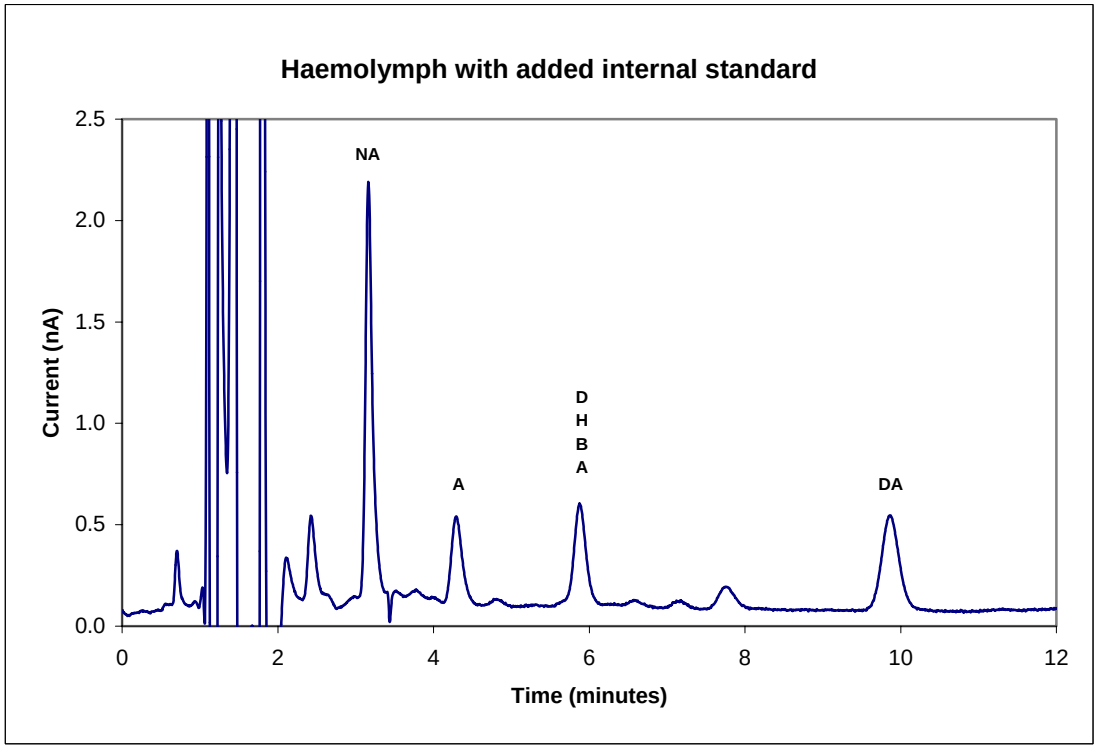


Figure 3. Oyster haemolymph with added internal standard (DHBA).

Experiment 2: Effect of temperature variation on circulating noradrenaline and dopamine concentration in oysters

The results show that when the oysters were transferred from 15^{o}C to 30^{o}C water their average NA concentrations remained at about 4ng mL^{-1} for the first 4 hours and then increased to more than 6ng mL^{-1} at 6 and 8 hours before decreasing to about 4ng mL^{-1} after 10 hours (Figure 4). In comparison, their average DA concentrations remained at low levels (0.28~0.52ng mL^{-1}) for the first 6 hours and then increased to 2.07±0.17ng mL^{-1} at 10 hours post immersion in 30^{o}C water (Table 1, Figure 5). Analysis of the data showed that temperature stimulus elicited significant dopamine responses ($p < 0.001$) in oyster haemolymph (Appendix 3: Table 1), while the response of noradrenaline to this stimulus was not significant ($p > 0.05$) (Appendix 3: Table 2).

The variation in NA levels among the three samples collected each sampling time was high, especially for the 0 and 8 hour samples (Table 1). One of the three 0 hour samples for NA was 8.01ng mL^{-1}, which was much higher than the concentration in undisturbed oysters reported by Lacoste *et al.* (2001c) for this species (1.61±0.3ng mL^{-1}). To determine the possible reasons for the high level and high variability of NA in the "undisturbed" oysters in this experiment further investigations were conducted in Experiment 3.

Table 1.
The effects of temperature treatment duration (30°C) on catecholamine levels in oyster haemolymph*. NA = noradrenaline, DA = dopamine.

Treatment duration (hours)	Catecholamine (ng mL^{-1}) (individual oyster) NA	DA	Catecholamine (ng mL^{-1}) (average ± SD, n = 3 oysters) NA	DA
0	2.75	0.21	4.25±3.28	0.52±0.34
	1.98	0.48		
	8.01	0.88		
4	3.84	0.50	4.20±0.92	0.33±0.15
	5.25	0.24		
	3.51	0.25		
6	4.36	0.34	6.37±3.54	0.28±0.12
	4.29	0.14		
	10.46	0.35		
8	3.08	1.67	6.83±7.58	1.72±0.08
	15.55	1.82		
	1.86	1.68		
10	5.38	1.92	4.55±2.57	2.07±0.17
	6.61	2.04		
	1.67	2.26		

* All samples were collected within 1.5 minutes with 22 gauge needles.
SD: standard deviation.

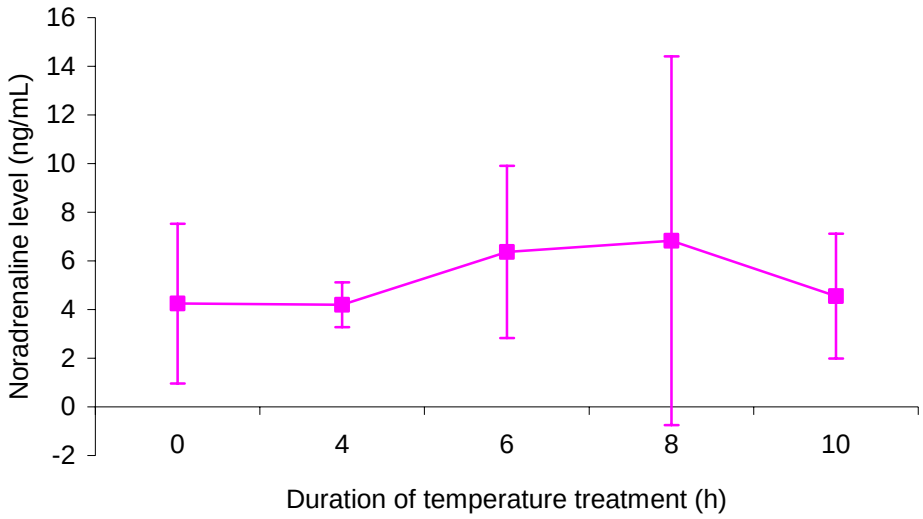


Figure 4. The effect of duration of time of oysters held at 30ºC on haemolymph noradrenaline levels (error bars are standard deviations, n = 3 oysters).

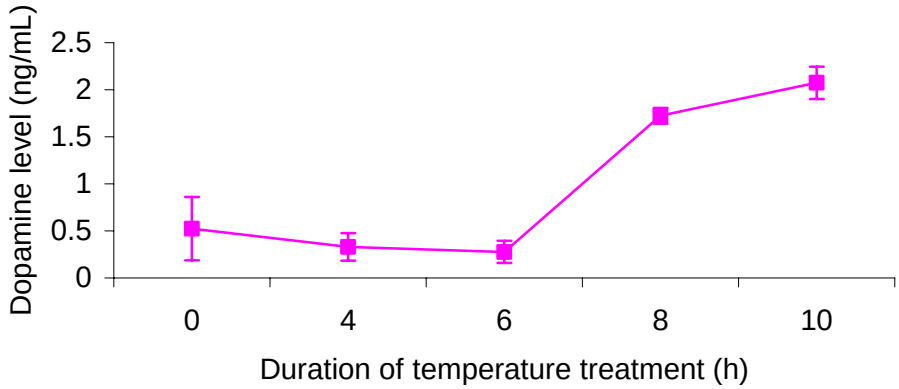


Figure 5. The effect of duration of time of oysters held at 30ºC on haemolymph dopamine levels (error bars are standard deviations, n = 3 oysters).

Experiment 3: Optimisation of the methods for oyster haemolymph sample collection

In the first trial only 22 gauge needles were used (the same as in Experiment 2). The concentrations of noradrenaline and dopamine in the circulatory system of individual oysters purchased from the seafood wholesaler and left undisturbed for 12 days in the aquarium room at SARDI ranged from 1.14 to 54.26ng mL^{-1} and from 0.10 to 4.58ng mL^{-1} respectively (Table 2 and Figure 6). Different combinations of opening and bleeding times were attained in haemolymph collections, which included combinations of short opening and short bleeding times (eg. animal ID 4 in Table 2), short opening and long bleeding times (eg. animal ID 14 in Table 2) and long opening and short bleeding times (eg. animal ID 26 in Table 2).

The short opening (less than 23 seconds) and long bleeding time (more than 54 seconds) combination produced very high haemolymph catecholamine levels (NA at 22.81±20.20ng mL^{-1} and DA at 0.79±0.36ng mL^{-1}, Trial 1 in Table 3). The average NA and DA concentrations from those samples collected with the short opening (less than 24 seconds) and short bleeding time (less than 30 seconds) combination (less than 60 second in total time: bleeding time + opening time + 7 second transaction time[1]) were 5.09±3.09ng mL^{-1} and 0.32±0.20ng mL^{-1} respectively (Trial 1 in Table 3). The average NA concentration in the haemolymph collected with a long opening (more than 120 seconds) and short bleeding (less than 30 seconds) time combination was 4.48±3.24ng mL^{-1}, similar to the samples collected with the short opening and short bleeding time combination (Trial 1 in Table 3). The average DA concentration in samples collected with a long opening (more than 120 seconds) and short bleeding (less than 30 seconds) time combination was 1.44±1.81ng mL^{-1} (Trial 1 in Table 3). The differences in NA concentrations between the samples collected with the short opening and long bleeding time combination, short opening and short bleeding time combination and the long opening and short bleeding time combination were close to significant ($p = 0.053$, Appendix 3: Table 3). The differences in DA concentrations between the samples collected with short opening and short bleeding time combination and the long opening and short bleeding time combination were not significant ($p > 0.05$, Appendix 3: Table 4).

[1] time taken for person opening the oysters to pass the oyster to person collecting the blood

Table 2
Effects of blood sample collection duration on catecholamine levels in Pacific oysters*. NA = noradrenaline, DA = dopamine.

Animal's ID	Blood collection duration (seconds)			Catecholamine (ng mL^{-1})	
	Opening time	Bleeding time	Total time	NA	DA
1	19	40	59	7.23	0.86
2	46	32	78	2.62	0.43
3	15	54	69	30.91	0.94
4	21	21	42	2.15	0.10
5	19	30	49	9.28	0.61
6	57	41	98	5.39	0.20
7	22	30	52	2.24	0.22
8	18	35	53	2.45	1.02
9	22	30	52	4.71	0.24
10	24	21	45	7.05	0.45
11	19	38	57	3.19	0.40
12	32	35	67	1.58	0.13
13	16	62	78	15.63	0.67
14	23	86	109	54.26	1.58
15	23	58	81	7.17	0.94
16	23	80	103	6.06	0.71
17	113	52	165	2.16	0.18
18	120	57	177	4.16	0.21
19	93	48	141	7.71	0.31
20	121	33	154	17.19	0.52
21	119	49	168	15.06	1.09
22	123	21	144	4.35	0.26
23	117	31	148	2.91	0.37
24	90	38	128	5.69	0.50
25	120	24	144	2.08	4.58
26	128	16	144	9.41	1.43
27	255	25	280	5.40	0.31
28	255	30	285	1.14	0.64
29	250	40	290	2.32	0.57
30	260	50	310	10.30	0.78

* 22 Gauge needles were used.

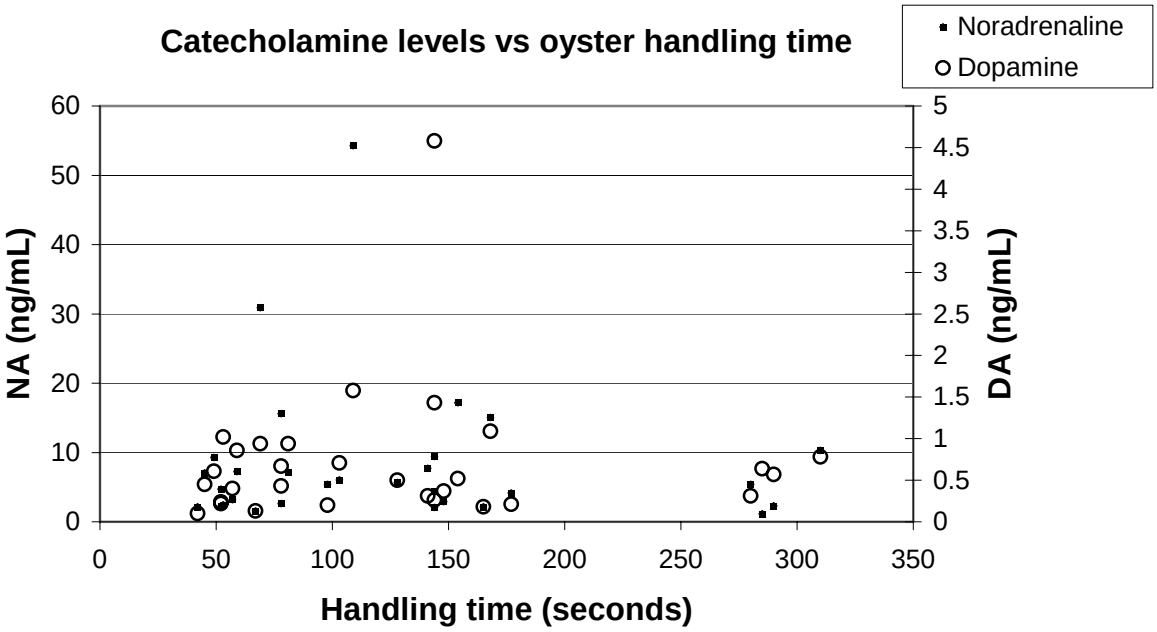


Figure 6. The effect of total handling time on noradrenaline and dopamine levels in oysters.

In the second trial two needle sizes (29 and 22 gauge) were used. The unused oysters from the first experiment were left undisturbed for another 8 days (20 days in total). Blood was then collected in less than 60 seconds (total blood collection time) from each oyster using either the 29 or 22 gauge. The average NA and DA concentrations in the haemolymph collected using 29 gauge needles were 1.63±1.35ng mL^{-1} and 0.57±0.34ng mL^{-1} respectively (Trial 2 in Table 3). The average NA concentration in the haemolymph collected using 22 gauge needles decreased from 4.65±2.97ng mL^{-1} in oysters that had been left undisturbed for 12 days to 3.32±1.49ng mL^{-1} in oysters that had been left undisturbed for 20 days (Trial 2 in Table 3). However this level is still much higher than the base level (1.61±0.30ng mL^{-1}) published by Lacoste *et al.*, (2001c). Statistical analysis showed that needle size had a strong effect on NA concentration in circulating oyster haemolymph. A significant difference in NA concentration ($p < 0.01$) was found between the haemolymph collected using the 29 gauge and 22 gauge needles (Appendix 3: Table 5).

Three needle sizes (18, 22 and 29 gauge) were used in the third trial to investigate the effects of needle size and blood collection time on catecholamine concentrations in oyster haemolymph. The average concentration of NA in the blood collected using 22 gauge needles was the same as in the second trial. The average concentration of NA collected using 29 gauge needles in this trial (2.76±1.36ng mL^{-1}) was higher than that in the second trial (1.63±1.35ng mL^{-1}) (Table 3). Although the average NA concentration obtained in the samples collected using 18 gauge needles was higher, the NA level of the second sample (1.35ng mL^{-1}, Table 4) was close to the published NA level for this species (1.61±0.30ng mL^{-1}; Lacoste *et al.*, 2001). This sample was collected within a very short bleeding time (< 6 seconds).

In the fourth trial the blood sampling protocol was dependent on needle size. The 29 gauge needle samples were divided into a 10-20 second bleeding group and a 21-30 second bleeding group and the 18 gauge needle samples were divided into a 4-6 second bleeding group and the 7-11 second bleeding group (Trial 4 in Table 3). The average NA concentrations in the haemolymph collected using 29 gauge needles were 1.60±1.75ng mL^{-1} and 1.78±0.75ng mL^{-1} for the 10-20 second bleeding group and the 21- 30 bleeding group respectively. The difference in NA concentration between the two time groups for the 29 gauge needle were not significant ($p > 0.05$, Appendix 3: Table 6). In comparison, average NA levels of 2.76±1.07ng mL^{-1} and 3.03±2.45ng mL^{-1} were obtained for the 4~6 second bleeding group and the 7-11 second bleeding group respectively using 18 gauge needles. As for the 29 gauge needle, the differences in NA between the two time groups for the 18 gauge needle were not significant ($p > 0.05$, Appendix 3: Table 7). The average DA concentrations in the haemolymph collected using 29 gauge needles were 0.42±0.19ng mL^{-1} and 0.55±0.05ng mL^{-1} for the 10-20 second bleeding group and the 21-30 second bleeding group, respectively. Average DA levels of 1.07±0.87ng mL^{-1} and 0.55±0.43ng mL^{-1} were obtained for the 4-6 second bleeding group and the 7-11 second bleeding group respectively using 18 gauge needles. As for NA, the differences in DA between the two time groups for both the 29 gauge needle and the 18 gauge needle were not significant ($p > 0.05$, Appendix 3: Tables 8 & 9, respectively).

Results from the fifth trial, showed that there was no significant effect of either needle size or needle shear force (end of syringe removed vs end of syringe not removed) and no needle size x shear force interaction on noradrenaline or dopamine levels ($p > 0.05$, Figures 7 and 8, Appendix 3: Tables 10 and 11).

Table 3.
Effects of blood sample collection method on catecholamine levels in Pacific oysters. NA = noradrenaline, DA = dopamine.

Experiment Date	**Trial** No	**Oyster batch** number[1]	**Number of days** undisturbed	**Needle** gauge	**Sample*** number	**Blood collection (seconds)** Opening time	Bleeding time	Total time	**Catecholamine (ng mL^{-1})** NA (average±SD)	DA
07/05/03	1	1	12	22	5 (individual)	18~24	21~30	49~60	5.09±3.09	0.32±0.20
					5 (individual)	120~255	21~30	144~285	4.48±3.24	1.44±1.81
					5 (individual)	15~23	54~86	69~109	22.81±20.20	0.79±0.36
15/05/03	2	1	20	29	5 (individual)			<60	1.63±1.35	0.57±0.34
				22	7 (individual)			<60	3.32±1.49	0.64±0.42
22/05/03	3	2	9	29	3 (pooled)	<15	16~40	31~60	2.76±1.36	0.45±0.05
				22	3 (pooled)	<15	14~27	28~46	3.61±2.08	0.94±0.85
				18	3 (pooled)	<15	5~15	21~31	4.32±2.76	0.56±0.14
19/06/03	4	3	21	29	3 (pooled)	<15	10~19	31~46	1.60±1.75	0.42±0.19
					3 (pooled)	<15	21~30	41~50	1.78±0.75	0.55±0.05
				18	3 (pooled)	<15	4~6	22~27	2.76±1.07	1.07±0.87
					3 (pooled)	<15	7~11	25~33	3.03±2.45	0.55±0.43

* Individual sample means that 0.5mL of blood was collected from individual oysters. Pooled sample means that each 0.6mL pooled sample was made up by mixing blood collected from three oysters, with 0.2mL from each individual.

[1] The first two batches of oysters were purchased from fish market, the third batch was provided by the Zippel Enterprises Pty Ltd.

SD: standard deviation

Table 4.
Effects of blood sample collection duration on catecholamine levels in Pacific oysters. NA = noradrenaline, DA = dopamine.

Experiment date	**Needle** gauge	**Sample** number	**Blood collection (seconds)*** Bleeding time	Total time	**Catecholamine (ng mL^{-1})** NA	DA
22/05/03	18	1	15	31	6.82	0.54
			6	21		
			13	30		
		2	5	24	1.35	0.71
			6	24		
			5	21		
		3	9	22	4.78	0.43
			8	24		
			6	32		

* The bleeding and total times used to collect haemolymph from individual oysters in each pooled sample. Each pooled sample was 0.6mL in volume and made up by mixing blood equally contributed from three oysters, with 0.2mL from each individual.

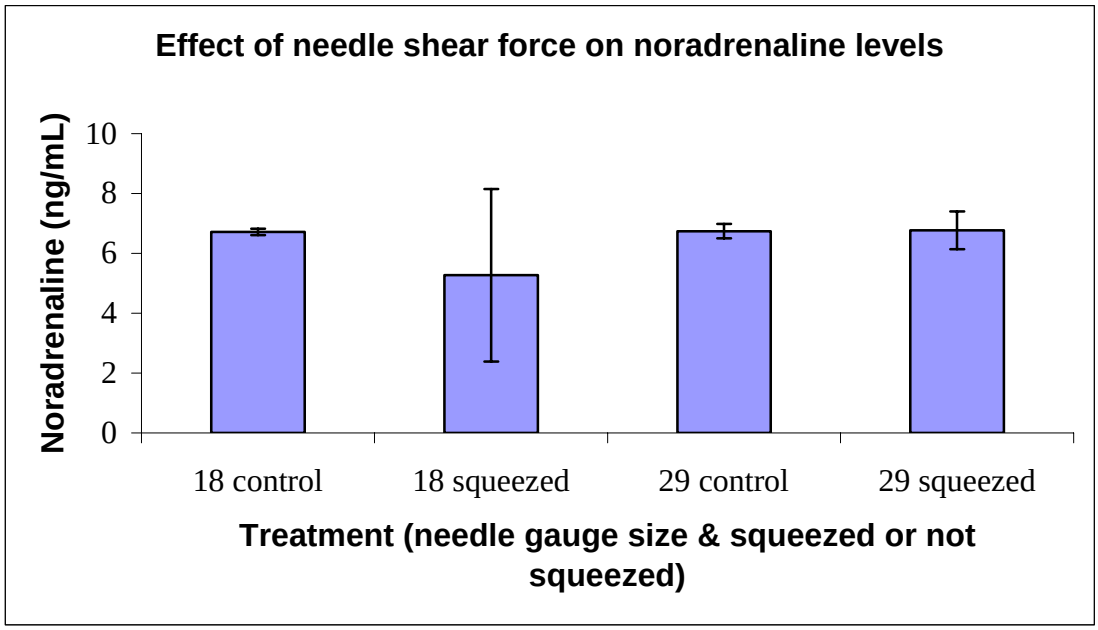


Figure 7 The effect of needle shear force on haemolymph noradrenaline levels. Error bars are standard deviations, n = 3 replicates.

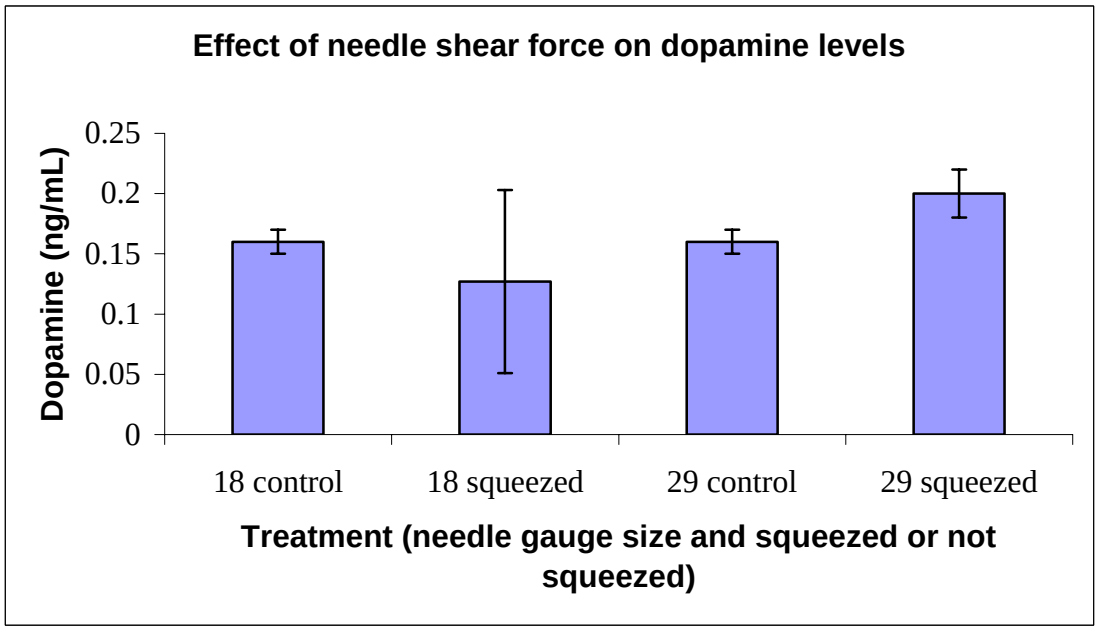
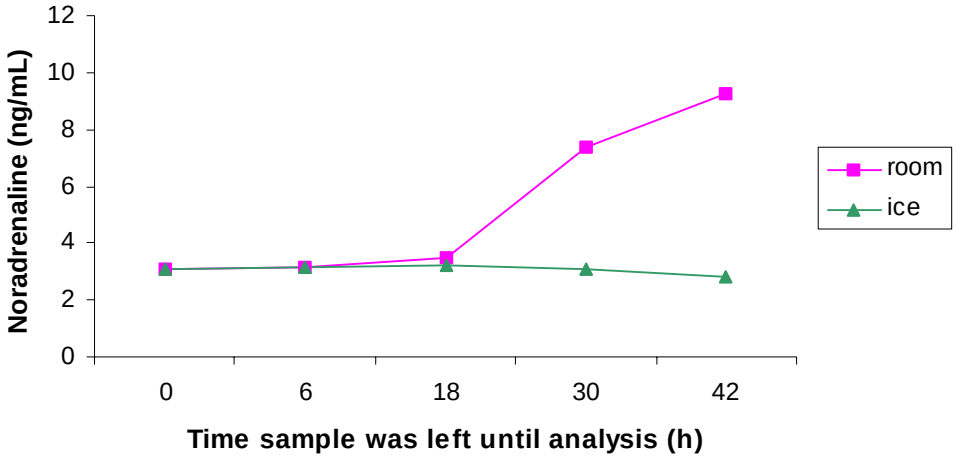


Figure 8 The effect of needle shear force on haemolymph dopamine levels. Error bars are standard deviations, n = 3 replicates.

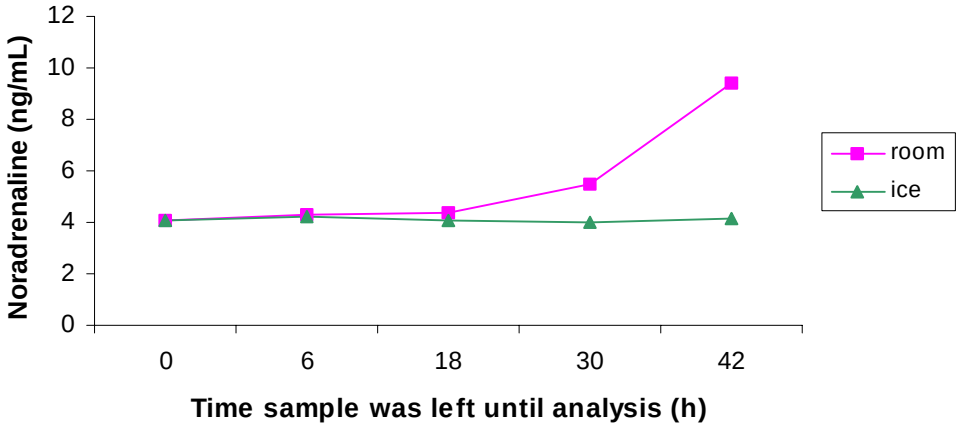
Experiment 4: Development of methods for stabilisation of catecholamines in oyster haemolymph samples

The catecholamine concentrations presented in Figures 9 and 10 show that NA and DA could be preserved for up to 42 hours using the preservative developed by the project. Virtually no change in either NA or DA concentrations occurred in the samples stored on ice over the course of the experiment (42 hours). The NA and DA levels in the samples kept at room temperature (18~19^{o}C) remained unchanged for the first 18 hours. After 18 hours the NA concentrations in all replicates increased gradually, and by 42 hours storage time at room temperature, the levels of NA and DA were twice or nearly twice as high as those recorded at 18 hours after blood sample collection (Figure 9A & B and Figure 10B).

A



B



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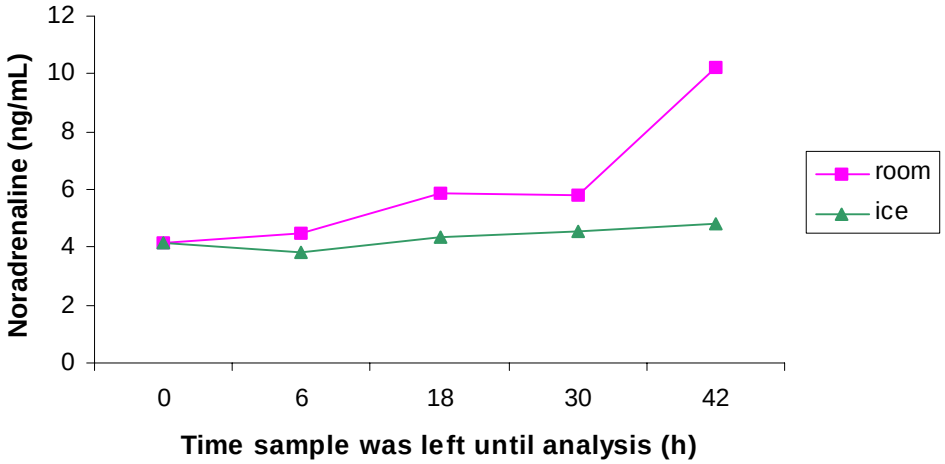
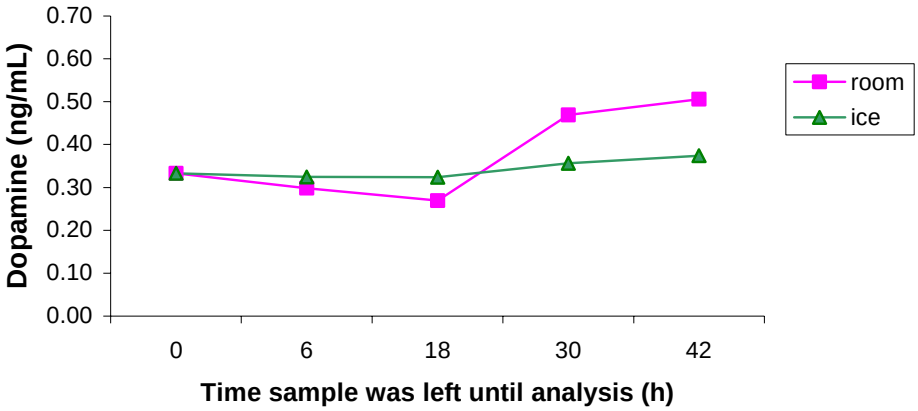


Fig 9. Samples preserved for different durations of time prior to preparation for HPLC analysis (noradrenaline). Samples were left in air (pink line) and on ice (green line). Figures A, B & C are 3 replicates.

A



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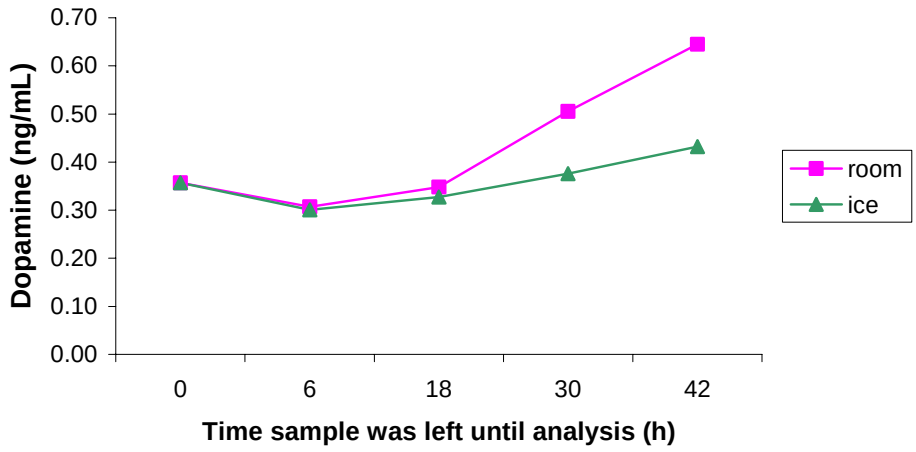

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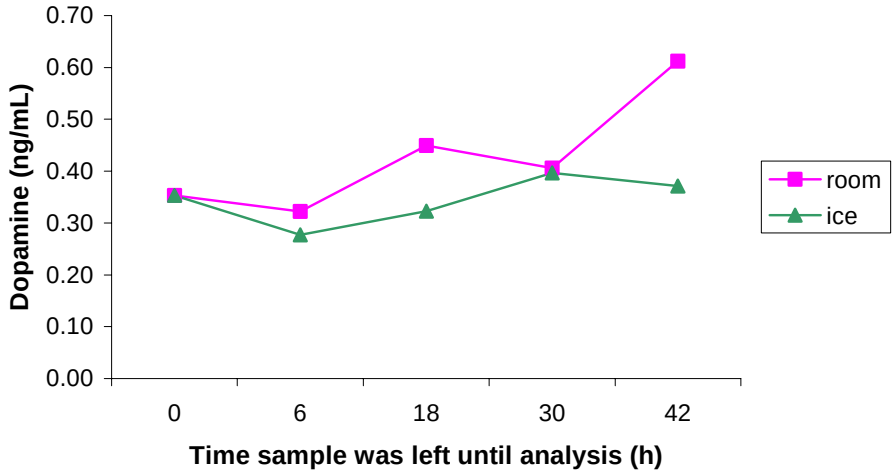

Fig 10. Samples preserved for different durations of time prior to preparation for HPLC analysis (dopamine). Samples were left in air (pink line) and on ice (green line). Figures A, B & C are 3 replicates.

DISCUSSION

Published data reports that the catecholamine response in oysters to stress, reflects both the intensity and duration of the stressor (Lacoste *et al*., 2001c) and that stress-induced neuroendocrine changes increase the susceptibility of juvenile Pacific oysters to *Vibrio splendidus*, which was considered responsible for the juvenile oyster summer mortalities in the Bay of Morlaix, France (Lacoste *et al*., 2001a,b). Given the negative correlation between stress and immune competence, determining the catecholamine response in oysters to different farming activities may provide valuable information that could be used to improve or modify existing techniques so they have the least possible effect on oyster health. However, before catecholamine levels in oysters from farms in remote areas in South Australia can be measured it is necessary to 1) evaluate the published methodology on oyster catecholamine quantification with the equipment available in South Australia and 2) establish method(s) to extend the half-life time of detectable forms of catecholamines in cell-free oyster haemolymph from the estimated 30 minutes (Lacoste *et al*., 2001c) to a duration that allows blood samples collected from remote farms in South Australia to be processed and analysed at SARDI without a change in catecholamine levels.

Experiment 1: Establishment of the standard noradrenaline (NA) and dopamine (DA) elution peaks by using liquid chromatography with electrochemical detection

The HPLC chromatograms presented in Figures 1, 2 and 3 and the results in Table 1 show that NA and DA can be detected and quantified with the equipment available at SARDI. The detection limits for the catecholamines were in the order of 0.01ng mL^{-1}, which is more than adequate to detect normal values of approximately 1ng mL^{-1} in oyster haemolymph.

The recovery of standards – dihydroxybenzylamine (DHBA) added to haemolymph after the preparation for HPLC analysis ranged from 75% to 80%, which are within the ranges reported in the paper published by Lacoste *et al*., (2001c) (70% to 90%).

The range in noradrenaline (1.14 to 54.26ng mL^{-1}) and dopamine concentrations (0.10 to 4.58ng mL^{-1}) detected in individual oyster cell-free haemolymph, using the methods developed in this project, are wider than the published catecholamine values for pooled blood samples for this species (Lacoste *et al*., 2001c). In addition, adrenaline was also detected in some samples in this project (Figure 3). Detection of this chemical was not reported by Lacoste *et al.*, (2001c).

Experiment 2: Effect of temperature variations on circulating noradrenaline and dopamine concentrations in oysters

To standardise sample collection and analysis it was decided at the start of this experiment that 0.5mL of haemolymph would be sampled from each oyster and then processed and analysed individually. It was found in preliminary experiments that it was difficult to collect 0.5mL blood from individual oysters in less than 1.5 minutes using 26 gauge needles. Although this method was used by Lacoste *et al*. (2001c) in their experiments it was anticipated at that time it would be very difficult to apply this method on commercial farms. Therefore a larger needle (22 gauge) was chosen for the experiments.

The purpose of Experiment 2 was to investigate the sensitivity of the methods developed in Experiment 1 in detecting variation in catecholamines in circulating haemolymph from oysters subjected to a mechanical stressor such as shaking. However, it was realised that the shaking revolutions used in the paper published by Lacoste *et al*., (2001c) 300 rpm and 100 rpm, were much higher than the revolutions used for oyster grading on commercial farms in South Australia. In addition, Lacoste *et al.'s* (2001c) experiment was conducted under water whereas the grading activities in South Australia are normally conducted in air on land-based facilities. Therefore, instead of rumbling, the stress used for this experiment was a temperature increase from 15^{o}C to 30^{o}C.

As in the paper published by Lacoste *et al.*, (2001c), temperature stimulus elicited significant dopamine responses ($p < 0.001$) in oyster haemolymph (Figure 5, Appendix 3: Table 1), while the response of noradrenaline to this kind of stimulus was not significant ($p > 0.05$) (Figure 4, Appendix 3: Table 2). Very high concentrations and very high variation in NA were detected in the 0 hour samples (undisturbed oysters). The possible reasons for the high concentration and high variation in NA might be due to the effects of needle size, blood collection time, oyster acclimation period, genetics etc. and will be discussed in the next section.

Experiment 3: Optimisation of the methods for oyster haemolymph sample collection

In the first trial it was logistically impossible to control the oyster blood sample collection time although the opening times could be manipulated to some extent. However, in order to provide information that could be used for the experimental designs in subsequent trials the blood sample collection times were recorded separately and then analysed accordingly.

From the data obtained in trial 1 (Table 3) it appeared that circulating catecholamine concentrations in oysters could possibly be affected by the bleeding times used in blood sample collection. In addition it also appeared that the DA levels in the oyster haemolymph might be affected by opening times. However, neither bleeding time nor opening time effects were found to produce significant effects on catecholamine levels (Appendix 3: Tables 3 & 4). The average NA concentration in the samples collected with short opening and short bleeding time combination was 5.09±3.09ng mL^{-1}, which is much higher than the concentration for the undisturbed oysters in the paper published by Lacoste *et al.*, (2001c) (1.61±0.30ng mL^{-1}). The reason for this

discrepancy might be due to either the oyster condition, differences in oyster size or age, genetics, or the needle size used for the blood collection because the periods used for animal acclimation were similar in both studies (12 days in this experiment vs 10 days in Lacoste *et al.'s* experiment (2001c)). In addition the blood collection time from individual oysters was shorter in this study (<60 seconds) than in Lacoste *et als*. (2001c) paper (<90 seconds).

In the second trial needle size was found to affect NA concentration in circulating oyster haemolymph (second trial in Table 3). A significant difference in NA concentration ($p < 0.01$) was found between the haemolymph collected using the 29 gauge and 22 gauge needles (Appendix 3: Table 5). In addition, the NA concentration in the blood collected using 29 gauge needles was similar in level to those from the undisturbed oysters in the paper by Lacoste *et al.*, (2001c). Comparison of the results for the 22 gauge needle between the first and second trials showed that the effect of oyster acclimation period on blood NA concentration was not significant. As discussed in Experiment 2, although lower circulating NA concentrations in undisturbed oysters could be obtained by using 29 gauge needles in blood sample collection, it was viewed at the time that it would be more difficult for farmers to collect 0.5mL of blood from individual oysters in less than 60 seconds using needles of this size. A possible solution to this would be to use 29 gauge needles but reduce the amount of blood needed to be collected from individual oysters from 0.5mL to 0.2mL and then pooling the blood collected from three individuals into a 0.6mL sample. It is also possible that if blood sample collection time using a larger needle (eg. 18 gauge) was reduced, similar catecholamine levels to those obtained using a 29 gauge needle could be obtained. Indeed, it did appear from the second sample results in trial three (NA, 1.35ng mL^{-1} and DA 0.71ng mL^{-1}, Table 4) that the concentration of catecholamines collected using an 18 gauge needle may be affected by bleeding time. Further experiments were conducted in the fourth trial to investigate this, however, the results did not support this hypothesis (Table 3).

Further experiments are required to verify the effects of combined needle size and blood sample collection time on catecholamine levels. Until this work has been conducted we recommend using 29 gauge needles based on our results from Experiment 3, Trial 2, which showed 29 gauge needles had significantly less effect on noradrenaline levels than 22 gauge needles. Using 29 gauge needles we were able to successfully collect 0.2mL of blood from individual oysters within 30 seconds. The blood from 3 oysters was then pooled to produce one 0.6mL sample, 0.5mL was the minimum required for catecholamine extraction.

Experiment 4: Development of a method for the stabilisation of catecholamines in oyster haemolymph samples

Measurable NA quantities decrease very rapidly in bivalve haemolymph. The estimated half-life of detectable forms of NA in Pacific oyster cell-free haemolymph is 25~30 min (Lacoste *et al.*, 2001b). This has been improved substantially by using the preservative developed in this project (Figures 9 & 10). The catecholamine concentrations in the oyster haemolymph stored on ice with the preservative added remained constant for up to 42 hours.

The catecholamine concentrations in oyster haemolymph stored at room temperature started to increase gradually after 18 hours and the concentration doubled at 42 hours for NA. The reason for these increases is not known. It has been reported that catecholamine binds to vertebrate serum proteins (Powis, 1975) and it may be possible that this hormone also binds to oyster haemolymph proteins.

Although the catecholamine concentrations in oyster haemolymph remained unchanged for 42 hours on ice with the preservative developed in this project, it is recommended that the blood samples should not be stored for more than 30 hours, because in our preliminary experiments the DA concentration in one sample increased slightly after 30 hours storage.

The published data and the data from this study showed that the response time of catecholamines to different stressors were different, with the fastest response time of approximately 15 minutes being obtained for shaking stress (Lacoste *et al*., 2001c) in comparison to about 8 hours for high temperature stress (this study and that of Lacoste *et al*., 2001c). The corresponding times for the catecholamines to return to normal levels were approximately 1 hour for shaking stress and 36 hours for high temperature stress (Lacoste *et al*., 2001c). In addition, the NA response was normally quicker than the DA response. Therefore, the timing of haemolymph collection will be very critical to the accuracy of any proposed tests on oysters in the future.

BENEFITS

This project has resulted in the development of methodologies for the measurement of catecholamines in oyster blood. This means that the stress induced in oysters from various farming activities including grading, sorting, and transportation can now be tested on farms around South Australia. As stress can negatively affect the immune system this will enable farmers to identify those activities that may predispose oysters to mortality. Action can then be taken to modify the farming practices so that the level of stress imposed on the oysters is reduced and consequently their susceptibility to pathogens minimised.

The adoption of the stress test developed in this project could benefit the oyster farmers through a reduction in mortality. As a result of increased oyster production through a reduction in mortality other beneficiaries include those businesses that service the oyster industry. These include companies who manufacture equipment used in oyster farming eg. grading equipment and baskets and those involved in oyster sales. Expansion of the industry due to increased production will increase in job opportunities in rural communities where farms are situated.

FURTHER DEVELOPMENT

The outcome of this project was the establishment of a protocol (Appendix 4) for oyster blood sample collection, preservation, transportation and analysis for catecholamines. The next stage in the development of this technology is to use it to measure the stress induced in oysters by various farming practices used on farms around South Australia. The practices inducing the most and least stress can then be identified. This information can be used by the oyster industry to modify their practices so they minimise the stress placed on their oysters thereby reducing the impact of farming practices on oyster health.

Before this test can be utilised to monitor catecholamine response in oysters to stresses from different farming practices, preliminary experiments will be required to determine the optimal time when the blood should be sampled for each different practice (source of stress). This is because the present research and published data indicate that the speed in catecholamine response varies with different kinds of stressors.

PLANNED OUTCOMES

As a result of the protocol developed in this project a tool now exists which can be used to assess and thus help improve the current farming and processing technologies used by the South Australian oyster growers so that they result in minimal stress to oysters. Use of low stress management technologies will reduce damage to the oysters and losses caused by stress related aetiologies. This should result in an increase in the consistency and quality of oysters for market and subsequently an increase in market confidence resulting in increased sales. In addition a tool for the measurement of stress could assist in selective breeding programs where the selection of reduced susceptibility to stress is viewed as a desirable trait by industry.

CONCLUSION

The two objectives of this project were achieved. The catecholamine concentrations in the haemolymph collected from Pacific oysters have been maintained at constant levels for at least up to 30 hours using the preservatives developed by this project. This could allow the monitoring of catecholamine response to acute stresses on the oyster farms far from the facilities required for catecholamine extraction and analysis. This is very important because most oyster farms in South Australia are in remote locations. The success of the project now opens the opportunity for oysters growers to improve their farming activities and product quality by adopting or developing low stress farming techniques. In addition, the preservative developed by this project could also provide opportunities to further study on the neuroendocrine biology in oysters.

As a significantly lower level of noradrenaline was found using a 29 gauge needle compared with a 22 gauge needle in this study, we recommend that 29 gauge needles be used in catecholamine experiments on oysters. In addition, 29 gauge needles are cheaper to purchase than other sizes because they are used by diabetics for insulin injections. We were able to easily collect 0.2-0.3mL of blood from individual oysters in 30 seconds using 29 gauge needles. As the effects of a longer duration of blood collection time (the time the needle was inside the oyster heart) are not known, we recommend collection time be kept below 30 seconds. This is because in Experiment 3, trial 1, the effect of blood collection time was nearly significant ($p=0.053$).

Based on the results from the experiments in this project a protocol for oyster blood sample collection was developed (Appendix 4) which includes the usage of the preservative developed in this project and the steps from opening of the oyster to when the haemolymph is sent off to the facility where the catecholamine extraction and analysis will be conducted. Before this protocol is adopted and used to monitor catecholamine response in oysters to any new kinds of stress, preliminary experiments will be required to determine the optimal time when the blood should be sampled. This is because the present research and published data indicate that the speed in catecholamine response varies with different kinds of stressors.

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APPENDIX 1: Intellectual Property

N/A

APPENDIX 2: Staff

Principal Investigator:	**Dr Xiaoxu Li** (South Australian Research and Development Institute, SA)
Co-investigators:	**Dr Peter Babidge** (South Australian Research and Development Institute, SA)
	Dr Meegan Vandepeer (South Australian Research and Development Institute, SA)
	Dr Richard Musgrove (South Australian Research and Development Institute, SA)
	Professor Hongsheng Yang (Institute of Oceanology, Chinese Academy of Sciences)
	Mr Gary Zippel (Chairman of the South Australian Research Council)
Technical Support:	**Mr Kriston Bott** (South Australian Research and Development Institute, SA)

APPENDIX 3: Statistical Analyses

Experiment 2: Effect of temperature variations on circulating noradrenaline and dopamine concentrations in oysters

Table 1 One-way analysis of variance to test for differences in dopamine response with different duration of temperature treatment.

Source	DF	Sum of squares	Mean square	F Value	P value
Time	4	8.62051	2.15513	57.89	< 0.001
Residual	10	0.37227	0.03723		
Total	14	8.99277			

Table 2 One-way analysis of variance to test for differences in noradrenaline response with different duration of temperature treatment.

Source	DF	Sum of squares	Mean square	F Value	P value
Time	4	19.03	4.76	0.27	0.891
Residual	10	176.37	17.64		
Total	14	195.41			

Experiment 3: Optimisation of the methods for oyster haemolymph sample collection

Trial 1

Table 3 One-way analysis of variance to test for differences among the short opening and long bleeding time, short opening and short bleeding time and long opening and short bleeding time combinations on noradrenaline levels.

Source	DF	Sum of squares	Mean square	F Value	P value
Time	2	1083.9	542	3.80	0.053
Residual	12	1711.8	142.7		
Total	14	2795.8			

Table 4 One-way analysis of variance to test for differences among the short opening and long bleeding time, short opening and short bleeding time and long opening and short bleeding time combinations on dopamine levels.

Source	DF	Sum of squares	Mean square	F Value	P value
Time	2	3.160	1.580	1.37	0.292
Residual	12	13.866	1.156		
Total	14	17.026			

Trial 2

Table 5 One-way analysis of variance to test for differences in noradrenaline as a response to collection of haemolymph with different needle sizes (29 and 22 gauge)

Source	DF	Sum of squares	Mean square	F Value	P value
Gauge	1	14.274	14.274	11.04	0.006
Residual	12	15.516	1.293		
Total	13	29.790			

Trial 4

Table 6 One-way analysis of variance to test for differences in noradrenaline levels between the 10-20 and 21-30 second bleeding time groups using a 29 gauge needle

Source	DF	Sum of squares	Mean square	F Value	P value
Time	1	0.0468167	0.04682	0.0258	0.8801
Residual	4	7.250667	1.81267		
Total	5	7.2974833			

Table 7 One-way analysis of variance to test for differences in noradrenaline levels between the 4-6 and 7-11 second bleeding time groups using a 18 gauge needle

Source	DF	Sum of squares	Mean square	F Value	P value
Time	1	0.112067	0.11207	0.0314	0.8680
Residual	4	14.282667	3.57067		
Total	5	14.394733			

Table 8 One-way analysis of variance to test for differences in dopamine levels between the 10-20 and 21-30 second bleeding time groups using a 29 gauge needle

Source	DF	Sum of squares	Mean square	F Value	P value
Time	1	0.02666667	0.026667	1.35694	0.3084
Residual	4	0.07846667	0.019617		
Total	5	0.10513333			

Table 9 One-way analysis of variance to test for differences in noradrenaline levels between the 4-6 and 7-11 second bleeding time groups using a 18 gauge needle

Source	DF	Sum of squares	Mean square	F Value	P value
Time	1	0.4108167	0.410817	0.8757	0.4024
Residual	4	1.8764667	0.469117		
Total	5	2.2872833			

Trial 5

Table 10 Two-way analysis of variance to test for the effect of needle size and needle shear force on noradrenaline.

Source	DF	Sum of squares	Mean square	F Value	P value
Model	3	4.881	1.627	0.743	
Gauge size	1	1.740	1.740	0.794	0.399
Squeezed or unsqueezed	1	1.505	1.505	0.687	0.431
Gauge size vs sq or unsq	1	1.635	1.635	0.746	0.413
Error	8	17.528	2.191		
Total	12	509.969			

Table 11 Two-way analysis of variance to test for the effect of needle size and needle shear force on dopamine.

Source	DF	Sum of squares	Mean square	F Value	P value
Model	3	0.008	0.003	1.705	
Gauge size	1	0.004	0.004	2.547	0.149
Squeezed or unsqueezed	1	0.00003	0.00003	0.021	0.888
Gauge size vs sq or unsq	1	0.004	0.004	2.547	0.149
Error	8	0.013	0.002		
Total	21	0.334			

APPENDIX 4: Protocol for blood sample collections

Protocol for collection of haemolymph from the Pacific oyster (*Crassostrea gigas*) for catecholamine analysis.

Materials (Figure 1)

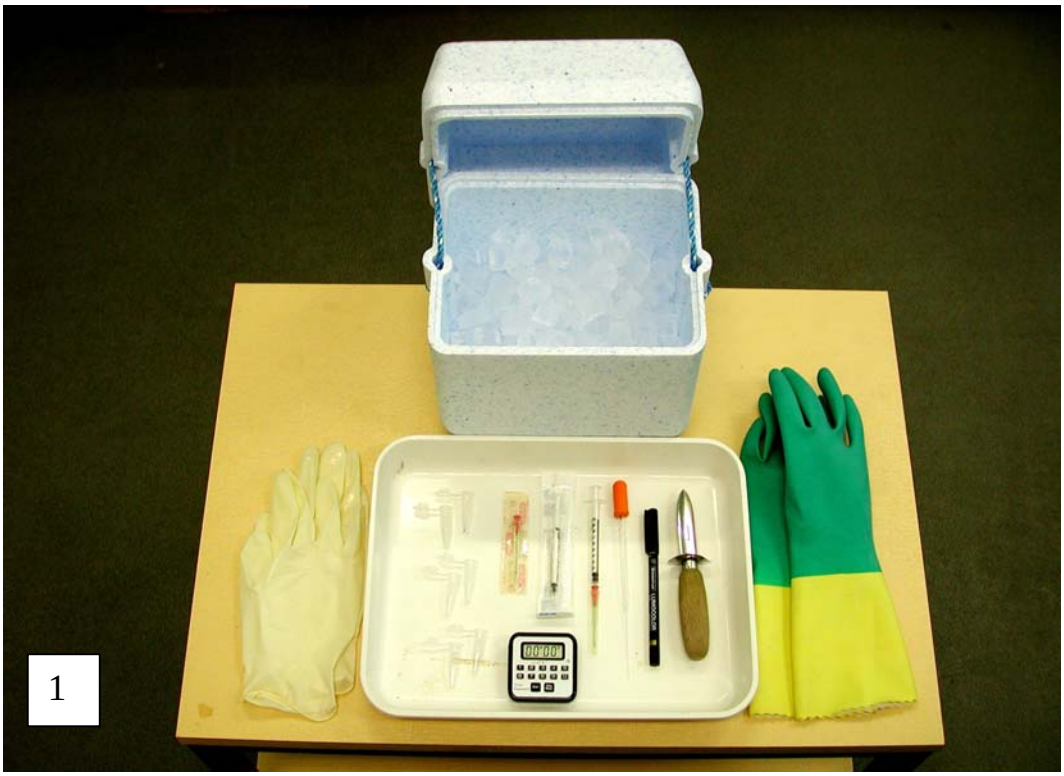
- Oyster knife
- Stopwatches x 2
- 1mL sterile syringes (1 syringe per replicate blood sample)
- 29g x 1.5 inch needles (1 needle per replicate blood sample)
- Rubber gloves
- Surgical gloves
- Styrofoam container filled with ice
- Standard disposable glass pasteur pipette
- Preservative solution provided by SARDI (which can be stored in -18^{o}C freezer for a maximum of 3 months or on ice in an esky for 3 days)
- Eppendorf tubes
- Permanent marker
- Needle and syringe disposal container

Methods

1. Take the preservative from the freezer and thaw it in running water. When thawed store it on ice in an esky.
2. Withdraw some of the additive (at least enough for 2 drops) from the stock solution provided by SARDI with the disposable glass pasteur pipette and then put the stock solution back into the Styrofoam container filled with ice.
3. With the pipette held in a vertical position, place 2 drops of additive into each of the Eppendorf tubes that will be used for sample collection (Figure 2).
4. Remove the oyster from the holding tank or the stressor.
5. Shuck the oyster by levering open the hinge and running the knife along the right valve to sever the adductor muscle (Figure 3). Remove the right valve to completely expose the oyster (Figures 4). The oyster should be shucked

within 15 seconds to reduce the effects of stress caused by opening on catecholamine levels.

6. The tissue attached to the adductor muscle should be severed exposing the pericardial cavity and the excess water removed from the shell by tipping the oyster on its side and allowing the water to run out. The oyster is now ready for haemolymph collection. Discard the animal if its pericardial cavity is broken.
7. Place the shucked oyster in the palm of one hand. In the other hand hold the 1mL syringe. Place the side of the hand holding the syringe along the edge of the oyster shell close to the pericardial cavity. This is to help stabilise the oyster when inserting the needle (Figure 5).
8. With the needle held on a 45° angle gently insert it inside the pericardial cavity just under the surface with the tip of the needle facing upwards (Figures 6 & 7). At the same time start the timer.
9. Use the thumb on the hand holding the syringe to draw the plunger back carefully. If part of the atrium or ventricle of the heart becomes lodged in the tip of the needle remove the needle slightly and then reinsert it back gently into the cavity. Once more than 0.2mL of haemolymph has been withdrawn remove the needle and stop the timer. Discard the haemolymph if less than 0.2mL is collected or the collection period is more than 30 seconds.
10. Adjust the haemolymph in the syringe to 0.2mL.
11. Squeeze the haemolymph into a 1.5ml Eppendorf tube and place the tube immediately on ice (Figure 8).
12. Pool the haemolymph collected from three individual oysters from the same holding tank or stressor together to produce one sample (0.6mL in total) for catecholamine analysis.
13. Mix the sample properly by shaking the Eppendorf tube.
14. Discard of the needle in an appropriate container after the completion of one sample collection (from three individuals).
15. Make sure that the samples reach the organisation(s) conducting the analysis as soon as possible because the samples have to be processed within 30 hours.



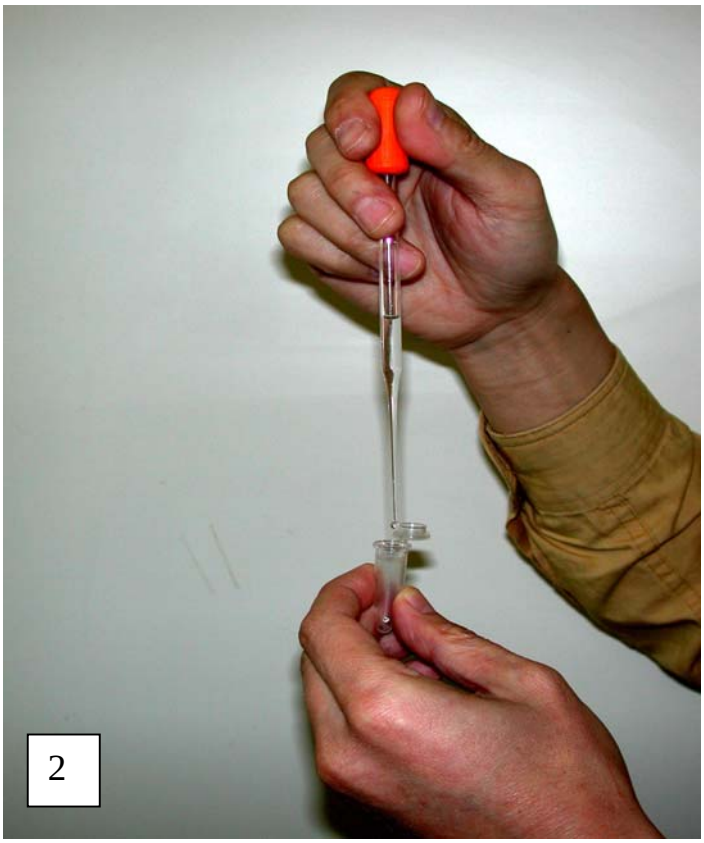
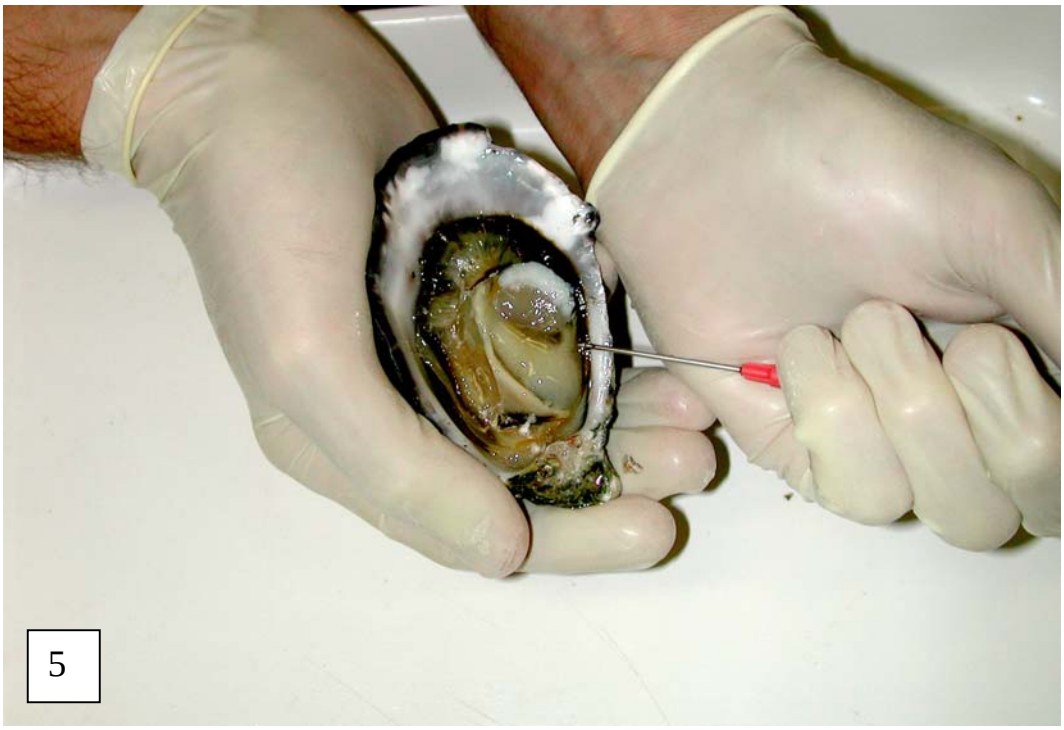


Figure 1: Setup of equipment needed for catecholamine extraction.
Figure 2: Adding of preservative to Eppendorf tubes

Figure 3: Opening of oyster.
Figure 4: Oyster ready for catecholamine extraction after residual water is tipped out of cavity.



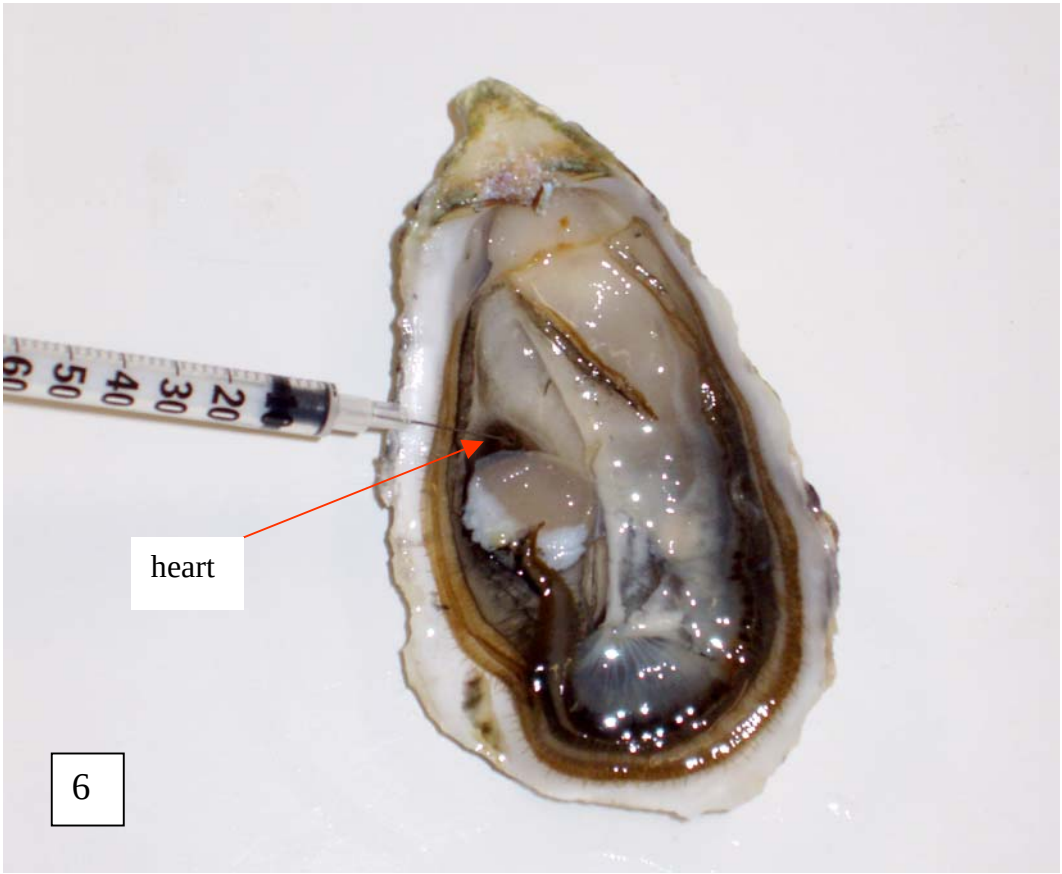
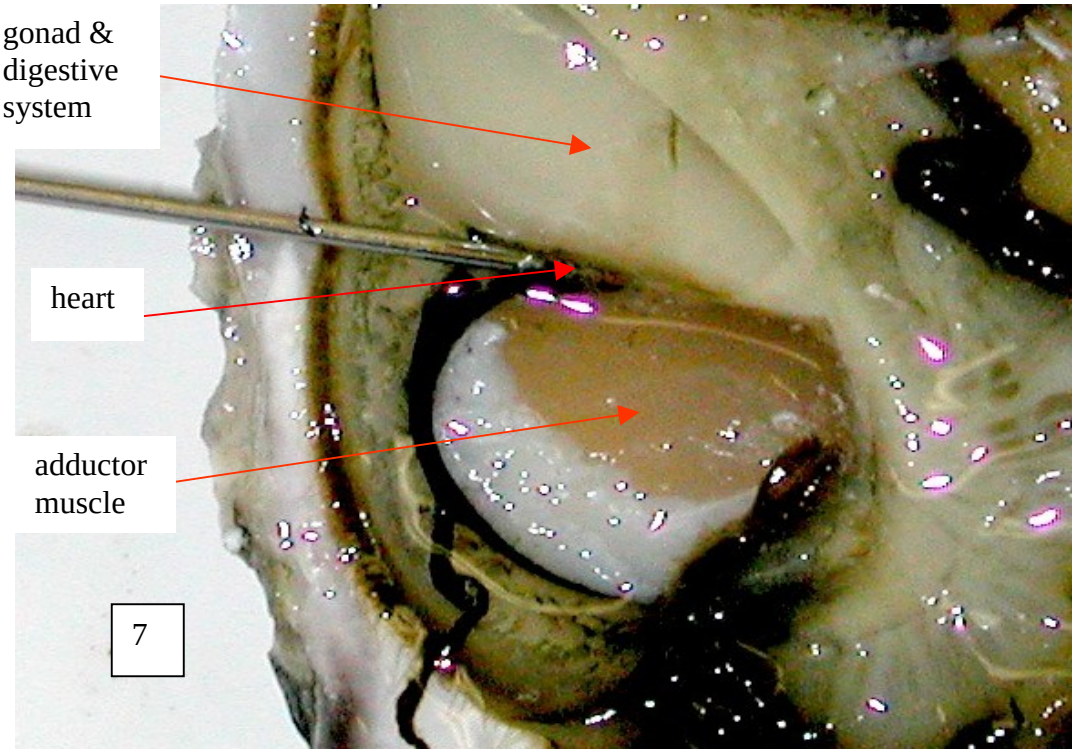


Figure 5: Holding of oyster and needle in position for catecholamine extraction
Figure 6: Insertion of needle into oyster heart and extraction of haemolymph.



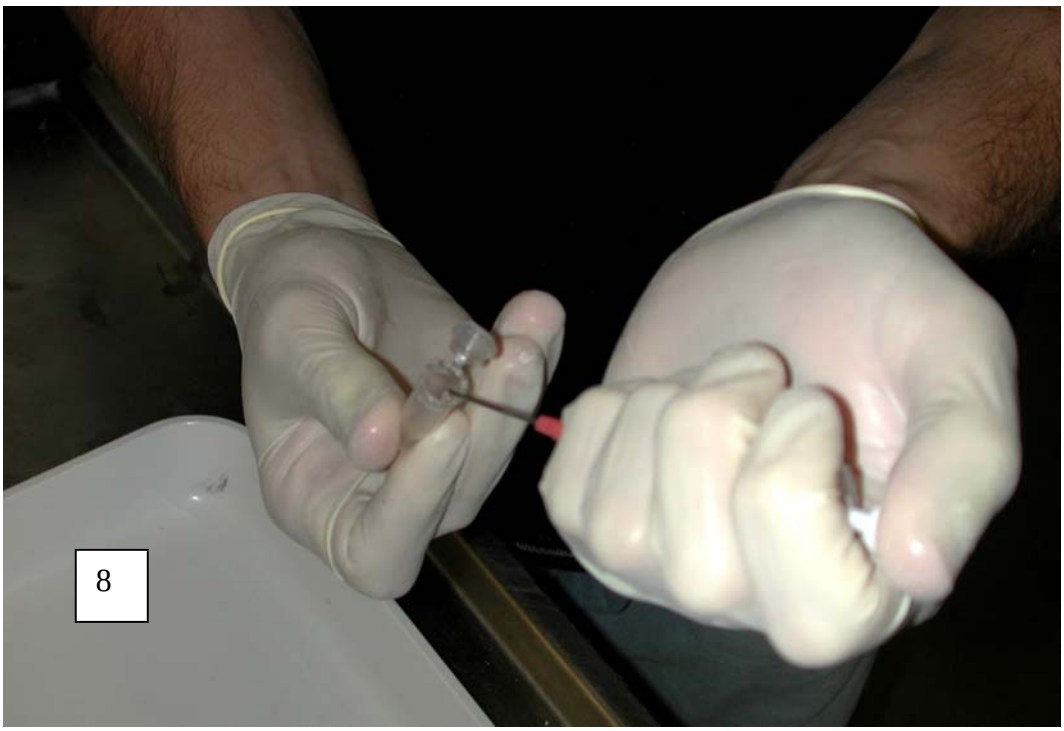


Figure 7: Insertion of needle into oyster heart and extraction of haemolymph.
Figure 8: Haemolymph sample placed into Eppendorf tube and then on ice.